

A multi-omic analysis of the photosynthetic  
endosymbioses of *Paramecium bursaria*

A DISSERTATION PRESENTED

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I, FINLAY MAGUIRE, CONFIRM THAT THE WORK PRESENTED IN THIS THESIS IS MY OWN. WHERE INFORMATION HAS BEEN DERIVED FROM OTHER SOURCES, I CONFIRM THAT THIS HAS BEEN INDICATED IN THE THESIS.

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# Publications

1. Maguire *et. al.* 2014, *Complex patterns of gene fission in the eukaryotic folate biosynthesis pathway* Genome Biology and Evolution **6** 2709-2720
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4. Richards *et. al.* 2015, *Molecular diversity and distribution of marine fungi across 130 European environmental samples* Proceedings of the Royal Society B **282** 20152243
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## *A multi-omic analysis of the photosynthetic endosymbioses of Paramecium bursaria*

### ABSTRACT

The photosynthetic endosymbioses between *Paramecium bursaria* and its green algal endosymbionts (*Chlorella variabilis*, *Chlorella vulgaris*, *Micractinium reisseri* and *Coccomyxa* sp.) have long been suggested to represent nascent endosymbiotic interactions as host and endosymbiont are believed to be able to exist and reproduce separately. Understanding the molecular systems underpinning these relationships would therefore provide a model system to investigate the process of photosynthetic endosymbioses before molecular co-dependence has become fixed (leading to genomic integration). To this end, the metatranscriptome of *P. bursaria*-*M. reisseri* during lit and dark conditions was recovered using single cell methods. This necessitated the development of novel techniques to optimise the assembly and the post-assembly attribution of transcripts to their originating organism. This work represents the first *de novo* single cell transcriptomic analysis of a multi-member eukaryotic system.

Metabolic function was then analysed in this system using both transcriptomic and metabolomics data. This identified potential roles for novel sugar, amino acid, and fatty acid interactions in the *M. reisseri* endosymbiosis. Additionally, *P. bursaria* SW<sub>1</sub> was determined to potentially form an obligate host of *M. reisseri* SW<sub>1</sub>-ZK.

Finally, this work also revealed a putative non-functional exogenous RNA induced RNAi system in *P. bursaria* potentially related to the absence of a factor associated with uptake of RNA from host vacuoles in both *P. bursaria* transcriptomes and a partial *P. bursaria* single cell genome. An analysis of the level of potential RNAi “cross-talk” collisions with the active host transcriptome suggests that the function of an exogenous RNA induced RNAi system in the presence of a eukaryotic endosymbiont may be deleterious.

Therefore, despite discovering several barriers to the utility of these systems for studying endosymbiotic evolution there is still benefit to their study. The “omic” resources and analyses presented here offer an important dataset to inform further analysis.

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# Nomenclature

$\mathbb{R}$	Real Numbers
$\mathbb{Z}$	Integers
$\mathcal{D}$	Training Data
$\theta$	Parameters
AA	Amino Acid
ABC	ATP-Binding Cassette
AdaBoost	Adaptive Boosting
AGO	Argonaute proteins
AIC	Akaike Information Criterion
ALE	Assembly Likelihood Evaluation
AMP	Adenosine Monophosphate
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide BLAST
BLASTP	Protein BLAST
BLASTX	BLAST against a Protein Database using a Translated Nucleotide Query
BLAT	BLAST-Like Alignment Tool
BLOSUM	Blocks Substitution Matrix
bp	Base Pair
CCAP	Culture Collection of Algae and Protozoa
CD-HIT	Clustering Database at High Identity with Tolerance
CDF	Cumulative Distribution Function
CDS	Coding DNA Sequence

CGAT	Computational Genomics Analysis and Training
CI	Continuous Integration
CNN	Convolutional Neural Network
CONCOCT	Clustering Contigs with Coverage and Composition
CPU	Central Processing Unit
CRF	Conditional Random Fields
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTAB	Cetrimonium bromide
CV	Cross-Validation
DAPI	4',6-diamidino-2-phenylindole
DBG	de Bruijn graph
DBSCAN	Density-Based Spatial Clustering of Applications with Noise
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DDBJ	DNA Data Bank of Japan
ddNTP	Dideoxy Nucleoside Triphosphate
DETONATE	<i>de novo</i> Transcriptome RNA-Seq Assembly with or without Truth Evaluation
DNA	Deoxyribonucleic Acid
dNTP	Deoxy Nucleoside Triphosphate
DPP	Dirichlet Process Prior
dsRNA	Double-Stranded RNA
DT	Decision trees
DV	Digestive Vacuole
EGT	Endosymbiotic Gene Transfer
EIC	Extracted-Ion Chromatogram
EM	Expectation-Maximisation
EPP	Eulerian Path Problem
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tags
ETE	Environment for Tree Exploration
eToL	Eukaryotic Tree of Life

ETS	External Transcribed Spacers
FAT	Fatty Acid Translocation
FDR	False Discovery Rate
FFT	Fast Fourier Transform
FN	False Negative
FP	False Positive
FRET	Fluorescence Resonance Energy Transfer
GAI	Genome Analyzer 2
GC	Guanine Cysteine
GC-	Gas Chromatography
GFF	General Feature Format
GFP	Green Fluorescent Protein
GI	Gene Identifier
GMM	Gaussian Mixture Model
GNU	GNU's not UNIX
GO	Gene Ontology
GTF	General Transfer Format
GTR	Generalised Time Reversible model
HAMAP	High-quality Automated and Manual Annotation of Proteins
HGT	Horizontal Gene Transfer
HMM	Hidden Markov Model
HPLC	High Performance Liquid Chromatography
HPP	Hamiltonian Path Problem
IDBA	Iterative de Bruijn Assembler
IDBA-MT	Iterative de Bruijn Assembler - Metatranscriptome
IDBA-MTP	Iterative de Bruijn Assembler - Metatranscriptome with Protein Alignment
IDBA-UD	Iterative de Bruijn Assembler - Uneven Sequencing Depth
IES	Internal Eliminated Sequences
iid	Independent and Identically Distributed
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside

IR	Infrared
ITS	Internal Transcribed Spacer
JGI	Joint Genome Institute
KAAS	KEGG Automatic Annotation Server
KAT	K-mer Analysis Toolkit
KDE	Kernel Density Estimate
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria Broth
LBA	Long Branch Attraction
LC-	Liquid Chromatography
LCA	Lowest Common Ancestor
LDA	Linear Discriminant Analysis
LECA	Last Eukaryote Common Ancestor
LG	Le-Gascuel model
LTS	Long-Term Support
MAC	Macronucleus
MAFFT	Multiple Alignment using Fast Fourier Transform
MCMC	Markov Chain Monte Carlo
MCMCMC	Metropolis-Coupled MCMC
MDA	Multiple Displacement Amplification
MDS	Multidimensional Scaling
MEGAN	Metagenome Analyzer
METLIN	Metabolite Link Database
MFS	Major Facilitator Superfamily
MIC	Micronucleus
miRNA	Micro RNA
ML	Maximum Likelihood
MLE	Maximum Likelihood Estimator
MNIST	Mixed National Institute of Standards and Technology
MP	Maximum Parsimony

MPP	Mitochondria and Plastid Porin family
mRNA	Messenger RNA
MS	Mass Spectrometry
MSA	Multiple Sequence Alignment
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NADH	Nicotinamide adenine dinucleotide (reduced)
NBRP	National BioResource Project
NCBI	National Center for Biotechnology Information
NCL	New Cereal Leaf-Prescott
ncRNA	Non-Coding RNA
ND	NanoDrop
NIST	National Institute of Standards and Technology
NJ	Neighbour-Joining
NMR	Nuclear Magnetic Resonance
NNI	Nearest Neighbour Interchange
NP	Non-deterministic Polynomial Time
NR	Non-Redundant
NTS	Non-transcribed Spacers
OD	Optical Density (OD <sub>600</sub> would be OD at 600 nanometer wavelength)
OLC	Overlap Consensus
ORF	Open Reading Frame
P	Polynomial Time
PAM	Protein Data Bank
PBCV	<i>Paramecium bursaria-Chlorella</i> Virus
PbMr	<i>Paramecium bursaria-Micractinium reisseri</i>
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PE	Paired-End
PFAM	Protein Families Database

PGM	PiggyMac
piRNA	Piwi-Interacting RNA
PP	Posterior Probability
PTGS	Post-Transcription Gene Silencing
PV	Perialgal Vacuole
QC	Quality Control
QDA	Quadratic Discriminant Analysis
qPCR	Quantitative Polymerase Chain Reaction
QQQ	Triple Quadrupole
QTOF	Quadrupole Time-of-Flight
QUAST	Quality Assessment Tool for Genome Assemblies
RAM	Random Access Memory
RBF	Radial Basis Function
RBM	Restricted Boltzmann Machine
RdRP	RNA-Dependent RNA polymerase
RFO	Raffinose Family Oligosaccharide
RNA	Ribonucleic Acid
RNAi	RNA-mediated Interference
RNase	Ribonuclease
RNN	Recurrent Neural Network
RSEM-EVAL	RNA-Seq by Expectation-Maximisation - Evaluation
RT	Reverse Transcription
RT-PCR	Reverse Transcription PCR
RVM	Relevance Vector Machine
SC	Single Cell
scnRNA	Scan RNA
SCT	Single-Cell Transcriptome
SDC	Single Digested <i>Chlorella</i>
SEECER	Sequencing Error Correction for RNA Reads
SGC	Single Green <i>Chlorella</i>

SGLT	Sodium-Glucose symporters
SI	International System of Units
siRNA	Short-Interfering RNA
SMART	Single Molecular Analysis Research Tool
SMRT	Single Molecule Real-Time
SNP	Single Nucleotide Polymorphism
SPR	Subtree-Prune-and-Regraph
SRA	Sequence Read Archive
SSF	Sodium-Solute Symporter Family
ssRNA	Single-Stranded RNA
SVM	Support Vector Machine
t-SNE	t-Distributed Stochastic Neighbour Embedding
TA	Thymine-Adenine
TCDB	Transporter Classification Database
TM	Transmembrane
TMHMM	Transmembrane Hidden Markov Model
TN	True Negative
ToL	Tree of Life
TP	True Positive
TPM	Transcripts Per Million
TST	Tonoplast Sugar Transporter
UK	United Kingdom
UTR	Untranslated Region
UV	Ultraviolet
VCS	Version Control System
WGD	Whole Genome Duplication
WTA	Whole Transcriptome Amplification
XCMS	X-Chromatography Mass Spectrometry Library

# Maths Notation

Throughout this thesis several standard mathematical conventions have been used (inline with the ISO80000-2 regulations for technical writing [ISO International Standard \(2009\)](#)).

Specifically (unless the object is a specific quantity with a pre-established symbol), matrices are denoted by boldface italic capital letters ( $\mathbf{A}$ ,  $\mathbf{B}$ ,  $\mathbf{X}$ ) and their elements by standard italic lower case letters indexed using subscripts (i.e.  $a_{ij}$  is the element  $i, j$  of the matrix  $\mathbf{A}$ ). Vectors are denoted by an arrow above standard italic lowercase letters ( $\vec{x}$ ,  $\vec{y}$ ,  $\vec{z}$ ) with their elements indicated in the same manner as matrix elements ( $x_i$  is the  $i$ th element of the vector  $\vec{x}$ ). Tensors of third and higher orders are represented by boldface italic sans serif capitals ( $\mathbf{T}$ ,  $\mathbf{L}$ ). Finally, scalars and variables which may be a tensor of any order are denoted by standard lowercase italic elements ( $k$ ,  $n$ ,  $p$ ).

Norms are used and denoted in standard linear algebra fashion e.g. the norm of a scalar is equivalent to its absolute value ( $\|n\| = |n|$ ). Unless stated otherwise all matrix and vector norms ( $\|\mathbf{A}\|$ ,  $\|\vec{y}\|$ ) are the  $\ell - 2$  (or Euclidean) norms. Otherwise the norm is indicated by a subset number in place of  $p$ :  $\|\vec{x}\|_p = (\sum_{i=1}^n |x_i|^p)^{\frac{1}{p}}$ . When applied to a set (e.g.  $x = \{0, 1, 2\}$ )  $|x|$  indicates the cardinality.

*Hofstadter's Law: It always takes longer than you expect,  
even when you take into account Hofstadter's law*

- Douglas Hofstadter: *Gödel, Escher, Bach: An Eternal*

*Golden Braid, 1979*

# 1

## Introduction

### 1.1 ENDOSYMBIOSIS

#### 1.1.1 WHAT IS ENDOSYMBIOSIS?

Endosymbiosis has proven one of the most fundamental processes in the evolution of the eukaryotic cell (Timmis et al., 2004; Lane, 2007; Martin and Herrmann, 1998; Archibald, 2015). It has both shaped the global climate and created the cellular context in which specialised multicellular organisms have evolved.

Endosymbiosis is a special case of symbiosis, which is the long-term stable interdependent living together (“sym/σύν” – together, “bios/βίωσις” – living) of two or more organisms to a point of mutual benefit (de Bary, 1869; Pound, 1893).<sup>1</sup> What differentiates endosymbiosis from symbiosis in general is that one partner (the endosymbiont) lives wholly inside (“endo/ἔνδον” - inside)

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<sup>1</sup>Many now expand this definition beyond mutualism to include other categories of biological interactions (table 1.1.1) (Leung and Poulin, 2008; O'Malley, 2015)

of another (the host). This “inside” can refer to symbionts either living intracellularly or within the tissues of multicellular organisms. However, it excludes niches such as the digestive tract of metazoa as this can be considered as an external surface of the host. These latter symbionts are occasionally termed ectosymbionts.

Interaction Name	Interaction Outcome
Mutualism	(+, +)
Antagonism	(+, -)
Competition	(-, -)
Commensalism	(+, o)
Amensalism	(-, o)
Neutralism	(o, o)

**Table 1.1.1:** An overview of the categories of biological interaction and the effect they have on the two interacting biological units, which may be anything from individual species to whole populations. The outcome column contains a tuple relating the effect an interaction has on a pair of interacting biological units. This “effect” is often assessed in terms of metrics such as individual fitness, population size and/or growth rate. Specifically, ‘+’ represents a positive, ‘-’ a negative, and ‘o’ a neutral outcome. Therefore, (+, +) indicates an interaction which is beneficial to both partners and (+, o) one that is beneficial to one partner and neutral to the other. Note: parasitism and predation are mechanisms by which an antagonistic interaction may take place (Abrams, 1987) in the same sense that endosymbiosis is a mechanism by which a mutualistic interaction can take place. In reality most interactions will not fall neatly into one of these categories and throughout its duration will often display characteristics of multiple categories (Leung and Poulin, 2008)

There is a considerable diversity of endosymbiotic relationships in nature. These relationships can encompass many different degrees of host-symbiont integration, interdependence and ecological interaction types (table 1.1.1). Even if we restrict ourselves to endosymbioses that are largely “mutualistic”<sup>2</sup> there is a broad range of characteristics.<sup>3</sup>

For example, in terms of interdependence of host and endosymbiont you can construct a spectrum (fig. 1.1.1) with “incidental” endosymbioses such as bacterial or fungal escape of digestion in macrophages at one extreme and at the other obligate systems such as the mitochon-

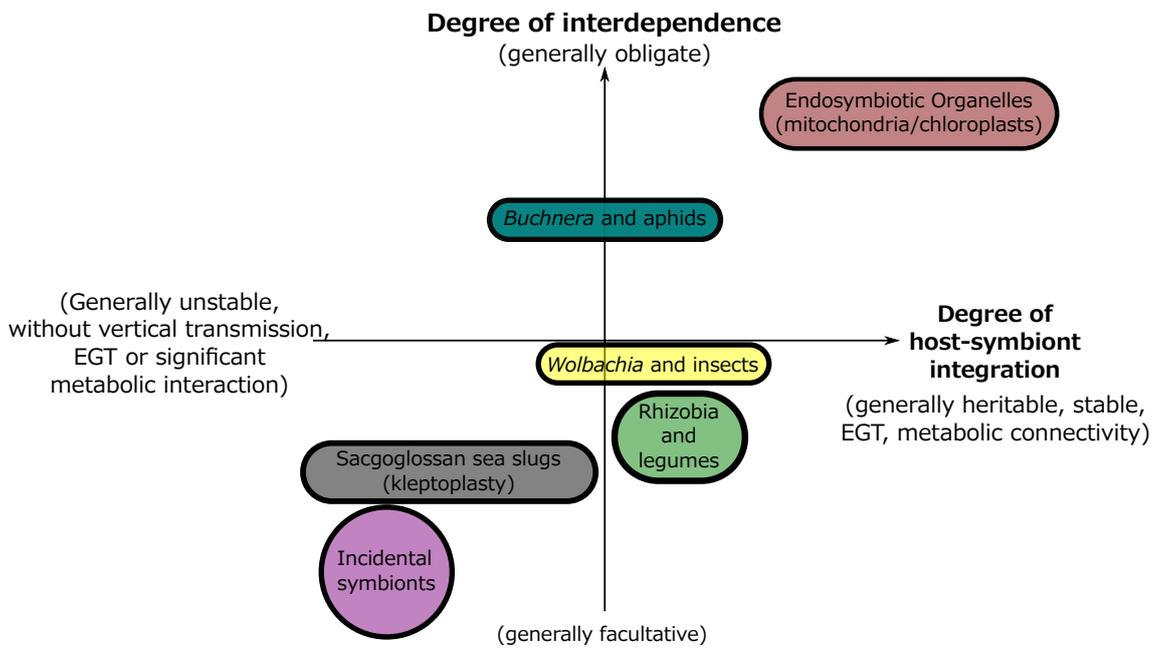
<sup>2</sup>Noting that the exact nature of a certain endosymbiosis is highly dependent on the specific ecological context at a particular point of time

<sup>3</sup>Attempting to classify endosymbioses leads us into the territory of a common motif of biology: the application of discrete schemas to continuous distributions of traits. These biological quantisations are prone to error (fuzzy delineations) and are constantly challenged by novel discoveries which exhibit a mosaic of category features. There are many examples of this such as the classification of mitochondria-related organelles (Maguire and Richards, 2014), types of biological interactions (see table 1.1.1), and the numerous species concepts (De Queiroz, 2007; Boenigk et al., 2012). That is not to say biological quantisation is without utility or is a futile task. Indeed, as long as there is a clarity to the application, basis and limitations of these schema then they form a critical (epistemological) framework upon which further research and communication can build (Boenigk et al., 2012). However, care must be taken not to forget that they do not reflect reality and can inadvertently obscure the grey areas (Leung and Poulin, 2008).

dria or chloroplast where host and symbiont are essentially a single organism (Margulis, 1998; Archibald, 2015). In the middle of such a spectrum you could find facultative endosymbioses where each partner is capable of, and does, live aposymbiotically for extended life phases e.g. Rhizobia soil bacteria and legume (Fabaceae) plants (reduction of atmospheric  $N_2$  to ammonia (Hirsch, 1992) in exchange for host-derived carbon sources such as malate and succinate (Prell and Poole, 2006)).

An endosymbiosis may be highly integrated in terms of metabolism, genome and life history while still only being moderately interdependent (such as the facultative Rhizobia nitrogen fixation which takes place in carefully controlled specialised root nodule structures (Crespi and Frugier, 2008)). Therefore, a second dimension could be applied to our approximate classification of endosymbiosis (fig. 1.1.1) representing the degree of host-endosymbiont integration. Generally interdependence and integration correlate reasonably well due to the increased selective pressure to minimise lethal aberrant interactions that comes with interdependence. This can be seen in the extreme of host-symbiont integration: that of the endosymbiotic organelles, which are so highly integrated they were only conclusively identified as being endosymbiotic and not part of the cell relatively recently (see (Archibald, 2014) for a brief historical overview).

Intracellular endosymbionts can be found inhabiting multiple host-compartments from nakedly in the cytoplasm, to host-derived vacuolar compartments (often from exo- and endocytic systems) e.g. (Kodama and Fujishima, 2009) along with a range of host organelles including the endoplasmic reticulum (Vogt, 1992), Golgi body (Cho et al., 2011), mitochondria (Sassera et al., 2006), chloroplast (Wilcox, 1986) as well as the nucleus (Schulz and Horn, 2015). Owing to the endosymbiotic origin of the chloroplast and mitochondria it becomes apparent that there can be multiple “layers” of endosymbiosis. A primary endosymbiont is an endosymbiont that is the direct endosymbiont of the host (e.g. the mitochondria to eukaryotes) whereas a secondary endosymbiont is the endosymbiont of an endosymbiont. The layers of endosymbioses can get impressively deep, for example, bacterial endosymbionts have been identified within the chloroplast stroma (cyanobacterial endosymbiont) of dinoflagellates (e.g. *Woloszynskia pascheri* (Wilcox, 1986)) In turn, dinoflagellate plastids have been discovered that are likely the product of tertiary endosymbioses (Gabrielsen et al., 2011) with higher-order events hypothesised in related groups (Stiller et al., 2014). Therefore, bacteria like this could be the endosymbiont of an



**Figure 1.1.1:** Plot demonstrating a fragment of the diversity of endosymbioses and specifically highlighting the possibility of a well integrated by facultative endosymbiosis. Host-symbiont integration is a rough measure of how connected the host and symbiont have become genomically, metabolically and in terms of life history. Whereas, interdependence is an approximate measure of the degree to which the relationship is necessary for life of organisms involved. It should be noted that both axes can be highly reliant on specific ecological and environmental context.

endosymbiont of an endosymbiont of an endosymbiont (quaternary) or higher.

With this considerable diversity it is perhaps not surprising that endosymbioses have been discovered featuring partners from all three domains of cellular life. However, with the exception of one extant Bacteria-Bacteria endosymbiosis (von Dohlen et al., 2001), typically the majority of known endosymbioses feature a eukaryotic host<sup>4</sup> but can include endosymbionts from all 3 domains. For example:<sup>5</sup>

- Eukaryote-Archaea (Moissl-Eichinger and Huber, 2011)

<sup>4</sup>There are however many examples of mutualistic symbioses which are Bacteria-Bacteria (e.g. biofilms (Watnick and Kolter, 2000)), Bacteria-Archaea (e.g. anaerobic methanotrophic archaea and sulphate-reducing bacteria likely responsible for a large proportion of global methane consumption (Boetius et al., 2000; Knittel and Boetius, 2009) and SM1 euryarchaea/*Thiothrix* sp. sulphide-oxidising bacteria (Henneberger et al., 2006; Wrede et al., 2012)), and at least one example of Archaea-Archaea (*Ignioccus hospitalis*/*Nanoarchaeum equitans* (Huber et al., 2002). Interestingly *Ignioccus* is the first identified case of an energised outer-membrane in double-membrane bound archaea or bacteria, a significant finding for the development of theories of eukaryogenesis (Küper et al., 2010))

<sup>5</sup>Although with all these examples it is important not to consider an endosymbiotic relationship in isolation from other endosymbionts present in the same host. There are examples where facultative “secondary endosymbionts” are able to compensate for the loss of an obligate endosymbiont (Koga et al., 2003). Symbiont-symbiont interactions have been found to play a role in determining which endosymbionts are capable of establishing themselves in a certain host and can even be capable of generating additional phenotypes e.g. the R-bodies of “killer” *Paramecium* species which may be a product of an interaction between the *Paramecium* host, *Caedibacter* and a bacteriophage (Schrallhammer and Schweikert, 2009).

- Methanogenic archaea within various ciliates species (e.g. *Plagioplya frontata*) (Fenchel and Finlay, 1992; Lange et al., 2005)
- *Cenarchaeum symbiosum* within the tissues of marine sponges (Preston et al., 1996; Wrede et al., 2012)
- Eukaryote-Bacteria
  - *Hartmannella* and its intranuclear endosymbiont *Candidatus Nucleicultrix amoebiphilia* (Schulz et al., 2014)
  - the most famous pairing of mitochondria and plastids
- Eukaryote-Eukaryote
  - The fungi *Diplodia mutila* which aids herbivory resistance in the palm *Iriarteia deltoidea* in lowlight conditions but becomes pathogenic if host is well lit (Álvarez Loayza et al., 2011)
  - Red alga derived plastids in brown algae (Dorrell and Smith, 2011)
  - Numerous examples of algal mediated acquired phototrophy in ciliates (Johnson, 2011)

Endosymbiosis is the one of the most significant evolutionary processes in eukaryotic cell. It offers a means for eukaryotes to benefit from the extensive metabolic diversity present in the bacterial and archaeal pangenome, especially the only known forms of primary energy production - photosynthesis and chemosynthesis (Wernegreen, 2012).

#### 1.1.2 PLASTID ENDOSYMBIOSES

Most molecular evidence currently points towards a single primary endosymbiotic event between a phagotrophic ancestral eukaryote (with mitochondria and developed endomembrane system (Rockwell et al., 2014)) and a cyanobacteria (blue-green algae) as giving rise to the Archaeplastida (that is the green algae, red algae, glaucophytes and land plants (Green, 2011)) and their double membrane bound plastids (Keeling, 2013). While this event is one of the most fundamental events in the evolution of life, in and of itself, it is only capable of explaining a small proportion

of the diversity of plastids across the eTOL (Keeling, 2013). Apart from one other putative primary endosymbiosis in *Paulinella chromatophora*<sup>6</sup> all other oxygenic phototrophs (as well as several non-photosynthetic but plastid bearing pathogens (Sato, 2011)) have arisen by secondary or higher order endosymbioses (Hoshina and Imamura, 2009).

Secondary endosymbioses are those in which another eukaryotic lineage has engulfed a primary plastid bearing algae before reducing and integrating them into a simulacrum of primary endosymbiosis, occasionally serially (Keeling, 2010). This and subsequent loss of membranes leads to the range of membrane layer numbers around plastids in various eukaryote lineages (Keeling, 2013). These secondary order plastid endosymbioses have occurred independently in divergent eukaryote lineages all over the eToL e.g. chloroarchniophytes and euglenids, and an unresolved number of times in the set of cryptomonads, haptophytes, stramenopiles, dinoflagellates and apicomplexans (Keeling, 2013). Additionally, there are an uncertain number of higher order endosymbioses in the dinoflagellates (Keeling, 2013).

Therefore, understanding the mechanisms and evolution of secondary photosynthetic endosymbioses would provide important insight into the evolution of a considerable number of eukaryotic lineages. Unfortunately, most extant examples feature endosymbioses within which metabolic co-dependence has already become fixed. This means the potential mechanisms through which the endosymbiosis may have originated are likely to be masked. Facultative systems such as the green algal endosymbionts of *Paramecium bursaria* offer a potential avenue to investigate secondary photosynthetic endosymbioses at an earlier stage i.e. before metabolic co-dependence has become fixed (while acknowledging the impossibility of interrogating events that have already occurred within the correct ecological context). Furthermore, as the ancestral protist involved in the primary plastid endosymbiosis likely exhibited a similar life style to serially phagotrophic *Paramecium* and would, initially at least, have also been mixotrophic (combining phagotrophy with phototrophy via the newly acquired plastid (Rockwell et al., 2014)) the study of the *Paramecium bursaria*-green algal systems offer a potential insight into this early and fundamental stage of eukaryote evolution.

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<sup>6</sup>A euglyhid amoeba with photosynthetic chromatophores that are vertically inherited, synchronised to host and bear a much stronger molecular and morphological resemblance to reduced cyanobacteria than the chloroplast of the Archaeplastida (Kies and Kremer, 1979; McFadden, 2014).

## 1.2 *PARAMECIUM BURSARIA*

*Paramecium* are large (50-330  $\mu\text{m}$ ) phagotrophic single-celled eukaryotes belonging to a genetically diverse (Prescott, 1994) sub-grouping of the alveolates known as the ciliates (see fig. 1.2.5). They have been studied since the invention of microscopy (Görtz and Fokin, 2009) (first recorded by a contemporary of van Leeuwenhoek, see fig. 1.2.1) and are some of the longest-standing model unicellular eukaryotes. They have been used to study everything from mutagenesis and developmental genetics, to genomics rearrangement and epigenetics (McGrath et al., 2014). Therefore, this system has a well-developed methodological (Sonneborn, 1970) and theoretical literature along with several available genomes (see fig. 1.2.3 for information on the genomes and their relative relationship to *P. bursaria*).

*Paramecium bursaria* “the green *Paramecium*” is distinguished from most<sup>7</sup> other *Paramecium* by the distinctive stable, heritable secondary photosynthetic endosymbiosis it maintains with several species of *Chlorella*, *Micractinium* and *Coccomyxa*. Each 100-160  $\mu\text{m}$  *P. bursaria* (Jennings, 1939) cell contains  $\sim$  300 endosymbiotic algae maintained in individual perialgal vacuoles (PV) around the cell cortex (Hoshina and Imamura, 2009).

Much like other ciliates, *Paramecium* are covered by cilia. These are minute hairlike biochemically heterogeneous organelles capable of sensing the environment and by beating in co-ordinated metachronal waves (Funfak et al., 2015) provide cellular locomotion and. In the case of phagotrophic ciliates like *Paramecium*, these cilia also play an important role forcing food bacteria towards the oral groove (cytopharynx) where they can be phagocytosed (Hamel et al., 2011; Aubusson-Fleury et al., 2015). *Paramecium* is also capable of rapid locomotion via the expulsion of trichocysts. These are defensive membrane-bound organelles containing a crystalline spike which can be rapidly ejected into the environment on fusion of trichocyst membrane with plasma membrane (Hamel et al., 2011).

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<sup>7</sup>There is at least one other species, *Paramecium chlorelligerum*, that harbours a different green alga (*Meyerella*) (Kreutz et al., 2012) and owing to the multiple origins of algal symbionts in *P. bursaria* (Hoshina and Imamura, 2009) and the general prevalence of mixotrophy in ciliates (Johnson, 2011) there are likely others yet to be discovered.

A



B

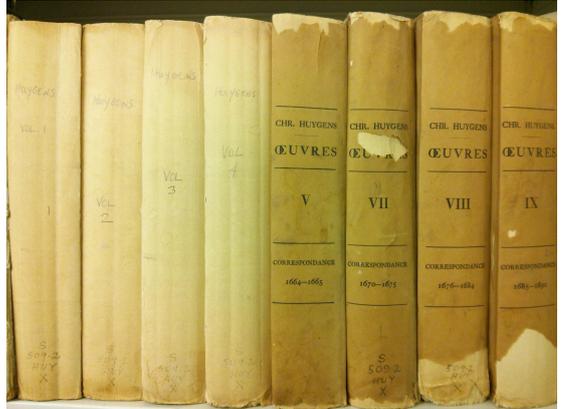
peu. Et par deux fois j'ay veu dans cette mefme eau un animal dix fois plus grand que ces autres qui avoit des pieds tout le long du corps, et estoit de cette forme.

Les 4 ou 5 pieds du costé A remuant fans cesse quand mefme l'animal estoit en repos. Il courroit vifte comme les autres, et se tournoit et pirouettoit dans l'eau. Hartfoecker m'affeure d'en avoir

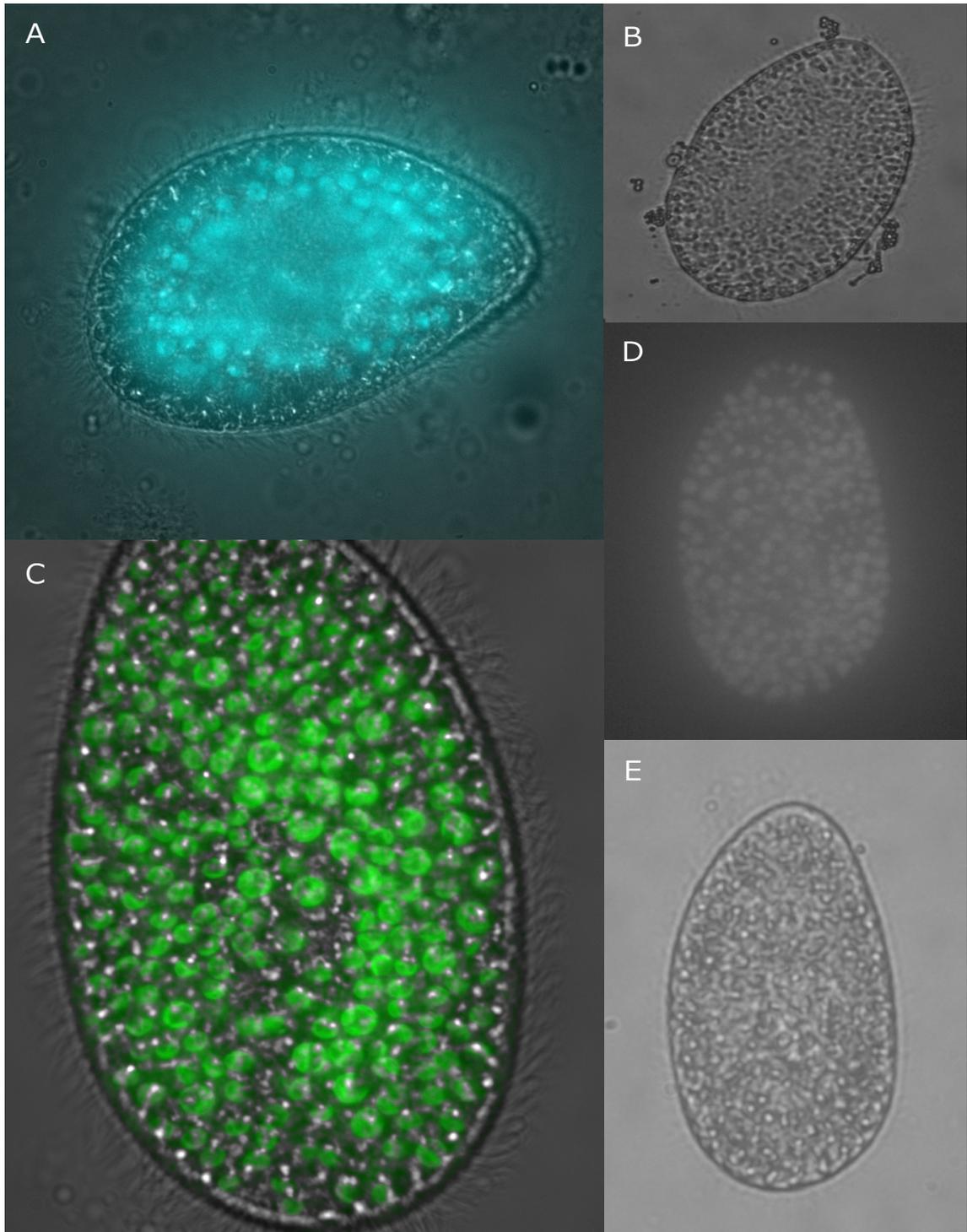
A

trouvè de la mefme efpece in femine corrupto.

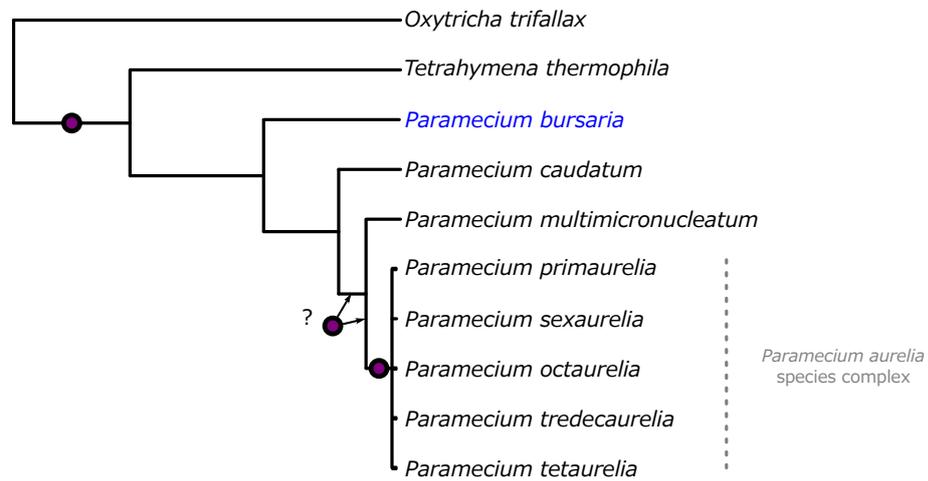
C



**Figure 1.2.1:** **A:** Carving of Christiaan Huygens (1629-1695), the prominent Dutch Golden Age mathematician and scientist and contemporary of Antoni van Leeuwenhoek, from a medallion by Jean-Jacques Clérion 1679 (reproduced from (Huygens, 1899)). **B:** Likely the first sketch of the micro-organism that we now know as *Paramecium* by Christiaan Huygens in a letter (No. 2133, 11th of August 1678) to his father Constantijn Huygens. An approximate translation of the accompanying text goes as follows "I have twice seen in this water an animal 10 times as large as the others and with feet all over its body and a narrow form. 4 or 5 feet stirred even when the animal was at rest. It moves as fast as the others, turning and spinning in the water. Hartfoecker thinks he may have discovered the same species in 'semine corrupto' (as a dried out husk?)." (reproduced from (Huygens, 1899)). **C:** 8 of the 10 volumes of the collected correspondences of Christian Huygens as prepared for the Dutch Society of Sciences and published from 1888-1905.



**Figure 1.2.2:** **A:** Composite fluorescence overlay of *Paramecium bursaria* CCAP 1660/12 captured on an Olympus IX73. **B, D, and E:** Greyscale light and chlorophyll fluorescence images of *P. bursaria* CCAP 1660/12 captured on a Nikon Eclipse 80i. **C:** Composite overlay of CCAP 1660/12 captured on an Olympus IX81 microscope. All samples were fixed in paraformaldehyde at a concentration of 4%.



**Figure 1.2.3:** Modified phylogeny redrawn from (Fokin et al., 2004; Aury et al., 2006; McGrath et al., 2014) showing the relative relations of ciliate species with genomic/transcriptomic resources and hypothesised WGD event locations with a purple dot. Specifically 2 strains of *Paramecium tetaurelia* (Aury et al., 2006), assemblies for *P. caudatum*, *P. multimicronucleatum*, and *P. aurelia* complex species *P. sexaurelia*, *P. primaurelia*, *P. octaurelia* and *P. tredecaurelia* on ParameciumDB (as of 05/03/2015) (Arnaiz and Sperling, 2011a). As well as *Tetrahymena thermophila* (Eisen et al., 2006) and *Oxytricha trifallax* (Swart et al., 2013).

Like other ciliates, including *Tetrahymena*, *Paramecium* deviates from the universal genetic code. Canonical stop codons TAA and TAG have been reassigned to produce glutamine therefore there is only one stop codon (TGA) but four glutamine codons (Salim et al., 2008).

Another defining feature of *Paramecium*, and ciliates in general, is a unique means of germline sequestration from somatic function in the form of “nuclear dimorphism” (Jahn and Klobutcher, 2002). Specifically, they have two types of nuclei, expression optimised highly polyploid somatic macronuclei (MAC) and largely silent diploid germline micronuclei (MIC) (Prescott, 1994).

During normal vegetative growth the MIC is densely packed with chromatin, is transcriptionally silent and undergoes mitosis as standard. Meanwhile, the MAC reproduces by a non-standard pinching process termed “amitosis”. This process appears to lack any mechanism to ensure equal segregation of chromosomes such as spindle fibres (Chalker et al., 2013). On the other hand, during sexual reproduction (in which two compatible *P. bursaria* exchange haploid MIC gametes generated by meiosis) the MAC degrades and must be reconstituted entirely from the newly formed heterozygous MIC (Jahn and Klobutcher, 2002) (see fig. 1.2.4). The exact number of MIC and MAC varies widely by species and genus however, *P. bursaria* contains a single large MIC which consists of 80 to several hundred chromosomes depending on the exact subspecies (Chen, 1940).

*P. bursaria* reaches sexual maturity after 50-100 fissions (Siegel and Larison, 1960) and will

conjugate with another *Paramecium bursaria* cell of compatible mating type and exchange haploid MIC gametes. Most *Paramecium* have a finite number of vegetative divisions and will die if they do not sexually reproduce (Chalker et al., 2013). Unlike all other studied *Paramecium*, *P. bursaria* does not only have 2 mating types and appears to have undergone gene duplication at two unlinked mating type loci. Different *P. bursaria* isolates display 2, 4 and 8 mating types (Phadke and Zufall, 2009) and form 4 or more mutually incompatible groups (Jennings, 1939). Sexually *P. bursaria* appears to have synclonal inheritance - a strictly Mendelian inheritance contrary to the more complex epigenetic patterns observed in other *Paramecium* species (that led to much of the early work on epigenetics) (Siegel and Larison, 1960; Phadke and Zufall, 2009). During conjugation there is minimal cytoplasmic exchange (with no exchange of endosymbionts) (Wichterman, 1946). Contrary to other *Paramecium* species, which undergo autogamy after 75 rapid replications (Sung et al., 2012) or 30-35 while starving (Berger, 1986), *Paramecium bursaria* has not been found to naturally undergo autogamy (Siegel, 1963; Yanagi, 2004) but it can be induced by treatment of methyl cellulose (Yanagi, 2004)

In *Paramecium* the haploid size and complexity of the MIC is greater than that of the MAC as a result elimination of approximately 20-30% of DNA during reconstitution of the MAC from the MIC. Eliminated sequences are known as internal eliminated sequences (IES) and are a mixture of transposon-related repetitive sequences and nongenic single-copy sequences resulting in a gene dense low-repeat MAC (Chalker et al., 2013). Similarly, the MAC has a greater number of shorter chromosomes than the MIC due to chromosomal fragmentation during DNA elimination by imprecise deletion of internal DNA segments followed by rejoining or telomere addition (Chalker et al., 2013). This process involves 3 steps, as observed in *P. tetraurelia*, and features a special class of small RNAs (scnRNAs) (Chalker et al., 2013):

- DNA amplification to high ploidy.
- DNA elimination pathway 1: accurate removal of short unique-copy elements (IES, internal eliminated sequences) that run through coding and non-coding sequences. This is achieved using bounding 5'-TA-3' dinucleotides to target double-stranded breaks and subsequent end-joining (Mayer and Forney, 1999; Bétermier, 2004)
- DNA elimination pathway 2: imprecise removal of large DNA regions (often containing transposable elements) in a manner similar to transposon silencing in other eukaryotes.

This process likely involves short ncRNAs targeting heterochromatin formation via histone methylation to induce fragmentation. This fragmentation is subsequently repaired via the addition of new telomeres (Duret et al., 2008).

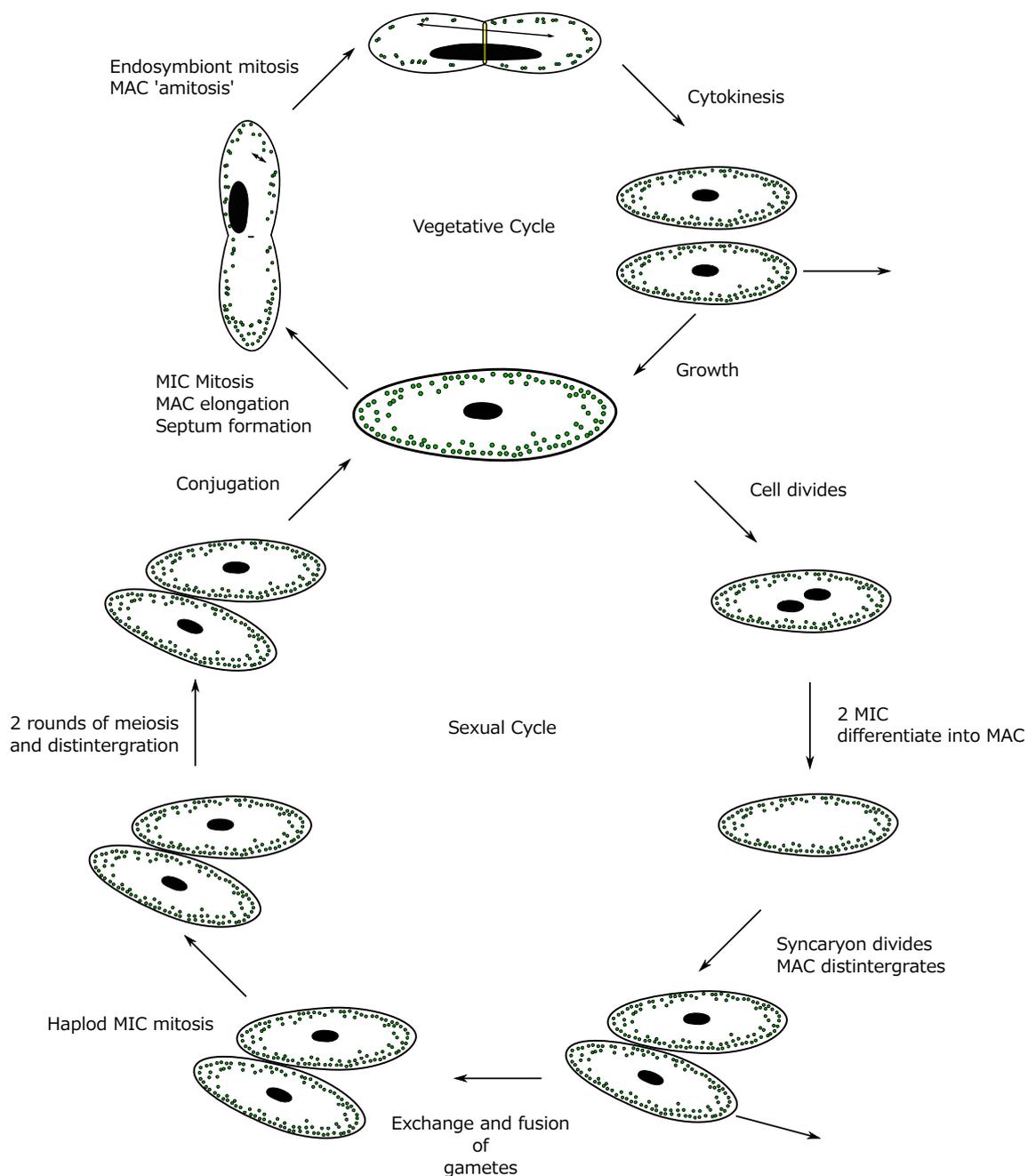
This process involves a special class of meiosis specific small RNAs (scnRNAs) which target aspects of DNA elimination (Chalker et al., 2013).

While the MIC appears to vary in size between subspecies of *P. bursaria* the MAC is roughly the same size and generally contains 10-30 times the amount of DNA than the diploid MIC (Cullis, 1972). Therefore, the MAC of *P. bursaria* is likely 20-60n (in contrast to the 800n MAC ploidy found in *P. tetraurelia* (Duret et al., 2008)). The MAC genome is likely to be somewhere between 20 and 100Mb and contain somewhere between 18,000 and 40,000 genes based on the size of the *Tetrahymena* (Eisen et al., 2006), *P. tetraurelia* (Aury et al., 2006), *P. caudatum* (McGrath et al., 2014) and *Oxytricha* (Swart et al., 2013) MAC genomes.

We can infer other likely features of the *P. bursaria* MAC genome from the *P. tetraurelia* sequencing project. Specifically, it is likely AT-rich (28% GC in *P. tetraurelia*), compact (78% coding density in *P. tetraurelia*), mostly repeat free with small intergenic regions and many short introns (e.g. 25 bp IES elements) (Aury et al., 2006). There is also likely to be evidence of at least one whole genome duplication (WGD) (an ancient WGD before the divergence of *Tetrahymena* and *Paramecium* clades but not the two most recent WGD (see fig. 1.2.3) giving rise to the *P. aurelia* complex) with a moderate level of conservation to gene synteny and duplicated gene retention (weakly correlating with a gene's GC%, expression level and functional class) (Aury et al., 2006; McGrath et al., 2014). *P. bursaria* is also likely to have a high level of replication fidelity and relatively low rate of base-substitution mutation, traits found in *P. tetraurelia*, as *P. bursaria* shares ciliate specific modifications to the active sites of B-family polymerases  $\alpha$ ,  $\zeta$ , and the proofreading exonuclease of DNA polymerase  $\epsilon$  believed to be adaptations to improve replication fidelity, a necessary adaptation when maintaining a separate germline (Sung et al., 2012).

There is no established methodology in *Paramecium* to transform the MIC genome so the only available reverse genetic methodology is that of gene knock-down with RNA interference (RNAi) (Marker et al., 2014). However, RNAi can be induced by one of two distinct but overlapping RNAi systems in *P. tetraurelia* (Marker et al., 2014). This is by microinjection of homologous transgenes (transgene-induced silencing) or by feeding *Paramecium* cells *Escherichia coli*

**Figure 1.2.4:** Figure redrawn and modified from (Duret et al., 2008). During normal vegetative growth *Paramecium bursaria* (and other *Paramecia*) divide by binary fission with the MAC elongating and “pinching” off in a process distinct from mitosis (known as amitosis) while the MIC undergoes mitosis. As the cell pinches before cytokinesis an unknown septum forms at the “neck”, this stops cytoplasmic streaming which induces the endosymbionts to begin to divide. Cytokinesis then occurs largely simultaneously in host and endosymbionts (Kadono et al., 2004; Takahashi et al., 2007). The sexual cycle involves conjugation of compatible mating types (taking around an hour and lasts 24-48 hours (Jennings, 1939)) which triggers two-rounds of meiosis of the MIC with one product disintegrating after each division so only a single haploid MIC remains. This undergoes mitosis to produce male and female gametes. Male gametes are then reciprocally exchanged between mating cells and fuse with the respective female gamete to create a syncaryon. Each syncaryon divides once and one product disintegrates before undergoing two subsequent divisions. Two products differentiate into MACs by programmatic reorganisation, conjugants split and a normal binary fission occurs restoring normal 1 MAC and 1 MIC (Siegel, 1963)



transformed to produce dsRNA corresponding to the target gene (Galvani and Sperling, 2002). Furthermore, natural exogenous ssRNA in food bacteria of *P. tetraurelia* has been observed to produce low levels of silencing, therefore this mechanism is a likely a form of natural gene regulation used by *Paramecium* (Carradec et al., 2015). As *P. bursaria* shares the initial ancient WGD with *P. tetraurelia* (Aury et al., 2006) based on *P. tetraurelia* genes that are the product of this WGD it currently or previously will have possessed: a pair of Dicer/Dicer-like proteins, 6 pairs of Piwi genes and a single RdRP (Marker et al., 2014). Therefore, RNAi is likely available in *P. bursaria* as a means of testing predictions generated through transcriptomic and genomic investigation.

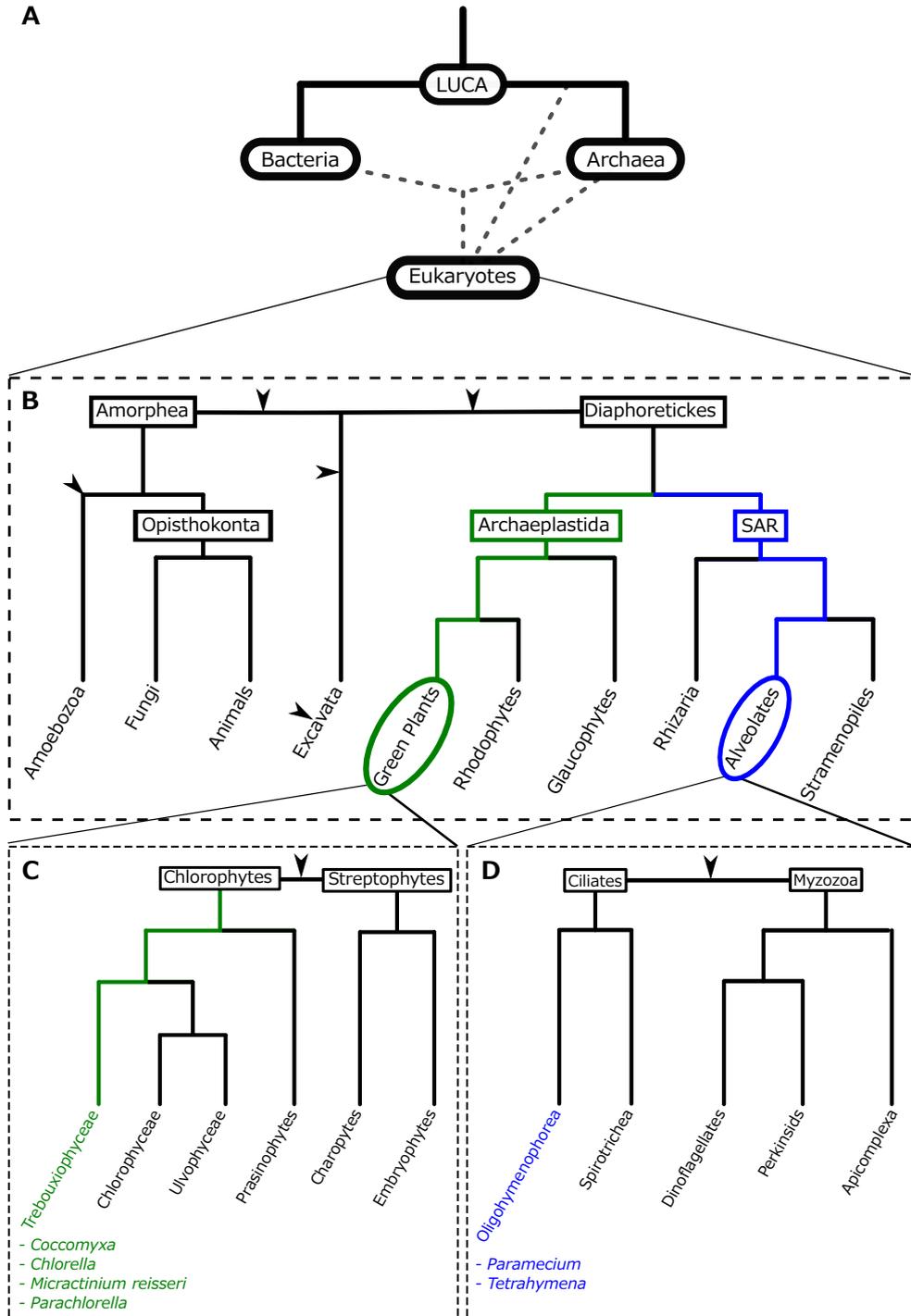
*Paramecium* appears to be particularly competent for endosymbioses with an array of over 60 genetically diverse putative endosymbionts described (Görtz and Fokin, 2009). This is no surprise as ciliates have been known to have bacterial (Görtz and Fokin, 2009), archaeal (Wrede et al., 2012) and eukaryotic (Kodama and Fujishima, 2009) endosymbionts. These endosymbionts range in degree from mutualist to parasitic and are cytoplasmic, endomicronucleic, endomacronucleic and/or perinuclear. As a serial phagotroph, *Paramecium* species are liable to infiltration by bacterial capable of escaping or resisting the phagosomal digestive process (Gortz, 1988). The *Paramecium bursaria* micronuclei frequently contains bacterial endosymbionts. The closed nature of reproduction has been suggested as a reason why endonucleobioses are common in *Paramecium* species (Görtz and Fokin, 2009) Some endosymbionts exhibit high levels of adaptation (no longer able to be found free-living and with evidence of the genome reduction distinctive of endosymbiosis) (Görtz and Fokin, 2009). The most frequently identified bacterial endosymbionts in German environmental samples are that of *Holospora caryophila*, *Holospora obtusa* and *Caedibacter caryophilus*. Several of these endosymbionts have been shown to require specific *Paramecium* genes for maintenance (Dohra et al., 1998).

### 1.3 *PARAMECIUM BURSARIA* - GREEN ALGAL ENDOSYMBIOSES

The *Paramecium*-green algal<sup>8</sup> endosymbioses are established when the algae is phagocytosed by the serially phagotrophic *Paramecium* and is then able to escape the digestive vacuole. For this

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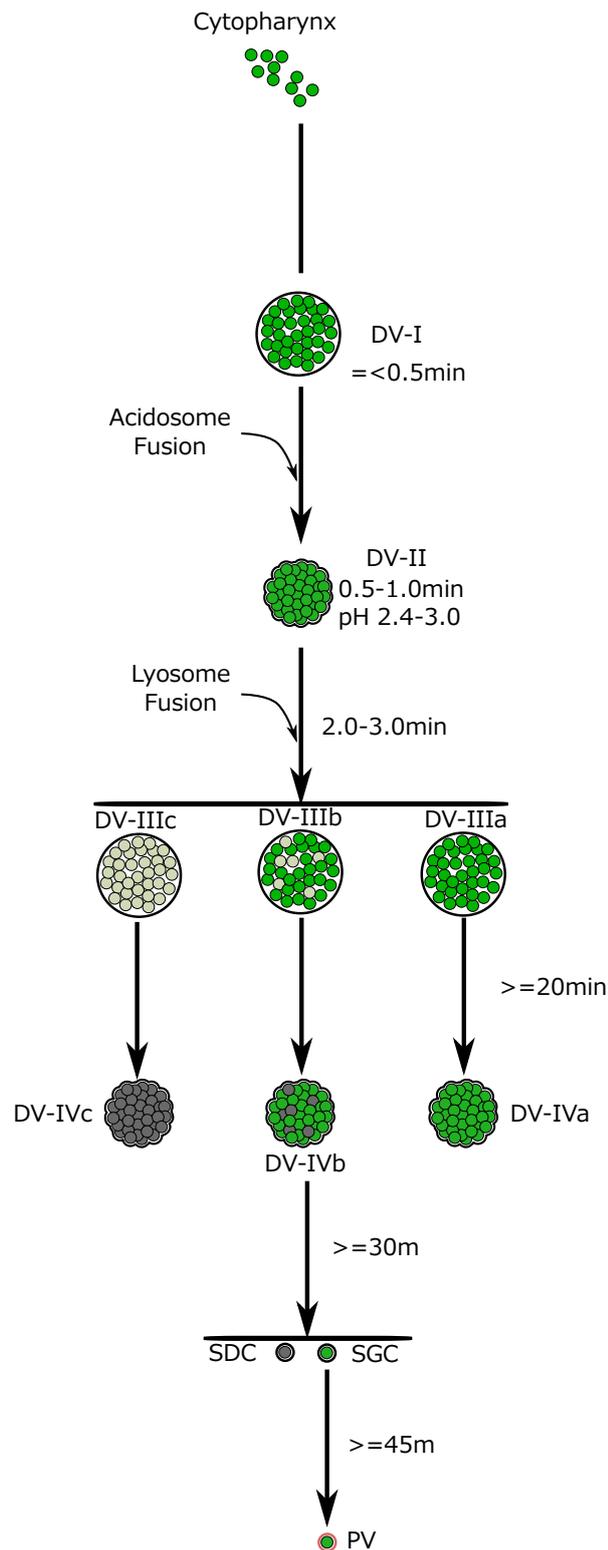
<sup>8</sup>These include *Chlorella*, *Micractinium* and *Coccomyxa* species as mentioned above. However, historically only *Chlorella* was described and as such most literature refers exclusively to *Chlorella*. Therefore, *Chlorella* is often best considered as a stand-in term for any of the green algal endosymbionts of *P. bursaria* (see Chapter 3 for a detailed review and analysis of this)



**Figure 1.2.5:** **A:** Schematic of the current best estimate of the tree of life demonstrating the 2D and 3D hypotheses, dashed lines indicate multiple potential branch location, arrowed lines demonstrate known endosymbiotic events (based on work reviewed in (Gribaldo et al., 2010)) **B:** Schematic of the current known eukaryotic portion of the tree of life (based on work reviewed in (Burki, 2014; Adl et al., 2013)), **C:** Schematic of phylogeny of the ciliates (based on work by (Bachvaroff et al., 2011) showing Oligohymenophorea containing *Paramecium* and *Tetrahymena* and sister group Spirotrichea containing *Euplotes* and *Oxytricha*. **D:** Schematic of phylogeny of the green algae (based on work reviewed in (Leliaert et al., 2012))

escape to take place, the endosymbiont must initially resist acidification caused by acidosome fusion with digestion vacuole. If the endosymbionts are able to resist this acidification they begin, through an unknown mechanism, to 'bud-off' from the initial phagosome into a new vacuole. This new perialgal vacuole (PV) is released into the cytoplasm and each PV contains an individual algal cell (Kodama and Fujishima, 2009) The PV appears resistant to lysosome fusion and further digestive steps suggesting molecular modification of the vacuole membrane (Johnson, 2011) These perialgal vacuoles then bind the host cortex and compete for attachment with host structures known as trichocysts (Kodama and Fujishima, 2012) in a region with low to no lysosome activity (Kodama and Fujishima, 2009) This suggests the observed resistance to lysosome fusion may be a by-product of localisation. As few as a single algal cell can infect the host (Weis and Ayala, 1976) however, the majority of algae are digested especially non-competent strains (Kodama et al., 2007). Furthermore, it has been established that *Chlorella* strains are fairly host-specific. For example, Summerer et al. (2007) showed that *Chlorella* isolated from other ciliates were able to establish endosymbioses with *P. bursaria* however, those isolated from cnidarian *Hydra* were not. This paper also demonstrated that *P. bursaria* favours its symbiotic partner over those isolated from other ciliates when given the choice. This suggests specific adaptations have taken place between host and endosymbiont (Summerer et al., 2007). Free-living *Chlorella* strains do rarely establish endosymbioses with *Paramecium* (Siegel and Karakashian, 1959), however they are generally only able to infect fewer *Paramecium* and establish much smaller endosymbiotic populations within the host than the symbiont strains (Siegel and Karakashian, 1959).

Once established, the symbiosis appears to be mutually beneficial with an observed flux of amino acids and CO<sub>2</sub> to the endosymbiont and oxygen and photosynthate (principally maltose) to the host as a function of light levels (Karakashian, 1963). The extent of this endosymbiosis is such that *Chlorella* is capable of supporting *Paramecium* in media without its typical bacterial food-stocks and conversely the *Paramecium* is capable of supporting the phototrophic *Chlorella* in the dark for ~2 weeks (or up to 51 endosymbiont cell divisions) suggesting considerable bi-directional nutrient flux (Siegel and Larison, 1960; Karakashian, 1963). It should be noted that for longer periods in the dark or when a bacteria-free culture is used in the dark the host will digest the endosymbionts (Parker, 1926). From an ecological perspective, this endosymbiosis can be considered as a means of acquired phototrophy (or mixotrophy), a tactic believed to be advanta-



**Figure 1.3.1:** Process by which some endosymbiont escape digestion and generate perialgal vacuoles (PV). The digestive vacuole (DV) transitions through 8 distinct stages during PV differentiation. Initially a spherical DV-I is formed from the cytopharynx. Within a minute acidosomes fuse and differentiate this to the acidic DV-II. Primary lysosome fusion triggers swelling and conversion to DV-III. At this stage endosymbiosis capable endosymbionts escape digestion by an undefined mechanism. DV-III return to near neutral pH and are categorised into 3 categories based on them containing totally bleached, partially bleached or unbleached algae. The DV-III condense into DV-IV following the same total, partial and undigested categories. Finally, DV-IVb undergo budding into a mixture of single digested *Chlorella* (SDC) and single green *Chlorella* (SGC). Finally, SGC translocate to the host cortex and further differentiate into PV (Kodama and Fujishima, 2009). Figure redrawn and modified from (Kodama and Fujishima, 2009) 40

geous for survival in patchy oligotrophic environments by providing fixed carbon to cover respiration requirements (Putt, 1990). This is largely supported by studies, such as Karakashian (1963), showing that with a sufficient concentration of bacterial feedstock in the media the growth rate of aposymbiotic *Paramecium* (“bleached”) and *Paramecium* with *Chlorella* endosymbionts are largely equal. This threshold is estimated to lie between  $10^6$  and  $10^7$  bacteria per ml. However, as this is generally a much greater concentration than found in the natural environments of *P. bursaria* the endosymbiosis offers a considerable adaptive advantage to the host (Karakashian, 1963). Temporary acquisition of phototrophy is estimated by some research (Raven, 1997) to be less energetically costly than the permanent maintenance of plastids (via endosymbiosis or kleptoplasty) within the host. Therefore, this indicates that this endosymbiosis likely provides other host benefits beyond just the energetics of acquired phototrophy. These include:

- Exploitation of low oxygen environments by the host (as the photosynthesising endosymbiont is capable of providing oxygen to the host (Reisser, 1980)).
- Photoprotection and protection against 257nm and 282nm UV radiation potentially via endosymbiont pigmentation and localisation to shield host nuclei (Sommaruga and Sonntag, 2009; Summerer et al., 2009; Miwa, 2009). This is especially important as the AT-rich *Paramecium* genome is likely prone to UV-damage via the formation of cyclobutane thymine dimers (Sommaruga and Sonntag, 2009).
- Protection against predation (Berger, 1980). The exact mechanism by which this occurs is unknown, however, it has been observed that mixotrophic ciliates are able to move in rapid ‘jumping’ movements. This is hypothesised as being an energetically costly escape reaction made possible by sugar-rich photosynthate mixotrophic ciliates gain from their algal endosymbionts (Pérez et al., 1997). Intriguingly, this protection against predation occurs despite endosymbiont displacement of trichocysts (defensive cellular structures) for attachment to the ciliate cortex (Kodama and Fujishima, 2011).
- Protection against undesired endosymbionts and/or parasites. Algae in *P. bursaria* form an antagonistic relationship with some bacterial endosymbionts but there is experimental evidence that *P. bursaria* can only be infected by bacteria and yeasts after *Chlorella* is eliminated (Gortz, 1982). This is consistent with bacterial symbionts having been repeatedly

identified as providing resistance to parasites in organisms such as the insects (Martinez et al., 2014).

- Protection against chemical toxins, for example symbiotic *Paramecium* have a much higher survival rate (96%) to 0.5 mM nickel chloride (NiCl<sub>2</sub>) than aposymbiotic *Paramecium* via an undetermined mechanism (Miwa, 2009)
- Increased thermotolerance (tested at 42°C) (Miwa, 2009), again, by unknown mechanisms but potentially related to the undefined means of perialgal vacuole attachment to the cell cortex.
- Protection against excessive oxidative burden (potentially due to endosymbiont dismutases and catalases) (Hörtnagl and Sommaruga, 2007) and hydrogen peroxide (hypothesised by Miwa as being due to the improved energetics of the symbiotic host) (Miwa, 2009).

In return, the endosymbiont also appears to gain several advantages including a generally much increased level of photosynthetic activity (Sommaruga and Sonntag, 2009):

- CO<sub>2</sub> from the host (Parker, 1926)
- Nitrogen supply (Johnson, 2011).
- Amino acids including L-glutamine (likely an important nitrogen source) and L-arginine, L-asparagine, L-serine, L-alanine and glycine (Kato and Imamura, 2009b).
- Host supplied mono- and divalent cations such as K<sup>+</sup>, Mg<sup>2+</sup>, and Ca<sup>2+</sup>. All of which have key roles in photosynthesis (Kato and Imamura, 2009b).
- Protection against *Paramecium bursaria* – *Chlorella* Virus (PBCV) (Yashchenko et al., 2012) a large isocahedral dsDNA, 330 kbp virus with 133-genes that lyses symbiotic *Chlorella* when isolated from the host (Van Etten et al., 1983). This potentially occurs by preventing contact between PBCV and the endosymbiont.
- Effective photo-accumulation and increased mobility (Niess et al., 1982a).

This exchange of materials between host and endosymbiont is regulated by an effective biochemical “bartering” system with numerous feedback cycles. For example, the release of endosymbiont photosynthate is dependent on  $\text{Ca}^{2+}$ . This ion is provided by the host and also has a role in the up-regulation of photosynthesis (as proxied by oxygen evolution) (Kato and Imamura, 2009b). Once photosynthate is released into the PV lumen endosymbiont  $\text{H}^+$ -ATPases are activated which allow the generation of the  $\text{H}^+$  gradient necessary for endosymbiont uptake of host-provided amino acids via a set of amino acid-proton symporters (in the same manner as (Camoni et al., 2006)) (Kato and Imamura, 2009b). This proton gradient will potentially lead to further photosynthate release due to observed pH-dependence of this (Kato and Imamura, 2009b). As we can see the more photosynthate supplied to the PV lumen, the greater the uptake of provided nitrogen sources. Intriguingly, from experiments using cycloheximide to selectively interrupt endosymbiont but not host protein synthesis it appears that the maltose exporter that is responsible for export of photosynthate from the PV lumen into the host cytoplasm is endosymbiont derived (Muscatine, 1967). However, unless photosynthesis is also inhibited (using DCMU) the build up of photosynthate without exportation in the PV triggers the swelling of the vacuole up to 25x its original size. This removes the vacuole from the region in which it is protected from lysosome fusion and leads to the digestion of the endosymbiont (Kodama and Fujishima, 2009). So, here we can see further regulation of the relationship – in which the endosymbiont is degraded if it does not release photosynthate to the host. This also demonstrates the importance of cytoplasmic localisation and the conditions in the PV to the control of this relationship.

On top of this system of secretion, uptake and feedback there have also been several other observed regulatory interactions between host and endosymbiont. The most apparent of these are the synchronising of cell division and circadian rhythms between host and endosymbiont. This integration is evidenced by the introduction of endosymbiotic *Chlorella* being sufficient to recover a circadian rhythm in arrhythmic *Paramecium* mutants (Miwa, 2009). This regulation of the timing of cell division for both members of the system appears well co-ordinated and takes place in such a way that neither host or endosymbionts outgrow one another (Kadono et al., 2004; Takahashi et al., 2007). The importance of regulation of endosymbiont distribution at host division is evidenced in the only natural aposymbiotic *P. bursaria* mutant which has an impairment in this mechanism and thus can't maintain endosymbionts stably (Tonooka and Watanabe, 2002).

## 1.4 CONCLUSION

In conclusion, understanding the mechanisms by which primary and secondary photosynthetic endosymbioses have occurred is one of the most significant outstanding problems in understanding the evolution of the eukaryotes. *Paramecium bursaria* and its endosymbiosis with green algae such as *Chlorella* offers a useful system to investigate secondary photosynthetic endosymbioses before metabolic co-dependence has become fixed. As both organisms seem highly prone to forming endosymbiotic relationships with multiple other organisms as a serial host and serial endosymbiont respectively it may be possible, by identifying the key molecular components of their relationship, to understand what factors contribute to such prolific endosymbioses. Furthermore, while there is considerable supporting literature and many established methodological techniques for working on these organisms individually and in endosymbiosis there have been relatively scant efforts using the latest -omics techniques and reverse genetics such as RNAi. Considering the historical role both organisms have played independently in our understanding of endosymbiosis<sup>9</sup> it is perhaps apt that further insight may be gleaned by applying the latest modern techniques to interrogate their relationship.

Therefore, the main aims of this research are to assess the utility of *P. bursaria* and its algal endosymbionts as models for the study of the evolution of endosymbiosis. Specifically, are they metabolically, transcriptomically and/or genomically tractable? Is this system amenable to “single cell” analysis? Can *M. reisseri* be separated from its *P. bursaria* as has been observed in other *P. bursaria-Chlorella variabilis* strains? Finally, can RNAi be used to test hypotheses generated using these “omic” analyses?

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<sup>9</sup>Margulis was strongly influenced and inspired by research conducted in organisms closely related to both *Paramecium bursaria* and *Micractinium reisseri*. Specifically, the discovery of Tracey Sonneborn of non-mendelian cytoplasmic inheritance in *Paramecium bursaria* (Sonneborn, 1950) and the multiple lines of evidence of the presence of DNA within the chloroplasts gleaned from several species of green algae related to *Micractinium reisseri* (*Spirogyra* (Stocking and Gifford Jr., 1959), *Chlamydomonas moewussii*, and *Chlorella ellipsoidea* (Ris and Plaut, 1962)).

Science is what we understand well enough to explain to a computer. Art is everything else we do.

- Donald Knuth: foreword to *A = B* by Petvosek, Wilf and Zeilberger

# 2

## Methods

### 2.1 MICROBIOLOGY

#### 2.1.1 STRAIN INFORMATION

During this project 3 *Paramecium bursaria* cultures have been used. These have been obtained from the UK Culture Collection of Algae and Protozoa (CCAP) and the Japanese National BioResource Project (NBRP). Specifically:

- CCAP 1660/12: *Paramecium bursaria* SW<sub>1</sub> with *Micractinium reisseri* SW<sub>1</sub>-ZK (Hoshina et al., 2010)
- CCAP 1660/13: *Paramecium bursaria* (unknown strain) with *Coccomyxa* CCAP 216/24<sup>1</sup>

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<sup>1</sup>This is a mixed culture containing both CCAP 1660/12 strain with *Micractinium* and the *Coccomyxa* bearing strain, the *Coccomyxa* endosymbiont has been further isolated in CCAP under the description CCAP 216/24 (pers. comm. Undine Achilles-Day CCAP)

- NBRP Yad1g1N: *Paramecium bursaria* Yad1g with *Chlorella variabilis* 1N<sup>2</sup>

Both CCAP cultures (1660/12 and 1660/13) were isolated from the same pond in Cambridge, UK (pers. comm. Undine Achilles-Day CCAP, Oban, Scotland) CCAP 1660/12 was the principal culture and all genomic, transcriptomic and metabolomic analyses were conducted using these cultures. Theoretically, these 3 cultures provide us with *Paramecium bursaria* strains harbouring members of 3 of the 4 species of green algal *Paramecium* endosymbiont (see Chapter 3 for more details).

### 2.1.1.2 MEDIA AND CULTURE CONDITIONS

All *P. bursaria* cultures were maintained in New Cereal Leaf-Prescott Liquid (NCL) medium:

- 4.3 g l<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O
- 1.6 g l<sup>-1</sup> KCl
- 5.1 g l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>
- 2.8 g l<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O
- 1 g l<sup>-1</sup> wheat bran

NCL medium is gravity filtered via GF/C paper and autoclaved before use (CCAP, 2012). Cultures were stored in an incubator at 15 °C with a 12:12 light:dark cycle. The incubator was lit using 2 x 21 W 865 daylight fluorescent tubes, producing 2000 lumen each. Cultures were sub-cultured approximately every 2 weeks using fresh NCL medium and were inspected using light microscopy to monitor health. No bacteria was added to cultures used prior to “omic” analyses but otherwise the medium was bacterised with *Klebsiella pneumoniae* SMC (strain donated by the Meyer Lab, Ecole Normale Supérieure, Paris, France) the day before use.

## 2.2 OMICS

“omic” technologies are those aimed at globally characterising a class of biomolecules within a specific biological sample (characterising the “-ome”). The major areas of this are genomics, transcriptomics, metabolomics and proteomics. Genomics aims to characterise DNA and generally

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<sup>2</sup>Yad1g1N host is mating type 1 and was created by mixing of isolated and cultured endosymbiont (*Chlorella variabilis* Clone 1 (known as 1N strain))

involves sequencing the genome, it is used to discover and describe genes (and non-coding DNA) and, by comparison with other genomic datasets, their evolution. Similarly, transcriptomics is orientated around the characterisation of the RNA present in a sample. This can include the canonical messenger RNA (mRNA) transcripts but also other RNA elements i.e. non-coding RNA (ncRNA) such as small interfering RNAs (siRNA) and micro RNAs (miRNA) and generally involves sequencing the RNA fraction of interest. Transcriptomics can be used to catalogue transcripts (and their variant splices), aid genome annotation, and/or assess transcriptional response to a given condition or cellular state (Wang et al., 2009). Metabolomics seeks, instead, to identify and quantify small biomolecules that make up the terminal and intermediate products of cellular metabolism e.g. carbohydrates, alcohols, and amino acids. Finally, proteomics characterises the proteins present in a sample. Typically, the metabolome and proteome are interrogated using various forms of mass-spectrometry. There are also a plethora of additional approaches which seek to characterise different subsets of these biomolecules e.g. epigenomics (epigenetic modification to DNA such as methylation and histone binding), glycomics (characterisation of cellular saccharides). Another frequently encountered term is that of “meta- ... -omics”. “Meta- ... -omics” is the application of specific “omic” method to a biological sample containing multiple organisms. For example, “metagenomics” has been used to investigate the cellular community composition of marine micro-eukaryotes (Cuvelier et al., 2010) and “metatranscriptomics” has been used to analyse the transcriptomes of the microbes present in the gut of metazoa (Perez-Cobas et al., 2013).

The utility of “-omic” approaches is that they allow a researcher to characterise a high proportion of a biological system’s function in a way that is faster, cheaper and requires less *a priori* knowledge of the system than more targeted approaches. For example, in order to estimate the abundance of all mRNA transcripts in a sample using specific approach such as qPCR would require sequence knowledge to design primers as well as an infeasible amount of reactions to acquire a characterisation comparable to that obtainable by a transcriptomic approach such as RNAseq. Additionally, due to being “non-targeted” (or rather less targeted) “omics” also removes one aspect of researched-induced bias caused by a conscious selection of molecule specific probes. By not considering elements of a system in isolation like the classic methodologically reductionist<sup>3</sup>

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<sup>3</sup>Epistemological reductionism: “explain all biology in terms of physics and chemistry” (Crick, 1966) i.e. biology is applied chemistry which is applied physics which is applied maths. Ontological reductionism: a biological system is only the sum total of its component molecules and their interactions. Methodological reductionism: examination of simple components can be used to understand complex system (Fang and Casadevall, 2011)

approaches “omics” can reveal complex systemic mechanisms/features (or at the extreme “emergent properties”) that would otherwise have been missed (Fang and Casadevall, 2011). Experimental design is very important when using of “-omic” platforms as the the number of biological replicates tends to be far smaller than the number of parameters/metabolites/transcripts being studied.

However, until relatively recently “omic” methodologies were restricted to specialised institutions and well characterised “model” organisms. While, *Paramecium bursaria* and green algae such as *Micractinium reisseri* could be considered “model” organisms throughout the early days of molecular biology, they are much less frequently studied in the genomics era (2000-today) particularly compared to organisms such as *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. Fortunately, due to the development and maturation of both technologies and databases the potential for functional and adaptive analysis of non-model organisms using combined “omics” (i.e. using genomics as a reference to guide subsequent transcriptomics) approaches e.g. (Muñoz Mérida et al., 2013; Feldmesser et al., 2014) has recently been demonstrated. Additionally, there are two other developments which make *P. bursaria*-*M. reisseri* (*PbMr*) amenable to “omic” analysis: *de novo* transcriptomics, which dispense with the need to generate accurate genomes in the relatively genomically intractable *Paramecium* e.g. (Kodama et al., 2014), and single-cell approaches which allow fine-grained analysis of the *Paramecium bursaria* - green algal relationship on a cell-by-cell basis.

It should be noted that care must be taken with “omics” approaches as they can easily become purely descriptive, and at worst generate models that lack any biological relevance (Fang and Casadevall, 2011). This concern holds for all systems-level approaches and has been frequently raised and discussed in the context of genomics (Dougherty, 2008). Therefore, it is crucial to supplement “omic” approaches with targeted methods in a way that compensates for the weakness of each type of method. Specifically, the systems approach should be used to generate novel and interesting hypotheses which can then be tested (and falsified) in isolation using reductionist methods (Casadevall and Fang, 2008). For example, “omics” methods could be used to create a model of inter-organism host-endosymbiont metabolism and targeted approaches such as RNAi could then be used to test hypotheses generated by this model i.e. testing that a particular transporter protein is responsible for the transfer of metabolites by knocking out that transporter and

observing the resultant phenotype: is the relationship perturbed in a predictable manner?

## 2.2.1 GENOMICS AND TRANSCRIPTOMICS

### 2.2.1.1 DNA SEQUENCING

In the majority of cases, genomics and transcriptomics are both synonymous with the sequencing nucleic acids. Earlier approaches, based upon the fluorescent marking of the hybridisation of DNA and/or RNA to arrays of short complementary probes e.g. genomic tiling arrays and the transcriptome microarrays (Mockler and Ecker, 2005), are of more limited utility. Relative to sequencing-based approaches these methods require relatively more prior knowledge of the organism and require a custom array to be designed for any novel system. Additionally, while microarrays can determine relative expression levels of transcripts by the comparison of the fluorescence intensity at given complementary probe(s) the continuous nature of this output, difficulty distinguishing alternative isoforms and more limited dynamic range (combined with previously mentioned limitations) has meant the sequencing of cellular transcripts (RNAseq) has largely supplanted microarrays (Wang et al., 2009). However, both SNP tiling arrays and microarrays do have the advantage of throughput and ease of analysis in situations where the host organism is well known and suitable arrays have already been designed and evaluated. For this reason they are still frequently encountered in specialist area of medical diagnostics.

While it is possible to directly sequence RNA transcripts (Ozsolak et al., 2009) most approaches first utilise a reverse transcription (RT) step to convert transcripts to cDNA. As ribosomal RNA makes up a sizeable proportion of RNA in the cell it is often necessary to enrich or select the RNA fraction of choice in order to minimise wasted effort when sequencing (Wilhelm and Landry, 2009). For eukaryotic mRNA enrichment this can be easily achieved by using poly-T primers during RT which selectively bind to the poly-adenylated tail of these messenger transcripts. However, for bacterial/archaeal work and transcriptomic analyses focusing on non-poly-adenylated transcripts such as ncRNAs/siRNAs/miRNAs etc. ribosomal depletion is used (O'Neil et al., 2013). This is a process by which ribosomal probes are attached to magnetic beads. Ribosomal RNAs bind to these probes and the magnetic beads can be used to partition the majority of ribosomal sequences away from the other RNA (O'Neil et al., 2013). This means that transcripts can be sequenced using the same methods and platforms as any other DNA sample

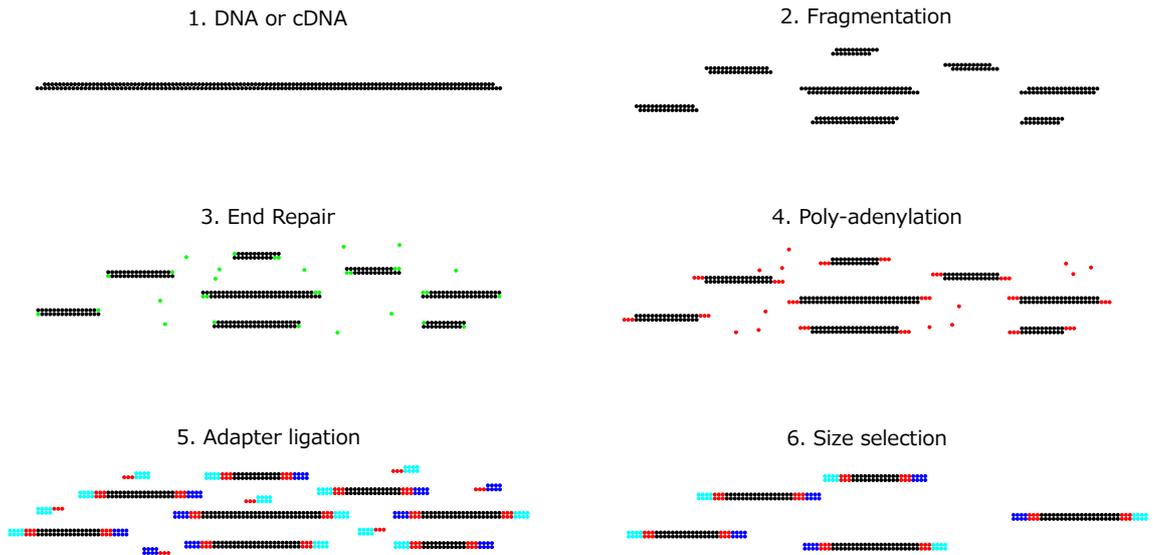
with analysis only diverging against post-sequencing. It should be noted that there are potential disadvantages to this reverse transcription step and it can potentially generate artefacts and biases in the analysis (as well as placing limitations on the quality and quantity of input RNA) (Ozsolak and Milos, 2011) however, the advantages of the more developed DNA sequencing technology outweighs these disadvantages.

These DNA sequencing technologies can largely be divided into 3 technological eras with today (2015) broadly at the transition between 2nd and 3rd generations.

1st generation (also known as Sanger) sequencing technology originated in 1970s with the work of Sanger & Coulson (Sanger and Coulson, 1975; Sanger et al., 1977a,b) which developed sequence determination via the principal of chain termination during synthesis and subsequent determination of relative fragment sizes. Briefly, by having 4 separate reactions in which DNA synthesis terminates on the incorporation of dideoxy nucleotides (ddNTP) corresponding to each of the 4 principal DNA bases (i.e. ddATP, ddGTP etc.) you can generate a series of DNA fragments of various sizes. Size fraction separation of these fragments via methods such as gel electrophoresis means the DNA sequence can be easily read from the fragment size distribution across the 4 ddNTP reactions (Sanger et al., 1977b). This technique was used to sequence the first DNA genome (bacteriophage  $\phi$ X174 (Sanger et al., 1977a)). The methodology was subsequently improved by use of fluorescently labelled ddNTPs by Leroy Hood, massively simplifying automation of the process (Smith et al., 1985, 1986). Further improvements followed throughout the 1990s and early 2000s such as capillary electrophoresis and other general throughput and length enhancements (Bonetta, 2006). Transcriptomic analysis was possible using Sanger sequencing by generating clone libraries from partial or complete cDNA and randomly sequencing clones (Adams et al., 1991; Gerhard et al., 2004). However, while this did allow resolution of different isoforms and could be used to aid annotation (Adams et al., 1991) it was not possible to investigate relative expression levels beyond a broad identification of highly expressed transcripts based on the proportion of the cDNA/EST library they made up. Sanger sequencing's main utility lies in high quality short fragment (300-1000 bp) sequencing to determine or confirm the sequence of specific DNA fragments such as vectors or PCR products (Bonetta, 2006; Tsiatis et al., 2010).

2nd generation sequencing emerged commercially in 2005 with the work of both George Church and 454 Life Sciences (Margulies et al., 2005) and featured reduced individual reaction

## Library preparation



**Figure 2.2.1:** A brief overview of library preparation for Illumina modified from (Mardis, 2008) and Illumina TruSeq kit documentation. DNA is fragmented before various steps (end-repair and poly-adenylation) to facilitate attachment of adapters. Finally, fragments with adapters are size-selected according to the specification of that particular sequencing run.

volumes, greater parallelisation (and so higher throughput), cell-free preparation without the need for time-consuming cloning of DNA fragments into bacterial vectors to generate clonal templates for sequencing, and direct sequencing detection obviating the need for size fractionation (Jaszczyszyn et al., 2014). These technologies generate huge amounts (on the order of  $10^6 - 10^9$  bp of relatively short (on the order of  $10^1 - 10^3$  bp) DNA sequences (reads) randomly sampled from the input (c)DNA.

Commercially available 2nd generation platforms include 454's GSFLX and GSJunior (now Roche), Ion Torrent's (now Life Technologies) Personal Genome Machine, Applied Biosystem's (now Life Technologies) SOLiD and Illumina's (formerly Solexa) HiSeq, MiSeq and older Gene Analyzer II (Nederbragt, 2013).

Although these platforms use a range of different implementations and tend to exhibit various different trade-offs (mainly in terms of number of reads and their respective lengths) they all largely follow the same basic process (Shendure and Ji, 2008):

1. Library generation: Randomly fragmenting input DNA into short fragments of a specific size followed by ligation of adapter sequences with some platforms allowing development of "paired-end" or "mate-pair" libraries in which each end of a fragment is sequenced separated with a known size unsequenced fragment aiding subsequent assembly (see fig. 2.2.1)

2. Clonal amplification: Generation of clonally identical spatially distinct clusters of DNA mainly via emulsion PCR (Dressman et al., 2003) (SOLiD, Ion Torrent, 454) or bridge PCR (Adessi et al., 2000; Fedurco et al., 2006) (Illumina) (see fig. 2.2.2)
3. Sequencing-by-synthesis: In which a complementary DNA strand is generated base by base via sequentially flooding and clearing a chamber with each dNTP and a polymerase (or ligase in the case of SOLiD). On incorporation of a base into a cluster a detectable signal is released such as emission of certain wavelengths of light detectable using optics (e.g. Illumina, 454, SOLiD) or release of hydrogen ion (e.g. Ion Torrent).

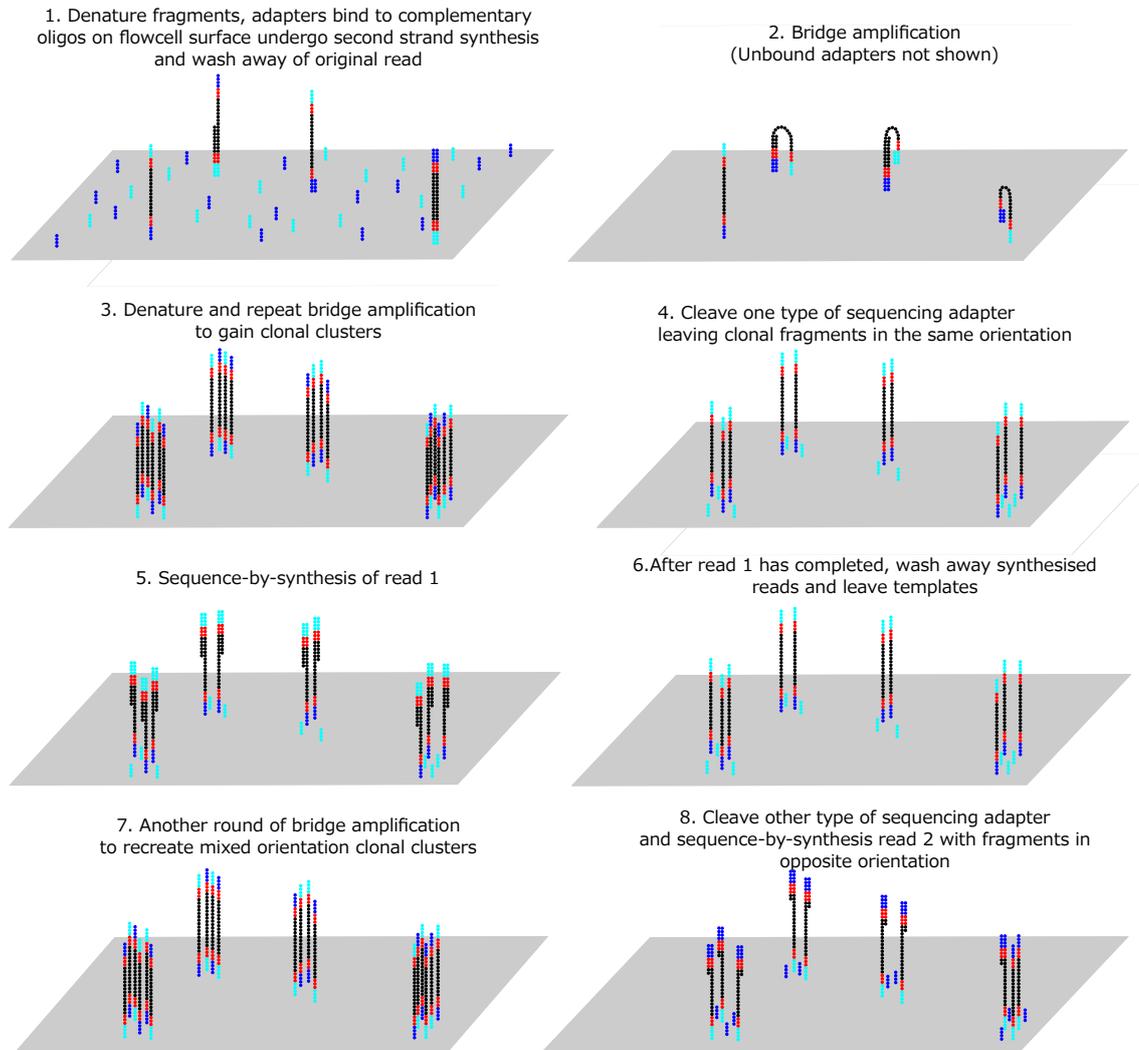
The explosion in sequencing throughput on 2nd-generation platforms has driven a massive decrease in per-base sequencing cost and the subsequent expansions in the amount of available data (e.g. the US National Center for Biotechnology's (NCBI) short-read archive (SRA)) has made both genomic and RNAseq analysis and annotation easier and more effective.

While 2nd generation sequencing has driven down per-base sequencing costs, the cost of library preparation has fallen more slowly (Blainey, 2013). For this reason, combined with the higher throughput, it has become common to multiplex different samples during sequencing runs. Multiple distinct samples can be sequenced in the same reaction (e.g. flowcell lane for Illumina platforms) by adding an indexed tag during library preparation. These tags can then be used to partition the reads back to their original separate samples after sequencing.

The current *de facto* standard in 2nd generation sequencing is that of the bridge amplification based (Shendure and Ji, 2008) Illumina platforms (Regalado, 2014) due to relatively low error rate ( $\leq 0.1\%$  (Glenn, 2011)), very high throughput (e.g. HiSeq2500 generates up to 400M 125 bp reads per run (1 Tbp of data) (Nederbragt, 2013)) and the lowest cost per Mbp ( $\leq \$0.04$  (Glenn, 2011)).

Finally, 3rd generation technologies are generally known as single-molecule sequencing. These platforms sequence individual DNA (or RNA molecules (Ozsolak et al., 2009)) without a potentially bias generating and error-prone amplification step. The first 3rd generation platform was that of the now defunct Helicos Bioscience's Helicoscope (Harris et al., 2008) based on breakthroughs in the resolution of fluorescence visualization using paired FRET methods (Braslavsky et al., 2003). Currently there is only one publically available platform: Pacific Biosciences (PacBio)

## Paired-End Illumina Sequencing



**Figure 2.2.2:** A brief overview of paired end sequencing in an Illumina flowcell after library preparation, derived from (Mardis, 2008) and Illumina. Briefly, prepared sequences with adapters are ligated to the flowcell and undergone bridge amplification to generate clonal clusters. These clusters are treated to ensure all fragments are in the same orientation before iterative sequencing-by-synthesis stages and washes. Finally, the clusters are regenerated and cleaved to sequence the opposite orientation (i.e. read 2 in paired end sequencing).

RS platform. PacBio operates on a similar principal of sequencing-by-synthesis as the 2nd generation platforms but uses fixed polymerases at the base of specially wave-guide structures allowing the detection of fluorescence from a single reaction instead of many parallel reactions in a clonal cluster. This produces few (compared to 2nd generation platforms) long (20kb and longer) reads but has a high cost and high error rate (14%) (Jaszczyszyn et al., 2014). Another platform, currently in testing, Oxford Nanopore's MinIon, reads individual strands of DNA through an array of pore proteins and determines the sequence at each pore based on the physical properties (impedance) of a particular set of bases.

Unfortunately, partly as an element of their relatively nascence and partly due to the poorer signal:noise of single molecule approaches compared to analysing large batches of identical DNA sequences, 3rd generation technologies have a relatively high error rate (Quail et al., 2012). Therefore, they are generally inadequate for eukaryotic assembly tasks in and of themselves (although that is changing as the platforms mature (VanBuren et al., 2015)). Where they have shown great utility is in conjunction with 2nd generation datasets as a scaffolding tool i.e. producing long noisy reads upon which more accurate but shorter reads can be assembled (Zhou et al., 2009). 3rd generation platforms are also highly useful in the resolution of structural variation and genomic repeats (Pendleton et al., 2015).

Therefore, all genomic and transcriptomic sequencing in this thesis has been performed using the 2nd generation Illumina GAII and HiSeq platforms due to their relative maturity, high-throughput, relatively accurate paired-end output making it currently the most amenable platform to effectively use *de novo* genomic and transcriptomics approaches. Additionally, Sanger sequencing has been used when accurate targeted sequencing was called for, such as investigating the taxonomic distribution of *Paramecium* green algal endosymbionts (see Chapter 3).

#### 2.2.1.2 BASE-CALLING

All sequencing platforms involve base-calling procedures in which the continuous primary sequencing data e.g. fluorescence with Illumina or current with IonTorrent is converted into a discrete nucleotide sequence. This is typically achieved using parametric modelling and machine learning approaches optimised by the platform manufacturer (Ledergerber and Dessimoz, 2011). Researchers using mature platforms such as Illumina typically rely on the default base-calling. However, there is much active research in improving these systems particularly for SMRT se-

quencing. These include various advanced signal processing and machine learning methods such as Conditional Random Fields (CRF) and Recursive Neural Networks (RNN).

The major 2nd and 3rd generation base-calling procedures output a FastQ formatted file. These are files containing the sequence of a read and a per-base quality score known as a Q score. These scores (Q) are calculated as a logarithmic relation to the base-called error probability ( $p$ )

$$Q = -10 \log_{10} p$$

and conversely:

$$Q = 10 \frac{-Q}{10}$$

Therefore  $Q = 30$  corresponds to 99.9% base call accuracy

#### 2.2.1.3 READ PRE-PROCESSING

Before assembly, there are several stages of read pre-processing that generally takes place for both genomic and transcriptomic studies. Typically, there are 4 key steps:

- Library quality control and contamination screening
- Trimming sequencing adapters and low probability reads
- Error corrections
- Digital normalisation

Library quality control (QC) and contamination screening is frequently done by as part of the standard sequencing facility diagnostics due to their utility in identifying technical problems. Problems with library preparation and sequencing can be detected by analysis of various metrics such as the distribution of per-base quality scores across sequences, distribution of low quality base reads across the flowcell and the presence of massively over-represented  $k$ -mers and sequences in the raw libraries. One of the most commonly used quality control tool is FastQC ([Andrews, 2015](#)) which performs tests and visualises these metrics and highlights aberrant values.

Identification of potential library problems can be used to attempt to ameliorate these problems, for example, if there are consistently low quality bases at the end of the reads then a harsher 3'

trimming step can be used. Alternatively, libraries with many problems may require re-sequencing. Contamination screening is also a highly important part of sequencing, especially for single cell approaches (see Chapter 4 for an in-depth analysis) as contaminant reads from non-target organisms can greatly complicate analysis and assembly of data.

While second-generation sequencing technologies have massively increased throughput on an individual read basis they exhibit a much higher error rate than earlier Sanger approaches with Illumina HiSeq reads showing 0.5 – 2.5% error rate (Kelley et al., 2010). Typically, Illumina errors are substitution errors (Yang et al., 2013a) and are distributed non-randomly across the read. The error rate increases from 5' to the 3' end of a given read (Liu et al., 2012). The presence of sequence error does lead to assembly error (Macmanes and Eisen, 2013). Sequencing error greatly complicates assembly graphs and increases the computational demands of graph traversal. Therefore, before assembly this error must be minimised. This generally involves two processes, read trimming and explicit error correction.

Read trimming serves two roles: the removal of contaminating sequencing adapters that may have infiltrated the library during sequencing, and the removal of low quality sequencing data. Low quality sequence data is removed as these reads/parts of reads are more likely to contain errors. There are many available tools for read-trimming e.g. Trimmomatic (Bolger et al., 2014), Sickle (Joshi and Fass, 2015), FASTX-toolkit (Gordon and Hannon, 2010), PRINSEQ (Schmieder and Edwards, 2011) and cutadapt (Martin, 2011). These generally fall into two algorithmic groups: running-sum based approaches e.g. Cutadapt and ERNE-FILTER and window-based e.g. FASTX, PRINSEQ, Sickle and Trimmomatic (Del Fabbro et al., 2013). Briefly, running-sum approaches involve the calculation of a score for each base,  $i$ , from 3' to 5':

$$s(i) = s(i + 1) + \varphi(i) - Q_{min}$$

where  $\varphi$  is a function returning the  $Q$  score for that base and  $Q_{min}$  is the minimum defined quality threshold. These  $s$  scores are then used to determine the start of the trimming point. Alternatively, window-based approaches generally calculate the average quality score across a sliding-window of  $n$  bases. If the score in a given window drops below the minimum average quality it is then trimmed.

Currently, there is no clear answer to the question 'which is the best trimming algorithm?'

This is due to many dataset-specific effects as well parameter-dependence (Del Fabbro et al., 2013). In general, most trimming tools have been found to largely perform equivalently across multiple RNAseq and DNAseq datasets and applications (see ‘File S2’ in Del Fabbro et al. (2013)). Due to its ease of use and maintenance of paired-read correspondence, Trimmomatic has been the primarily used trimming tool throughout this thesis.

Traditionally, most projects use conservative trims which only accept reads and bases above a high threshold such as an average score of  $Q_{30}$  e.g. (Looso et al., 2013). However, recently, there have been empirical studies suggesting the optimal approach is permissive trimming (e.g.  $q \geq 2$ ) followed by explicit error correction (MacManes, 2014).

Error correction of Illumina sequencing reads has been acknowledged as an increasingly important step in the creation of both high quality genomic (Schatz et al., 2012) and transcriptomic (Macmanes and Eisen, 2013) assemblies. This pre-process step generally operates on the assumption that errors are infrequent and random. An error at a given position can thus be identified and corrected by comparison to the sequences of the other reads which are sample from the same region (Yang et al., 2013b). Specifically, if the majority of other reads feature the same sequence but a single read has a single substitution this is more likely to be product of sequencing error than biological diversity.

This is most typically achieved using probabilistic approaches after decomposition of reads into  $k$  sized substrings (known as  $k$ -mers). These  $k$ -mers are then analysed via spectral techniques and the construction of hamming graphs (e.g. (Nikolenko et al., 2013)) to identify related low-abundance and likely erroneous  $k$ -mers. Some approaches will also integrate sequence alignment and quality score features. However,  $k$ -mer approaches are generally more computationally efficient and more effective at removing sequencing error than quality score based approaches (Zhang et al., 2014). See (Molnar and Ilie, 2014) for a review of error correction algorithms.

Error correction is more difficult for transcriptomic datasets (and single cell data) because the assumption of uniform coverage is not true for these datatypes (Macmanes, 2015). Therefore, correction of these datasets generally relies on explicitly probabilistic approaches that avoid this assumption by analysing relative abundances and methods such as bayesian subclustering of the hamming graphs (Nikolenko et al., 2013).

The final form of read-processing is arguably a variant of error correction, it is known as digital

normalisation (Brown et al., 2012). This simply involves the elimination of redundant read data in a given library. As short-read sequencing involves the random sampling of the transcriptome or genome there is a high level of redundant sequencing where many reads are derived from the same template. The number of reads that map to a given portion of a template is known as the coverage. While the high levels of coverage are necessary for accurate assembly reconstruction it generates a computational burden and increases the problem of sequencing error. Therefore, by normalising coverage by progressively filtering out the most abundant reads it is possible to minimise sampling variation and generate much smaller libraries that still contain nearly the same amount of information (Brown et al., 2012). This means the computational demands of assembly are much lower and thus it is easier to tune assembly parameters.

#### 2.2.1.4 ASSEMBLY

Assembly is the process by which reads are combined to recapitulate the transcripts or chromosomes they were sampled from. There are two main approaches to both genome and transcriptome assembly - referenced and *de novo*. A referenced assembly consists of the alignment of processed reads to a prior assembly or reference sequence using specialised short-read aligners such as Bowtie or BWA (Langmead and Salzberg, 2012). Unfortunately, these are reliant on the availability of pre-existing genomic or transcriptomic resources for the organisms being analysed. However, even if the reference is divergent, referenced assembly may still produce a higher quality assembly than *de novo* methods alone (Vijay et al., 2013). For referenced transcriptome assembly there are multiple tools that conduct post-processing of mapping data to account for features of transcripts such as alternative isoforms and spliced out intronic sequences (Kim et al., 2013).

On the other hand, *de novo* assembly algorithms don't require a prior reference sequence and form two main groups: Overlap-Layout-Consensus (OLC) methods and de Bruijn graph (DBG) methods.

OLC are conceptually relatively simple, a graph is constructed based on the overlap of sequencing reads determined using standard pair-wise alignment algorithms. Each graph vertex represents a read and an edge connecting a pair of vertices indicates overlap between those reads. Therefore, assembly is a process of finding a hamiltonian path (HPP) (i.e. the path that visits each vertex exactly once) across the OLC graph. Unfortunately, calculating overlaps is computationally demanding and hamiltonian paths are difficult to discover (NP-complete) (Karp, 1972).

Alternatively, de Bruijn graph assembly involves the decomposition of reads into  $k$ -mer sets. These  $k$ -mers are then assembled into graphs based on  $k - 1$  overlaps with other  $k$ -mers. However, in DBGs the vertices represent the  $k - 1$  overlaps with the  $k$ -mers themselves forming the edges. This simplifies the problem of assembling contigs from finding hamiltonian cycles in the graph ( $NP$ -complete complexity) to that of the eulerian cycle (i.e. visit each edge once) problem (EPP) ( $P$  complexity). This is a computationally much simpler problem as it has far fewer degrees of freedom. This is because all edges (and therefore all non-disjoint vertices) must be visited whereas in the HPP not all edges will necessarily be used. Furthermore, the existence of solution to the EPP within a graph (or subgraph) can be easily determined by observing whether that a) the graph is connected and b) the order of only  $\leq 2$  vertices is odd (i.e. an even number of edges connected to them). This reduced computational complexity therefore allows assembly of much larger datasets (Compeau et al., 2011).

Generally OLC assembly is limited by a requirement for fast and accurate overlap calculations and alignment whereas the de Bruijn approaches requires robust error correction (Palmer et al., 2010). As de Bruijn graph generation relies on exact  $k - 1$  matches errors exponentially increase the number of possible graph traversal paths. More traversal paths means greater graph complexity and thus increased risk of error as well as higher computational demands (Pop, 2009). Some modern assemblers utilise paired-end read information directly in the generation of these graphs (Bankevich et al., 2012), however, most assemblers just use this information to post-process assembled contigs using various heuristic methods.

No one assembler will produce the optimal assembly for every dataset, indeed often the best assemblies are generated by combining multiple assemblies. Therefore, for all genomic and transcriptomic analyses in this thesis I will use multiple assembly approaches and implementations.

#### 2.2.1.5 THE PROBLEM WITH PLOIDY

One important complication in the assembly of eukaryotic genomes relative to bacterial or archaeal sources is the issue of highly heterozygous polyploid genomes. This is problematic as the de Bruijn graphs constructed during assembly rapidly increase in complexity when reads from heterozygous samples become incorporated (Kajitani et al., 2014a). This is because  $k$ -mers derived from heterozygous regions of homologous chromosomes will partition the assembly graph into bubbles that cannot be easily or accurately resolved by most assemblers expecting only limited

structural variation during assembly. Previously, attempts to work around this included inbreeding to generate homozygous lines or fosmid based approaches.

Specialised genome assemblers have been produced to address this problem e.g. Platanus. By using a range of approaches such as  $k$ -mer autoextension, merging haplotypes at both contig assembly and scaffolding steps, and incorporation of various heuristics in bubble resolution these assemblers have performed reasonable well on both highly and lowly heterozygous genomes (Bradnam et al., 2013; Kajitani et al., 2014b).

Likewise, transcriptome assembly complexity rapidly increases with the number of alleles expected per gene, ploidy, heterozygosity, presence of complex gene families and alternative splicing. This is particularly problematic in the *PbMr* system owing to the massive ploidy of the host *Paramecium bursaria* and the numerous whole genome duplications in its relatively recent evolutionary history (McGrath et al., 2014). This also explains the difficulties in using sister species, as the most sequenced *Paramecium* genus species are the aurelia complex which have undergone 2 WGD since divergence with *P. bursaria* (McGrath et al., 2014).

### 2.3 MACHINE LEARNING AND STATISTICAL PATTERN RECOGNITION

Machine learning is a field of computer science devoted to the challenge of developing and applying algorithms capable of automatically inferring and utilising patterns in data (Murphy, 2012). A commonly used formal definition of machine learning: “A computer is said to learn from experience  $e$  with respect to some class of tasks  $t$  and performance measure  $p$ , if its performance at tasks  $t$ , as measured by  $p$ , improves with experience  $e$ .” (Mitchell, 1997). Machine learning encompasses techniques and methods from various areas including statistics, optimisation/control engineering, neuroscience and artificial intelligence. Applications range in complexity from simple linear regression to deep convolutional neural networks (CNN) with millions of free parameters running on dedicated super-computers (Wu et al., 2015) which are capable of beating human-performance on complex image classification tasks e.g. IMAGENET (Russakovsky et al., 2014; He et al., 2015).

Typically, we seek to set the parameters ( $\theta$ ) of a function in such a way that another property is minimised. For example, in linear regression the aim is to find parameters of a straight line  $h_{\theta}(x) = \vec{\theta} \vec{x}$  (assuming  $x_0 = 1$  and  $\theta_0$  is the intercept) which minimise the distance between the

line and the dependent variable ( $y$ ). In this case  $x$  and  $y$  are most commonly vectors, however, more complicated regression tasks will involve higher order tensors. The distance/error is calculated using something known as the cost function e.g. for the sum of squares distance measure:

$$J(\theta) = \frac{1}{2}m \sum_{i=1}^m (h_{\theta}(x_i) - y_i)^2$$

(where  $m$  is the number of  $x, y$  pairs in the dataset for linear regression). Most algorithms will seek to minimise the value of this cost function  $J(\theta)$  with respect to the parameters of the original function  $h_{\theta}(x)$ . Typically, this is achieved using a variety of algorithmic optimisation techniques. The most prevalent of these are gradient descent based methods in which the value of  $\theta$  is modified in the direction of the gradient of the cost function (determined using the partial derivative of  $J$  with respect to  $\theta$ :  $\frac{\partial J}{\partial \theta}$ ).

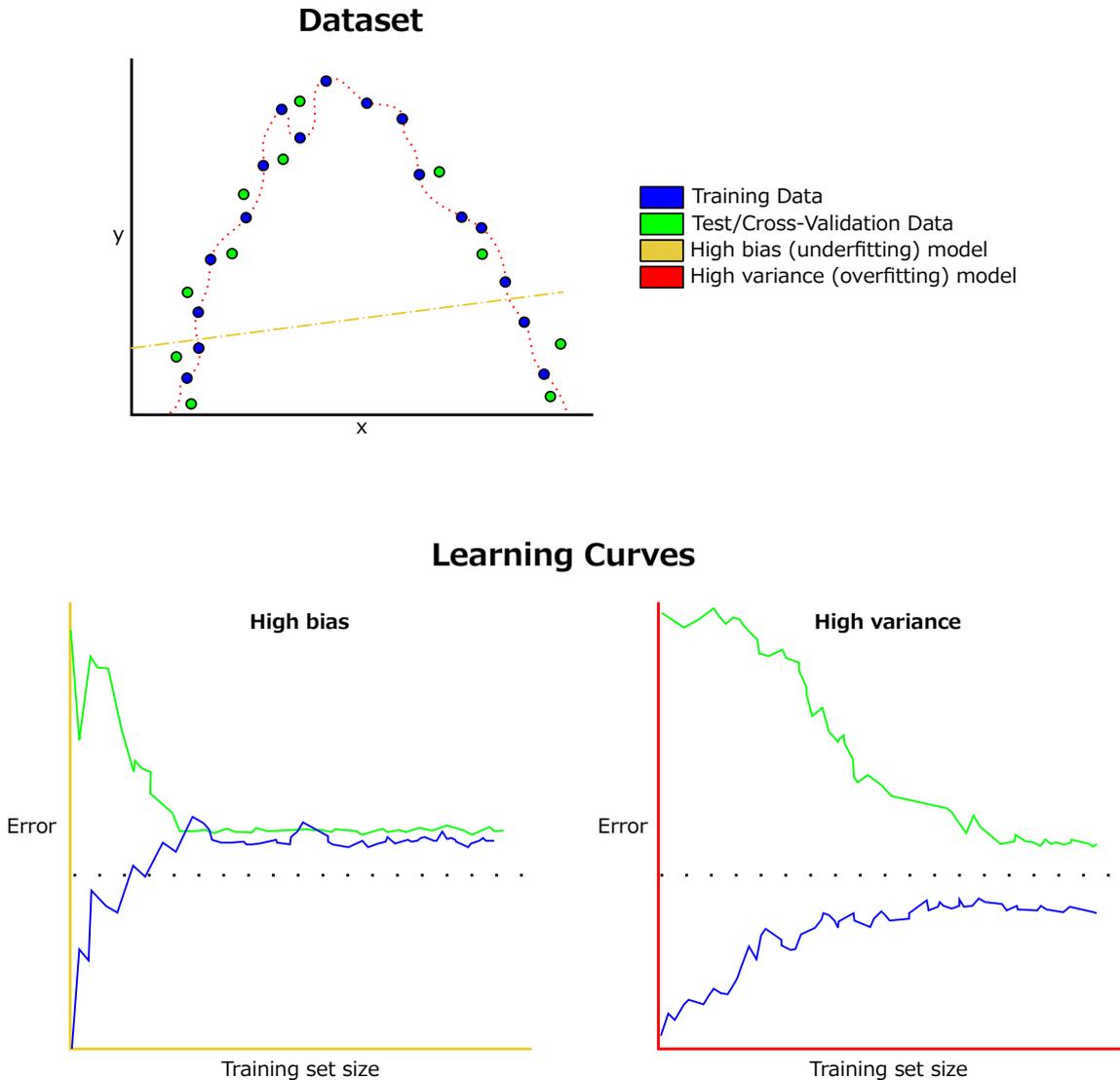
In an ideal world, the best machine learning model trained using our data will generalise well for novel data generated from the same underlying process which generated the training data. This is known as generalisability and it plays into the concept of ‘fit’. A model that minimises its particular cost function on the training dataset has been fitted to that dataset, however, it is possible for the model to fit the training data in such a way that it has low error on the training data but performs incredibly poorly when applied to new data from the same process. This is typically the case when a model has overfit the data.

The classic example of this is fitting a line to a set of points using a high degree polynomial (fig. 2.3.1). This polynomial will perfectly pass through all the points but is likely to be a worse predictor for the value of some new data than a much simpler model which might appear to have a worse fit to the original training data. Likewise, a model that is misspecified or cannot fit the training data well e.g. the training data follows a non-linear distribution but the model is linear, is known as underfitted. Underfitted models will perform poorly on both the training and test data.

As constantly assessing generalisability using the test data essentially makes this part of the training data (and thus will lead to overfitting) almost all machine learning analyses use the principal of cross-validation. Cross-validation is the partitioning of the training dataset to create a validation dataset which can be used as a proxy test set.

No single model will perform best for all tasks (to paraphrase and simplify Wolpert and McCreedy’s “No Free Lunch Theorem” (Wolpert, 1996)), there are no shortcuts in machine learning

(and many other areas) or optimisation. Therefore, testing different models (and hyperparameter values) using cross-validation is key to generating a useful model. Another important way to prevent overfitting is to introduce regularisation in the cost function i.e. a term which penalises higher model complexity.



**Figure 2.3.1:** Plot showing a high bias (underfit) model in yellow and a high variance (overfit) model in red. Below are learning curves corresponding to each of these respectively. Learning curves show the effect of different training set sizes on the training and test error of misspecified models. Overfitted models show a large gap between test and training errors, they fit to the training data well but don't generalise to new data (i.e. test data). Underfitted models show a very high training error and little difference between test and training data as the model is too simple to fit the training data at all.

Machine learning is typically divided into 2 main subsets depending on the nature of the dataset involved: supervised learning (e.g. classification and regression) and unsupervised learning (e.g. clustering, density estimation and dimensionality reduction). There are also approaches that blend features of both supervised and unsupervised learning known as semi-supervised learn-

ing as well as an alternative idea known as reinforcement learning built on the premise of the psychology of behaviour and the indirect reward of trial and error approaches (Bishop, 2006).

### 2.3.1 SUPERVISED LEARNING

In supervised (also referred to as predictive) learning the principal aim is to learn a mapping between inputs/features  $x$  and outputs/response  $y$  from a set of inputs and their corresponding expected output. This is known as the training set i.e.  $\mathcal{D} = (x_i, y_i) \quad \forall i \in n$  where  $n$  is the cardinality (size) of the training set (Murphy, 2012). A supervised learning algorithm thus seeks to approximate  $y = f(x)$  where  $f$  is an unknown function. This estimated function  $\hat{y} = \hat{f}(x)$  would then generally be applied to new data known as the test data for which the expected outputs are not known (i.e.  $(x_i, y_i) \notin \mathcal{D}$ ). In other words,  $i$  samples comprised of  $j$  dimensions each can be arranged as an  $i \times j$  matrix  $\mathbf{X}$  and the outputs to a vector  $\vec{y}$  of length  $i$ . Therefore supervised learning can be formulated as a means of identifying a mapping ( $\hat{f}$ ) between  $\mathbf{X}$  and  $\vec{y}$ :

$$\begin{bmatrix} x_{0,0} & \cdots & x_{0,j} \\ \vdots & \ddots & \vdots \\ x_{i,0} & \cdots & x_{i,j} \end{bmatrix} \xrightarrow{\hat{f}} \begin{bmatrix} y_0 \\ \vdots \\ y_i \end{bmatrix}$$

Supervised learning is further subdivided into two approaches depending on the nature of the expected outputs: classification and regression.<sup>4</sup>

In regression the desired outputs are real-valued (or ordinal) i.e.  $y_i \in \mathbb{R}$  and we seek to estimate a particular output quantity for a specific input. The simplest example of this would be the 2-dimensional linear regression problem mentioned above in which we are determining the parameters of a line (gradient/weight and intercept/bias) which best fits the training dataset ( $\mathcal{D}$ ) composed of pairs of  $x$  and  $y$  values. Once this line has been found we can use it to predict the value of  $\hat{y}_i$  for data in the test set ( $x_i \notin \mathcal{D}$ ).

On the other hand, in classification the expected outputs are categorical or nominal variables such as class labels like “host” and “endosymbiont” ( $y_i \in \text{host, endosymbiont, } \dots, C$ ). These classifications can be binary (two possible outputs i.e.  $y = \{0, 1\}$ ), multiclass ( $|y| > 2$ ), or multilabel (similar to multiclass but outputs aren’t mutually exclusive, i.e. an input have multiple

<sup>4</sup>It is worth noting that the somewhat confusingly named “logistic regression” is typically a form of classification.

labels ) (Murphy, 2012).

Supervised learning algorithms can also be either probabilistic or non-probabilistic and generative or discriminative. Probabilistic functions will return a probability distribution associated with possible class labels or regression values whereas non-probabilistic approaches will only return the most likely class label or value. Generative algorithms, such as Naive Bayes or Restricted Boltzmann Machines (RBMs), seek to model the process by which the output data was generated from the input i.e. learn the joint probability  $p(x, y)$  and make predictions on that basis via Bayes Theorem:

$$\begin{aligned} p(x, y) &= p(y|x)p(x) \\ p(x, y) &= p(x|y)p(y) \\ p(y|x)p(x) &= p(x|y)p(y) \\ p(y|x) &= \frac{p(x|y)p(y)}{p(x)} \end{aligned} \tag{2.1}$$

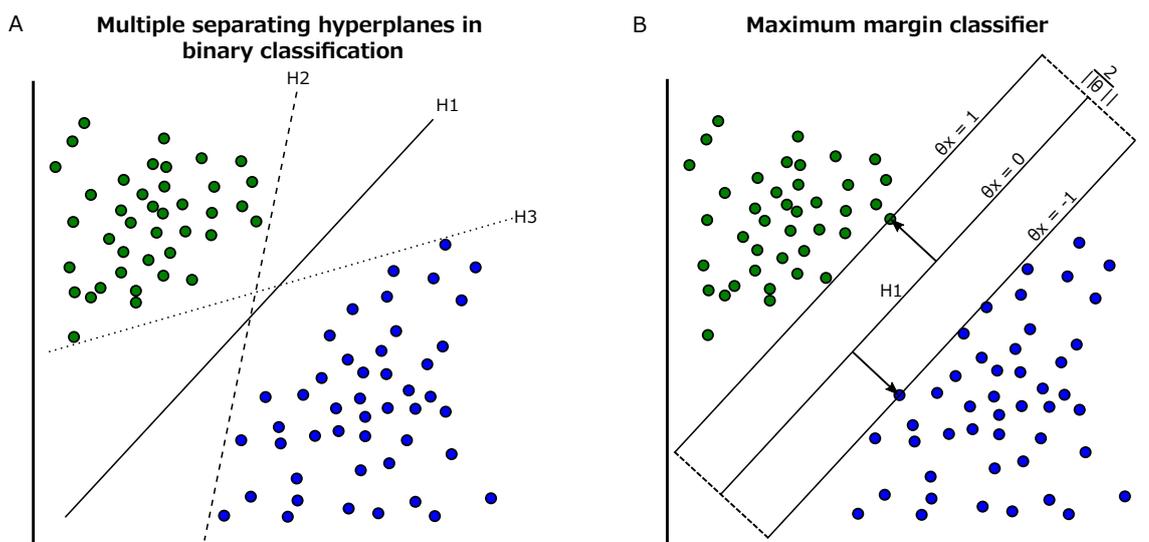
In other words, for classification problems a generative model would determine the statistical distribution of individual classes whereas discriminative models (such as logistic regression/linear classifiers) would just determine the boundaries between them. Generative models often perform better on small training sets by preventing overfitting whereas discriminative classifiers perform better as the training set grows (Ng and Jordan, 2002).

### 2.3.1.1 SUPPORT VECTOR MACHINES

Support Vector Machines (SVMs) are a type of sparse kernel maximum-margin supervised classification algorithm. With the innovation of the kernel trick in 1992 (Boser et al., 1992) and soft-margins in 1993 (not published until Cortes and Vapnik (1995)) SVMs have been among the most successfully applied classification algorithms (Fernández-Delgado et al., 2014). Only relatively recently have they begun to lose ground to the deep learning methods such as deep convolutional neural networks e.g. LeNet (LeCun et al., 1998) exemplified by the defeat of SVMs by the LeNet on the MNIST digit recognition dataset (Hinton and Salakhutdinov, 2006; Bengio et al., 2007, 2013).

The goal of SVMs is to learn a hyperplane which separates two sets of labels in the dataset. Note, for multiclass classification, a series of one-vs-all classifiers are typically trained (that is for

$k$  classes,  $k$  SVMs are trained each classifying between label  $k$  and all other labels). However, not all possible hyperplanes that could separate the labels will necessarily generalise well to novel data (and this generalisation is the ultimate goal of supervised learning). Therefore, it is necessary to determine a way to select the hyperplane which should generalise best and to do this in a manner that will be relatively efficient especially with high dimension datasets. This optimal hyperplane for separable classes can be demonstrated to be the hyperplane which maximises the margin between the two classes (Vapnik and Kotz, 1982). In other words, the optimal boundary is the one that has the largest possible distance from each class (while still separating them). Conceptually, the positioning of this boundary is only dependent on the relatively small subset of the training data  $\mathcal{D}$  that is near the boundary and it would be inefficient to consider all points when placing the decision boundary. For this reason, SVMs can define the decision boundary in terms of the namesake support vectors and can reformulate their cost function in a more efficient constrained way.



**Figure 2.3.2:** A: Demonstration of 3 valid decision boundaries in a 2D classification problem, B: The optimal boundary (H1) is that which maximises the separation of different classes. This optimal boundary can be defined in terms of support vectors. The bias/intercept has been folded into  $\theta$  directly.

A naive formulation of this problem is simple specifically we are trying to find a linear model  $f(x) = \theta_0 + \theta^T x$  which can be simplified to  $f(x) = \theta x$  if we assume that the first element of  $x$  is fixed to 1. We thus want to minimise  $J$  in terms of  $\theta$  to find the largest margin that correctly labels all the training data (in other words is constrained). Fortunately, due to geometry the margin is property of the norm of  $\theta$  i.e.  $\|\theta\|$  but we use  $\frac{1}{2}\|\theta\|^2$  for mathematical convenience. Therefore:

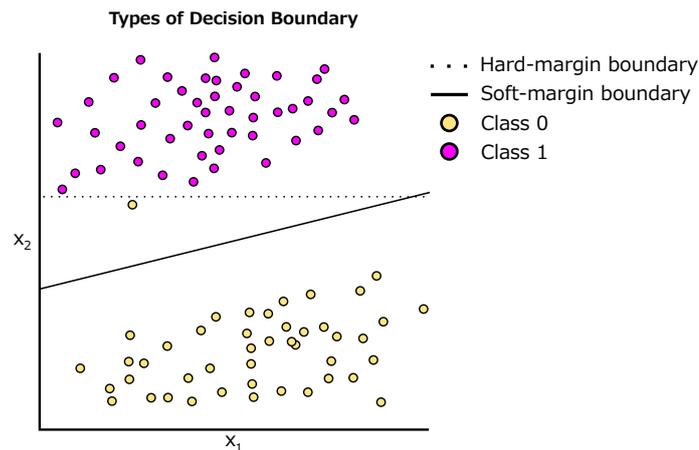
$$\operatorname{argmin}_{\theta} J(\theta) = \frac{1}{2} \|\theta\|^2 \quad \text{s.t.} \quad y_i(\theta x_i) \geq 1 \quad \forall i$$

In reality, this cost function would be converted to a constrained optimisation problem using Lagrange multipliers and reformulated using the Lagrangian dual form.

The 2nd major enhancement of SVMs is that of soft-margins (Cortes and Vapnik, 1995). Soft-margins are a way of allowing a degree of misclassification if doing so would increase the size of the margin that can be generated. Specifically, a user defined penalty constant  $c$  is specified and added to the cost function penalising the degree of misclassification  $\xi$ , e.g.:

$$\operatorname{argmin}_{\theta} J(\theta) = \frac{1}{2} \|\theta\|^2 + c \sum_{i=1}^n \xi_i \quad \text{s.t.} \quad y_i(\theta x_i) \geq 1 - \xi_i \quad \forall i$$

This can improve robustness to outlier data and generally improve generalisability by keeping the margin as large as possible.



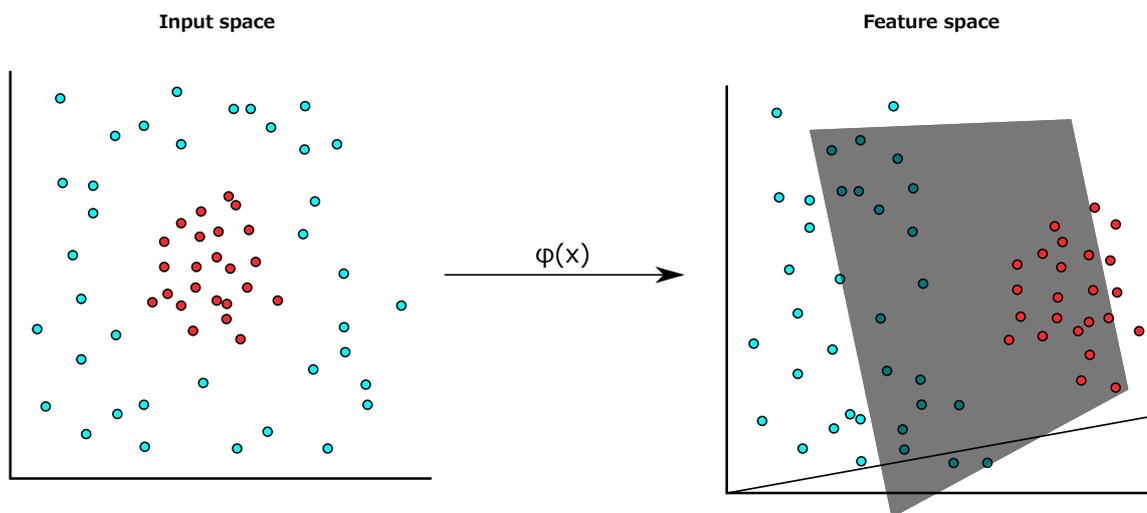
**Figure 2.3.3:** Demonstration of the utility of a soft-decision boundary to improve the overall fit of a decision boundary by allowing a degree of misclassification during training. The dotted line represents a hard-margin classifier which must have a much smaller margin to correctly classify the class 0 outlier. On the otherhand by accepting this misclassification the soft-margin boundary allows a much wider margin.

Finally, the 3rd major advantage of SVMs is that despite nominally being linear classifiers they can effectively classify data which is not linearly separable in the input dimensions using the kernel trick. Conceptually, a kernel function is used to transform data from the input dimensions to a higher dimensional space in which the data is linearly separable. These transformed feature spaces can have incredibly high number of dimensions (in the case of popular kernels like radial basis function, an infinite number of dimensions). Explicitly transforming data in this way would

be computationally intensive so instead the “kernel trick” is used, where instead of explicitly transforming all the data into the feature space it is done implicitly by computing the inner product of all pairs of data points (fig. 2.3.4). This is a lot more efficient and precludes the computationally intensive step of converting the data into the new, potentially infinite, co-ordinate space. Radial basis function (RBF) is an example of a commonly used kernel ( $\sigma$  forming a hyperparameter):

$$K(x_i, x_j) = \exp\left(-\frac{\|x_i - x_j\|^2}{2\sigma^2}\right)$$

Even with the kernel trick, operations on every pair of points can become infeasible for large datasets due to the combinatorial explosion in necessary operations as the dataset increases in size. However, in the same way that the decision boundary parameters are determined using only a subset of the training data (i.e. the support vectors) the kernel trick only needs evaluated on a subset of points near the decision boundary. This is the reason SVMs are sometimes referred to as sparse kernel methods.



**Figure 2.3.4:** A kernel transform can allow SVM to produce non-linear classification boundaries by mapping the data to a higher dimensional space in which they are linearly separable. This is known as the kernel trick and the key to its efficiency in SVM is that it is only evaluated for those sets of points near the decision boundary.

The advantages of SVM is that they are somewhat resistant to the curse of dimensionality i.e. they are effective with large numbers of features even if the number of features is greater than the size of the training set. By using support vectors, the kernel trick, and Lagrange bound optimisation they are relatively fast and memory efficient to train and as classification only depends on the location of the decision boundary very fast to test. Additionally, in simple form, finding the

hyperplane of an SVM is a true convex optimisation therefore is guaranteed to always find the global optimum (this guarantee does break with more complex kernels and soft-margins). The major disadvantage is not natively generating probabilistic output (i.e. attaching a probability to a certain classification). However, this can be achieved using methods like Platt Scaling or the related Relevance Vector Machine (RVM) algorithm. The other disadvantage is that hyper-parameters such as the misclassification penalty for soft-margins ( $c$ ) and kernel choice (and its parameters) need chosen, typically this is solved by training using a grid-search of permutations of these parameter settings and selecting the best model via cross-validation. However, there is both theoretical and empirical evidence that either random search ([Bergstra and Bengio, 2012](#)) or Bayesian optimisation ([Eggenberger et al., 2013](#)) are more efficient means of selecting hyper-parameter values.

### 2.3.2 UNSUPERVISED LEARNING

The other main form of machine learning is that of unsupervised or descriptive learning. In which the training dataset has no provided output labels i.e.  $\mathcal{D} = \{x_i \mid \forall i \in n\}$  and  $y \notin \mathcal{D}$  (where again  $n$  is the cardinality of this training dataset). In other words, we just have our dataset and have no additional information. This is slightly more difficult problem as it lacks an obvious error metric like supervised learning (i.e. difference between actual output and expected output) but is important and useful tool to try to discover patterns in datasets.

There are two major groups of unsupervised learning algorithms, the first of which is clustering algorithms such as  $k$ -means that seeks to partition a dataset into a set of groups (see [fig. 2.3.5](#) for more details). The other major group of unsupervised algorithms are those used for visualisation and/or dimensionality reduction. Dimensionality reduction is a way of projecting a multi-dimensional dataset into a lower number of dimensions in a way that still corresponds to “shape” of the data in the original number of dimensions.

Formally, dimensionality reduction seeks to take a set of data ( $\mathcal{D}$ ) and convert it to a lower dimension form  $\mathcal{Y}$  known as a map  $\mathcal{Y} = \{y_i \mid \forall i \in n\}$  with each individual  $x_i$  in  $\mathcal{D}$  represented by a corresponding map point  $y_i$ . It also seeks to do this in a way that maintains as much of the structure found in the original data as is possible ([Maaten and Hinton, 2008](#)). Therefore, if two data points are similar in the original dimensions they should still be similar in the map  $\mathcal{Y}$  (and the inverse). Some dimensionality reduction approaches are well known in biology, specifically: prin-

principal component analysis (PCA) (Hotelling, 1933) and multidimensional scaling (MDS) (Torgerson, 1952) which both aim to identify hidden features within the dataset that can explain a high degree of the variation.

As ever different methodologies have a range of pros and cons, with some better at preserving global structure (e.g. isomap) and others local data structures (e.g. local linear embedding) and so on. One of the most recent innovations in this area is that of  $t$ -distributed stochastic neighbour-embedding (t-SNE) in which the similarity of data points in the input space is modelled as pairwise probabilities using Gaussian distributions. These probabilities are then translated into positions in the map  $\mathcal{Y}$  and similarities re-calculated using Student's  $t$ -distributions. The position and variance of these points and distributions respectively is then optimised by minimising the difference between the similarity probabilities in the input space and on the map as assessed by metrics such as Kullback-Leibler (KL) divergence (Maaten and Hinton, 2008).

### 2.3.2.1 K-MEANS

$k$ -means clustering is a non-probabilistic unsupervised learning method in which we seek to partition data points in multidimensional space into  $k$  clusters. It is often used to initialise Gaussian mixture models (GMMs).

Specifically, given a set of  $n$  observations  $x = \{x_1, \dots, x_n\}$  partition each point ( $x_n$ ) into  $k$  clusters, where typically  $x$  is a large matrix.

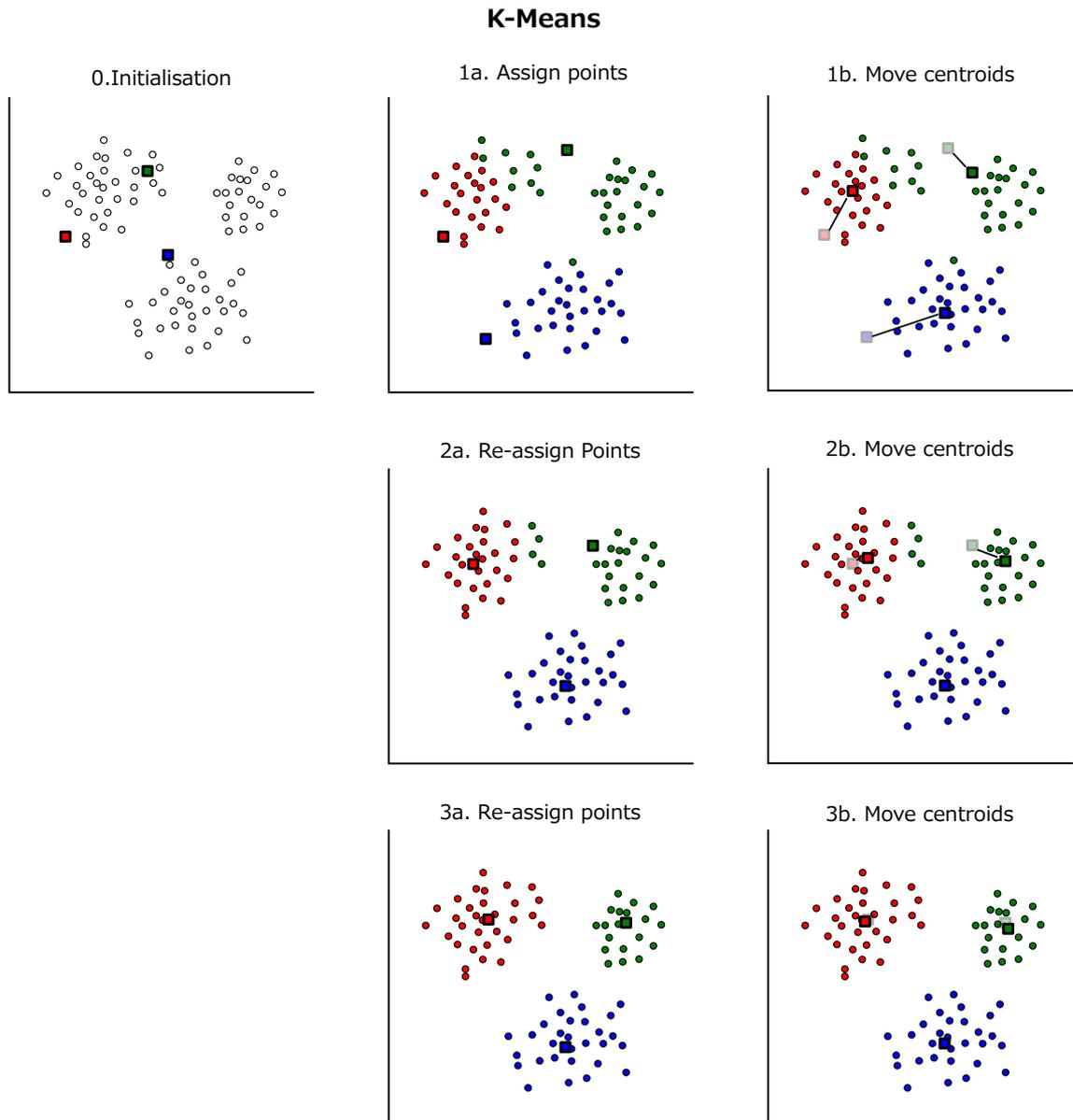
A cluster can be intuitively considered as a group of observations/points which are “closer” to one another than to other observations and the  $k$ th cluster can be defined by its centroid. So, with  $k$ -means clustering we actually seek the set of  $k$  cluster centroids  $\mu$  which minimise the sum of squares distances of each data point from its closest cluster centroid (Bishop, 2006).

If we define a 1-of- $k$  coding scheme with  $r_{nk} \in \{0, 1\}$  as a binary variable that is 1 when  $x_n$  has been assigned to cluster  $k$  (with centroid  $\mu_k$ ) and 0 otherwise then we can define an objective cost function ( $J$ ) that represents the sum of squares distances of each data point  $x_n$  from its assigned cluster centroid  $\mu_k$ .

$$J = \sum_{i=1}^n \sum_{j=1}^k r_{ij} \|x_i - \mu_j\|^2 \quad (2.2)$$

Therefore, the goal of  $k$ -means clustering is to find values for  $r_{nk}$  and  $\mu_k$  that minimise this

linear function eq. (2.2). (Bishop, 2006)



**Figure 2.3.5:** Demonstration of the  $k$ -means algorithm applying 3 rounds of Expectation-Maximisation (EM) to cluster a group of 2 dimensional samples. The 3 cluster centroids (represented by coloured rectangles) are randomly initialised in 0 before undergoing 3 rounds of EM. This involves the successive assignment of samples to their nearest centroids (1a, 2a, 3a) and then the movement of the centroids to the center of the points currently assigned to that centroid (1b, 2b, 3b). Assignment of a given sample to a centroid is indicated by a shared colouring and centroid relocation by an arrow with a faded version showing the initial location.

The standard algorithm proceeds in two alternating steps following the initialisation of  $\mu$  with starting cluster centroid locations (Forgy, 1965; Lloyd, 1982):

1.  $\operatorname{argmin}_{r_{nk}} J$  i.e. minimise eq. (2.2) w.r.t the assignment of points to clusters while keeping the cluster centroids fixed.

2.  $\operatorname{argmin}_{\mu} J$  i.e. i.e. minimise eq. (2.2) w.r.t the position of the cluster centroids while keeping the assignment of points to centroids fixed.

Step 1 roughly corresponds to the expectation step in the expectation-maximisation (EM) algorithm and is trivially achieved by assigning each point to the cluster represented by the nearest centroid or formally:

$$r_{nk} = \begin{cases} 1, & \text{if } k = \operatorname{argmin}_j \|x_n - \mu_j\|^2 \\ 0, & \text{otherwise} \end{cases}$$

Step 2 roughly corresponds to the maximisation step in EM is can be determined by taking the partial derivative of  $J$  w.r.t  $\mu$  setting it to 0 and solving for  $\mu$ :

$$\begin{aligned} \frac{\partial J}{\partial \mu_k} &= 2 \sum_{i=1}^n r_{ik} (x_i - \mu_k) \\ 0 &= 2 \sum_{i=1}^n r_{ik} (x_i - \mu_k) \\ \mu_k &= \frac{\sum_{i=1}^n r_{ik} x_i}{\sum_i r_{ik}} \end{aligned} \tag{2.3}$$

In other words set  $\mu_k$  to the mean of all data points  $x_n$  assigned to cluster  $k$  (thus  $k$ -means) (Bishop, 2006)

These two steps are repeated until a specified maximum number of iterations are reached or no points change cluster assignment during step 1.

$k$ -means has many modifications and improvements such as refining the initialisation of the clusters by the Bradley-Fayyad method (clustering random samples of the dataset and then  $k$ -means clustering the resulting clusters) (Bradley and Bradley, 1998) or over-clustering (running more than  $k$ -means clustering with more than the specified number of clusters and merging clusters at the end to generate the correct number of clusters). One of the most recent and promising improvements is that of “ying-yang”  $k$ -means clustering which gains a moderate speed-up over the conventional algorithm by minimising the number of distance calculation required. This is achieved by creating upper and lower bound distance filters using the triangle inequality (i.e.  $d(a, b) \leq d(a, c) + d(b, c)$  where  $d$  is a function that calculates the distance between 2 points) (Ding et al., 2015).

An efficient implementation of the  $k$ -means algorithm is available in the MLPACK C++ Ma-

chine Learning library (Curtin et al., 2013). While very efficient and effective,  $k$ -means has some limitations, it requires a user specified number of clusters and therefore diagnostics to check for obvious misspecification in the number clusters. Information criterion can be used to determine the optimal number of clusters. Additionally, it is not guaranteed to discover the global optimal clusters (can converge to local optima). This can be amortised by running multiple times with different initialisations.

## 2.4 PHYLOGENETICS

Phylogenetics is an effective tool (if there is sufficient signal/resolution) to investigate the evolutionary ancestry of biological sequence data. It can be used to identify how closely related a given pair of sequences are, as well as indicate what the sequence most likely looked like in a shared common ancestor (ancestral node reconstruction). Phylogenetic methods also allow estimation of evolutionary processes such as selection pressure, migration, genome reduction, and horizontal gene transfer. In the context of endosymbiosis, phylogenetics can be used to determine evolutionary ancestry of the genes recovered in a transcriptome and to aid identification of the likely origin (host, endosymbiont, contaminant) of these transcripts. Additionally, it can pinpoint potential horizontal gene transfer events between host and endosymbiont by searching for single gene/transcript phylogenies that have an incongruent branching pattern compared to established species trees. Finally, it can be used to aid identification of the putative function of novel transcripts by comparison to other transcripts of known function from databases such as genbank.

Phylogenetics can be defined as a means of arranging a set of character sequences into an optimal hierarchical branching tree structure reflecting some measure of relatedness between the sequences. Usually, these trees will have variable branch lengths that are product of a measure of divergence between the connected nodes.

Typically, these sequences take the form of protein or DNA sequences<sup>5</sup> and the measure of relatedness is some proxy for evolutionary distance ranging from simple distance measures e.g. the Hamming distance  $d$ , which can be defined for two sequences (in the form of two vectors  $\vec{x}$

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<sup>5</sup>Strictly phylogenetics refers to the study of molecular sequence data although the same methods are applicable to non-molecular characters such as morphological traits (and occasionally originated in this domain) as well as any other set of discrete data vectors. It has even been applied to fields such as linguistics (Atkinson and Gray, 2005).

and  $\vec{y}$  of length  $k$ ) as the sum of the number of positions the sequences differ:

$$d = \sum_{i=0}^k (1 - \delta_{x_i, y_i})$$

Note that  $\delta$  is the Kronecker delta function:

$$\delta_{ij} = \begin{cases} 1, & i = j \\ 0, & i \neq j \end{cases}$$

to more complicated probabilistic estimations based on observed data. A character is an element of a sequence such as an individual base or amino acid, homologous characters are those in separate sequences that are descended from a common ancestor. As they were the first molecular sequences easily available much of the early work in molecular phylogenetics was conducted using protein sequences e.g. (Eck and Dayhoff, 1966; Fitch and Magoliash, 1967).

This phylogenetic estimation can be a non-trivial process (especially with more complex measures of relatedness) as the number of possible trees rapidly increases with the number of sequences. Specifically, for rooted and unrooted trees the number of possible trees  $n$  increases by the number of taxa  $t$  as follows:

$$\text{For rooted phylogenies : } n = \prod_{i=2}^t (3i - 3)$$

$$\text{For unrooted phylogenies : } n = \prod_{i=2}^{t-1} (3i - 3)$$

However, the key stages in a phylogenetic analysis are that of sequence sampling (selection of sequences for inclusion in the analysis), alignment (in which homologous sites in the sampled sequences are aligned with one another), masking (in which sites which are evolutionarily informative – can be determined to be homologous but also non-invariant are selected), model selection (in which the best fitting evolutionary model is selected or calculated) and finally, phylogenetic reconstruction (in which the tree is generated that minimises some measure e.g. most likely tree for probabilistic models or most parsimonious).

One implication of most current phylogenetic methods is that they implicitly assume a branch-

ing tree structure is the best representative of the evolutionary process that is being modelled. However, as the discovered prevalence of horizontal gene transfer has increased it is becoming clear that in some cases a network like structure may in fact be more appropriate.

Most analyses in this thesis are conducted using amino acid sequences. DNA is more likely to display a compositional bias and independence of sites is often severely violated due to the structure of codons. Amino acids have more states so are less susceptible to back mutations than DNA and are easier to align.

#### 2.4.1 SEQUENCE SAMPLING

Sequence sampling, the selection and identification of sequences for initial inclusion in a phylogenetic analysis, is arguably the most important stage in phylogenetic analysis. Any biases introduced here will propagate throughout the rest of the analysis. While some biases can be mitigated to lesser and greater extents by careful application of various methods in the following stages, there is a degree of fundamental truth in the statement “garbage in - garbage out”.

The aim of proper taxon sampling is to maximise phylogenetic accuracy and to allow testing of specific hypotheses. Phylogenetic accuracy is usually considered in terms of consistency (as data increases the analysis tends towards the correct tree), efficiency (how quickly does this convergence occur), and robustness (how sensitive is the phylogeny to violation of assumptions in reconstruction) (Nabhan and Sarkar, 2012). Typically, sequence sampling will be conducted from the basis of a single seed sequence which will be used to query existing databases using alignment tools such as BLAST and HMMs to attempt to discover potentially homologous sequences from different organisms.

The main issues caused by poor taxonomic sampling in molecular phylogenetics are that of conflicting phylogenetic signals, inadequate rate of evolution to resolve relationships of interest, and violations of assumptions e.g. expectation of a uniform distribution of traits (Nabhan and Sarkar, 2012).

Generally, increased taxon sampling has a strong positive effect on phylogenetic accuracy (Zwickl and Hillis, 2002). However, it can also lead to a situation where there are too many sequences to efficiently reconstruct a phylogeny. Care must also be taken not to unintentionally bias datasets by removing any sequences that are considered “problematic” especially when conflicting phylogenetic signal or model violations can be biologically informative. Therefore, it is

usually necessary to include borderline error-generating sequences within a phylogeny initially and to iteratively remove them before repeating the phylogenetic inference. Unfortunately, the reduction of the input sequences to a representative subset by heuristics and/or naive clustering can generate biases of their own. However, tools exist that utilise taxonomic database information to automatically find a set of sequences of a specified cardinality/size which displays the maximum possible taxonomic diversity for that set size (Zhou et al., 2014).

Another source of bias in sequence sampling is the usually heuristic choice of outgroup taxa. Most contemporary models of phylogenetic inference only infer unrooted trees. Therefore, it is common practice to “root a tree” by selecting a set of sequences from known evolutionarily distance organisms to form an outgroup. If this outgroup is correctly recovered (monophyletically) the root can be placed between it and the other sequences in the phylogeny (Yang and Rannala, 2012). However, choice of outgroup can change implications which may be drawn from a phylogeny regardless of methodology used to infer it (Milinkovitch et al., 1996). Care must be taken to ensure the selected outgroup doesn’t actively distort the accuracy of inference of the rest of the phylogeny regardless of the issue of root placement (Milinkovitch and Lyons-Weiler, 1998).

#### 2.4.2 MULTIPLE SEQUENCE ALIGNMENT

The goal of multiple sequence alignment (MSA) is to align sets sequences such that evolutionarily homologous residues occupy the same column. In other words, any given column in the alignment theoretically should contain amino acid or nucleotide residues that derive from the same common ancestor and have evolved in each sequence lineage. It is also possible that insertion or deletion events have taken place and a particular residue is absent in the ancestral node or sequence lineage.

This is a non-trivial computational problem which has been proven to have an NP-complete<sup>6</sup> computational complexity (Wang and Jiang, 1994). Specifically, the optimal alignment of  $n$  sequences has a complexity of  $O(l^n)$  for  $n$  sequences of length  $l$  (Sievers et al., 2011).

Due to this complexity, the majority of MSA algorithms implement heuristic approaches in order to get, if not the optimal solution, a sufficiently good one in a reasonable amount of time.

Typically, MSA algorithms start by generating the sets of all pairwise alignments using estab-

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<sup>6</sup>A decision problem for which an answer can be verified in polynomial time by a non-deterministic Turing machine and to and from which any NP-hard problem can be translated (Karp, 1972).

lished pairwise alignment algorithms. Pairwise alignment algorithms are almost all based upon a pair of “Ur-algorithms” with different goals: Needleman-Wunsch, a global alignment algorithms (which attempts to maximise alignment quality over entire sequence lengths) (Needleman and Wunsch, 1970) and Smith-Waterman, a local alignment algorithm (which is optimised towards producing high quality alignments in sub-strings) (Smith and Waterman, 1981). While early MSA algorithms were typically largely derived from Needleman-Wunsch most modern algorithms seek to combine optimisation of local and global alignments. The distances used in these pairwise alignments will typically be “scored” based upon which matches or alignments are more frequent substitutions (e.g. Leucine and its isomer Isoleucine or Adenine to its fellow purine base Guanine (transition)) are positively scored. Alternatively, gaps (extension of a gap is typically less penalised than creating a gap) or unlikely changes (e.g. the transversion of Adenine to Cytosine or Glutamine to Cysteine) are penalised. This will generally be codified in a substitution matrix e.g. the PAM (Dayhoff et al., 1978), BLOSUM (Henikoff and Henikoff, 1992) amino acid matrices and their numerous subsequent derivations and improvements.

The mostly widely heuristic used to go from these series of pair-wise alignments to a useful MSA is that of progressive-alignment (Feng and Doolittle, 1987) (implemented in tools such as CLUSTAL W (Thompson et al., 1994)). This involves building the pairwise alignment scores into a distance matrix which summarises the relative divergence of each pair of sequences. From this matrix a “guide-tree” is generated using simple neighbour-joining methods (in which a tree is built by recursively clustering the least dissimilar sequences (Saitou and Nei, 1987)). Sequences are then progressively aligned using their branching order within this guide-tree (Thompson et al., 1994). This drastically reduces the  $O(l^n)$  complexity to approximately  $O(n^2)$  (Sievers et al., 2011). While there have been various improvements and alternative approaches created such as merging both local and global alignment (Notredame et al., 2000), rapid identification of homologous regions using Fast Fourier Transforms (FFT) (Kato et al., 2002), iterative refinement of alignments (Edgar, 2004b) and use of Hidden-Markov Models (Eddy, 1995).

There have been compelling arguments as early as 1991 that MSA in isolation from phylogenetic inference is inherently flawed as the consideration of evolutionary processes (only really done during phylogenetic inference) is key in the objective weighting and assessment of potential alignments (Thorne et al., 1991). Therefore, the phylogeny and MSA should be jointly inferred

(Thorne et al., 1991; Redelings and Suchard, 2005; Bouchard-Côté and Jordan, 2013). This approach also minimises the risk of conscious or subconscious researcher bias towards alignments and subsequent phylogenies that support their pre-conceived ideas. This approach has been attempted using interesting probabilistic programming approaches i.e. BALI-phy (Suchard and Redelings, 2006). Unfortunately, it is still far too slow a process to infer phylogenies in this manner on large or even moderately sized datasets. This means, that for now, independent MSA estimation is here to stay, at least until computational resources and algorithmic development has continued until these more theoretically satisfying approaches become feasible.

Therefore, throughout this thesis, two progressive/iterative alignment tools will be used: Kalign2 (Lassmann and Sonnhammer, 2005; Lassmann et al., 2009) for high-throughput analyses and iteratively refined MAFFT7 (Katoh et al., 2002, 2005; Katoh and Standley, 2013) for individual accuracy critical phylogenetic analyses. Kalign is a very high-speed and relatively accurate (Thompson et al., 2011) progressive alignment tool that uses an efficient and fast Wu-Manber approximate string-matching algorithm to calculate sequence distances (Lassmann and Sonnhammer, 2005). MAFFT, with iterative refinement, is a relatively slow but highly accurate MSA alignment method (Thompson et al., 2011) that incorporates all pairwise alignment information when refining instead of using heuristics to approximate pairwise sequence differences like most approaches.

#### 2.4.3 MASKING

Unfortunately, MSA is far from perfect, especially with the faster algorithms necessary for larger datasets and higher throughput. Therefore, it is often necessary to trim alignments to manually fix any obviously misaligned residues, and remove any ambiguously aligned or absent sites. This has been demonstrated to improve phylogenetic accuracy (Talavera and Castresana, 2007).

However, manual masking can also be a major source of researcher-bias as well as a painstaking process. For this reason, there are tools that attempt to automate this process. They typically score each column independently with criteria including number of absent character states, how similar/variable the character is and if there are multiple putative alignments - how likely is that column to be found in multiple different MSAs. These criteria can then be used to mask out certain columns based on certain thresholds and trade-offs between the length of the alignment and inclusion of low-scoring columns. TrimAL is an example of a tool that automates the masking process using this sort of methodology (Capella-Gutiérrez et al., 2009).

Similarly to MSA, for high-throughput analyses I will use TrimAL whereas for individual accuracy critical analyses masking will be done manually using the graphical tool Seaview (Gouy et al., 2010).

#### 2.4.4 SUBSTITUTION MODEL SELECTION

While the simplest means of phylogenetic inference - parsimony i.e. finding the tree that requires the fewest sequence changes - does not require any explicit model of sequence evolution (see section 2.4.5), all other means of phylogenetic inference do (Le and Gascuel, 2008).

A substitution model is, in its simplest sense, the same as the PAM and BLOSUM matrices used in pairwise and MSA. They are a means of scoring and weighting the significance of different character changes e.g. is an A to a G a more evolutionarily rare state change for a given dataset than an A to a T.

Substitution models typically assume neutrality, independence and finite sites. With the probability of substitution rates having an independent and identically distributed (i.i.d) (Hasegawa et al., 1985). This measure of distance can be naive models where rates of change between character states and the frequency of each state is equal (i.e.  $p(x \rightarrow y) \quad \forall x \forall y \in G, C, T, A$  where  $x \neq y$ ) (Jukes and Cantor, 1969)) to models fully parameterised in terms of character frequency and rates of change by the masked alignment e.g. the generalised time-reversible (GTR) model (Tavare, 1986). While models like GTR can feasibly be fully parameterised with DNA sequence data due to DNA's relatively few character states it is usually necessary to use empirically-defined models for amino acid datasets. These are substitution matrices that have been determined using the empirically observed substitution rates for various amino acids changes in many large MSAs (Le and Gascuel, 2008).

Unfortunately, a single substitution model will rarely hold true over an entire alignment with the rate of evolution varying both across and within sites (heterogeneity and heterotachy). The frequency of character states also frequently changes across a phylogeny. It is important to control for these phenomena, because, as mentioned earlier, violation of model assumptions can decrease phylogenetic accuracy.

The most frequent violation that is controlled for is allowing the rate of substitution to vary

across sites by using a  $\Gamma$  distribution:

$$\text{var}_{\Gamma} = \frac{\alpha}{\beta^2}$$
$$\mu_{\Gamma} = \frac{\alpha}{\beta}$$

with a given shape  $\alpha$  and trivial scale factor  $\beta$  depending on the dataset to scale rates at each site. For datasets that have a high degree of rate heterogeneity a low valued  $\alpha$  produces a broad distribution of rates, whereas a high value will generate a narrow distribution for datasets with low rate heterogeneity (Yang, 1993). For reasons of computational efficiency  $\Gamma$  is typically approximated as a discrete distribution of 4 to 8 categories of equal probability (Yang, 1994). A more limited version of this is the invariant sites model in which sites are divided into 2 classes, one considered invariable while the other has normal substitution rates applied (Hasegawa et al., 1985).<sup>7</sup>

Unfortunately, these models still assume other model parameters namely the equilibrium frequencies and relative rates are the same across sites (but just scaled). However, some models have been proposed with multiple rate matrices (Lartillot and Philippe, 2004) and state frequencies can be defined at each site (Bruno, 1996) but needs lots of taxa (Lartillot and Philippe, 2004). Another alternative is the CAT model which is a mixture model with  $k$  classes each containing a different state frequency. If  $k = n$  then this is similar to the aforementioned Bruno’s model, however, generally  $k < n$ . A probabilistic process known as a Dirichlet Process Prior (DPP) is used to assign columns to various state frequency classes and simultaneously determines the optimal value of  $k$  during this process (Lartillot and Philippe, 2004). An alternative to this approach is explicitly partitioning a masked alignment and generating a model and state frequencies for each partition, some consider this equivalent to a CAT model depending akin to preferences for fixed-effects vs random-effects models (Yang and Rannala, 2012). However, automated partitioning using a DPP has the advantage of not requiring arbitrary user-defined partitions, which could be a source of bias.

Finally, the rate of evolution can vary even with a site itself (a process known as heterotachy) especially when large numbers of divergent taxa are included in a masked alignment. One model modification which attempts to control for this is that of the concomitantly variable codon or “covarion” model (Penny et al., 2001). It allows sites to switch between “on” and “off” across the tree with the proportion of sites in the relative states determined at each site (Zhou et al., 2010).

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<sup>7</sup>This can also be used with  $\Gamma$  and is approximately equivalent to the addition of another discrete  $\Gamma$  category.

Generally, simpler models such as the “null” parsimony model or basic models that don’t account for complex evolutionary phenomena are more susceptible to artefacts such as long-branch attraction (LBA) (Yang, 1996).<sup>8</sup>

However, in the grand tradition of “no-free lunch”, there is no universally best model for all datasets. Therefore, it is necessary to test multiple competing models using a provided MSA. Typically, these models are then compared for their fit to the observed data using information criterion (Sullivan and Joyce, 2005) such as Akaike’s (AIC) which assess fit while penalising model complexity in a standard regularisation trade-off ( $AIC = 2k - 2\ln(l)$  where  $k$  is the number of parameters and  $l$  the model likelihood (Akaike, 1974)). Other criteria include corrected AIC (Sugiura, 1978), Bayesian Information Criteria (Schwarz, 1978) and Decision Theoretic criteria (Minin et al., 2003) based approaches (Sullivan and Joyce, 2005).

Throughout this thesis, I will use two tools which incorporate these various criteria to infer the best fitting model depending on the input data. ProtTest3 (Abascal et al., 2005; Darriba et al., 2011) will be used for analyses involving protein sequences and jModelTest2 (Posada, 2008; Darriba et al., 2012) for phylogenetic inference of DNA datasets.

#### 2.4.5 PHYLOGENETIC INFERENCE

##### 2.4.5.1 DISTANCE AND PARSIMONY METHODS

The simplest phylogenetic inferences are those of distance matrix methods. Distance matrix methods (Fitch and Magoliash, 1967) work on the basis of generating a matrix representing the pairwise distances of each sequence using the selected substitution model and inferring a phylogeny from this. The simplest case would be searching tree space for the optimal tree using a standard least-squares criteria between actual and expected branch lengths (i.e. distances) (Fitch and Magoliash, 1967; Cavalli-Sforza and Edwards, 1967).

However, the most common is that of neighbour-joining which begins with the distance matrix and a star topology tree in which all leaf node branches are connected to a single shared central node. Then:

1. Find the closest two branches in the distance matrix

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<sup>8</sup>LBA is a distorting effect in which long branches (rapidly diverging) are incorrectly placed close to one another regardless of actual shared homology. This is due to the increased chance of rapidly diverging sequences to share independently acquired residues (Bergsten, 2005).

2. Join the closest pair into a single branch with a new internal node connected to central node
3. Generate a new distance matrix consisting of the distances of each leaf from this new internal node.
4. Repeat the process with the new matrix (Nei, 1987)

It works on the assumption that the true tree has the smallest expected length (minimum evolution) and a short tree that has similar topology can be achieved using the fast simple agglomerative algorithm. NJ is one of the best distance methods and is more reliable than maximum-parsimony which can be asymptotically inconsistent While already efficient (possibly efficient as possible) NJ can be made more efficient using effective heuristics to search tree space (Kumar, 1996) as well as improvements where variance is minimised instead of pure distance improving performance in datasets with high substitution rates e.g. BIONJ (Gascuel, 1997).

Distance methods are very fast but can perform very poorly for divergent sequences with large sampling errors as they don't generally account for variance in distance estimates (Yang and Rannala, 2012).

Parsimony approaches (Camin and Sokal, 1965) on molecular sequences (Eck and Dayhoff, 1966) seek to infer the maximum parsimony (MP) tree. That is the tree which requires the smallest number of character changes (has the best tree score). Where the tree score is the sum of all character lengths (the minimum number of changes for each site in the alignment). Any site that is invariable is not informative for generation of a parsimony tree. MP has no explicit assumptions relative to other methods however, this also means it is difficult to build in prior knowledge of sequence evolution when generating a tree. It also fails when multiple substitutions have occurred at the same site or with parallel changes in two long branches and therefore is especially prone to long-branch attraction (Felsenstein, 1978). The popularity of parsimony methods has declined with discoveries that they can produce statistically inconsistent phylogenies (Felsenstein, 2001). Interestingly, maximum likelihood inference under the no common mechanism model (which involves independent branch-length estimation for every site and branch) will result in the same phylogeny as parsimony (Tuffley and Steel, 1997). Therefore, arguably parsimony can be a special case of likelihood.

#### 2.4.5.2 MAXIMUM LIKELIHOOD

Maximum likelihood (ML) methods seek to discover the maximum-likelihood estimates (MLEs) of the tree parameters (topology  $\tau$ , branch length  $\theta_l$  and usually substitution model parameters  $\theta_\mu$ ) for the data i.e. MLE of  $l(\tau, \theta_l, \theta_\mu)$ .

These MLEs are estimated numerically using standard iterative optimisation algorithms. They were developed relatively early in molecular phylogenetics using relatively simple models (Neyman, 1971) but more efficient implementations, e.g. (Felsenstein, 1981), and increased computational power has made them one of the more popular means for phylogenetic inference.

Generally, an ML approach will sequentially perturb a starting tree topology (often BIONJ or simple ML tree itself) using branch swapping operations such as Nearest-Neighbour Interchanges (NNI) or Subtree-Prune-and-Regraph (SPR) where whole subtrees are removed and reattached to a different part of tree. SPR is slower but less prone to get caught in local optima than NNI and thus will lead to higher likelihood phylogenies overall (Criscuolo, 2011). Expectation-maximisation or the Newton-Raphson method can then be used to find the MLE for branch length and model parameters. For example, PhyML uses an initial BIONJ and standard hill-climbing which perturbs topology and branch lengths simultaneously.

The advantage of ML approaches is that they have explicit model assumptions (which can therefore be tested), are relatively robust to model misspecification, are relatively efficient in a phylogenetic sense, and can make use of sophisticated evolutionary models. They can thus compensate for many pathological data features (heterotachy, state and rate heterogeneity within and across sites) (Yang and Rannala, 2012). Almost all published phylogenies are Bayesian or ML (or ideally both) for this reason. Unfortunately, ML inference is also relatively slow to calculate especially in comparison to distance methods.

In order to get an estimate for the robustness of a particular phylogenetic ML inference, the masked alignment can be repeatedly resampled (bootstrap samples) with replacement and phylogenies regenerated. Each node can then be scored based on the proportion of these bootstrap samples in which it is recapitulated (Felsenstein, 1985). A similar approach, known as jackknifing, uses random subsets of the alignment instead of samples (Miller, 1974; Lapointe et al., 1994). Finally, approximate likelihood-ratio tests (aLRT) can be used on branches to give support values by comparing the likelihood of existence of a given branch compared to its non-existence

(Anisimova and Gascuel, 2006). There is considerable literature evaluating the pros and cons of different support schemes. However, bootstrap supports are the *de facto* method of inferring a variance of phylogenetic error (Stamatakis et al., 2008) as they are both simple and conservative (but are computationally expensive) (Anisimova and Gascuel, 2006). It should be noted that all of the above methods of determining phylogenetic robustness can be applied to distance and parsimony methods as well.

In this work, for high-throughput analyses FastTree2 was used due to its considerable greater speed relative to ML inference tools (Price et al., 2010). For individual phylogenetic analyses ML trees were inferred using RAxML8 (Stamatakis, 2014) and non-rapid bootstrap supports.

#### 2.4.5.3 BAYESIAN INFERENCE

Bayesian inference is, as the name suggests, based on the Bayes theorem (eq. (2.1)). Specifically, the most probable tree is recovered based on the posterior probability:

$$p(\tau, \theta_l, \theta_\mu | D) = \frac{p(\tau, \theta_l, \theta_\mu) p(D | \tau, \theta_l, \theta_\mu)}{p(D)}$$

where  $p(\tau, \theta_l, \theta_\mu)$  are the prior probability for model parameters (topology  $\tau$ , branch length  $\theta_l$ , substitution model  $\theta_\mu$ ) and  $p(D | \tau, \theta_l, \theta_\mu)$  is the likelihood of the data given a certain set of parameters with  $p(D)$  as the marginal probability.

Due to the computational difficulty directly calculating the marginal likelihood (integrated over all possible parameter values in all dimensions) phylogenetic inference uses a process known as Markov-Chain Monte-Carlo (MCMC) to sequentially randomly sample the posterior probability distribution. Conceptually, these can be considered as random walkers on the probability distribution that are more likely to accept new movements that increase the likelihood than decrease it.

An advantage of Bayesian inference is that the posterior probability (PP) of a given node means “support” values are built-in to the inference and additional bootstrapping is unnecessary. Unfortunately, posterior probabilities are sensitive to model violations and have been found to not be very conservative estimators of branch support (Simmons, 2003) (although again there has been considerable work comparing Bootstraps to PP (Anisimova and Gascuel, 2006)). Additionally, the prior distribution in Bayesian Inference allows information that is already known

about the dataset to effectively be built-in to the inference. This potentially improves phylogenetic accuracy.

All in-depth individual phylogenetic analyses presented in this thesis were inferred using both Bayesian (via MrBayes3 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012)) and ML methods. Phylogenies are then presented with both PP and bootstrap support values on each node inferred using both methodologies.

## 2.5 INFORMATICS LANGUAGES AND HARDWARE

### 2.5.1 LANGUAGES AND LIBRARIES

Several programming languages and a range of libraries were used throughout this PhD depending on the suitability of a particular tool for a task. The full details of the specific tools used for the main analyses are outlined during the description of these analyses, however, the tools used for prototyping as well as those used for smaller tasks not covered in detail are omitted elsewhere.

Languages and libraries were chosen depending on their best fit for a particular task. Performance sensitive code such as those dealing with large datasets (e.g. high-throughput sequencing libraries or image data) were principally conducted using the C++ language in line with the C++11 standard (ISO International Standard, 2011)

The main C++ libraries used in addition to the C++11 standard library were:

- Seqtk - fastq/a sequence parsing library (Li, 2015)
- MLPACK - a high-performance machine learning library (Curtin et al., 2013)
- OpenCV3 - widely used computer vision library (Bradski, 2000)
- Armadillo - numerical computation library (Sanderson, 2010)

The majority of tasks were accomplished using the high-level python language (python2.7 or python3.4 depending on the application). In addition to the standard library, the numerical computation libraries numpy and theano, machine learning library scikit-learn, statistical and scientific libraries scipy and pandas, the bioinformatics libraries scikit-bio and biopython, and plotting libraries holoviews, matplotlib, and seaborn were all used extensively. Frequent use was made of literate programming offered by environments such as the ipython notebook (recently renamed jupyter).

The statistical programming language R (R Core Team, 2015) was used for some data analysis and visualisation primarily using `ggplot2` (Wickham, 2009) and `dplyr` (Wickham and Francois, 2014). This was primarily done using the R-Studio (<http://www.rstudio.com/>) integrated development environment) and R-markdown (Allaire et al., 2014).

All code was version controlled using `git` (<http://git-scm.com/>) and remotely hosted using `github` (<https://github.com/>) and `bitbucket` (<https://bitbucket.org/>) services. Unit tests were automatically run on synchronisation ('push') with these remote servers using the Travis (<https://travis-ci.org/>) Continuous Integration (CI) service.

Incidental scripting was done using `zsh` and `bash` languages and all code was written using `vim` in an `st` terminal.

### 2.5.2 HARDWARE

All analyses were conducted on either the lab cluster (running Ubuntu Server LTS 12.04 and 14.04 (<http://www.ubuntu.com/>):

- PowerEdgeM910 with 2 x Intel Xeon CPU E6510 @ 1.73GHz, 512GB RAM
- PowerEdgeM910 with 2 x Intel Xeon CPU E7-4807 @ 1.87GHz, 512GB RAM
- PowerEdgeM620 with 2 x Intel Xeon CPU E5-2650 v2 @ 2.60GHz, 512GBRAM

Or on two workstations both running continuously updated versions of Arch Linux (<https://www.archlinux.org/>).

- Apple MacPro with 2 x Intel Xeon CPU E5520 @ 2.27GHz, 16GB RAM
- Dell Precision T7500 with 2 x Intel Xeon E5620 @ 2.4GHz, 48GB RAM

*Taxonomy is described sometimes as a science and sometimes as an art, but really it's a battleground*

- Bill Bryson: *A Short History of Nearly Everything*

# 3

## Endosymbiont diversity

### 3.1 INTRODUCTION

#### 3.1.1 ENDOSYMBIONT TAXONOMY AND CLONALITY

Over 50 strains of green algal photobionts have been identified in *Paramecium bursaria* species (Hoshina et al., 2010, 2004; Hoshina and Imamura, 2009; Summerer et al., 2008; Vorobyev et al., 2009). These form at least four distinct species groups, believed to be represented in the following cultures:

- *Micractinium reisseri* (e.g. former “European” group endosymbionts such as those attributed to CCAP 1660/12)
- *Chlorella variabilis* (e.g. former “American” group endosymbionts such as *Chlorella variabilis* NC64A)
- *Chlorella vulgaris* (e.g. the endosymbiont attributed to CCAP 1660/10)

- *Coccomyxa* sp. (e.g. the endosymbiont attributed to CCAP 1660/13)

These species display a polyphyletic distribution within the green algae providing evidence for multiple separate origin events for the *P. bursaria* endosymbiosis (Hoshina and Imamura, 2008, 2009) Furthermore, there is emerging evidence, in the form of intron HGTs and ITS2 sequencing data that strains of *P. bursaria* are capable of hosting double and triple co-habitations of different photobiont species (Hoshina, 2012). Therefore, before an effective analysis can take place of an endosymbiotic system it is important to carefully define the species (singular or plural) involved.

Unfortunately, the systematics of the Chlorophyta has experienced a relatively high degree of flux, with multiple redefinitions even since the initial use of molecular phylogenetics of ribosomal sequences (Hori et al., 1985; Gunderson et al., 1987) in the 1980s (Leliaert et al., 2012; Hoshina et al., 2010). The algal endosymbionts of *Paramecium bursaria* in particular have gone through a range of names and classifications starting with *Zoochlorella* in 1882 and through various species of the genus *Chlorella* (Hoshina et al., 2010).

Initially, all symbiotic algae were named as single *Chlorella paramecii* species but this name was rejected and *Chlorella variabilis* was defined (Shihira and Krauss, 1965) but this was in turn rejected and fell out of use. Later, the first discovery of the existence of multiple distinct strains of photobiont was published (Douglas, 1986). With this came the understanding that the endosymbionts of *P. bursaria* are likely to be divergent but not distinct species to other described free-living *Chlorella* (Hoshina et al., 2010).

To add further confusion to the system, the most recently accepted terms defined species of endosymbiont merely as “American” and “European” leading to several misidentifications (Kodama et al., 2007; Hoshina et al., 2010). Recently, these two organisms have been redescribed as distinct species *Chlorella variabilis* and *Micractinium reisseri* respectively (Hoshina et al., 2010). Therefore, care must be taken when reading older literature to distinguish the earlier less well-defined *C. variabilis* from the modern usage.

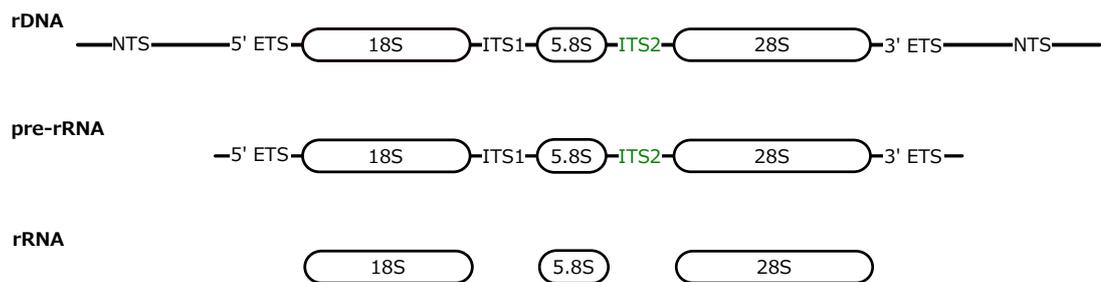
Another source of complication in the systematics of the photobionts are the cases of mislabelling and loss of cultures by culture collections. For example, the initial culture from which the original *Chlorella variabilis* was described from was lost and a supposedly identical culture from a different collection was found to have wildly different biochemical properties (Hoshina et al., 2010). These complications and confusions add to the importance of accurate endosymbiont

species identification.

### 3.1.2 ITS2 TAXONOMIC PROFILING

The most widely accepted means of rapidly taxonomically profiling Archaeplastida (and indeed a range of eukaryote species) is that of nuclear ribosomal internal transcribed spacer 2 (ITS2) (see fig. 3.1.1) barcoding. ITS2 has shown particular utility in the identification and separation of closely related green algal species (Buchheim et al., 2011; Heeg and Wolf, 2015) due to being universal, reliably amplifiable and highly variable (Hershkovitz and Lewis, 1996).

ITS2 barcoding has been recommended as a superior marker to other universal Archaeplastida DNA barcodes such as the *rbcL* (Chen et al., 2010). The conserved nature of the flanking 5.8S and 18S sequences allows near universal primers to be designed which efficiently amplify ITS2 sequences unlike the broadly distributed but highly variable *rbcL* (Buchheim et al., 2011).



**Figure 3.1.1:** Structure of Eukaryotic nuclear ribosomal DNA. rRNA genes exist in tandem repeats separated by nontranscribed spacers (NTS). These NTS are composed of internally transcribed spacers (ITS) and externally transcribed spacers (ETS). ITS2 is highlighted in green and forms an effective taxonomic barcode at sequence level for eukaryotic species analyses. The ITS2 secondary structure shows a greater level of conservation and can be used to investigate lower distance systematic relationships. Figure was redrawn from (Shi, 2005).

In many species the rDNA cistron is present in multiple copies as tandem head-to-tail repeats varying in copy number from one or two copies to thousands (Torres-Machorro et al., 2010). While these copies are frequently homogeneous there are many organisms that display intranuclear variation (Buchheim et al., 2011). For example, alveolates have been discovered with variant rDNA copies (Stern et al., 2012; Galluzzi et al., 2004). At different points in the life cycle of *Plasmodium* spp. (Nishimoto et al., 2008) there is expression of SSU rRNA gene paralogues with up to 11% difference (McCutchan et al., 1988; Chambouvet et al., 2015). Similarly, chlorophytes have previously displayed heterogeneity in rDNA copies (Pillmann et al., 1997; Fama et al., 2000). Therefore, care must be taken not to assume intranuclear homogeneity in phylogenetic analysis

of ITS2 sequences (Buchheim et al., 2011)

This said, ITS2 sequencing will identify the endosymbiont species in the CCAP 1660/12, Yad1g1N and CCAP 1660/13 cultures. It will also offer a method by which the clonality within the photobiont populations can be investigated. By amplifying and sequencing a large number of ITS2 fragments from the same culture there is a reasonably good chance that all the ITS2 level diversity will be sampled. If, on analysis, these sequences form multiple clades or display divergent groupings this could be strong evidence for a multiple photobiont co-habitation within the *P. bursaria* host.

Finally, one last means in which we sought to gain additional insight into the host-endosymbiont system was through the use of multiple-displacement amplification (MDA) based sequencing (Lasken, 2007). Due to difficulties in obtaining sufficient culture densities and the prevalence of putative sources of contamination within the culture, bulk genome sequencing was considered to be prone to major difficulties. Therefore, MDA offered a way in which we could further investigate this question of photobiont clonality while also generating a resource with potential use for further analysis. For example, searching for potentially biologically significant genes that are present in the genome but are not transcribed during endosymbiosis. The utility of this genomic resource hinges on our ability to partition recovered genomes/contigs into the originating host and endosymbiont genomes. It is particularly important to do this and effectively discard contaminant contigs derived from bacteria (food and symbionts) and viruses associated with the host.

### 3.1.3 ISOLATION OF *P. BURSARIA*

One avenue that is important for an effective analysis of a host-endosymbiont system is the ability to analyse the partners in isolation. This can be used to test individual hypotheses regarding each partner and allows controlled reintroduction experiments to be undertaken. Unfortunately, the majority of extant, well-characterised endosymbioses display metabolic co-dependence and therefore, host and endosymbiont cannot be isolated without one or other dying (i.e. they form an obligate relationship as supposed to a facultative one).

Fortunately, there have been numerous studies that have investigated the separation of host and symbiont in *P. bursaria* - green algal systems e.g. (Hosoya et al., 1995; Achilles-Day and Day, 2013b; Karakashian, 1963). Most recently, the only transcriptomic analysis of this system by Ko-

dama and Fujishima (2014) investigated the differential global metatranscriptome profile of *P. bursaria* Yad1g strain with and without its *Chlorella variabilis* 1N endosymbiont (Kodama and Fujishima, 2014). While, this is a different strain of both host and endosymbiont to the SW1-ZK strains in the CCAP1660/12 culture (*P. bursaria* and *Micractinium reisseri*) reproduction of this endosymbiont clearing offers a potential avenue by which to further investigate and, combined with RNAi, to test the functional underpinning of this relationship.

There have been several published methods for clearing endosymbionts from host cells namely, the herbicide paraquat (Hosoya et al., 1995), culturing under constant dark (Karakashian, 1963), herbicide DCMU (Kodama and Fujishima, 2009), X-ray (Wichterman, 1948), and cycloheximide (Weis, 1984; Kodama et al., 2007). Therefore, we attempted three of these methods: specifically paraquat, cycloheximide, and constant darkness treatments with bacterial feeding in order to clear endosymbionts from the host *Paramecium*.

## 3.2 AIMS

In this chapter I will determine the exact algal endosymbiont strains present in the principal *Paramecium bursaria* cultures used throughout this thesis and their relationships relative to one another and to other green algae.

I will also use this data and single cell genomics to investigate whether the algal endosymbiont present in the *Paramecium bursaria*-*Micractinium reisseri* CCAP 1660/12 strains form a clonal population.

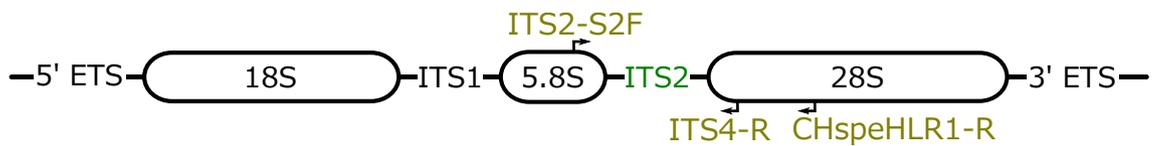
Finally, I will discuss the attempts to remove the endosymbiont in the *Paramecium bursaria* CCAP 1660/12 strain from the host.

## 3.3 METHODS

### 3.3.1 TAXONOMIC ANALYSIS

#### 3.3.1.1 ITS2 SEQUENCING

*Paramecium bursaria* CCAP 1660/12 and *Paramecium bursaria* CCAP 1660/13 cultures were maintained in New Cereal Life (NCL) media at 18 °C with 12:12 hour light/dark cycle. In order to mitigate the risk of sequencing free-living algae in the CCAP 1660/13 culture, ITS2 sequences



**Figure 3.3.1:** Schematic diagram showing the location of the forward (ITS2-S2F) and reverse primers (CHspeHLR1R, ITS4-R) used for the amplification of ITS2 sequences in this study. CHspeHLR1R binds within the 28S whereas ITS4-R binds closer to the 5' end of the 28S. Both primer sets recover the full ITS2 sequence.

were acquired from both pure culture samples and carefully purified samples. Purification involved successive filtering and washing steps of isolated cells in sterile NCL media. Specifically, filtration using a 10  $\mu\text{m}$  filter, washing off, re-suspension and 3 serial subcultures in sterile NCL media.

ITS2 sequences were amplified using 2 pairs of primers: ITS2-S2F primer (“ATGCGAT-ACTTGGTGTGAAT”) binding to conserved 5.8S sequences from (Chen et al., 2010) with the CHspeHLR1R (“CACTAGACTACAATTCGCCAGCC”) reverse primer specific to chlorophyte 28S (Hoshina et al., 2004) and the ITS4 primer (“TCCTCCGCTTATTGATATGC”) (White et al., 1990) (see fig. 3.3.1). The reason for the dual primer approach was that it was observed in the smaller biological samples created during the cleaning process that the ITS2-S2F - CHspeHLR1R primer pair wasn’t amplifying ITS2 very efficiently therefore the alternate primer pair was used.

PCR conditions used were 94 °C for 5 min followed by 40 cycles of 30 s at 94 °C, 30 s at 56 °C and 45 s at 72 °C. This was followed by a final elongation step of 10 min at 72 °C.

PCR products were then cleaned up, cloned, sequenced and processed using the same protocol as (Maguire et al., 2014). Briefly, the successfully amplified PCR products were gel-purified (Wizard SV Gel and PCR Clean-Up kit, Promega). These products were then TA-cloned using Agilent’s PCR StrataClone Cloning kit, blue-white screened and 5 clones selected for each PCR product. Clones were then externally Sanger sequenced using the M13Rev primer at MWG Eurofins. Flanking vector and primer sequences were removed, sequences trimmed to areas of high chromatograph quality and ambiguously defined bases corrected using Sequencher (Gene Codes, 2015).

From the 3 *Paramecium bursaria* CCAP 1660/12 biological replicates 14, 9, and 11 ITS2 sequences were obtained respectively. Similarly, from the 2 *Paramecium bursaria* CCAP 1660/13 biological replicates 8 were obtained from sequences obtained from the culture directly, and 10

from the purified, washed samples (7 using ITS<sub>2</sub>-S<sub>2</sub>F-ITS<sub>4</sub> primers and 3 using ITS<sub>2</sub>-CHspe). Additionally, 5 ITS<sub>2</sub> sequences were acquired from the Yad1g1N culture following the same protocol.

In order to mitigate the risk of sequencing error masquerading as true sequence divergence any sequences found in later phylogenetic analysis to demonstrate single nucleotide changes from the consensus of its clade placement was resequenced at MWG Eurofins in reverse using M13 Uni. Specifically, these were ITS-B18, ITS-2, ITS-19, ITS-B6, ITS-B3, ITS-A7, ITS-6, ITS-B15, ITS-10, ITS-9, ITS-15, and ITS-1.

See appendices (section A.1) for a full listing of trimmed sequences and representative gel images of the cloning products.

### 3.3.1.2 PHYLOGENETICS

ITS<sub>2</sub> sequences used in (Hoshina et al., 2010), (Hoshina and Fujiwara, 2013) were retrieved from genbank. The trimmed sequences and the established database sequences were then aligned using MUSCLE (Edgar, 2004a). This alignment was manually masked in the graphical SeaView (Gouy et al., 2010) package. jModelTest2 (Guindon and Gascuel, 2003; Darriba et al., 2012) was then used to pick an appropriate substitution model. Finally, phylogenies were inferred using the maximum likelihood method via RAxML version 8 (Stamatakis, 2014) with 1,000 bootstrap replicates. Similarly, MrBayes (Huelsenbeck and Ronquist, 2001) was used to infer the phylogeny using the bayesian framework. MrBayes used 2 independent runs of 4 Monte-Carlo Markov-Chains (MCMC) for 3,750,000 generations (at which point the 2 runs were considered to have converged, as determined in Tracer v1.4 (Rambaut and Drummond, 2007)). Trees were estimated from the MCMC results with a burn-in of 250,000 generations. Trees were then visualised and support values combined using TreeGraph2 (Stöver and Müller, 2010).

Trees were rooted using a Microthamniales outgroup composed of:

- *Trebouxia gigantea* AJ249577.2
- *Trebouxia arboricola* SAG219-1a Z68705.1,
- *Trebouxia jamesii* Hp-MT1 AJ511357.1
- *Trebouxia impressa* AJ249576.1

- *Trebouxia corticola* AJ249566.1
- *Trebouxia higginsiae* AJ249574.1.

### 3.3.2 SINGLE CELL GENOMICS

#### 3.3.2.1 DNA EXTRACTION

Individual *P. bursaria* CCAP 1660/12 cells were removed from culture and washed three times in a successive series of 10  $\mu$ l drops of sterile modified New Cereal Leaf-Prescott (NCL) medium to minimise prokaryotic contamination from bacterial foodstocks in the culture media. Cells were added to a final 10  $\mu$ l drop of sterile water before being added to a microcentrifuge tube.

DNA was then extracted using a cetrimonium bromide (CTAB) based method adapted from (Winnepenninckx et al., 1993). In brief, 748.5  $\mu$ l of CTAB extraction buffer (at 37°C and 100  $\mu$ l beads (Sigma, 425600  $\mu$ m; acid washed) was added and the tube vortexed for 5 minutes. The tube was incubated for 50 minutes at 37°C, vortexed again for 5 minutes and incubated for 50 minutes at 60°C. This was to ensure lysis of the endosymbiont's chitinous cell wall. DNA was extracted three times with phenol/chloroform/isoamylalcohol (25:24:1, pH 8), washed with 70% ethanol and re-suspended in 2.5  $\mu$ l TE (pH 8). Whole-genome amplification of purified genomic DNA was performed using the multiple-displacement amplification based (MDA) Qiagen REPLI-g Single Cell Kit. The REPLI-g amplified gDNA was purified using a QIAamp DNA mini kit and eluted in 100  $\mu$ l elution buffer.

#### 3.3.2.2 SEQUENCING

Five prepared libraries were put forward for sequencing (Pb-3, Pb-4, Pb-6, Pb-7 and Pb-8). Samples were multiplexed and were rapid sequenced in an Illumina HiSeq 2500 in 150 bp paired-end mode.

#### 3.3.2.3 READ PRE-PROCESSING

Trimmomatic (Bolger et al., 2014) was used to trim sequencing adapters (using sequences provided by Exeter Sequencing Service) via the ILLUMINACLIP setting. Reads were then quality trimmed at a minimum average SLIDINGWINDOW quality thresholds of Q5 and Q30.

Q<sub>5</sub> and Q<sub>30</sub> trimmed reads were then error corrected using BayesHammer (Nikolenko et al., 2013) as built into the SPAdes assembler (Bankevich et al., 2012).

Trimmed and error corrected libraries were also then digitally normalised (Brown et al., 2012) to a coverage of 20 and with a *k*-mer size of 25. Following this, *k*-mers were abundance filtered (Zhang et al., 2014, 2015) using the Khmer package (Crusoe et al., 2015).

#### 3.3.2.4 ASSEMBLY

Assemblies were then generated using the following sets of data:

- Q<sub>5</sub> trimmed reads with error correction
- Q<sub>30</sub> trimmed reads with error correction

The following assemblers were used:

- SPAdes assembler (Bankevich et al., 2012; Nurk et al., 2013)
- SPAdes assembler with “careful” thresholding (runs MismatchCorrector and minimises the risk of indels)
- MEGAHIT (Li et al., 2015b)
- Platanus (Kajitani et al., 2014a)

#### 3.3.2.5 ASSEMBLY ASSESSMENT

Assemblies were assessed and compared using the QUality ASsessment Tool for genome assembly (QUAST) (Gurevich et al., 2013) and key assembly metrics were compared (N<sub>50</sub>, N<sub>90</sub>, contig number and length and total assembly size).

#### 3.3.2.6 CONTIG BINNING

Contigs were subsequently cut into 10kb fragments for consistency in binning and taxonomic assignment and obviate the difficulties aligning very long sequences. Reads were then mapped back onto the final assembly using Bowtie2 (Langmead and Salzberg, 2012)

Using the metagenomic binning tool, CONCOCT (Alneberg et al., 2014) contigs were binned into clusters based on sequence composition and coverage features (derived from mapping data).

Coverage features were derived from a coverage and linkage table generated via CONCOCT scripts built around BEDTools (Quinlan and Hall, 2010; Quinlan, 2014), Picard and Samtools (Li et al., 2009) based parsing of the bowtie2 alignment files. Clustering was conducted using a Gaussian Mixture Model (GMM) (Bishop, 2006) and the number of clusters determined through variational Bayesian inference (Corduneanu and Bishop, 2001).

All CONCOCT analyses were completed using a provided pre-configured Docker Image (Merkel, 2014), a form of lightweight distributable process isolation container. This was downloaded from DockerHub (<https://hub.docker.com/r/binpro/concoct/>) on 2015-10-25.

Additionally, the cut contigs were taxonomically assigned using TAXAssign (<https://github.com/umerijaz/TAXAssign>) against the NCBI nt database. The BLAST database was downloaded using `update_blastdb.pl` script ([http://www.ncbi.nlm.nih.gov/blast/docs/update\\_blastdb.pl](http://www.ncbi.nlm.nih.gov/blast/docs/update_blastdb.pl)) and TAXAssign was run in parallel (using GNU parallel (Tange, 2011)) with a maximum of 10 reference matches per contig a minimum percentage identity for assignment to a given taxonomic level of 60, 70, 80, 95, 95, and 97 for Phylum, Class, Order, Family, Genus and Species respectively.

CONCOCT clusters were then evaluated using the taxonomic assignments from TAXAssign via the provided `validate.pl` script.

Finally, another attempt at taxonomic assignment using a custom ORF based pipeline was attempted:

- ORFs with a minimum size of 300 were called using *Tetrahymena* and Universal encodings from contigs over 500 bp
- ORFs were then clustered at 90% identity using CD-HIT
- Diamond BLASTP searches were then done against the NR protein database
- Taxonomy was assigned to each contig based on the lowest common ancestor of all its ORFs with hits (via the `lca_mapper.sh` accessory script in MEGAN)
- Contigs were then binned based on the identity of this taxonomic assignment:
  - Endosymbiont contigs were all those assigned to Archaeplastida or a descendent node.

- Host contigs were all those assigned to Aveolata or a descendent node.
- Eukaryote was a super group containing all Eukaryote assigned contigs
- Contaminant contigs were all those assigned as Bacterial
- Viral contigs were all those assigned as Viral sequences.

### 3.3.2.7 VARIANT CALLING

A variant calling on the binned genomic contigs was attempted to assess the clonality of the endosymbiont. Briefly, the top 10 longest contigs binned as “endosymbiont” were retrieved and Q5 trimmed, error corrected reads aligned to them using bowtie2 and output to BAM files for each library. Library BAMs were then combined using samtools “mpileup” with a minimum mapQ threshold of 5. All potential variants with a mapping depth of 0 were filtered out. SNPs were called from this filtered mpileup file using a custom perl script designed for the wheat genome project (pers. comms. Hall, Neil). This SNP calling used a coverage cutoff of 10%.

Called SNPs were then visualised and statistics calculated using R.

### 3.3.3 ENDOSYMBIONT ELIMINATION

CCAP 1660/12 and Yad1g1N1 cultures in NCL media with were treated under the following conditions to attempt to remove the endosymbiont. *P. tet aurelia* were used as a control culture and was given the same treatment.

Paraquat was added at both  $1\text{mgul}^{-1}$  and  $0.5\text{mgul}^{-1}$  concentrations. Cultures were maintained under normal 12:12 lit:dark conditions at  $15^{\circ}\text{C}$ . Cultures were inspected daily using light microscopy and assessed for “bleaching” (i.e. loss of green appearance due to death of chlorophyll bearing alga).

Cycloheximide was added to cultures at both  $1\text{mgul}^{-1}$  and  $10\text{mgul}^{-1}$ , again cultures were maintained under standard 12:12 lit:dark condition and  $15^{\circ}\text{C}$ . Cultures when looking clear were sub-cultured and resuspended in NCL without cycloheximide.

Cultures were maintained in the dark without a lit phase at  $15^{\circ}\text{C}$  and inspected every 2 weeks for clearing. This was to prevent providing too much light and further encouraging endosymbiont growth.

## 3.4 RESULTS

### 3.4.1 ITS<sub>2</sub> PHYLOGENY

The ITS<sub>2</sub> phylogeny demonstrates a clear and well supported relationship in all samples between the CCAP 1660/12 and CCAP 1660/13 endosymbionts and the species described as *M. reisseri* (see fig. 3.4.1). Additionally, the Yad1g1N endosymbiont is positively identified as *C. variabilis*. This phylogeny in general is consistent with previous ITS<sub>2</sub> phylogenies (Hoshina et al., 2010).

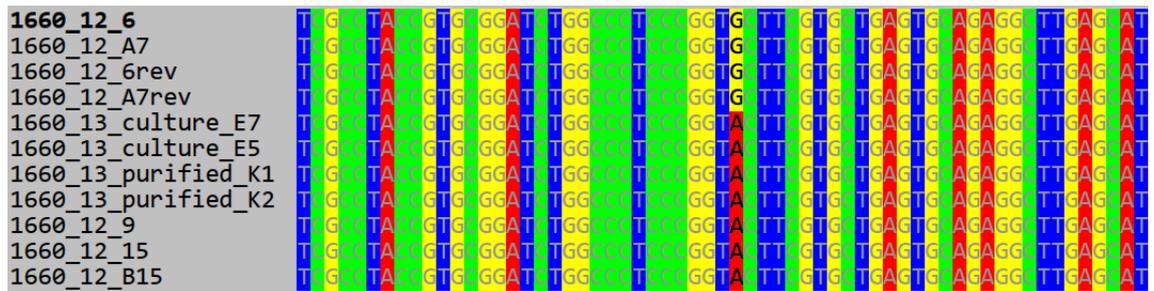
The ITS<sub>2</sub> sequences of the CCAP 1660/12 culture demonstrated a variety of SNPs but never more than a single SNP difference from the basal *M. reisseri* polytomy. These SNPs were grouped into 3 categories: 4 different SNPs that were not found in the reverse complement and therefore represent likely sequencing error (1660-13-purified-K4, K8, K6 and K7), 3 different SNPs that were found in both forward and reverse sequencing (1660-12-B6, 1660-12-19 and 1660-12-18) and therefore represent either true diversity or PCR error and finally 1 SNP that was found in forward and reverse sequencing and in two separate PCR reactions from different biological replicates (1660-12-A7 and 1660-12-6). This featured a single base change from A to G (see fig. 3.4.2) at position 126 in the full masked alignment. This SNP could be the result of intranuclear variation of the ITS<sub>2</sub> in the multicopy rDNA array.

With the exception of these SNPs the sequences were identical to 3 from previously sequenced *M. reisseri* endosymbionts, specifically CCAP 211/83 culture with *P. bursaria* Pbi host (AB206547.1), the SW1-ZK symbiont from a *P. bursaria* PB-SW1 host (AB506070.1), and TP-2008b from the SAG241.80 culture (FM205851.1) (see fig. 3.4.1).

This polytomy as the sister clade to other *Micractinium pusillum* taxa was highly supported in both ML and Bayesian phylogenies (91.3% of bootstraps and with a posterior probability of 1.00). There was similarly high support for the separate branching of these sequences from the clade containing the *C. variabilis* and *C. vulgaris* endosymbionts (87.3%/1.00) and the existence of a clade comprising these 3 endosymbionts to the exclusion of any *Coccomyxa* sequences was well supported (89.3%/0.95).

Sequences from the Yad1g1N culture formed a clade with high support with other *Chlorella variabilis* species including NC64A. This support the identification of the endosymbiont in this





**Figure 3.4.2:** Alignment showing the sole SNP (at pos 126 in masked ITS2 alignment) that is likely to represent true diversity. This indicates that the endosymbiont population is largely clonal with a small marginally divergent sub-population that has possibly arisen during the endosymbiosis itself. Alternatively, this represents intranuclear variation of the ITS2 across the genomic copies.

Sample	Raw PE Reads	Q30 Trimmed PE Reads	Q5 Trimmed PE Reads
Pb-3	$3.523 \cdot 10^7$	$1.951 \cdot 10^7$	$2.737 \cdot 10^7$
Pb-4	$3.228 \cdot 10^7$	$2.606 \cdot 10^7$	$3.035 \cdot 10^7$
Pb-6	$3.291 \cdot 10^7$	$2.437 \cdot 10^7$	$2.962 \cdot 10^7$
Pb-7	$4.023 \cdot 10^7$	$2.642 \cdot 10^7$	$3.404 \cdot 10^7$
Pb-8	$3.869 \cdot 10^7$	$2.613 \cdot 10^7$	$3.246 \cdot 10^7$

**Table 3.4.1:** Summary of the number of surviving reads for Q5 and Q30 trims in each library

culture as *C. variabilis* 1N.

### 3.4.2 SINGLE CELL GENOMES

#### 3.4.2.1 SEQUENCING AND PRE-PROCESSING

The number of remaining reads in each library after trimming at a minimum average sliding window quality threshold of 30 and 5 can be found in table 3.4.1.

After error correction the combined Q30 trimmed libraries comprised  $1.218 \cdot 10^8$  paired end reads. Similarly, the Q5 trimmed libraries comprised  $1.538 \cdot 10^8$  reads.

#### 3.4.2.2 ASSEMBLY

Assemblies were compared using generated contigs and QUAST. Assembly statistics were tabulated to allow comparison (table 3.4.2). As we are interested in recapitulating as much genomic sequence as possible from this complex metagenome but not necessarily to generate “clean” polished closed genome assemblies, the fact that the Q30-SPAdes assembly generated both the longest total assembly (over twice the size of the nearest assembly even when considering only contigs over 1 kbp) as well as the highest N50 and within 2 kbp of the longest contig of all assemblies

Assembly	Q30-MegaHit	Q30-Platanus	Q30-SPAdes-Careful	Q30-SPAdes	Q5-SPAdes
# contigs ( $\geq 0$ bp)	131,057	<b>25,789</b>	73,698	127,976	94,384
# contigs ( $\geq 1000$ bp)	14,960	<b>486</b>	13,301	21,808	12,614
Total length ( $\geq 0$ bp)	73,350,696	6,289,036	73,691,706	<b>142,281,712</b>	81,478,234
Total length ( $\geq 1000$ bp)	28,064,499	2,191,750	52,704,642	<b>105,269,748</b>	58,162,565
# contigs	41,221	<b>776</b>	24,923	42,180	24,109
Largest contig	13847	78624	207156	207157	<b>209,873</b>
Total length	46,095,605	2,398,106	60,731,204	<b>119,241,116</b>	66,064,486
GC (%)	38.81	33.68	37.78	37.85	39.27
N50	1,246	6,386	4,949	<b>7,163</b>	6,334
N75	769	<b>2,875</b>	1,845	2,277	2,188
L50	10,444	<b>112</b>	2,937	3,530	2,241
L75	22,405	<b>249</b>	7,974	11,103	6,716

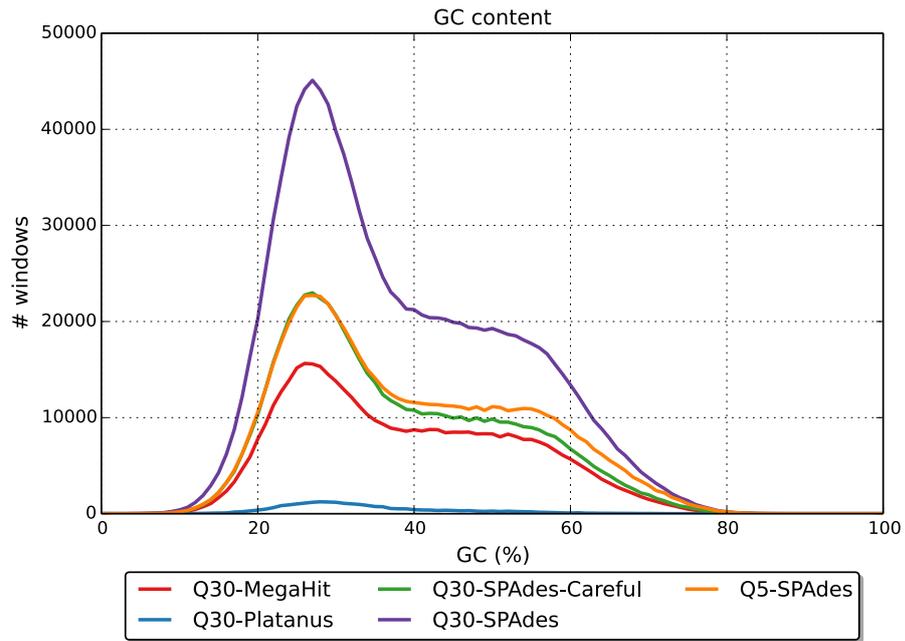
**Table 3.4.2:** Assembly statistics generated by an analysis of contigs using QUAST. Best values are highlighted in bold. All statistics are based on contigs of size  $\geq 500$  bp, unless otherwise noted (e.g. "# contigs ( $\geq 0$  bp)" and "Total length ( $\geq 0$  bp)" include all contigs). N50 and N75 are the minimum contig length at which all contigs of that length are larger comprise 50% and 75% of the total assembly size. Similarly, L50 and L75 are the number of contigs that are summed for a given N50 and N75 (i.e. lower is better). The highest values for each metric across the assemblies is emphasised in bold. This table shows that Q30 Platanus assembly generated the fewest and longest contigs overall, however the Q30-SPAdes assembly generated the longest assembly by a considerable margin with the highest N50.

(generated by Q5-SPAdes) was compelling.

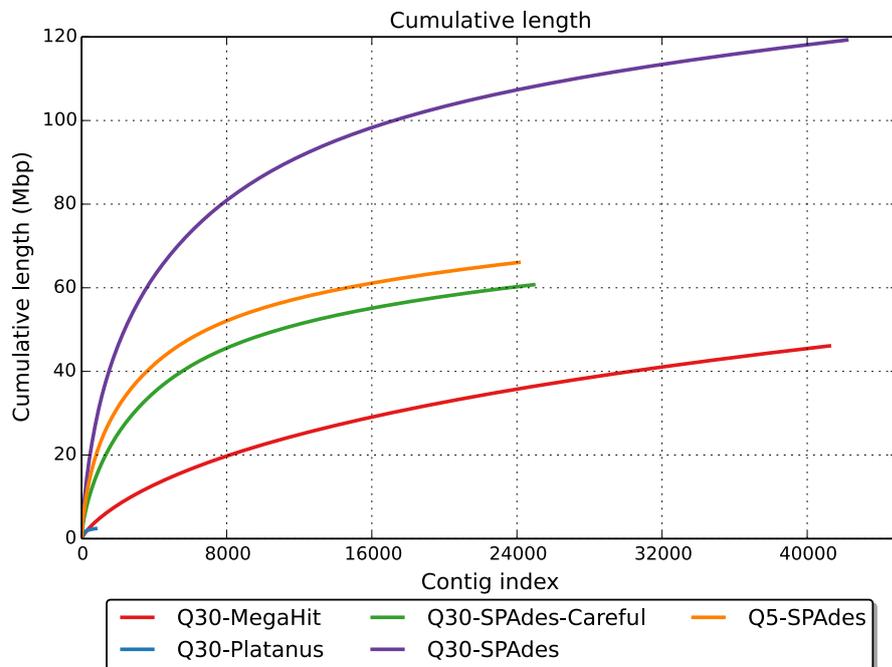
Generally, the SPAdes assemblers out-performed Platanus and MegaHit, likely due to being specifically designed for MDA based data. Note, that all assemblies were completed with BayesHammer corrected reads so the difference in performance cannot be attributed to this aspect of the assembly pipeline.

Plots of assembly GC (fig. 3.4.3), cumulative length (fig. 3.4.4) further support Q30-SPAdes as both the longest assembly but an assembly with similar GC profile to the other assemblies and contig length distribution. Finally, the plot of X's indicates that Q30-SPAdes isn't a disproportionately highly gapped assembly with low numbers of X's found in its longest contigs. It should be noted, however, that it does have a higher proportion of X's in shorter contigs than the other assemblies (fig. 3.4.5).

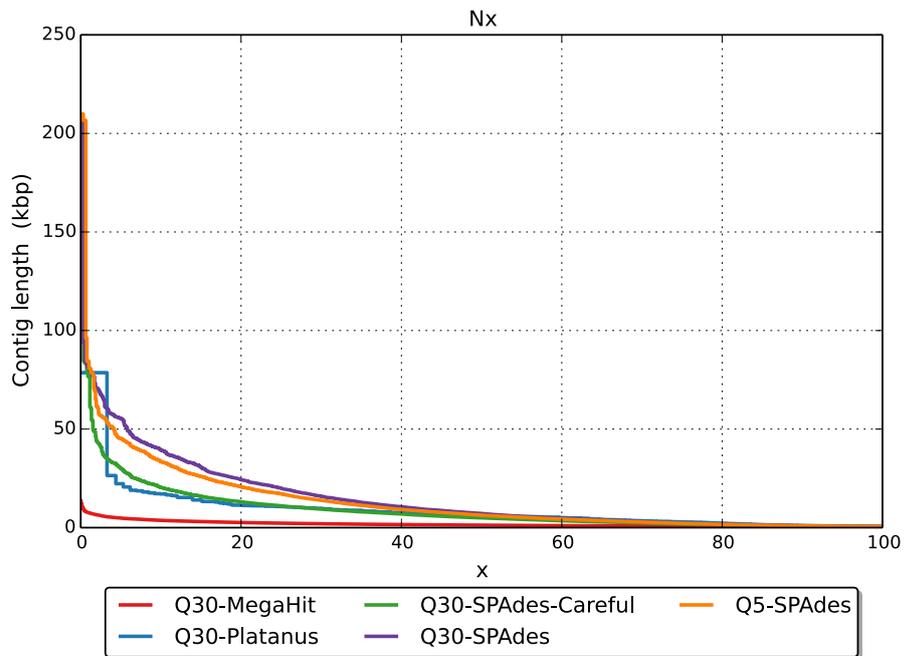
Therefore, Q30-SPAdes assembly was selected for further analysis and size filtered to exclude all contigs shorter than 500 bp to give 21,090 contigs.



**Figure 3.4.3:** GC densities of the compared genome assemblies. As expected all display a clear peak around 30% representing that the majority of the assemblies by length contigs are likely to be derived from the AT rich *Paramecium bursaria* host. The overall height of the Q30-SPAdes peak reflects the relative size of this assembly. Peaks around 50% GC may reflect endosymbiont contigs and possibly bacterial contamination.



**Figure 3.4.4:** The cumulative length of contigs as a function of contig number. Again, this plot reflects that Q30-SPAdes generated the largest assembly by a considerable margin. Platanus failed to recover many contigs found in other assemblies.



**Figure 3.4.5:** The number of X (i.e. gap) in the assembled contigs as a function of their length. This demonstrated that generally few Xs were assembled - however, it should be noted that these are contigs and not assembly scaffolds and thus fewer Xs would be expected.

### 3.4.2.3 BINNING

From the selected Q30-SPAdes assembly, the 21,090 contigs were cut to 10kb fragments for decomposition to generate 64,852 contigs. 18,277 of these 64,852 contigs were successfully given a phylum level assignment, table 3.4.3.

Contigs were clustered into 34 unique clusters by Concoct. These taxonomic assignments were then used to validate the 34 contig clusters generated in Concoct (visualised in fig. 3.4.6) by considering them as the “ground-truth”.

Recall that Precision and Recall can be defined as follows:  $Precision = \frac{TP}{TP+FP}$  and  $Recall = \frac{TP}{TP+FN}$  where  $TP$  are True Positives and  $FP$  and  $FN$  are False Positives and Negatives respectively (see table 3.4.4 for an explanation of what these terms mean in the context of clustering).

CONCOCT assigned clusters were relatively precise (0.912608) therefore there were relatively few  $FP$  i.e. the majority of clusters contained contigs with the same taxonomic assignments.

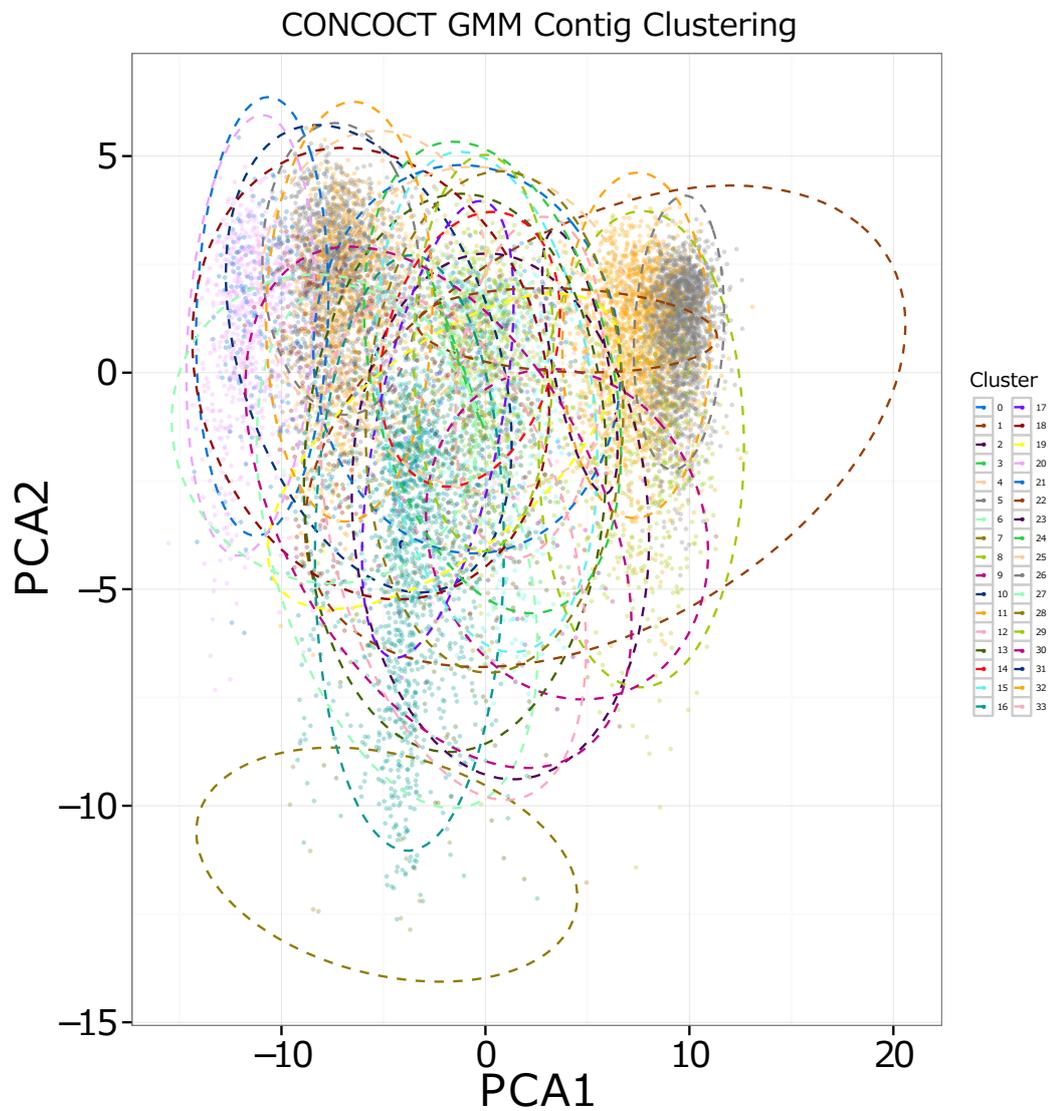
However, recall was relatively poor (0.542250) suggesting a fair number of  $FN$  i.e. contigs with the same taxonomic assignments were not confined to a single cluster and were spread over

Source Group	Number of Contigs	Total Length	Phylum-Level Breakdown
<b>Host</b>	13	38,209	Intramacronucleata
-	2	140	Apicomplexa
-	1	163	Colponemidia
<b>Endosymbiont</b>	12	2,758	Chlorophyta
-	12	3,987	Streptophyta
-	1	1,674	Cyanobacteria
<b>Bacterial Contamination</b>	16,230	13,435,718	Proteobacteria
-	468	669,751	Firmicutes
-	329	135,928	Actinobacteria
-	128	68,337	Bacteroidetes/Chlorobi group
-	1	241	Deinococcus-Thermus
<b>Eukaryotic Contamination</b>	605	640,915	Ascomycota
-	380	206,354	Chordata
-	74	38,529	Arthropoda
-	12	3,623	Basidiomycota
-	7	2,150	Nematoda
-	1	61	Platyhelminthes
-	1	102	Cnidaria
<b>Unknown</b>	540	345,834	Unclassified

**Table 3.4.3:** Summary of taxonomic assignments via TAXAassign grouped into putative “source groups” reflecting the most probable source of 10kb chunked contigs of that specific taxonomic provenance. Of note, is the disproportionate number of contigs from contaminating sources. Specifically, bacteria such as Firmicutes and potential user contaminant in the form of Chordate assigned contigs.

-	Positive	Negative
True	Contigs with <b>same</b> taxonomic assignment are assigned to the <b>same</b> cluster	Contigs with <b>different</b> taxonomic assignments are assigned to <b>different</b> clusters
False	Contigs with <b>different</b> taxonomic assignments are assigned to the <b>same</b> cluster	Contigs with the <b>same</b> taxonomic assignment are assigned to <b>different</b> clusters

**Table 3.4.4:** A contextual explanation of True and False Positive and Negatives in the context of contig binning/clustering. Top left indicates what a True Positive (TP) means in this context, bottom left a False Positive (FP). Similarly Top Right explains a True Negative (TN) and Bottom Right a False Negative (FN)



**Figure 3.4.6:** A low dimensional Principal Component representation of genomic contig cluster assignments. Clusters are assigned via a Gaussian Mixture Model (GMM) based on sequence compositional and coverage features as implemented in CONCOCT. Unfortunately, as can be observed clusters are both poorly distinguished even in the dimensions of the 2 principal components (PCA1 and PCA2) and there are many clusters (34). This figure highlights that the single cell metagenome decomposition is poorly resolved and noisy.

Bin	Number of Contigs	Total Size (in bp)
Endosymbiont	782	1,767,324
Host	3,451	24,294,611
Other Eukaryotic	1,646	7,237,238
Bacterial and Unknown	15,211	26,342,475

**Table 3.4.5:** Results of the customised taxonomic binning, note the far less conservative binning compared with TAXAssign. This analysis only consisted of contigs over 500 bp in length

main clusters.

The  $F_1^1$  score for CONCOCT clustering was therefore 0.680389 under the assumption that TAXAssign represents the ground-truth.

It is also worth noting that the 34 clusters had a relatively high level of mutual information (Normalised Mutual Information of 0.332022 and a Rand Index of 0.499741) suggesting many small but highly similar clusters were created. This level of similarity combined with the poor recall suggests a greater number of clusters were inferred than was present in the taxonomic assignment ground-truth. This is likely due to the variational inference of cluster numbers being partially reliant upon sequencing coverage features. As MDA is known to generate very uneven coverage due to amplification biases this likely explains the erroneous clustering.

Therefore, clustering and TAXAssign binning methods were abandoned and the custom ORF based pipeline bins (table 3.4.5) used for variant calling.

#### 3.4.2.4 VARIANT CALLING

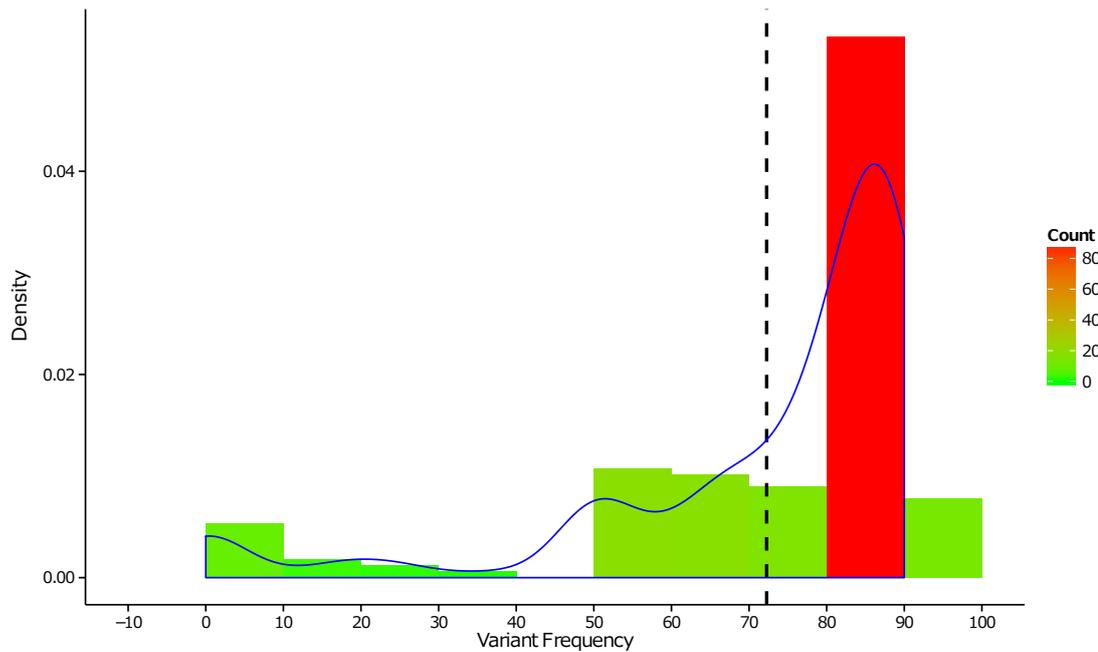
The variant calling demonstrated that the majority of potential variants were present in almost all endosymbiont genomes (fig. 3.4.7). Indeed, the highest number of variants ( $\geq 75$ ) were present in 80 – 90% of endosymbionts.

#### 3.4.3 ELIMINATION OF ENDOSYMBIONT

All elimination analyses focused on the *P. bursaria*-*M. reisseri* CCAP 1660/12 strain. After 1 week  $10\mu\text{gml}^{-1}$  paraquat treated CCAP 1660/12 were partially bleached with few visible green *M. reisseri* present in the cells under light microscopy. Unfortunately, after 2 weeks, and despite regular

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$^1F_1 = 2 * \frac{(\text{precision} * \text{recall})}{(\text{precision} + \text{recall})}$



**Figure 3.4.7:** Histogram summary of variant frequency in the longest 10 custom binned “endosymbiont” contigs (total length 269.755 bp). Variant frequency is the percentage of total chromosomes in the sample that share that variant and y axis shows the density (blue line). The vertical dotted line indicates the mean of the sample and the heatmap shows absolute counts in the histogram bins. This figure shows that the majority of variants are present in the majority of endosymbiont cells within the host.

feeding, all CCAP 1660/12 treated with paraquat appeared to die with lysis of the *Paramecium*.

To assess whether this phenotype was due to a too great concentration of paraquat the experiment was repeated at a lower concentration ( $1\mu\text{gml}^{-1}$ ). Unfortunately, this led to the same process of gradual bleaching of the CCAP 1660/12 cultures followed by their death. The difference being at the lower concentration this occurred over a 6 week time frame instead of 2 weeks.

A similar pattern was observed with both concentrations of cycloheximide where  $10\mu\text{gml}^{-1}$  treatment led to a reduction in endosymbiont abundance by 90% after 1 week followed promptly by host death. The lower concentration  $1\mu\text{gml}^{-1}$  displayed the same pattern but over a 6 week period.

Finally, with subculturing and feeding cultures maintained in constant darkness did lead to gradual bleaching over 4-8 weeks. However, after 10 weeks the cultures died with no visible *P. bursaria* cells.

## 3.5 DISCUSSION

### 3.5.1 CCAP 1660/12 AND CCAP 1660/13 CONTAIN LARGELY CLONAL *M. REISSERI* SYMBIONTS

Phylogenetic analysis demonstrates that the endosymbiont present in the CCAP 1660/12 and CCAP 1660/13 is *M. reisseri*. The ITS<sub>2</sub> sequences derived from these two cultures across five different biological replicates (using both primer sets and with or without extra purification steps to minimise contamination from any algae present in the media) were all identical (with the exception of individual SNPs) to 3 separate previously published *M. reisseri* ITS<sub>2</sub> sequences. That this formed a well supported clade with other *Micractinium* sequences and was clearly a distinct grouping from the other *P. bursaria* endosymbiotic green algal species further supports the identity of the 1660/12 and 1660/13 endosymbionts as *M. reisseri*.

While 8 different SNPs were identified in the ITS<sub>2</sub> sequences, these never occurred in the same sequence and half are easily attributable to sequencing error as they couldn't be recapitulated in reverse sequencing of the same clone. Of the remaining 4 SNPs that were validated as not being sequencing error, only 1 was discovered in separate PCR reactions and biological replicates and thus can putatively be attributed to genuine biological diversity and not merely PCR error (ITS<sub>2</sub>-6 and ITS<sub>2</sub>-A7, A to G transition). Therefore, on the basis of ITS<sub>2</sub> sequences we cannot say the endosymbionts in CCAP 1660/12 and 1660/13 form a clonal population. However, a single SNP in the hypervariable ITS<sub>2</sub> region represent very recent and minor divergence. The most likely explanation is that this represents the emergence of a slightly modified line of endosymbionts within the clonal endosymbiont population of the CCAP 1660/12 culture or intranuclear variation within a single clonal population. The distribution of SNP variants on endosymbiont binned contigs supports this hypothesis. This is because the majority of SNPs were detected to be present in 73% or more of endosymbiont chromosomes. Due to the uniformity of the ITS<sub>2</sub> sequences there is no evidence of multi-strain photobiont co-habitation as described by (Hoshina, 2012).

It should be noted that the majority of ITS<sub>2</sub> based studies make use of the secondary structure (predicted using tools such as RNAstructure (Mathews et al., 2004)) in inference (Schultz and Wolf, 2009). This increases reliability of phylogenetic inference (Keller et al., 2008) allows ITS<sub>2</sub> to be used to distinguish higher taxonomic levels (Coleman, 2003), and plays a role in re-

solving the thorny problem of species determination (Müller et al., 2007). However, as the endosymbiont species ITS2 secondary structures have already been extensively investigated (e.g. (Hoshina and Imamura, 2008; Hoshina et al., 2010)) and are generally better suited for analysis of more divergent taxa, it was considered unnecessary to conduct structural analysis for taxonomic analysis of these endosymbionts.

### 3.5.2 RELIABILITY OF CULTURE COLLECTIONS

One clear result and point worth raising is that contrary to previous studies (accession AB260896.1 (Hoshina and Imamura, 2008)) and CCAPs culture description CCAP 1660/13 does not contain a *Coccomyxa* endosymbiont and contains an identical *M. reisseri* endosymbiont to the CCAP 1660/12 culture. Unfortunately, on communication with CCAP it emerged that the 1660/12 strains in their collection are no longer available and that CCAP 1660/13 had apparently become overgrown by free-living *Coccomyxa*. Therefore it is likely that the previous finding of *Coccomyxa* “endosymbionts” in CCAP 1660/13 (Hoshina and Imamura, 2008) represents accidental contamination and sequencing of the free-living *Coccomyxa* also present in the culture.

The identical nature of the CCAP 1660/12 and CCAP 1660/13 endosymbioses is perhaps not surprising when it is emphasised that these cultures were isolated from the same pond (Cambridge, UK) by CCAP.

This demonstrates the necessity of not taking culture collection labels and taxonomic assignments on faith. It is critical to thoroughly determine that all received cultures actually contain the organism.

### 3.5.3 MDA METAGENOMES ARE NON-TRIVIAL

The biases induced by MDA in single cell genomes are known to be formation of chimeric sequences and the amplification of undesired contaminant sequences (Binga et al., 2008). Additionally, despite a theoretical basis that the amplification coverage bias should be random (Hosono et al., 2003) there is evidence disputing this in practice (Ellegaard et al., 2013b). The magnitude of this bias is related to the starting quantity of DNA (Ellegaard et al., 2013a). Fortunately, there does not appear to be any bias related to GC (Ellegaard et al., 2013a). An increase in the number of starting cells to the range of a few hundred to a few thousand cells has been observed to improve amplification considerably (Ellegaard et al., 2013a). Unfortunately, increasing the number

of cells in the case of CCAP 1660/12 *P. bursaria* - *M. reisseri* system would likely compound issue with bacterial contamination due to both a greater sample volume leading to greater inclusion of food bacteria living in the media and an increase in the number of partially digested bacterial (and viral) symbionts associated with the host.

SPAdes, by far, generates the best assemblies of complex MDA-based metagenomes of the assembly tools trialled. This cannot be attributed to the effective read error correction implemented as part of SPAdes via BayesHammer as all assemblies were completed on BayesHammer error corrected reads. The performance of SPAdes is likely attributable to two factors: it is specifically designed to handle MDA-based single cell assemblies and thus is highly tolerant of the coverage variability observed and secondly it is the lone genome assembler that effectively utilised paired-end data during assembly. The vast majority of assemblers will only utilise this data in ad-hoc post-assembly heuristic operations to improve contigs and scaffold the dataset. On the other hand, SPAdes generates the assembly DBG using siamese rectangular graphs that incorporate both forward and reverse reads and their respective insert. In future, it may be worth re-analysing this data using other MDA-specific tools such as HyDA to assess their performance. Additionally, likelihood/probabilistic methods such as CGAL (Rahman and Pachter, 2013) or ALE (Clark et al., 2013) could be applied to the problem of genome assembly assessment instead of somewhat arbitrary individual metrics.

The relative performance of Q30-SPAdes with and without the “careful” setting is interesting. This setting minimises the risk of mismatch and indels found in the assembly. This led to assembly with statistics relatively similar to that of the Q5-SPAdes assembly. However, on correspondence with the developers of SPAdes it emerged that there was a bug in this setting in the version of the assembler used within this study leading it to be highly conservative and discard many assembled contigs that were unlikely to be mismatches.

Finally, the poor performance of CONCOCT suggests that coverage and composition are not effective metrics by which to decompose an MDA-based metagenome into constituent “bins”. The poor recall and high similarity indices between the clusters suggests that a greater number of clusters were inferred than was present in the ground truth of the taxonomic assignments. This likely represents the effect of biased amplification in MDA (therefore heterogeneous variable coverage) on the variational inference of the number of clusters and the utility of the coverage feature

in general. This means, therefore, in MDA-based metagenomes standard metagenomic binning pipelines that are reliant on coverage metrics (even partially as in the case of CONCOCT) are not effective.

This problem is somewhat symptomatic of the current state of the tool ecosystem for MDA-based eukaryotic metagenomes. The few MDA-orientated analysis tools focus on the assembly of bacterial systems whereas the majority of the metagenomic tools are based on features and metrics such as coverage that are only consistent in conventional non-MDA bulk genomic studies. Ideally, future research will improve the ease of analysis and assembly of datasets such as this.

#### 3.5.4 METABOLIC CO-DEPENDENCE IN THE CCAP 1660/12 SYSTEM

Due to the repeated failure to create endosymbiont free *Paramecium* hosts from the CCAP 1660/12 cultures using 3 of the major accepted methodologies (cultivation in darkness (Karakashian, 1963), paraquat (Hosoya et al., 1995; Tanaka et al., 2002) or cycloheximide (Weis, 1984)) we are forced to address the possibility that the *Micractinium reisseri* endosymbiont and *P. bursaria* system in CCAP 1660/12 and CCAP 1660/13 cultures forms an obligate system. By some unidentified mechanism, metabolic co-dependence may have become fixed in this culture.

Cycloheximide does partially inhibit host protein synthesis (Weis, 1984; Kodama et al., 2007; Kodama and Fujishima, 2008, 2009) therefore it is possible that in the host strain found in the CCAP 1660/12 culture that this partial inhibition is lethal to both host and endosymbiont. However, the failure of this method in conjunction with the paraquat, a herbicide which theoretically should only affect the endosymbiont, and constant dark culturing suggests it is potentially the loss of the endosymbiont that is lethal to the host cells (as adequate bacterial foodstocks were included in these cultures).

The one major method that wasn't attempted was the use of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) an established blocker of photosystem II (van Gorkom, 1974). However, DCMU has previously been found to be mildly toxic in *P. bursaria*, affecting the sexual reproduction system (Miwa, 2009) therefore, this would have proven unlikely to show different results in either the case of a particularly "sickly" host strain or obligate endosymbiosis.

This result indicates the presence of key differences between the current state of this endosymbiosis and the previously studied *C. variabilis* endosymbiosis studied by (Kodama and Fujishima, 2014). Therefore, a comparative analysis of these systems could theoretically shed light on the

mechanism by which metabolic co-dependence has become fixed in one system. Alternatively, this difference may just reflect the nature of two different, independently acquired endosymbioses with different species and strains of both host and endosymbiont.

Another avenue of study that we did not investigate was that of isolation of endosymbiont into free-living cultures (Achilles-Day and Day, 2013a). This would allow us to establish whether green algae such as *Micractinium* that have obligate hosts are themselves obligate endosymbionts. There is some evidence pointing towards this in nature, with the widespread predation of *M. reisseri* and *C. variabilis* by their specific PBCV virotypes as well as the relative paucity of natural free-living strains of these species. To my knowledge, there has only been a single isolated and characterised free-living *M. reisseri* (Abou-Shanab et al., 2014) example and no *C. variabilis* examples. However, this said, algae have previously been isolated from the CCAP 1660/13 culture (Achilles-Day and Day, 2013a). We have demonstrated via ITS<sub>2</sub> sequencing that the endosymbionts in CCAP 1660/13 are the same as those in CCAP 1660/12. Therefore, if these isolated algae are actually endosymbionts (as supposed to the free-living *Coccomyxa* sp. that overgrew the culture shortly after this study was published) then the *M. reisseri* endosymbiont is capable of living without the host and is not an obligate endosymbiont despite *P. bursaria* being an obligate host.

### 3.6 CONCLUSIONS

Therefore, on the basis of ITS<sub>2</sub> sequencing the CCAP 1660/12 culture endosymbiont is a strain of *Micractinium reisseri*. Additionally, the CCAP 1660/13 endosymbiont has been misclassified as a strain *Coccomyxa* and is the same *Micractinium reisseri* species found in the CCAP 1660/12 culture. I have confirmed the Yad1g1N endosymbiont as being *C. variabilis* 1N. Despite poor performance in genome assembly, the evidence of the genomes and ITS<sub>2</sub> data seem to indicate that this endosymbiont forms a clonal or near clonal population within the CCAP 1660/12 endosymbiont. Similarly, on the basis of ITS<sub>2</sub> diversity the Yad1g1N culture contains a clonal 1N endosymbiont population. At a minimum, a single strain of *M. reisseri* comprises the sole green algal endosymbiont in the CCAP 1660/12 and 1660/13 cultures although there may be intranuclear variation of the ITS<sub>2</sub> or it may be actively evolving as evidenced by a small divergent sub-population.

Finally, the *Micractinium reisseri* endosymbiont in cultures CCAP 1660/12 CCAP 1660/13 has potentially become metabolically co-dependent with the host. The host appears incapable of survival without the endosymbiont, therefore, it is important to attempt to identify the differences between the demonstrably facultative relationship between the Japanese Yad1g1N strains (used in (Kodama and Fujishima, 2014)) and the putatively obligate CCAP 1660/12. Identifying these differences may pinpoint the mechanism by which metabolic co-dependence becomes fixed in *P. bursaria* - green algal endosymbioses.

"Look on my works, ye Mighty, and despair!"

- Percy Bysshe Shelley: *Ozymandias*, 1818

# 4

## Transcriptomic analysis of the *Paramecium bursaria* and *Micractinium reisseri* endosymbiosis

### 4.1 INTRODUCTION

The *Paramecium bursaria*-*Micractinium reisseri* (*PbMr*) endosymbiosis conveys phototrophy (Karakashian, 1963), numerous photobiological traits (e.g. (Berk et al., 1991; Saji and Oosawa, 1974; Nakajima and Nakaoka, 1989; Niess et al., 1982b; Iwatsuki and Naitoh, 1988; Summerer et al., 2009)), partially reviewed in (Sommaruga and Sonntag, 2009)) and its establishment and maintenance is dependent on photosynthetic activity and enigmatic light-induced factors (Karakashian, 1963; Hosoya et al., 1995; Kodama et al., 2007; Kodama and Fujishima, 2014). Therefore, a relatively unbiased global metatranscriptomic profile of host and endosymbiont in both lit and dark conditions would potentially identify key transcripts which play a role in the establishment, maintenance, and characteristics of this endosymbiosis.

“Dual-RNAseq” is a form of transcriptomics which characterises transcripts in a small number of defined organisms simultaneously (Westermann et al., 2012). It has proven an effective method in several studies investigating host-chloroplast interactions (Nowack et al., 2011; Jiggins et al., 2013; Xiang et al., 2015), and host-pathogen systems (Tierney et al., 2012; Kawahara et al., 2012; Jones et al., 2014; Hayden et al., 2014). It differentiates itself from both standard metatranscriptomics, such as those common in microbial ecology (Poretsky et al., 2005; Aliaga Goltsman et al., 2014), by being conducted on samples of known, or mostly known composition, and from classical transcriptomics by not depending on axenic samples.

*Paramecium bursaria* and its green algal endosymbionts form a system well-posed for “dual-RNAseq” analysis. Firstly, there is a plethora of literature on the physiology and behaviour of host and endosymbiont, both together and individually (e.g. (Iwatsuki and Naitoh, 1988), see (Kato and Imamura, 2009b) and the Introductory Chapter for more details), presenting a key resource by which results can be contextualised. Additionally, transcriptomic analysis has proven feasible in reasonably close relatives of both host (Arnaiz et al., 2010; Kolisko et al., 2014) and endosymbiont (Guarnieri et al., 2011; Rowe et al., 2014; Bashan et al., 2015). Even more promisingly, there has been an analysis of the host-endosymbiont system (although in a different strain: Yad1g1N) (Kodama et al., 2014). Unfortunately, this study focused only on the expression pattern of the host alone with and without its endosymbiont and discarded endosymbiont derived data during analysis.

This said, the *PbMr* system does also present some severe difficulties in terms of its transcriptomic tractability. Specifically, the system is highly genomically and transcriptomically complex with *P. bursaria*’s ciliate nuclear dimorphism and high order polyploidy (Raikov, 1995), there is sexual reproduction in both host (Jennings, 1939) and endosymbiont species (Blanc et al., 2010), and the system has a large range of GC biases (Kodama et al., 2014). Therefore, care must be taken to optimise sequencing, and assembly methods, to mitigate these complications.

These difficulties are compounded by the lack of available reference genomes for either *Paramecium bursaria* or *Micractinium reisseri* and thus necessitating *de novo* transcriptome assembly. However, the utility of sequenced genomes from divergent ciliate species (i.e. *Tetrahymena thermophila* (Eisen et al., 2006), *Paramecium tetaurelia* (Aury et al., 2006) and *Paramecium caudatum* (McGrath et al., 2014)) and endosymbiotic green algae *Chlorella variabilis* NC64A (Blanc et al., 2010)

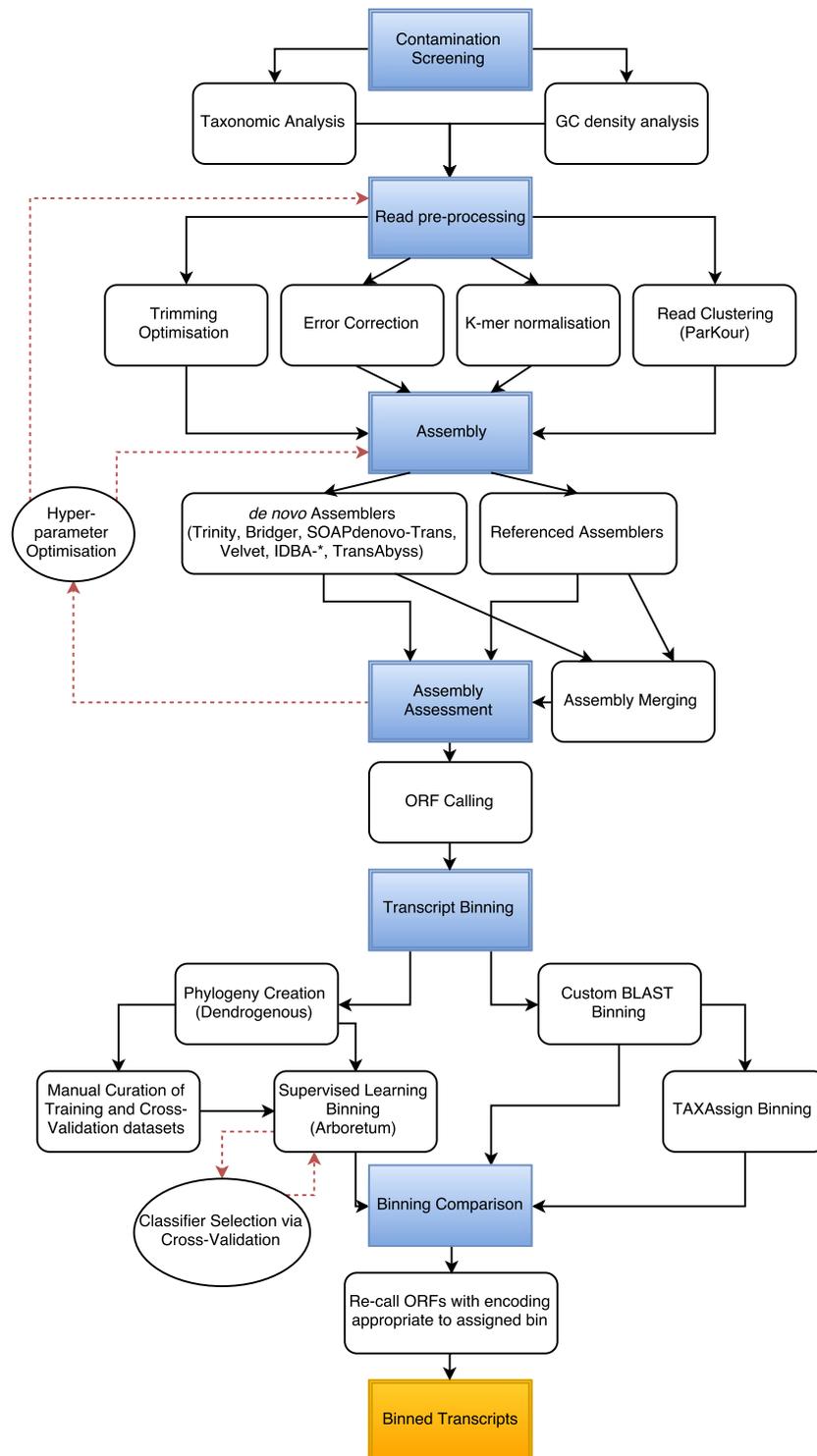
and *Coccomyxa subellipsoidea* C-169 (Blanc et al., 2012) (see fig. 1.2.3 in the Introductory Chapter and fig. 3.4.1 in Chapter 1 for respective phylogenetic context of these genomes) as references for assembly was investigated. It should also be noted that the existing *Paramecium bursaria* (Kodama et al., 2014), *Paramecium duboscqui* (Kolisko et al., 2014) and *Chlorella vulgaris* (Guarnieri et al., 2011) transcriptomes mentioned above were successfully recapitulated *de novo* (without a reference genome).

The mixotrophic nature of the host *Paramecium* (Dolan, 1992) means there are partially digested bacterial prey species, as well as numerous associated bacteria (Görtz and Fokin, 2009; Fokin and Görtz, 2009; Schrollhammer and Schweikert, 2009) and viruses (Van Etten et al., 1983) which all present potentially obfuscating sources of contamination in the analysis of host-endosymbiont interaction. Therefore, it is key to effective analysis of this system to develop methods that minimise the effects of contamination at all stages of analysis. To address this, we investigated methods to reduce contamination during library preparation such as washing steps, cell picking and single cell sequencing techniques; methods to screen and/or filter sequenced libraries for contaminants before inclusion in assembly and methods to effectively sort assembled transcripts into bins relating to their likely originating organisms (i.e. “host”, “food” or “endosymbiont” derived).

To this end, bulk RNAseq libraries from cultured *PbMr* were sequenced using 76 bp paired-end reads and the Illumina Gene Analyzer II platform taking care to minimise contamination by filtering and washing cultures and carefully assessing culture health to maximise the number of healthy *PbMr* sequenced. Unfortunately, due to limitations in the maintainable culture density of the *Paramecium bursaria* CCAP 1660/12 and thus the quantity of extractable mRNA it was necessary to pool all day and night replicates into a single pair of day and night libraries.

While this provided sufficient material for sequencing it precluded accurate inference of differential expression between day or night by masking the biological replicates (Auer and Doerge, 2010). We, therefore, also sequenced a set of 3 (followed later by an additional 5) dark and 3 light biological replicates using single-cell RNAseq (sc-RNAseq) methods. This also allowed a finer-grain control over cell selection and potentially a method to reduce culture based contamination.

While reasonably new, sc-RNAseq has shown a lot of promise in well characterised systems such as human cell cultures (Bengtsson et al., 2005; Shalek et al., 2013) and *Saccharomyces cerevisiae* (Lipson et al., 2009) and there are high expectations of their utility for “dual-RNAseq”



**Figure 4.1.1:** A flowchart summarising the full analysis and transcript binning of the *PbMr* single cell transcriptome data. Key stages are indicated by square boxes and blue colouring, individual analyses are shown in rounded square boxes and iterative parameter optimisation steps are shown in circles and highlighted with red-dotted dependency arrows.

(Westermann et al., 2012). sc-RNAseq addresses the key difficulties of analysing unculturable or poorly culturable organisms (Murray et al., 2012) and investigating cell-cell heterogeneity in

expression patterns (Raj and van Oudenaarden, 2008; Shalek et al., 2013). Uninvestigated this heterogeneity (either from biological and/or genomic variance or just the stochasticity of gene expression) can lead to a Yule-Simpson effect (Yule, 1903; Simpson, 1951), where the false amalgamation of distinct expression patterns in previously cryptic but distinct cellular subpopulations can generate a spurious expression pattern contrary to either subpopulation.

There are a range of possible sc-RNAseq methods, however, we used Qiagen's Repli-G Whole Transcriptome Amplification (WTA) MDA-based kit. This was due to MDA being well established and characterised in single cell genomics e.g. (Spits et al., 2006), having a simple methodology not requiring additional equipment, and claims suggesting that MDA is more successful at recovering transcripts from a wide range of abundance levels than other methods i.e. recovers many lowly expressed transcripts<sup>1</sup>. Unfortunately, despite the publication of empirical comparisons of single cell transcriptomic methods (Wu et al., 2014), Qiagen's Repli-G WTA MDA-based kit has yet to be directly assessed relative to other approaches and thus its performance has not been independently verified. Briefly, this method involves the ligation of reverse transcribed cDNAs using oligo-dT primers (after lysis and removal of gDNA) before MDA by a  $\phi$ 29 DNA polymerase with a 5'-3' exonuclease proofreading activity (Korfhage et al., 2015) (reducing error-rate of amplification to  $9.5 \cdot 10^{-6}$  errors per nucleotide (Paez et al., 2004) compared with  $10^{-4}$  to  $10^{-5}$  for Taq (Tindall and Kunkel, 1988; Eckert and Kunkel, 1990)).

Unfortunately, despite its utility sc-RNAseq generates a new set of difficulties. First and foremost, there has only been a single published use of sc-RNAseq, to my knowledge, in non-model unicellular eukaryotes. This study by (Kolisko et al., 2014), briefly addressed the issues of bias, contamination and gene discovery effectiveness in a set of model and non-model eukaryotes and constitutes an important proof-of-concept. However, it also used a different sc-RNAseq approach (SMRT), focused on single organisms, and didn't address, in-depth, the optimal way to process, assemble and utilise single cell datasets from protists. While some work has been done investigating the optimal pre-processing of bulk RNAseq datasets, e.g. (Macmanes and Eisen, 2013; Macmanes, 2015), the effect of different trims and error correction on sc-RNAseq has yet to be characterised. There are also some early indications that cryptic bacterial contamination from samples and/or reagents in sc-RNAseq can be particularly problematic (Kolisko et al.,

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<sup>1</sup><https://www.qiagen.com/gb/shop/sample-technologies/rna-sample-technologies/total-rna/repli-g-wta-single-cell-kit/> as of 2015/08/25

2014). This further increases the importance of library screening and post-assembly transcript binning.

## 4.2 AIMS

Therefore, this chapter will investigate the optimal use of 2nd generation bulk and sc-RNAseq libraries in characterising a complex reference-free system. Specifically, it will look at the screening of RNAseq libraries for contamination before assembly, the optimal preprocessing (partitioning, trimming, digital normalisation and error correction), assembler and assembly parameters (including the utility of divergent reference genomes from related species) in recapitulation of host and endosymbiont transcripts. Finally, I will address the problem of the attribution of recovered transcripts into their appropriate likely originating organism.

## 4.3 METHODS

### 4.3.1 SAMPLE PREPARATION AND SEQUENCING

#### 4.3.1.1 BULK TRANSCRIPTOME RNA PREPARATION

For bulk transcriptomic analyses CCAP 1660/12 cells were harvested in a way to minimise contamination from bacterial prey species in the culture. Aliquots consisting of  $\sim 10^6$  cells were strained through 40  $\mu\text{m}$  sieves, filtered on 10  $\mu\text{m}$  nylon filters, before finally being filtered on 8  $\mu\text{m}$  TETP polycarbonate filters using a low-pressure filtration pump. Collected samples were either immediately quick-frozen in liquid nitrogen for storage ( $-20^\circ\text{C}$  for short-term storage and  $-80^\circ\text{C}$  for longer storage) or harvested by centrifugation. In order to investigate the two main metabolic states of the symbiosis (i.e. under light conditions during active photosynthesis and in the dark when no photosynthesis is taking place) samples were extracted 5 hours into the light and dark phase of the 12:12 hour day-night cycle.

To ensure extracted RNA was representative of healthy and interacting host and endosymbionts care was taken to minimise the number of dead/dying cells from which RNA was extracted. In order to do this, a subsample was taken from each culture during the process of harvesting and scored for dead/dying cells. Cell assays were formed by taking 1-2 ml of each harvest cell pellet and fixed using 40  $\mu\text{l}$  Lugol's solution (0.5 g  $\text{I}_2$  and 1g KCl in 8.5ml of MilliQ water). Dead/dying

cells were identified as broken or puckered cells and counted using light microscopy. Samples containing > 10% dead/dying cells were discarded and no RNA extracted from them.

In order to lyse collected samples, cells were washed from the filter or the pellet was resuspended in 1 ml TriReagent (Sigma) heated to 60 °C. Cells were vortexed with sterile 300 µm glass-beads for 15 s, incubated at room temperature for 10 min, vortexed for 15 s, quick-frozen in liquid nitrogen and stored at -20 °C before further processing. Samples were defrosted, vortexed for 15 s, placed in a heat-block set to 60 °C for 10 minutes while continuing to be vortexed, removed from heating and vortexed again for 15 s. RNA was extracted by adding 0.2 µl of chloroform to the glass-bead-trizol-sample solution, shaking for 15 s, incubating for 5 minutes at room temperature and centrifuging at 12,000g for 15 min at 4 °C. The upper-phase was then transferred to an RNase-free 1.5 ml tube and an equal volume (~0.5 ml) of isopropanol was added before shaking for 15 s. The isolated RNA was then incubated at -20 °C for 10 min (up to several hours) before being collected as a pellet using a centrifuge at 10,000g for 10 min at 4 °C (supernatant was discarded). The RNA pellet was then washed with 1 ml of 75% ethanol and centrifuged twice at 10,000g for 10 minutes at 4 °C with the supernatant being discarded after each centrifugation. The pellet was then dried before being resuspended in 100 µl of RNase-free water. The RNA was cleaned further using the Qiagen RNeasy clean-up kit before being assessed for quality using ND-1000 (NanoDrop) and BioAnalyzer (Agilent).

#### 4.3.1.2 SINGLE CELL RNA PREPARATION

For single cell transcriptomics, a “cell-picking” approach was used in which *P. bursaria* cells (from the CCAP1660/12 culture) were inspected on an inverted light microscope before being picked using an orally aspirated drawn-glass Pasteur pipette (Garcia-Cuetos et al., 2012). In order to minimise contamination from food bacteria present in the media these picked cells were washed 3 times by serial transfer to 10 µl droplets of sterile NCL media. The washed cell was then transferred to a 10 µl droplet of sterile water. Cells were picked 5 hours into both the lit and dark phase of the 12:12 hour day-night cycle identically to the bulk analyses. As cells were picked individually, health status could be exhaustively assessed during picking and therefore the subsampling and scoring method used to check the status of cells in bulk preparations was unnecessary.

cDNA was generated and amplified using the MDA-based Qiagen REPLI-g WTA Single Cell Kit (Korfhage et al., 2015) with additional cell disruption steps. Specifically, cells were transferred

from their respective 10  $\mu$ l droplets of sterile water to a PCR tube containing 6  $\mu$ l water and 4  $\mu$ l lysis buffer. Due to the robust chitin cell walls of *M. reisseri* (Kapaun and Reisser, 1995) it was important to ensure thorough cell lysis. Therefore, samples underwent mechanical disruption by bead beating (Sigma, 425-600  $\mu$ m, acid-washed) followed by freeze-thaw via submersion in liquid nitrogen for 5 s. In order to compare disruption methods, extractions and amplifications were also conducted using just lysis buffer, bead beating and vortexing (i.e. without freeze-thaw), and just the lysis buffer. Samples were then quantified using a ND-1000 (NanoDrop) and as extraction methods produced near identical DNA concentrations the maximal disruptive method of freeze-thaw, beat beating, vortexing and lysis buffer described above was used for further purification and library preparation. The samples were then vortexed for 1 min before a gDNA removal step.

mRNA was selectively amplified and reverse transcribed to cDNA using poly-A selection (i.e. oligo-dT) primers to prevent amplifying ribosomal sequences. Prior to MDA by a  $\phi$ 29 DNA polymerase cDNA were ligated into long fragments due to lower MDA efficiency for short fragments (Korfhage et al., 2015). This reduces size-dependent amplification bias but could potentially lead to the creation of chimeric transcripts in which paired-reads cross boundaries of adjacently ligated cDNA transcripts.

The amplified cDNA was then purified using a QIAamp DNA mini kit and eluted in 100  $\mu$ l elution buffer. This kit operates by binding the DNA to a QIAamp membrane in a spin column followed by successive washing steps to remove impurities such as remaining proteins and cations. This led to the creation of 3 dark cDNA libraries (Dark1-2, Dark1-3, Dark1-5) and 3 light libraries (Light1-9, Light1-10, Light1-11).

Due to low quantities of eukaryote identifiable reads in the initial 3 sequenced single cell dark libraries a set of additional single cell extractions were conducted. These followed the same protocol as above but also featured an additional final PCR-based screening of synthesised cDNA using primers specific for *Paramecium* Bug22 sequence. Bug22 is a highly conserved ciliary protein found in a large number of organisms including the ciliates (Smith et al., 2005b; Laligne et al., 2010), green algae (Keller et al., 2005; Laligne et al., 2010; Meng et al., 2014), higher plants (Hodges et al., 2011), and animals (Mendes Maia et al., 2014). Therefore, this was used as a marker for *Paramecium* derived cDNA. Primers used were Bug22BFW and Bug22BREV (table 4.3.1) under standard PCR conditions.

Primer	Sequence
Bug22BFW	GCATTCTAGACCAATCTGGCTTTCTGTCAA
Bug22BREV	GCATTTCGAATTGAGGCTCTAAATCTTCTTCTCA

**Table 4.3.1:** Sequences of the Bug22 primers used to screen cDNA

Five (Dark2-2, Dark2-3, Dark2-6, Dark2-7, Dark2-8) samples with bands of appropriate size were then taken forward for library preparation and sequencing.

#### 4.3.1.3 LIBRARY PREPARATION

For both bulk and single cell preparations each cDNA sample was fragmented in 130  $\mu$ l 1xTE buffer on the Covaris E220 with a target size of 225bp (duty factor of 10%, 200 cycles per burst, peak incident power of 175, 200 s at 7 °C). Fragment sizes were checked on a BioAnalyzer (Agilent) 7500 DNA chip. cDNA was then concentrated using a GeneRead kit column with an elution in 35  $\mu$ l. Fragmentation step was then repeated 3 times (110 s) until majority of cDNA in each library was between 200-250 bp.

cDNA ends were then end-repaired, adenylated and adapters ligated using the NEXTFlex (Bioo scientific) sequencing kit according to the manufacturer's instructions and using NEBNext (New England Biolabs) indices. Also following the NEXTFlex kit instructions, MgNa bead purification was done before and after PCR amplification using NEBNext reagents. Finally, prepared libraries were size selected using a Blue Pippin machine at a size selection of 350 bp (range 315-385 bp).

A final bioanalyzer step was conducted with individual library concentrations ranging from 0.66-4.09 nmol.

#### 4.3.1.4 SEQUENCING

The bulk day and night library were paired-end (PE) 76 bp sequenced using an Illumina Genome Analyzer II by the Exeter University Sequencing Service. The two libraries were sequenced on separate flowcells (Bulk-Light, Bulk-Dark).

Single cell libraries were paired-end 150 bp using an Illumina HiSeq 2500 by Exeter Sequencing Service. 3 dark (Dark1-2, Dark1-3, Dark1-5) and 3 light (Light1-9, Light1-10, Light1-11) samples were multiplexed sequenced on a single flowcell lane. The 5 additional dark samples (Dark2-2, Dark2-3, Dark2-6, Dark2-7, Dark2-8) were multiplexed and sequenced on a single flowcell lane

in a separate sequencing run.

#### 4.3.2 LIBRARY CONTAMINATION SCREENING

##### 4.3.2.1 TAXONOMIC ANALYSIS

Sequenced libraries were initially screened using the standard metrics implemented in the FastQC to check for standard sequencing issues such as flowcell defects, library degradation, and adapter read-through (Andrews, 2015).

To further investigate potential contamination, a taxonomic profile and GC% probability density was determined for each library.

The former was conducted using a custom tool dubbed “DueyDrop” which functions as follows. Briefly, for each library 5 batches of 10,000 PE reads were sampled using the reservoir sampler (Vitter, 1985) implemented in Heng Li’s seqtk library (Li, 2015). While 5 batches of 10,000 reads should be equivalent to 50,000 random samples by using batches and changing the random seed any potential problems from poor randomisation implementation was achieved. Furthermore, the batches allowed easy comparison of the consistency of taxonomic profiles. These randomly sampled reads were subsequently aligned to NCBI’s Protein NR RefSeq database (Pruitt et al., 2007) using the efficient short-read optimised BLASTX implementation of DIAMOND (Buchfink et al., 2015) (at a expectation of  $e^{-5}$ ) and top hits for each read retained. Gene identifiers (GI) were extracted from these top hits and queried against a local copy of the NCBI taxonomy database (Federhen, 2012) to recover a hit taxonomic lineage for each read that aligned to a sequence within NR database. These lineages were then interactively tallied at several different taxonomic levels (e.g. domain level - eukaryote vs bacteria, or lower level - viridiplantae vs ciliate) and variances calculated. Results were then tabulated and libraries compared to assess whether any libraries appeared aberrant. This whole analysis was repeated for both untrimmed reads and reads quality trimmed to a high quality threshold of an average Q30 over a sliding window of size 4 using Trimmomatic (Bolger et al., 2014) to assess the impact trimming has on this profiling. Taxonomic profiles were additionally visualised in Krona (Ondov et al., 2011) using the tabular BLAST hit import functionality.

Scripts used to conduct this analysis are available in the following github repository:

<https://github.com/fmaguire/dueydrop>

To determine how representative profiles created using small subsamples consisting of <1% of reads are to profiles of entire libraries a similar analysis was done using full libraries. All libraries were pre-trimmed at the harsh threshold of the Q30 sliding window discussed above. The forward read from each trimmed library was used in a similar DIAMOND based BLASTX search however all hits were retained. Multiple hits for a given read were collapsed into a single lowest common ancestor (LCA) using the LCA algorithm (Gabow and Tarjan, 1985) implemented in MEGAN (via the “mtools” package) (Huson et al., 2007; El Hadidi et al., 2013). LCA were then summarised and tabulated using a script in the CGAT collections (`lca2table.py`) (Sims et al., 2014) and visualised using Krona (Ondov et al., 2011).

On the basis of the resultant taxonomic profiles libraries were excluded or included from downstream preprocessing and assembly. The libraries selected for inclusion during these analyses are referred to as the “taxonomically filtered” single cell libraries.

#### 4.3.2.2 GC DENSITY ESTIMATES

Each library’s GC% probability density was estimated from per-read GC proportions (calculated using `awk` (Aho et al., 1987)) via Kernel Density Estimation (KDE) (Rosenblatt, 1956; Parzen, 1962) (implemented in the `seaborn` package (Waskom et al., 2015)). This involved a standard Gaussian kernel and a bandwidth determined by “Scott’s normal reference rule” (Scott, 1979). Again this analysis was repeated with both untrimmed and Q30 trimmed reads.

#### 4.3.3 OPTIMISING READ PRE-PROCESSING

##### 4.3.3.1 TRIMMING

To investigate the optimal trimming parameters for single cell libraries, random subsamples were trimmed using a range minimum quality thresholds and then the effects investigated by mapping against 3 draft *de novo* transcriptomes.

Specifically, 5000 PE reads were randomly sampled without replacement from each of the raw FASTQ libraries using the streaming reservoir sampling (Vitter, 1985) algorithm implemented in Heng Li’s `seqtk C` library (Li, 2015). To guarantee that pairing was maintained the same random seed was used for the left and right read of each library and incremented between libraries.

Trimmomatic (Bolger et al., 2014) was run on these samples with adapter clipping (ILLUMINA-CLIP) using sequencing service provided fasta file of adapters, a maximum mismatch count

of 2, a palindromic clip threshold of Q<sub>35</sub> and a simple clip threshold of Q<sub>15</sub>, a sliding window quality trim of size 4 and average window quality thresholds of Q<sub>0</sub>, Q<sub>2</sub>, Q<sub>5</sub>, Q<sub>10</sub>, Q<sub>15</sub>, Q<sub>20</sub>, Q<sub>25</sub>, Q<sub>30</sub>, Q<sub>35</sub>, and Q<sub>40</sub>. Finally, a minimum length of 40 bp filtering criteria was used after other trimming operations had been applied.

The trimmed samples were then mapped to three different *de novo* draft transcriptome assemblies using bowtie2 (Langmead and Salzberg, 2012) with minimum and maximum insert sizes of 37 bp and 1161 bp (derived from library preparation fragment size distribution and histograms of mapped insert sizes for untrimmed reads against bulk reference).

These 3 draft assemblies were a “baseline” bulk RNASeq transcriptome reference consisting of a Trinity (Haas et al., 2013) assembly of the light and dark bulk libraries preprocessed to remove low quality bases (<Q<sub>20</sub>) and adapters using FastQ-MCF (Aronesty, 2013); and two Trinity assemblies of the taxonomically filtered sc-RNASeq libraries previously trimmed at an average window quality threshold of Q<sub>5</sub> and Q<sub>30</sub> respectively.

For each library and set of quality thresholds the total number of concordantly mapping (i.e. forward and reverse PE reads mapped to transcripts within the range of the insert sizes used) reads was recorded. This heuristic measure was chosen because the number of concordantly mapping reads generally correlates with the assembly quality (MacManes, 2014). The proportion of surviving reads which mapped was not used as a metric because this could be spuriously inflated in cases where a particular set of trimming parameters has caused the majority of reads to be discarded.

The number of concordantly mapping reads were tallied and plotted in seaborn for each library, reference transcriptome and set of trimming parameters. The shape of this line was then used to determine the optimal quality threshold to use for further assembly.

Scripts used to conduct this are available in my thesis scripts github repository: [https://github.com/fmaguire/thesis\\_scripts/tree/master/chapter\\_2\\_assembly\\_and\\_binning/trimming\\_optimisation](https://github.com/fmaguire/thesis_scripts/tree/master/chapter_2_assembly_and_binning/trimming_optimisation)

#### 4.3.3.2 GC PARTITIONING OF READS

To assess the utility of pre-assembly read partitioning an unsupervised clustering tool was created: Paired Arrangement of Reads via *K*-means On Unlabelled Reads (parKour). This C++ tool implements a fast and efficient *k*-means clustering of reads based on the dual features of GC% in forward and reverse paired reads and was designed to exploit the wildly differing GC biases of *P*.

*bursaria* and *M. reisseri*.

ParKour operates as follows:

1. Parse user input of paired FASTQ files (i.e. a file of forward and file of reverse reads) and specified number of clusters.
2. Simultaneously iterate over the pair of FASTQ files calculating the GC% for each pair of reads. GC% tallies are then loaded into an Armadillo 2 by  $n$  matrix (Sanderson, 2010) where  $n$  is the total number of PE reads.
3. Bradley-Fayyad  $k$ -means (Bradley and Bradley, 1998) clustering as implemented in the MLPACK library (Curtin et al., 2013)
4. Re-read the two input FASTQs assigning them to output files based on the assigned cluster of the pair.

GNUplot (Williams et al., 2010) was used to visualise classification and cluster assignment.

This approach was attempted using a range of expected clusters from 2 to 5.

Scripts used to conduct this are available in a github repository: <https://github.com/fmaguire/parKour>

#### 4.3.3.3 ERROR CORRECTION

The effect of error correction on assemblies involving single cell libraries was assessed by applying two different error correction algorithms to the screened, trimmed reads before assembly. These were a Bayeshammer (Nikolenko et al., 2013) implemented as part of the SPAdes genome assembler (Bankevich et al., 2012) and optimised for MDA-based single cell genomic data, and SEECER (Le et al., 2013) which is optimised for RNAseq (but not necessarily sc-RNAseq data). The impact of each of these error correction algorithms at the read level was assessed as well as their subsequent impact on downstream assembly metrics, particularly RSEM-EVAL likelihood score as will be expanded upon below in the description of assembly assessment.

#### 4.3.3.4 $k$ -MER NORMALISATION AND TRIMMING

Taxonomically screened sc-RNAseq libraries trimmed at a minimum sliding window quality threshold of Q30 and bulk libraries were  $k$ -mer normalised and trimmed using the Khmer package (Cru-soe et al., 2015)

Specifically, reads were interleaved (Döring et al., 2008) and then digitally normalised using diginorm (Brown et al., 2012) with a  $k$ -mer size and coverage cut-off of 20. Low abundance and likely erroneous  $k$ -mers were then filtered relative to the read coverage i.e. low abundance  $k$ -mers were removed from high coverage reads but would be more likely to be retained for low coverage reads (Zhang et al., 2015, 2014).

Filtered data was then assembled using Trinity (with minimum  $k$ -mer coverage of 2) and the subsequent assembly partitioned into transcript families in Khmer (Pell et al., 2012).

The final assemblies were then compared to un-normalised and  $k$ -mer trimmed assemblies (see section 4.3.4.1 for details).

#### 4.3.4 ASSEMBLY

Referenced and *de novo* assemblies were attempted using a range of assemblers and assembly parameters.

Firstly, trimmed bulk and taxonomically filtered single cell libraries were mapped to *Chlorella* NC64A, *Coccomyxa* C169, *Tetrahymena thermophila* and *Paramecium caudatum* macronuclear (MAC) genomes. The former pair being the closest available genomes to the endosymbiont and the latter to the host. Mapping was done using the TopHat2 spliced aligner (Kim et al., 2013) against the genomes and was supplemented with and without annotated ORF information (in the form of gtf). GTF files were generated from best available gene annotations in the form of GFF files using gffread (part of cufflinks). Cufflinks (Trapnell et al., 2011) was then used to extract isoforms from the spliced alignments.

For *de novo* assembly, assemblies were conducted using following assemblers with default settings unless specified otherwise:

- Trinity v2.0.6 (Grabherr et al., 2011) with and without a minimum  $k$ -mer coverage of 2
- SOAPdenovo-Trans v1.03 (Xie et al., 2014) with  $k$ -mer sizes of 20, 32, 64, and 80
- TransAbyss v1.5.3 (Robertson et al., 2010) with  $k$ -mer sizes 20, 32, and 64
- Velvet v1.2.10 (Zerbino and Birney, 2008) and Oases v0.2.08 (Schulz et al., 2012) with  $k$ -mer size of 21, a minimum  $k$ -mer coverage of 2 and a minimum transcript length of 100.
- Iterative de Bruijn Graph Assembler (IDBA)-tran (Peng et al., 2010, 2012, 2013)

- IDBA-MTP (Leung et al., 2014), IDBA-UD (Peng et al., 2012), IDBA-MT (Leung et al., 2013) workflow.
- Bridger (Chang et al., 2015).

Trinity was used for all further downstream assembly optimisation due to its performance and consistency. Specifically, a minimum  $k$ -mer coverage of 1-3 were attempted as well as various combinations of libraries (i.e. bulk and screened sc-RNAseq libraries) and also sequencing data from Kodama's previously published *P. bursaria* bulk RNAseq analysis (Kodama and Fujishima, 2014).

To assess the utility of combining assemblies as discussed in (Nakasugi et al., 2014), the best assemblies from Bridger and Trinity (as assessed below) were combined using the Evidential-Gene `tr2aacds` pipeline (Gilbert, 2013). Additionally, the best assemblies from all assemblers that ran to completion i.e. Bridger, Trinity, SOAPdenovo-Trans, Transabyss and IDBA-tran were also combined and assessed.

#### 4.3.4.1 ASSEMBLY ASSESSMENT

Resultant assemblies were compared using standard assembly statistics (e.g. contigs number and size, bases assembled) as implemented in the `trinitystats.pl` perl script supplied with Trinity (Haas et al., 2013) and TransRate (Smith-unna et al., 2015). Additionally, the reference free probabilistic assembly assessment RSEM-EVAL package (part of DETONATE) (Li et al., 2014) was used to estimate likelihood scores for various completed assemblies.

#### 4.3.4.2 ORF CALLING

ORFs were called from assembled transcripts using TransDecoder (Haas et al., 2013) with a minimum protein size of 100 amino acid residues.

TransDecoder operates as follows:

1. All ORFs are found in transcripts by identification of sequences between a start codon and an in-frame stop codon. Partial ORFs are also identified as sequences between the 5' transcript terminus and a stop codon or a start codon and the 3' transcript terminus.
2. The top 500 longest of these ORFs are selected and used to train a reading-frame specific 5th-order Markov model.

3. All of the ORFs are then scored for each reading frame as a sum of the per-base log odd scores (log probability of a given base and reading frame given its preceding 5 bases normalised by the relative frequency of that nucleotide across all transcripts).
4. The highest scoring reading frame is retained as a candidate.
5. Any of the initial ORFs with homology to proteins in PFAM and Swissprot (as determined by HMMR and BLASTP (minimum e-value of  $1e^{-5}$ )) are also retained.

*Paramecium* uses an alternative genetic code in which two universal stop codons (UAA, UAG) are reassigned to glutamine. For the purposes of initial BLAST based binning ORFs were called and translated using both universal encoding and this alternative code. However, for the later BLAST-based bin accuracy verification purposes and subsequent automated phylogeny based binning all ORFs were initially only called using the alternative ciliate encoding. The ciliate encoding was used instead of universal because spuriously extended transcripts were considered favourable to falsely truncated ones. This greatly reduced redundancy in the later binning analyses.

#### 4.3.5 TRANSCRIPT BINNING

##### 4.3.5.1 INITIAL BLAST BASED BINS

Initially, 10,000 randomly chosen, translated transcripts from an earlier iteration of the assembly process were binned into their predicted source - Host (H), Endosymbiont (E), Food (F) and Unknown (U).

Each of the assembled transcripts were used as a BLASTP query against a database consisting of the following predicted proteomes: *Chlorella* NC64A, *Chlamydomonas reinhardtii*, *Coccomyxa* C169, *Paramecium tetraurelia*, *Tetrahymena thermophila*, *Arabidopsis thaliana*, *Homo sapiens* (helping to identify contamination), *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Bacillus cereus* ATCC 14579, *Escherichia coli* 536, *Escherichia coli* O157 H-7, *Salmonella typhimurium* LT2 and *Escherichia coli* K-12 (the last five genome datasets helping to identify food bacterial genes). Then initial bins were determined as follows:

- Endosymbiont (E): Transcript's highest scoring BLAST hit at an expectation of  $\leq e^{-50}$  was to *Coccomyxa*, *Chlamydomonas* or *Chlorella*. Or transcript's highest scoring hit at  $e^{-20}$

was one of those species and the longest likely coding region in the transcript was using the universal codon table.

- Host (H): Transcript's highest hits at  $\leq e^{-50}$  were to *Paramecium tet aurelia* or *Tetrahymena thermophila*. Or highest hit at  $e^{-20}$  was one of those species and longest likely coding region was using the *Tetrahymena* codon table.
- Food (F): Transcript's highest scoring BLAST hit at an expectation of  $\leq e^{-50}$  was to one of the *E. coli* species or *Salmonella*. Or transcript's highest scoring hit at  $e^{-20}$  was one of those species and the longest likely coding region in the transcript was using the universal codon table.
- Unknown (U): highest scoring hits to *Arabidopsis*, *Homo sapiens*, *Saccharomyces* or *Schizosaccharomyces* or any sequence not fitting into the above categories.

The accuracy of the BLAST based binning was then determined by generating phylogenies using the method described below. Resultant phylogenies were then manually parsed and assessed for phylogenetic congruence with their bin. For example, do host binned sequences predominantly branch with other ciliate sequences? Do endosymbiont binned sequences mainly branch with Archaeplastida sequences?

#### 4.3.5.2 AUTOMATED PHYLOGENY GENERATION PIPELINE - DENDROGENOUS

To rapidly generate phylogenies an established lab tree generation pipeline, known as "Darren's Orchard" (Richards et al., 2009) was modified and ported to python3 from perl5. This new pipeline "Dendrogenous" takes in a multi-fasta set of inputs and a set of genomes to search against. For each input sequence:

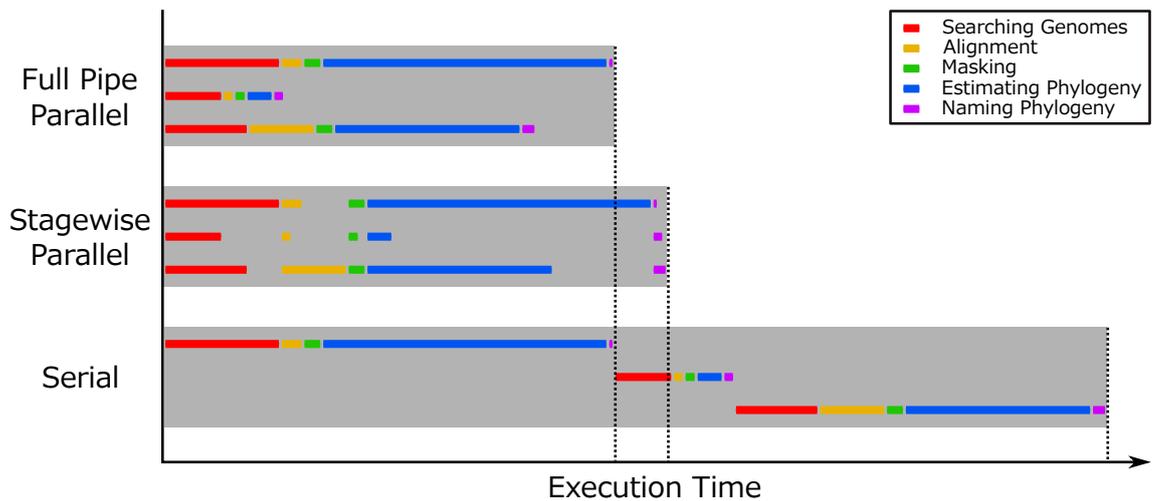
1. The user specified genome database is queried using BLASTP.
2. The results are parsed and a fasta file of putative homologues is created, with inputs that have fewer than a specified number of hits (default of 5) ejected.
3. A multiple sequence alignment (MSA) is created from this fasta using Kalign (chosen for its speed) (Lassmann et al., 2009).

4. This MSA is then masked automatically to remove ambiguous sites using TrimAL (Capella-Gutiérrez et al., 2009) and masked alignments with fewer than a specified number of sites (default of 30) are ejected from the pipeline.
5. A rapid maximum-likelihood phylogenetic tree is generated using FastTree2 (Price et al., 2010).
6. Finally, encoded taxonomic information is recovered from the “cider” database of the original “Darren’s Orchard” pipeline and the trees are named with full species names.

The two key improvements are that of full and efficient parallelisation of the tree generation process (see fig. 4.3.1) and increased use of filestreams to pass data between pipeline stages. This latter modification reduces costly and slow file reading and writing operations.

In the process of creating this modified phylogenetic pipeline I upgraded the general purpose python phylogenetic toolkit ETE (Huerta-Cepas et al., 2010) to support python3. As ETE is an open source project I submitted these changes to the maintainer and they have subsequently been merged into the master. These changes compose a significant proportion of the latest major release version of this toolkit (<https://github.com/jhcepas/ete/pull/105>).

40 genomes covering the diversity of the tree of life, with a particular focus on green algal and ciliate representatives, were selected for this phylogenetic generation: *Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, *Ostreococcus tauri*, *Micromonas pusilla* CCMP1545, *Chlorella variabilis* NC64A *Chlorella vulgaris* C-169, *Physcomitrella patens*, *Saccharomyces cerevisiae* S288C, *Neurospora crassa* OR74A, *Homo sapiens*, *Mus musculus*, *Dictyostelium discoideum*, *Paramecium caudatum*, *Paramecium tetraurelia*, *Tetrahymena thermophila macronucleus*, *Oxytricha trifallax*, *Toxoplasma gondii*, *Guillardia theta*, *Bigeloviella natans*, *Emiliana huxleyi* CCMP1516, *Aureococcus anophagefferens*, *Ectocarpus siliculosus*, *Schizosaccharomyces pombe*, *Bacillus cereus* ATCC 14579, *Escherichia coli* str. K-12 substr. MG1655, *Escherichia coli* O157 H7 str. Sakai, *Salmonella enterica* subsp. *enterica* serovar *Typhi* str. CT18, *Amycolatopsis mediterranei* U32, *Aquifex aeolicus* VF5, *Borrelia burgdorferi* B31, *Chlamydophila pneumoniae* CWL029, *Chlorobium tepidum* TLS, *Deinococcus radiodurans* R2, *Caulobacter crescentus* CB15, *Sulfolobus islandicus* M.14.25, *Nanoarchaeum equitans* Kin4-M, *Haloferax mediterranei* ATCC 33500, *Methanococcus maripaludis* S2, and *Cenarchaeum symbiosum* A.



**Figure 4.3.1:** An explanatory plot showing 3 different possible architectures for a tree generation pipeline. The least efficient design (the serial architecture) makes no use of multiprocessing and takes significantly longer to complete execution. Stagewise parallelism (e.g. all alignments for each input sequence are run side-by-side and masking begins once the last sequence has finished alignment) is moderately more efficient but a single slow stage for one input sequence can hold up the whole pipeline and leave resources idle. Additionally, by running many of the same type of process at the same time, each with similar resource requirements, the risk of hardware bottlenecks is increased compared to a more heterogeneous load. Finally, fully parallel runs each input sequence through the pipeline stage-by-stage separately from all other inputs to the pipeline. This architecture proves most efficient, preventing blocking and allowing effective use of computational resources.

#### 4.3.5.3 AUTOMATED PHYLOGENETIC TRANSCRIPT BINNING - ARBORETUM

In order to automate phylogeny based transcript binning the 10,000 manually verified phylogenetic bins from the initial BLAST based binning and analysis were used as a training dataset for supervised classification. The cardinalities of each label in this training set was relatively balanced (i.e. all within the same order of magnitude) 1975 endosymbiont phylogenies, 2600 host, 3456 food, and 1969 unknown.

The supervised classification was implemented in a script called “Arboretum”. For each phylogeny “Arboretum” generates a vector using the following algorithm:

1. Parses the phylogeny and identifies the  $k$  (default of 10) closest (based on branch length) leaf nodes to the seed transcript used to generate the phylogeny.
2. A vector ( $\vec{z}$ ) of length  $p$  (where  $p$  is the number of class labels) is 0-initialised.
3. Then, for each of these  $k$  closest leaves:
  - The species is queried taxonomically using the NCBI taxonomy local database implemented in the ETE toolkit.

- A set of user defined look-up filters is used to identify what “type” of species this is in terms of the defined class labels. For example, sequences from ciliates could be defined as “host-like” and those from Archaeplastida as “endosymbiont-like” etc.
  - The reciprocal phylogenetic distance between this leaf node and the seed transcript node is then added to corresponding index in  $\vec{z}$ . Therefore, if this leaf has been determined as having the same type as class label 2 in the look-up filters (for example) this reciprocal distance will be added to  $z_2$ .
4. The magnitude of each dimension in vector  $\vec{z}$  then represents the summed reciprocal distance between the node containing the transcript sequence and all of the nearest branches that have been identified as being indicative of a certain class.

Concretely, we can define our class labels as:

$$\vec{l} = \{ \text{“endosymbiont”}, \text{“host”}, \text{“food/bacterial”}, \text{“unknown”} \}$$

Noting that in practice they will be represented as integers using a 1-of- $n$  encoding. Similarly,  $\vec{a}$  is a  $k + 1$  length vector consisting of the transcript node and the  $k$  nearest terminal nodes to it:

$$\vec{a} = \{ \text{transcript node}, \text{node}_1, \dots, \text{node}_k \}$$

If  $\gamma(x, y)$  is a function which returns the phylogenetic distance between two nodes  $x$  and  $y$ ,  $\psi(x)$  is a function which represents the ‘look-up’ filters (i.e. if terminal node  $x$  is an Archaeplastida species  $\psi(x)$  will return the “endosymbiont” label), and  $\delta_{ij}$  is the Kronecker delta<sup>2</sup>, then for a given phylogeny the length  $p$  vector ( $\vec{z}$ ) is defined as follows:

$$\vec{z}^T = \begin{bmatrix} \sum_{i=1}^k \left( \frac{1}{\gamma(a_o, a_i)} * \delta_{\psi(a_i), l_o} \right) \\ \vdots \\ \sum_{i=1}^k \left( \frac{1}{\gamma(a_o, a_i)} * \delta_{\psi(a_i), l_p} \right) \end{bmatrix}$$

---


$${}^2\delta_{ij} = \begin{cases} 1, & i = j \\ 0, & i \neq j \end{cases}$$

These vectors are then stacked to form matrix  $X$  with dimensions  $t \times q$  where  $t$  is the number of phylogenies and  $q$  is the number of class labels.

Training data was visualised using Radial Visualisation (RadViz) (Hoffman et al., 1997; Fayyad et al., 2001). RadViz is a form of radial co-ordinate visualisation that non-linearly maps a set of  $N$ -dimensional points onto a plane for easy 2D visualisation. This mapping operates on the physical principle of “springs” anchored evenly around a unit circle with “spring” stiffness determined by the normalised 0 – 1 value of that dimension for that point. Each point therefore rests at the point of mechanical equilibrium between the “springs” (Novakova, Lenka and Stepankova, 2006).

1,000 vectors from this training set were held out to form the test set and all models were then trained using 5-fold cross-validation (CV) on the remaining 9,000 training vectors. We evaluated Support Vector Machines (SVMs) with both linear and radial basis function (RBF) kernels (Vapnik and Lerner, 1963), naive Bayes,  $k$ -neighbours, Decision Trees (DT) (Quinlan, 1986), DTs ensembles in a Random Forests (Breiman, 2001) and Extremely Randomised Trees (ExtraTrees) (Geurts et al., 2006), adaptively boosted (AdaBoost) DTs (Freund and Schapire, 1997), Linear Discriminant Analysis (LDA) and Quadratic Discriminant Analysis (QDA). Models were trained and hyperparameters were optimised using Bayesian optimisation implemented in the HPOlib library (Eggenesperger et al., 2013; Komer et al., 2014) over the CV-folds. Finally, each model was assessed using the held out test set and performance was evaluated by inspection of label-wise classification reports containing various metrics e.g. label F1-scores and confusion matrices.

The best performing model and hyperparameters were then used to classify the remaining unlabelled phylogenies.

#### 4.3.5.4 TAXAASSIGN COMPARISON

To assess the performance of supervised learning and phylogeny based system (Arboretum) described above a stand-alone sequence identity binning tool TAXAssign (<https://github.com/umerijaz/TAXAassign>) was run against the 70,605 CDS sequences.

TAXAssign queried each CDS against the entire NCBI nt database. The nt BLAST database was downloaded using `updateblastdb.pl` script ([http://www.ncbi.nlm.nih.gov/blast/docs/update\\_blastdb.pl](http://www.ncbi.nlm.nih.gov/blast/docs/update_blastdb.pl)) and TAXAssign ran BLASTN in parallel (using GNU parallel (Tange, 2011)) with a maximum of 10 reference matches per CDS and a minimum necessary

percentage identity for assignment to a given taxonomic level of 60, 70, 80, 95, 95, and 97 for Phylum, Class, Order, Family, Genus and Species respectively.

Results were then tabulated and compared with the Dendrogenous-Arboretum assignments.

## 4.4 RESULTS

### 4.4.1 LIBRARY CONTAMINATION SCREENING

Libraries were screened for inclusion in assemblies by inspection of their taxonomic profiles (see table 4.4.1 and table 4.4.2) as determined by DueyDrop and their GC% probability densities (via KDE).

The GC density estimates of the single cell libraries show a clear bimodal GC density with a high 70GC% peak (fig. 4.4.1) in all dark single cell libraries. With the exception of Dark1-2 and Dark2-3 this high GC peak is a greater density than the expected peak 30-50GC% (from known GC% found in genomes of sequenced relatives of both host and endosymbiont).

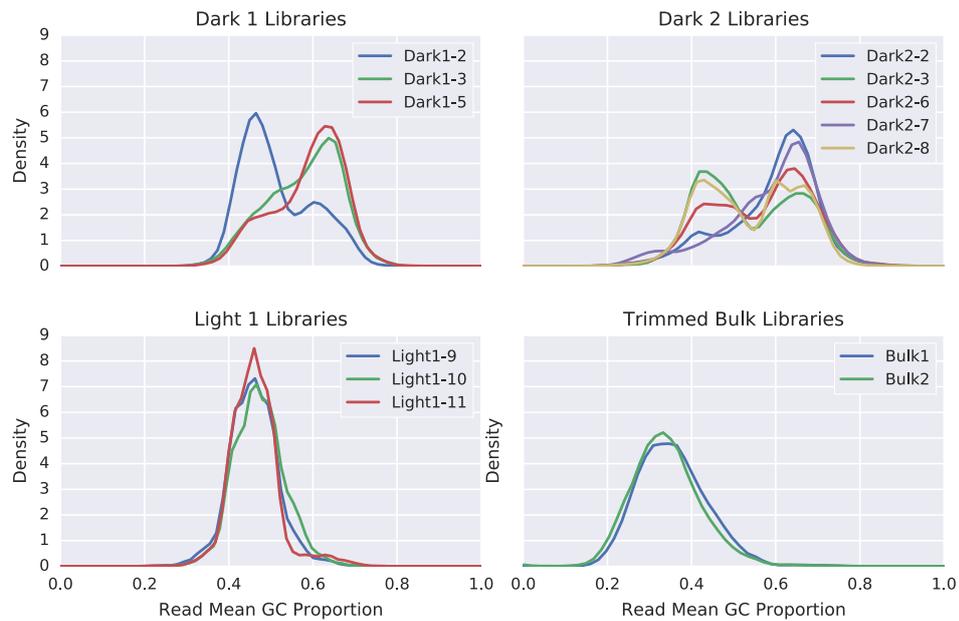
When these KDE are compared to the densities estimated from the Q20 trimmed bulk reads (bottom right pane in fig. 4.4.1) and raw bulk RNAseq reads from (Kodama et al., 2014) (see fig. 4.4.2) it is apparent that this high GC% peak is likely originating from a high GC% bacterial contaminant in the Dark single cell libraries.

One other observation when comparing the bulk RNAseq analyses to the single cell libraries is that the main GC peak is slightly lower in the bulk (and Kodama dataset), around 30GC% versus 45-50GC%. This possibly indicates a greater proportion of reads deriving from the low GC% *Paramecium bursaria* host and fewer from the 50GC% endosymbiont in bulk libraries relative to single cell libraries.

By comparing the results of the KDE GC analysis with and without read trimming it is apparent that trimming of reads makes nearly no difference in the density estimates. The KDE of Q30 sliding window trimmed single cell reads in fig. 4.4.3 is nearly identical to that of the raw reads fig. 4.4.1.

The taxonomic profiles of single cell (table 4.4.1) and bulk libraries (table 4.4.2) generated by DueyDrop are summarised in the tables below. It is readily apparent that Dark1-3, Dark1-5,

## Kernel Density Estimates of Read GC Proportion

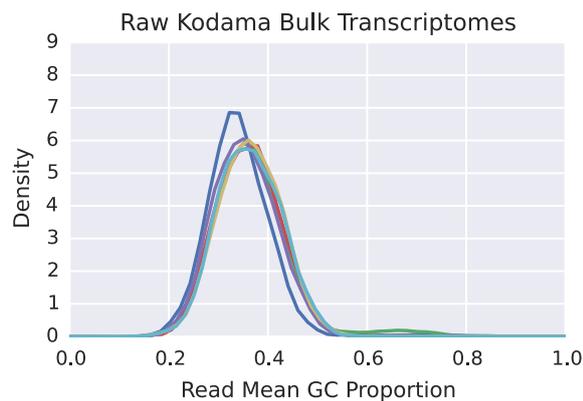


**Figure 4.4.1:** Probability densities of per-read GC proportions for the raw data (apart from pre-trimmed bulk explained previously) from each sequenced library. Densities were derived using Kernel Density Estimation implemented in Seaborn. Dark 1 (Dark1-2, Dark1-3, and Dark1-5) and Light 1 (Light1-9, Light1-10, Light1-11) were sc-RNaseq from the first round of SCTs sampled during the mid-dark and light culture phases. Similarly, Dark 2 (Dark2-2, Dark2-3, Dark2-6, Dark2-7, Dark2-8) were the libraries sampled in the dark from the second round of SCT. Bulk1 and Bulk2 are the bulk RNAseq libraries sequenced under lit and dark conditions. The bulk and single cell light libraries demonstrate similar shaped distributions although the bulk has a greater proportion of low GC% reads potentially representing more *Paramecium* derived data. All single cell dark libraries demonstrate a bimodal density with up to the majority of reads deriving from an unknown high 70% GC population. The dark single cell libraries exhibiting a relatively larger peak at 70% GC than at 40-50%GC (i.e. Dark1-3, Dark1-5, Dark2-2, Dark2-7) were the same libraries which were identified as potentially contaminated in taxonomic screening (see table 4.4.1).

Dark2-2, and Dark2-7 display an aberrantly low number of reads aligning to known alveolate (or even eukaryote) sequences. Forward and reverse reads within a library display similar profiles with a slightly lower proportion of hits in the reverse reads. This can likely be attributed to the lower read quality found in reverse reads relative to forward reads in paired-end Illumina sequencing.

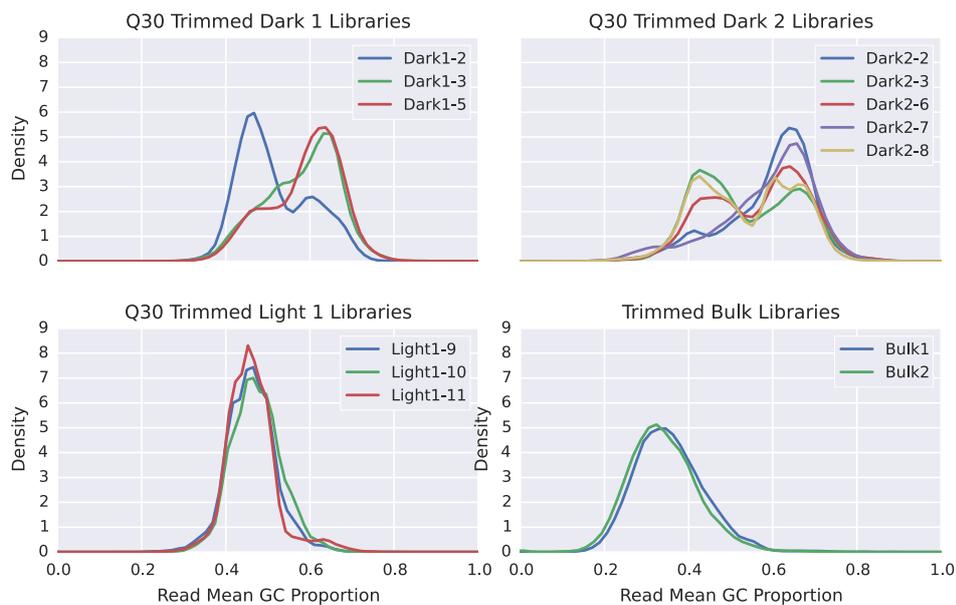
The bulk libraries demonstrate a very low level of hits compared to single cell libraries (see table 4.4.2), to the point where if they were single cell libraries they would be taxonomically excluded. However, it should be noted that the bulk libraries were sequenced on a Gene Analyzer II and are on average half the length of single cell reads (76 bp vs 150 bp). Due to the difficulty align-

## GC Proportion KDE of Kodama Read



**Figure 4.4.2:** Probability density of the per-read GC proportion for 6 raw libraries derived from (Kodama et al., 2014) transcriptome analysis of a different *P. bursaria* species (Yad1g) with and without its *Chlorella variabilis* 1N endosymbiont. Individual libraries are indicated in the key using their DDBJ accession. This dataset displays densities relatively similar to the bulk RNAseq conducted in this project - “Trimmed Bulk Libraries” in fig. 4.4.1.

## GC Proportion KDE of Trimmed Read



**Figure 4.4.3:** Probability densities of per-read GC proportions for trimmed reads. To ensure probability densities estimated in fig. 4.4.1 weren't biased by low quality ambiguous reads the same analysis was repeated using reads trimmed using a sliding window approach with a stringent average quality threshold of Q30. In all cases the densities produced appear near identical to the analysis of the raw data.

ing short reads to references the difference between libraries may be attributable to this alone. Additionally, the vast majority of the lower number of hits do align to eukaryote (and alveolate) taxa consistent with an non-contaminated library.

SCT Library	PE	Eukaryote	Bacteria	Alveolate	Viridiplantae	Total Hits
<i>Light1-9</i>	R1	51.89 +/- 0.45	9.37 +/- 0.26	25.15 +/- 0.71	7.45 +/- 0.33	69.49 +/- 0.37
	R2	51.75 +/- 0.25	8.82 +/- 0.24	24.85 +/- 0.56	7.49 +/- 0.21	68.75 +/- 0.29
<i>Light1-10</i>	R1	46.35 +/- 0.56	15.72 +/- 0.46	22.96 +/- 0.24	6.94 +/- 0.26	68.73 +/- 0.30
	R2	46.12 +/- 0.83	15.14 +/- 0.48	23.13 +/- 0.38	6.99 +/- 0.37	68.73 +/- 0.30
<i>Light1-11</i>	R1	58.28 +/- 0.47	3.62 +/- 0.12	28.68 +/- 0.43	8.20 +/- 0.40	71.38 +/- 0.49
	R2	57.74 +/- 0.27	3.50 +/- 0.10	28.23 +/- 0.36	8.41 +/- 0.31	70.42 +/- 0.20
<i>Dark1-2</i>	R1	28.64 +/- 0.51	22.88 +/- 0.61	12.23 +/- 0.28	4.93 +/- 0.19	60.31 +/- 0.49
	R2	28.29 +/- 0.24	21.06 +/- 0.21	12.13 +/- 0.28	4.87 +/- 0.34	57.65 +/- 0.35
<b><i>Dark1-3</i></b>	R1	9.48 +/- 0.43	25.07 +/- 0.42	2.15 +/- 0.13	2.60 +/- 0.27	41.43 +/- 0.68
	R2	8.89 +/- 0.19	23.11 +/- 0.52	2.13 +/- 0.16	2.45 +/- 0.18	38.50 +/- 0.46
<b><i>Dark1-5</i></b>	R1	5.56 +/- 0.19	23.99 +/- 0.44	1.07 +/- 0.07	2.89 +/- 0.11	36.72 +/- 0.33
	R2	4.94 +/- 0.21	21.75 +/- 0.53	1.02 +/- 0.11	2.33 +/- 0.17	33.06 +/- 0.52
<b><i>Dark2-2</i></b>	R1	12.32 +/- 0.25	9.81 +/- 0.19	3.73 +/- 0.16	4.33 +/- 0.17	27.65 +/- 0.47
	R2	11.53 +/- 0.15	9.00 +/- 0.17	3.67 +/- 0.22	3.74 +/- 0.12	25.71 +/- 0.39
<i>Dark2-3</i>	R1	32.07 +/- 0.31	7.43 +/- 0.15	12.81 +/- 0.21	4.71 +/- 0.21	48.42 +/- 0.53
	R2	32.47 +/- 0.24	6.68 +/- 0.21	13.11 +/- 0.43	4.58 +/- 0.12	47.92 +/- 0.28
<i>Dark2-6</i>	R1	24.11 +/- 0.28	8.55 +/- 0.11	9.04 +/- 0.35	5.27 +/- 0.15	41.69 +/- 0.45
	R2	22.89 +/- 0.55	7.44 +/- 0.17	8.74 +/- 0.49	4.36 +/- 0.24	38.85 +/- 0.58
<b><i>Dark2-7</i></b>	R1	9.96 +/- 0.24	16.89 +/- 0.27	4.22 +/- 0.24	2.83 +/- 0.17	37.06 +/- 0.40
	R2	8.77 +/- 0.18	15.00 +/- 0.43	3.94 +/- 0.14	2.16 +/- 0.11	32.86 +/- 0.29
<i>Dark2-8</i>	R1	28.24 +/- 0.48	4.45 +/- 0.13	12.00 +/- 0.32	4.69 +/- 0.06	40.50 +/- 0.37
	R2	28.22 +/- 0.47	4.30 +/- 0.22	11.98 +/- 0.37	4.32 +/- 0.24	40.05 +/- 0.22

**Table 4.4.1:** Taxonomic profiles of raw single cell libraries generated using “DueyDrop”. All values are percentage of reads mapping to that category +/- the standard deviation between sample replicates. The analysis was conducted for both forward and reverse reads from each library (indicated as R1 and R2 in the paired-end (PE) column). Libraries highlighted in bold were those excluded from subsequent analysis on the basis of their very low numbers of reads identifiable as eukaryotic (or specifically alveolate or Archaeplastida). All forward and reverse read pairs display similar profiles to one another suggesting the problem of “MDA chimeras” may be minor.

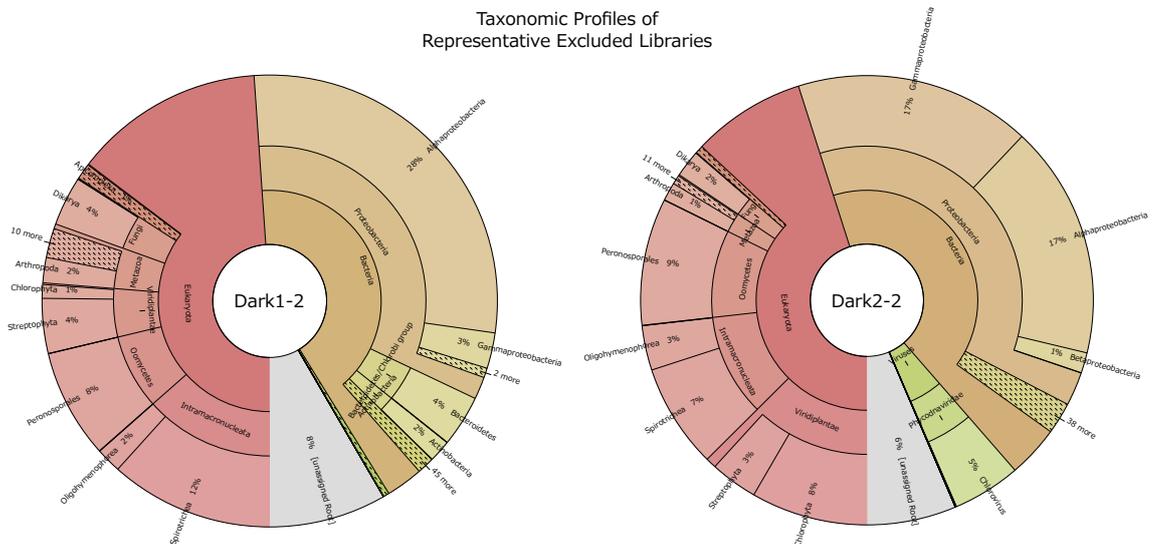
Bulk Library	PE	Eukaryote	Bacteria	Alveolate	Viridiplantae	Total Hits
<i>Light</i>	R1	9.66 +/- 1.55	0.18 +/- 0.13	6.28 +/- 1.41	0.86 +/- 0.3	10.10 +/- 1.48
	R2	9.62 +/- 0.81	0.26 +/- 0.09	6.58 +/- 0.36	1.04 +/- 0.41	10.16 +/- 0.95
<i>Dark</i>	R1	4.90 +/- 0.78	0.36 +/- 0.11	3.14 +/- 0.58	0.50 +/- 0.16	5.40 +/- 0.93
	R2	5.50 +/- 1.25	0.22 +/- 0.19	3.82 +/- 0.81	0.50 +/- 0.12	6.02 +/- 1.22

**Table 4.4.2:** Taxonomic profile of the two trimmed (Q20) bulk transcriptome libraries generated using “DueyDrop”. All values are the percentage of reads mapping to that taxonomic category +/- the standard deviation between sampling replicates. The analysis was conducted for both forward and reverse reads from each library (indicated as R1 and R2 in the paired-end (PE) column). Overall only a very small number of bulk reads could be assigned to any taxonomic class by “DueyDrop”.

To identify the likely source of the high GC% contamination and to assess how representative the taxonomic profiling of small  $\leq 1\%$  random subsamples of reads where to full scale analyses Krona was used to create interactive hierarchical plots of the taxonomic profiles<sup>3</sup> From this, Rhizobia species are the most prevalent high GC% species found in the libraries with this high 70GC% peak in the KDE plots and therefore are the most likely source of this particular aspect

<sup>3</sup>Accessible at [http://finlaymagui.re/dueydrop\\_analysis](http://finlaymagui.re/dueydrop_analysis)

of contamination.



**Figure 4.4.4:** Krona visualisation of taxonomic profiles of two representative single cell libraries (Dark1-2, Dark2-2) that were excluded from further analysis due to aberrant profiles (typically large proportion of reads being assigned to Bacteria (yellow) than Eukaryota (red)). Note that nearly 50% of each library is identified as bacterial.

Therefore, small random subsamples are representative of the full library and read-level taxonomic assignment can be used to screen single cell libraries for contamination.

#### 4.4.2 READ PRE-PROCESSING

##### 4.4.2.1 TRIMMING OPTIMISATION

The optimal trimming threshold was determined by a combination of read mapping statistics against 3 preliminary reference assemblies as well as the impact on resultant *de novo* assemblies at that threshold.

A rapid decrease in the number of concordantly mapping PE reads (i.e. within insert distance of one another) was observed above a Q<sub>30</sub> quality threshold. This proves true regardless of the reference assembly being mapped to (see fig. 4.4.7). Q<sub>30</sub> relative to Q<sub>20</sub> appears to induce a very slight decrease in total number of mapping reads but not drastically so.

Additionally, naive assemblies in Trinity of taxonomically screened single cell libraries at different sliding window quality threshold trims of Q<sub>5</sub>, Q<sub>20</sub>, and Q<sub>30</sub> (table 4.4.3) were created. These show that more permissive trims (Q<sub>5</sub> and Q<sub>20</sub>) lead to a greater number of assembled



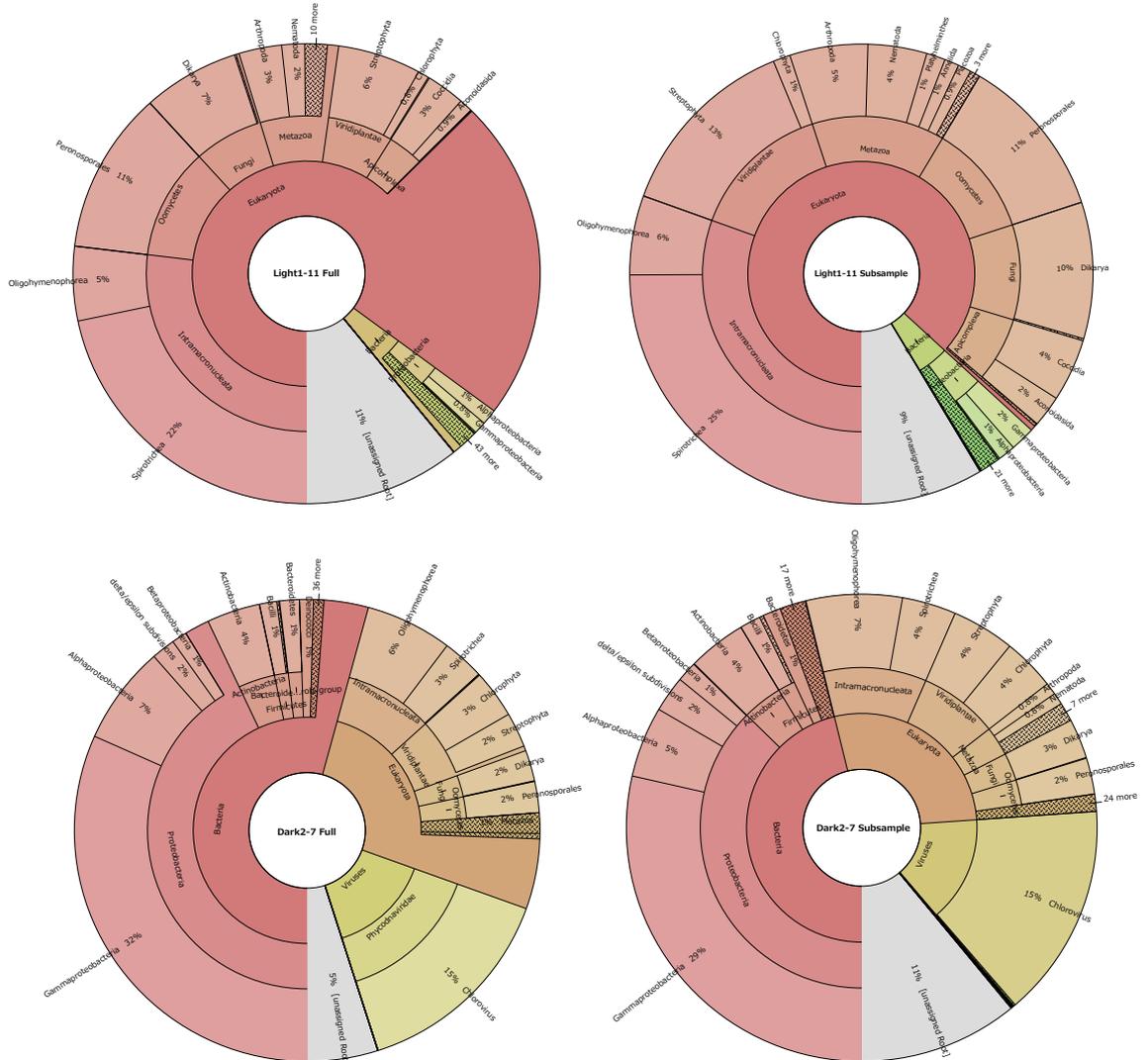
**Figure 4.4.5:** Krona visualisation of the taxonomic profiles of representative RNAseq libraries (Bulk1, Dark2-3, and Light1-9) that were retained in the analysis after taxonomic screening. The key thing this figure shows is that in retained libraries the vast majority of reads were identified as eukaryotic (red) in origin.

bases and transcripts but the likelihood of these assemblies are also lower than that generated using the more conservative Q<sub>30</sub> trim. However, it should be noted that the difference in the number and size of assembled transcripts at different thresholds was less than was found using different assemblers and assembly parameters.

Trim Threshold	Number of Transcripts	Bases Assembled	Assembly Likelihood ( $-\log$ )
Q <sub>5</sub>	112,182	52,511,552	$-3.168 * 10^{10}$
Q <sub>20</sub>	107,955	50,809,686	$-3.015 * 10^{10}$
Q <sub>30</sub>	99,784	47,313,963	$-2.832 * 10^{10}$

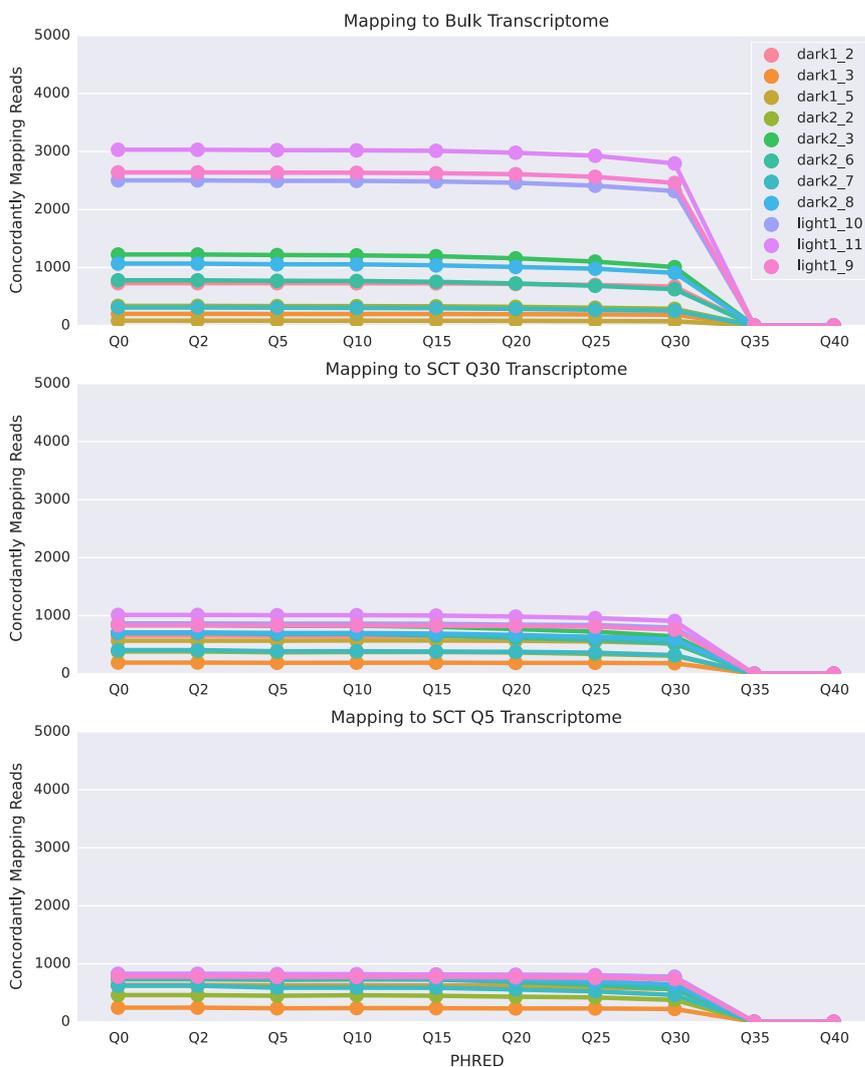
**Table 4.4.3:** Comparison of Trinity assemblies of taxonomically screened single cells reads (no bulk reads) at 3 different sliding window minimum average quality trimming thresholds. Trimming largely does not cause a major difference between assemblies in terms of number of contigs recovered or overall assembly likelihoods. Harsher (Q<sub>30</sub>) trims result in slightly smaller but slightly more likely assemblies than permissive trims (Q<sub>5</sub>).

Taxonomic Profiles from Subsamples Compared To Full Analyses



**Figure 4.4.6:** Comparison of taxonomic profiles derived from small  $\leq 1\%$  random subsamples of libraries compared to profiles generated using the full library. Light1-11 and Dark2-5 are used as representative examples as they display the trends common for all single cell libraries. All subsamples demonstrated taxonomic profiles with relatively similar proportions to full analyses. For example, in the Light1-11 subsample of reads with hits the proportion of eukaryote to Bacteria was 87:4 % vs 85:4% of the root for the full analysis. Similarly the ratios for Dark2-7 shown eukaryote to bacteria are 26:54 for full analysis and 28:46 for subsample. The key difference is the assignment of a greater proportion of reads to intermediate taxonomic levels in the full analyses due to the difference in resolution of multiple hits per read. Principally, the full library analyses retain all hits and assign level based on a lowest common ancestor algorithm whereas the subsample analysis just uses the top hit.

### Comparison of Trimming Parameters on Mapping



**Figure 4.4.7:** Assessment of the optimal minimum average quality threshold in Trimmomatic's sliding window (size 4) trim. Plots display the number of concordantly mapping reads (i.e. the forward and reverse read map to assembly at a distance of approximately their insert) at a range of different trimming thresholds. 5000 randomly sampled PE reads from each single cell library are mapped against 3 different reference assemblies. The key finding is above a threshold of Q30 there is a huge decrease in the number of mapping reads.

Library	Number of raw PE Reads	Number of Q <sub>30</sub> trimmed PE Reads
Dark1-2	$6.460 * 10^7$	$3.355 * 10^6$
Dark2-3	$2.243 * 10^7$	$1.478 * 10^7$
Dark2-6	$2.431 * 10^7$	$1.443 * 10^7$
Dark2-8	$2.761 * 10^7$	$1.866 * 10^7$
Light1-9	$1.524 * 10^7$	$1.382 * 10^7$
Light1-10	$1.614 * 10^7$	$1.478 * 10^7$
Light1-11	$1.474 * 10^7$	$1.334 * 10^7$

**Table 4.4.4:** Summary of the library size of the taxonomically selected single cell libraries before and after trimming at a minimum average SLIDINGWINDOW quality threshold of Q30. Of interest, Dark1-2 was generally of poor quality and thus was disproportionately minimised by trimming. Additionally, the two bulk RNAseq libraries were trimmed at Q20 in FastQ-MCF resulting in total library sizes of  $2.458 * 10^7$  and  $2.779 * 10^7$  respectively.

Therefore, due to increasing the assembly likelihood while only very marginally decreasing the number of contigs and mapping reads relative to more permissive trims Q<sub>30</sub> was determined to be the optimal trimming threshold. It can be considered from this data that Q<sub>30</sub> forms a maximum feasible stringency for trimming.

#### 4.4.2.2 GC PARTITIONING

GC partitioning was conducted on Q<sub>30</sub> trimmed reads using *k*-means clustering as implemented in the parKour tool described above to attempt to remove GC% rich contamination from single cell libraries.

The two different clustering schemes attempted using 2 and 3 target clusters. Additionally, both clustering schemes were also run with an initial overclustering factor of 3 i.e. parKour originally found 6 and 12 clusters and then merged them to produce the target 2 and 3 clusters respectively. Therefore, over-clustering made a minimal effect on cluster centroids and read assignment.

Unfortunately, as might have been foreseen, the resultant assemblies from individual read clusters displayed high levels of fragmentation regardless of the clustering regime used. For example, in the case of the 2 cluster (without over-clustering) and subsequent individual Trinity-based assemblies resulted in 268,806 transcripts of marginally shorter average length than the equivalent un-pre-partitioned assembly (99,784 transcripts).

The same pattern, consistent with assembly fragmentation, was observed when only dark sin-

Clustering Scheme	Centroids	Number of Reads Assigned
2	(0.6674, 0.6177)	57.3M
	(0.4557, 0.4393)	81.6M
2 (over-clustering)	(0.6672, 0.6168)	57.7M
	(0.4555, 0.4392)	81.2M
3	(0.5363, 0.5092)	44.0M
	(0.6924, 0.6394)	43.3M
	(0.4231, 0.4096)	51.6M
3 (over-clustering)	(0.5365, 0.5090)	43.9M
	(0.6921, 0.6396)	43.6M
	(0.4235, 0.4098)	51.7M

**Table 4.4.5:** Final cluster centroids and number of reads assigned to each cluster in parKour using various run settings. Centroids are the mid-point of each cluster, therefore in the 2 cluster scheme “parKour” identified one cluster of reads centred around 66.74% GC for the forward read and 61.77% for the reverse read. Note that overclustering made a minimal impact on cluster location.

gle cell libraries were clustered using 2 or 3 clusters. Therefore, GC-based pre-assembly read partitioning proved incapable of improving the assembly of this highly heterogeneous RNAseq dataset.

#### 4.4.2.3 ERROR CORRECTION

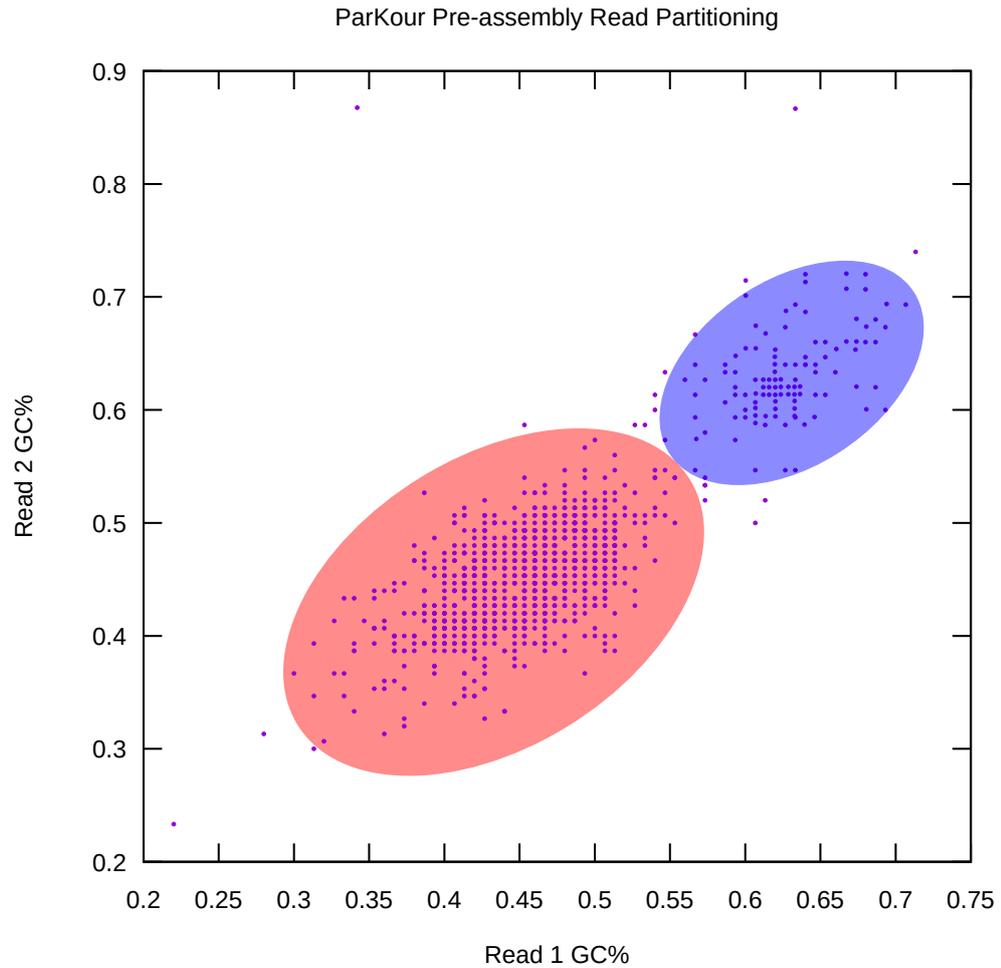
Error correction was attempted on both lightly trimmed ( $Q_5$ ) and harshly trimmed ( $Q_{30}$ ) taxonomically selected SCT reads.

Bayeshammer, as implemented in the Spades genome assembler, even on permissively trimmed ( $Q > 5$ ) reads corrected only a maximum 0.0007% of reads in the 7 taxonomically selected SCT libraries. As this affected on the order of 10s of reads it was not considered worth pursuing this tool further.

SEECER, an RNAseq specific error correction tool was used to correct lightly trimmed ( $Q_5$ ) and harshly trimmed ( $Q_{30}$ ) SCT reads. Approximately, 5.37% of  $Q_5$  trimmed SCT reads were corrected in SEECER and 0.51% of  $Q_{30}$  trimmed SCT reads were corrected.

Trinity assemblies of taxonomically selected single cell libraries (without bulk libraries) were then compared with and without SEECER error correction (see table 4.4.6).

As can be observed, error correction of SCT reads made minimal effect to the overall likelihood of assemblies for this dataset even using only lightly trimmed reads. Error corrected  $Q_5$  trimmed reads actually performed worse than  $Q_{30}$  trimmed reads without error correction. Ad-



**Figure 4.4.8:** Visualisation of GC-based paired read  $k$ -means clustering on a small random subset of all single cell transcriptome reads. 2 initial centroids were specified without an overclustering factor and approximate final centroids (0.6674, 0.6177) and (0.4557, 0.4393) are indicated by highlighted areas. 57.3M and 81.7M were assigned to each respective cluster. Comparison to other clustering regimes can be found in table 4.4.5. This supports the finding of the fig. 4.4.1 that there is a clear and identifiable cluster of high GC% reads present in the sample and it is possible to identify and group these reads using unsupervised learning.

Trim Threshold	Number of Transcripts	Bases Assembled	Assembly Likelihood ( $-\log$ )
Q5	112,182	52,511,552	$-3.168 \cdot 10^{10}$
Q5 SEECER Corrected	111,853	51,847,128	$-3.147 \cdot 10^{10}$
Q30	99,784	47,313,963	$-2.912 \cdot 10^{10}$
Q30 SEECER Corrected	96,494	46,312,469	$-2.995 \cdot 10^{10}$

**Table 4.4.6:** Naive Trinity assembly of Q5 and Q30 trimmed taxonomically selected single cell libraries with and without SEECER error correction. While assembly likelihood increases after error correction for Q5 trimmed reads it is still lower than Q30 uncorrected. For Q30 trimmed reads error correction marginally decreases assembly likelihood.

ditionally, Q30 trimmed reads generated marginally less likely assemblies with error correction than without.

Therefore, error correction was considered ineffective for this dataset and thus was not used

for further analysis. Instead, we elected to use uncorrected, taxonomically selected, Q<sub>30</sub> trimmed reads from this point on.

#### 4.4.2.4 DIGITAL NORMALISATION

Digital normalisation and removal of likely erroneous *k*-mers (i.e. low abundance) via Khmer reduced the total input reads from the Q<sub>30</sub> trimmed taxonomically filtered SCT and bulk libraries from  $2.912 \cdot 10^8$  to  $8.473 \cdot 10^6$  paired reads.

Of those,  $6,231 \cdot 10^6$  derive from the bulk and  $2.253 \cdot 10^6$  from single cell libraries. Therefore, as Q<sub>30</sub> trimmed single cell libraries comprised  $9.318 \cdot 10^6$  paired end reads and bulk libraries consisted of  $52.377 \cdot 10^6$  reads digital normalisation and abundance filtering resulted in a retention of 2.418% of single cell PE reads and 11.891% of bulk PE reads.

Of these surviving single cell PE reads  $9.762 \cdot 10^5$  were from the 3 selected light libraries (Light1-9, Light1-10, and Light1-11) and  $1.277 \cdot 10^6$  were derived from the dark libraries (Dark1-2, Dark2-3, Dark2-6, and Dark2-8). Therefore, abundance filtering and digital normalisation did not disproportionately remove light or dark single cell reads.

This Khmer based pre-processing had a very positive effect on assembly likelihoods. The standard Trinity assembly improved in likelihood by an order of magnitude while assembling more transcripts of near equal length (based on median contig length). The Khmer processed assembly marginally increased median contig length at the expense of a lower N<sub>50</sub>.

Preprocessing	Number of Transcripts	Bases Assembled	Contig N <sub>50</sub>	Median Contig	Assembly Likelihood ( $-\log$ )
Q <sub>30</sub> and Bulk	127,508	83,264,944	851	411	$-2.832 \cdot 10^{10}$
Q <sub>30</sub> and Bulk with Khmer processing	147,902	92,395,841	789	423	$-1.224 \cdot 10^9$

**Table 4.4.7:** Trinity assemblies (with  $-\text{min-kmer-cov } 2$ ) of Q<sub>30</sub> trimmed, taxonomically selected single cell and bulk libraries with and without Khmer digital normalisation and *k*-mer abundance filtering. Khmer pre-processing improved the assembly likelihood by an order of magnitude, and significantly increased the total size of the assembly while only having a marginally negative effect on contig N<sub>50</sub>s.

As Khmer pre-processing both significantly improved assembly run time as well as the overall assembly quality (as assessed in the Trinity assembly comparison metrics above table 4.4.7) digitally normalised and *k*-mer abundance filtered bulk and taxonomically selected Q<sub>30</sub> trimmed

SCT were determined to be the optimal pre-processing for this dataset.

#### 4.4.3 ASSEMBLY

##### 4.4.3.1 REFERENCED ASSEMBLY

Referenced assembly using the divergent *Chlorella NC64A*, *Coccomyxa subellipsoidea C-169*, *Tetrahymena thermophila*, *Paramecium caudatum* genomes as references was largely ineffectual. Of all bulk and SCT reads only 0.3 and 0.4% mapped to the algal references respectively. Similarly, only 0.6 and 0.9% of reads mapped to the related ciliate genomes. This level of mapping is on the order of random chance. Of the reads which mapped, a high proportion (73 – 82%) mapped non-uniquely. This suggests mapping was occurring in low complexity regions and is an artefact for the most part instead of biological significance.

The addition of gene junction annotation files for the reference genomes to improve spliced mapping only improved the percentage of reads mapping by 0.05 – 0.3 percentage points. With so few reads mapping, any attempt to class transcripts from this using cufflinks resulted in 10 – 23 total transcripts.

Therefore, referenced assembly using divergent related genomes proved impossible for this dataset.

##### 4.4.3.2 DE NOVO ASSEMBLY

The results of the initial assembler comparison using the combined assembly of Q30 trimmed taxonomically selected SCT libraries (Light1-9, Light1-10, Light1-11, Dark1-2, Dark2-3, Dark2-6, Dark2-8) and bulk libraries are shown in table 4.4.8.

Critically, Oases, the IDA-MTP/UD/MT pipeline and SOAPdenovo-Trans at higher  $k$ -mer values all failed to run to completion correctly with the dataset. In the case of Oases and SOAPdenovo-Trans at higher  $k$ -mer values this was due to exhaustion of system memory and in the case of IDBA-MTP/UD/MT workflow an unresolved coding error resulting in repeated segmentation faults.

However, Trinity and Bridger both consistently generated assemblies of approximately equal size (100-130,000 contigs of rational sizes: N50s of 700-850 and mean and median contig sizes of 600-660 and 410-470) across a variety of assembly parameters (not shown). Furthermore, they

Assembler	Parameters	Number of Contigs	Bases Assembled	Assembly Likelihood – log
SOAPdenovo-Trans	K23	374,325	$7.64 \cdot 10^7$	$3.778 \cdot 10^{10}$
	*K64	-	-	-
	*K80	-	-	-
TransAbyss	K20	3,272,137	$1.722 \cdot 10^8$	-
	K32	853,079	$1.321 \cdot 10^8$	-
	K64	376,280	$9.755 \cdot 10^7$	-
	Merged	3,055,851	$2.71 \cdot 10^8$	$-3.113 \cdot 10^{10}$
Oases*	-	-	-	-
IDBA-tran	-	54,113	$2.7 \cdot 10^7$	$-4.589 \cdot 10^{10}$
IDBA-MTP/UD/MT**	-	-	-	-
Trinity	min_kmer_cov 2	127,508	$8.326 \cdot 10^7$	$-2.832 \cdot 10^{10}$
Bridger	K25	114,582	$9.707 \cdot 10^7$	$-2.587 \cdot 10^{10}$

**Table 4.4.8:** *De novo* combined assemblies of Q30 trimmed taxonomically selected single cell libraries and bulk libraries (but not digitally normalised or *k*-mer abundance filtered) with a range of assemblers and parameters. *k*-mer size used for assemblers with that option are indicated in the Parameters column e.g. K23 indicates a 23-mers. Bridger and Trinity outperformed other assemblers in terms of assembly likelihood and rational contig numbers and sizes. \* indicates assemblies programs that failed to run to completion due to insufficient computational resources (despite using a server with 500GB of memory) \*\* indicates assemblies which failed due to coding errors in the application.

both consistently generated the assemblies with the greatest likelihoods (from RSEM-EVAL), and ran most computationally efficiently.

Trinity and Bridger assemblies using digitally normalised and *k*-mer abundance filtered, taxonomically selected, Q30 trimmed, single cell and bulk libraries performed even better in terms of assembly likelihood and read incorporation.

Assembler	Parameters	Contigs	Bases Assembled	Assembly Likelihood (– log)
Bridger	K19	102,686	$8,209 \cdot 10^7$	$-1.729 \cdot 10^9$
	K25	113,106	$9.866 \cdot 10^7$	$-1.183 \cdot 10^9$
	K31	112,391	$8.941 \cdot 10^7$	$-1.143 \cdot 10^9$
Trinity	Minimum <i>k</i> -mer Coverage of 1	176,097	$1.113 \cdot 10^8$	$-1.214 \cdot 10^9$
	Minimum <i>k</i> -mer Coverage of 2	147,902	$9.239 \cdot 10^7$	$-1.238 \cdot 10^9$

**Table 4.4.9:** Assembly summaries of Q30 trimmed taxonomically selected SCT and bulk reads after digital normalisation and *k*-mer abundance filtering. Parameters used in the assembly indicates any special parameter settings used in the assembly i.e. K19 indicates a *k*-mer size of 19 was used.

Smaller *k*-mer values (19-mer) performed worse in the case of the Bridger assembly with the optimal assembly in terms of contig number and size was the *k*-mer size of 25. This was slightly lower in terms of likelihood than the 31-mer Bridger assembly. The digitally normalised and filtered Trinity assemblies generated much larger assemblies overall but still produced good likelihoods.

#### 4.4.3.3 ASSEMBLY COMBINATION

Two assemblies were combined using the `tr2aacds.pl` script in EvidentialGene and a minimum CDS size of 100. The first consisted of all successfully completed assemblies of non-normalised/filtered reads i.e. SOAPdenovo-Trans, TransAbyss (multiple  $k$ -mer assembly merged using built-in tool), IDBA-tran, Trinity and Bridger in table 4.4.8. The second, of the 3 Bridger digitally normalised assemblies and two Trinity assemblies described in table 4.4.9.

Assembly	Input Contigs	Collapsed Contigs	Assembly Likelihood ( $-\log$ )
Non-normalised Assemblies	3,726,379	46,063	$-4.347 * 10^{10}$
Normalised Assemblies	652,182	53,628	$-1.823 * 10^9$
<b>CD-HIT 90% meta-clustering</b>	99,691	94,628	$-5.133 * 10^{10}$

**Table 4.4.10:** Summary of merged multi-assemblies. Collapsed contigs is the number of contigs found in the merged set by the EvidentialGene pipeline. The level of assembly reduction and redundancy removal is high and, at first appearance, is impressively consistent between meta-assemblies despite differences in preprocessing. However, CD-HIT metaclustering at 90% identity shown at the bottom demonstrated that there was very little overlap between these two minimised assemblies. Even the merged normalised assemblies generated a meta-assembly of lower overall likelihood than the best individual constituent assemblies.

The combination of all non-normalised assemblies produced a surprisingly small set of contigs, however, both assemblies also had lower likelihoods than any of their constituent assemblies. It is of interest that despite generating similar numbers of contigs there was next to no overlap between the two combinations as assessed by clustering using CD-HIT at a similarity of 90%.

Therefore, the assembly selected for downstream binning and analysis was Bridger assembly of bulk and library screened single cell normalised and  $k$ -mer abundance filtered reads with a  $k$ -mer size of 31 as it displayed the best likelihood while maintaining assembly statistics within expected ranges.

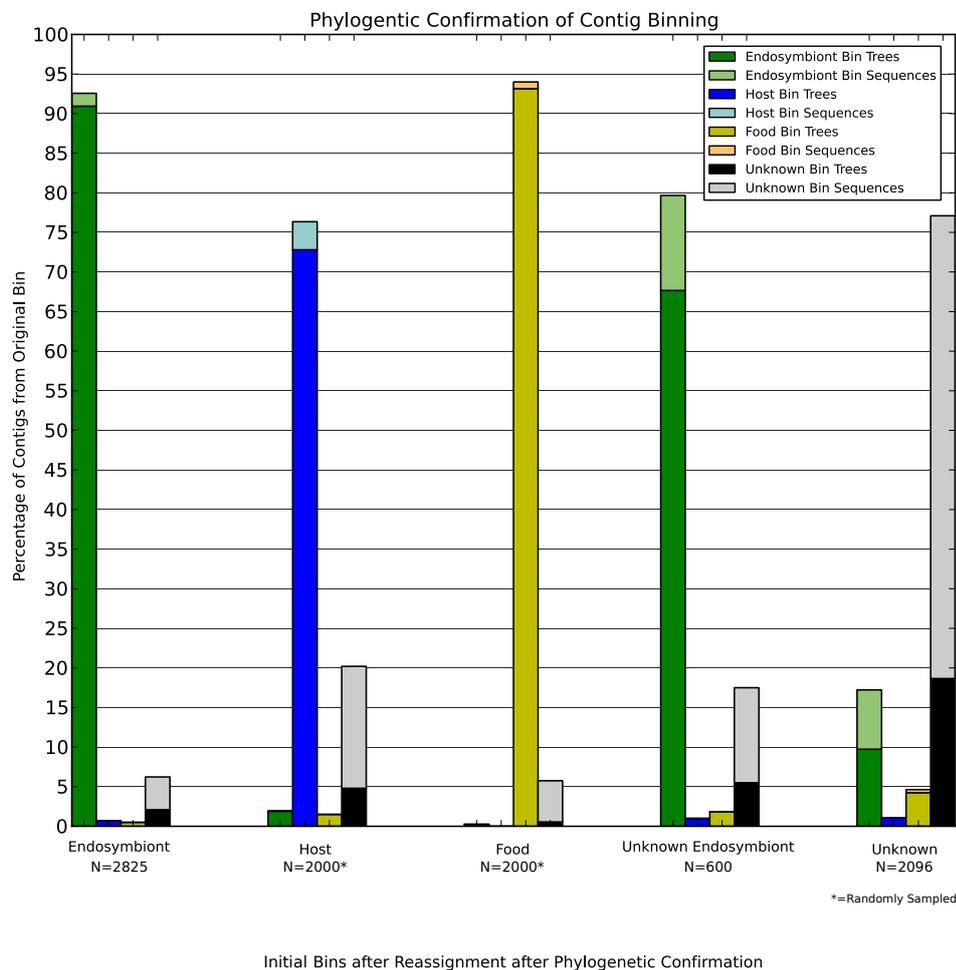
#### 4.4.4 BINNING

##### 4.4.4.1 ORF CALLING

From the 112,391 contigs in the final selected assembly (31-mer Bridger Normalised and Taxonomically Selected SCT and Bulk) - 1,005,370 ORFs longer than 30 amino acids were identified using a *Tetrahymena* encoding. Using the 500 longest of these ORFs to train a Markov Model and removing shorter ORFs that lay entirely within a longer ORF resulted in a final set of 70,605 ORFs.

#### 4.4.4.2 PERFORMANCE OF BLAST-BASED BINNING

10,000 of these ORFs were randomly selected and used to search the NCBI nr database with BLASTP with an expectation of  $1e - 5$ . Based on the taxonomic provenance of the top-hit these ORFs were assigned to a particular originating bin. The initial identification and binning of recovered transcripts into host and endosymbiont categories was tested using this phylogenetic approach. The results of this analysis is plotted in fig. 4.4.9. This demonstrates that the initial bin identifications were accurate for endosymbiont ( $\sim 92\%$ ) and food ( $\sim 94\%$ ) derived transcripts.



**Figure 4.4.9:** Preliminary analysis of change in binning after manual phylogenetic confirmation. These results demonstrate that individual top BLAST hits is a sub-optimal means of identifying transcript bins as they are partially inconsistent with full phylogenetic analyses. Furthermore, this error is unevenly distributed across initial bins i.e. BLAST bins perform worse for potential Host transcripts than they do for other bins. This analysis was based on an earlier iteration of the assembly and ORF calling.

#### 4.4.4.3 PHYLOGENY-BASED BIN CLASSIFICATION

The 70,095 transdecoder called peptide sequences were then run through the automatic phylogeny generation pipeline (“Dendrogenous”) against the 40 representative genomes described above. Of these, 38,193 had no BLAST hit against any genome database sequence and thus were not used to generate phylogenies. A further 9,335 had less than 4 hits and thus were not used to generate phylogenies but were taxonomically sorted based on the BLAST hit binning criteria to give 8,574 “host” sequences, 258 “endosymbiont”, 395 “food” and 108 “unknown”. An additional 9 sequences had insufficient numbers of sites when masking to generate a phylogeny ( $\leq 30$ ). Finally, 10 phylogenies were malformed due to a latent bug in FastTree2. Therefore, 22,672 phylogenies were successfully generated and named from the input sequences.<sup>4</sup>

The training dataset and test datasets were visualised to ensure that the training dataset (generated during a previous iteration of these analyses) was representative of the test dataset. These plots demonstrate a possible under representation of “Unknown” and/or “Food” samples (fig. 4.4.10) but do reflect a training dataset that largely encompasses a good quantity of the same feature space as the test dataset (fig. 4.4.11).

Numerous classification algorithms were fitted to this training dataset and hyperparameters were efficiently optimised using random search and Bayesian optimisation on the cross-validation folds. The average F-1 scores across classes were tallied and compared revealing *k*-neighbours the most effective classification algorithm for this dataset (fig. 4.4.12).

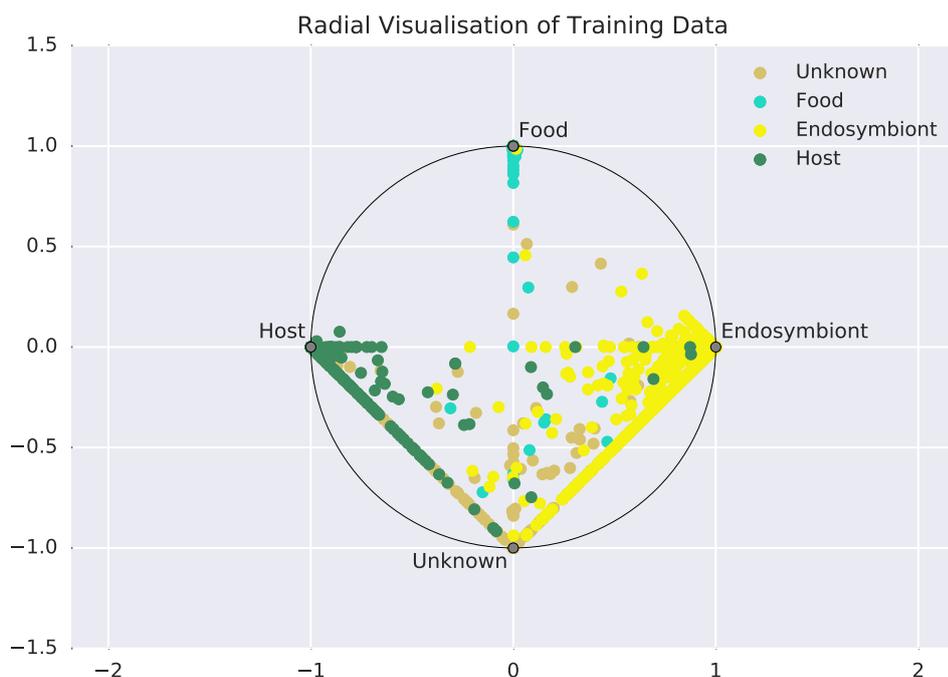
As can be seen in the confusion matrix (and manual parsing of the classification reports from each classifier (see appendix section A.2.2) *k*-neighbours (like the majority of classifiers) poorly classified “Unknown” samples but largely performed well (0.89 – 0.9 for each class (table 4.4.11).

When the trained K-Neighbours model was used to classify the unlabelled 22,672 phylogenies: 415 were “endosymbiont”, 2253 “unknown”, 19476 “host” and 531 “food”.

Therefore, of the 70,095 called ORFs in total there were: 28,050 were “host” derived, 673

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<sup>4</sup>In speed testing “Dendrogenous” proved very efficient at rapidly generating phylogenies with its fully parallelised mode capable of generating 100 phylogenies randomly selected transcripts against 41 genomes in an average 2:22.50 minutes. The same pipeline run serially took an average of 23:41.39 minutes and the stage-wise parallel was very marginally faster at an average of 21:45.02 minutes.



**Figure 4.4.10:** Radial Visualisation of Manually Parsed Training Data. All input features are normalised to unit magnitudes. Each point represents a single training sample (i.e. phylogeny) and its relative proximity to the cardinal points of the unit circle represents a the number of closely related taxa considered part of that “class”. Unknown and Food classes can be seen to be particularly problematic and poorly partitioned. represents the

“endosymbiont”, 40446 “unknown” and 926 “food”.

#### 4.4.4.4 PERFORMANCE RELATIVE TO TAXASSIGN

TAXAssign performed relative poorly at taxonomic classification/binning of transcripts. Of 70,605 CDS sequences only 2,043 (2.893%) were assigned a phylum level taxonomic identity (table 4.4.12). This can be contrasted with the 29649/70605 or 41.99% classified using the phylogeny and supervised classification system.

## 4.5 DISCUSSION

### 4.5.1 LIBRARY SCREENING IS A KEY STAGE IN SC-RNASEQ

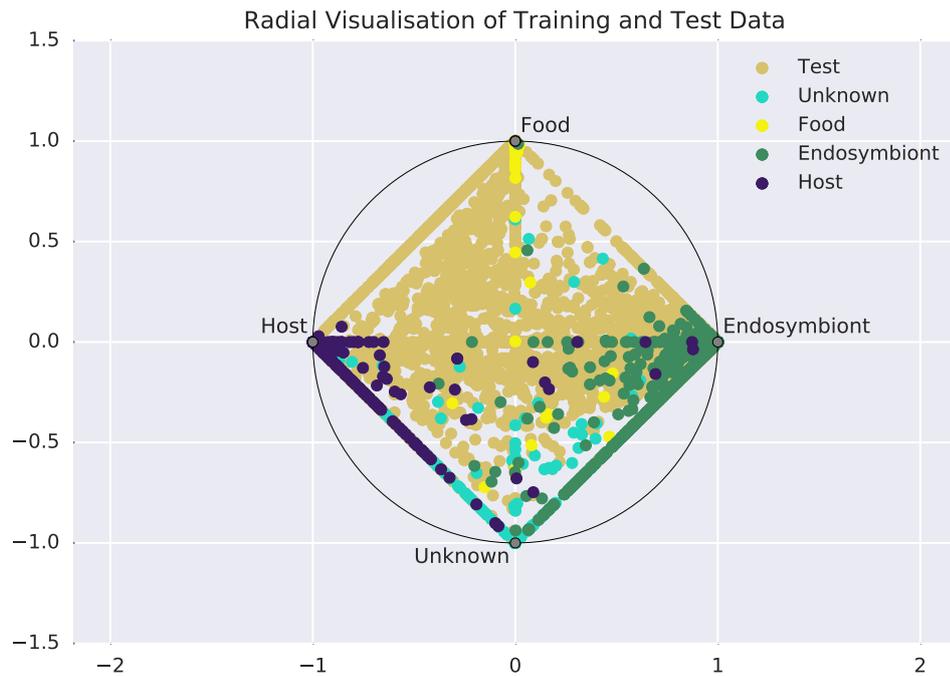
Despite evidence that nanoscale methods can greatly reduce levels of contamination (Blainey and Quake, 2011), the taxonomic profiling conducted here indicates a high level of bacterial (and viral) contamination in the sc-RNAseq. Therefore, much as library contamination is one of the

Label	Precision	Recall	F1-Score	Support
“Unknown”	0.96	0.84	0.90	156
“Food”	0.98	0.99	0.99	426
“Host”	0.90	0.99	0.98	787
“Endosymbiont”	0.97	0.99	0.89	359
average / total	0.95	0.96	0.95	1728

**Table 4.4.11:** Classification report of a trained and optimised  $k$ -neighbours classifier using a leaf size of 30, minkowski distance metric and 50 neighbours. Note the poor performance on “Unknown” samples but generally good ( $\geq 90\%$ ) on other labels. This can likely be explained by the “miscellaneous” nature of this label and the diverse phylogenies that comprise it.

Class	Phylum	Sequences Assigned
“Host”	Intramacronucleata	97
“Endosymbiont”	Streptophyta	101
	Chlorophyta	58
	Cyanobacteria	1
	Proteobacteria	1270
“Food”	Firmicutes	80
	Actinobacteria	35
	Bacteroidetes/Chlorobi	29
	Chordata	365
“Unknown”	Chlorovirus	94
	Arthropoda	7

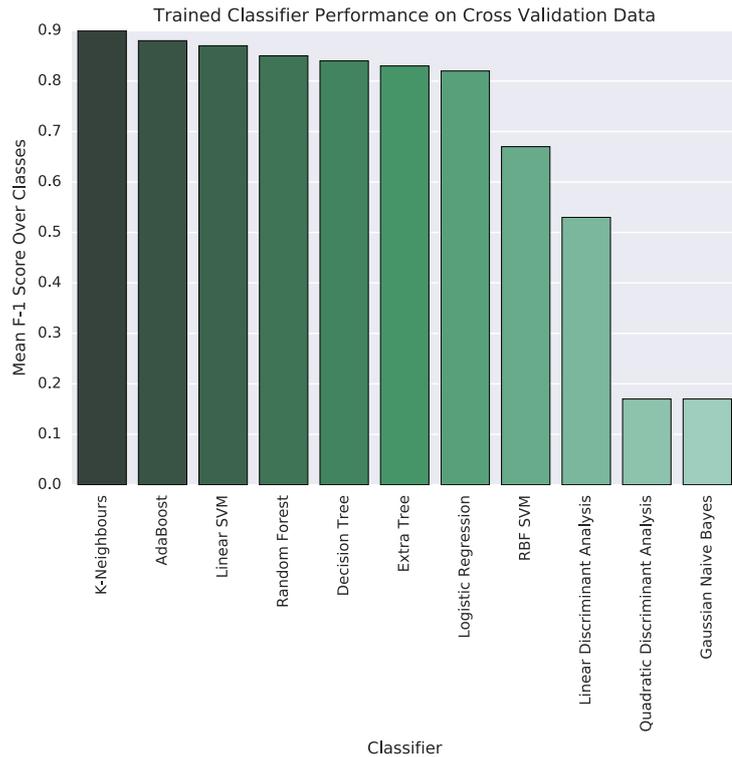
**Table 4.4.12:** Phylum level TAXAssign assignments for (2, 043/70, 605) CDS called from the Bridger 31-mer assembly. Only 2.893% were assigned using this method relative to 39.72% for the phylogenetic supervised learning (Dendrogenous-Arboretum) method. Therefore, this demonstrates how well this method works relative to conventional binning approaches like TAXAssign.



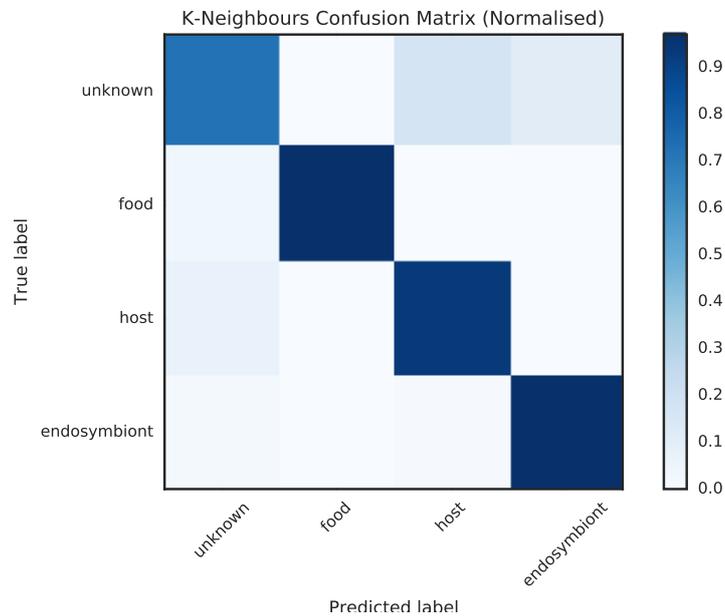
**Figure 4.4.11:** Radial Visualisation of Test Data and Training Data. All input features are normalised to unit magnitudes. Each point represents a single training sample (i.e. phylogeny) and its relative proximity to the cardinal points of the unit circle represents a the number of closely related taxa considered part of that “class”. Test shows the position of all unlabelled phylogenies. This plot shows where the training data is poorly sampled - specifically phylogenies that only contain “host” and “food” taxa or “host” and “endosymbiont” taxa. Therefore, these phylogenies in the test data may prove problematic to easily classify.

key issues with single cell genomics (Blainey, 2013; Lusk, 2014), it is also highly important in SCT. This is in concordance with the findings of (Kolisko et al., 2014), in which enigmatic, bacterial contamination was a problem in single cell eukaryotic transcriptomes.

Single cell methods are particularly prone to contamination issues from reagents, laboratory environment and enigmatic nucleic acids within the biological samples themselves. This is due to the low-input concentration and high amplification necessary in these approaches (Blainey, 2013) leading to enrichment of non-target sequences, especially bacterial contaminants present around or within the *P. bursaria* host. It is critical to identify and discard highly contaminated libraries in *de novo* assemblies as contaminant reads severely complicate the assembly graph thus increase the computational difficulty and reduce the accuracy of the DBG path resolution. This was highlighted by observations in the preliminary stages of this project that the inclusion of certain (SCT) libraries would increase assembly run-time and lead to the generation of fragmented transcripts relative to assemblies without those libraries.



**Figure 4.4.12:** Average F1-scores of each classification algorithm attempted. Note, that *k*-neighbours performed the best and there was particularly poor performance in QDA and Naive Bayes.



**Figure 4.4.13:** Normalised confusion matrix for *k*-neighbours. These plots highlight the problematic classes in the cross-validation dataset. The heatmap corresponds to the proportion of samples classified with a given predicted label compared with their true labels. “Host” samples are accurately classified however a small number are erroneously classified as “Unknown”. Similarly, “Unknown” samples are relatively poorly classified in general.

Taxonomic profiling also reveals other features of the dataset that wouldn't necessarily be obvious otherwise. For instance, our profiles demonstrated systematically low levels of Viridiplantae related reads across both bulk and sc-RNAseq libraries and in both lit and dark conditions. Despite care being taken to ensure thorough lysis of the chitinous *Micractinium* cells during RNA extraction and a ratio of  $\sim 300 : 1$  endosymbiont to host cell ratio this may be related to lytic inefficiencies (Korfhage et al., 2015) or potentially just relatively lower endosymbiont transcriptional activity relative to host and associated bacteria species. Finally, it is possible that due to the endosymbiont being largely provisioned by the host it may be relatively transcriptionally inactive and thus relatively fewer transcripts can be recovered.

Intriguingly, taxonomic profiling of the bulk libraries showed a very high percentage of reads mapping to no sequence in the nr protein database. This was significantly more than in the sc-RNAseq libraries. While this finding is concerning it is likely to be an artefact of the older sequencing platform the bulk data was generated on. These paired reads were sequenced via the GAII and were half the length of the HiSeq2500 reads used for the SCTs. Shorter reads and a relatively higher technical error rate on this platform may have played a role in this marked decline in recognisable reads. Despite this, the relative proportion of bacterial reads to eukaryote reads among the recognisable reads is much lower for bulk libraries than SCT. This does, however, suggest that read length is highly important to accurate contamination screening/taxonomic profiling.

Taxonomic profiling of reads/libraries proved surprisingly robust to trimming. The profiles generated in “DueyDrop” were largely identical regardless of whether the input library had been trimmed or not (even at high stringency thresholds). This indicates that screening can occur before the computationally expensive trimming process without loss of accuracy. The results presented here also demonstrate that the profile generated from a relatively small random subsample of a library is largely consistent with that of the profile generated from the library as a whole.

“DueyDrop” could potentially be improved in such a way that an entire library can be screened quickly instead of just subsamples. Briefly, this would involve quantifying exact  $k$ -mer matches between library reads and a pre-generated database of known taxa using efficient probabilistic hashing data structures such as a bloom filter, or more likely count-min sketch and an efficient  $k$ -mer counting library such as Jellyfish (Marçais and Kingsford, 2011). This would have a ma-

for speed advantage compared to the BLASTX-based method of “DueyDrop” and would thus allow entire libraries to be checked in reasonable time. A similar approach to this has been implemented as a service by <https://www.onecodex.com/>, although this focuses specifically on screening for medically relevant taxa. However, it would require laborious workarounds to handle  $k$ -mers shared between multiple taxa in the database, translation of reads and/or database sequences into a matching sense and form (e.g. protein), and use of a locality-sensitive hash function to handle scenarios where there is no exact  $k$ -mer match. This latter issue is particularly problematic for libraries consisting of transcripts from poorly sampled sections of the tree of life where exact matches would become commensurately rarer as sampling sparsity increases. While still affected by this problem, the BLASTX/Diamond approach implemented in the “DueyDrop” scripts are relatively more robust to these problems due the explicit probabilistic modelling of sequence divergence built into the BLAST alignment algorithm (e.g. e-values). Other potential improvements to “DueyDrop” would be to incorporate some degree of automatic clustering of phylogenetic profiles using unsupervised learning and possibly manifold embedding, potentially including a form of anomaly detection to discover contaminated libraries. Robustness of taxonomic inference for each profile can also be improved by taking all hits instead of just the top one and resolving conflicting phylogenetic signal using a lowest common ancestor algorithm over all the hits.

#### 4.5.2 COMBINING SINGLE CELL AND BULK TRANSCRIPTOME DATA CREATES NEW CHALLENGES

Contrary to previous studies in optimising conventional RNAseq assemblies where: permissive trims (MacManes, 2014), error correction (Macmanes and Eisen, 2013; Macmanes, 2015) and the combination of multiple assemblies (Nakasugi et al., 2014) have been demonstrated as effective tactics in recapitulating a comprehensive set of transcripts *de novo*, MDA-based SCT and bulk datasets such as the one investigated above exhibit different properties. It is important to pick a trimming threshold which minimises sequence error (mostly substitutions (Yang et al., 2013b)), as these result in assembly of spurious sequences, but doesn't discard reads necessary to complete transcripts (Macmanes and Eisen, 2013; MacManes, 2014). For the *P. bursaria* - *M. reisseri* bulk and SCT dataset the optimal trimming threshold - based on both mapping statistics to preliminary assemblies and final assembly likelihoods proved to be a harsh threshold of Q30.

Following a similar theme, despite numerous indications that error correction is important

for improving the accuracy of genomic and transcriptomic assemblies using Illumina reads (e.g. (Molnar and Ilie, 2014; Macmanes, 2015)) in the case of this dataset error correction appeared to have a minimal effect with few reads being corrected and downstream assemblies being largely equivalent to those without error correction. In fact, permissively trimmed (Q<sub>5</sub>), error corrected assemblies were shown to have lower likelihoods and smaller assembly sizes than more conservatively trimmed assemblies (Q<sub>30</sub>). It should be noted that SEECER, while an RNAseq specific error correction tool is not optimised for single cell MDA-based datasets, and Bayeshammer is not optimised for transcriptomic data. Therefore, the poor performance of error correction in this dataset might be a consequence of the lack of error correction tools designed for MDA-based sc-RNAseq datasets. It will likely prove beneficial, as datasets of this type become more prevalent, to develop tools for this specific use case combining the most effective features of the MDA aware BayesHammer and the RNAseq optimised SEECER. This said, there are several other available Illumina RNASeq error correction tools which were not trialled, SEECER was chosen in accordance to the recommendations based on dataset and hardware heuristics (> 50M reads and the availability of a high memory system) (Macmanes, 2015) but as we've demonstrated the limitations of such heuristics on new types of data it might be worth investigating these alternative tools further.

Finally, merging multiple assemblies proved a sub-optimal strategy with all merged assemblies generating lower likelihood assemblies than the best individual assembly. While not merging assemblies might mean fewer transcripts are recovered (especially assemblies at a range of *k*-mer sizes as short *k*-mers generally recover lower expression transcripts and vice versa for long *k*-mers) the much higher likelihoods meant the best performing individual assemblies (Bridger using 31-mers, Q<sub>30</sub> trimmed taxonomically selected SCT and bulk libraries) were preferred.

These results suggest that MDA-based single cell transcriptomic datasets do not behave in a qualitatively similar way to bulk RNAseq in terms of pre-processing and assembly parameters. This means care must be taken incorporating advice and heuristics derived from studies based on analysis bulk RNAseq datasets e.g. (Macmanes and Eisen, 2013; MacManes, 2014; Macmanes, 2015; Nakasugi et al., 2014). As further studies using MDA SCTs are completed e.g. (Kolisko et al., 2014) a greater understanding of the optimal analysis of this type of dataset will emerge.

### 4.5.3 PRE-ASSEMBLY READ PARTITIONING IS NON-TRIVIAL

As we expect the *PbMr* metatranscriptome to contain predominantly a highly AT-rich organism, *Paramecium*, (ranging from 24.1 to 28.2%GC in *P. aurelia* species complex and *P. caudatum* (Aury et al., 2006; McGrath et al., 2014)) and a GC-rich organism, *Micractinium*, (*Chlorella variabilis* NC64A genome is approximately 67.1%GC, the highest found in a sequenced eukaryote genome (in 2010) (Blanc et al., 2010)), the utility of pre-assembly read partitioning was assessed. This GC pattern was supported by the clear bimodal GC distribution that can be observed in fig. 4.4.1. However, under careful observation it was apparent that the bimodal GC distribution was more attributable to the presence of a GC-rich contaminant such as Rhizobiales (Peralta et al., 2011). Therefore, in practice pre-read partitioning was mainly attempted to try to remove these contaminant reads from screened libraries. Theoretically, accurate pre-assembly read partitioning could transform a complex assembly graph into two relatively simpler assembly tasks. As well as simplifying path resolution accuracy this method could speed up assembly considerably and thus allow more iterations to optimise other assembly parameters.

This pre-assembly partitioning has been tried with mixed success in other meta-omic analyses e.g. Dröge and Mchardy (2012). However, a lack of fast efficient tools to accomplish this led to the creation of “parKour”. The developed GC partitioning package proved very effective at rapidly and relatively computationally efficient clustering of PE RNAseq data. ParKour generated clusters with centroids reasonably where they may be expected from inspection of per read GC probability densities (see fig. 4.4.3) i.e. partitioning out the GC rich potentially contaminant reads (likely from *Rhizobia* bacterial species). Unfortunately, in the case of this dataset, clustering proved ineffective at improving assembly accuracy and fully removing groups of contaminant reads with large GC skews. The likely explanation for this is that even 150 bp paired end reads are too short to consistently statistically demonstrate the GC-AT bias of the originating organism. This means any partitioning is likely to remove a significant number of reads necessary to complete transcripts due to local variation in AT bias. The high number of shorter contigs is indicative of the kind of assembly fragmentation that would be expected in this situation.

However, the relative efficiency and theoretical benefits of this type of clustering indicates there may be some potential to utilising a similar but less naive approach in future work. It may be possible to combine “DueyDrop” and “ParKour” to allow read-level screening and partitioning

of reads on the basis of taxonomic profile and compositional characteristics such as GC% and tetramer frequencies. This could improve resolution of clusters and decrease the observed contig fragmentation effect while performing accurate taxonomic screening.

Other improvements could include the consideration of alternative clustering algorithms such as  $k$ -medoids (Kaufman and Rousseeuw, 1987) with more robust outlier stability or large scale database clustering algorithms such as DBSCAN (Ester et al., 1996) or BIRCH (Zhang et al., 1996) (allowing non-convex clusters). Silhouette coefficients and analysis<sup>5</sup> (Rousseeuw, 1987) can be incorporated to aid determination of the expected number of clusters when it cannot be determined *a priori* from inspecting the data as well as validation of generated clusters. Unfortunately, other validation and analysis systems are somewhat limited due to the lack of ground truth labelling available. Alternatively, a variational Bayes approach could be implemented to determine the optimal number of clusters e.g. CONCOCT (Alneberg et al., 2014). Finally, memory efficiency can be improved by use of streaming clustering algorithms (e.g. those discussed in O’Callaghan et al. (2002)) in which all data does not require to be loaded into a matrix at a given time and can be clustered as they are parsed.

#### 4.5.4 DIGITAL NORMALISATION GREATLY IMPROVES ASSEMBLIES

Digital normalisation, a method to remove redundant read data from libraries and thus reduce the computational burden of assembly (Brown et al., 2012), was also investigated for this dataset and found to be a highly effective strategy in improving assemblies of mixed bulk and MDA SCT data.

Interestingly, some have argued that error correction is a special case of digital normalisation (Krasileva et al., 2013). This is supported by the fact that many error correction algorithms operate on similar principal of attempting to remove low abundance  $k$ -mers from input datasets.  $k$ -mers with a low abundance are more likely to be due to sequencing errors than representing novel biological diversity. This said digital normalisation has the potential to spuriously discard true variation that is merely undersampled in our libraries due to the high level of contamination.

This hypothesis is somewhat supported by the disproportionate retention of bulk reads relative to noisy single cell reads. However, within the context of the single cell reads the more

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<sup>5</sup> $s = \frac{b-a}{\max(a,b)}$  where  $s$  is Silhouette coefficient,  $a$  is the mean distance between a sample and all other points in the same class and  $b$  is the mean distance between a sample and all other points in the next nearest cluster (Pedregosa et al., 2011). Therefore, the Silhouette coefficient acts as a measure of cluster definition.

highly contaminated dark reads were retained in roughly equal proportion to the light reads. This suggests that MDA derived data may also just display a greater quantity of low-level sequencing error.

It should be noted that the digitally normalised and  $k$ -mer abundance filtered assemblies also incorporated more bases overall than the equivalent assembly using the full libraries therefore resultant assemblies were not just high confidence (and likelihood) subsets of the initial input data.

One factor that has not been adequately analysed in the context of this work is that of sequencing depth. Future studies will need to carefully consider sufficient sequencing depth given the noise and prevalence of contamination in MDA based data.

#### 4.5.5 ASSEMBLY AND ASSEMBLY ASSESSMENT

While we have demonstrated that some progress can be made identifying optimal pre-processing parameters using measures such as mapping metrics it is very difficult to identify the parameters (preprocessing or otherwise) which will lead to the “best” *de novo* assembly without actually generating the assembly. Assembly can be considered an example of Wolpert and Macready’s “No Free Lunch Theorems” (Wolpert and Macready, 1995, 1997) as (in the case of *de novo* assembly) it is fundamentally a hamiltonian/eulerian cycle search problem (equivalent in the de-Bruijn formulation) and therefore any two assembly implementations (in different assemblers and/or with different parameters) should ultimately be equivalent across all possible input datasets.<sup>6</sup> For this reason, it is necessary to try assembly using a variety of assembly hyperparameter values and indeed a range of both *de novo* and referenced assemblers.

Unfortunately, the task of identifying the “best” *de novo* transcriptome assembly is also a non-trivial task (Neil and Emrich, 2013). Many widely used assembly assessment metrics have been shown to be inconsistent measures in simulated sequencing data, especially those metrics related to individual contigs (theoretically different transcript splices). Metrics such as average length and N50 prove consistent across both simulated sequencing depth and read lengths i.e. they improve towards (Neil and Emrich, 2013). Furthermore, the number of possible metrics is greatly reduced if assessment is mainly conducted in a reference-free manner (Li et al., 2014). As

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<sup>6</sup>This should be taken with a pinch of salt, a proof of this theorem applied to the case of assembly is beyond both the scope of this thesis and my abilities.

the majority of assemblies were *de novo* and the suitability of the related but divergent genomes proved lacking it was necessary to restrict to reference-free assembly assessments. Therefore, a model-based reference-free assembly scoring algorithm (RSEM-EVAL (Li et al., 2014)) was, along with standard (if imperfect) metrics, used to evaluate different assemblies in this study. The assumption of the accuracy of the RSEM-EVAL likelihood is a strong one, and deserves careful re-consideration in further work.

In terms of referenced assembly using divergent relatives, it is safe to conclude that despite other findings that even divergent (up to 15%) genomes can generate transcriptomes of higher-quality than *de novo* (Vijay et al., 2013), the potential references are too divergent in the case of the *PbMr* to be of any utility.

Overall, a comparison of *de novo* assemblies using a range of assemblers and parameters on “optimally” pre-processed read data demonstrated the clear superiority of both “Bridger” and “Trinity”. Trinity comes with the advantages of being a generally better developed tool that interlocks effectively via several plugins and utility scripts with other tools and analysis pipelines.

However, despite being relatively newer and consisting of a less mature and tested codebase, Bridger proved to be a slightly more effective assembly tool overall. Unfortunately, coding problems and a lack of public active development means it is non-trivial to successfully use this tool. In the process of implementing the above analyses it was necessary to fix several bugs present in the assembler. These upgrades were merged into the code and are available on GitHub ([https://github.com/fmaguire/Bridger\\_Assembler](https://github.com/fmaguire/Bridger_Assembler)). Hopefully rehosting this code on a public development and collaboration platform (as well as adding continuous integration) will spur further development of this promising tool.

Interestingly, despite strong evidence supporting the need to combine assemblies, due to the size of the disjoint sets of transcripts recoverable from different algorithms and parameter choices (Lowe et al., 2014), assembly merging systematically led to worse overall assemblies with this dataset (as assessed by RSEM-EVAL likelihood scores). The likelihood of the merged assemblies were worse than the best individual constituent assemblies.

#### 4.5.6 BINNING

Even once a good assembly has been generated it is still necessary to identify the likely originating species of a given transcript i.e. host, endosymbiont, food bacterial contaminant or other

contaminant. While a successful partitioned pre-assembly strategy may simplify this process it would still be sensible to confirm bins using downstream analyses that use full length assembled transcripts. Rough, approximate bins were generated using a simple “top BLAST hit” approach following ORF calling (using *Tetrahymena* and Universal encodings) against a set of representative predicted proteomes. In order to assess how accurate these bins were likely to be, 10,000 were randomly selected and rapid maximum-likelihood phylogenies were generated using the transcript sequence as a seed to search the entire RefSeq protein nr database. This was accomplished using “Dendrogenous”, a rewritten and modified version of a pipeline originally known as “Darren’s Orchard” which first appeared in (Richards et al., 2009). Phylogenies were manually assessed to check whether the resultant topology was congruent with the BLAST based binning i.e. are supposedly “endosymbiont” transcripts branching principally with Archaeplastida taxa. However, due to the slow largely manual nature of this phylogeny assessment process it would be infeasible to repeat this for all transcripts generated from a single assembly, let alone investigating several such assemblies.

Therefore, this became a fundamental classification problem with the 10,000 manually verified phylogenies forming a handy training dataset for supervised learning. To determine the best performing classification algorithm and hyperparameters for this dataset an automated search was conducted using bayesian optimisation. This was then converted to a binning script named “Arboretum”.

High throughput phylogeny generation, parsing and supervised classification is a more sensitive and powerful way in which to bin transcripts into their likely originating organisms provided a reasonable level of *a priori* knowledge of the system at hand. This demonstrably operates better than established although simpler approaches such as TAXAssign or top BLAST hit. While classification accuracy (and F-1) is sub-optimal for “Food” and “Unknown” bins it (table 4.4.11) a decent level of precision and recall for the target bins of “Host” and “Endosymbiont” was achieved using “Arboretum”.

However, this classification is still a work in progress and could potentially be improved by the addition of anomaly detection in place of the catch-all (and subsequently poorly classified) “Unknown” classes. Furthermore, there are several potential possible improvements in the classification itself that could be made. Specifically, unsupervised clustering pre-training could potentially

forgo the need for laborious manual generation of the training dataset and minimise the difficulties in handling these aberrant phylogenies. Additionally, an AutoML bagging estimator such as one of those implemented in the AutoML project (Eggenesperger et al., 2013), a variational autoencoder pre-processing followed by a deep neural network classifier or using a phylogeny specific kernel function (Vert, 2002) in a Gaussian process or SVM system all offer potential algorithmic improvements. Finally, incorporation of more sequence related features such as  $k$ -mer coverage,  $n$ -mer frequencies and composition into each samples may help greatly improve the fidelity of classification.

## 4.6 CONCLUSION

In conclusion, for this dataset the optimal pre-processing was determined to be careful taxonomic screening of input libraries, followed by trimming at a high (Q30) threshold and subsequent digital normalisation and low-abundance  $k$ -mer filtering. The optimal assemblies were generated using larger (25-31  $k$ -mer) sizes and utilised the Bridger (and to lesser extent Trinity) assembly algorithms. While pre-assembly read partitioning proved ineffective in this implementation, in future a less naive method that incorporates both read-level taxonomic data and compositional information could potentially improve assemblies of complex eukaryotic metatranscriptomes, especially those that combined bulk and single cell RNAseq data. In general, MDA-based single cell datasets have been shown to be noisy and thus difficult to analyse. Potentially it may be advisable to limit their use to systems with robust references or to use much greater sequencing depths.

Finally, I have demonstrated that BLAST based transcript binning alone is ineffective at accurately binning transcripts. Fast, automated phylogeny generation and the subsequent use of supervised learning (particularly large ensemble models such as Random Forests and those AutoML algorithms) can potentially improve the quality of such binning. Further work in the implementation of unsupervised clustering of generated phylogenies could conceivably forgo the laborious process of manually generating a training dataset.

Points arising in this analysis:

- It is possible to generate a functional working transcriptome combining bulk and MDA based RNAseq (see table 4.6.1)

<b>Transcript Bin</b>	<b>Number of Transcripts</b>	<b>Called ORFs</b>
Endosymbiont	8,975	4,275
Host	18,793	17,920
Food	18,516	-
Unknown	66,107	-

**Table 4.6.1:** Summary of transcriptome assembly and binned sequences

- sc-RNAseq libraries generated from dark samples are problematic.
- Binning methodologies may prevent easy finding of novel genes due to a lack of homology.

*“All models are wrong, but some are useful”*

- George E.P. Box & Draper: *Empirical model-building  
and response surfaces*, 1987

# 5

## Metabolic integration

### 5.1 INTRODUCTION

The linking of metabolism between host and endosymbiont is a fundamental stage in endosymbiotic integration (Bhattacharya et al., 2007; Karkar et al., 2015). Complementation of the respective metabolic deficiencies/limitations in host and endosymbiont allow exploitation of novel niches and provide the key selective benefits of endosymbiosis (Hoffmeister and Martin, 2003).

In order to identify putative metabolic integration it is necessary to identify the primary “points of contact” between the metabolic networks of host and endosymbiont. These “points of contact” comprise two major classes of proteins, transporters and secreted proteins. In the *P. bursaria* systems we are mainly interested in host and endosymbiont transporters which localise to the perialgal vacuole (PV) membrane and the outer-membrane of the endosymbiont. Similarly, we are interested in the host and endosymbiont proteins which are secreted into the PV lumen.

We can also investigate metabolic integration through the annotation and analysis of known

metabolic pathways in host and endosymbiont from the binned transcriptome sequences. This allows identification of pathways being expressed while in the endosymbiotic relationship and potentially identify sites of metabolic integration between host and endosymbiont e.g. (Russell et al., 2013).

Finally, a characterisation of metabolites present in the system can be used to further interrogate host and endosymbiont metabolic function. A metabolomics based-approach has the benefit of directly characterising the metabolites themselves and thus determining biological activity at the top functional level. Therefore, relative to analyses relying on abstracted measures such as transcription levels or gene copy number, novel information about cellular dynamics can be revealed. This is particularly important in cases of cryptic regulatory systems that break-down direct mapping from genes to transcripts to proteins to metabolites. By using both targeted and untargeted metabolomics approaches it is possible to survey the combined pool of host and endosymbiont metabolites both qualitatively and quantitatively. These inferences can then be correlated with other data such as the presence of transcripts involved in the synthesis or degradation of these metabolites.

By utilising these three separate streams of metabolic analysis: comparative transcript annotation and mapping, directed identification and analysis of transporters and secreted proteins and metabolomics, we maximise the strength of any inferences and reduce the chance of false negatives. First, I will summarise what is currently known about the metabolic integration of *P. bur-saria* and its green algal endosymbionts before discussing each of the analytical approaches that will be taken in more detail.

#### 5.1.1 METABOLISM OF HOST AND ENDOSYMBIONT

The most obvious point of metabolic integration in any endosymbiosis featuring a photosynthetic partner is that of the flow of photosynthates from endosymbiont to host. This is believed to primarily be in the form of carbohydrates such as maltose (Muscatine, 1967). In return, the host facilitates increased rates of photosynthesis in the endosymbiont (Sommaruga and Sonntag, 2009), via supply of  $\text{CO}_2$  (Parker, 1926), one or several forms of nitrogen (Johnson, 2011), and mono- and divalent cations such as  $\text{K}^+$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$ . All of which have key roles in photosynthesis (Kato and Imamura, 2009b).

#### 5.1.1.1 PHOTOSYNTHATE

The transfer of maltose, glucose, fructose and malate from endosymbiont to host has been observed previously using radiolabelling e.g. (Brown and Nielsen, 1974). Furthermore, green algae strains competent to form endosymbioses can be induced (via modifying pH) to release significantly more photosynthate (in the form of  $\sim 95\%$  maltose) than strictly free-living strains (on the order of 5.4 – 86.7% vs. 0.4 – 7.6% of total photosynthate) (Muscatine et al., 1967). Interestingly, the export of photosynthate from the PV to the host cytoplasm may be dependent on a transporter derived from the *C. variabilis* 1N in the *P. bursaria* Yad1g endosymbiosis (Kodama and Fujishima, 2008).

In terms of the uptake of saccharides, *C. variabilis* F36-ZK endosymbiont strains seem incapable of directly utilising sucrose, maltose, glucose or fructose in free-living culture (Kamako et al., 2005; Kato and Imamura, 2009b). Glucose, does promote growth however, via an apparent sensing pathway that leads to the up-regulation of amino acid importers (Kato and Imamura, 2009b). On the other hand, the free-living *C. vulgaris* strains have an inducible system for active hexose uptake (Tanner et al., 1974). In order of highest to lowest uptake rate, the free-living *C. vulgaris* took up sucrose, glucose and maltose but not fructose (Kato and Imamura, 2009b).

#### 5.1.1.2 NITROGEN

Nitrogen is the most transferred material between host and endosymbiont after carbon (Kato and Imamura, 2009b). There has been considerable research and interest in exactly what form this nitrogen exchange takes (Kato et al., 2006; Kamako et al., 2005; McAuley, 1986).

*C. variabilis* (both NC64A and the Japanese F36-ZK) have been found to be able to use amino acids effectively as a nitrogen source but only minimally utilise ammonium ( $NH_4^+$ ) and are incapable of effective nitrate ( $NO_3^-$ ) or nitrite ( $NO_2^-$ ) use (Kamako et al., 2005; Kato and Imamura, 2009b). Similar patterns have been observed in *M. reisseri*, although all strains tested could utilise nitrate and 3/4 could use nitrite to greater or lesser degrees (Kessler and Huss, 1990). On the other hand, free-living species such as *Parachlorella kessleri* can effectively utilise all of the nitrogen sources mentioned (Kato and Imamura, 2009b) (although amino acid utilisation has to be induced with glucose treatment (Cho et al., 1981)).

In terms of amino acids as a nitrogen source there isn't a high degree of correlation between

the ability to uptake an amino acid and its usage (Kato and Imamura, 2009b). For example, while *C. variabilis* F36-ZK can uptake all 20 amino acids, only 6 (L-arginine, L-asparagine, L-glutamine, L-serine, L-alanine) were found to promote growth (Kato et al., 2006). This was despite some of these 6 being taken up at lower rates than some non-utilised amino acids such as L-proline, L-cysteine or L-leucine (Kato et al., 2006).

Similarly, *C. variabilis* NC64A was found to have stimulated growth in the presence of L-arginine and L-glutamine, whereas another *C. variabilis* strain, 3N813A, used every amino acid apart from L-lysine and L-glutamic acid (McAuley, 1986; Kato and Imamura, 2009b).

The free-living *C. vulgaris* NIES-227 was found to not utilise any amino acid apart from low levels of uptake of L-arginine (Kato et al., 2006). Therefore, even within the *C. variabilis* strains there is a range of traits in terms of amino acid uptake and utilisation.

Kinetic analyses and competitive assays indicate three amino acid transport systems in *C. variabilis* F36-ZK, a general amino acid transporter for all amino acids except L-alanine, a basic transporter for L-arginine and L-lysine and a specialised L-alanine transporter (Kato and Imamura, 2009a,b). All of these are constitutively functioning, active, amino-acid symporters (Kato and Imamura, 2009a,b).

As *P. bursaria* cannot import nitrate (Albers et al., 1982) the heterogeneous loss of nitrate and nitrite utilisation in several endosymbiotic strains is perhaps not surprising (Kato and Imamura, 2009b). Without host nitrate uptake there is no pressure to maintain enzymes necessary for this pathway as an endosymbiont. This reduced selection pressure for nitrate and nitrite utilisation may explain the presence of low-activity mutant Nitrate Reductase (NR) and Nitrite Reductase (NiR) in the two *C. variabilis* strains (Kato and Imamura, 2009b).

#### 5.1.1.3 CATIONS

The final major group of host-endosymbiont transferred metabolites are those of mono- and divalent cations. Specifically  $Ca^{2+}$ ,  $Mg^{2+}$  and  $K^{+}$  (Kato and Imamura, 2009b). All of these have key roles in photosynthesis. Interestingly,  $Ca^{2+}$  has also been found to inhibit amino acid uptake (Kato and Imamura, 2008). As glucose has been found to increase amino acid uptake, some researchers have hypothesised that the relative concentration of photosynthates and  $Ca^{2+}$  in the PV lumen defines a photosynthate-amino acid barter system between endosymbiont and host (Kato and Imamura, 2009b).

## 5.1.2 IDENTIFYING DIRECT POINTS OF CONTACT

### 5.1.2.1 TRANSPORTER PROTEINS

The most important group of proteins in the control and evolution of metabolic integration is that of host and endosymbiont transporter proteins. This is due to their role in facilitating diffusion and active transport across the lipid membranes that exist between host and endosymbiont.

Without transporters many metabolically important large uncharged polar molecules (e.g. carbohydrates, amino acids) and charged molecules (e.g. the various biologically relevant cations and anions such as  $H^+$ ) are incapable of significant rates of diffusion across membranes even in the presence of high concentration gradients. Therefore, the presence of transporters is vital to facilitating any interaction involving these groups of metabolites.

Several metabolites are, however, capable of unfacilitated diffusion across lipid membranes at significant rates. These include important respiratory gases such as  $O_2$  and  $CO_2$ , hydrophobic compounds like benzene and small uncharged polar molecules (e.g.  $H_2O$  and ethanol) (Cooper, 2013; Alberts, 2015). Despite this, transporter proteins have evolved to facilitate even more rapid diffusion of some of these metabolites e.g. aquaporins (Agre et al., 1993).

Finally, certain transporters can provide the ability to actively transport metabolites against concentration gradients. This involves the expenditure of energy (typically in the form of ATP) to directly pump compounds or generate an opposing gradient which can be exploited (primary vs secondary active transport).

There are 5 functional groups of transporters (Saier et al., 2014):

- Channel/Pore types which catalyse diffusion of metabolites along concentration gradients e.g. porins and the Mitochondria and Plastid Porin (MPP) family.
- Electrochemical Potential-driven transporters which use a carrier-mediated process to catalyse uniportation (single metabolite) or cotransportation (two species in the same direction, symportation, or two species in opposite direction, antiportation). These make use of chemiosmotic gradients but generally do not directly make use of cellular energy molecules such as ATP. However, many make use of a gradient/potential generated by the active transport of solutes by another complex, in this case they can be referred to as secondary active transporters. Electrochemical Potential-driven transporters are a very large family and

include the ubiquitous major facilitator superfamily (MFS) and Cation Diffusion Facilitator (CDF) families.

- Primary active transporters which use a direct source of chemical, electrical or light energy such as ATP, voltage or photon to drive transport against concentration gradients. Transporters of this type form many of the components fundamental to life as they allow an organism to decouple itself directly from environmental and intracellular gradients. They include rhodopsins, ATP-binding Cassette (ABC) Superfamily, and the general Secretory Pathway (Sec) family.
- Group translocators, which modify a substrate during transportation e.g. polysaccharide synthesis during secretion in the Polysaccharide Synthase/Exporter family and the Fatty Acid Group Translocation (FAT) family which can acylate fatty acids during transport.
- Transmembrane Electron Carriers which transport single electrons from a donor to an acceptor across a membrane. The major groups of these include the cytochrome and Photosystem I complexes.

This analysis will focus on transporters of the first 4 classes. In the case of *P. bursaria* and its endosymbiont we are particularly interested in the transporters of the host perialgal vacuole membrane and those of the outer membrane of the green algal endosymbiont.

Therefore, the first step to the successful analysis of the metabolic integration of host and endosymbiont is the accurate identification of transporter proteins present in their respective binned transcriptome sequences. By both identifying these proteins and qualitatively investigating their day:night expression, targets can be generated for further analysis. Specifically, validation of function and analysis of localisation to the PV and/or algal outer membrane.

Transporter proteins can be identified primarily via annotation of transmembrane (TM) domain motifs and homology searches to previously identified transporters (Saier et al., 2006, 2009, 2014). All transporters feature at least 1 TM helix, usually considerably more (von Heijne, 2006). However, as not necessarily every sub-unit of a transporter will contain a TM domain and as our assemblies consist of numerous partial transcripts it is necessary to not rely exclusively on TM prediction to discover transporter proteins.

#### 5.1.2.2 SECRETED PROTEINS

The next major class of proteins involved in endosymbiosis are those which are secreted in the PV lumen. Host and endosymbiont derived proteins targeted to the lumen of this vacuole are responsible for generating the local environment for endosymbiosis. This is fundamental to the exchange of metabolites between host and endosymbiont as this is the cellular context in which that exchange must occur. Furthermore, in the establishment of endosymbiosis secreted effectors from the endosymbiont are likely responsible for the modification of a phagosomal vesicle into the perialgal vacuole.

One particularly interesting example to identify would be the hypothesised endosymbiont derived transporter exported to the PV membrane that is responsible for export of photosynthate to the host cytoplasm (Kodama and Fujishima, 2008).

The identification of secreted proteins relies on the analysis of signal peptides and/or a range of standard classification algorithms based on sequence and compositional features. Signal peptides are short 15-30 amino acid N-terminal sequences that determine targeting of proteins to cellular compartments (Schatz and Dobberstein, 1996; Rusch and Kendall, 1995).

SignalP (Nielsen et al., 1997) has proven the most effective method of predicting the presence of signal peptides (Lee et al., 2009; Petersen et al., 2011). This method uses a standard feed-forward artificial neural network with 8-41 hidden units (depending on whether the organism is eukaryotic, gram positive or gram negative) trained with back-propagation.

Subcellular localisation of a given protein can also be inferred using tools such as the WoLF PSORT (Horton et al., 2007). This tool implements a standard  $k$ -neighbours classifier trained on localisation labelled proteins from SwissProt and uses PSORTII (Nakai and Kanehisa, 1992; Nakai and Horton, 1999; Horton and Nakai, 1997) and iPSORT (Bannai et al., 2002) derived sequence features and automatic inference of weightings (Horton et al., 2006).

In addition to these tools, there are many other systems that have been designed to predict protein localisation. In order to maximise the probability of successful identification of secreted proteins I have created a consensus ensemble classifier that incorporates predictions from several of these tools, principally SignalP, TMHMM, TargetP, ChloroP, and WoLFPSort.

### 5.1.3 METABOLIC MAPPING

Metabolic pathways form the functional backbone of all biological processes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999; Okuda et al., 2008; Kanehisa et al., 2014) and MetaCyc (Caspi et al., 2007) databases form an important resource for contextualising genomic and transcriptomic scale results into these networks. The utility of this approach is emphasised by the presence of numerous tools and analytical pipelines to explore these databases e.g. (Okuda et al., 2008; Nakao et al., 1999; Karp et al., 2002, 2010; Antonov et al., 2008; Klukas and Schreiber, 2007).

By comparing the relatively complete predicted peptides sets from the sequenced endosymbiotic algae *C. variabilis* NC64A and *Coccomyxa subellipsoidea* C-169 genome projects to the predicted endosymbiont binned peptides from the transcriptomes of *M. reisseri* and *C. variabilis* 1N it is possible to infer which endosymbiotic metabolic pathways are active in the latter two endosymbionts. Furthermore, identification of pathways unique to individual algae can identify potential distinct adaptations to endosymbiosis in that algae.

## 5.2 AIMS

The principal aim of this chapter is to identify likely points of metabolic integration between host and endosymbiont to generate targets for subsequent targeted mass spectrometry, RNAi and qPCR based validation experiments. This will be achieved by:

- Identifying transporter proteins present in the endosymbiont binned transcripts from the CCAP1660/12 RNAseq analysis and analysing them for qualitative expression across day and night.
- Identifying secreted proteins present in the endosymbiont binned transcripts from the CCAP1660/12 RNAseq analysis and analysing them for qualitative expression over day and night.
- Comparative analysis of metabolic pathways between host and endosymbiont relative to sequenced green algal genomes.
- A pilot untargeted global metabolomic profile of the system and comparison of day to night.

- Targeted quantitative analysis of amino acid concentrations between day and night.

## 5.3 METHODS

### 5.3.1 TRANSPORTER ANALYSIS

#### 5.3.1.1 TRANSPORTER IDENTIFICATION PIPELINE

Transporters were identified in the 4 sets of sequences (*C. variabilis*, *M. reisseri*, *C. vulgaris* and *C. subellipsoidea*) using the following set of pipe-lined filters:

1. Transmembrane (TM) domains were predicted for each sequence using an HMM approach implemented as part of TMHMM2 (Sonnhammer et al., 1998; Krogh et al., 2001) and sequences predicted to contain at least 1 TM domain were extracted.
2. These sequences were then used to search a PFAM database of profile HMMs (Eddy, 1995) via HMMER3's hmmscan utility (Eddy, 1995; Johnson et al., 2010; Eddy and R, 2011; Mistry et al., 2013) and sequences with a hit to a PFAM domain at an independent e-value of  $1e^{-5}$  were retained.
3. These hits were then finally filtered for PFAM domains which mapped to transporter families classified by the Transporter Classification Database (TCDB) (Saier et al., 2006, 2009, 2014) mapping files.

Additionally, to ensure thorough discovery of all *M. reisseri* transporters, *M. reisseri* binned sequences were BLASTP-ed against the nr protein database with an e-value of  $1e^{-3}$  and a maximum of 20 hits. InterproScan (Zdobnov and Apweiler, 2001) was then used to further annotate these proteins incorporating results from BlasProDom (Servant et al., 2002), FPrintScan (Attwood et al., 1994), HMMER (Mistry et al., 2013) scans against the PIR (Barker et al., 1998), PFAM (Bateman, 2002), SMART (Schultz et al., 1998), PANTHER (Thomas, 2003) and TIGRFAM databases (Haft, 2003), ProfileScan (Gribskov et al., 1988), HAMAP (Lima et al., 2009), PatterScan, SuperFamily (Gough and Chothia, 2002), SignalP (Petersen et al., 2011), TMHMM (Sonnhammer et al., 1998), Gene3D (Buchan et al., 2002), Phobius (Käll et al., 2007) and Coils. Results were then mapped to GO terms (Ashburner et al., 2000; Harris et al., 2004) and annotated via BLAST2GO (Conesa et al., 2005).

Finally, all proteins annotated to have a GO term associated with “transport” and “transport activity” (specifically GO:0006810 and its child terms) were extracted.

This set was then filtered for the presence of TM domains and to remove organelle related transporters such as cytochrome system and photosystems as these are unlikely to localise anywhere other than the mitochondria and chloroplast.

### 5.3.2 SECRETOME PREDICTION

A conservative set of secreted proteins were predicted using the following consensus ensemble classifier:

1. Signal peptides were detected using SignalP4.1 and mature sequences created for each sequence with a signal peptide
2. Sequences detected to have a TM domain (by TMHMM) within either the mature sequence or full length for proteins without signal peptides were discarded.
3. Signal peptides were re-added to mature sequences and the remaining sequences were then filtered using for those predicted as secreted by TargetP1.1
4. These sequences were then filtered down to those which had extracellular localisation in WoLFPSORT 0.2
5. Finally, for the endosymbiont, secreted protein found to have a Chloroplast targeting signal (via ChloroP1.1) were removed.

In addition to this, a larger permissive set was generated without the TMHMM filtering step and retaining all proteins predicted to be extracellular or have a signal peptide targeting for secretion.

### 5.3.3 QUALITATIVE EXPRESSION ANALYSIS

Kallisto (Bray et al., 2015) was used to pseudoalign and estimate abundances for all taxonomically screened single cell libraries (4 dark and 3 light) to the called “endosymbiont” binned CDS sequences from the *P. bursaria* CCAP 1660/12 transcriptome (see Chapter 4).

Kallisto doesn't align reads to references in the same manner as conventional short read alignment algorithms such as Bowtie2 (Langmead and Salzberg, 2012). Instead of specifically mapping a read to a set of co-ordinates it instead determines which transcripts are compatible with the alignment of a given read. This is achieved via decomposition of transcripts in de-Bruijn graphs and fast  $k$ -mer hashing to compare reads to transcript graph nodes in constant time. These  $k$ -compatibility classes are then used with bootstrapped expectation-maximisation to estimate transcript quantification and determine uncertainty (Bray et al., 2015).

Results were visualised and analysed using "sleuth" and the seaborn plotting library (Waskom et al., 2015).

#### 5.3.4 METABOLIC MAPPING ANALYSIS

First, predicted proteomes were obtained or generated for *Coccomyxa subellipsoidea* C-169, *Chlorella variabilis* NC64A, *Chlorella variabilis* 1N, and *M. reisseri*.

For *M. reisseri* the endosymbiont binned sequences from the transcriptomic sequencing project discussed in the previous chapter were used. *Coccomyxa subellipsoidea* C-169 genome project (Blanc et al., 2012) version 2.0 JGI annotated proteins (created 12-01-2014) were downloaded from JGI's Phytozome v10.3.1 (Goodstein et al., 2012). Similarly, the "best" annotated proteins from version 1 of the *Chlorella variabilis* NC64A genome project (Blanc et al., 2010) were downloaded from JGI's genome portal (Grigoriev et al., 2011; Nordberg et al., 2014)

However, to obtain *C. variabilis* 1N endosymbiont peptides a reassembly and binning of raw sequencing data from (Kodama et al., 2014) was conducted (discussed below).

Once all sequences were acquired they were annotated using KEGG orthology. This was achieved using the KEGG Automatic Annotation Server (KAAS) (Moriya et al., 2007) single-directional best hit with both BLAST and GHOSTZ (Suzuki et al., 2014, 2015) method against the following 40 gene sets: *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Glycine max*, *Vitis vinifera*, *Oryza sativa*, *Ostreococcus lucimarinus*, *Ostreococcus tauri*, *Micromonas* sp. RCC299, *Cyanidioschyzon merolae*, *Galdieria sulphuraria*, *Saccharomyces cerevisiae*, *Candida albicans*, *Neurospora crassa*, *Aspergillus nidulans*, *Coccidioides immitis*, *Schizosaccharomyces pombe*, *Ustilago maydis*, *Encephalitozoon cuniculi*, *Monosiga brevicollis*, *Dictyostelium discoideum*, *Acanthamoeba castellanii*, *Plasmodium falciparum* 3D7, *Cryptosporidium hominis*, *Tetrahymena thermophila*, *Paramecium tetraurelia*, *Phaeodactylum tricorutum*, *Emiliana huxleyi*, and *Guil-*

*lardia theta*.

KEGG annotations were then plotted onto KEGG metabolic networks and compared to identify key aspects of differences between the algal species as well as between the host and endosymbiont metabolic networks.

#### 5.3.4.1 *CHLORELLA VARIABILIS* 1N ASSEMBLY

232.3M 100 bp paired-end reads from (Kodama et al., 2014)'s bulk RNAseq transcriptome of *Paramecium bursaria* Yad1g1N (syngen 3, mating type 1) bearing *Chlorella variabilis* 1N endosymbionts were downloaded from the DNA Data Bank of Japan (DDBJ) (Tateno et al., 2002; Kaminuma et al., 2011) in Sequence Read Archive (SRA) format (Leinonen et al., 2011; Kodama et al., 2012) (accession DRA000907 Kodama et al. (2014)).

These reads were then converted to fastq using “fastq-dump” using the SRA Toolkit (National Center for Biotechnology Information, 2011). Reads were then trimmed for sequencing adapters using ILLUMINACLIP and SLIDINGWINDOW with a window size of 4 and a minimum average quality of 5 in Trimmomatic (Bolger et al., 2014).

Following this, reads were error-corrected using SEECER with a  $k$ -mer size of 25 and default settings otherwise (entropy of 0.6 and a cluster log-likelihood of -1) (Le et al., 2013). Error-corrected reads were digitally normalised using a  $k$ -mer size of 25 and a coverage of 20 (Brown et al., 2012) and low abundance  $k$ -mers in high coverage reads were filtered (Zhang et al., 2014, 2015) using the Khmer software package (Döring et al., 2008; Crusoe et al., 2015).

Assemblies were completed in a modified/fixed version of Bridger 2014-12-01 (Chang et al., 2015) (available at [https://github.com/fmaguire/Bridger\\_Assembler](https://github.com/fmaguire/Bridger_Assembler)) and Trinity v2.0.6 (Grabherr et al., 2011; Haas et al., 2013) both with  $k$ -mer sizes of 25.

An alternative Trinity assembly was also completed using SLIDINGWINDOW Q30 trimmed reads without normalisation or error correction.

Assemblies were compared using RSEM-EVAL (Li et al., 2014) and the best overall assembly selected on the basis of likelihood. ORFs were called from the best assembly using universal and *Tetrahymena* encodings via TransDecoder (Haas et al., 2013) retaining the best scoring sequences and those with HMMR hits to PFAM and BLASTP hits to the SwissProt database.

Phylogenies were generated for each sequence using the same approach and pipeline described in Chapter 4. These phylogenies were subsequently classified using the same trained  $k$ -Neighbours

supervised learning algorithm. Any sequence that didn't have enough BLAST hits in the genomes used to generate a phylogeny (5) were parsed based on what hits were retrieved. Those with no hits were classified as "unknown" and those with hits were classified based on the origin of those hits e.g. hits to green algae and plant genomes were considered "endosymbiont" and so on.

Finally, the ORF bins for host and endosymbiont from both encodings were manually combined and reconciled to generate transcript bins. The transcripts binned into "host" and "endosymbiont" were subsequently recalled as ORFs using the appropriate transdecoder encodings.

### 5.3.5 METABOLOMICS

Three mass spectrometry analyses were conducted to investigate the presence/absence and relative abundances of polar and non-polar metabolites. Specifically, GC-QTOF to principally identify metabolites such as carbohydrates, LC-QTOF in positive and negative ionisations to profile general metabolites.

Finally, a targeted mass spectrometry analysis was conducted using LC-QQQ to quantitatively assess the concentration of free amino acids in the host-endosymbiont system during the day and night.

#### 5.3.5.1 UNTARGETED LC-QTOF PROFILING

Sample preparation for mass spectrometry followed standard protocols. Briefly, 5 biological replicates were sampled from *P. bursaria* CCAP 1660/12 cultures at the midpoint of both the day and night cycles. Samples were then dried, flash-frozen in liquid nitrogen, and homogenised using a cell disruptor.

For each sample, 10 mg was dissolved in 400  $\mu$ l of a solution of 80 % MeOH containing 7.2 mg ml<sup>-1</sup> of an umbelliferone internal standard. This solution was kept on ice and vortexed for 30 s every 10 min for 30 min. Samples were sonicated in ice cold water for 15 minutes and then centrifuged at 13 krpm for 10 min. Retaining the supernatant in a separate tube, the pellet was resuspended in 400  $\mu$ l 80 % MeOH and vortexing, sonication and centrifugation steps repeated. The two supernatants were combined and filtered through a 0.2  $\mu$ m syringe filter (Chromacol). Samples were sub-divided into two a 5  $\mu$ l of each was loaded into an Agilent 1200 Series HPLC with a 3.5  $\mu$ m, 2.1  $\times$  150 mm Eclipse Plus C18 Agilent column. One sample was then analysed using a positive electron spray ionisation and the other a negative ionisation on an Agilent 6520 accurate mass

quadrupole time of flight (QTOF) mass spectrometer. Data was captured using the standard Agilent data acquisition software and converted from “.d” format to the open mzXML format (Pedrioli et al., 2004). The same process was repeated for 7 blank samples under both ionisation conditions as well as an apigenin QC standard.

Samples were analysed primarily using the XCMS R package (Smith et al., 2006; Tautenhahn et al., 2012b). Peak features were detected in each samples using the centWave algorithm with a 30ppm tolerated m/z deviation, minimum peak width of 10 and maximum peak width of 60 (Tautenhahn et al., 2008). Peaks were aligned with a 0.025 m/z width overlap, and a maximum bandwidth 5 retention time deviation. Using the aligned peaks, the retention time deviation between samples were calculated. The samples were then realigned correcting for retention time deviation and integrated using fillPeaks.

In order to determine differential presence of globally detected metabolites unpaired Welch’s *t*-tests were conducted comparing the 5 day samples to the 5 night samples. Welch’s tests were used as they don’t assume equal sample sizes or variances between the two groups (Welch, 1947).

P-values from this were corrected for multiple comparisons using false discovery rates (FDR). FDR is a less conservative correction than, the classic family-wise error rate correction, Bonferroni adjustment<sup>1</sup> but allow maintenance of a greater proportion of statistical power with a slightly elevated risk of Type-I errors.

Features were then annotated using the METLIN metabolite database (Smith et al., 2005a; Sana et al., 2008; Tautenhahn et al., 2012a). Extracted ion base peak chromatograms were manually inspected for each significantly expressed feature and any that weren’t clear distinct peaks were discarded. Any sample without an annotation against METLIN was similarly discarded. Finally, annotations were manually parsed and samples with implausible annotations (e.g. chemotherapeutic drugs) discarded.

### 5.3.5.2 UNTARGETED GC-QTOF PROFILING

Standard sample preparation was conducted with 5 light and 5 dark biological replicates.

Agilent “.d” proprietary format outputs were converted to mzXML as above. The majority of the analysis pipeline was conducted using the metaMS R library (Wehrens et al., 2014). Briefly,

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<sup>1</sup>P-value threshold  $\alpha$  is adjusted relative to the number of comparisons ( $n$ ). Specifically, significance is determined as a P-value  $\leq \frac{\alpha}{n}$ .

<b>Preprocessing</b>	<b>PE Reads</b>
<b>Raw Reads</b>	$2.323 \cdot 10^8$
<b>Q30 Trimmed</b>	$1.75 \cdot 10^8$
<b>Q5 Trimmed</b>	$2.127 \cdot 10^8$
<b>Q5 Error Corrected</b>	$2.021 \cdot 10^8$
<b>Q5 Digital Normalisation</b>	$1.09 \cdot 10^7$
<b>Q5 <i>k</i>-mer abundance filtering</b>	$1.055 \cdot 10^7$

**Table 5.4.1:** Summary of read pre-processing stages for the Kodama library. This table further emphasises the massive amount of redundancy that digital normalisation removes from the assembly. The minimal effect of *k*-mer abundance filtering is likely due to a relative redundancy in this pre-processing from the error correction step.

this involved standard peak picking using the default XCMS algorithm (Smith et al., 2006) followed by clustering into pseudospectra using CAMERA (Kuhl et al., 2012). Pseudospectra were then annotated against the NIST and METLIN databases using a combination of spectral and retention time features. Finally intensity and relative abundances were calculated and tested using FDR corrected Welch's *t*-tests.

#### 5.3.5.3 TARGETED QUANTITATIVE AMINO ACID ANALYSIS

5 Day and 5 Night samples were prepared for liquid chromatography using the same approach as the untargeted LC-QTOF analysis. In addition to this, the Day<sub>1</sub> and Night<sub>1</sub> samples were analysed at both 2x and 0.5x titrations. 3 blank samples were run as well as standards consisting of a complete amino acid mix from Sigma at 0.5μM and 4 samples consisting of Asparagine-Glycine-Tryptamine and Leucine-Glutamine-Lysine respectively.

After chromatography samples were ionised using electrospray ionisation and analysed using multiple reaction monitoring optimised for amino acids with an Agilent Technology 6410B enhanced sensitivity triple quadrupole mass spectrometer (QQQ).

Results were analysed using the Agilent Quantitative Analysis software package with peaks normalised in respect to the umbelliferone standard.

Assembly	Contigs	Likelihood ( $-\log$ )
<b>Trinity Q5 Normalised</b>	101,957	$1.216 \cdot 10^9$
<b>Bridger Q5 Normalised</b>	62,504	$1.285 \cdot 10^9$
<b>Trinity Q30</b>	53,938	$5.619 \cdot 10^9$

**Table 5.4.2:** Summary of resultant assemblies of sequencing data derived from (Kodama and Fujishima, 2014). As the Trinity Q5 Normalised assembly had the best likelihood while generating the greatest number of contigs it was used for downstream analyses.

Bin	Number of Transcripts
Food	3,873
Endosymbiont	8,627
Host	53,295
Unknown	36,162

**Table 5.4.3:** Summary of transcript binning for the Q5 Trinity assembly of Yad1g1N. A much greater proportion of transcripts were assigned to host and endosymbiont for this assembly than in the single cell based assemblies previously conducted.

## 5.4 RESULTS

### 5.4.1 *P. BURSARIA-C. VARIABILIS* YAD1G1N ASSEMBLY

A complete *de novo* assembly was conducted of sequencing data from (Kodama and Fujishima, 2014) based on experiences assembling *P. bursaria-M. reisseri* CCAP 1660/12 (see Chapter 4). Briefly, all samples were combined, trimmed to Q5, error corrected, digitally normalised and abundance filtered. Another dataset was prepared just using adapter trimming and a quality threshold of Q30. Assemblies conducted with these datasets using both Trinity and Bridger *de novo* assemblers were evaluated using RSEM-EVAL and the optimal assembly chosen on score.

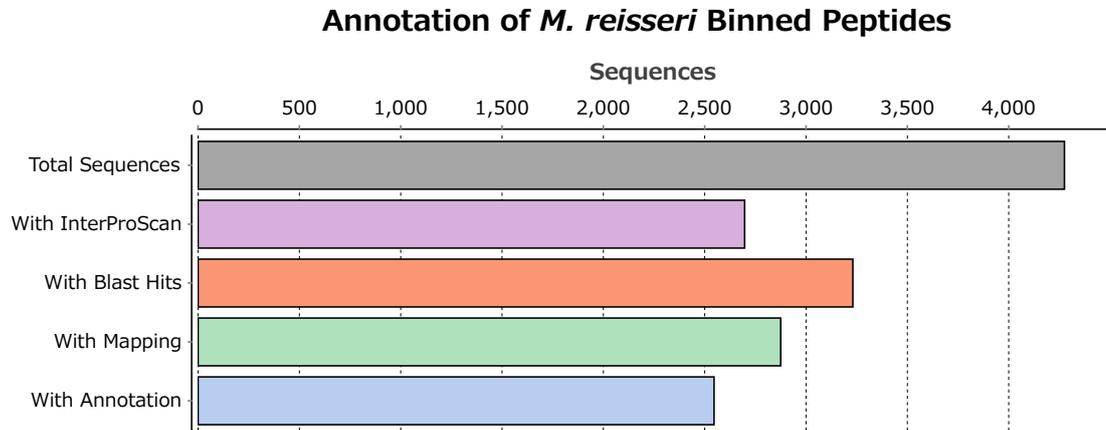
Therefore, the optimal assembly chosen for further analysis was the Trinity Q5 normalised assembly. From the 101,957 transcripts 193,906 ORFs were called using *Tetrahymena* encoding and 20,875 universal. These were subsequently binned using the same approach as used in previous chapter.

Finally, “Host” and “Endosymbiont” binned transcripts were re-ORF called using the appropriate encodings to result in a host ORF bin of 61,239 sequences and an endosymbiont bin of 5,565 peptides.

These are relatively reasonable dataset sizes especially when considered against the source of the raw sequencing data. Specifically, the analysis of (Kodama et al., 2014), which involved the elimination of endosymbionts from the culture. Therefore, only half of the libraries theoretically

	<i>C. variabilis</i> 1N	<i>M. reisseri</i> CCAP 1660/12	<i>C. variabilis</i> NC64A	<i>C. subellipsoidea</i> C-169
Peptides	5,565	4,275	9,791	9,629
1+ TM domains	695	419	1,722	1,709
1+ TM and TCDB	251	185	690	697

**Table 5.4.4:** Summary of the sizes of the complete transporter complements identified in the various algal sequence sets. The two genome based predicted proteomes generated much larger predicted sets of proteins (*C. variabilis* NC64A and *C. subellipsoidea* C-169).



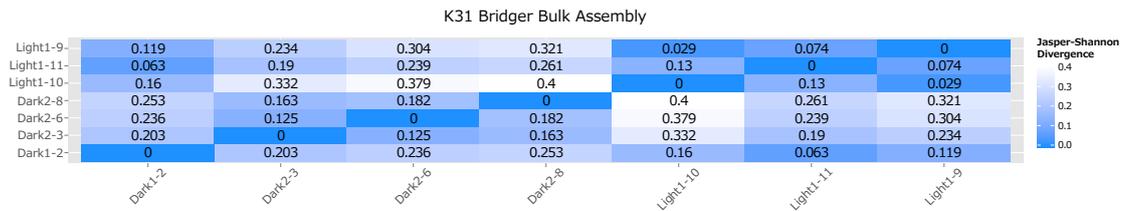
**Figure 5.4.1:** Summary of Endosymbiont Bin Annotation. This shows that the majority but not all transcripts were successfully annotated.

contained *C. variabilis* sequences so the recovery of the majority of the predicted proteome is surprising.

#### 5.4.2 TRANSPORTER IDENTIFICATION

Based on the results of the transporter identification pipeline the genome based predictions from *C. variabilis* NC64A and *Coccomyxa* led to a much larger set of candidate transporter proteins than those from the two binned transcriptomes. However, smaller subsets represent all candidate transporters that are actively being transcribed during endosymbiosis rather than just all those present in the genome. Therefore, this is not necessarily problematic and could be symptomatic of the endosymbiont exhibiting a streamlined metabolism inside the host. The *M. reisseri* predictions were then supplemented using standard annotation pipelines (fig. 5.4.1).

From the GO based annotations there were 427 proteins associated with the GO term for transmembrane transport (GO:0006810). These overlapped with approximately half of the TM/TCDB identification (93/185 proteins). 133 of the 427 GO term annotated transporters had at least 1 TM domain (and this 133 contained all 93 shared TM/TCDB and GO based identifications as would be expected).



**Figure 5.4.2:** Comparison of similarity of taxonomically screened single cell libraries. This shows how relatively divergent each library is and demonstrates the high level of noise. The dark and light groups are only very faintly visible. This further supports the use of read-mapping inferences as qualitative rather than quantitative data for MDA-based SCT.

On manual inspection, a large number of GO based annotations were partial/subunits or could only very tenuously be referred to as transporters. Due to this only those with a TM domain were retained and added to the list of candidate transporters. This resulted in a list of 225 putative transporters for *M. reisseri* CCAP 1660/12. These were then manually filtered to remove obviously organelle related groups, specifically cytochromes/electron transport chain proteins and photosystem related proteins leading to a final set of 161 putative transporter proteins.

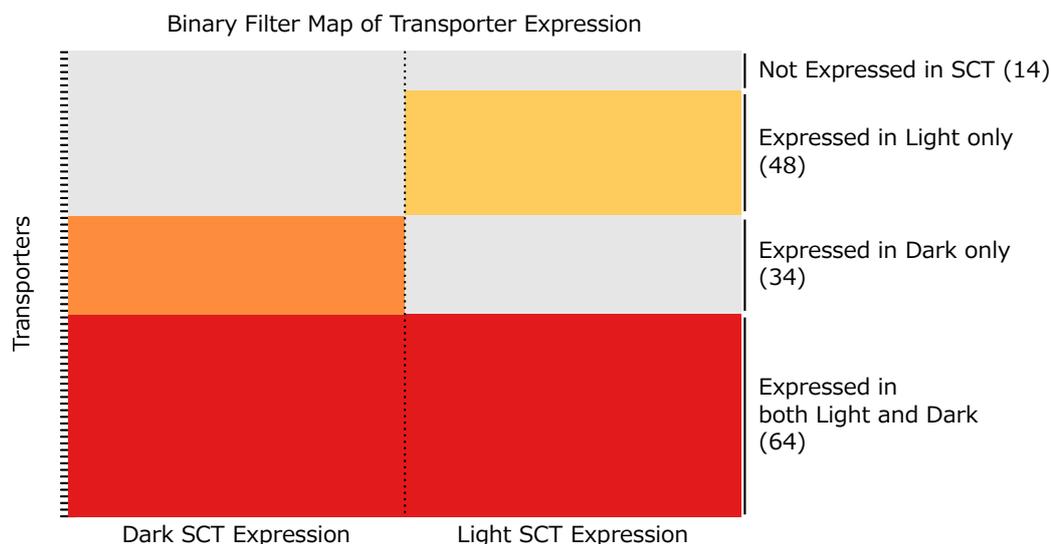
In terms of candidate sugar transporters, this set includes 7 MFS transporters, a hexose sugar transporter, a sugar-phosphate translocator, a sweet1 sugar transporter a nucleotide-sugar transporter, a mannose-6-phosphate isomerase, and a fucose permease. There were also 2 polyol transporters e.g. a glycerol-3-phosphate transporter and an inositol transporter. Candidate amino acid transporters included 8 amino acid permeases.

### 5.4.3 SUBSET OF TRANSPORTERS EXPRESSED IN DAY AND NIGHT

Using the nucleotide CDS sequence of the called peptides identified as transporters reads from each of the 7 taxonomically screened single cell libraries were pseudo-mapped to them using Kallisto. Due to the compositional/coverage biases of MDA-based single cell transcriptomics Kallisto statistical inference was likely to be spurious and relate to the well-documented coverage biases of MDA (see fig. 5.4.2). Therefore, a simple presence/absence filter was implemented for the single cell libraries where an estimated Transcripts per million (TPM) was above 0 for at least 1 biological replicate in each condition.

Name	Description/Annotation	Top BLASTP Hit Accession	Top Hit Species
comp1093_seq1 m.1645	Uric acid-xanthine permease	ref XP_005848091.1	<i>Chlorella variabilis</i>
comp34246_seq0 m.33953	Uric acid-xanthine permease	ref XP_005848091.1	<i>Chlorella variabilis</i>
comp11781_seq0 m.10145	Signal recognition particle protein 3	ref XP_005846072.1	<i>Chlorella variabilis</i>
comp35891_seq0 m.35196	Sensory protein	ref WP_027033724.1	<i>Mesorhizobium loti</i>
comp13652_seq0 m.12349	Phagocytic receptor 1b	ref XP_009389646.1	<i>Musa acuminata</i>
comp12191_seq1 m.10601	Inorganic phosphate transporter	ref XP_005852067.1	<i>Chlorella variabilis</i>
comp23811_seq0 m.24460	Inorganic pyrophosphatase	ref WP_028786954.1	<i>Terrimonas ferruginea</i>
comp34406_seq1 m.34111	Potassium transporter 2-like	ref XP_011399197.1	<i>Auxenochlorella protothecoides</i>
comp25846_seq0 m.26580	Protein trigalactosyldiacylglycerol chloroplastic	ref XP_005845784.1	<i>Chlorella variabilis</i>
comp14064_seq0 m.12814	Sugar transport protein 10-like	ref XP_005842790.1	<i>Chlorella variabilis</i>
comp15360_seq0 m.14352	Drug metabolite transporter superfamily	ref XP_005851889.1	<i>Chlorella variabilis</i>
comp16603_seq1 m.16010	Adenine guanine permease	ref XP_005850398.1	<i>Chlorella variabilis</i>
comp17473_seq0 m.17096	gpr1 fun34 family protein	ref XP_005848680.1	<i>Chlorella variabilis</i>
comp18033_seq0 m.17793	Hypothetical upf0065 protein	ref WP_019198042.1	<i>Afpia birgiae</i>
comp21389_seq0 m.21756	Proton phosphate symporter	ref XP_011398136.1	<i>Auxenochlorella protothecoides</i>
comp22779_seq0 m.23394	Na <sup>+</sup> solute transporter	ref XP_011396846.1	<i>Auxenochlorella protothecoides</i>
comp22990_seq0 m.23626	Inositol transporter 4-like	ref XP_005846641.1	<i>Chlorella variabilis</i>
comp23135_seq0 m.23792	MFS transporter	gb ESW58454.1	<i>Pseudomonas fluorescens BBc6R8</i>
comp26434_seq0 m.27099	ATPase p	ref WP_026773962.1	<i>Sediminibacterium sp. OR43</i>
comp26454_seq0 m.27109	Plasma membrane iron permease	ref XP_005844294.1	<i>Chlorella variabilis</i>
comp2716_seq0 m.2808	Amino acid permease 6	ref XP_011400870.1	<i>Auxenochlorella protothecoides</i>
comp2716_seq1 m.2811	Amino acid permease 2	ref XP_011400870.1	<i>Auxenochlorella protothecoides</i>
comp2716_seq2 m.2815	Amino acid permease 2	ref XP_011400870.1	<i>Auxenochlorella protothecoides</i>
comp30376_seq0 m.30648	Amino acid permease 2	gb KPF41572.1	<i>Rhizobium sp. AAP43</i>
comp85197_seq0 m.68822	Amino acid permease	ref XP_005846503.1	<i>Chlorella variabilis</i>
comp43295_seq0 m.41027	Lysine transporter?	ref XP_005847284.1	<i>Chlorella variabilis</i>
comp29655_seq0 m.30102	ABC transporter permease	ref WP_044404984.1	<i>Rhodospseudomonas palustris</i>
comp27137_seq0 m.27822	ABC transporter ATP-binding protein	gb ACFo1145.1	<i>Rhodospseudomonas palustris TIE-1</i>
comp51985_seq0 m.48090	ABC permease ATP-binding family protein	gb EFD03879.1	<i>Propionibacterium acnes</i>
comp13567_seq0 m.12197	ABC transporter	ref XP_005849318.1	<i>Chlorella variabilis</i>
comp32752_seq0 m.32669	Membrane AAA-metalloprotease	ref XP_001697103.1	<i>Chlamydomonas reinhardtii</i>
comp72377_seq0 m.62224	ABC transporter permease	ref WP_046827962.1	<i>Afpia massiliensis</i>
comp52706_seq0 m.48773	ABC transporter permease	ref WP_046827962.1	<i>Afpia massiliensis</i>
comp27304_seq0 m.27991	Peptide ABC transporter permease	ref WP_009735611.1	<i>Bradyrhizobiaceae bacterium SG-6C</i>
comp58314_seq0 m.53037	Protein transport protein sec61 subunit alpha isoform partial	ref XP_005843596.1	<i>Chlorella variabilis</i>
comp49211_seq0 m.45885	ATP-binding protein	ref WP_051503901.1	<i>Shingomonas jaspis</i>
comp15817_seq0 m.15012	Transmembrane 9 superfamily member 3	ref XP_005845268.1	<i>Chlorella variabilis</i>
comp39178_seq0 m.37743	Transmembrane 9 superfamily member 3-like	ref XP_011395771.1	<i>Auxenochlorella protothecoides</i>
comp49560_seq0 m.46210	Transmembrane 9 superfamily member 4	ref XP_005847406.1	<i>Chlorella variabilis</i>
comp29161_seq0 m.29630	Sulphate transport system	gb AGZ19377.1	<i>Chlorella sp. ArMoo29B</i>
comp35923_seq0 m.35211	Membrane protein	ref XP_005844583.1	<i>Chlorella variabilis</i>
comp36025_seq0 m.35298	MFS transporter	ref WP_012790048.1	<i>Chitinophaga pinensis</i>
comp39264_seq0 m.37815	TctA transporter	ref WP_027575203.1	<i>Bradyrhizobium sp. WSM1743</i>
comp40136_seq0 m.38461	NaDH dehydrogenase subunit 3	ref YP_009049981.1	<i>Chlorella sorokiniana</i>
comp40842_seq0 m.39088	Adenine guanine permease azg1	ref XP_005850398.1	<i>Chlorella variabilis</i>
comp43747_seq0 m.41450	Vesicle-associated membrane protein 726	ref XP_005843431.1	<i>Chlorella variabilis</i>
comp44082_seq0 m.41788	Serine threonine protein kinase	ref WP_053333544.1	<i>Gemmatimonas phototrophica</i>
comp44147_seq0 m.41832	Calcium-transporting ATPase endoplasmic reticulum-type	ref XP_005847889.1	<i>Chlorella variabilis</i>
comp45947_seq0 m.43343	Cyclic nucleotide-binding protein	ref XP_005848599.1	<i>Chlorella variabilis</i>
comp46264_seq0 m.43542	V-type proton ATPase 16 kDa proteolipid subunit	ref XP_005848107.1	<i>Chlorella variabilis</i>
comp46589_seq0 m.43815	V-type proton ATPase subunit A3-like	ref XP_005849093.1	<i>Chlorella variabilis</i>
comp50679_seq0 m.47029	ER lumen protein-retaining receptor	ref XP_005845363.1	<i>Chlorella variabilis</i>
comp51446_seq0 m.47612	AMP-dependent synthetase	ref WP_019199842.1	<i>Afpia birgiae</i>
comp60279_seq0 m.54339	Presenilin-domain-containing partial	gb KDD71768.1	<i>Helicospiridium sp. ATCC 50920</i>
comp64356_seq0 m.57092	Zinc transporter	ref XP_007512557.1	<i>Bathycoccus prasinos</i>
comp64706_seq0 m.57251	ATPase P	ref WP_034225211.1	<i>Actinotalea ferrariae</i>
comp85939_seq0 m.69120	Glycosyl transferase/Callose synthase	ref XP_011395511.1	<i>Auxenochlorella protothecoides</i>
comp66975_seq0 m.58878	Fucose permease	ref WP_022830286.1	<i>Cytophagales bacterium</i>
comp59167_seq0 m.53685	Mannose-6-phosphate isomerase	ref WP_052557275.1	<i>Gemmata sp. ILL30</i>
comp69839_seq0 m.60536	Iron/manganese transporter	ref WP_022832831.1	<i>Cytophagales bacterium B6</i>
comp76549_seq0 m.64487	Iron/manganese transporter	gb AEW00627.1	<i>Niastella koreensis GR20-10</i>
comp78298_seq0 m.65658	Upf014 membrane protein star2	ref WP_035836204.1	<i>Cryobacterium roopkundense</i>
comp80077_seq0 m.66613	Diguanylate cyclase	ref WP_009735916.1	<i>Bradyrhizobiaceae bacterium SG-6C</i>
comp9596_seq0 m.8023	Tricarboxylate transport membrane protein	ref WP_014280238.1	<i>Paenibacillus terrae</i>

**Table 5.4.5:** A complete list of the 64 putative transporters present in both Light and Dark SCT libraries (at least one of each) with their annotation/description



**Figure 5.4.3:** A binary filter heatmap that displays the 4 groups of transporter proteins identified in the *P. bursaria-M. reisseri* transcriptome. Specifically, it shows the way that transporters fall into different expression “groups” and their relative sizes. In detail, 34 transporters are expressed only in dark single cell libraries, 48 expressed only in light libraries, the 64 expressed in both and the 14 only recovered in the bulk libraries.

#### 5.4.4 SECRETED PROTEINS

Using the secretome prediction consensus ensemble classifier in conservative and permissive settings resulted in a putative secretome of 24 and 249 proteins respectively. Unfortunately, the permissive group contained a significant number of transporters so the analysis focused on the conservative consensus secreted proteins.

While many of these have no clearly defined function two of the most interesting secreted proteins here relate to carbohydrate metabolism. Specifically, “comp65133\_seqo|m.57547” which

Secreted Protein Name	Annotation/Description	Top BLASTP Hit Accession	Top Hit Species
comp10940_seqo m.9287	Unknown	-	-
comp11029_seqo m.9365	Unknown	-	-
comp23584_seq1 m.24185	Unknown	-	-
comp13389_seqo m.11956	Unknown membrane component	ref WP_004718904.1	<i>Acinetobacter guillouiae</i>
comp13389_seq1 m.11962	Unknown membrane component	ref WP_004718904.1	<i>Acinetobacter guillouiae</i>
comp15590_seqo m.14704	Hypothetical protein	ref XP_005845446.1	<i>Chlorella variabilis</i>
comp19575_seqo m.19598	SOUL heme-binding protein	ref XP_005646909.1	<i>Coccomyxa subellipsoidea</i>
comp19875_seqo m.19979	DDB1- and CUL4-associated factor 12-like	ref XP_005848716.1	<i>Chlorella variabilis</i>
comp24544_seqo m.25243	Peptide ABC transporter substrate-binding protein	gb KJC48675.1	<i>Bradyrhizobium</i> sp. LTSP849
comp25746_seqo m.26477	Chloroplast precursor	-	-
comp26585_seqo m.27218	Hypothetical protein	ref XP_005846166.1	<i>Chlorella variabilis</i>
comp30431_seqo m.30722	Predicted protein	-	-
comp31345_seqo m.31491	Outer membrane protein	ref WP_008612716.1	<i>Joostella marina</i>
comp41497_seqo m.39631	$\alpha$ -l-fucosidase	ref WP_041886110.1	<i>Pedobacter</i> sp. NL19
comp45018_seqo m.42444	Dipeptidyle peptidase	ref XP_003740022.1	<i>Metaseiulus occidentalis</i>
comp45978_seqo m.43356	Glycoside hydrolase	ref WP_049876385.1	<i>Sorangium cellulosus</i>
comp48174_seqo m.45141	Recombinase	gb AAM94959.1	<i>Volvox carterii</i> f. <i>nagariensis</i>
comp48206_seqo m.45162	Glutathione peroxidase	ref WP_022832669.1	<i>Cytophagales bacterium</i> B6
comp50890_seqo m.47213	type i polyketide synthase	ref XP_005650993.1	<i>Coccomyxa subellipsoidea</i>
comp56156_seqo m.51365	SNase-like nuclease	gb AIP99476.1	<i>Ornithobacterium rhinotracheale</i> ORT-UMN 88
comp57702_seqo m.52483	Unknown	-	-
comp59306_seqo m.53753	soma ferritin-like	gb AAN63032.1	<i>Branchiostoma lanceolatum</i>
comp60645_seqo m.54526	Hypothetical protein	ref XP_005851273.1	<i>Chlorella variabilis</i>
comp6932_seq1 m.5603	Hypothetical protein	gb EKE16659.1	Uncultured bacterium
comp65133_seqo m.57547	Raffinose synthase	ref XP_005845739.1	<i>Chlorella variabilis</i>

appears to be homologous (expectation of  $1.38e-10$ ) to Raffinose synthase, and “comp41497\_seqo|m.42444” which is a fucosidase.

The secretion of a glutathione peroxidase is also intriguing. Peroxidases protect from oxidative damage therefore, this suggests there may be oxidative stress within the PV lumen. Finally, the membrane components could either be a misprediction and proteins integral to the algal outer membrane. Alternatively, they could be some of the hypothetical algal derived factors that integrate into the PV membrane (Kodama and Fujishima, 2009).

#### 5.4.5 METABOLIC MAPS

Comparison of the *M. reisseri* metabolic map against the combined maps of the other 3 green algal datasets here (*C. variabilis* 1N, *C. variabilis* NC64A, and *C. subellipsoidea*) reveals some unique genes transcribed in *M. reisseri* that are expressed while an endosymbiont.

Specifically, several subunits of carotenoid biosynthesis were expressed in *M. reisseri* during endosymbiosis. Carotenoid liposomes have previously been implicated in the prevention of phototoxicity in *Paramecium caudatum* (Rich et al., 1992). This means it is possible that these carotenoids could play a role in the observed photoprotective phenotype. Additionally, there are numerous aspects of amino acid metabolism and degradation only present in *M. reisseri* compared to the other algal endosymbionts. For example, lysine degradation pathway components, glutaminases (necessary for glutamine, D-glutamate degradation and the metabolism of alanine and aspartate), and urea cycle components. Finally, there appears to be missing components of fatty acid catabolism in *M. reisseri* relative to the other endosymbionts. The degradation products of fatty acids have been found to inhibit *Chlorella* growth (Ikawa et al., 1997) meaning the partial loss of the fatty acid degradation pathways may be highly deleterious. This might explain the complete loss despite the relatively small phylogenetic distance between *M. reisseri* and the other endosymbiotic green algae.

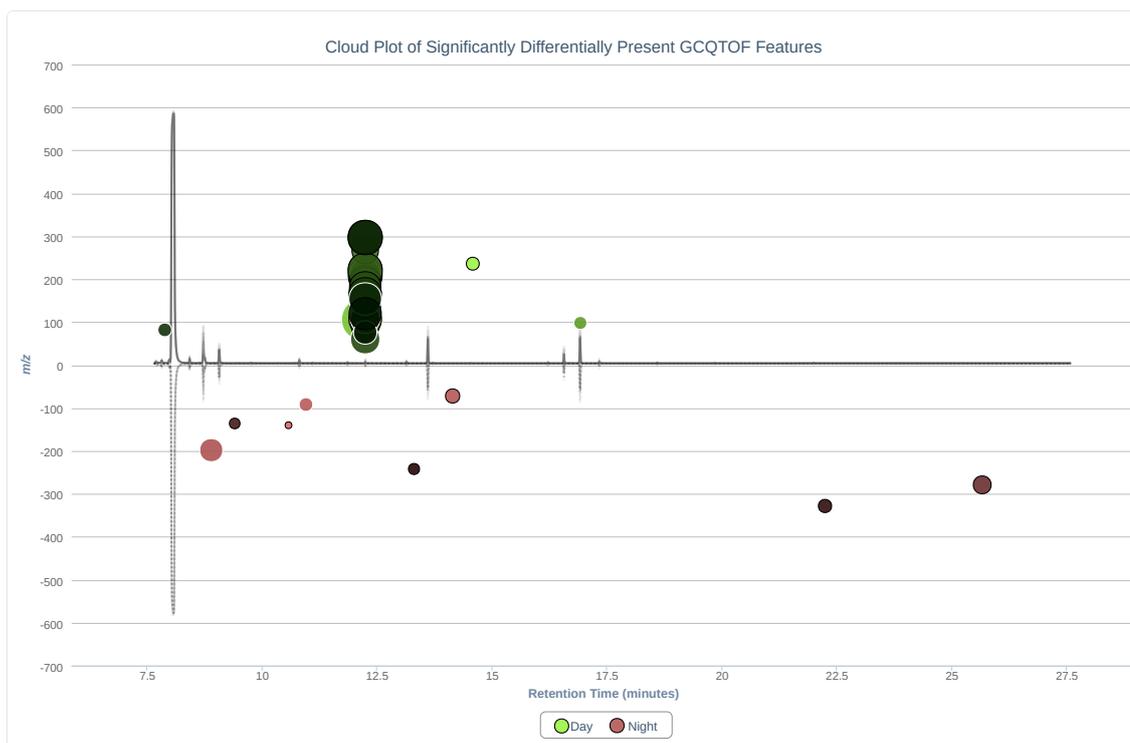
#### 5.4.6 METABOLOMICS

##### 5.4.6.1 GLOBAL PROFILING

The global metabolic profiling had mixed results. Very few metabolites were identified in the GC-QTOF analysis and of those very few were the target carbohydrates. Inspection of cloud plots for



**Figure 5.4.4:** KEGG maps contrasting the putative metabolism of the endosymbiont binned sequences with the metabolism of each of the other 3 green algal species (*C. variabilis* NC64A and 1N, and *Coccomyxa subellipsoidea* C-169).



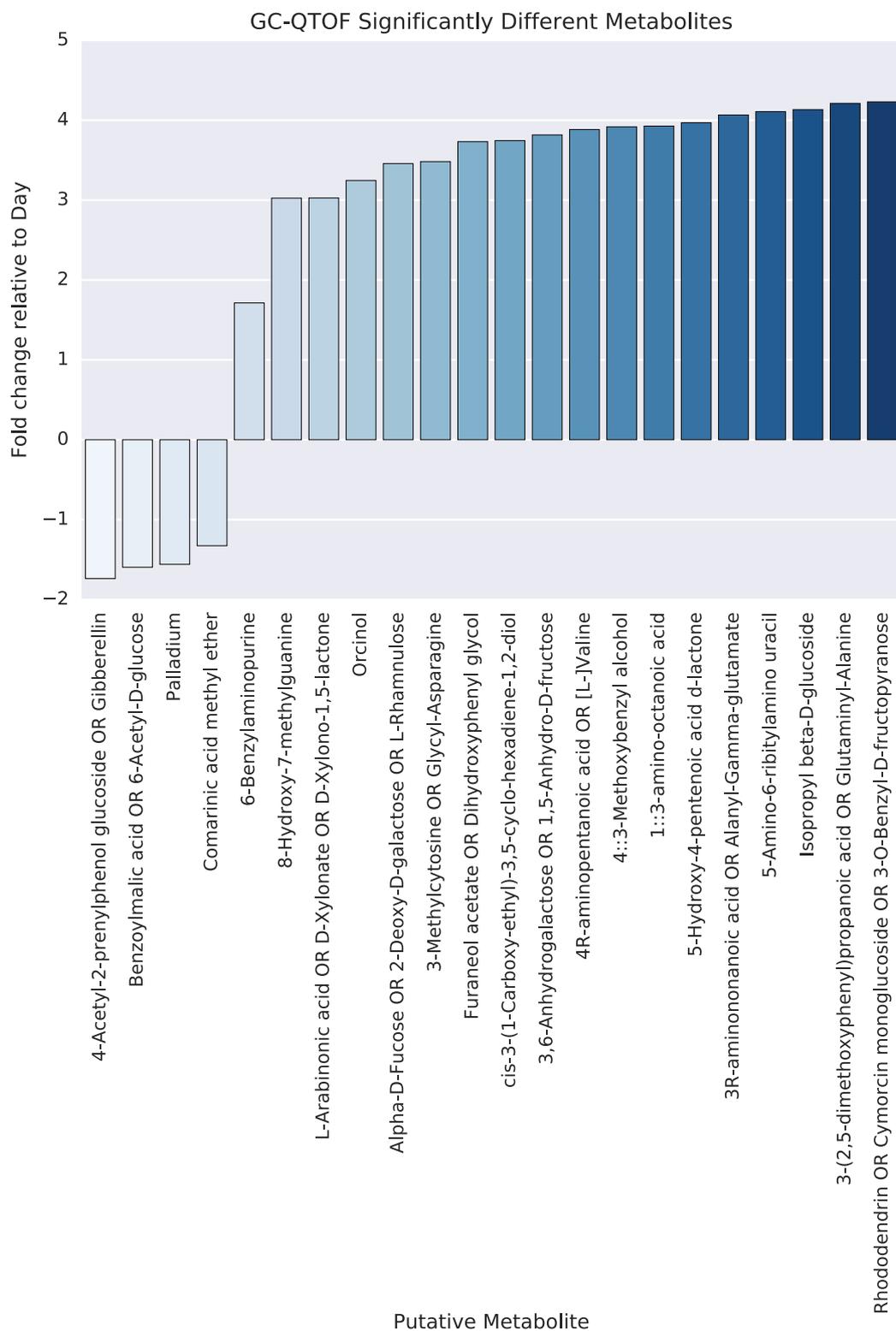
**Figure 5.4.5:** Cloud plot showing for the GC-QTOF analysis. The radius of a given point reflected its fold change. Data is filtered to those 50 points with significantly different expression (FDR corrected P-value of 0.01 shown by depth of colour). The poor separation of components across retention time suggests that GC could be re-calibrated to optimise the separation of these metabolites

this dataset (fig. 5.4.5) indicates poor calibration of GC/MS capture parameters.

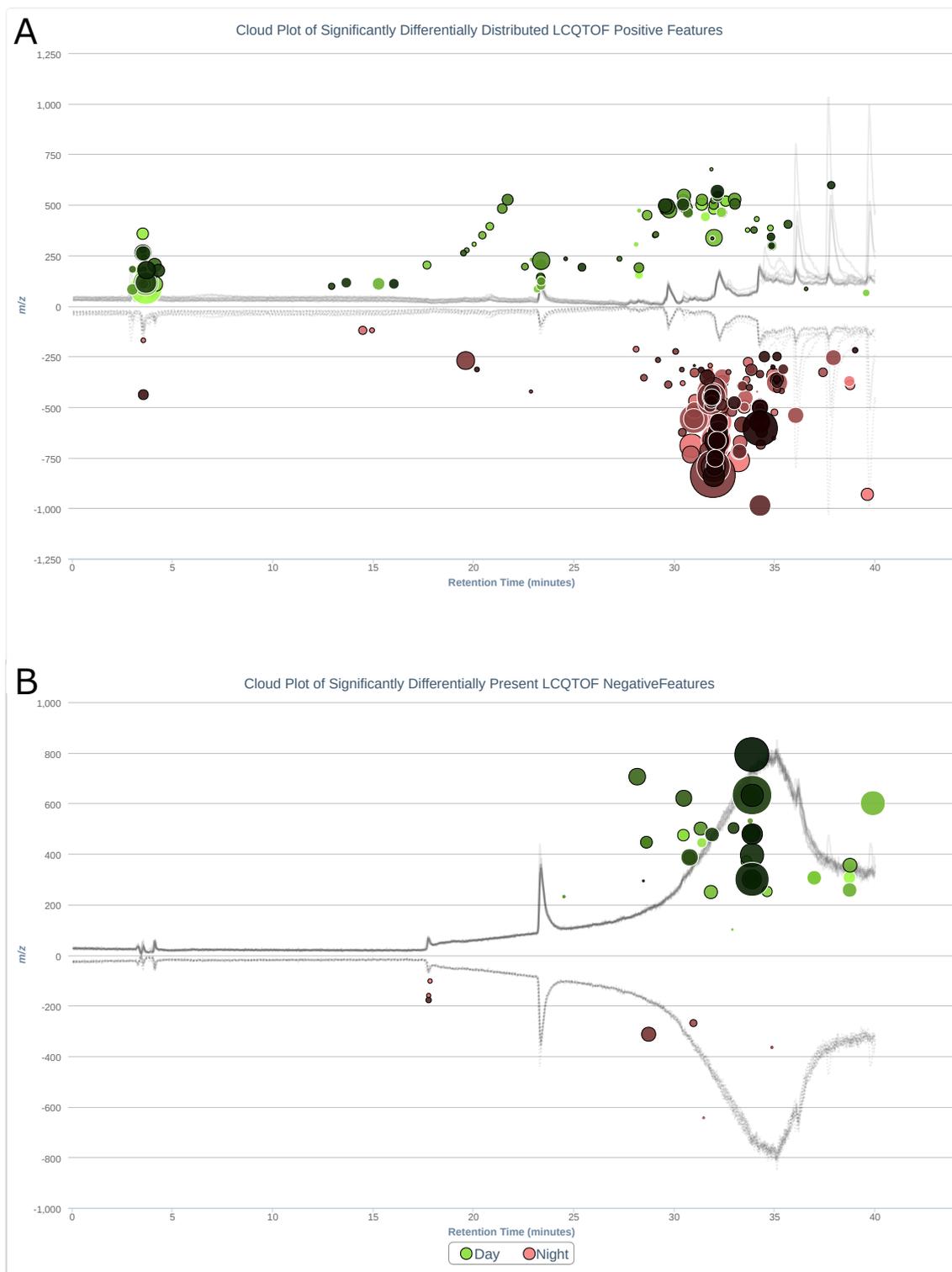
However, 3 carbohydrates were identified in this dataset (fig. 5.4.6), including a putative decreased concentration of acetyl-glucose during the day. On the other hand, a putative galactose/fructose and a fucose/galactose/rhammucose peak demonstrated significant increases in abundance during the day with fold changes between 3 and 4.

LC-QTOF seemed to be more calibrated - identifying a much greater number of metabolites (fig. 5.4.8, fig. 5.4.9, fig. 5.4.10) with better separation (fig. 5.4.7).

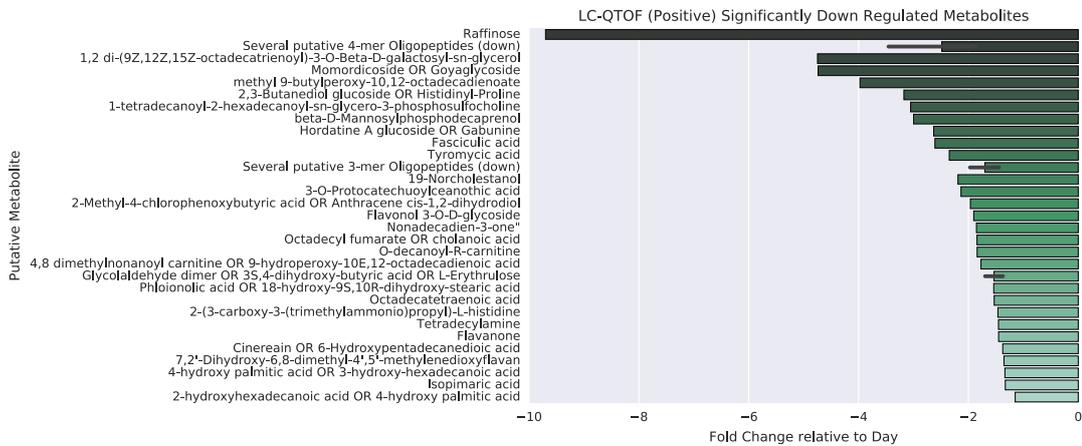
Several amino acids and oligopeptides were identified as having significantly different day/night concentrations in the LC-QTOF analyses. However, the most significant peaks were that of a hugely decreased abundance of Raffinose (fold change of 9.7) during the day. On the other hand, the polyols Arabitol/Xylitol were present at higher quantities (1.52-2.24 fold) and arabinose (2.7 fold) during lit conditions.



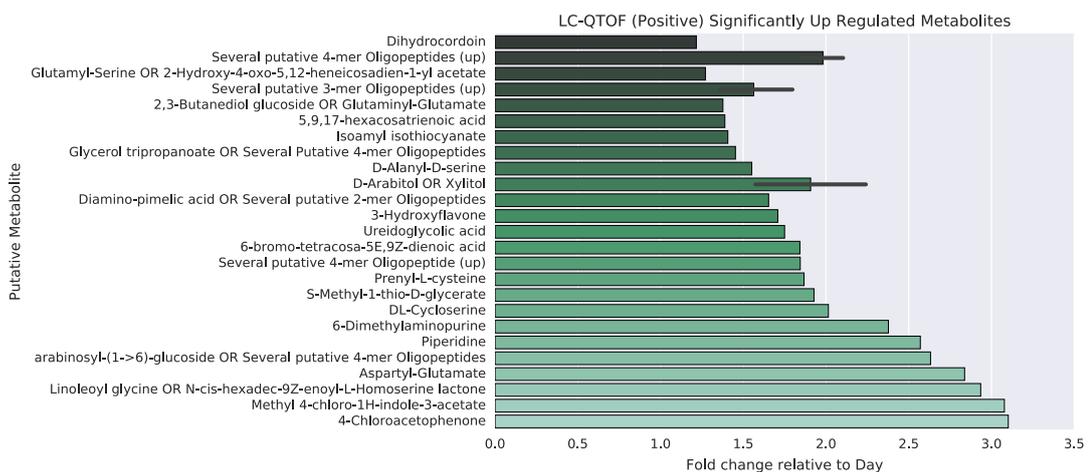
**Figure 5.4.6:** Plot showing the fold change of the 23 putatively identifiable significantly differently present metabolites from GC-QTOF. 5 peaks were discarded after inspection of the EIC. 8 were discarded as there was no sensible annotation, 14 had no annotations.



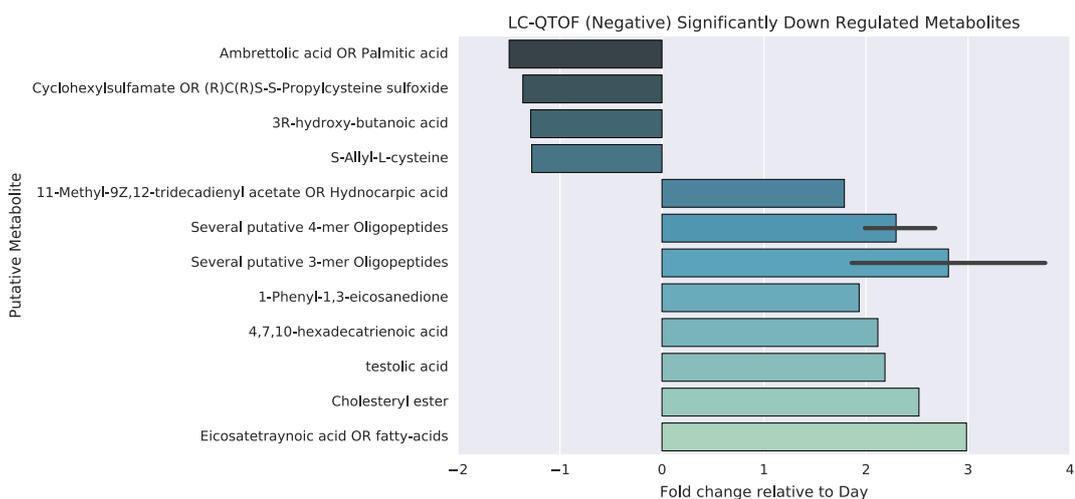
**Figure 5.4.7:** LC-QTOF cloud plots with **A** showing the positive polarity analysis and **B** showing the negative polarity. The radius of a given point represents its fold change, with larger radius indicating a larger fold change. Data is filtered only to include points with significantly different expression and the depth of colour indicates the P-value. Contrary to the GC-QTOF analysis LC-QTOF shows a relatively good separation of metabolites. This plot also emphasises that far more metabolites were detected and discovered differentially expressed under positive ionisation.



**Figure 5.4.8:** LC-QTOF positive ionisation detected metabolites that were significantly more abundant at night relative to the day. Of the 254 positive significantly different present metabolites, 19 were removed after manual inspection of peaks, 95 were removed due to having no METLIN hits.



**Figure 5.4.9:** LC-QTOF positive ionisation detected metabolites that were significantly more abundant in the day relative to night. 254 positive significantly different present metabolites, 19 were removed after manual inspection of peaks, 95 were removed due to having no METLIN hits.



**Figure 5.4.10:** LC-QTOF negative ionisation metabolites identified as significantly higher or lower concentration between day and night. 43 significantly different present metabolites were identified, 3 were removed after manual inspection of peaks, 17 were removed due to having no METLIN hits.

#### 5.4.6.2 TARGETED AMINO ACID ANALYSIS

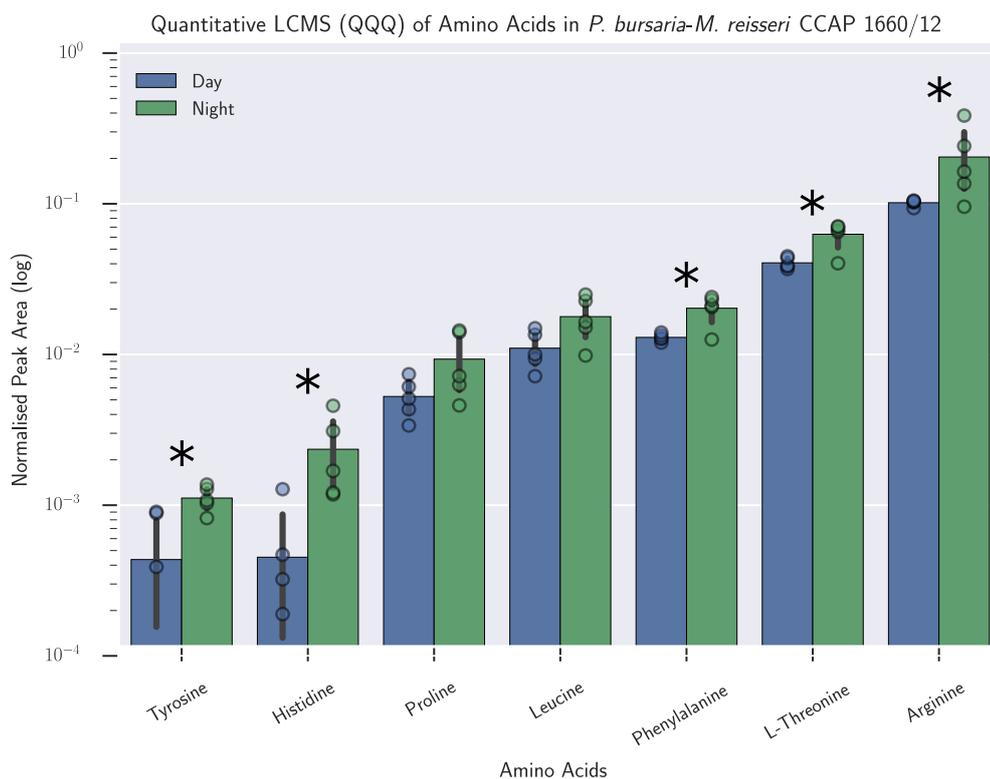
The targeted quantitative analysis of the amino acids in the system between day and night also had mixed results. The majority of amino acids were not reliably recovered and calibration curves could not be fitted. Therefore, several key amino acids remain unprofiled. The relatively large concentration of arginine as well as significant day-night differences suggests that this may be an important nitrogen source for *M. reisseri* during endosymbiosis.

## 5.5 DISCUSSION

### 5.5.1 NOVEL SUGARS IMPLICATED IN THE ENDOSYMBIOSIS

One of the key findings supported by multiple lines of evidence is the existence of roles for sugars not previously implicated in the function of *P. bursaria* - green algal endosymbioses. Specifically, arabinose, raffinose and fucose.

Fucose is identified as having a significantly higher concentration during the day and the endosymbiont expresses a fucose permease in both lit and dark conditions. Arabinose is similarly significantly at greater abundance the day however, no arabinose transporter was directly identified in the endosymbiont transcriptome. It is possible that one of the MFS group transporter identified is capable of uptake of this compound though.



**Figure 5.4.11:** LC-QQQ analysis of amino acid abundances. Normalised Peak Areas Calibration was conducted using Day1 and Night1 samples at two titrations, as well as Asn-Gln-Tryptamine and Sigma AA mixes. Calibration and quantitative analysis failed for the following amino acids: Glutamic Acid, Tryptamine, Asparagine, Tryptophan, Isoleucine, Methionine, Valine, Serine, Glutamate, Glutamine, Aspartic acid, Cysteine or Lysine. Significant concentration differences between day and night (as determined via Welch's 't' are indicated by an asterisk).

Raffinose was both detected at significantly lower abundance during the day in the metabolomics and a raffinose synthase predicted as a secreted protein. An uptake transporter for raffinose was not identified in the endosymbiont but again it is possible that one of the MFS transporter may be capable of the uptake of this compound. Alternatively, the host could encode a transporter for raffinose uptake into the host cytoplasm. A transporter that was directly identified in the endosymbiont was that for inositol. As raffinose synthase function involves the production of inositol and raffinose from galactinol and sucrose (Caspi et al., 2007) this suggests that inositol is either taken up by the endosymbiont or pumped into the PV lumen.

It seems clear that raffinose plays some significant role in the endosymbiosis especially as it is likely to be synthesised within the PV lumen itself due to the putatively secreted peptide. Raffinose has been associated with cold shock in *Parachlorella kessleri* (formerly *C. vulgaris*), accumulating during cold exposure and disappearing after returning to normal temperatures. Specifically, it has also been directly associated with cryoprotection of thylakoid membranes (Lineberger, 1980). Raffinose and another Raffinose Family Oligosaccharide (RFO) stachyose are also generated in gymnosperms during the cold season (Kandler and Hopf, 1982). Interestingly, raffinose has been found to inhibit growth under isosmotic conditions in a *C. vulgaris* (Setter and Greenway, 1979).

Therefore, it is not immediately clear what role raffinose may play in the endosymbiosis. I present 2 hypotheses: firstly, that it may be involved in the stability and maintenance of the PV membrane due to its previously implicated role in cryoprotection of thylakoid membranes and secondly, that it may form a way in which the endosymbiont can “sequester” released carbohydrates from the host by converting them to a format the *P. bursaria* host cannot uptake. This doesn't directly explain the significantly high concentration of raffinose at night relative to day, however, this could relate to the storage of photosynthate in the form of raffinose in the absence of active photosynthesis.

It is worth noting that the identification of other secreted proteins related to the hypothetical synthesis and metabolism of complex sugars in the PV could have been missed due to poor terminal resolution of transcripts during sequencing. This is particularly problematic for bulk RNAseq and low terminal coverage and thus less trust-worthy data was identified in a preliminary analysis of this type of transcriptomic data. Theoretically, due to the ligation step, MDA based sc-RNAseq

shouldn't have this issue. However, it seems likely the general noise and biases of MDA may have rendered a reduced positional bias as a moot issue.

#### 5.5.2 ALTERNATIVE EXCHANGED AMINO ACIDS IN *P. BURSARIA*-*M. REISSERI*

The failure of accurate quantification in the second round of LC-QQQ spectrometry for the majority of amino acids is problematic. However, this targeted approach still revealed useful information regarding the relative abundance of certain amino acids.

Particularly, both the high concentration of arginine as well as its significantly differential abundance between day and night indicates that this amino acid may well form a major component of host provided nitrogen for *M. reisseri*. This is in concordance with previous findings suggesting the importance of this amino acid in the *C. variabilis* endosymbiosis (Kato et al., 2006). The presence of elements of arginine metabolism pathways such as the urea cycle in the transcriptome also supports this hypothesis.

Despite not have been implicated in previous analyses as one of the key amino acid nitrogen sources, the identification of both high quantities and differential abundance of threonine and phenylalanine suggests that these amino acids may play a role in the host-endosymbiont barter system of *P. bursaria*-*M. reisseri*. Additionally, the unique presence of lysine, glutamine and D-glutamate degradation pathways in *M. reisseri* relative to other green algae suggests that these amino acids also comprise an element of the host-derived nitrogen supply. The differential abundances of the amino acids as well as differential numbers of reads mapping to putative amino acid transporters indicates a potential light-dependent amino acid uptake mechanism in the endosymbiont.

This markedly different behaviour in *M. reisseri* relative to the other algal endosymbionts suggests that the feeding experiment results by (Kato et al., 2006) and (Kato and Imamura, 2009b) need to be re-evaluated for *M. reisseri*. This also adds further evidence of a broad diversity of endosymbiotic relationships and traits among the various algal endosymbioses of *P. bursaria*.

Unfortunately, a failure to accurately quantify and calibrate for several amino acids means this targeted metabolomic analysis is incomplete. Of particular interest, is the remaining 5 amino acids implicated in *C. variabilis* F36-ZK's endosymbiosis.

There is also a potential supply of oligopeptides to the endosymbiont. This is evidenced by the presence of a partial oligopeptide transporter combined with the observed high concentrations of

various 3- and 4-mer oligopeptides. Previous studies focusing exclusively on endosymbiont utilisation and uptake of individual amino acids (e.g. those reviewed in (Kato and Imamura, 2009b)) may have missed on the role of these in host-endosymbiont nitrogen flux. This, therefore, merits further analysis.

In terms of other nitrogen sources, i.e. nitrate and nitrite: NR and NiR are both present in the *M. reisseri* binned transcriptome. This supports findings that *M. reisseri* can utilise nitrate and nitrite. However, it is possible that these enzymes are non-functional such as the mutants present in *C. variabilis* NC64A and F36-ZK.

### 5.5.3 POTENTIALLY MISSING TRANSPORTERS AND SECRETED PROTEINS

The identification and analysis of secreted and transporter proteins, as well as the metabolic mapping are fundamentally reliant on the quality and completion of the host and endosymbiont binned transcriptomes. Transcripts may be missing from these bins either due to failure to assemble, cryptic MDA biases, or erroneous binning into bins other than host or endosymbiont.

It is a cause for concern that there are a number of endosymbiont secreted and transporter proteins which have top BLASTP hits against bacterial species. On inspection many of these do have other hits to green algal species at slightly lower expectation therefore binning could be working as intended. However, bacterial contamination of the endosymbiont bin is a very real possibility. Further studies should confirm the identity of these proteins using manual in-depth phylogenetics instead of the high-throughput and potentially error prone method used in transcript binning.

There is evidence that binned endosymbiont transcriptomes are incomplete in the large disparity in predicted transporter set sizes between the genomes and transcriptome based datasets. However, we are not necessarily interested in all the transporters and secreted proteins that the endosymbiont is capable of producing but merely those that it is producing while an endosymbiont. A high level of transcription as an endosymbiont suggests that the function of given protein plays a significant role in symbiosis. Therefore, theoretically the only major group of factors that are both involved in endosymbiosis and systematically absent from these binned transcripts are those of effectors related to the establishment of endosymbiosis that are not expressed during the rest of the endosymbiotic relationship.

One set of proteins in which erroneous binning may be particularly problematic is that of

proteins which have recently undergone endosymbiotic gene transfer (EGT) between endosymbiont and host (Timmis et al., 2004) or that have been horizontally acquired from other sources. In the former case, this is well observed phenomenon that has resulted in the eventual loss of the endosymbiont in the majority of algal secondary endosymbiotic organelles as genes are transferred to the host nucleus (Keeling and Palmer, 2008; Archibald, 2005; Timmis et al., 2004; Keeling, 2004). It is unknown and difficult to determine to what extent the unusual nuclear dimorphism and germline sequestration of the host will effect this form of transfer. However, hypothetically this should present a barrier to such transfers. As some *M. reisseri* and *Chlorella* endosymbionts have been demonstrated as capable of free-living and metabolic co-dependence has putatively not become fixed it is unlikely that EGT has occurred between host and endosymbiont as extensively as that observed in established photosynthetic organelles. Fortunately, the binning method used means that while some peptides may have been falsely assigned to wrong bin, all “host” and “endosymbiont” ORFs that were either so novel they lacked any homology to known proteins or were recently acquired from bacteria were still included in this analysis, just not necessarily attributed to the correct partner.

As for the latter case of HGT from other sources, this will lead to the misclassification of proteins into the “food” or “unknown” bin and thus their discard. This is potentially problematic as there is evidence for bacterially acquired hexose-phosphate transporters playing a key role in the establishment of primary plastid endosymbiosis (Price et al., 2012; Karkar et al., 2015). There is also evidence of the acquisition of a bacterial polyamine biosynthesis pathway within the host *Paramecium* (Li et al., 2015a).

Ideally, future work could expand the component analyses over the “unknown” and “food” binned sequences in combination with synteny analyses using genomic sequences to investigate and identify potential horizontally acquired transporters that may play a role.

Another issue with the binning approach used is the possibility of totally novel transporter (and other proteins) not being classified due to the dependence of the binning on homology to known sequences. Therefore, totally novel proteins would not have been identified as “host” or “endosymbiont”. Unfortunately, this problem could only properly be resolved with a genome sequence for both host and endosymbiont which was outside the scope of this analysis.

Finally, there are two additional difficulties specific to transporter and secretome prediction.

In terms of secreted proteins, without knowledge of *P. bursaria*'s intracellular trafficking system it is not possible to easily infer which host peptides are secreted into the PV. For this reason, analysis of secreted proteins focused on the endosymbiont bin as the secretion signal are generally better conserved and established.

In the case of transporters the risk of false positives where sensors are misidentified as transporters is fairly high. Only a minimal (as few as a single amino acid) divergence is sufficient to convert a transporter protein to non-transporting sensor proteins (Lalonde et al., 1999; Bianchi and Díez-Sampedro, 2010). However, sensor proteins are likely to play important roles in the function of this endosymbiosis so accidental identification may not be a major issue but it does mean transporter activity needs to be verified for all predictions.

Prediction methods used to identify transporter and secreted proteins could also be further improved by more careful application of state-of-the-art classification algorithms. Prediction of protein secretion could be improved using recent approaches such as recursive neural network models like long-short term memory networks (Hochreiter and Schmidhuber, 1997; Greff et al., 2015). These are well suited to arbitrary length sequence data so wouldn't require work-arounds to accommodate variable length signal peptides. They are also capable of representation learning so the identification of sequence features that best predict localisation would be unnecessary. Alternatively, existing prediction tools could be combined in a more sophisticated way than the conservative consensus ensemble used in this analysis. For example, the various predictors could be combined using Bayesian model combination (Monteith et al., 2011).

However, even with these improved predictions, it is still best to consider all analyses of these transcriptomes as proof of presence but not proof of absence of any component. Furthermore, any identified protein should be validated individually using immunolocalisation and (q)PCR based analyses.

#### 5.5.4 METABOLOMICS SHOWS PROMISE

The pilot application of metabolomics demonstrated mixed results. There was poor performance of GC/MS with a failure to comprehensively profile carbohydrates. Several compounds strongly implicated in the endosymbiosis were not detected e.g. maltose, and glucose. This was potentially due to the miscalibration of gas chromatography leading to poor separation of components.

While LC/MS analyses did prove relatively successful, a careful validation of metabolites of

interest using multi-reaction monitoring and tandem spectrometry would be necessary to make firm predictions. Additionally, advanced novel techniques such as nanoscale secondary ion mass spectrometry combined with microscopy and isotope labelling could theoretically allow direct analysis of metabolites present in the PV (Kopp et al., 2015; Legin et al., 2014).

The targeted quantitative LC/MS of amino acids needs further optimisation and re-running due to the inability to fit calibration curves to the majority of amino acids. Despite this, the technique did prove effective at identifying a potential role for several amino acids not previously implicated in this endosymbiosis.

Finally, one more improvement to the metabolomics analyses would include more advanced hypothesis testing than the corrected unequal variance *t*-test used e.g. Kurschke's Bayesian BEST algorithm (Kruschke, 2013). This has the advantage of a Bayesian inference which can be made robust to multiple comparisons without need for extensive correction procedures via standard multi-level approaches (Gelman et al., 2009).

## 5.6 CONCLUSION

This analysis of host-endosymbiont metabolic integration has led to some promising results. Namely, discovering quantitative data supporting the mechanism by which the host likely provides a nitrogen source to the endosymbiont. Specifically, a novel group of amino acids may well be used in *M. reisseri* endosymbiosis: lysine, d-glutamate, threonine, and phenylalanine, as well as the previously implicated arginine and glutamine (Kato and Imamura, 2009b). Additionally, potential novel roles for carbohydrates previously not associated with *P. bursaria* endosymbioses, specifically fucose, arabinose and raffinose have been identified. Unfortunately, poor resolution and identification of carbohydrates in GC/QTOF prevents a thorough analysis of endosymbiotic carbohydrate metabolism. Finally, *M. reisseri* appears not to express elements of fatty acid degradation present in the other algal endosymbionts. This is potentially interesting as fatty acid metabolism has previously been identified as a key conserved function of plastids (Donaher et al., 2009). However, further work is needed to confirm both the role and localisation of these compounds and their facilitators.

Ultimately, the key finding from this analysis is that *M. reisseri* exhibit a range of adaptations to endosymbiosis that are distinct from previously studied algal strains such as *C. variabilis*.

*In biology, nothing is clear, everything is too complicated, everything is a mess, and just when you think you understand something, you peel off a layer and find deeper complications beneath. Nature is anything but simple.*

- Richard Preston: *The Hot Zone*, 1994

# 6

## RNAi in *P. bursaria*

### 6.1 INTRODUCTION

#### 6.1.1 RNAi

Post-transcriptional gene silencing (PTGS) is a highly useful experimental technique in reverse genetic analyses. The most widely used PTGS experimental method is that of RNA-mediated interference (RNAi) of gene expression (Fire et al., 1998). It has extensively been used in the study of model eukaryotic organisms (Morf et al., 2013; Batista and Marques, 2011; Matthew, 2004; Ketting, 2011; Chang et al., 2012).

RNAi covers a set of evolutionarily conserved systems across the eukaryotes with various mechanisms of action in which the expression of particular transcripts are regulated via several classes of transcribed small non-coding RNA (ncRNA) such as short-interfering (siRNA), micro (miRNA) and Piwi-interacting (piRNAs) (Carthew and Sontheimer, 2009).

These systems likely originated as a form of defence against viruses and transposons (Water-

house et al., 2001; Buchon and Vaury, 2006) and were present in some form in the last universal eukaryotic ancestor (LECA) (Cerutti and Casas-Mollano, 2006; Shabalina and Koonin, 2008). Many eukaryotes utilise these small RNA-mediated gene silencing pathways in the regulation of their own cell expression patterns (Wu and Belasco, 2008). Despite its ancestral nature there has been considerable diversification of this process, its function and mechanism (Ketting, 2011). Indeed, even within the same organism, different points of the life cycle may use different RNAi systems (Flemr et al., 2013).

Generally RNAi pathways involve the generation of 21-28nt siRNAs from some form of RNA precursor such as dsRNA (although ssRNA systems exist) via the function of the RNAase III Dicer (Bernstein et al., 2001) or a related protein. These short RNAs are then bound by Argonaute proteins which act alone or as part of a complex to silence the expression of sequences homologous to the siRNA (Ketting, 2011). This silencing isn't just limited to the post-transcriptional endonucleolytic degradation of mRNA transcripts but can also involve transcriptional inhibition and DNA elimination (Marker et al., 2014). The one unifying element of all discovered RNAi pathways is that of the central role argonaute (AGO) proteins play (Ketting, 2011). They are formed of two subclasses: the Ago and Piwi subfamilies (Peters and Meister, 2007) with a range of functions and complex-forming behaviours (Ender and Meister, 2010). The magnitude of the silencing response is occasionally amplified by the generation of more copies of the trigger dsRNA by RNA-dependent RNA polymerases (RdRPs) (Arp et al., 2007). On the other hand, RdRPs can also sometimes directly generate the siRNAs (Aoki et al., 2007; Ketting, 2011). The last universal eukaryotic ancestor (LECA) likely contained at least one Ago and one Piwi family Argonaute protein, a Dicer and an RdRP (Cerutti and Casas-Mollano, 2006).

The other main form of RNAi system present in eukaryotes is that of miRNA based systems. These are differentiated by miRNAs being encoded by dedicated genes and displaying partial complementarity to their targets whereas siRNAs are generated from exogenous dsRNAs (i.e. environmental dsRNA from viral infection or phagocytosed bacteria (Whangbo and Hunter, 2008)) or transgenes, as described above, and involve full or near full complementarity (Shabalina and Koonin, 2008).

On top of this, there are piRNA based systems, which are involved in germline based transposon silencing (Iwasaki et al., 2015), and the ciliate specific scan RNA (scnRNA) system. This is

involved in the elimination of internal eliminated sequences (IESs) during macronuclear (MAC) regeneration (Mochizuki and Gorovsky, 2004; Chalker et al., 2013).

Experimentally, the existence and function of these systems permits a researcher to introduce dsRNA homologous to an RNA transcript of interest and trigger targeted cell-wide RNAi of that transcript. Unfortunately, there are several problems with RNAi as a general method. Many organisms lack active RNAi systems (although such systems can occasionally be induced (Alibu et al., 2005)). On top of this, RNAi requires accurate sequence data to design the precursors and, therefore, necessitates some form of sequencing. The main difficulty, however, is that of off-target effects. These are caused when a provided RNA precursor induces RNAi in more than just the target transcript. These can lead to enigmatic phenotypic outcomes that are then falsely attributed to the initial target. Avoidance of off-target effects requires a complete genome and/or transcriptome to check a prospective siRNA against during the design stage. This further increases the epistemological burden of attempting RNAi in a novel system.

Finally, RNAi does not necessarily induce total silencing of a given transcript and low-levels of transcription may still occur. This, conceivably, can be sufficient to maintain the non-knock down phenotype. This allows a researcher to falsely conclude a non-relationship between a given transcript and phenotype.

#### 6.1.2 RNAi IN *PARAMECIUM*

In addition to the ciliate specific scnRNA system, siRNA based pathways have been discovered in the two principal ciliate model organisms: *Tetrahymena thermophila* (Collins and Lee, 2006; Yao and Chao, 2005) and *Paramecium tet aurelia* (Galvani and Sperling, 2001, 2002). There are two established methods for inducing RNAi in *Paramecium tet aurelia*: microinjection and transformation of the MAC with high-copy transgenes lacking 3' untranslated region (UTR) (Galvani and Sperling, 2001) and the introduction of dsRNA by either microinjection or feeding using transformed dsRNA expressing bacteria (Galvani and Sperling, 2002).

In the transgene pathway, the 3' truncation leads to the production of aberrant sense and antisense transcripts (Galvani and Sperling, 2001; Marker et al., 2010; Beisson et al., 2010a). Based on the identified required components (see table 6.1.1), aberrant transcripts are processed by a Dicer protein (Dcr1) (Lepere et al., 2009) and a putative RdRP complex formed of an RdRP (Rdr2) and a nucleotidyl transferase (Cid2) (Marker et al., 2014) into 23nt siRNA (Lepere et al.,

Pathway	Component	Function
transgene-induced siRNA	Rdr3 Ptiwi14	RdRP Piwi
both pathways	Rdr2 Dcr1 Ptiwi13 Cid2	RdRP Dicer Piwi Nucleotidyl transferase
exogenous dsRNA-induced siRNA	Rdr1 Cid1 Ptiwi12 Ptiwi15 Pds1	RdRP Nucleotidyl transferase Piwi Piwi Import of dsRNA?

**Table 6.1.1:** Summary of the components identified as necessary to the function of both primary siRNA RNAi pathways in *P. tetaurelia* as identified by forward genetic screens in (Marker et al., 2014).

2009). A putatively non-catalytic RdRP (Rdr3) also plays an undefined role in the generation of primary ( $1^\circ$ ) siRNAs from transgene pre-cursors (Marker et al., 2010, 2014). Finally, two Argonaute Piwi proteins (Ptiwi13 and Ptiwi14) (Bouhouche et al., 2011) are involved in targeting post-transcriptional silencing via mRNA cleavage (Bouhouche et al., 2011; Marker et al., 2014).

Alternatively, the exogenous dsRNA pathway can be induced by either microinjection directly into the MAC (for a transient 48 hour long silencing) or by continued feeding with a bacteria experimentally modified to generate dsRNA. Typically, this involves an *E. coli* with an IPTG-inducible T7 polymerase and deficiency for RNase III transformed with a plasmid containing a T7 promoter and the sequence homologous to the target transcript (Fire et al., 1998; Timmons et al., 2001; Galvani and Sperling, 2002). Importantly, there is some evidence that this pathway also is activated at low levels by ssRNA from normal food bacteria (Carradec et al., 2015).

RNA precursors are likely processed by Dcr1 (Lepere et al., 2009), and then two hypothetical RdRC (Cid1-Rdr1, Cid2-Rdr2) (Marker et al., 2010, 2014) are involved in the generation of  $1^\circ$  siRNA. The second RdRC (or specifically Rdr2) is also involved in the generation of low levels of secondary ( $2^\circ$ ) siRNA which spread along the full length of target mRNA (i.e. 3'-to-5' and 5'-to-3' transitivity) in primarily antisense form (Carradec et al., 2015). These  $2^\circ$  siRNAs don't appear to play a significant role in silencing themselves (contrary to similar systems in *C. elegans* where they form the principal targeter of silencing (Sijen et al., 2007; Pak and Fire, 2007)) (Carradec et al., 2015). Three Piwis play a role in targeting silencing. Ptiwi13 hypothetically loads the  $1^\circ$  siRNA and targets cleavage of cytoplasmic mRNA (Bouhouche et al., 2011), while Ptiwi12 and

Ptiwi15, based on their homology to nuclear Piwi proteins, (Marker et al., 2014; Carradec et al., 2015; Bouhouche et al., 2011) may be involved with 2° siRNA communication with the MAC (Carradec et al., 2015). One final protein that has been identified as necessary for the function of feeding based dsRNA-induced RNAi is an uncharacterised novel *P. tetaurelia* complex protein (Pds1) (Marker et al., 2014). It has been hypothesised that Pds1 may play a role in the export of RNA from the food vacuole (Carradec et al., 2015). Therefore, theoretically microinjected dsRNA should induce RNAi even in the absence of this protein as the microinjection circumvents the need for Pds1 facilitated dsRNA import.

Many of these components are a product of the 3 whole genome duplication events in the evolution of the *Paramecium* clade (McGrath et al., 2014). As *P. bursaria* shares only the first *Paramecium* clade whole genome duplication event with *P. tetaurelia* it is expected it should contain the RNAi components identified as belonging to WGD1 (or have secondarily lost them) (McGrath et al., 2014). This is believed to include a single RdRP gene, 6 Piwi genes, and 2 Dicer genes (Marker et al., 2014).

If it is possible to experimentally induce RNAi in *P. bursaria* SW1 (from CCAP 1660/12 culture) specific hypotheses as to the necessity of hypothetically important endosymbiotic components can be tested. For example, what is the effect on endosymbiosis of the inhibition of certain host-derived transporters?

Similarly, what components of core *P. tetaurelia* RNAi pathways can be identified in the *P. bursaria* SW1 (see Chapter 4) and *P. bursaria* Yad1g transcriptomes (see Chapter 5)? Does *P. bursaria* express these components during endosymbiosis? If they don't is there evidence of them in the partial *P. bursaria* SW1 genome (see Chapter 3)?

### 6.1.3 RNAi “CROSS-TALK”

Evidence has emerged of the role RNAi plays in numerous host-pathogen (Nowara et al., 2010; LaMonte et al., 2012; Weiberg et al., 2013; Buck et al., 2014) and host-symbiont (Helber et al., 2011; Koch et al., 2013) relationships. This has led some authors to suggest that siRNA and RNAi mechanisms can form communication systems between diverse organisms and even across domains (Liang et al., 2013; Knip et al., 2014; Weiberg et al., 2015).

The evidence of natural food bacteria ssRNA induced RNAi “cross-talk” in *P. tetaurelia* (Carradec et al., 2015) implicates that this process may also take place in *P. bursaria*. In addition to

also being a serial phagotroph, *P. bursaria* acts as a host to numerous bacterial and green algal endosymbionts. For this reason it is not inconceivable that such a mechanism of cross-talk may play role in these endosymbioses.

Therefore, it may be informative to investigate the quantity and targets of potential cross-talk between host and endosymbiont in terms of “collisions” i.e. matching 23nt RNA strings between host and endosymbiont transcripts bins. Contextualising these values across the diversity of the tree of life is important. “Collision” levels have implications for the regulation and expression of exogenous dsRNA RNAi pathways by the host.

## 6.2 AIMS

The goal of this chapter is to investigate both the practical and theoretical utility of RNAi systems in *P. bursaria*. Specifically:

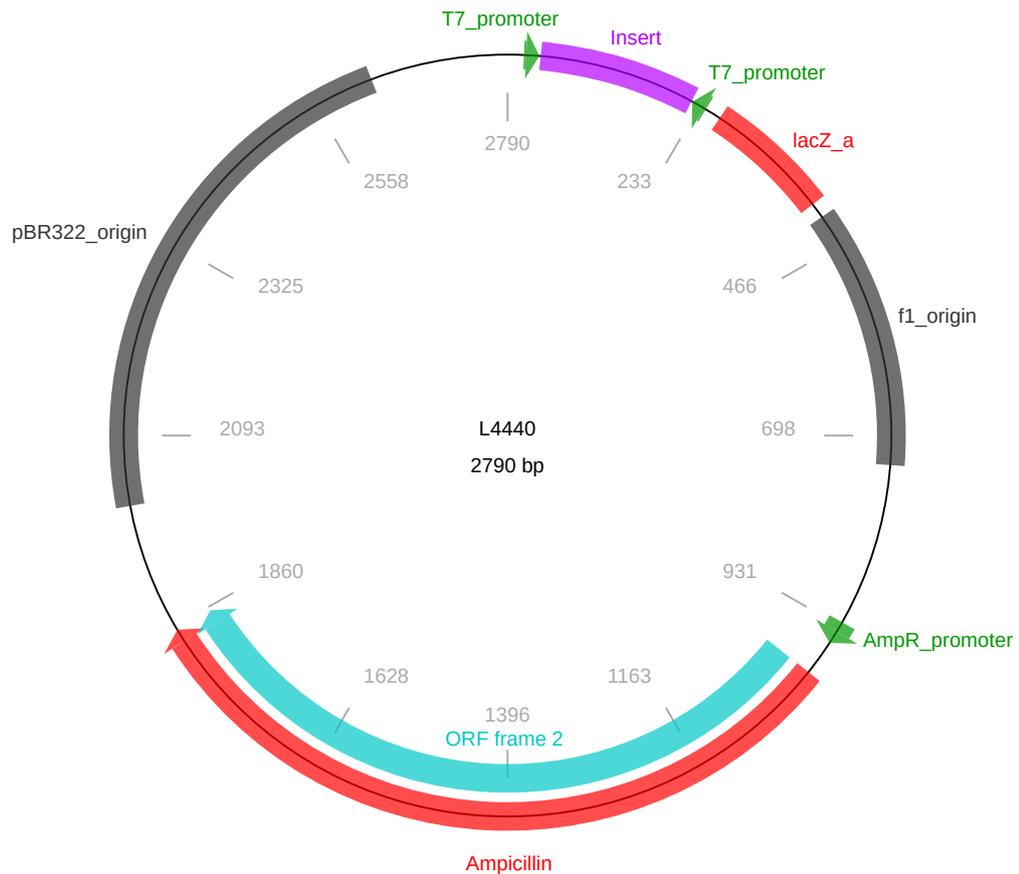
- Is *P. bursaria* capable of microinjection or feeding based exogenous dsRNA siRNAi?
- What components, previously identified as necessary, of these pathways are present and expressed in *P. bursaria*?
- To what degree could potential RNAi “cross-talk” occur in *P. bursaria* and is this elevated compared to what might be faced in *Paramecium* species without eukaryotic endosymbionts?

## 6.3 METHODS

### 6.3.1 RNAi CONSTRUCTS

All RNAi methods were based on previously published protocols, specifically ([Galvani and Sperling, 2001, 2002](#); [Beisson et al., 2010b](#)).

Six different constructs were created featuring genes whose knock-down induces known phenotypes in *P. tetraurelia* (see table 6.3.1). All inserts were designed in the same manner, firstly *P. tetraurelia* sequences were taken from the *P. tetraurelia* genome. These were then used as BLASTN queries against the entire unbinned *P. bursaria*-*M. reissieri* SW1-ZK transcriptome. The insert sequences were then determined using the returned *P. bursaria* sequences. Each insert was cloned



**Figure 6.3.1:** Schematic map of the L4440 vector used for the RNAi experiments. Site of the insert is highlighted in purple with the T7 promoters shown in green and the ampicillin marker in red. Figure was adapted from the unpublished Fire Lab *C. Elegans* vector kit via Addgene (<https://www.addgene.org/1654/>).

into an L4440 vector featuring two convergent T7 promoters and an ampicillin resistance marker (shown in fig. 6.3.1).

#### 6.3.1.1 RNAI FEEDING

For feeding experiments the vectors were transformed into *E. coli* HT115-DE3. This strain is deficient for RNase III and features an IPTG inducible T7 polymerase under the control of a Plac promoter. Method used was as described in (Beisson et al., 2010b). RT-PCR using standard

Gene	Function	RNAi phenotype in <i>P. tetaurelia</i>	Vector Design	Reference
<i>epi2</i>	Epiplasmin	"Monstrous" cells	500 bp via <i>Pst</i> I and <i>Hind</i> III	(Damaj et al., 2009)
NSF	Membrane fusion factor	Lethal	500 bp via <i>Pst</i> I and <i>Hind</i> III	(Galvani and Sperling, 2002)
pTMB.422c	Binding protein	Lethal	500 bp via <i>Pst</i> I and <i>Hind</i> III	(Nowack et al., 2011)
<i>bug22</i>	Basal body/ciliary protein	Slow swimming and death	313 bp via <i>Xba</i> I and <i>Hind</i> III	(Laligne et al., 2010)
BBS7	Ciliary ion transport	Fewer, shorter cilia	486 bp via <i>Xho</i> I and <i>Hind</i> III	(Valentine et al., 2012)
PGM	PGM endonuclease	Post-autogamous cells unable to resume normal growth	500 bp via <i>Pst</i> I and <i>Hind</i> III	(Baudry et al., 2009)

**Table 6.3.1:** Details of RNAi vectors used in dsRNA experiments. All constructs were cloned into a L4440 vector and used an Ampicillin resistance markers.

methods was conducted on the transformed cultures to confirm expression of the dsRNA.

Bacterial pre-cultures were started using a single colony picked from an LB plate containing  $50 \mu\text{g l}^{-1}$  ampicillin and  $12.5 \mu\text{g l}^{-1}$  tetracycline. This picked colony was grown overnight in LB medium with the same antibiotics. The overnight culture was then diluted 50 fold and grown with shaking at  $37^\circ\text{C}$  up to an  $OD_{600}$  of 0.4 to 0.6. IPTG was then added at a concentration of 0.4mM and shaken for 3 hours at  $37^\circ\text{C}$ . 30 ml of this culture was centrifuged for 2 minutes ( $3100 \times g$ ), then the supernatant removed and the pellet washed twice in *Paramecium* growth medium. The pellet was resuspended in *Paramecium* medium with 0.4mM IPTG,  $100 \mu\text{g l}^{-1}$  ampicillin and adjusted to a final  $OD_{600}$  of 0.1.  $1 \mu\text{l}$  of beta-sitosterol (at  $4 \text{ mg ml}^{-1}$  in ethanol) was added to each 5 ml of medium.

For the actual feeding, 10 ml *P. bursaria* CCAP 1660/12 culture were centrifuged at  $800 \times g$  for 10 minutes and re-suspended in 1 ml of supernatant. 9 ml of the induced bacterised media was then added. The sample was then incubated in a tissue culture flask at  $27^\circ\text{C}$ . Feeding was repeated for each day of analysis.

#### 6.3.1.2 RNAI MICROINJECTION

Microinjection used the same protocol as described in (Beisson et al., 2010a) but only tested the PGM and *epi2* constructs. Briefly, the circular plasmid is linearised using a unique restriction site, and purified using phenol:ethanol extraction and a purification column. It is then dissolved in  $H_2O$  at a minimum concentration of  $5 \text{ mg ml}^{-1}$ . Cells were washed twice by picking with a micropipette in Dryl-BSA wells before being placed into individual droplets on a glass cover slip and covered in paraffin oil. The cover slip was then placed onto a microscopy stage and a microinjector used under  $10\times$  magnification to inject linearised construct directly into the macronucleus.

Microinjection controls were also conducted in which GFP was injected into the MAC and the fluorescence observed. Successful microinjection would feature fluorescence localised to the MAC.

#### 6.3.2 ANALYSIS OF RNAI PATHWAY

##### 6.3.2.1 SURVEY FOR RNAI COMPONENTS IN *P. BURSARIA*

Using the canonical seed sequences identified in *P. tetaurelia* by (Marker et al., 2014) (see table 6.3.2) the entire assembled *P. bursaria*-*M. reisseri* SW1-ZK transcriptome predicted ciliate

Gene	<i>P. tet aurelia</i> Accession	Length
Rdr1	PTETG8500012001	4319
Rdr2	GSPATG00036857001	4162
Rdr3	GSPATG00006401001	3292
Cid1	PTETG9100013001	1051
Cid2	PTETG13400003001	1083
Pds1	PTETG600032001	2084
Dcr1	GSPATG00021751001	5394
Ptiwi12	GSPATG00001709001	2315
Ptiwi13	PTETG4800007001	2483
Ptiwi14	PTETG16300003001	2428
Ptiwi15	GSPATG00005370001	2315

**Table 6.3.2:** Table of the RNAi pathway components identified by (Marker et al., 2014). Includes their “canonical” accession in the *P. tet aurelia* MAC genome.

encoded peptides and *P. bursaria*-*C. variabilis* Yad1g1N transcriptome predicted ciliate encoded peptides with BLASTP and a minimum expectation of  $1e^{-5}$ .

Finally, if a component could not be identified in the transcriptomes it was additionally searched for in the assembled *P. bursaria*-*M. reisseri* SW1-ZK genomic contigs (over 500 bp) using TBLASTX with a minimum expectation  $1e^{-5}$ . This would theoretically allow the identification of present but non-expressed components.

Additionally, the other sequenced *Paramecium* genomes were searched using BLASTP via ParamediumDB (Arnaiz et al., 2007; Arnaiz and Sperling, 2011b). Specifically, *P. caudatum* (McGrath et al., 2014), *P. biaurelia*, *P. primaurelia*, *P. sexaurelia* and *P. multimicronucleatum*. Finally, *T. thermophila* (Eisen et al., 2006) and *Oxytricha trifallax* (Swart et al., 2013) predicted proteins were searched to form outgroups during phylogenetic analysis.

#### 6.3.2.2 PHYLOGENETIC ANALYSIS OF RNAI PATHWAY

Peptide sequences were aligned using MAFFT (Kato et al., 2002) and manually masked in Seaview (Gouy et al., 2010). Sequences that were too divergent to align were removed, or in the case of the RdRPs: the alignment of Rdr1 and Rdr2 was split from that of Rdr3 to form two separate alignments. Similarly, sequences that were identical to one another were removed at this stage.

Substitution models were fitted using ProtTest3 on the basis of the Bayesian Information Criterion (BIC) (Darriba et al., 2011). Phylogenies were then generated using RAXML with 1000 non-rapid bootstraps and MrBayes with 2 runs of 4 MCMCMC chains run for 2,000,000 gener-

ations or until convergence. MCMC convergence was checked and burn-in determined using Tracer (Rambaut and Drummond, 2007). Sequences forming long branches were removed after inspection of these phylogenies and the alignment, masking, model prediction and phylogeny generation steps were repeated.

### 6.3.2.3 STRUCTURAL PREDICTION AND FUNCTIONAL ANALYSIS

The structure of Pds<sub>1</sub> was predicted from the *P. tetaurelia* protein sequence (PTETP600032001) using RaptorX (Källberg et al., 2012). This prediction used default settings and was based on a weighted combination of physicochemical features of the amino acids sequence, 3D structural alignments, entropic modelling and domain prediction (Källberg et al., 2012). The predicted structure was then plotted from the PDB file using PyMOL (Delano, 2002).

Using the PDB structure from RaptorX, functional prediction was attempted using ProFunc (Laskowski et al., 2005), CombFunc (Wass et al., 2012) and PredictProtein (Rost et al., 2004) all with default settings.

### 6.3.3 DSRNA CROSS-TALK ANALYSIS - “EDICER”

In order to investigate the prevalence of “cross-talk” between host and endosymbiont a tool to analyse short sequence collisions between two sets of transcripts was created. “eDicer” is built around the Jellyfish *k*-mer counter (Marçais and Kingsford, 2011) and the *k*-mer Analysis Toolkit (KAT) (Clavijo et al., 2015). Using efficient *k*-mer hashing it allows the identification of shared *k*-mers between two sets of sequences. As Dcr<sub>1</sub> in *P. tetaurelia* generates 23nt fragments, by identifying the number of shared 23-mers between two datasets e.g. the set of host and endosymbiont transcripts we can identify the number of potential RNAi “collisions” or cross-talks between the two species.

For each comparison between a set of query transcripts and a reference set of transcripts (endosymbiont transcripts in this case) the following values were calculated and tabulated:

- Number of *k*-mers in the query, in other words the length of the query in *k*-mers:

$$\frac{\sum_{n=1}^s \text{len}(x_n)}{w}$$

for a set *x* containing *s* transcripts and using a *k*-mer size of *w*.

- Number of unique  $k$ -mers in the query, the non-redundant length of the query.
- Number of shared  $k$ -mers (“collisions”) between query and subject bin.
- Number of unique shared  $k$ -mers between query and subject bin.
- Shared  $k$ -mers normalised by subject length in  $k$ -mers.
- Shared  $k$ -mers normalised by the subject length in unique  $k$ -mers.
- Shared unique  $k$ -mers normalised by subject length in  $k$ -mers.
- Shared unique  $k$ -mers normalised by the subject length in unique  $k$ -mers.

In order to contextualise the number of collisions between the 2 host and 2 endosymbiont transcript sets I analysed the collisions between the two sets of endosymbiont transcripts with several other datasets.

These datasets were composed of predicted or sequenced transcriptomes from the following groups: bacteria, archaea, eukaryotes, green algae, and the ciliates. Sequences were selected to sample the breadth of the sequenced diversity of each group as fully as possible.

Specifically, 3 ciliate transcript sets were used *Paramecium tetraurelia*, *Tetrahymena thermophila* and *Oxytricha trifallax* along with 5 green algae *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea* C-169, *Chlorella variabilis* NC64A, *Micromonas pusilla* RCC299, and *Ostreococcus lucimarinus*. The eukaryote dataset was composed of 58 transcript sets (section A.3.1), the bacterial 130 (section A.3.2), and the archaea 89 (section A.3.3).

Tabulated values were then analysed statistically using standard python tools outlined in the methods chapter.

In the course of this work improvements made to KAT were submitted and merged back into the core KAT development codebase (<https://github.com/TGAC/KAT>).

## 6.4 RESULTS

### 6.4.1 INDUCTION OF RNAi

There was a total failure to induce RNAi related phenotypes in feeding experiments for any of the vectors. Despite observation for several days, continuous refeeding with transformed *E. coli* (and

RT-PCR proof of dsRNA expression) none of the *P. bursaria* CCAP 1660/12 cultures displayed any altered phenotypes as a consequence of feeding experiments.

Microinjection was attempted using just the PGM and epiz constructs due to time constraints. *P. bursaria* tended to burst after a single injection attempt. As a control, an attempt was also made to inject GFP into the MAC. Unfortunately, despite numerous attempts it was never possible to observe fluorescence localised to the MAC. This suggests microinjection was never successfully achieved.

## 6.4.2 RNAI PATHWAY COMPONENTS

### 6.4.2.1 DCR1

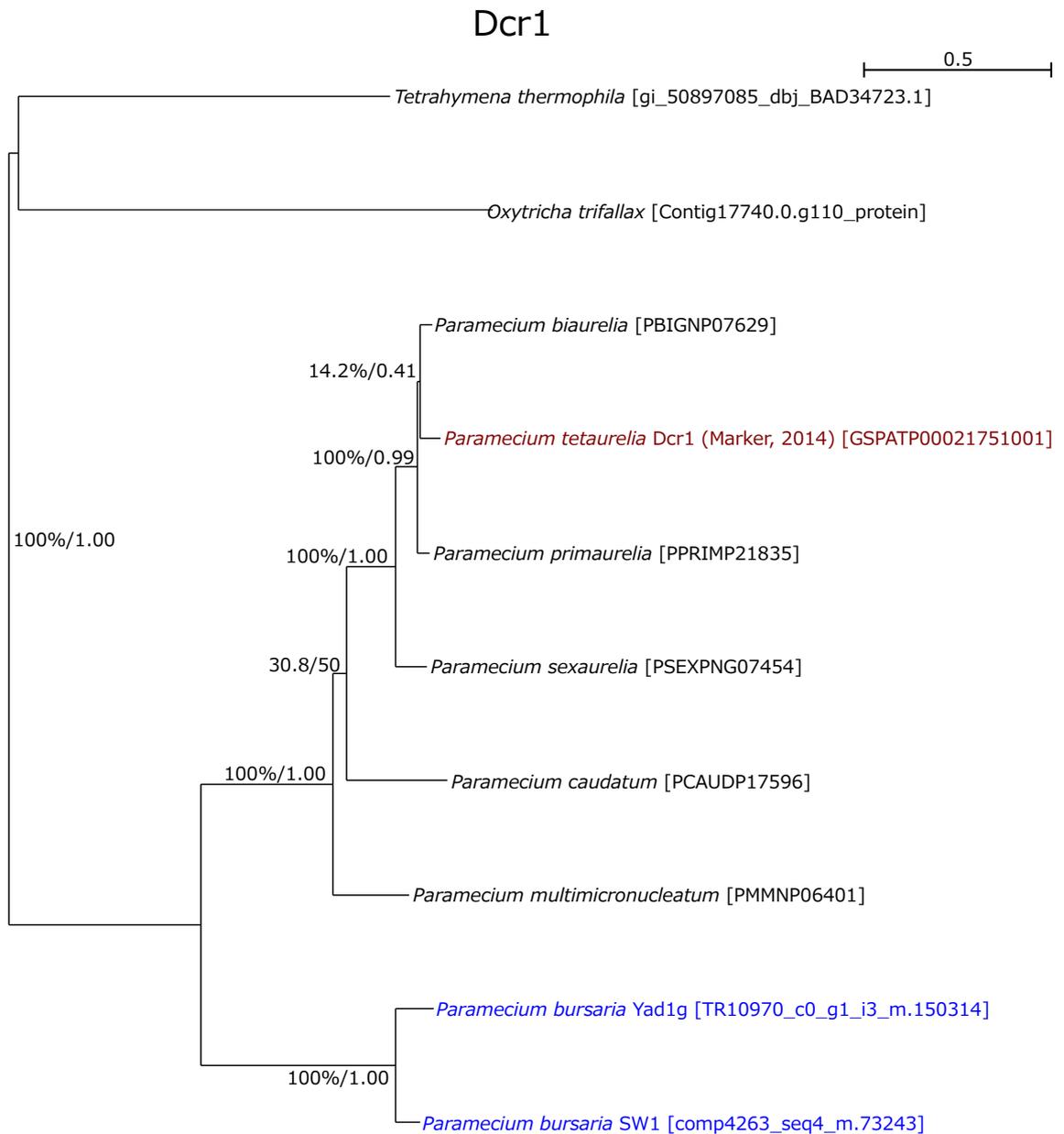
A single Dcr1 orthologue was clearly identified in each of the two *P. bursaria* host transcriptomes. Phylogenetic analysis (fig. 6.4.1) recovered a phylogeny matching the established ciliate taxonomy (Aury et al., 2006; Fokin et al., 2004; Swart et al., 2013) with strong support for *P. bursaria* as the the outgroup to the rest of the *Paramecium* clade.

### 6.4.2.2 PDS1

There were no hits for Pds1 in any of the 3 *P. bursaria* datasets (both transcriptomes and the genome). There were homologues in each of the other *Paramecium* species i.e. *P. sexaurelia*, *P. biaurelia*, *P. primaurelia*, *P. multimicronucleatum* and *P. caudatum* but not *T. thermophila* or *O. trifallax* (fig. 6.4.2).

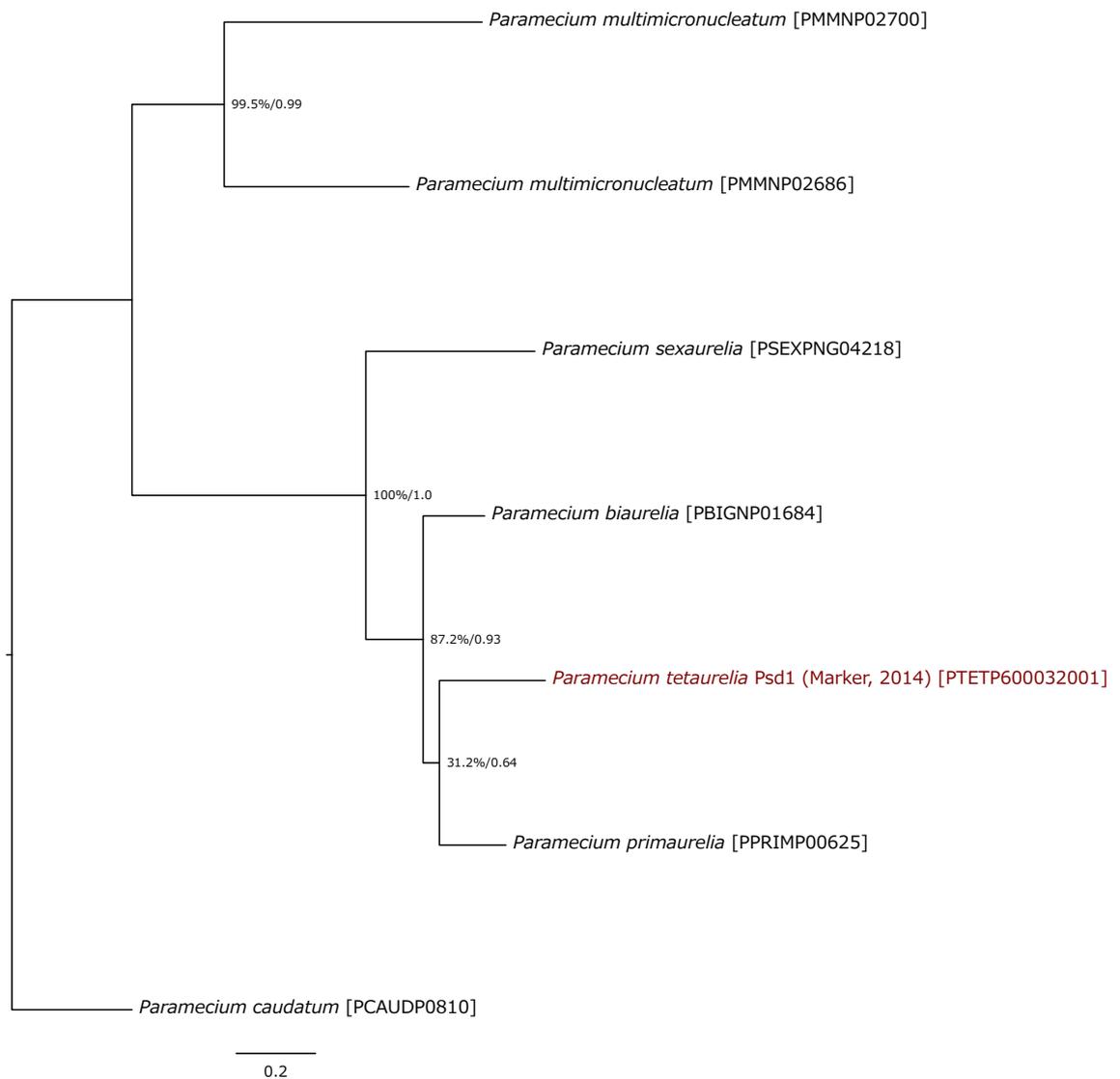
As this protein has no assigned function based on sequence homology (Marker et al., 2014; Carradec et al., 2015) but potentially plays an important role in the uptake of RNA from vacuoles at attempt was made to infer function by structural analysis.

The structure of Pds1 (fig. 6.4.3) was predicted from the *P. tetraurelia* sequence via RaptorX (Källberg et al., 2012). Unfortunately, no function could be assigned to this structure using ProFunc (Laskowski et al., 2005), CombFunc (Wass et al., 2012) or PredictProtein (Rost et al., 2004). No structural hits were found against known enzyme active sites, ligand-binding sites or DNA-binding templates in ProcFun. Therefore, no function could be assigned to this protein.

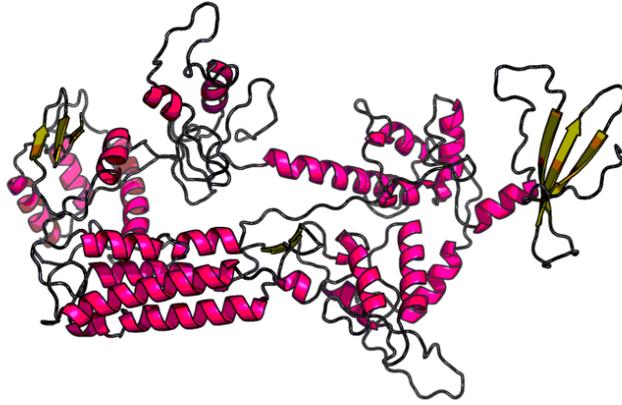


**Figure 6.4.1:** Dcr1 Phylogeny (1196 sites) inferred using RAxML with LG+G+F and 1000 non-rapid bootstraps. Bayesian PP were inferred using MrBayes with 2 runs of 4 chains run for 2,000,000 generations (5% burn-in) and the LG+G model. *P. bursaria* peptides are highlighted in blue whereas *P. tetaurelia* components identified by (Marker et al., 2014) are indicated in red. Phylogeny is largely consistent with established ciliate phylogenies.

# Pds1



**Figure 6.4.2:** Pds1 Phylogeny (424 sites) inferred using RAxML with VT+G+F and 1000 non-rapid bootstraps. Bayesian PP were inferred with MrBayes (2 runs of 4 chains for 2,000,000 generations, 5% burn-in) with VT+G and annotated. This phylogeny is consistent with the general taxonomy of the ciliate clade.



**Figure 6.4.3:** RaptorX Predicted Structure of *P. tetraurelia* Pds1 protein (PTETP600032001). No functional annotations could be made using this structure.

#### 6.4.2.3 CID

Two Cid orthologues were identified in each *P. bursaria* transcriptome. Additionally, a closely related Cid3 orthologue was identified in the other *Paramecium* sequences.

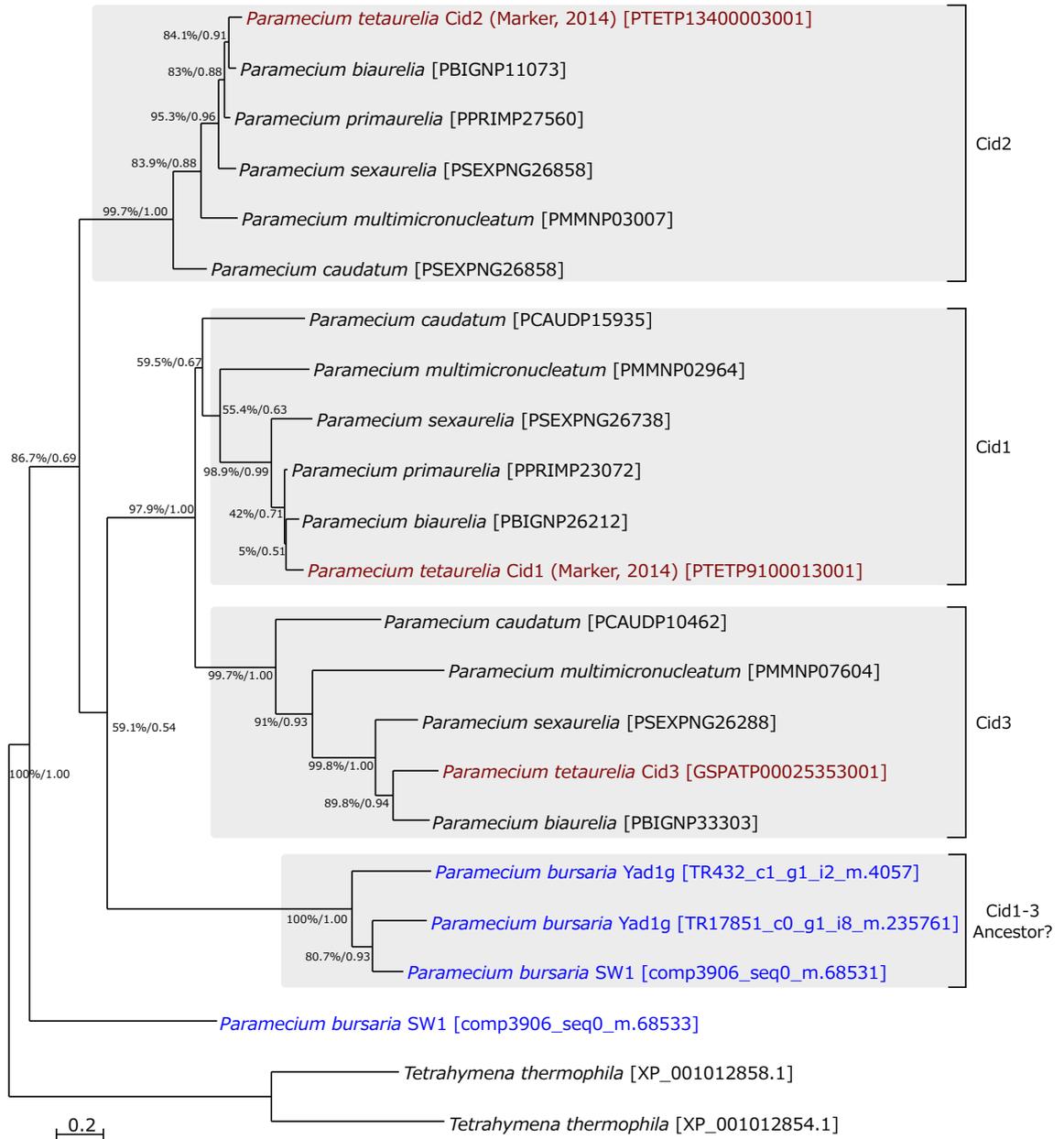
Phylogenetic analysis (fig. 6.4.4) showed an unclear picture with both the Yad1g Cid peptides and 1 of the SW1 peptides branching with moderate support (86.7% bootstraps and a PP of 0.69) within a clade composed of Cid1 and Cid3. Unfortunately, there was poor support (59.1%/0.54) for the *P. bursaria* sequences forming a sister to these clades, therefore their exact placement is unclear. Additionally, the other SW1 orthologue branched as the outgroup to all remaining Cid with high support (100%/1.00).

This suggests that the orthologues present in Yad1g and one of the orthologues in SW1 may be the unduplicated ancestor to Cid1 and Cid3 (named Cid1-3 for convenience).

In general this phylogeny is consistent with a scenario in which a single ancestral Cid has undergone duplication resulting in Cid2 and a Cid1-3 ancestor either before or after the branching of *P. bursaria*. If this divergence occurred before this speciation then Cid2 has been lost in *P. bursaria*. Regardless, there is a clear subsequent duplication and divergence of the Cid1-3 ancestor into the modern Cid1 and Cid3 between the *P. bursaria* and *P. caudatum* branches.

Interestingly, the timing of these events and the presence of all 3 Cid homologues in *P. caudatum* (which shares the single ancient WGD with *P. bursaria*) suggests that this pattern is not directly related to the polarised position of WGD in *Paramecium*.

## Cid



**Figure 6.4.4:** Phylogeny of Cid1, Cid2, and Cid3 (268 sites) inferred using RAxML with rtREV+G and 1000 non-rapid bootstraps. Bayesian PP were inferred using MrBayes (2,500,000 generations with 2 runs of 4 chains and a 10% burn-in). Phylogeny shows a potential orthologue of an ancestral pre-divergence version of Cid1 and Cid3 (named Cid1-3) in *P. bursaria* Yad1g and SW1 and an uncertain Cid orthologue possibly related to Cid2 (depending on timing of the Cid1-3 and Cid2 divergence in *P. bursaria* SW1).

#### 6.4.2.4 RDR

Two putative sequences were identified by Rdr1 and Rdr2 searches in each *P. bursaria*. Phylogenies of these sequences (fig. 6.4.5) indicate that *P. bursaria* has an orthologue of Rdr2 (strong support (99.9%/1.00) of an outgroup to the Rdr2 sequences). The other sequences from both transcriptomes branch basally to the other *Paramecium* RdRPs with moderate/weak support and may nor may not be an orthologue of Rdr1.

The Rdr3 analysis didn't identify any hits in the *T. thermophila* or *O. trifallax* outgroups but did find an orthologue in both *P. bursaria* transcript sets. These branched as a sister to the other *Paramecium* with strong support suggesting that they may be orthologous to the *P. tetaurelia* Rdr3. The phylogeny recapitulated the taxonomy of the ciliates (fig. 6.4.6). However, the lack of homology to the other RdRs indicates that this Rdr may have been an independent innovation arising basally to the *Paramecium* clade and is unrelated to Rdr1 and Rdr2.

#### 6.4.2.5 PIWI

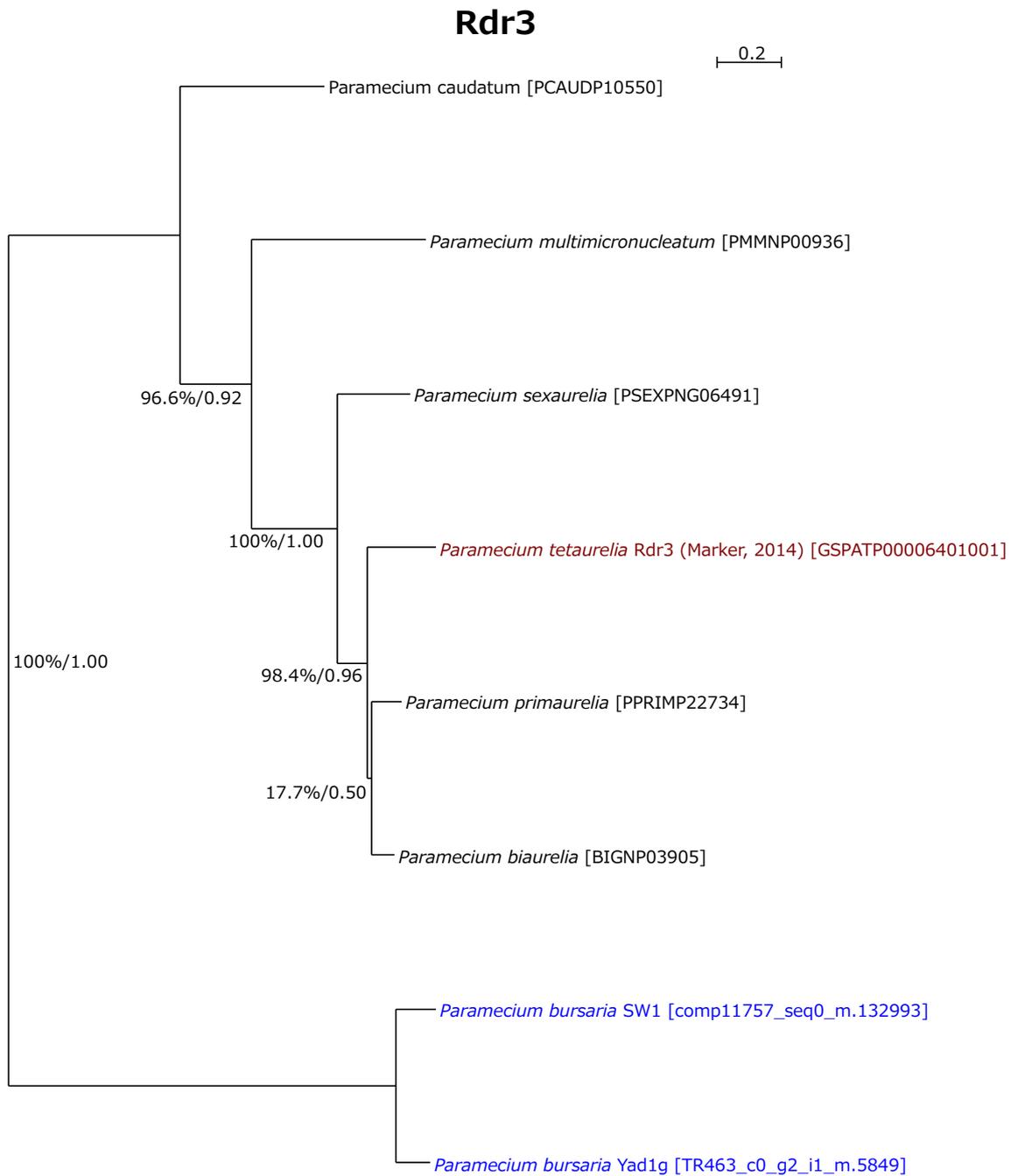
There were a large number of Piwi detected homologues across the datasets. Specifically, 16 Piwi in the *P. bursaria* SW-1 transcriptome and 5 in the partial genome, and 17 in the *P. bursaria* Yad1g transcriptome. Due to the large size of this family, large number of paralogues and relatively short sequences, phylogenetic inference of these sequences proved largely intractable. There are Piwi homologues present in *P. bursaria* although their exact relation and function is unknown.

#### 6.4.3 DSRNA COLLISIONS

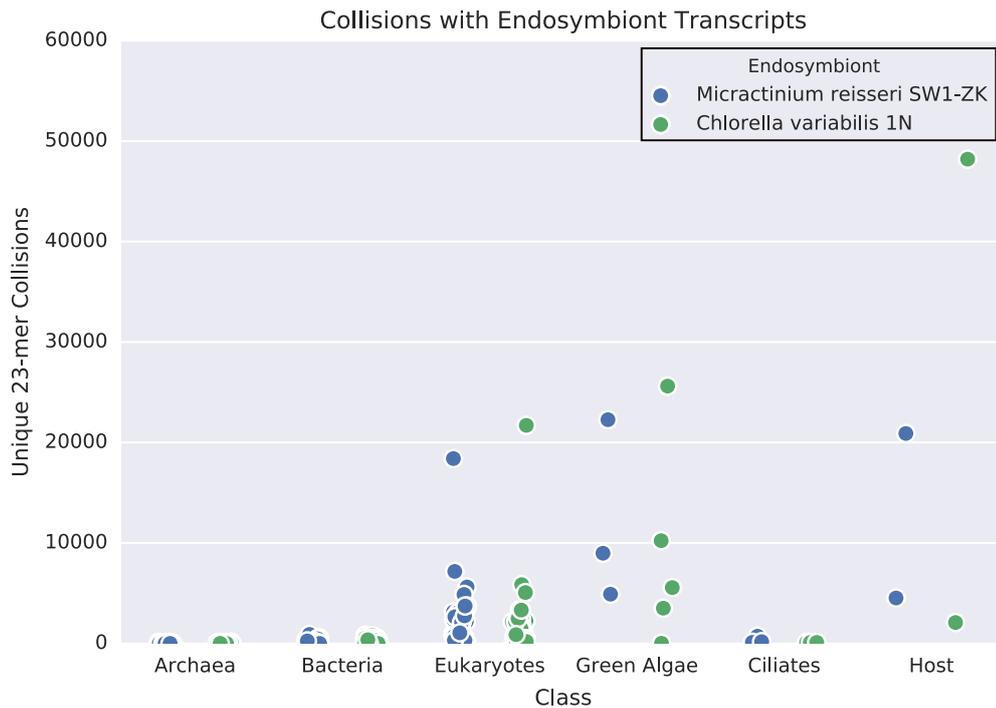
The total number of unique collisions between the endosymbionts and the various classes of subject transcriptomes were plotted (fig. 6.4.7). This showed next to no collisions with Archaea, moderate levels of total collisions with Bacteria and generally higher levels of collision with the Eukaryotes. The elevated number of collisions with eukaryotic transcriptomes is not surprising given their phylogenetic position and gene content. However, the difference in total collisions between the Archaea and Bacteria is potentially interesting. It is possible this reflects the sequencing bias in Archaea towards extremophiles which often have compositional adaptations.

Additionally, as might be expected from their close relationship with the green algal endosym-





**Figure 6.4.6:** Phylogeny of Rdr3 (432 sites) inferred using RAxML with JTT+G+F and 1000 non-rapid bootstraps. Bayesian PP were inferred using MrBayes (2,000,000 generations with 2 runs of 4 chains and a 5% burn-in) and annotated onto the RAxML phylogeny. Phylogeny shows the presence of a likely Rdr3 in *P. bursaria*.

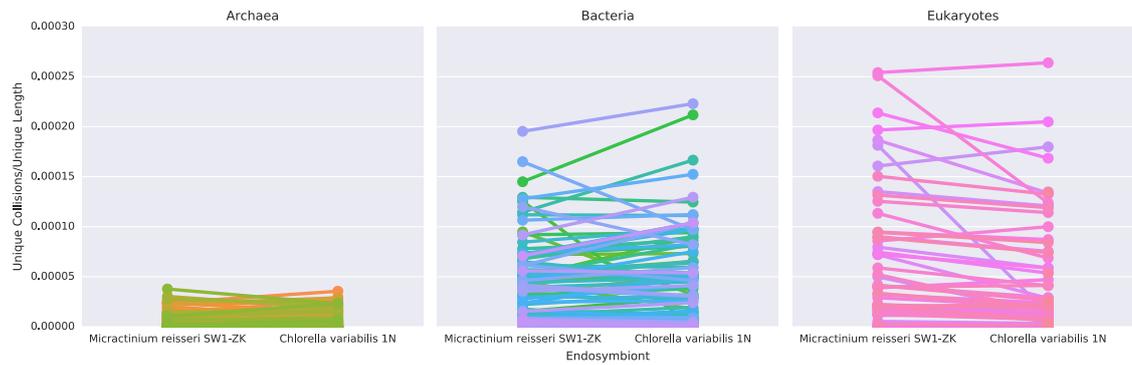


**Figure 6.4.7:** The number of unique 23-mer collisions between transcripts from the *C. variabilis* 1N and *M. reisseri* SW1-ZK endosymbionts and different classes of subject transcripts. The y-axis is truncated to better separate the classes however, the the only taxa that had more than 6000 unique collisions were *Coccomyxa variabilis* NC64A and *Chlorella subellipsoidea* C-169 , *Arabidopsis thaliana*, *Chlamydomonas reinhardtii* and transcripts from the two host bins *P. bursaria* SW1 and *P. bursaria* Yad1g. This suggests RNAi-cross talk could be occurring between the host and endosymbiont and would likely occur at higher rates than occurs between *Paramecium* and their bacterial prey.

bionts some of the most frequent collisions were with the other green algae. Interestingly, the three ciliate species displayed a generally low number of collisions but there was moderate to high levels of collision against the two *P. bursaria* host transcriptomes. This potentially indicates a higher level of collision between the active host genome rather than its total genome.

The collision level was relatively consistent for both endosymbionts when compared to the same subject (fig. 6.4.8). However, there are visible exceptions where a given subject transcriptome has far more collisions against one of two endosymbiont transcripts than the other. This can be seen in the line in the pair-plots with sharp angles instead of being close to level.

This is particularly obvious in the comparison of collisions between the host endosymbiont pairs (fig. 6.4.9). There is a considerable difference in collisions for the *C. variabilis* 1N endosymbiont from the Yad1g1N culture with next to no collisions against the *P. bursaria* from the other

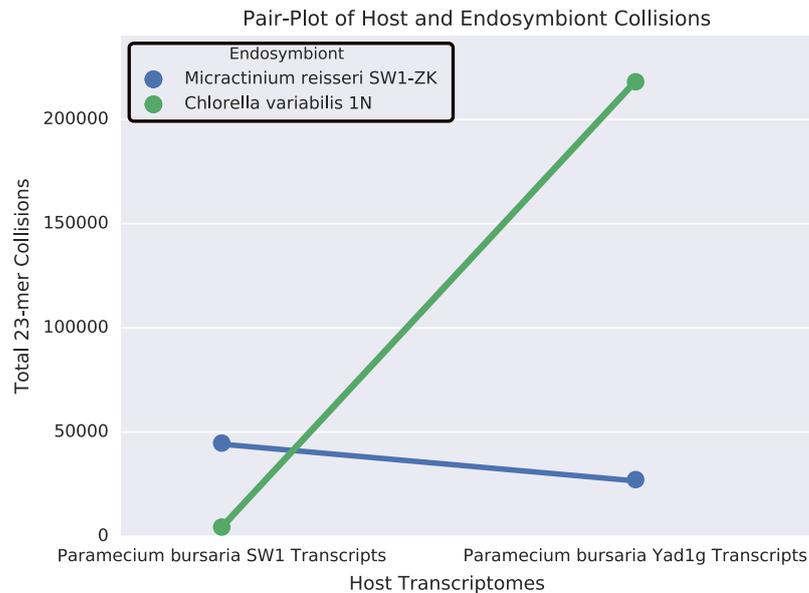


**Figure 6.4.8:** Pair-plot of the normalised collisions in Archaea, Bacteria and Eukaryotes showing the relative consistency of the number of hits between the two endosymbionts. Lines join the number of collisions to the same subject transcriptome in the different endosymbiont transcriptomes. Colour is merely for illustration purposes and has no significant meaning. The plot shows that overall the collisions are relatively consistent but there are aberrations where one endosymbiont has far more collisions to a given subject than the other.

culture but a high level of collisions against its own host. Interestingly, *M. reisseri* was relatively consistent across both hosts with only slightly more hits to its own host. This suggests a potential problem in the binning of endosymbiont and host transcripts, especially in the *P. bursaria*-*C. variabilis* Yad1g1N transcriptome.

In order to test to what degree the number of collisions was related to the length of the subject query, a simple linear regression was conducted (fig. 6.4.10). This demonstrates there is at least a partial linear dependence between the length of the query and the number of collisions as would be expected.

Normalising the unique number of collisions by the unique length of the subject predicted transcriptome led to some interesting results (fig. 6.4.11). Specifically, collisions with eukaryotic taxa were largely reduced to being on-par with collisions against Bacteria. While collisions against the ciliates relatively disappearing, there was still considerable levels of potential RNAi cross-talk against the host transcriptomes and green algae.



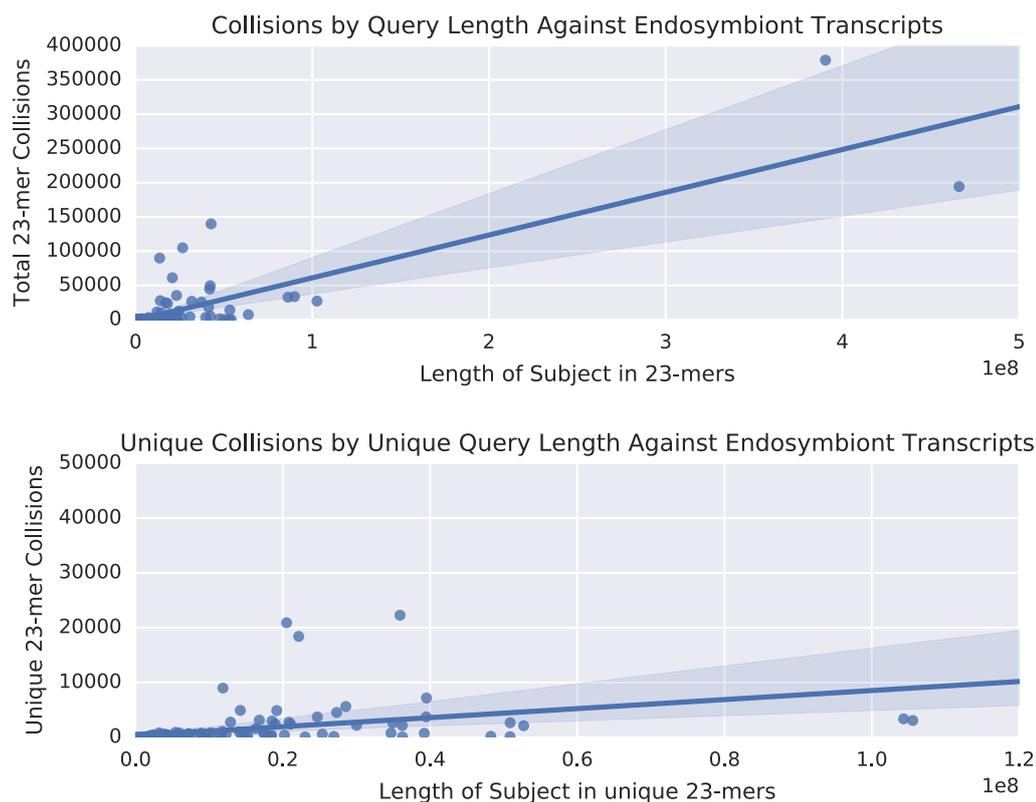
**Figure 6.4.9:** Pair-plot of the total number of collisions between the two endosymbiont transcriptome sets and the two host transcriptome sets. *M. reisseri* has a relatively consistent number of collisions against both hosts, however, the *C. variabilis* transcriptome has a significantly higher number of collisions against its own endosymbiont. This suggests potential issues with the binning of transcripts in the Yad1g1N transcriptome.

## 6.5 DISCUSSION

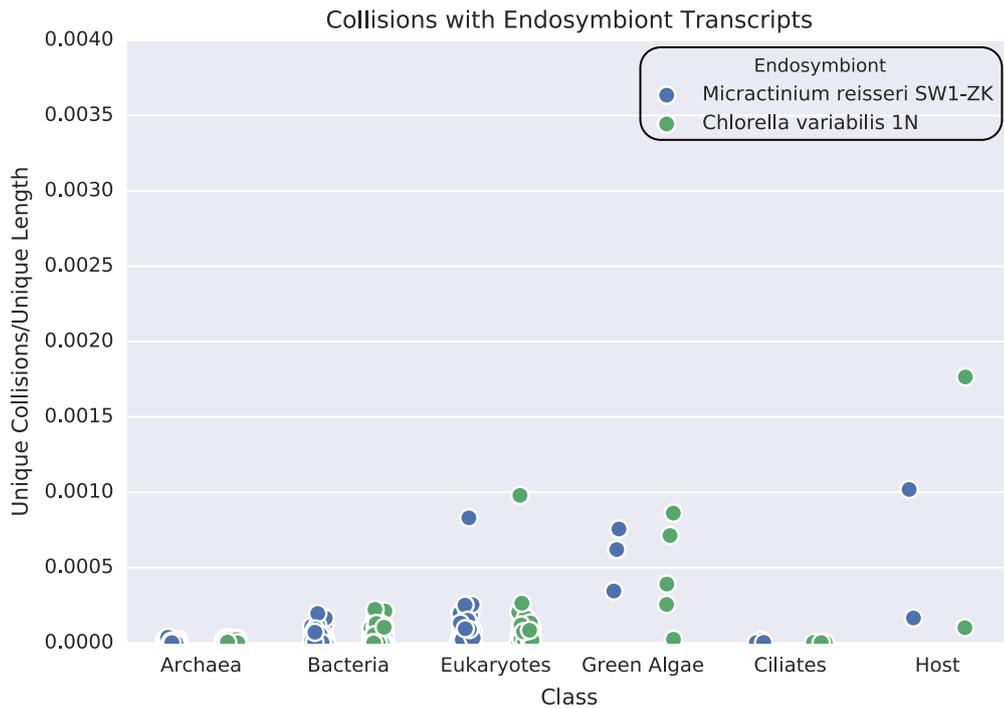
### 6.5.1 NO dsRNA RNAi INDUCIBLE PHENOTYPES IN *P. BURSARIA* SW1

Despite numerous attempts, all feeding experiments failed to induce any of the RNAi knockout phenotypes. RT-PCR tests (not shown) with the Bug22 and BBS7 constructs demonstrated that the *E. coli* was successfully transformed and could inducibly express the dsRNA. This indicates that dsRNA is either incapable of escaping the digestive vacuole or that *P. bursaria* does not have an active pathway for exogenous dsRNA induced RNAi.

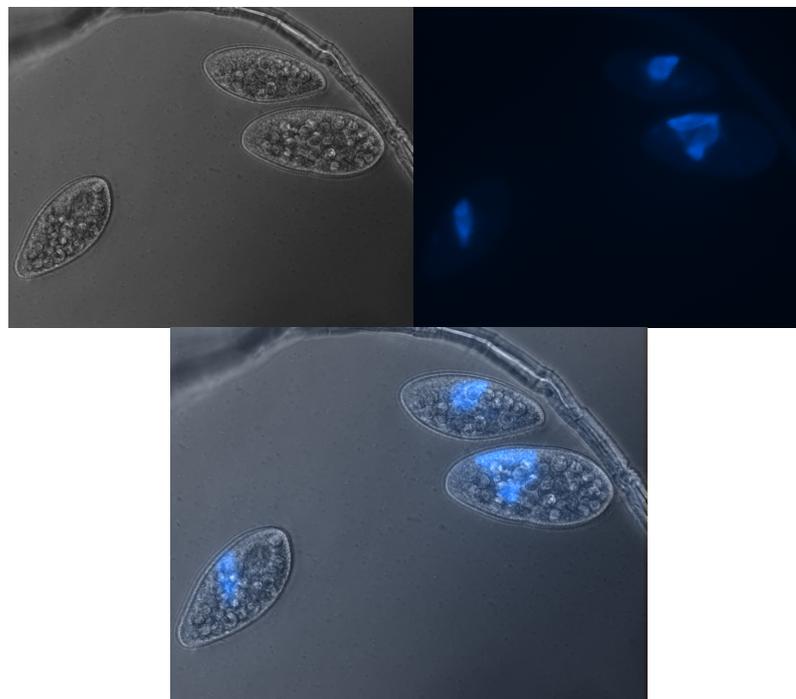
The potential failure of microinjection of dsRNA directly into the MAC to elicit RNAi phenotypes would support the absence of an active dsRNA induced RNAi pathway. However, the high methodological difficulty involved in identifying and injecting the MAC without lysing the cell (fig. 6.5.1) means microinjection may have only failed to induce RNAi due to failure to correctly microinject *P. bursaria*. *P. bursaria* was particularly prone to lysis relative to *P. tetraurelia* and there were greater issues with trichocysts blocking the microinjector. Attempts made to inject GFP into the MAC also failed to generate fluorescence localised to the MAC. This indicates that the failure of microinjection is primarily technical and cannot be used to make inferences about the state of the RNAi pathway in host.



**Figure 6.4.10:** Linear Regressions of the relationship between the number of collisions as query length increases. Features points from collisions with both *C. variabilis* 1N and *M. reissieri* SW1-ZK. Dark blue cloud indicates 95% confidence intervals. The top plot shows total collisions against total length whereas the bottom analysis only looks at unique collisions against unique length so theoretically ameliorates the effect of repetitive transcripts/many isoforms. Both show a clear if noisy (correlation coefficients of 0.18085 and 0.02811 respectively) linear relationship and thus support that the size of a transcriptome plays a role in the number of collisions.



**Figure 6.4.11:** The number of collisions between transcripts from the *C. variabilis* 1N and *M. reisseri* SW1-ZK endosymbionts and different classes of subject transcripts normalised by the unique length of those subjects.



**Figure 6.5.1:** DAPI stained *P. bursaria* MAC to demonstrating how difficult it is to accurately identify the location of the MAC for microinjection. Top left panel shows 3 *P. bursaria* cells under light microscopy as they appear when attempting microinjection. Top right shows fluorescence from DAPI staining to demonstrate where the MAC nuclei are location. Bottom panel is an artificial overlay of the light microscopy and DAPI staining.

Unfortunately, the alternative transgene RNAi methodology (Galvani and Sperling, 2001) (which was not attempted) also involves microinjection of the MAC with the transgene construct itself. Therefore, even if the transgene pathway is present and active it may still not be possible to reliably induce RNAi in *P. bursaria*.

#### 6.5.2 MISSING COMPONENTS OF RNAI PATHWAY IN *P. BURSARIA*

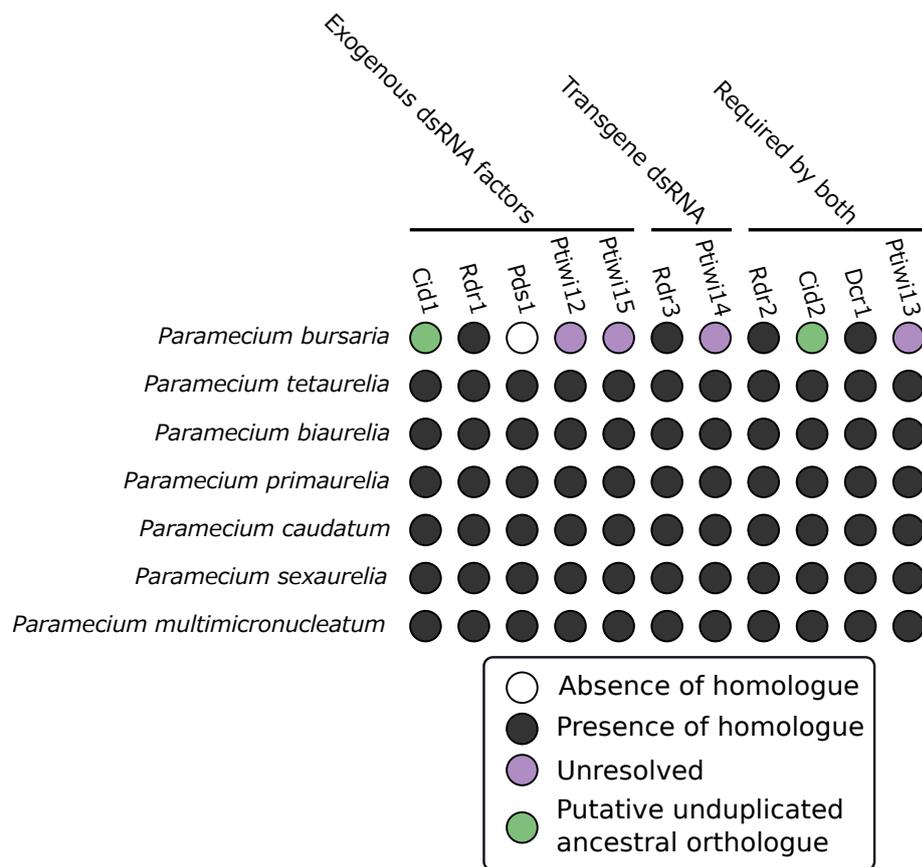
An investigation into the RNAi pathway components identified by (Marker et al., 2014) revealed the absence of one required component for the exogenous dsRNA pathway (Pds1) and, depending on the function of a putative ancestral Cid protein, the absence of a factor in the common pathway or just exogenous dsRNA pathway (fig. 6.5.2).

Pds1 is totally absent outside of the post-*P. bursaria* *Paramecium* clade. A phylogenetic analysis of Pds1 sequences (fig. 6.4.2) recapitulated the established *Paramecium* taxonomy (fig. 1.2.3). This suggests that Pds1 was either acquired after the divergence of *P. bursaria* and *P. caudatum* or was lost in *P. bursaria*. The pattern of paralogues would more likely support the former scenario. The lack of paralogues (with the exception of a terminally duplicated *P. multimicronucleatum* copy) in the *P. aurelia* complex species is interesting. As the presence of Pds1 in *P. caudatum* indicates that this should have undergone duplication during the two subsequent WGD events. Potentially, this represents serial losses in these species.

It is possible that Pds1 is present and just hasn't been recovered in the partial transcriptomes because it is not being transcribed (or is transcribed at a very low level) during endosymbiosis. It is also possible it is missing in the partial genome due to the incompleteness of this data. However, the combination of being missing in all 3 datasets as well as any non-*Paramecium* ciliates indicates that it is likely not present in *P. bursaria*.

The Cid proteins are difficult to resolve, either *P. bursaria* has an undiverged ancestral version of the Cid proteins or has a Cid1-3 ancestor and has secondarily lost Cid2. This depends on the timing of the Cid2 and Cid1-3 divergence, if it occurred after the branching of *P. bursaria* from the rest of the clade then the latter scenario is more likely and vice versa.

If the latter scenario is true then Cid2 has been lost and if the *P. bursaria* RNAi pathways require the same components as *P. tetraurelia* this may mean both the transgene and dsRNA pathways might not be active. Additionally, if the Cid1-3 ancestor or the ancestral undiverged Cid



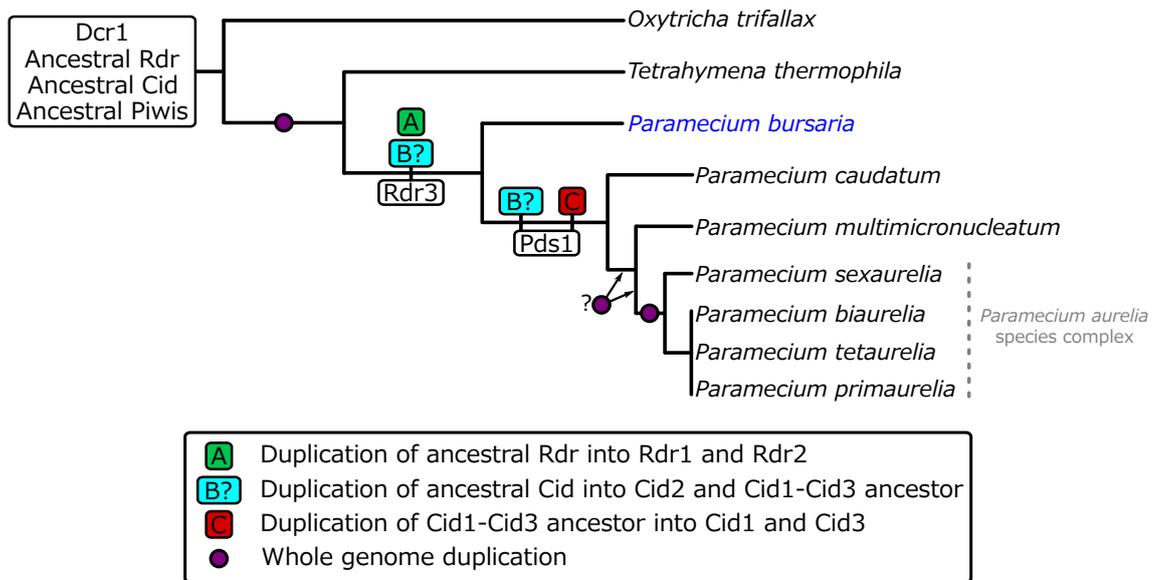
**Figure 6.5.2:** Coulson plot showing the absence/presence of RNAi pathway factors identified in *P. tetaurelia* (Marker et al., 2014) across the *Paramecium* clade.

does not have the same function as *P. tetaurelia*'s Cid1 then the exogenous dsRNA pathway may not be active in *P. bursaria*.

The unresolved Piwis also represent potential issues with the RNAi pathway but the only way to thoroughly investigate the roles of these proteins would be targeted mutagenic screening or a similar approach.

The low levels of sequence homology suggests independent innovation of Rdr3 and potentially sheds doubt on its relationship to the ancestral Rdr that this analysis confirms was likely present in was present in the first WGD (Marker et al., 2014).

Interestingly, the general disposition of paralogues across the *Paramecium* clade does not recapitulate the three established WGD events in this group. For example, there is a relatively consistent number of paralogues of all components, especially RdRPs and Cid proteins in *P. caudatum* despite this clade having undergone the same number of WGD as *P. bursaria* and not sharing the two recent WGD with the majority of the remaining *Paramecium*



**Figure 6.5.3:** Diagram showing the *Paramaecium* clade with *Tetrahymena* outgroup showing the putative evolutionary scenarios behind the currently observed distributed of RNAi factors.

### 6.5.3 ENDOSYMBIONT “COLLISION” HYPOTHESIS

Hypothetically, *Paramaecium bursaria* may have deactivated/lost feeding induced RNAi (specifically the uptake of RNA from vacuoles) as a consequence of the greater levels of potentially deleterious cross-talk between it and its eukaryotic green algal endosymbionts. As an exogenous RNAi response is not essential for viability in *P. tetaurelia* (Marker et al., 2014) loss of this system in *P. bursaria* may not have a high fitness cost.

On first glance, the high levels of collisions between endosymbiont transcripts against host and eukaryote classes in general support this hypothesis (fig. 6.4.7). However, there were low levels of collisions between endosymbiont transcripts and ciliate transcripts indicating that the collision levels observed between host and endosymbiont require an additional explanation. The first scenario is that the active host transcriptome during endosymbiosis is not representative of all possible host transcripts and features a much higher level of collision with the endosymbiont. The second scenario is that the levels of collisions between host and endosymbiont actually reflects misbinning of endosymbiont transcripts as belonging to the host. A comparison of the collisions of each endosymbiont against its own host and the other host *P. bursaria* (fig. 6.4.9) suggests that binning error may explain some of this difference and that the binning the Yad1g1N transcriptome has potentially more issues than the SW1-ZK (CCAP 1660/12) transcriptome. This might be explained by the fact that only half the sequenced libraries in Yad1g1N actually contained the

*C. variabilis* 1N endosymbiont as this dataset originated from an analysis of transcriptomic profiling of *P. bursaria* with and without its endosymbiont (Kodama et al., 2014).

There is a linear relationship between the unique size of a transcriptome in terms of 23-mers and the number of collisions (fig. 6.4.10). This is expected as a longer set has a higher probability of a random match by chance. When the number of collisions is normalised by the length of the subject (fig. 6.4.11) the level of collisions from eukaryotes is largely on-par with those of the Bacteria. However, as eukaryotes have larger genomes the un-normalised number of collisions is more reflective of biological reality. Indeed, the size and diversity of their transcriptome may be the reason why eukaryotic cross-talk is potentially more problematic than that of bacterial cross-talk.

It might be interesting to test the linear relationship between phylogenetic relatedness and the number of *k*-mer collisions. For example, phylogenetic distances between the endosymbiont and various taxa could be derived from an established published multi-gene analysis that includes these species and regression conducted using this as a feature.

One down-side of the efficient *k*-mer hashing based “eDicer” design is that it only finds exact matches. RNAi has been found to not always require exact sequence matches to induce knock-down (Elbashir et al., 2001) therefore, this analysis potentially misses a large amount of near-identical collisions. Fortunately, it is probably a relatively safe assumption that the number of identical collisions correlates strongly with the number of near-identical collisions.

Finally, while this may not have thoroughly resolved the question of matches “eDicer” may form a useful tool in the rapid screening of off-target effects in the RNAi analyses in different organisms. It is considerably more efficient than than cutting and alignment based methods such as that offered on ParameciumDB (Arnaiz and Sperling, 2011a).

## 6.6 CONCLUSIONS

RNAi induced phenotypes could not be created in *P. bursaria* SW<sub>1</sub> from CCAP1660/12 via feeding experiments. The absence of Pds1 in *P. bursaria* offers a potential explanation for this as this protein has been implicated in playing some undefined role in the uptake of RNA (dsRNA or ssRNA) from digestive vacuoles (Carradec et al., 2015). As “eDicer” identified that there are a large number of 23-mer collisions between *P. bursaria* transcripts and eukaryotic transcriptomes (espe-

cially the green algal endosymbionts) the loss/deactivation of the uptake of RNA from digestive (or potentially perialgal) vacuoles could be a consequence of having a eukaryotic endosymbiont in *P. bursaria*. The relatively lower levels of RNAi cross-talk between *Paramecium* and bacterial endosymbionts and/or food species may prove less deleterious than eukaryotic cross-talk.

Due to Pds1 only likely being involved in uptake from a food vacuole it is possible that dsRNA could still be induced by direct microinjection. Similarly, microinjection of transgenes may still be possible. Unfortunately, microinjection has proven difficult technically in *P. bursaria*. Further optimisation of the experimental method and training is required to thoroughly test the activation or deactivation of injected dsRNA or transgenes.

Alternatively, the potential ancestry of the Cid proteins in *P. bursaria* may indicate a deactivation/absence of RNAi by the pathways identified by (Marker et al., 2014) in *P. bursaria* depending on the relative functionality of this ancestral form. Further analysis of RNAi systems in *P. bursaria* and other *Paramecium* species would be required to thoroughly answer this question.

“a typical symbiotic *Chlorella* strain common to all *P. bursaria* strains does not exist”

- Reisser et al. (1988)

# 7

## Conclusions and Recommendations

There were 3 main objectives to this research:

- Assessing the utility of *P. bursaria* and its endosymbioses with green algae as a model organism for the study of the evolution of endosymbiosis.
- Generating “omic” resources to inform further analysis of this system.
- Investigating the utility and feasibility of current MDA-based single cell genomic and transcriptomic sequencing methods in the analysis of complex multi-member single-celled eukaryotic systems.

Review of literature established that *Paramecium bursaria* and its 4 algal endosymbionts: *Chlorella variabilis*, *Chlorella vulgaris*, *Coccomyxa* sp., and *Micractinium reisseri* make theoretically good model organisms for the study of secondary photosynthetic endosymbioses. This is due to them being believed to share:

- a well-developed background literature.
- facultative endosymbioses allowing elimination of the endosymbiont and re-introduction experiments.
- the potential of a functional and tractable RNAi system for hypothesis testing via gene transcription knock down.
- easily culturable and diverse available cultures.

However, with the exception of one transcriptomic analysis (Kodama et al., 2014) there have been no analyses of these systems using contemporary transcriptomic, metabolomic or genomic techniques.

Unfortunately, an investigation of elimination of the endosymbiont in the *P. bursaria* -*M. reisseri* culture revealed that this endosymbiosis may, in fact, represent an obligate system. Specifically, the *P. bursaria* SW<sub>1</sub> host may be an obligate host of an algal endosymbiont. Even though three separate elimination methods and a range of treatment concentrations were attempted (to minimise the risk that the host death was related to a susceptibility to a specific treatment) all methods resulted in the same eventual host death.

It is uncertain from these results whether the *M. reisseri* algal endosymbionts are similarly obligate on their host. This could be tested by assessing whether an axenic culture of *M. reisseri* can be established. One method of attempting this could be to exploit the robustness of the chitinous cell wall of the algae relative to the *Paramecium* membrane. Gentle agitation would allow the lysis of the endosymbiont without the lysis of a significant number of the endosymbionts. The difficulty in this would be optimising the culture conditions for *M. reisseri* as these endosymbiont algae are known to be relatively fastidious (Hoshina and Imamura, 2009).

If this further work does result in finding that *P. bursaria* and *M. reisseri* do form a mutually obligate system then the case that metabolic co-dependence has become fixed in these strains can be strongly argued. To test

As the Yad1g1N culture has previously been established as a facultative endosymbiosis further work should use the Yad1g1N and CCAP1660/12 transcriptomes created here to attempt to identify key differences between them. Identifying these differences may pinpoint the mechanism by which metabolic co-dependence becomes fixed in *P. bursaria* - green algal endosym-

bioses.

The ITS<sub>2</sub> sequence analysis of the CCAP 1660/12, and CCAP 1660/13 cultures revealed that these cultures likely contained the same *M. reisseri* endosymbiont and not a *Coccomyxa* endosymbiont as described in the culture collection. The identity of the Yad1g1N endosymbiont as *C. variabilis* 1N was also confirmed. These results emphasised the necessity of phylogenetic analyses to robustly identify the members of a system of study. This work analysing the endosymbionts should ideally but supplemented by an analysis of the host taxonomy. This could be achieved in the various *Paramecium bursaria* strains by a similar analysis targeting the single MIC copy of the rDNA.

This ITS<sub>2</sub> analysis also established that the photobionts in the CCAP 1660/12, CCAP 1660/13 and NBRP Yad1g1N cultures most likely form clonal photobiont cultures within their host and that none of the cultures show evidence of multiple species of photobiont. This suggests that clonal photobiont samples do potentially exist in nature as samples such as the CCAP 1660/12 and 1660/13 cultures were collected directly from the environment. As the Yad1g1N culture creation involved the isolation and purification of the 1N endosymbiont, clearing of the Yad1g host and then their subsequent reintroduction the observed ITS<sub>2</sub> sequence similarities suggests that the photobiont undergo little divergence and remain largely homogeneous within the host.

Unfortunately, the utility of the single cell metagenome to further test endosymbiont clonality was limited due to the high level of bacterial contamination. New tools and methods need to be developed to process and cluster MDA genomic contigs in the absence of reference genomes due to the unreliability of coverage as a feature. The existence of this type of method optimised for *de novo* assembled eukaryotic data would greatly aid the analysis of complex interacting eukaryotic systems using MDA based genomics. One approach could be to utilise blanket normalisation methods and base the variational inference off the normalised coverage and compositional features. However, by using a blanket coverage threshold instead of a relative one there is the potential to discard a significant portion of sequencing data.

In combination the results from the first results chapter shed doubt on some of the arguments supporting the utility of *Paramecium* in the study of endosymbiosis. Specifically, that it is not necessarily facultative and metabolic dependence, if not necessarily co-dependence, has become fixed in at least one species. Additionally, due to this observed diversity between the individual

endosymbiotic relationships combined with the taxonomic turmoil and previous mislabelling means that the utility of the reference literature is reduced. Any data from literature prior to the establishment of molecular taxonomy in these species needs to be carefully revisited and verified before it can be effectively used to contextualise any “omic” analyses.

In-depth analysis of the optimisation of pre-processing, filtering, assembly, and binning of single cell transcriptomics revealed that it was possible to generate and assemble single cell RNAseq datasets of complex eukaryotic systems. Previous work has shown the potential utility of *de novo* SCT in eukaryotic micro-organisms (Kolisko et al., 2014). However, this work represents the first analysis using SCT to investigate a non-axenic eukaryotic system. Particularly, this is also the first analysis of a pair of interacting eukaryotic partners using single cell methods in the absence of reference genomes. This analysis identified that GC% based pre-assembly read partitioning is ineffectual for these datasets, but taxonomic screening is highly necessary to minimise the levels of bacterial contamination. This data also emphasises the utility of phylogenetically informed transcript binning processes instead of relying exclusively on naive top BLAST hit approaches. This work also determined that current recommended practices in bulk RNAseq, such as digital normalisation and error correction, are still highly useful techniques in the analysis and assembly of SCT datasets. However, specific findings regarding optimal settings for these tools need to be re-evaluated for sc-RNAseq data.

Future work could consider the utility of phylogenetically aware kernels e.g. (Vert, 2002) in the classification of transcript bins. Pre-assembly read partitioning should also be revisited and the benefit of incorporating additional sequence feature such as composition and coverage data investigated in this form of pre-processing.

An analysis of the endosymbiont metabolism via expressed transporters and secreted proteins revealed novel aspects of amino acid usage by *M. reissieri* as well as the potential synthesis of complex saccharides such as raffinose and arabinose within the PV lumen. Metabolomic data supporting these hypotheses were also presented. While the untargeted metabolomic profiling does require further optimisation, particularly GC-QTOF analysis of carbohydrate metabolism, these approaches showed themselves as highly useful in supplementing transcriptomic data. Further targeted mass spectrometry is required to confirm the differential abundances of raffinose, arabinose. Additionally, the targeted amino acid analysis requires revisited due to a failure to fit

calibration curves to the peaks generated by the majority of the amino acids.

Comparison of the active metabolic network in *M. reisseri* during endosymbiosis to that of *C. variabilis* 1N and the total metabolic capacity of *C. variabilis* NC64A and *Coccomyxa subellipsoidea* also revealed unique traits. Specifically, *M. reisseri* does not express aspects of fatty acid degradation present in the other endosymbionts. Additionally, it has distinct amino acid degradation pathways that are congruent with the identified alternative amino acid usage in this species.

The discovery of novel metabolic traits further supports the potential utility for single cell transcriptomics and bulk metabolomic analysis for identifying the underlying molecular function of a given endosymbiotic relationship. Unfortunately, it also further underlines the diversity and variability displayed between different *P. bursaria* - green algal systems.

Finally, an analysis of RNAi in *P. bursaria* revealed a potentially inactive/absent dsRNA induced RNAi system in *P. bursaria* SW<sub>1</sub> (CCAP 1660/12). The common pattern of presence and absence of the previously identified components of the RNAi pathways in both *P. bursaria* transcriptomes suggests that this system is likely to be inactive or missing in *P. bursaria*. The most significantly missing factor is that of the *Pds1* gene that has been implicated in the uptake of RNA from the digestive vacuole. As this has been discovered to occur at low and natural levels in *P. tet aurelia* during normal feeding (Carradec et al., 2015) the potentially deleterious presence of eukaryotic algal endosymbionts may offer an explanation for the deactivation/absence of this system.

An “in-silico” study of the number of potential siRNA “collisions” between the active *Paramecium* transcriptomes and other eukaryotic transcriptomes (particularly those belonging to the endosymbionts) supported this hypothesis. Relatively more collisions occur between the host and eukaryotic transcriptomes than do versus bacterial ones. The greater number of collisions increases the chance of deleterious cross-talk taking place and thus likely increases the fitness cost of maintaining this system in the presence of eukaryotic endosymbiont. Additional work needs done to assess the exact nature of these collisions, particularly between host and endosymbiont.

The results in this thesis presented above (namely the discovery evidence of numerous specialised adaptations in each host-algal system and obligacy) raise an interesting question: why is there a lack of evidence evidence of tighter integration in this system? Specifically, the type of genomic integration displayed in other endosymbionts such as EGT.

Firstly, the protein import systems considered necessary for extensive EGT to start taking place are more complicated in the cases of secondary and tertiary endosymbioses than in basic plastids due to the increased number of membranes that may need to be traversed. This is especially true for import directly to the secondary plastid from the host (Hirakawa et al., 2012). Secondly, the unusual nuclear dimorphism of the host *P. bursaria* and alternative codon usage may prove a barrier to the vast majority of EGT activity.

For successful transfer to take place between host and endosymbiont it would be necessary for the gene to transfer not just from the endosymbiont to the transcriptionally active host MAC but to the germline MIC. Even then integration into the MIC would have to occur in such a way that it would be correctly spliced and duplicated during the conversion of the MIC back to the MAC. Compounding this with sexual reproduction further decreases the probability of effective integration. It is notable that the prototypical hosts of the endosymbiotically “promiscuous” green algae - *Chlorella*, *Coccomyxa* and *Micractinium* all display germline sequestration either through the aforementioned dimorphism in *P. bursaria* or via standard metazoan germlines in the case of *Hydra* (Kawaida et al., 2013) and the kleptoplastic sacoglossan sea slugs (Yellowlees et al., 2008).

Future work could attempt to use the genomic contigs generated here and/or further sequencing to pin-point examples of endosymbiont genes being present in host contigs and vice versa. Then, due to the rate of chimeric contigs in MDA, PCR and Sanger sequencing could be used to confirm any putative EGTs.

Another interesting angle of investigation of these systems is analysing what host and endosymbiont transcripts are not expressing during endosymbiosis. This was partially analysed by (Kodama et al., 2014) however, the partial genome and transcriptome could be used to further answer this question. Specifically, all genes present in the genome assembly could be identified and annotated using standard annotation pipelines and then the transcriptomes surveyed for their presence. Any gene present in the genome that is not observed in the transcriptomes of endosymbiosis could shed further light on the function and evolution of these systems.

Ultimately, this work has identified the diverse, complex and distinct set of traits that different *P. bursaria*-green algal endosymbioses display and demonstrated that, while still nascent, single cell methodologies can be amenable to the analysis of complex multi-member eukaryotic systems that lack prior genomic references. Unfortunately, both due to the diversity of the systems

discovered in the *P. bursaria*-algal endosymbioses and the inability to induce RNAi, the utility of the *Paramecium bursaria* as a general model for the evolution of co-dependence is less than initially believed. However, future work in these systems using the “omic” resources generated in this thesis as a base dataset could help us understand how such mechanistic endosymbiotic diversity is possible even in closely related host and endosymbionts species. Understanding the answer to this question would greatly improve our understanding of the evolution of endosymbiosis and ultimately the eukaryotic cell.

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# Appendices

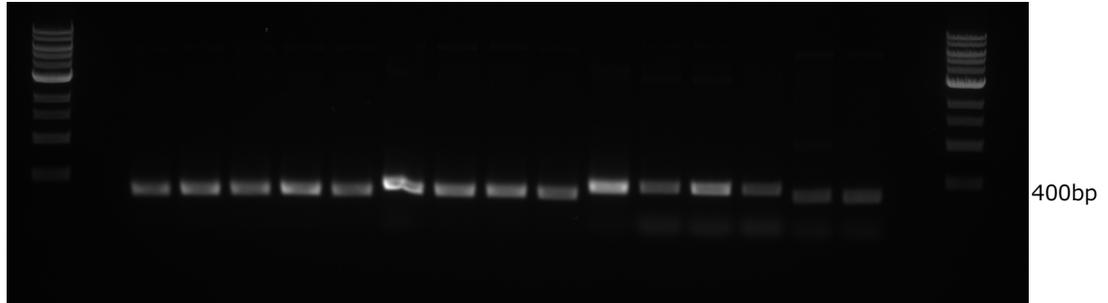
**A**

Supplementary Data

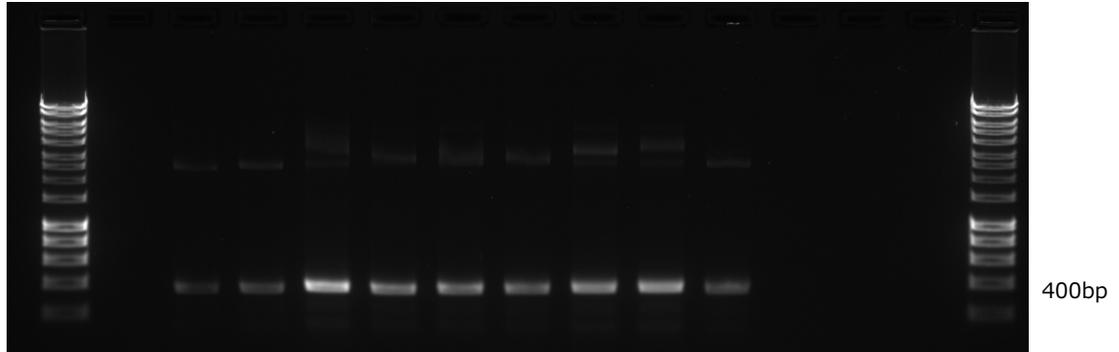
## A.1 ITS<sub>2</sub> SEQUENCING

### A.1.1 PCR GELS

CCAP 1660/13 Purified and Cultured PCR



CCAP 1660/12 A and B PCR



**Figure A.1.1:** Gel image of the PCR products from initial ITS<sub>2</sub> PCR in CCAP1660/12 initial reactions (1660/12 1-20) as well as CCAP1660/13 purified and culture reactions. As all PCR products were within expected size range of 400bp products were pooled for cloning. No gel pictures are available for other reactions.

### A.1.2 ITS<sub>2</sub> SEQUENCES

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CGGCTGAAGTGCAGAGGCTTGAGCATGGACCCCGTTTGCAGGGCAATGGCTTGGT  
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GCAGGAACCGGGGCGTCAAACCTCCGGCGTCTCACACTTTCGACCTGAGCTCAGG  
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>1660\_12\_B16

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CGGCTGAAGTGCAGAGGCTTGAGCATGGACCCCGTTTGCAGGGCAATGGCTTGGT  
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GCAGGAACCGGGGCGTCAAACCCCGGCGTCTCACACTTTCGACCTGAGCTCAGG  
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>1660\_12\_B17

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GTGAGTGC GGATCTGGCCCTCCCGGCTCCGACTGAACTCGTTCAGCCATCCGGGT  
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GCAGGAACCGGGGCGTCAAACCCCGGCGTCTCACACTTTCGACCTGAGCTCAGG  
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AGCCTCC

>1660\_12\_B18

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GCAGGAACCGGGGCGTCAAACCCCCGGCGTCTCACACTTTCGACCTGAGCTCAGG  
CAAGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAATAAACTAACT  
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AGCCTCC

>1660\_12\_B18rev

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>1660\_12\_B20

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GCAGGAACCGGGGCGTCAAACCCCCGGCGTCTCACACTTTCGACCTGAGCTCAGG  
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AGCCTCC

>1660\_13\_culture\_E3

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>1660\_13\_culture\_E4

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GCAGGAACCGGGGCGTCAAACCCCGGCGTCTCACACTTTCGACCTGAGCTCAGG  
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AGGATGCCCTTAGTAACGGCGAGCGAACC GG GCAAAGCCCAACTTGAAAATCCCC  
AGCCTCC

>1660\_13\_culture\_E5

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>1660\_13\_culture\_E6

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>1660\_13\_culture\_E7

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>1660\_13\_culture\_E8

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>1660\_13\_culture\_E9

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AGGATGCCCTTAGTAACGGCGAGCGAACC GG GCAAAGCCCAACTTGAAAATCTCC  
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>1660\_13\_culture\_E10

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GCAGGAACCGGGGCGTCAAACCCCGGCGTCTCACACTTTGACCTGAGCTCAGG  
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CGGCTGAAGTGCAGAGGCTTGAGCATGGACCCCGTTTGCAGGGCAATGGCTTGGT  
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>1660\_13\_purified\_K2

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>1660\_13\_purified\_K3

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>1660\_13\_purified\_K4

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>1660\_13\_purified\_K5

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>1660\_13\_purified\_K6

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>1660\_13\_purified\_K7

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>1660\_13\_purified\_K8

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>1660\_13\_purified\_K9

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>1660\_13\_purified\_K10

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>Yad1g1N\_8T3

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## A.2 ARBORETUM CLASSIFIER COMPARISON

### A.2.1 GENOMES USED

Genomes used in transcript binning pipeline. Genomes were chosen to be a representative of the sampled diversity of the eukaryotic tree of life as possible:

- *Arabidopsis thaliana*
- *Chlamydomonas reinhardtii*
- *Ostreococcus tauri*
- *Micromonas pusilla* CCMP1545
- *Chlorella variabilis* NC64A

- *Chlorella vulgaris* C-169
- *Physcomitrella patens*
- *Saccharomyces cerevisiae* S288C
- *Neurospora crassa* OR74A
- *Homo sapiens*
- *Mus musculus*
- *Dictyostelium discoideum*
- *Paramecium caudatum*
- *Paramecium tetraurelia*
- *Tetrahymena thermophila macronucleus*
- *Oxytricha trifallax*
- *Toxoplasma gondii*
- *Guillardia theta*
- *Bigeloviella natans*
- *Emiliana huxleyi* CCMP1516
- *Aureococcus anophagefferens*
- *Ectocarpus siliculosus*
- *Schizosaccharomyces pombe*
- *Bacillus cereus* ATCC 14579
- *Escherichia coli* str. K-12 substr. MG1655
- *Escherichia coli* O157 H7 str. Sakai
- *Salmonella enterica* subsp. *enterica* serovar *Typhi* str. CT18

- *Amycolatopsis mediterranei* U32
- *Aquifex aeolicus* VF5
- *Borrelia burgdorferi* B31
- *Chlamydomphila pneumoniae* CWL029
- *Chlorobium tepidum* TLS
- *Deinococcus radiodurans* R2
- *Caulobacter crescentus* CB15
- *Sulfolobus islandicus* M.14.25
- *Nanoarchaeum equitans* Kin4-M
- *Haloferax mediterranei* ATCC 33500
- *Methanococcus maripaludis* S2
- *Cenarchaeum symbiosum* A

#### A.2.2 CLASSIFICATION REPORTS

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.96	0.84	0.90	156
food	0.98	0.99	0.99	426
host	0.90	0.99	0.94	787
endosymbiont	0.97	0.99	0.98	359
avg / total	0.96	0.96	0.95	1728

**Table A.2.1:** KNeighborsClassifier(algorithm='auto', leaf\_size=30, metric='minkowski', metric\_params=None, n\_neighbors=50, p=2, weights='uniform')

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.94	0.85	0.90	156
food	0.98	1.00	0.99	426
host	0.92	0.96	0.94	787
endosymbiont	0.96	0.99	0.98	359
avg / total	0.95	0.95	0.95	1728

**Table A.2.2:** LinearSVC(C=1.0, class\_weight=None, dual=True, fit\_intercept=True, intercept\_scaling=1, loss='squared\_hinge', max\_iter=1000, multi\_class='ovr', penalty='l2', random\_state=None, tol=0.0001, verbose=0)

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.43	0.94	0.59	156
food	0.93	0.12	0.22	426
host	0.95	0.85	0.90	787
endosymbiont	0.99	0.97	0.98	359
avg / total	0.83	0.70	0.65	1728

**Table A.2.3:** SVC(C=1.0, cache\_size=200, class\_weight=None, coef0=0.0, degree=3, gamma=0.0, kernel='rbf', max\_iter=-1, probability=False, random\_state=None, shrinking=True, tol=0.001, verbose=False)

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.90	0.83	0.86	156
food	0.97	0.98	0.98	426
host	0.89	0.93	0.91	787
endosymbiont	0.98	0.99	0.99	359
avg / total	0.94	0.94	0.94	1728

**Table A.2.4:** DecisionTreeClassifier(class\_weight=None, criterion='gini', max\_depth=None, max\_features=None, max\_leaf\_nodes=None, min\_samples\_leaf=1, min\_samples\_split=2, min\_weight\_fraction\_leaf=0.0, random\_state=None, splitter='best')

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.84	0.83	0.83	156
food	0.97	0.98	0.97	426
host	0.87	0.93	0.88	787
endosymbiont	0.97	0.97	0.97	359
avg / total	0.92	0.92	0.92	1728

**Table A.2.5:** ExtraTreeClassifier(class\_weight=None, criterion='gini', max\_depth=None, max\_features='auto', max\_leaf\_nodes=None, min\_samples\_leaf=1, min\_samples\_split=2, min\_weight\_fraction\_leaf=0.0, random\_state=None, splitter='random')

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.89	0.91	0.90	156
food	0.98	1.00	0.99	426
host	0.95	0.92	0.94	787
endosymbiont	0.97	0.96	0.97	359
avg / total	0.95	0.95	0.95	1728

**Table A.2.6:** RandomForestClassifier(bootstrap=True, class\_weight=None, criterion='gini', max\_depth=None, max\_features='auto', max\_leaf\_nodes=None, min\_samples\_leaf=1, min\_samples\_split=2, min\_weight\_fraction\_leaf=0.0, n\_estimators=10, n\_jobs=1, oob\_score=False, random\_state=None, verbose=0, warm\_start=False)

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.90	0.83	0.86	156
food	0.98	1.00	0.99	426
host	0.88	0.93	0.90	787
endosymbiont	0.98	0.97	0.98	359
avg / total	0.94	0.94	0.94	1728

**Table A.2.7:** AdaBoostClassifier(algorithm='SAMME.R', base\_estimator=None, learning\_rate=1.0, n\_estimators=50, random\_state=None)

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.31	0.99	0.47	156
food	0.99	0.79	0.88	426
host	1.00	0.04	0.08	787
endosymbiont	0.00	0.00	0.00	359
avg / total	0.58	0.47	0.38	1728

**Table A.2.8:** LDA(n\_components=None, priors=None, shrinkage=None, solver='svd', store\_covariance=False, tol=0.0001)

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.60	0.03	0.06	156
food	0.72	1.00	0.84	426
host	0.57	0.90	0.70	787
endosymbiont	0.99	0.93	0.96	359
avg / total	0.73	0.73	0.65	1728

**Table A.2.9:** QDA(priors=None, reg\_param=0.0)

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.60	0.03	0.06	156
food	0.66	1.00	0.79	426
host	0.59	0.84	0.69	787
endosymbiont	0.99	0.93	0.96	359
avg / total	0.71	0.72	0.64	1728

**Table A.2.10:** GaussianNB()

	<b>precision</b>	<b>recall</b>	<b>f1-score</b>	<b>support</b>
unknown	0.98	0.66	0.79	156
food	0.97	1.00	0.98	426
host	0.78	1.00	0.88	787
endosymbiont	0.98	0.99	0.99	359
avg / total	0.93	0.92	0.91	1728

**Table A.2.11:** LogisticRegression(C=1.0, class\_weight=None, dual=False, fit\_intercept=True, intercept\_scaling=1, max\_iter=100, multi\_class='ovr', penalty='l2', random\_state=None, solver='liblinear', tol=0.0001, verbose=0)

## A.3 eDICER

### A.3.1 EUKARYOTE TRANSCRIPTOMES

eDicer Eukaryote Predicted Transcriptomes used:

- *Acanthamoeba castellanii* str. Neff
- *Aplanochytrium kerguelense* PBS07
- *Arabidopsis thaliana*
- *Aurantiochytrium limacinum*
- *Batrachochytrium dendrobatidis* JAM81
- *Bigelowiella natans*
- *Blastocystis hominis*
- *Bodo saltans*
- *Caenorhabditis elegans*
- *Capsaspora owczarzaki*
- *Chlamydomonas reinhardtii*
- *Chondrus crispus*
- *Ciona intestinalis*
- *Cryptococcus neoformans* var. *grubii* H99
- *Cryptosporidium parvum*
- *Cyanidioschyzon merolae*
- *Cyanophora paradoxa*
- *Dictyostelium discoideum*
- *Drosophila melanogaster*

- *Ectocarpus siliculosus*
- *Emiliana huxleyi* CCMP1516
- *Entamoeba histolytica*
- *Fonticula alba*
- *Giardia intestinalis*
- *Guillardia theta*
- *Homo sapiens*
- *Hyaloperonospora arabidopsidis*
- *Klebsormidium flaccidum*
- *Laccaria bicolor*
- *Monosiga brevicollis*
- *Mortierella verticillata* NRRL 6337
- *Mus musculus*
- *Naegleria gruberi*
- *Nannochloropsis gaditana*
- *Neurospora crassa* OR74A
- *Ostreococcus lucimarinus*
- *Perkinsus marinus*
- *Phaeodactylum tricornutum*
- *Physcomitrella patens*
- *Phytophthora ramorum*
- *Plasmodium falciparum*

- *Populus trichocarpa*
- *Reticulomyxa filosa*
- *Rozella allomycis* CSF55
- *Salpingoeca* sp. ATCC 50818
- *Schizosaccharomyces pombe*
- *Sphaeroforma arctica* jp610
- *Takifugu rubripes*
- *Tetrahymena thermophila macronucleus*
- *Thalassiosira pseudonana*
- *Thecamonas trahens* ATCC 50062
- *Toxoplasma gondii*
- *Trichomonas vaginalis* G3
- *Trichoplax adhaerens*
- *Trypanosoma brucei*
- *Tuber melanosporum*
- *Ustilago maydis*
- *Vitrella brassicaformis* CCMP3155

#### A.3.2 BACTERIAL TRANSCRIPTOMES

eDicer Bacteria Predicted Transcriptomes used:

- *Acidimicrobium ferrooxidans* DSM 10331
- *Nocardia farcinica* IFM 10152
- *Frankia alni* ACN14a

- *Propionibacterium acidifaciens* DSM 21887
- *Kitasatospora setae* KM-6054
- *Bifidobacterium longum* NCC2705
- *Collinsella tanakaei* YIT 12063
- *Rubrobacter xylanophilus* DSM 9941
- *Conexibacter woesei* DSM 14684
- *Moorella thermoacetica* ATCC 39073
- *Aquifex aeolicus* VF5
- *Persephonella marina* EX-H1
- *Desulfurobacterium thermolithotrophum* DSM 11699
- *Hydrogenobacter thermophilus* TK-6
- *Chthonomonas calidirosea* T49
- *Fimbriimonas ginsengisoli* Gsoil 348
- *Armatimonadetes bacterium* CSP1-3
- *Bacteroides fragilis* 3\_1\_12
- *Flavobacterium psychrophilum* JIP02\_86
- *Salinibacter ruber* DSM 13855
- *Chlorobium tepidum* TLS
- *Chlorobium luteolum* DSM 273
- *Chloroherpeton thalassium* ATCC 35110
- *Prosthecochloris aestuarii* DSM 271
- *Ignavibacterium album* JCM 16511

- *Melioribacter roseus* P<sub>3</sub>M-2
- *Phycisphaera mikurensis* NBRC 102666
- *Isosphaera pallida* ATCC 43644
- *Blastopirellula marina* DSM 3645
- *Chlamydia trachomatis* 434/Bu
- *Waddlia chondrophila* WSU 86-1044
- *Lentisphaera araneosa* HTCC2155
- *Opitutus terrae* PB90-1
- *Akkermansia muciniphila* ATCC BAA-835
- *Anaerolinea thermophila* UNI-1
- *Caldilinea aerophila* DSM 14535
- *Chloroflexus aurantiacus* J-10-fl
- *Dehalococcoides mccartyi* 195
- *Ktedonobacter racemifer* DSM 44963
- *Thermomicrobium roseum* DSM 5159
- *Gloeobacter violaceus* PCC 7421
- *Nostoc punctiforme* PCC 73102
- *Acaryochloris* sp. CCMEE 5410
- *Synechococcus elongatus* PCC 6301
- *Synechococcus* sp. JA-3-3Ab
- *Chroococcidiopsis thermalis* PCC 7203
- *Pleurocapsa* sp. PCC 7327

- *Fischerella muscicola* PCC 7414
- *Calditerrivibrio nitroreducens* DSM 19672
- *Deferribacter desulfuricans* SSM1
- *Denitrovibrio acetiphilus* DSM 12809
- *Flexistipes sinusarabici* DSM 4947
- *Deinococcus radiodurans* R1
- *Truepera radiovictrix* DSM 17093
- *Meiothermus silvanus* DSM 9946
- *Thermus aquaticus* Y51MC23
- *Acidobacterium capsulatum* ATCC 51196
- *Fibrobacter succinogenes* subsp. *succinogenes* S85
- *Chitinivibrio alkaliphilus* ACh1
- *Bacillus subtilis* B7-s
- *Lactobacillus acidophilus* NCFM
- *Streptococcus suis* BM407
- *Halanaerobium praevalens* DSM 2228
- *Coprobacillus* sp. 3\_3\_56FAA
- *Mitsuokella multacida* DSM 20544
- *Anaerococcus hydrogenalis* ACS-025-V-Sch4
- *Thermoanaerobacter ethanolicus* JW 200
- *Cetobacterium* sp. ZOR0034
- *Fusobacterium nucleatum* 13\_3C

- *Ilyobacter polytropus* DSM 2926
- *Leptotrichia wadei* Fo279
- *Sebaldella termitidis* ATCC 33386
- *Nitrospira defluvi*
- *Thermodesulfobivrio islandicus* DSM 12570
- *Leptospirillum* sp. Group I
- *Caulobacter crescentus* CB15
- *Agrobacterium tumefaciens* WRT31
- *Hyphomonas* sp. 25B14\_1
- *Parvularcula bermudensis* HTCC2503
- *Wolbachia pipientis* wAlbB
- *Sphingobium* sp. AP49
- *Acetobacter pasteurianus* 386B
- *Bordetella avium* 197N
- *Neisseria bacilliformis* ATCC BAA-1200
- *Azoarcus* sp. BH72
- *Nitrosomonas eutropha* C91
- *Methylobacillus flagellatus* KT
- *Aeromonas hydrophila* NJ-35
- *Escherichia coli* str. K-12
- *Pseudomonas aeruginosa* PAO1
- *Xanthomonas oryzae* ATCC 35933

- *Alcanivorax* sp. DG881
- *Vibrio fischeri* SR5
- *Shewanella baltica* BA175
- *Bdellovibrio bacteriovorus* HD100
- *Desulfarculus baarsii* DSM 2075
- *Myxococcus xanthus* DZ2
- *Desulfovibrio vulgaris* DP4
- *Geobacter sulfurreducens* KN400
- *Campylobacter jejuni* 10186
- *Helicobacter pylori* 35A
- *Nautilia profundicola* AmH
- *Nitratifractor salsuginis* DSM 16511
- *Sulfurovum lithotrophicum*
- *Brachyspira innocens* ATCC 29796
- *Leptospira interrogans* serovar Bim str. P2529
- *Borrelia burgdorferi* B31
- *Spirochaeta thermophila* DSM 6578
- *Treponema denticola* ATCC 33520
- *Synergistes* sp. 3\_1\_syn1
- *Aminomonas paucivorans* DSM 12260
- *Dethiosulfovibrio peptidovorans* DSM 11002
- *Anaerobaculum mobile* DSM 13181

- *Pyramidobacter piscolens* W5455
- *Acholeplasma laidlawii* PG-8A
- *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1
- *Kosmotoga olearia* TBF 19.5.1
- *Petrotoga mobilis* SJ95
- *Fervidobacterium islandicum*
- *Caldisericum exile* AZM16c01
- *Desulfurispirillum indicum* S5
- *Dictyoglomus thermophilum* H-6-12
- *Elusimicrobium minutum* Pei191
- *Gemmatimonas aurantiaca* T-27
- *Gemmatimonadetes bacterium* KBS708
- *Nitrospina gracilis* 3/211
- *Mariprofundus ferrooxydans* PV-1
- *Thermodesulfatator atlanticus* DSM 21156
- *Thermodesulfobacterium thermophilum* DSM 1276
- *Caldithrix abyssi* DSM 13497

### A.3.3 ARCHAEA TRANSCRIPTOMES

eDicer Archaea Predicted Transcriptomes:

- *Acidianus hospitalis* W1
- *Acidilobus saccharovorans* 345-15
- *Aciduliprofundum boonei* T469

- *Aeropyrum pernix* K1
- *Archaeoglobus fulgidus* DSM 4304
- *Caldisphaera lagunensis* DSM 15908
- *Caldivirga maquilingensis* IC-167
- *Candidatus Korarchaeum cryptofilum* OPF8
- *Candidatus Nitrosopelagicus brevis*
- *Cenarchaeum symbiosum* A
- *Desulfurococcus fermentans* DSM 16532
- *Ferroglobus placidus* DSM 10642
- *Ferroplasma acidarmanus* fer1
- *Fervidicoccus fontis* Kam940
- *Halalkalicoccus jeotgali* B3
- *Haloarcula hispanica* ATCC 33960
- *Halobacterium salinarum* R1
- *Haloferax mediterranei* ATCC 33500
- *Halogeometricum borinquense* DSM 11551
- *Halomicrobium mukohataei* DSM 12286
- *Halopiger xanaduensis* SH-6
- *Haloquadratum walsbyi* DSM 16790
- *Halorhabdus utahensis* DSM 12940
- *Halorubrum lacusprofundi* ATCC 49239
- *Haloterrigena turkmenica* DSM 5511

- *Halovivax ruber* XH-70
- *Hyperthermus butylicus* DSM 5456
- *Ignicoccus hospitalis* KIN4 I
- *Ignisphaera aggregans* DSM 17230
- *Metallosphaera sedula* DSM 5348
- *Methanobacterium formicicum* DSM 3637
- *Methanobrevibacter ruminantium* M1
- *Methanobrevibacter* sp. AbM4
- *Methanocaldococcus fervens* AG86
- *Methanocella conradii* HZ254
- *Methanococcoides burtonii* DSM 6242
- *Methanococcus maripaludis* S2
- *Methanocorpusculum labreanum* Z
- *Methanoculleus bourgensis* MS2
- *Methanofollis liminatans* DSM 4140
- *Methanohalobium evestigatum* Z-7303
- *Methanohalophilus mahii* DSM 5219
- *Methanolobus psychrophilus* R15
- *Methanomethylovorans hollandica* DSM 15978
- *Methanoplanus limicola* DSM 2279
- *Methanopyrus kandleri* AV19
- *Methanoregula formicicum* SMSP

- *Methanosaeta thermophila* PT
- *Methanosalsum zhilinae* DSM 4017
- *Methanosarcina acetivorans* C2A
- *Methanosphaera stadtmanae* DSM 3091
- *Methanosphaerula palustris* E1-9c
- *Methanospirillum hungatei* JF-1
- *Methanothermobacter marburgensis* str. Marburg
- *Methanothermococcus okinawensis* IH1
- *Methanothermus fervidus* DSM 2088
- *Methanotorris igneus* Kol 5
- *Nanoarchaeum equitans* Kin4-M
- *Natrialba magadii* ATCC 43099
- *Natrinema pellirubrum* DSM 15624
- *Natronobacterium gregoryi* SP2
- *Natronococcus occultus* SP4
- *Natronomonas pharaonis* DSM 2160
- *Nitrosopumilus maritimus* SCM1
- *Picrophilus torridus* DSM 9790
- *Pyrobaculum arsenaticum* DSM 13514
- *Pyrococcus yayanosii* CH1
- *Pyrolobus fumarii* 1A
- *Salinarchaeum* sp. Harcht-Bsk1

- *Staphylothermus hellenicus* DSM 12710
- *Sulfolobus islandicus* M.14.25
- *Thermococcus barophilus* MP
- *Thermofilum pendens* Hrk 5
- *Thermogladius cellulolyticus* 1633
- *Thermoplasma acidophilum* DSM 1728
- *Thermoplasmatales archaeon* BRNA1
- *Thermoproteus uzoniensis* 768-20
- *Thermosphaera aggregans* DSM 11486
- *Vulcanisaeta distributa* DSM 14429

**B**

Publications

# B.1 COMPLEX PATTERNS OF GENE FISSION IN THE EUKARYOTIC FOLATE BIOSYNTHESIS PATHWAY

GBE

GENOME BIOLOGY AND EVOLUTION

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## Complex Patterns of Gene Fission in the Eukaryotic Folate Biosynthesis Pathway

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Data deposition: Novel sequence data generated as part of this study has been deposited at GenBank under the accession AFW17812.1.

### Abstract

Shared derived genomic characters can be useful for polarizing phylogenetic relationships, for example, gene fusions have been used to identify deep-branching relationships in the eukaryotes. Here, we report the evolutionary analysis of a three-gene fusion of *folB*, *folK*, and *folP*, which encode enzymes that catalyze consecutive steps in de novo folate biosynthesis. The *folK-folP* fusion was found across the eukaryotes and a sparse collection of prokaryotes. This suggests an ancient derivation with a number of gene losses in the eukaryotes potentially as a consequence of adaptation to heterotrophic lifestyles. In contrast, the *folB-folK-folP* gene is specific to a mosaic collection of Amorphea taxa (a group encompassing: Amoebozoa, Apusomonadida, Breviatea, and Opisthokonta). Next, we investigated the stability of this character. We identified numerous gene losses and a total of nine gene fission events, either by break up of an open reading frame (four events identified) or loss of a component domain (five events identified). This indicates that this three gene fusion is highly labile. These data are consistent with a growing body of data indicating gene fission events occur at high relative rates. Accounting for these sources of homoplasy, our data suggest that the *folB-folK-folP* gene fusion was present in the last common ancestor of Amoebozoa and Opisthokonta but absent in the Metazoa including the human genome. Comparative genomic data of these genes provides an important resource for designing therapeutic strategies targeting the de novo folate biosynthesis pathway of a variety of eukaryotic pathogens such as *Acanthamoeba castellanii*.

**Key words:** phylogenetics, comparative genomics, pterin biosynthesis, Diaphoretickes.

### Introduction

The resolution of ancient phylogenetic relationships is proving a difficult task (Philippe and Laurent 1998; Philippe 2000; Dagan and Martin 2006). Rare genomic characters such as: Insertions and/or deletions within open reading frames (ORFs), intron distribution, and gene fusions are potentially useful tools for polarizing evolutionary relationships and rooting trees (Jensen and Ahmad 1990; Philippe et al. 2000; Rokas and Holland 2000). In these cases, assuming parsimony, the logic proceeds that taxa A and B possess a rare genomic character, whereas taxa C and D do not, therefore taxa A and B are likely to be monophyletic to the exclusion of taxa C and D.

The process of gene fusion and domain recombination is itself an important evolutionary process, leading to: Acquisition of new gene functions (Doolittle 1995), biochemical channeling (Miles et al. 1999), coregulation, colocalization, and potentially promoting the fixation of horizontally transferred genes (Andersson and Roger 2002; Yanai et al. 2002; Slot and Rokas 2010, 2011) see also (Lawrence and Roth 1996; Lawrence 1999; Walton 2000). The corollary with investigating gene fusions is that they are also subject to homoplasy in the form of: Horizontal gene transfer (HGT) (Andersson and Roger 2002; Yanai et al. 2002), separation (gene fission), gene duplication with differential loss of subsections of the

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gene (also a form of gene fission), total gene loss (Nakamura et al. 2007; Leonard and Richards 2012), or convergent evolution (Nara et al. 2000; Stover et al. 2005).

Folate is an essential metabolite involved in the biosynthesis of: Adenine and thymidine bases, methionine and histidine amino acids, and formyl-tRNA (Brown 1971). Many plants, protists, Fungi, Bacteria, and Archaea manufacture folate de novo (Cossins and Chen 1997; Levin et al. 2004; de Crecy-Lagard et al. 2007) principally via a double-branched pathway involving the pterin and pABA branches which feed into the step mediated by the enzyme encoded by *folP* (the pathway is illustrated in fig. 1 with gene and protein names listed). In the plant *Arabidopsis thaliana* many steps, including the proteins encoded by *folK-folP*, are localized to the mitochondria, whereas the enzymes that catalyze pABA synthesis are localized within the plastid organelle (de Crecy-Lagard et al. 2007). Folate salvage systems are also known from a range of taxa, where pterin and pABA-glutamate fragments produced by folate breakdown are fed into curtailed versions of the pathway (Orsomando et al. 2006; de Crecy-Lagard et al. 2007). For example, in some metazoans the core of the pathway is bypassed by folic acid uptake from food (Cossins 2000; Lucock 2000), leaving only the requirement for: Dihydrofolate reductase (DHFR) and thymidylate synthase (TS) (see figs. 1 and 2). Antifolate drugs (e.g., sulfonamides and sulfones) targeting the DHPS step in the pterin branch (encoded by *folP*) are therefore important antimicrobial agents (Lawrence et al. 2005) because host animals do not encode the equivalent metabolic trait. Additionally, drugs targeting the latter steps of the pathway (e.g., methotrexate which inhibits DHFR) are used in chemotherapy to target cancer cells (Huennekens 1994; Cossins and Chen 1997).

The genes that encode the folate biosynthesis enzymes DHFR and TS are fused in many eukaryotes (Stechmann and Cavalier-Smith 2002) resulting in synthesis of a two domain multifunctional protein. This character has been suggested to be an anciently derived synapomorphy uniting the “bikont” clade (Stechmann and Cavalier-Smith 2002, 2003), a group of “ancestrally biciliate eukaryotes” including the: Stramenopiles, Alveolata, Rhizaria (known collectively as the SAR supergroup), Excavata, Cryptophyta, Haptophyta, and Archaeplastida. However, several eukaryotic subgroups appear to have lost either the fused or unfused DHFR and TS-encoding genes (Simpson and Roger 2004; Roger and Simpson 2009) (fig. 2) making this an unreliable character for polarizing evolutionary relationships. In addition, the “bikont” grouping has been revised and these taxa, with the exception of the Excavata, are now grouped within Diaphoretickes (Adl et al. 2012). We also note that Cavalier-Smith has abandoned this rooting system (Cavalier-Smith 2010) in favor of a root within the Excavata (Simpson 2003) rendering the “bikonts” paraphyletic. Furthermore, although myosin II was thought to be exclusive to Amoebozoa and Opisthokonta taxa (Richards and Cavalier-Smith 2005) this gene architecture is found in Heterolobosea

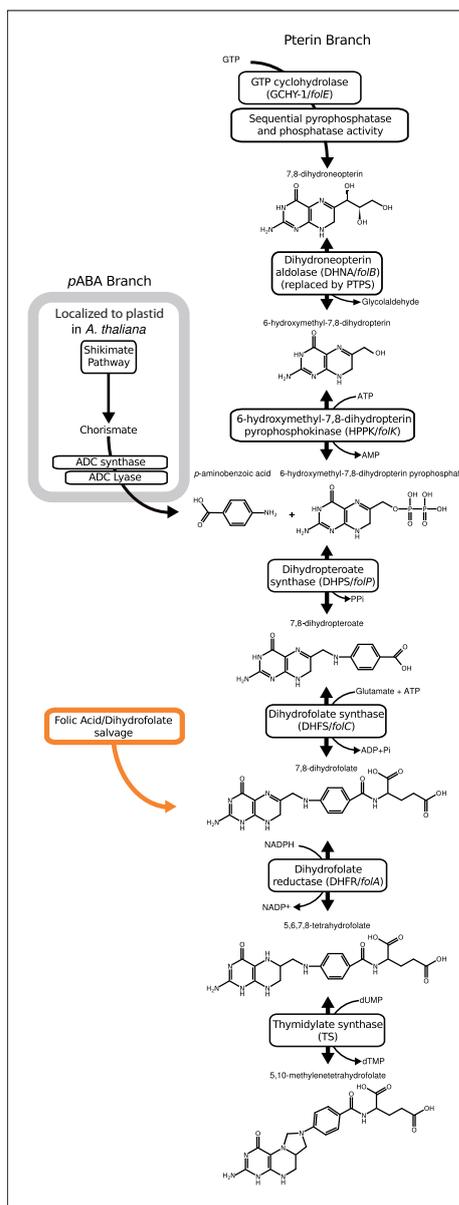
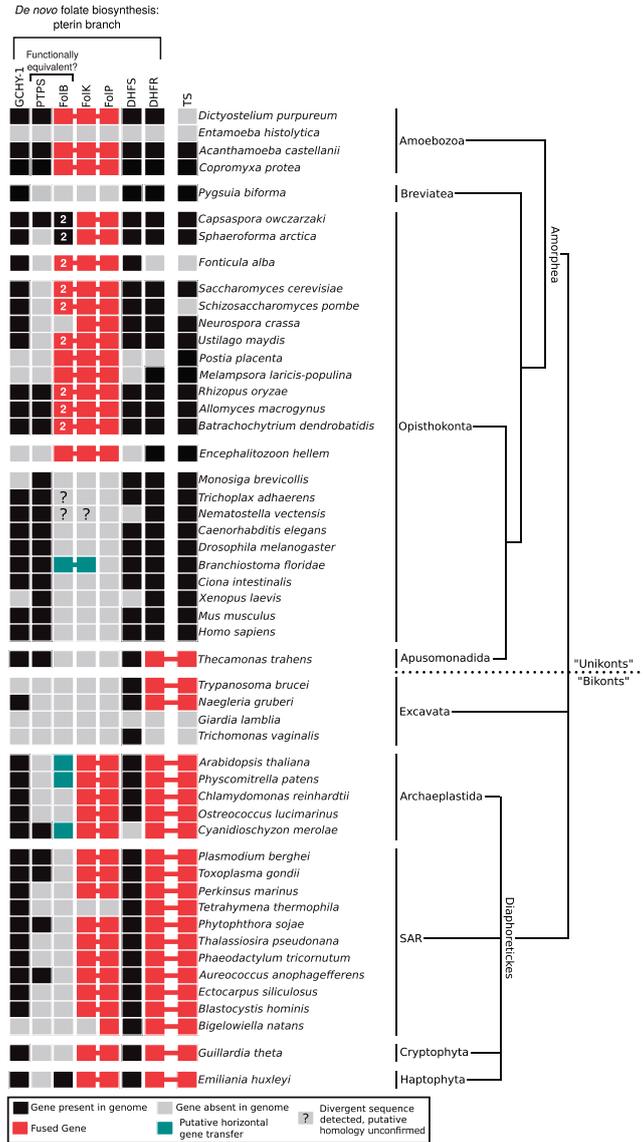


Fig. 1.—Part of the folate biosynthesis pathway with intermediate chemical states of the pathway illustrated. Protein and gene names that encode each step of the pathway are given.

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**Fig. 2.**—Presence, absence, and fusion state of putative folate pathway encoding genes across the eukaryotes. Taxonomic distribution of the pterin branch of the folate biosynthesis pathway. The red boxes and connecting lines indicate a gene fusion, black boxes represents presence of a putative homologue, and gray indicates gene not identified in the genome sequence data. Amoebozoa and Opisthokonta were formerly referred to as the “unikonts,” and likewise SAR, Excavata, and Archaeplastida were formerly referred to as the “bikonts.” Note that the putative *foB* of *Trichoplax adhaerens* and the putative *foB-foK* fusion of *Nematostella vectensis* were removed from phylogenetic analyses due to poor alignment of these sequences, as such their provenance and evolutionary ancestry remains questionable and are therefore indicated by a question mark at the relevant position.

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(Excavata) (Fritz-Laylin et al. 2010). This suggests a different or deeper ancestry of myosin II. Alternatively, this distribution pattern may be the result of HGT (Berney C, personal communication) with additional examples of HGT-derived genes shared by Heterolobosea and Amoebozoa (Andersson 2011; Herman et al. 2013) supporting the idea that HGT between these groups has played a role. However, an amended version of the “bikont” and “unikont” bifurcation recently gained some direct support using a rooted multigene phylogenetic analysis of genes derived through the mitochondrial endosymbiosis (Derelle and Lang 2012), but also see He et al. (2014) for an alternative tree topology derived from a similar analytical approach.

In 2005, Lawrence et al. published the structure of three components of the *Saccharomyces cerevisiae* folate biosynthesis pathway; a triple domain gene fusion, encompassing the DHNA, HPPK, and DHPS enzymes encoded by *folB*, *folk*, and *folP* genes—steps 3, 4, and 5 in pterin biosynthesis pathway (Lawrence et al. 2005) (fig. 1). Interestingly, gene fusions are common in secondary metabolic networks, for example, the shikimate pathway that forms the prerequisite to the pABA branch of folate biosynthesis is encoded by numerous variant gene fusions (Campbell et al. 2004; Richards et al. 2006) and genes which encode key enzymes of the pABA branch of folate biosynthesis are often found fused (de Crecy-Lagard et al. 2007). Here, we report a phylogenomic analysis of gene fusion characteristics in the pterin folate biosynthesis pathway across the eukaryotes. We use these data to investigate the evolutionary ancestry of the three-domain pterin biosynthesis gene fusion, identifying: a diversity of gene fusion architectures, gene fission events, and a number of gene losses. Using these results, we evaluate this three gene fusion character as synapomorphy for the monophyletic grouping of the Opisthokonta and Amoebozoa finding a high incidence of homoplasy.

## Materials and Methods

### *Cloning and Sequencing of Folate Triple Domain Gene Fusion from Acanthamoeba castellanii* cDNA

Using the partially assembled genome reads of the *Acanthamoeba castellanii* sequencing project (available at the Baylor College of Medicine—<https://www.hgsc.bcm.edu/microbiome/acanthamoeba-castellani-neff>, last accessed October 3, 2014), we designed a range of overlapping polymerase chain reaction (PCR) primers (Marshall 2004) to target different domain sections of the three folate biosynthetic genes *folB*, *folk*, and *folP* (see [supplementary table S1, Supplementary Material](#) online). *Acanthamoeba castellanii* Neff strain was grown axenically in a modified M11 defined media (Shukla et al. 1990) without folate ([supplementary table S2, Supplementary Material](#) online) to encourage the transcription of folate biosynthesis pathway genes. Cells were collected and suspended in 1 ml of trizol reagent

(Invitrogen) and RNA extracted using the single-step acid guanidinium thiocyanate–phenol–chloroform protocol as described by Chomczynski and Sacchi (Chomczynski and Sacchi 1987). The cDNA was then synthesized using the AffinityScript kit with random hexamers (Stratagene). PCR amplification for target folate biosynthesis genes was conducted using Master Mix (Promega, containing 3 mM MgCl<sub>2</sub>, 400 μM of each dNTP, and 50 U/ml of Taq DNA polymerase) to create a 25 μl PCR reaction mix (12.5 μl of Master Mix, 1 μl each primer (10 μM), 9.5 μl of Milli-Q pure water (Millipore), and 1 μl of template cDNA). *Acanthamoeba* cDNA was diluted to approximately 100 ng/μl using spectrophotometry (NanoDrop ND-1000). Thermocycling followed an initial incubation at 95 °C for 5 min, and cycling conditions details in [supplementary table S1, Supplementary Material](#) online followed by a 72 °C–5 min elongation step. See [supplementary table S1, Supplementary Material](#) online, for details of PCR primers used. Successfully amplified PCR products were gel-purified (Wizard SV Gel and PCR Clean-Up kit, Promega) and cloned using TA-cloning (PCR StrataClone Cloning Kit, Agilent Technologies). Five clones were selected from each PCR reaction and externally sequenced using the M13/pUC vector primers via Sanger sequencing (Cogenic Beckman-Coulter sequencing service, High Wycombe). The flanking vector sequences were removed; the sequences trimmed to areas of high chromatograph quality and ambiguously defined bases corrected. The overlapping sequences were then assembled into contigs using Sequencher (Gene Codes) version 4.10.1 program (<http://www.genecodes.com/>) producing a high-confidence consensus sequence for a partial ORF for the *folB*, *folk*, and *folP* gene fusion (GenBank Acc: AFW17812.1). These data demonstrate that the *folB*, *folk*, and *folP* genes are transcribed as a single three-domain gene fusion. It should be noted that subsequently a draft genome and predicted proteome of *Acanthamoeba* has been released (Clarke et al. 2013), which contains the same gene fusion of near identical sequence (513/514 identities with no gaps—GenBank Acc: XP\_004341460). The full-length gene derived from the genome sequence was used for the subsequent *folB*, *folk*, and *folP* phylogenetic analyses.

### Survey of Additional Protist Taxa Using RNA-Seq Data

We used the *Dictyostelium purpureum* (XP\_003290941) *folB*, *folk*, and *folP* three gene fusion and *Bacillus cereus* single domain unfused-genes (*folB*—NP\_829975.1, *folk*—ZP\_03233543.1, *folP*—ZP\_07056868.1) as a search query to identify putative homologues using the basic local alignment search tool (tBLASTn) against a set of protistan RNAseq “in-house” data sets. This data set included the unicellular opisthokont *Fonticula alba*, the amoebozoan *Copromyxa protea*, and the breviate *Pygusua biforma* (PCbi66). From these data, we were able to identify components of the *folB*, *folk*, and *folP* genes from *Fonticula* and *Copromyxa*,

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but not in the breviate *P. biforma* (PCbi66). Phylogenomic analysis demonstrates that breviate flagellates are related to opisthokonts and the Apusomonadida (Brown et al. 2013).

For these RNAseq projects, total RNA was isolated using Tri-reagent (Sigma) following the protocol supplied by the manufacturer. Construction of cDNA libraries and Illumina RNAseq was performed by the Institut de Recherche en Immunologie et Cancérologie of Université de Montréal (Canada) for *Copromyxa protea* (strain CF08-5), the BROAD Institute (Boston) for *F. alba* (strain ATCC 38817), and Macrogen (South Korea) for the *P. biforma* (PCbi66). Raw sequence read data were filtered based on quality scores with the fastq\_quality\_filter program of FASTXTOOLS ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)), using a cutoff filter (a minimum 70% of bases must have quality of 20 or greater). Filtered sequences were then assembled into clusters using the Inchworm assembler of the TRINITY r2011-5-13 package (Grabherr et al. 2011). The *F. alba* assembly is available via the BROAD Institute; however, the other two assemblies are currently unreleased (manuscript in preparation). All unmasked protein alignments are included as [supplementary material](#), [Supplementary Material](#) online, on GitHub (DOI: 10.5281/zenodo.11716) as MASE files which includes the alignment mask information (generated by SeaView [Galtier et al. 1996]).

## Comparative Genomics and Phylogenetic Analysis

Using BLASTp and tBLASTn (Altschul et al. 1990) we initially searched NCBI GenBank, the Joint Genome Institute (<http://genome.jgi-psf.org/>), and the Broad Institute (<http://www.broadinstitute.org/>) genome databases (as of November 2013) using three separate folate biosynthesis domains from *B. cereus* (*folB*—NP\_829975.1, *folK*—ZP\_03233543.1 and *folP*—ZP\_07056868.1) and the *D. purpureum* (XP\_003290941) *folB*, *folK*, and *folP* three gene fusion divided into the three-domain regions. Care was taken to survey the major eukaryotic, archaeal, and bacterial groups; to this end additional BLAST searches were conducted using multiple start seeds from diverse taxa to check for alternative sequence hits. The amino acid sequences gathered for each domain were run through the REFGEN tool (Leonard et al. 2009). The multiple sequence comparison by log-expectation program (v3.8.31) (Edgar 2004) was used to produce a multiple sequence alignment for each domain (*folB*, *folK* and *folP*). Alignments were then manually corrected and masked in SeaView (version 4.2.4) (Galtier et al. 1996). Sequences that caused an unacceptable loss of putatively informative sites (due to the sequence nonalignment or not masking well) or that formed long branches in preliminary analysis were removed. Duplicate entries from closely related taxa, for example, highly similar sequences from different representativeness of the same bacterial or fungal genus (e.g., *Escherichia*, *Bacillus*, and *Aspergillus*) or multiple highly similar genes

from the same genome (sister branches on preliminary phylogenetic trees) were removed from the alignments.

Phylogenetic analysis was conducted using both Bayesian and maximum-likelihood methodologies with the model of amino acid substitution selected using ProtTest3 (version 3.2.1—[Darriba et al. 2011]—see [supplementary figs. S1–S7](#), [Supplementary Material](#) online). Sequences shown to form long branches in the phylogenetic analysis were removed from the alignment to reduce the risk of long-branch attraction artifacts (Felsenstein 1978; Philippe 2000), for example, the Microsporidian: *Encephalitozoon hellem* ATCC 50504 *folB-folK-folP* gene fusion—XP\_003887200, and *Plasmodium berghei* *folK-folP* gene fusion—XP\_15149005 from the *folK* alignment, and the analyses rerun. The phylogenies were calculated using parallelized-PHREADS RAXML (version 7.7—Stamatakis 2006) with 1,000 (nonrapid) bootstrap replicates and using the substitution matrix and gamma distribution identified using ProtTest3 (version 3.2.1) (Yang 1996; Darriba et al. 2011). In a subset of these analyses invariant sites were also included as a model parameter (in accordance with ProtTest3 recommendations), see the figure legends for [supplementary figures S1–S7](#), [Supplementary Material](#) online, for more details of the models used. Bayesian phylogenies were also reconstructed using MrBayes (version 3.2). Each analysis was conducted as two independent runs of four metropolis-coupled Markov chain Monte Carlo [MCMCMC] chains and continued until convergence of these runs as determined using the Tracer (version 1.5) (Rambaut and Drummond 2007). Burn-in was then also determined using Tracer. The program TREEMAMER (Leonard et al. 2009) was then run on the resulting tree files in order to restore the correct taxa names from the REFGEN tags used during phylogenetic processing. These analyses were also repeated using the same methods but focusing on a reduced taxon data set and a concatenation of the *folK* and *folP* alignments to tests for improved topology support for key nodes ([supplementary figs. S4–S7](#), [Supplementary Material](#) online).

## Results

### Diversity of Gene Fusions in the Folate Biosynthesis Pathways

At the core of pterin branch of the folate biosynthesis pathway are three genes (*folB*, *folK*, and *folP*) that encode sequentially acting enzymes: DHNA, HPPK, and DHPS (fig. 1). In some fungi these are found as a single gene encoding a three-domain protein (e.g., *S. cerevisiae*: GenBank accession NP\_014143.2—[Lawrence et al. 2005]) suggesting that gene fusion has played a role in the pterin branch of folate biosynthesis. To investigate the evolutionary ancestry of this gene fusion, we conducted comparative genomics of these three domains. These analyses demonstrated a discontinuous distribution across the eukaryotes suggesting a complex

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pattern of gene loss (fig. 2). We identified four different domain architectures, as defined by PFAM searches (Bateman et al. 2004), of the eukaryotic folate biosynthesis protein sequences sampled: 1) *folB-folB-folK-folP* found in a range of fungi and the opisthokont sorocarpic protist *F. alba*; 2) *folB-folK-folP* found in Amoebozoa, the basidiomycete fungi *Postia placenta*, *Coprinopsis cinerea*, and *Melampsora laricis-populina*, and the microsporidian *E. hellem*, (excluded from phylogenetic analysis because it formed a long branch in the phylogenies, like many other microsporidian sequences [Hirt et al. 1999]); 3) *folB-folK* found in two metazoans; and 4) *folK-folP* found in a subset of ascomycete fungi, *Puccinia graminis*, *Capsaspora owczarzewski*, *Sphaeroforma arctica*, and a diverse range of Diaphoretickes (fig. 2).

In many Diaphoretickes groups, including SAR, Cryptophyta, and the Excavata, we could not identify a *folB* gene using standard BLAST similarity searches (fig. 2). To confirm this result, we used a five iteration PSI-BLAST search using both the *B. cereus folB* gene and the *folB* domain of the *D. purpureum folB-folK-folP* gene fusion as a search seed against the NCBI GenBank nonredundant (NR) protein database (performed both as a general search and a search restricted to eukaryotic taxa). These analyses failed to identify any additional putative *folB* encoding genes in the eukaryotic genomes available in the GenBank NR database.

Pyruvoyltetrahydropterin synthase (PTPS) has been suggested to represent a functional replacement of the DHNA enzyme (*folB*) (Pribat et al. 2009). To investigate the possibility that this gene has functionally replaced *folB* in the Diaphoretickes and Excavata, or other eukaryotic groups, we searched the eukaryotes for the presence of genes with similar sequence characteristics across the genomes sampled (fig. 2). These analyses identified no clear pattern of PTPS/*folB* presence/absence, providing no support for this hypothesis that PTPS is acting as a like-for-like functional replacement of *folB* across the eukaryotes.

## Phylogenetic Analyses of the *folB*, *folK*, and *folP* Domains

To further investigate the evolutionary ancestry of the gene fusion character, we calculated individual phylogenies for the three pterin biosynthesis domains with both comprehensive and reduced taxa alignment sampling. The results of these phylogenies are shown in [supplementary figures S1–S6, Supplementary Material](#) online, with all six trees demonstrating low levels of topology support while many features of the eukaryotic sections of the tree topologies are inconsistent with established multigene phylogenetic trees (e.g., Rodriguez-Ezpeleta et al. 2005; Hampl et al. 2009; Derelle and Lang 2012; Torruella et al. 2012; Brown et al. 2013). This is typical of single-gene phylogenetic analysis using limited numbers of amino acid alignment characters (i.e., 78, 102, 175, 110, 102, 236 amino acid characters for [supplementary figs. S1–S6, Supplementary Material](#) online, respectively) and which

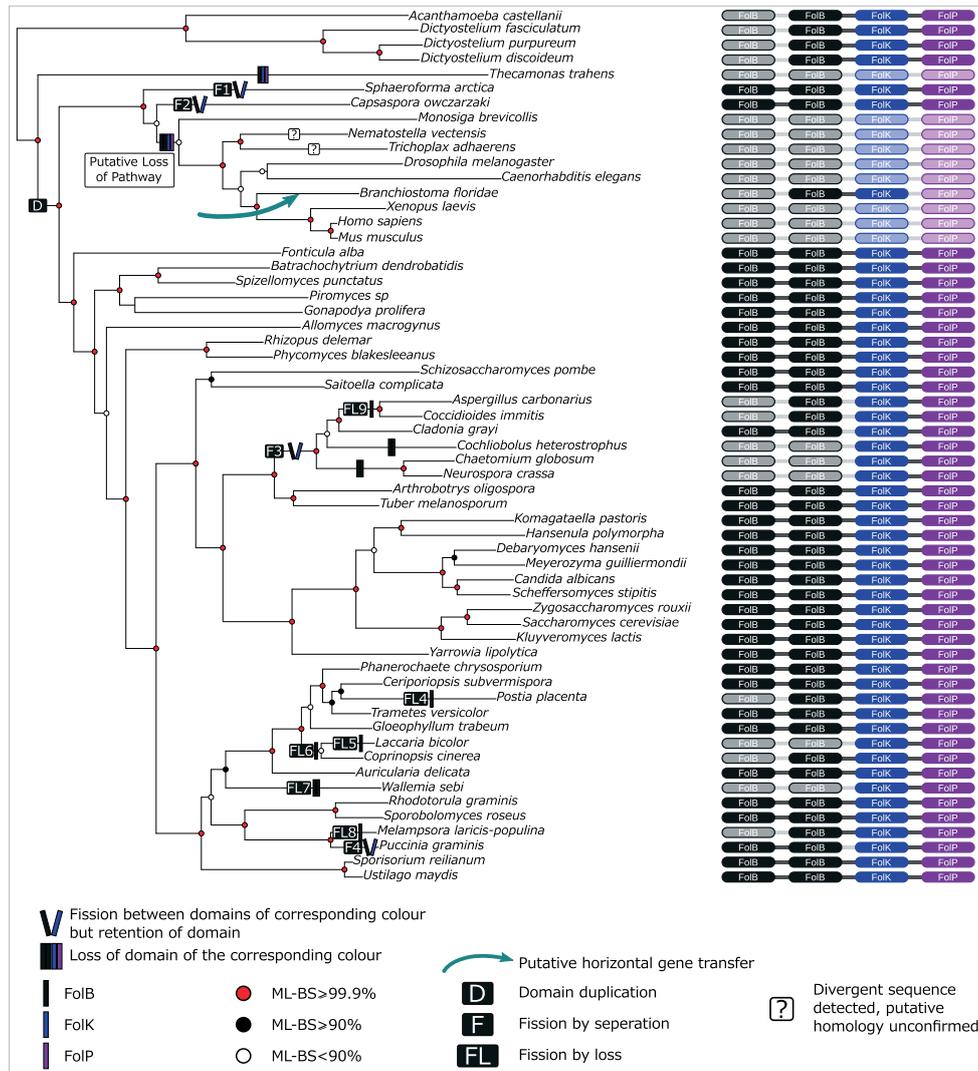
encompasses ancient and divergent evolutionary groups. These alignment character numbers do not compare favourably to multigene analyses where it has been shown that in excess of 5,000 amino acid alignment characters are required to robustly resolve the Archaeplastida (Rodriguez-Ezpeleta et al. 2005). Although interestingly, Hampl et al. (2009) demonstrated that a low number of genes are sufficient to recover monophyly of the Opisthokonta branching sister to the Amoebozoa.

Our analyses identified a *folB-folK* gene fusion in the metazoan *Branchiostoma floridae* genome assembly branching with a phylogenetic cluster of prokaryotes with moderate support within the comprehensive *folK* phylogeny (1/94% support for a grouping with *Planctomyces maris*—[supplementary fig. S2, Supplementary Material](#) online) and weak support in the reduced taxa *folK* analysis (0.939/27%—[supplementary fig. S5, Supplementary Material](#) online). The comprehensive *folB* phylogeny also shows the *Br. floridae folB-folK* gene fusion branching with prokaryote taxa with weak support (0.614/13%—[supplementary fig. S1, Supplementary Material](#) online). Collectively, these trees suggest that the *Br. floridae folB-folK* branching relationship is consistent with HGT into the *Br. floridae* genome or, alternatively, contamination of this genome project with a prokaryotic sequence. To explore these possibilities further, we found the genome sequence contig containing the *Br. floridae folB-folK* gene (GenBank acc: AC150408.2) demonstrating that the prokaryote like *Br. floridae folB-folK* gene is located in a 180,427 bp contig adjacent to genes that show standard patterns of animal sequence similarity. Analysis of the *B. belcheri* transcriptome demonstrated that an orthologue of the *Br. floridae folB-folK* gene is transcribed. Taken together these data suggest that the *Br. floridae folB-folK* gene is located on native source genome and it is not contamination. Therefore, it is likely to be a prokaryotic-derived HGT into this animal genome. However, it is interesting that an animal lineage could maintain only the first part of a pathway despite lacking the *folP* gene, whereas many other animal lineages have lost the entire pathway. Further to these data, we detected a putative *folB* gene in *Trichoplax adhaerens* and a putative *folB-folK* fusion gene in *Nematostella vectensis*. However, these genes were removed from further analyses due to difficulty in alignment of these sequences, as such their provenance and evolutionary ancestry remains questionable as noted on figures 2 and 3. These data suggest a partial folate biosynthesis pathway, or a pathway involving an alternative gene encoding the *folP* step present in *Branchiostoma*. Furthermore, we see evidence of incomplete pathways in other organisms, for example, the red alga *Cyanidioschyzon* lacks an identifiable standard *folP* gene (fig. 2).

Monophyly of the three-domain gene fusion would signify that the *folB-folK* gene fusion was the product of a single evolutionary event. However, this relationship was not resolved with strong support in these analyses with only the *folB*

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**Fig. 3.**—Phylogeny of the Apusomonadida, Breviata, Opisthokonta, and Amoebozoa demonstrating variation in the *folB-folK-folP* fusion gene. Tree topology was calculated using a concatenated alignment of conserved genes identified in (Torruella et al. 2012) and represents the best-known likelihood tree from 100 ML searches in RAxML (PROTCAT+LG) with 1,000 nonrapid bootstraps. ML-BS is an abbreviation of maximum likelihood bootstrap values, *FolB-folK-folP* fusion gene domain architecture of taxa included is listed down the right column, and fusion state is denoted by the presence/absence of connecting lines. Inferred gene/domain losses are shown as shadow domains. See key for guide to tree topology support values and character state changes. Domain duplication is indicated as (D) in a box of the appropriate domain colour, fission by domain loss events are denoted as (FL5–9) and specific fission events as (F1–4). Total losses of complete ORFs are not illustrated. Note that the putative *folB* of *Trichoplax adhaerens* and the putative *folB-folK* fusion of *Nematostella vectensis* were removed from phylogenetic analyses due to poor alignment of these sequences, as such their provenance and evolutionary ancestry remains questionable and are therefore indicated by a question mark at the relevant position.

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phylogenies demonstrating a monophyletic grouping of the three domain *folB-folK-folP* gene fusions (both as *folB-folK-folP* and *folB-folB-folK-folP*) with weak topology support (i.e., 0.539/19% and 0.991/37% support [supplementary figs. S1 and S4, Supplementary Material online, respectively]). Importantly, we note that the only members of the Diaphoretickes and Excavata (formerly the “bikonts”) possessing a putative *folB* gene are the Archaeplastida and that the *folB* gene of this eukaryotic group branches separately from the other eukaryotes within a clade of bacterial genes with moderate-to-strong posterior probability/bootstrap support (supplementary fig. S1: 0.992/82%, Supplementary Material online and supplementary fig. S4: 1.000/94%, Supplementary Material online) suggesting a separate evolutionary ancestry of this gene to that of the Opisthokonta and Amoebozoa. Given the taxonomic distribution of the *folB* gene across the Archaeplastida (supplementary figs. S1 and S4, Supplementary Material online), this xenologue is most likely to have been derived either by an ancient horizontal gene transfer from a bacterial source into the Archaeplastida lineage or via the cyanobacterial endosymbiosis that gave rise to the plastid organelle, a process that has been suggested to lead to the acquisition of a number of genes of mixed bacterial ancestry (Brinkman et al. 2002; Martin et al. 2002). Using the *A. thaliana folB* gene, we searched for evidence of subcellular localization using the “cell eFP browser” ([http://bar.utoronto.ca/cell\\_efp/cgi-bin/cell\\_efp.cgi?ncbi\\_gi=15229838](http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi?ncbi_gi=15229838), last accessed October 3, 2014) which suggested this gene product was localized to the cytosol or the mitochondria (supplementary table S3, Supplementary Material online). However, because the Archaeplastida *folB* is not an orthologue of the Opisthokonta/Amoebozoa version and no additional Diaphoretickes and Excavata *folB* orthologues are currently available, our *folB* phylogenetic analysis does not represent a strict test of the monophyly of the *folB-folK-folP* gene fusion within the eukaryotes.

Finally, in an attempt to improve tree resolution and to identify a resolved phylogeny, we conducted a concatenated phylogenetic analysis of the *folK* and *folP* genes (supplementary fig. S7, Supplementary Material online). This analysis again recovered a tree with low topology support values and taxonomic relationships inconsistent with established eukaryotic phylogenetic relationships (Rodríguez-Ezpeleta et al. 2005; Hampl et al. 2009; Derelle and Lang 2012; Torruella et al. 2012) and therefore provided no additional data to test the monophyly of *folB-folK-folP* three-domain gene fusions.

## *folB* Tandem Duplication in the Early Opisthokonta

Focusing on the “Opisthokonta and Amoebozoa *folB-folK-folP*” cluster, a clade specifically encompassing the *folB-folB-folK-folP* and *folB-folB* gene architectures found in Fungi, *F. alba*, *Sp. arctica*, and *C. owczarzaki* (fig. 2) forms with weak support in the reduced analysis (0.852/37%—supplementary fig. S4, Supplementary Material online). The taxon distribution

of this character suggests that the *folB* tandem exon-duplication represents a novel genetic character that arose in the last common ancestor of the opisthokonts followed by the loss of these genes in Metazoa and some other opisthokont taxa (figs. 2 and 3). We can identify this pattern because multigene phylogenies place the *Sp. arctica* and *C. owczarzaki* branch sister to the choanoflagellates and metazoans (Torruella et al. 2012), so parsimoniously the *folB-folB* gene duplication predated the diversification of the major Opisthokonta clades (see fig. 3). The distribution of the Opisthokonta *folB* duplication therefore provides a character that infers the *folB-folK* fissions within the opisthokonts are nested events (see fig. 3—F1–4 fission events) and the ancestral Opisthokonta possessed a *folB-folK* gene fusion.

## Evidence of Gene Fission in the *folB-folK-folP* Gene Fusion

Our gene fusion character distribution analysis identifies nine fission events either by loss of one or two domains or by separation of the *folB-folB-folK-folP* fusion in the opisthokonts (fig. 3). Specifically, these events involve: Fission to form *folB-folB* and *folK-folP*, on the *Sp. arctica*, *C. owczarzaki*, and *Pu. graminis* branches (fig. 3, fission events F1, F2, and F4) and within the Pezizomycotina before the divergence of: *Aspergillus carbonarius*, *Coccidioides immitis*, *Cochliobolus heterostrophus*, *Cladonia grayi*, *Chaetomium globosum*, and *Neurospora crassa* (fig. 3, fission event F3). Furthermore, these data identify loss of one or both *folB* domains on five occasions in the branches leading to the basidiomycetes: *Co. cinerea*, *Laccaria bicolor*, *Walleimia sebi*, *Po. placenta*, and *M. laricis-populina* (fig. 3, fission by loss events, FL: 5–9) and the branch leading to the ascomycetes *As. carbonarius* and *Co. immitis*. In all nine cases, we reconfirmed the gene architectures by examining gene alignments and the synteny of each candidate fission gene in the relevant genome assemblies.

## Discussion

### Distribution of Putative Folate Biosynthesis Gene Homologues and Adaptation to Folate Heterotrophy

Using a comparative genomic and phylogenetic approach, we have identified the taxonomic distribution of a three protein domain encoding gene fusions in the pterin branch of the folate biosynthesis pathway. In the absence of strong phylogenetic signal demonstrating eukaryote-to-eukaryote HGT our analyses identified multiple gene loss events in different eukaryotic groups (e.g., Metazoa and Excavata), suggesting that the capacity to manufacture folate de novo has been lost on multiple occasions within the eukaryotes. This is consistent with adaptation of these lineages to acquiring folate or folate intermediates from food sources and/or host organisms. Specifically, the comparative genomic data demonstrate that a complete pterin branch is absent from the Metazoa

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sampled, consistent with the hypothesis that animals acquire folate using “intact folate salvage” from digested food (Luccock 2000), putatively maintaining the last two or three steps of the biosynthesis pathway to facilitate salvage of folic acid (figs. 1 and 2). A similar pattern of gene presence/absence was identified for the *Trypanosoma* (Excavata), *Naegleria* (Excavata), and *Thecamonas* (Apusomonadida) genomes, suggesting that these protists acquire folate, or precursors of folate (e.g., folic acid), by salvage from external sources. We can therefore infer that these heterotrophic characteristics have resulted in concordant loss of the de novo folate biosynthesis. Likewise the absence, or near absence, of the entire folate biosynthesis pathway in *Entamoeba*, *Trichomonas*, and *Giardia* suggests a dependence on hosts or phagocytosed food for provision of intact folate, as such inhibiting folate synthesis as a therapeutic target is not viable for these parasitic protists, but inhibition of uptake transporters of intact folate may offer an alternative therapeutic strategy.

In many Diaphoretickes genomes (e.g., taxa from the SAR group and Cryptophyta) both *folK* and *folP* genes were present, but a putative homologue of the *folB* gene was not identified. These results suggest that this part of the pathway is absent from these taxa or performed by a highly divergent or nonhomologous gene family. A paralogue of *folB*: *folX* has been identified in *Escherichia coli* with 30% identical amino acid residues. This protein was classified as an epimerase and performs the equivalent aldolase type reaction with less than 1% velocity as the DHNA encoded by the *Ec. coli folB* gene (Hausmann et al. 1998) suggesting this paralogue is not functionally equivalent. Comparative genomic analysis of the distribution of *folB* gene in prokaryotes identified many phylogenetically disparate groups without an identifiable putative homologue (de Crecy-Lagard et al. 2007) leading these authors to make two suggestions: 1) the enzyme that catalyses this step is encoded by a uncharacterized putative transaldolase gene often found to cluster in the same operons as *folK*, and/or 2) because other taxa lacked the *folB* gene and a putative alternative transaldolase-encoding gene; a currently unidentified gene family must encode this enzyme (de Crecy-Lagard et al. 2007). Later work then showed some evidence that the *folB* in many bacteria has been replaced with a functionally equivalent six-PTPS (Pribat et al. 2009). Analysis of eukaryotic genomes demonstrates many eukaryotic protists lacking an identifiable *folB* or PTPS encoding gene, suggesting that a currently unidentified functionally equivalent but phylogenetically dissimilar gene may encode an enzyme that catalyses this step.

## Gene Fusion as an Adaptation for Folate Biosynthesis

Our data identified a number of variant gene fusions in pterin branch of the folate biosynthesis genes. These included a gene consisting of three domains and therefore the likely product of two distinct gene fusion events. Our comparative genomic survey suggests that this characteristic is only found in

opisthokont taxa including the: Fungi, *F. alba*, Microsporidia, and a range of Amoebozoa (e.g., *Dictyostelium*, *Acanthamoeba*, and *Copromyxa*). Moreover, two domain variations of these gene fusion forms were identified in a range of eukaryotes (fig. 2). Gene fusions have been identified elsewhere in the folate biosynthesis pathway (Stechmann and Cavalier-Smith 2002, 2003; de Crecy-Lagard et al. 2007) suggesting that gene fusion has been an important process in the evolution of the eukaryotic folate biosynthesis, possibly as a consequence of selection for: Cotranscription, colocalization, promotion of metabolic channeling, or a general improvement of enzyme kinetics (Welch and Gaertner 1975; Meek et al. 1985; Ivanetich and Santi 1990; Miles et al. 1999; Richards et al. 2006). This pattern is consistent with other secondary metabolic pathways that are also localized in the cytosol and show complex patterns of gene fusion (e.g., Nara et al. 2000; Stover et al. 2005; Richards et al. 2006).

A genome database search identified fragmenting fragments of the *folB-folK-folP* genes in the *Ac. castellanii* sequencing project (Baylor College of Medicine—<https://www.hgsc.bcm.edu/microbiome/acanthamoeba-castellani-neff>, last accessed October 3, 2014) and within the recently completed genome sequence (Clarke et al. 2013). To confirm that this was a bona fide *folB-folK-folP* triple domain gene fusion, we performed nested PCR on cDNA derived from an axenic culture of *Ac. castellanii* Neff strain grown in folate-limiting conditions (GenBank Acc: AFW17812.1). This work confirmed that *Ac. castellanii* transcribes a single gene fusion encoding the *folB-folK-folP* domain architecture and provides evidence of active folate biosynthesis via a complete pterin branch in *Ac. castellanii*. *Acanthamoeba* can cause keratitis infection of the cornea linked to use of contaminated contact lenses (Radford et al. 1995). These data suggests the potential for antimicrobial agents that inhibit pterin branch of folate biosynthesis (e.g., sulfonamides and sulfones) as therapeutic treatment for *Acanthamoeba* keratitis or as an additive to eye-care and contact lens solutions to prevent infections. Exploiting metabolic differences between *Acanthamoeba* and the human host is a potentially important avenue to identify new antimicrobials and limit toxic effects (Roberts and Henriquez 2010), particularly in the eye. For example, sulphadiazine has been used to target different metabolic pathways for the successful inhibition of *Acanthamoeba* growth in vitro (Mehlotra and Shukla 1993) and encouraging reports of its use in vivo have been made in experimentally induced *Acanthamoeba* meningoencephalitis in mice (Rowan-Kelly et al. 1982) and in granulomatous amoebic encephalitis in AIDS patients (Seijo Martinez et al. 2000).

## Phylogenetic Evidence for Frequency of Gene Fusion and Fission Events

We conducted a series of phylogenetic analyses to investigate if the gene fusion characters were monophyletic and identify

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any cases of gene fissions. Our results demonstrate the presence of a complex pattern of gene loss (discussed above). Comparisons of the distribution of different folate fusion genes to the established Opisthokonta phylogeny (James et al. 2006; Torruella et al. 2012) combined with individual domain phylogenetic analyses suggest a minimum of nine gene fission events (five by fission through domain loss [deletion] and four by fission through separation and retention of a separate genes encoding the constituent domains) (fig. 3). These suggest that gene fissions occur at a high rate in this pathway and *folB-folK-folP* gene fusions are not stable characters. This is consistent with a growing body of data demonstrating that the process of gene/domain separation is an important factor in gene evolution (Kummerfeld and Teichmann 2005; Nakamura et al. 2007; Leonard and Richards 2012).

Next, we used phylogenetic analysis to polarize the ancestry of the *folB-folK-folP* gene fusion. Our phylogenetic analysis generally proved inconclusive, because we failed to recover tree resolution and specifically because there is no *Diaphoretickes* and *Excavata* orthologue of the Amoebozoa and Opisthokonta *folB* gene. Taken together the phylogenies, therefore, do not constitute an appropriate test of the monophyly of the three-domain gene fusion clade (i.e., Amoebozoa and Opisthokonta). Furthermore, as the individual folate pathway gene phylogenies were generally unresolved, it is possible that undetected cases of hidden paralogy, multiple *folB* tandem duplications, and HGT may have occurred in the evolution of this pathway. HGT is especially a concern as some literature suggests that gene clustering increases the possibility that genes become fixed by selection once they have undergone transfer. This is because they lead to the acquisition of functional modules, either as an operon and/or gene fusions (e.g., Andersson and Roger 2002; Slot and Rokas 2010, 2011). Such factors would therefore act to further complicate the evolution of this pathway, but at present are hard to quantify using single-gene phylogenies. As we saw no additional evidence for HGT other than that discussed (i.e., ancestral acquisition of the *folB* gene in the Archaeplastida and acquisition of a *folB-folK* gene fusion in *Branchiostoma* from a likely prokaryotic source), we use the more parsimonious interpretation of vertical inheritance to explain the gene distribution observed.

The phylogenies provided no strong support for the paralogy and convergent evolution of the three-domain gene fusion in the Amoebozoa and Opisthokonta. Therefore, in the absence of strong signal to support an alternative hypothesis and based on current taxonomic distribution of this character, we currently favour the null hypothesis that the *folB-folK-folP* three-domain gene fusion is monophyletic and arose once and before the diversification of the opisthokonts and amoebozoans. We do acknowledge that alternative hypotheses involving fissions and loss in the *Diaphoretickes* and *Excavata* taxa, or convergent gene fusions in the Amoebozoa

and Opisthokonta taxa are only slightly less parsimonious given current data. This is an important concern as our data demonstrated that this gene fusion is not a stable character, subject to frequent gene fission and partial and total gene loss. Consequently, perhaps the overriding message of this work is that rare-derived genomic characters, such as gene fusions, can be noisy and therefore these data should not be applied to resolving evolutionary relationships without testing their ancestry and susceptibility to homoplasy.

## Supplementary Material

Supplementary figures S1–S7 and tables S1–S4 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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# COMPLEX PATTERNS OF GENE FISSION IN THE EUKARYOTIC FOLATE BIOSYNTHESIS PATHWAY

Gene Fissions in Eukaryotic Folate Biosynthesis

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## Dispatches

## Organelle Evolution: A Mosaic of 'Mitochondrial' Functions

An ancient endosymbiosis of an  $\alpha$ -proteobacterium produced a diverse range of organelles including mitochondria. Reconstruction of the *Pygsuia biforma* proteome adds to the mosaic of functional systems present in mitochondrial-related organelles and demonstrates the role of horizontal gene transfer.

Finlay Maguire<sup>1,2,\*</sup>  
and Thomas A. Richards<sup>3</sup>

The endosymbiosis that gave rise to mitochondria is one of the key evolutionary innovations that marks the eukaryotic cell [1]. This organelle evolved from the endosymbiosis of an  $\alpha$ -proteobacterium prior to the divergence of all known eukaryotes and canonically acts as the main site of aerobic ATP generation in many organisms [2]. Mitochondria also play fundamental roles in several other aspects of cellular metabolism including apoptosis, amino acid metabolism, pyruvate decarboxylation, and the biosynthesis of folate, phospholipids, heme, and iron-sulphur clusters [3–5]. A diverse range of organelles known as mitochondria-related organelles (MROs), originating from the same endosymbiotic event, have been identified in disparate anaerobic and microaerophilic lineages across nearly every major phylogenetic subdivision of the eukaryotes (Figure 1A) [5–7]. As reported in this issue of *Current Biology*, Stairs and colleagues [3] have added to this complexity by characterising the putative proteome of an MRO from *Pygsuia biforma*, a recently discovered bryozoan species which branches below the radiation of the fungi and animals [8]. *Pygsuia* further complicates the categorisation of MROs as it encodes a mosaic tapestry of organellar functions and demonstrates that this large family of organelles has no core conserved proteome.

We now have a multitude of terms to describe these sibling organelles, e.g. MROs, mitochondria-like organelles, mitochondrial-derived organelles, hydrogenosomes, mitosomes, cryptons, and hydrogen-producing mitochondria. Attempts to

classify these diverse organelles phylogenetically have been abandoned as they have a broad and punctate distribution across the eukaryotes (Figure 1A). This suggests that MROs are likely the product of multiple independent evolutionary modifications of the same ancestral organelle. That said, Müller and colleagues have proposed a functional classification in which MROs are split into five classes on the basis of energy metabolism [6]: aerobic mitochondria (Class 1), canonical or 'text-book' mitochondria which use oxidative phosphorylation to generate ATP with oxygen as the terminal electron acceptor (e.g. *Saccharomyces cerevisiae*, *Arabidopsis thaliana*); anaerobically functioning mitochondria (Class 2), which generate ATP but use alternative electron acceptors such as fumarate or nitrate (e.g. *Ascaris lumbricoides*, *Trypanosoma brucei*); hydrogen-producing mitochondria (Class 3), which possess an electron transport chain and generate hydrogen while producing ATP via substrate-level phosphorylation (e.g. *Nyctotherus ovalis*, *Blastocystis* sp.); hydrogenosomes (Class 4), which produce hydrogen but do not possess an electron transport chain and can produce ATP via substrate-level phosphorylation (e.g. *Trichomonas vaginalis*, *Piromyces* sp.); and mitosomes (Class 5), organelles which do not produce ATP and lack any components of the electron transport chain (e.g. *Giardia intestinalis*, *Entamoeba histolytica*, *Encephalitozoon cuniculi*) [6].

The predicted proteome of the *P. biforma* MRO demonstrates that this organelle is difficult to place in the above schema as it contains both cardiolipin and phospholipid biosynthesis pathway enzymes [3]

which have only been detected previously in aerobic mitochondria (Class 1). Furthermore, this proteome indicates that the organelle has features of Class 2 anaerobic mitochondria, Class 3 hydrogen-producing mitochondria and Class 4 hydrogenosomes. Specifically, it has the protein repertoire to perform hydrogenosomal-like hydrogen production and pyruvate oxidation as well as partial components of an electron transport chain (such as quinol-reduction) [3]. This complicates functional classification, as this organelle appears to span Classes 2, 3 and 4 while containing elements common to Class 1. However, *P. biforma* is not the only MRO which defies easy categorisation; for instance, the *Mastigamoeba balamuthi* MRO has a partial electron transport chain while also generating hydrogen [9], placing it in a grey area between hydrogen-producing mitochondria and hydrogenosomes (Class 3 and 4) [5]. Another potential issue with this form of functional classification is where to classify MROs capable of facultatively modifying their energy metabolism, e.g. *Chlamydomonas reinhardtii* (which can act anaerobically [10]) or the *Trypanosoma brucei* mitochondrion (which utilises an alternative oxidase with a highly truncated electron transport chain and does not generate ATP when in the bloodstream of its host [11]). Collectively these data demonstrate, perhaps unsurprisingly, how difficult it is to apply a discrete classification to such a mosaic of organelles.

As energetics metabolism is likely the defining reason behind the acquisition and, in most cases, the maintenance of this endosymbiotic organelle, Müller and colleagues' classification has great utility when considering MROs purely from the perspective of energetics [1,12]. However, problems emerge when the scheme is generalised beyond its initial formulation, principally because energy generation is not a conserved unifying characteristic of MROs. Therefore, these sibling organelles

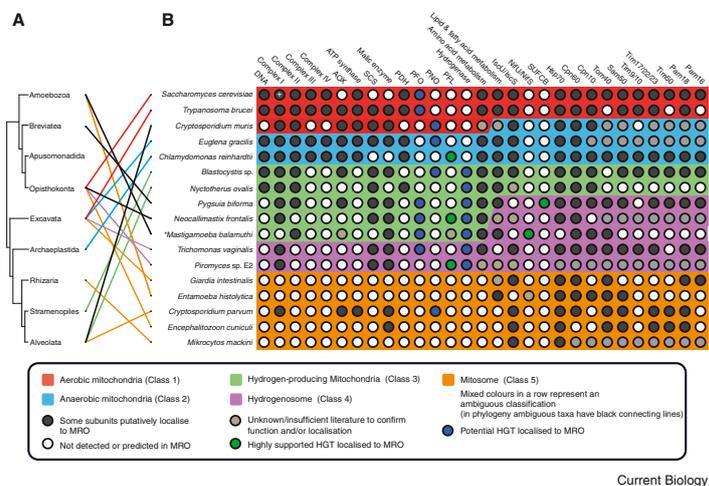


# ORGANELLE EVOLUTION: A MOSAIC OF MITOCHONDRIAL FUNCTIONS

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show complex patterns of gain and loss (Figure 1B), for example, gain of fumarate and nitrate based respiration [5]. The paper by Stairs *et al.* also provides another interesting example of function acquisition. Namely, the *Pygusua* MRO proteome has acquired a rhodoquinone biosynthesis protein. This potentially allows the completion of the *Pygusua* TCA cycle by producing a low electron potential quinone that would allow complex II to function as a fumarate reductase [3], like a Class 2 mitochondrion.

So if energy generation isn't a common feature of all MROs, are there any other alternative possibilities? Prime candidates would include the localisation and function of endosymbiotically derived chaperonin proteins (e.g. mtHsp70, mtCpn60), protein import machinery (e.g. Tim/Tom/Sam/Pam), and the biosynthesis of iron-sulphur clusters via the ISC system. However, in various MRO lineages there are clear examples of the loss of these features (Figure 1B) suggesting in the right circumstances these systems are also dispensable [9,13,14]. Even the ISC system, widely believed to be one of the essential conserved features of MROs, has been lost in *Entamoeba histolytica* [15] and *M. balamuthi* [16]. These amoeba have acquired an alternative means of iron-sulphur cluster biosynthesis in the form of an  $\alpha$ -proteobacterial-derived nitrogen fixation system [16,17]. Stairs and colleagues have also demonstrated the loss of the ISC system in the *P. biforma* MRO as well as the acquisition of an analogous archaeal sulphur-mobilisation protein[s] (SUF) via horizontal gene transfer (HGT) from *Blastocystis* sp. and/or Methanomicrobiales archaea. The SUF MRO protein is encoded by a gene fusion and possesses an amino-terminal MRO-targeting sequence. Localisation of this protein to the MRO was confirmed using fluorescence-microscopy methods, and this contrasts with *Blastocystis* sp. where the protein is localised in the cytosol [3]. This means ISC has been functionally replaced by this non-homologous HGT-acquired sulphur mobilisation system [3]. These data demonstrate that the conserved ISC MRO system, the only known function of some MROs (e.g. *Encephalitozoon cuniculi* [18]) and the only essential function of the yeast mitochondrion [19], is liable to loss.



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Figure 1. Mosaic of functions in mitochondria-related organelles.

(A) A sketch of the major groupings of the eukaryotic tree of life demonstrating the taxonomic membership (via lines) of various MRO-bearing lineages linked to (B). Phylogeny is a simplified version of the tree shown in [8]. The connecting lines are coloured to match the respective 'Class' of MRO sensu Müller [6] (see the key) with black lines linking to those lineages of ambiguous 'Class'. (B) A dot plot derived from similar tables in [5] and [6] showing the presence of a subset of proteins suggested/demonstrated to localise/function in MROs (some homologous proteins are not indicated as they putatively function in the cytosol of the taxa listed). Organisms are grouped by coloured boxes corresponding to their 'Class' as shown in the key. Strongly supported HGT events are highlighted in green with hypothesised HGTs in blue. Reference detail for data summarised in Figure 1 can be found in the form of a table at <http://richardslab.exeter.ac.uk/?p=212>. \**M. balamuthi* data are derived from a Sanger sequencing based EST project and thus proteins with relatively low expression such as the membrane translocases may not be detected. \**S. cerevisiae* complex 1 is potentially a non-canonical NADH oxidoreductase. (AOX, alternative oxidase; SCS, succinyl-coA synthetase; PDH, pyruvate dehydrogenase; PFO, pyruvate:ferredoxin oxidoreductase; PNO, pyruvate:NADP oxidoreductase; PFL, pyruvate formate lyase; ISC, iron-sulphur cluster biosynthetic system; NIF, nitrogen fixation biosynthetic system; SUF, sulphur mobilisation biosynthetic system; HSP, heat-shock protein; CPN, chaperonin; TOM, translocase of the outer membrane; TIM, translocase of the inner membrane; SAM, sorting and assembly machinery; PAM, presequence translocase associated motor.)

However, even in the rare cases of loss, the iron-sulphur cluster appears to be replaced with a system of analogous function. This implies that an ability to make proteins containing iron-sulphur clusters is a cellular necessity and that this process requires compartmentalisation in most eukaryotes [3,19] in contrast to prokaryotes.

The work of Stairs and co-authors [3] emphasises the prevalence of lineage-specific re-modelling of MROs as well as the role HGT plays in this process. *Pygusua* demonstrates that MROs do not display sets of discrete functional traits but instead are a product of a complex mosaic of diverse functions and evolutionary origins. Taking these data together (Figure 1), what emerges is a glimpse of an

ancestral organelle functioning in cellular energetics, facultatively anaerobic, aerobic and which may [12] or may not have [20] had hydrogenosomal functions.

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## Neuroethology: Self-Recognition Helps Octopuses Avoid Entanglement

How an octopus performs complex movements of its eight sucker-studded arms without entanglement has been a mystery. A new study has found that self-recognition of the octopus's skin by its suckers inhibits reflexive grasping of its own arms, simplifying the mechanisms needed to generate intricate arm behavior.

Robyn J. Crook and Edgar T. Walters\*

A couple learning to dance soon realizes how easy it is for eight active limbs to become entangled. The challenge to an octopus is far greater because each of its eight supple arms can bend in almost any direction from any point along its boneless length. Worse, the arms have numerous suckers that reflexively grasp whatever they touch. But with a few hundred million years more time than that available to human dancers to solve the limb entanglement problem, the octopus has evolved a solution based on a mechanism more familiar to immunologists than to neurobiologists: chemical self-recognition. In a study reported in this issue of *Current Biology*, Neshet et al. [1] demonstrate that a cue in the skin of octopus inhibits sucker attachment, helping to avoid inadvertent grasping of its own arms as

each arm performs its graceful routines.

Unlike human couples who struggle to synchronize movements commanded by just two brains, an octopus effectively has nine brains that have their own agendas: each of its eight arms has a large and relatively complete nervous system, which seems barely to communicate with the other arms [2,3]. The central brain sends general executive commands to all the arms at once, but these messages lack detailed instructions, leaving the individual arms remarkable autonomy to control their own movements [4,5]. Central encoding of arm position appears to be lacking; for example, somatotopically arranged sensory and motor representations of the octopus body within its brain are absent [6]. And while octopuses can learn to use visual feedback to guide an arm to a specific location [7], visual control of more than one arm at a time

is not apparent. So, without the brain or eyes telling each arm where it is and where the seven others are, some sort of local sensing and control are needed.

In a series of systematic experiments using the common octopus (*Octopus vulgaris*), Neshet et al. [1] first showed that the suckers of amputated octopus arms recognize skin from the same species. Suckers attached avidly to abiotic surfaces and to potential food items, but the suckers of amputated arms neither grasped skin of their own arm nor of other arms from the same octopus or other octopuses. Strong evidence for species recognition by individual suckers came from offering to amputated arms a petri dish containing a semi-circular slice of isolated skin covering half the glass: the suckers attached firmly to the glass, but adjacent suckers touching the skin refused to attach. What is the cue that tells an octopus sucker to avoid skin from its own species? Avoidance did not occur when the researchers presented amputated arms with skinned pieces of octopus arm, indicating that cues for species recognition are in the skin. Presentation of various skin extracts suggested that the cue molecules are hydrophobic, but their identity remains a mystery.

The new findings of Neshet et al. [1] are the first evidence for the use of a



## B.3 DIVERSE MOLECULAR SIGNATURES FOR RIBOSOMALLY ACTIVE PERKINSEA IN MARINE SEDIMENTS

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### RESEARCH ARTICLE

### Open Access

# Diverse molecular signatures for ribosomally 'active' Perkinsea in marine sediments

Aurélie Chambouvet<sup>1\*</sup>, Cédric Berney<sup>2,3</sup>, Sarah Romac<sup>2,3</sup>, Stéphane Audic<sup>2,3</sup>, Finlay Maguire<sup>1</sup>, Colombar De Vargas<sup>2,3</sup> and Thomas A Richards<sup>4</sup>

#### Abstract

**Background:** Perkinsea are a parasitic lineage within the eukaryotic superphylum Alveolata. Recent studies making use of environmental small sub-unit ribosomal RNA gene (SSU rDNA) sequencing methodologies have detected a significant diversity and abundance of Perkinsea-like phylotypes in freshwater environments. In contrast only a few Perkinsea environmental sequences have been retrieved from marine samples and only two groups of Perkinsea have been cultured and morphologically described and these are parasites of marine molluscs or marine protists. These two marine groups form separate and distantly related phylogenetic clusters, composed of closely related lineages on SSU rDNA trees. Here, we test the hypothesis that Perkinsea are a hitherto under-sampled group in marine environments. Using 454 diversity 'tag' sequencing we investigate the diversity and distribution of these protists in marine sediments and water column samples taken from the Deep Chlorophyll Maximum (DCM) and sub-surface using both DNA and RNA as the source template and sampling four European offshore locations.

**Results:** We detected the presence of 265 sequences branching with known Perkinsea, the majority of them recovered from marine sediments. Moreover, 27% of these sequences were sampled from RNA derived cDNA libraries. Phylogenetic analyses classify a large proportion of these sequences into 38 cluster groups (including 30 novel marine cluster groups), which share less than 97% sequence similarity suggesting this diversity encompasses a range of biologically and ecologically distinct organisms.

**Conclusions:** These results demonstrate that the Perkinsea lineage is considerably more diverse than previously detected in marine environments. This wide diversity of Perkinsea-like protists is largely retrieved in marine sediment with a significant proportion detected in RNA derived libraries suggesting this diversity represents ribosomally 'active' and intact cells. Given the phylogenetic range of hosts infected by known Perkinsea parasites, these data suggest that Perkinsea either play a significant but hitherto unrecognized role as parasites in marine sediments and/or members of this group are present in the marine sediment possibly as part of the 'seed bank' microbial community.

**Keyword:** 454 pyrosequencing, *Perkinsus*, *Parvilucifera*, Food web, Protist, Parasite

#### Background

Environmental DNA (eDNA) analyses have demonstrated that diversity records are missing significant data regarding protists (reviewed in: [1-3]). Parasitic protists are a key component of food webs, yet the role and diversity of these groups is often unknown (e.g. [4-6]). The protist 'superphylum' Alveolata includes numerous polyphyletic groups of parasites [7], for example: Apicomplexa, Perkinsea (also named perkinsids or Perkinsozoa) and Syndiniales

(including both marine alveolate group I and II [also sometimes called MALVI & MALVII a]) [4,8].

Molecular surveys have shown that Perkinsea-like sequences can be diverse and abundant in freshwater lakes, suggesting this group plays an important role in freshwater food webs [5,9-11]. However, most freshwater Perkinsea have still not been characterised ecologically or morphologically, with one exception, a recently identified Perkinsea-like protist linked to local mortality events of the Southern Leopard frog *Rana sphenocephala* in the USA in 2003 [12]. Analysis of the SSU rDNA sequence of this protist suggests that this infectious agent branches close to the Perkinsea in SSU rDNA phylogenies within

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a cluster consisting of only freshwater environmental sequences [12-14]. With the exception of the Perkinsea associated with frog infections, all morphological descriptions and cultured representatives of the Perkinsea are derived from two marine genera: *Perkinsus* and *Parvilucifera*.

*Perkinsus* is a group of parasites infecting molluscs and includes *P. marinus*, the main cause of mortality of bivalves leading to the economically important shellfish disease 'Dermo' [15]. *Parvilucifera* spp. are known to infect up to 26 different dinoflagellates, playing a role in species succession, for example, infecting dinoflagellates that cause red-tides [16]. Taken together these data suggest that the Perkinsea phylum is a diverse group of parasites infecting a wide range of species such as: molluscs, amphibians and dinoflagellates [14].

Numerous clone library surveys of eukaryotic diversity in marine waters have now been published (e.g. [17-22]) yet only a few Perkinsea sequences have been identified. Specifically, only nine sequences belonging to Perkinsea that are distinct from either *Perkinsus* or *Parvilucifera* cluster groups are currently available in GenBank (May-2013). To our knowledge, most of the environmental surveys of marine environments have, however, focused on sub-surface or deep chlorophyll maximum (DCM) water column samples, with only a few studies sampling sediments (e.g. [18,23,24]). As such, marine sediments are often thought of as a 'black box' in terms of microbial diversity and function [25], lacking in eukaryotic-specific molecular surveys (e.g. [18,23]). Furthermore, the majority of these publications use clone library survey methods and therefore give only a partial view of biodiversity. In contrast second-generation sequencing of environmental sequence tags theoretically allow deeper surveys of microbial biodiversity allowing the detection of low abundance microbes [26,27]. In this paper we use second generation sequencing methods to evaluate the diversity of the Perkinsea in multiple marine environments and test the hypothesis that the Perkinsea are hitherto under sampled group in marine environments.

## Results and discussion

### Processing of sequence data

Using 454 pyro-sequencing, we investigated the diversity of Perkinsea in a selection of European marine samples, sequencing the V4 region of the SSU rDNA [27] using both rDNA and rRNA as template. A similar DNA-based approach has been used to investigate freshwater Perkinsea [14]. We obtained sequence data from samples collected in four European coastal sites (Figure 1A), including sediment and multiple size filtrates from the sub-surface and the DCM water column samples (using a plankton net for the 2000-20  $\mu\text{m}$  fraction and sequential filtration for 3-20  $\mu\text{m}$  and 0.8-3  $\mu\text{m}$  fractions). All V4 sequence reads (~380 bp) were assigned to eukaryotic taxonomic groups

using a custom-built pipeline developed by the BioMark consortium (see [28]). This analysis identified 271 sequences preliminarily classified as Perkinsea.

The taxonomic affiliation of these sequences was checked using phylogenetic analyses of a SSU rDNA dataset comprising representative alveolate groups (Additional file 1: Figure S1 and Additional file 2: Table S1) and environmental sequences retrieved from GenBank (Additional file 3: Table S2). Of the 271 sequences initially identified as Perkinsea, alignment based analyses and phylogeny confirmed that 265 individual 454 'tag' sequences were not chimeras [29] and branched with known Perkinsea sequences. Moreover, a large number of the 265 sequences were highly similar and so were clustered at 99% identity resulting in 150 unique V4 sequences branching within, or close to, Perkinsea taxonomic groups (Additional file 1: Figure S1). Table 1 summarises the provenance of the sequences sampled and provides information regarding the total % of Perkinsea sequences within each V4 sequence dataset, which ranges from 0.244% to 0.006% of the 454 sequencing effort from each of the environments sampled.

### Diversity within marine Perkinsea

To investigate the diversity and environmental distribution of the Perkinsea-like sequence tags, we conducted a phylogenetic analysis focusing on the V4 region and including the 150 sequence clusters identified (Figures 2 and 3). The regions flanking the variable V4 region are relatively conserved, while V4 stems and loops are variable [27,30]. The phylogeny was derived from a masked alignment of 330 characters and included a mixture of sites with fast and slow patterns of variation. As our analysis was limited to the V4 region the deep and intermediate nodes of the phylogeny are poorly resolved so that the tree is only helpful for demonstrating the diversity of Perkinsea-like sequences and not the internal topology of the Perkinsea group, consistent with the aim of this study.

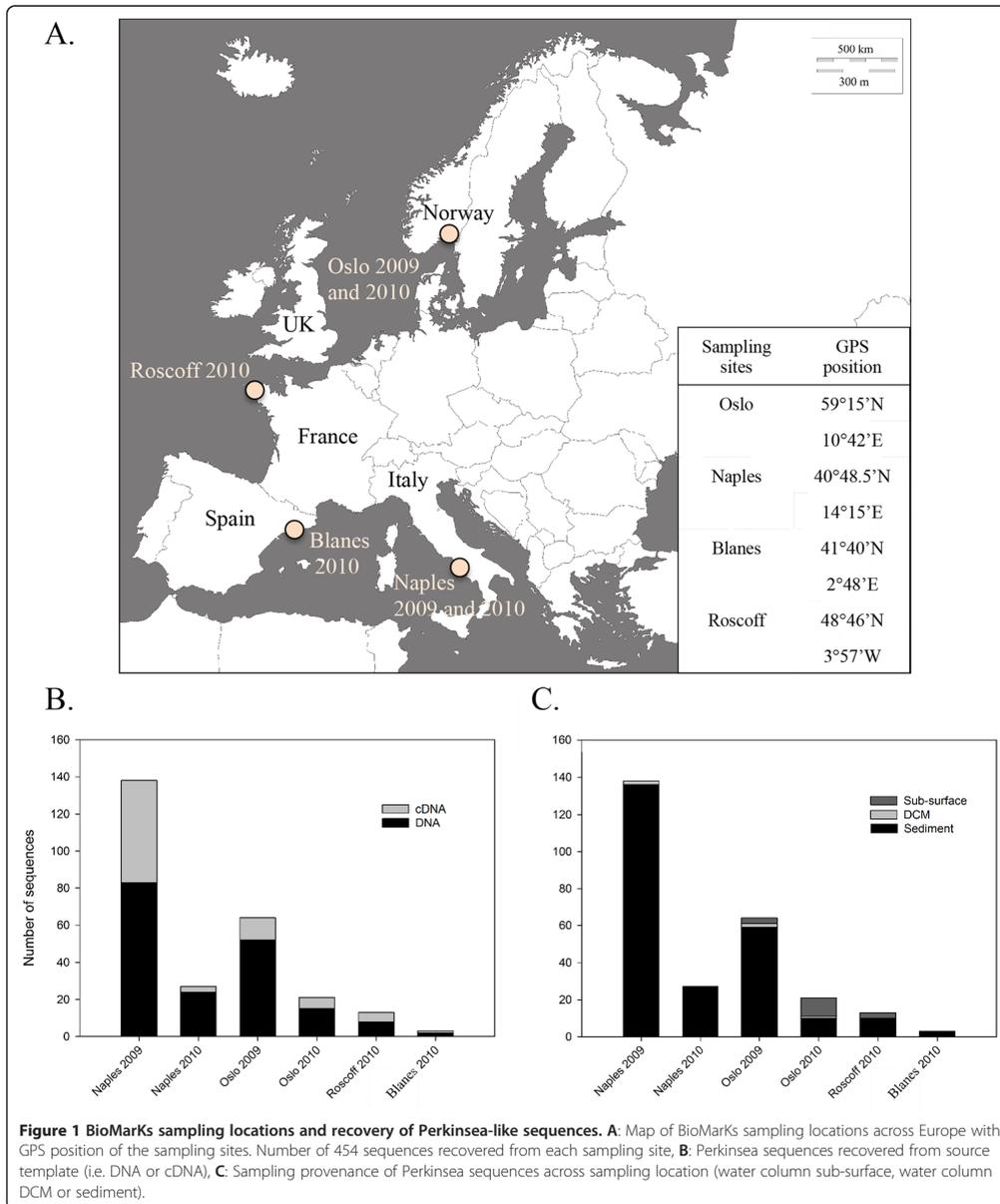
To identify a conservative picture of Perkinsea diversity we classified the sequence diversity into 'cluster-groups' on the basis of two restrictive criteria: 1) moderate topology support (>0.6/60%/60%) and 2) possession of two sequences from separate samples. Using this approach we identified 38 phylogenetic clusters labeled as cluster 1-38 on Figures 2 and 3 in addition to the morphologically characterised *Perkinsus* and *Parvilucifera* groups. 30 of these clusters represent previously undescribed marine diversity-groups. Additionally, 42 unique sequence clusters (28% - labeled with circles on the right column in Figures 2 and 3) were not grouped into 'cluster-groups' using our classification criteria.

The 30 new marine cluster groups show no more than 97% sequence identity between each group, suggesting they constitute taxonomically distinct groups. In contrast, the four described species that belong to *Perkinsus* spp.

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have highly similar SSU rDNA sequences (>98%, [31]). If the pattern of SSU rDNA variation in *Perkinsus* species is consistent across the wider diversity detected here, then these 30 cluster groups are likely to represent a diversity

of forms with distinct biological and/or ecological traits, i.e. given the biology of the known *Perkinsea* species the diversity of sequences detected here putatively represent parasites infecting a range of host organisms.

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**Table 1 Summary of samples used for 454 sequencing**

Geographic site	Year	Depth	Size fraction	DNA	RNA
Naples	2009	Sediment	Total	84 (0.243%)	54 (0.264%)
		DCM <sup>a</sup>	0.8-3 μm	2 (0.008%)	0
		Sub-surface	-	0	0
Naples	2010	Sediment	Total	24 (0.141%)	3 (0.043%)
		DCM <sup>a</sup>	-	0	0
		Sub-surface	-	0	0
Oslo	2009	Sediment	Total	50 (0.145%)	7 (0.024%)
		DCM <sup>a</sup>	0.8-3 μm	1 (0.006%)	0
			3-20 μm	1(0.007%)	0
		Sub-surface	0.8-3 μm	3 (0.032%)	0
Oslo	2010	Sediment	Total	9 (0.056%)	1 (0.008%)
		DCM <sup>a</sup>	0.8-3 μm	1 (0.007%)	0
		Sub-surface	0.8-3 μm	3 (0.019%)	0
			20 μm-total	3 (0.021%)	4 (0.034%)
Barcelona	2010	Sediment	Total	1 (0.105%)	1 (0.023%)
		DCM <sup>a</sup>	-	0	0
		Sub-surface	-	0	0
Roscoff	2010	Sediment	Total	8 (0.112%)	2 (0.069%)
		DCM <sup>a</sup>	-	0	0
		Sub-surface	0.8-3 μm	3 (0.027%)	0

The table includes the number of Perkinsea sequences recovered while the figures given in the brackets correspond to the percentage of Perkinsea sequences compared to the total sequencing effort for each sample.

<sup>a</sup>Deep chlorophyll maximum.

## Marine freshwater transitions

A growing body of literature has addressed the frequency in which protist groups have spread between marine and freshwater environments (e.g. [32,33]) with varying perspectives on the number and relative 'ease' of these transitions dependant on the group studied and the criteria used for identifying these transitions [14,32]. As described by Bråte *et al.* in 2010, marine-freshwater transitions are likely to have occurred during the diversification of the Perkinsea [14]. Our phylogenetic analyses identified the distribution of sequences recovered from both marine and freshwater environments demonstrating eight putative transitions on the phylogeny (five into freshwater and 3 into marine environments - Figures 2 and 3). However, we note that only three of these transitions are resolved in our V4 phylogenies with bootstrap support in excess of 50%. As such, additional sequencing from a range of environments combined with robust multi-gene phylogenetic analyses is required to characterise the frequency of freshwater-marine environmental transitions within the Perkinsea.

## The majority of marine Perkinsea diversity is recovered from sediments

244 (92%) of the V4 sequences classified as Perkinsea were recovered from sediment. Moreover, 27% of the

total sequencing effort were sampled from RNA derived cDNA libraries (Figure 1B,C and Table 1) suggesting that a significant proportion of the Perkinsea sequences were recovered from ribosomally active and intact cells. A large proportion of published environmental sequences are derived from DNA, a method that potentially detects dead organisms or extracellular DNA [34]. This is an issue arising from eDNA sequence surveys of sediment/soil environments. In contrast, extracellular RNA is thought to be less stable so that the use of rRNA can be useful for identifying ribosomally active microbes, inferring intact cells, but not distinguishing between active, senescent, dormant or encysted cells [35]. Therefore, we cannot exclude the possibility that the Perkinsea detected here are in a dormancy period or 'dying' whilst still maintaining transcription of a detectable RNA profile. However, these analyses identify a diverse range of ribosomally active Perkinsea in marine sediments, while in contrast recovering very little evidence of Perkinsea in the water column.

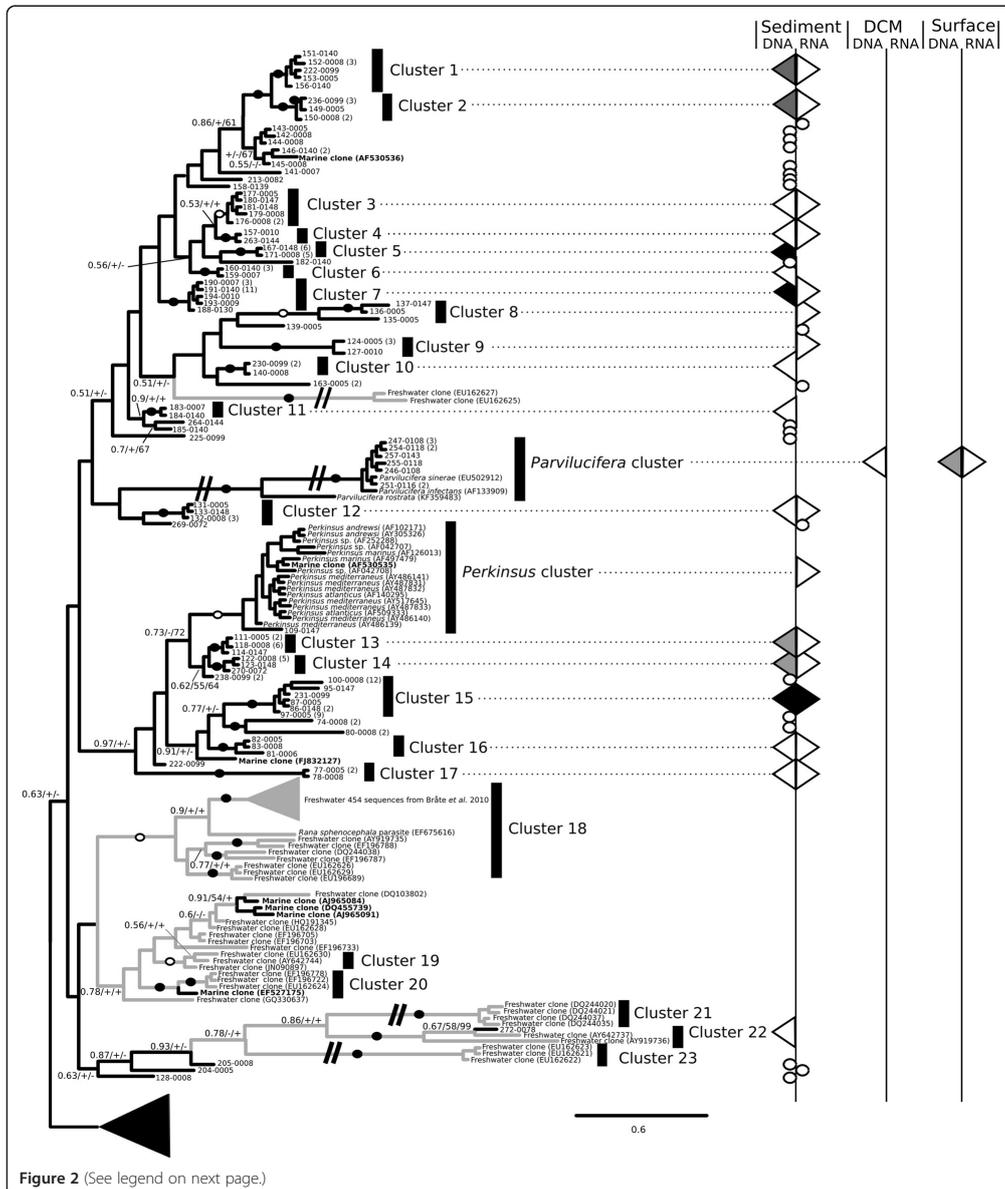
## Conclusions

It has previously been suggested that Syndiniales, including Marine Alveolata group I and II, predominate as parasitic protists in marine waters [36] while it has been suggested

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**Figure 2 Bayesian phylogenetic tree of Perkinsea diversity using the V4 region of SSU rDNA gene (part 1) and provenance of the Perkinsea BioMarKs sequences.** Subsection of the phylogenetic tree is shown (see Figure 3 for the rest of the phylogeny). Bayesian posterior probability, Maximum Likelihood bootstrap (1,000 replicates), and LogDet distance bootstrap (1,000 replicates) values are added at each node using the following convention: support values are summarised by black circles on the branch when support are equal to or higher than 0.9/80%/80% and ringed circles when bootstrap values are between 0.6/60%/60% and 0.9/80%/80%. When the bootstrap value is below 60%, a "+" is added if the topology of the tree is recovered in the ML and LogDet analyses. A "-" is shown when these tree topologies are not consistent. Nine sequences of Dinoflagellata and five sequences of Marine Alveolata group II were used as an outgroup. Branches shortened by 1/2 are labelled with a double slashed line. The black and grey branches on the tree indicate marine and freshwater lineages respectively. Distribution and provenance of sequences across RNA and DNA derived libraries are illustrated down the right columns as shaded triangles if they represent a cluster group. Number in brackets refers to multiple identical sequence reads from the same sample. Circles are used to represent the provenance of a single environment unique sequence cluster. The colour of triangles designates the number of sequences recovered from each location (surface, DCM and sediment) or rDNA/rRNA for each cluster group. White represent between 0 and 5 sequences, Grey between 6–10 and black higher than 10. For correspondence between the 17 freshwater cluster groups identified by Bråte *et al.* (2010) and the 5 freshwater cluster groups identified in our analyses see Additional file 4: Table S3.

that in freshwater environments Perkinsea might play analogous roles in terms of diversity and abundance [5,8,14]. The data presented here indicate that the situation is not so clear-cut and supports the conclusion that Perkinsea are a hitherto under-sampled, diverse, widespread and active group in marine sediments, although the abundance and diversity appears to be somewhat lower than that observed for Syndiniales in marine water column samples [4,8,10,36]. In contrast, the Perkinsea, apart from previously described groups, appear largely absent in the four European marine water columns sampled here. Although we note that absence in the water column may be an artefact produced by: 1) limited detection of Perkinsea by the sequencing methods employed here which is likely to be abundance-dependant and therefore prone to miss low abundance groups, and 2) an incomplete sampling of the environments, for example exclusion of certain size fractions, time series, and sampling across a diversity of abiotic gradients.

These results are based on 454 methods targeting a broad spectrum of eukaryotes followed by bioinformatics extraction of Perkinsea-like sequences. Such approaches can lead to partial detection of target groups, dependant on level of sequence saturation achieved and comprehensiveness of the primers selected. In reality achieving single gene-marker primers that allow comprehensive sampling combined with sample saturation is experimentally difficult, unless a narrow group is targeted. As such, future work should incorporate a multiple -group specific- primer approach in order to improve sampling of Perkinsea diversity and map their environmental distribution. A major challenge of future work is to elucidate the ecological roles of this diversity of Perkinsea putative parasites revealed by eDNA surveys.

## Methods

### Sampling

Four European coastal stations were sampled (Figure 1A) as part of the work of the BioMarKs consortium (<http://biomarks.scrol.fr>): offshore Oslo (Norway, GPS position 59°15'N, 10°42'E), Naples (Italy, GPS position 40°48.5'

N, 14°15'E), Blanes near Barcelona (Spain, GPS position 41°40'N, 2°48'E) and Roscoff (France, GPS position 48°46'N, 3°57'W). Each station was sampled over three depths (sediment, DCM and sub-surface) using the same sampling protocol (as described in [37] - Figure 1A). Environmental conditions and sampling area are described in [28]. Briefly, 30 to 50 litres of seawater were collected at the sub-surface and DCM either using a plankton net (for the fraction between 20–2,000 µm) or using Niskin bottles (for sampling of fractions less than 20 µm) coupled to a CTD sensor. Water samples were then size-fractionated using different pore size polycarbonate filters of 142 nm diameter (20 µm, between 3–20 µm and finally between 0.8–3 µm). Each filter was flash frozen and stored at –80°C for further analysis. Sediment samples were taken from the sediment core. Small aliquots of the surface sediment material (~1 cm<sup>3</sup>) were frozen and stored at –80°C for molecular analysis.

### DNA/RNA extraction and 454 tag sequencing

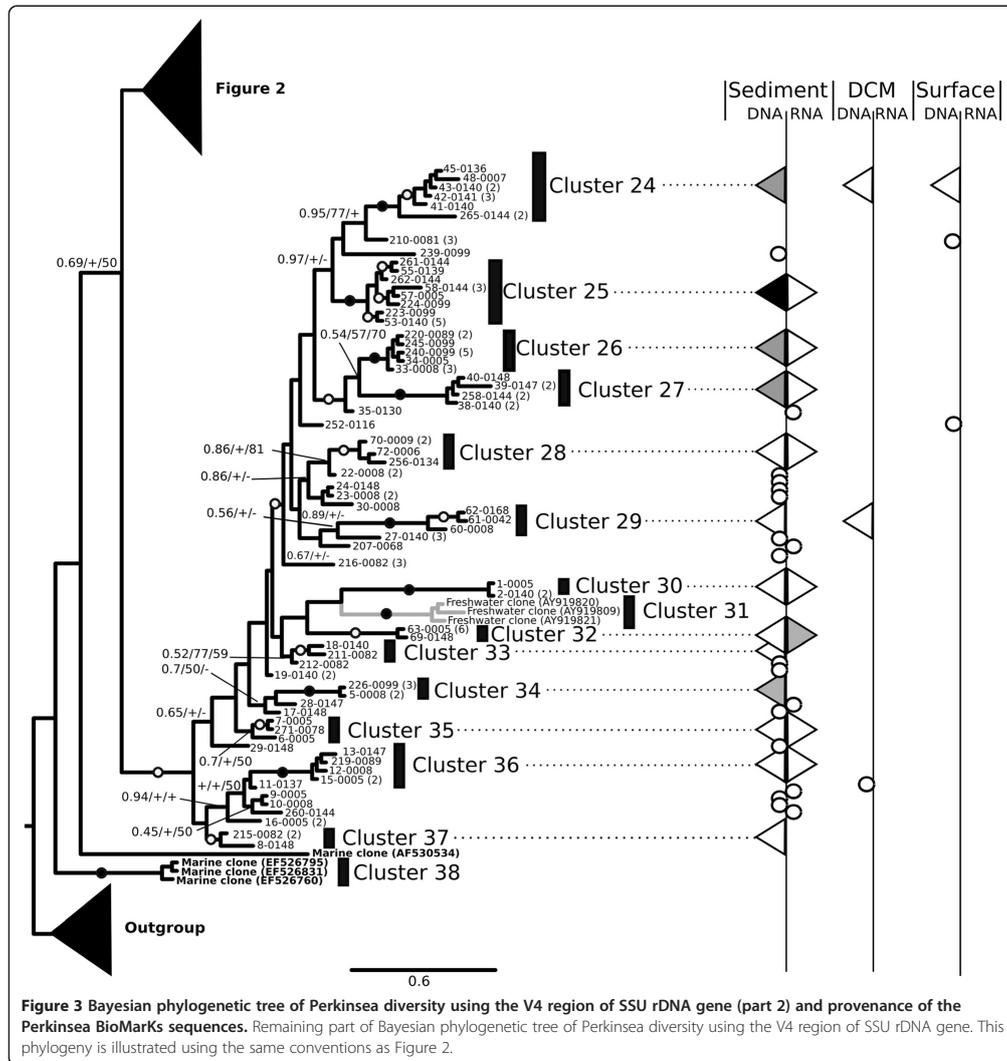
For water column samples, DNA and RNA were extracted simultaneously using the NucleoSpin RNA L kit (Macherey-Nagel, Düren, Germany). For sediment samples, DNA and RNA were isolated using the PowerMax Soil DNA Isolation kit and the PowerSoil total RNA Isolation kit (MoBio, USA). DNA and RNA quality were confirmed using gel electrophoresis (1.5% agarose gels) and quantified using a NanoDrop ND-1000 Spectrophotometer. To avoid contamination by DNA in the RNA extractions, DNase from the TurboDNA kit (Ambion, Carlsbad, CA, USA) was used to remove traces of DNA. Extracted RNA (100 ng) was reverse transcribed into cDNA using random primers and the Superscript III RT kit (Invitrogen, Carlsbad, CA, USA) following the protocol outlined by the manufacturer.

Universal eukaryotic primers TAREuk454FWD1 (5'-CCA GCASCYGC GGTAATTCC-3') and TAREukREV3 (5'-AC TTTCGTTCTTGATYRA-3') were used to sample the V4 region (~380 bp) of the SSU rDNA [27] using polymerase chain reaction (PCR) amplification. The primers were

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adapted for 454 sequencing with an A-adapter-tag forward and a B-adapter reverse as outlined in the 454 sequencing instructions. PCRs were performed in 25  $\mu$ l mixtures of 1X Master Mix fusion High Fidelity DNA polymerase (Finnzymes, Thermo Scientific, Espoo, Finland), 0.35  $\mu$ M of each primer, 3% dimethyl sulfoxide and 5 ng of template DNA or cDNA. PCR reactions consisted of an initial denaturation step at 98°C for 30s, followed by 10 cycles of: 10s at 98°C, 30s at 53°C and 30s at 72°C and then 15 cycles of 10s at 98°C, 30s at 48°C and 30s at 72°C. All PCR products

were conducted in triplicate, checked using agarose gel electrophoresis (1.5% agarose gels), pooled and purified using NucleoSpinExtract II (Macherey-Nagel, Düren, Germany), eluted in 30  $\mu$ l of water, and quantified using NanoDrop ND-1000 spectrophotometer. A final quantity of 200 ng of PCR product was then selected for 454 sequencing. Amplicon sequencing was carried out using a 454 GS FLX Titanium system (454 life sciences, Branford, USA) installed at Genoscope (<http://www.genoscope.cns.fr>), France.

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## Analysis of 454 reads of the V4 area SSU and phylogenetic analysis

Only reads with exact forward and reverse primer sequences and an estimated sequence error of  $\leq 0.1\%$  were retained for further analysis. Reads were assigned to taxonomic groups by co-clustering of sample sequences with those from a custom-built reference SSU rDNA database PR<sup>2</sup> [38] truncated to the V4 region. Reads were assigned to the Perkinsea when they were more similar to a reference Perkinsea sequence than to any other sequence in the PR<sup>2</sup> database, in terms of global alignment identity. This process identified 271 sequences tentatively classified as Perkinsea (each sequence has been labelled with a sequence number followed by the sequencing ID, see Additional file 5: Table S4 for details).

All existing Perkinsea SSU rDNA sequences (both environmental and from cultured organisms) plus a selection of 31 published sequences that encompass all the other major Alveolata lineages were recovered from the NCBI non-redundant nucleotide database and assembled into a reference dataset of 67 sequences (Additional file 2: Table S1 and Additional file 3: Table S2). The Perkinsea 454 V4 sequences were aligned to the previous reference dataset using Muscle [39], as implemented through the multiple alignment-editing program Seaview [39,40]. The alignment was then improved manually, with particular attention to the V4 region. Ambiguously aligned characters were masked and excluded from the alignment prior to phylogenetic analysis. A preliminary tree was used to identify long-branch or highly novel sequences that could potentially represent chimerical sequences. Candidate chimerical sequences were investigated further by visual inspection of the alignment according to methods described by Berney and co-authors [29]. Of the 271 putative Perkinsea 454 tags identified from the Biomarks dataset, 265 marine V4 454 sequences branched with the Perkinsea and were retained for the final analyses (Table 1 and Additional file 5: Table S4). All 271 sequences are available in the European nucleotide archive (<https://www.ebi.ac.uk/ena>) under accession numbers PRJEB5698. We have included the six putative chimeras in the submission so these can be checked historically, these six sequences have the reference numbers 37–0005, 54–0139, 134–0005, 138–0140, 206–0147 and 268–0287.

Two datasets were then created, with different alignment masks: 1) a dataset encompassing the complete SSU rDNA sequence alignment and including a wide selection of Alveolata lineages (Alveolata SSU dataset composed of 1,437 positions and 377 sequences) and 2) a second dataset restricted to an alignment mask of the V4 region and focusing only on the Perkinsea phylotypes including sequences from Bråte *et al.* [14] (Perkinsea V4 dataset; 330 positions and 351 sequences). As the 454 sequences only encompassed the V4 region, for the first alignment, all

missing positions in the Alveolata SSU dataset were encoded as gaps (consistent with the approach used in [14]). Prior to phylogenetic analyses we used the program Modelgenerator v0.85 [41] to determine the best model parameters for the two datasets. For the Alveolata SSU dataset a general time reversible model was selected with a discrete gamma distribution of the substitution rates (8 categories) and a proportion of invariable sites of 0.14 (GTR +  $\Gamma$  + I; gamma distribution shape parameter of 0.32). For the Perkinsea V4 dataset a GTR +  $\Gamma$  model was selected, with a gamma distribution shape parameter of 0.32 and 8 rate categories.

We then conducted Bayesian analyses using MrBayes v3.2.1 [42]. For both datasets we used the covarion parameter and a  $\Gamma$  rate correction with  $nst = 6$  (equivalent to the GTR substitution model). The chains were run for 5,000,000 generations with two replicate tree searches both with 4 chains with a heat parameter of 2. Trees were sampled every 250 generations. In both analyses the MrBayes runs reached a stationary phase by 500 generation samples, and so the first 500 samples were discarded (as the burnin), and a consensus topology calculated from the remaining trees. For both analyses, the covarion model was compared to the non-covarion via Bayesian model comparison. This should be done using Bayes factors (the ratio of the respective marginal likelihoods for the two models) [43]. Unfortunately, the high dimensionality of parameter space makes the marginal likelihood term computationally intractable to evaluate directly. Therefore, the simplest, if somewhat imperfect, method of estimating the marginal likelihood is that of the modified [44] harmonic mean estimator [45] as implemented in the Trace package v1.4 [46] using 1,000 bootstrap pseudo-replicates. These analyses demonstrate that the use of covarion parameters produced an improved tree search (Additional file 6: Table S5).

For both datasets, support for the tree topology was evaluated by the bootstrap method and using the Bayesian posterior probabilities (PP) from the MrBayes runs [42]. Bootstrap support values (BV) were estimated using RAxML v7.0.3 [47], with 1,000 pseudo-replicates. For the Perkinsea V4 dataset, we also conducted a LogDet distance analysis [48] with 1,000 pseudo-replicates, as implemented in the Seaview [40] tree calculation module. This extra analysis was included to account for the possibility of compositional biases in the sequences [49]. We did not conduct LogDet analysis for the Alveolata SSU dataset because the large number of missing characters resulted in poor bootstrap results (which was not an issue for the likelihood and Bayesian analyses).

## Additional files

**Additional file 1: Figure S1.** Bayesian phylogeny of Alveolata SSU sequences based on the analysis of 98 sequences of 1470 bp and 265 partial sequences from BioMarks V4 sequencing project (~278 bp in length).

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Posterior probability values and Maximum Likelihood bootstrap values were added at each node (pp/ML bootstrap support). Support values are summarised by black circles on the branch when they are equal to or higher than 0.90/80% and white circle when bootstrap values are between 0.6/60% and 0.9/80. Three ciliates sequences were used as the outgroup. Taxon names are consistent with Bråte *et al.* 2010. MA corresponds to Marine Alveolates. Arrow identifies the monophyletic Perkinsea clade. Complex clusters of 454 sequences have been reduced to representative triangles, see Figures 2 and 3 for complete phylogenetic data.

**Additional file 2: Table S1.** Details of published sequences of 18S rDNA used in phylogenetic analysis.

**Additional file 3: Table S2.** Published environmental sequences of 18S rDNA belonging to Perkinsea (Alveolata) used in phylogenetic analysis. Sequences sampled from marine environments are highlighted in grey.

**Additional file 4: Table S3.** Correspondence between newly described clusters from the present study and the previous studies [14].

**Additional file 5: Table S4.** Description of the V4 sequences ID labeling. Each sequence has been labeled by a unique number was given in Figures 2 and 3 (named X) followed by the sampling ID labeling.

**Additional file 6: Table S5.** Bayesian model comparison for method selection in phylogenetic inference.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

For the BioMark's project, SR carried out the molecular work and SA the global bioinformatics analyses. CdV and TAR are PIs on the BioMark's project. AC and CB constructed the sequence alignment. AC and TAR analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

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## B.4 CRYPTIC INFECTION OF A BROAD TAXONOMIC AND GEOGRAPHIC DIVERSITY OF TADPOLES BY PERKINSEA PROTISTS



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# Cryptic infection of a broad taxonomic and geographic diversity of tadpoles by *Perkinsea* protists

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The decline of amphibian populations, particularly frogs, is often cited as an example in support of the claim that Earth is undergoing its sixth mass extinction event. Amphibians seem to be particularly sensitive to emerging diseases (e.g., fungal and viral pathogens), yet the diversity and geographic distribution of infectious agents are only starting to be investigated. Recent work has linked a previously undescribed protist with mass-mortality events in the United States, in which infected frog tadpoles have an abnormally enlarged yellowish liver filled with protist cells of a presumed parasite. Phylogenetic analyses revealed that this infectious agent was affiliated with the *Perkinsea*: a parasitic group within the alveolates exemplified by *Perkinsus* sp., a “marine” protist responsible for mass-mortality events in commercial shellfish populations. Using small subunit (SSU) ribosomal DNA (rDNA) sequencing, we developed a targeted PCR protocol for preferentially sampling a clade of the *Perkinsea*. We tested this protocol on freshwater environmental DNA, revealing a wide diversity of *Perkinsea* lineages in these environments. Then, we used the same protocol to test for *Perkinsea*-like lineages in livers of 182 tadpoles from multiple families of frogs. We identified a distinct *Perkinsea* clade, encompassing a low level of SSU rDNA variation different from the lineage previously associated with tadpole mass-mortality events. Members of this clade were present in 38 tadpoles sampled from 14 distinct genera/phylogroups, from five countries across three continents. These data provide, to our knowledge, the first evidence that *Perkinsea*-like protists infect tadpoles across a wide taxonomic range of frogs in tropical and temperate environments, including oceanic islands.

frog decline | emerging disease | parasite | alveolates | molecular diversity

It is widely recognized that amphibians are among the most threatened animal groups: for example, in 2008, 32% of species were listed as “threatened or extinct” and 42% were listed as in decline ([www.iucnredlist.org/initiatives/amphibians/analysis](http://www.iucnredlist.org/initiatives/amphibians/analysis); accessed October 29, 2014) (1, 2). The main causes of amphibian decline have been identified as habitat loss, environmental change, and the introduction of nonnative species (e.g., refs. 3–6). Emerging infectious diseases have also been shown to play a key role in many amphibian declines: for example, the chytrid fungal pathogen *Batrachochytrium dendrobatidis* has caused mass mortality events (MMEs) in Australia, in Europe, and across the Americas (e.g., refs. 7–9). MMEs have also been associated with infection by *Ranavirus* in, for example, the United Kingdom (UK), United States (US), and Canada (10, 11). Recent work has linked local MMEs in the United States with the infection of larval frogs (tadpoles) of the genera *Lithobates* and *Acris* by a protist (*SI Appendix, Fig. S1*) (12, 13). In 2006, histological examinations of tadpole tissues revealed the presence of thousands of small

spherical cells preferentially infecting livers of tadpoles of the Southern Leopard Frog (*Lithobates sphenoccephalus*, formerly *Rana sphenoccephala*) sampled from an MME in Georgia (United States) (12). Small subunit (SSU) ribosomal DNA (rDNA) PCR and direct-amplicon sequencing, combined with phylogenetic tree reconstruction, showed that a lineage of protists closely related to *Perkinsus*, a parasite of marine bivalves (14), was the likely infectious agent (12).

*Perkinsea* alveolates were first described as being affiliated with the Apicomplexa (14, 15), which includes important human pathogens such as *Toxoplasma gondii* and *Plasmodium* spp. (the causative agents of malaria). Phylogenetic analysis has shown that *Perkinsea* are a deeply divergent sister-group of dinoflagellate alveolates (16). Only three representative groups of *Perkinsea* were previously described morphologically and taxonomically: *Perkinsus* spp., parasites of marine bivalves (e.g., oysters and clams), *Parvilucifera* spp., parasites of dinoflagellates, and *Rastromonas subtilis* (previously *Cryptophagus subtilis*), parasites of cryptophyte algae (17–20). However, environmental sequence

### Significance

Amphibians are among the most threatened animal groups. Population declines and extinctions have been linked, in part, to emerging infectious diseases. One such emerging disease has been attributed to *Perkinsea*-like protists causing mass mortality events in the United States. Using molecular methods, we evaluated the diversity of *Perkinsea* parasites in livers sampled from a wide taxonomic collection of tadpoles from six countries across three continents. We discovered a previously unidentified phylogenetically distinct infectious agent of tadpole livers present in a broad range of frogs from both tropical and temperate sites and across all sampled continents. These data demonstrate the high prevalence and global distribution of this infectious protist.

Author contributions: A.C. and T.A.R. designed research; A.C. and D.J.G. performed research; D.J.G., M.J., A.K.D., and G.B.B.-S. contributed new reagents/analytic tools; A.C., G.L., F.M., T.M.D.-B., and T.A.R. analyzed data; A.C., D.J.G., M.J.Y., M.W., and T.A.R. wrote the paper; and D.J.G., M.J., M.J.Y., A.K.D., T.M.D.-B., G.B.B.-S., and M.W. collected field samples.

The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database. For a list of accession numbers, see *SI Appendix*.

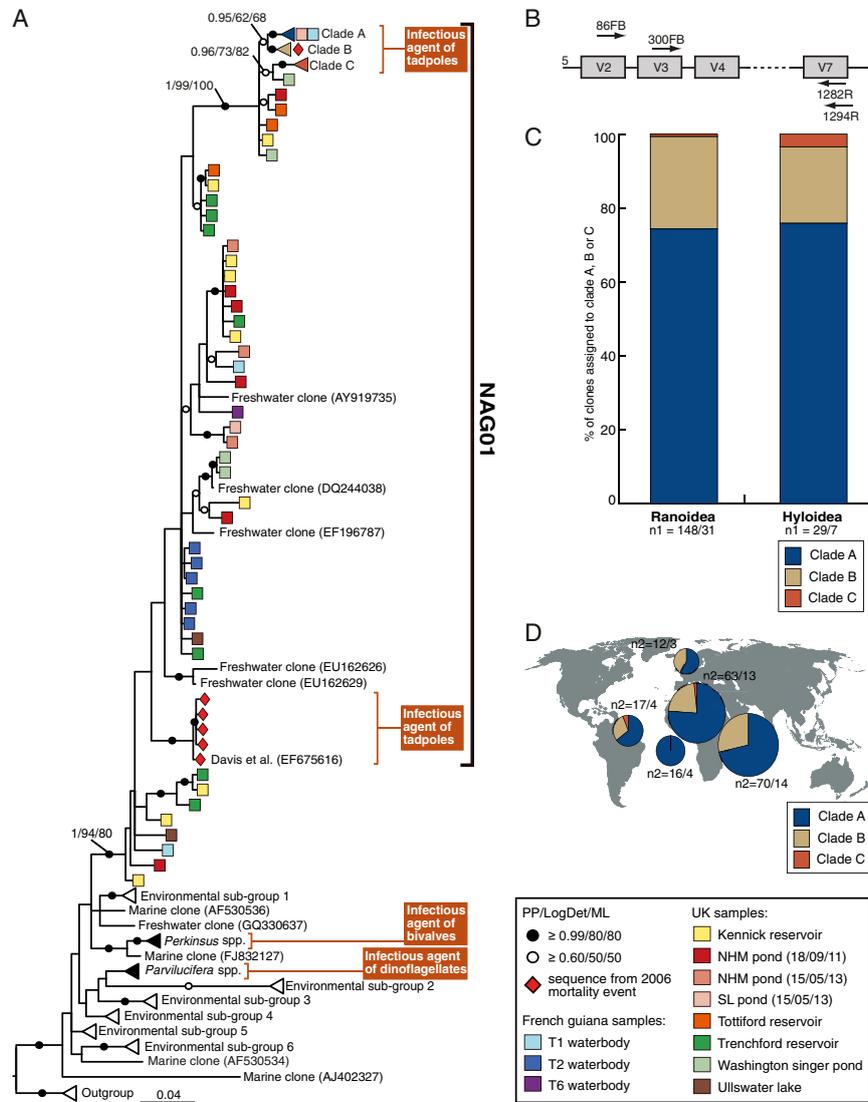
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ECOLOGY

# CRYPTIC INFECTION OF A BROAD TAXONOMIC AND GEOGRAPHIC DIVERSITY OF TADPOLES BY PERKINSEA PROTISTS

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**Fig. 1.** (A) Phylogenetic tree of *Perkinsea* SSU rDNA sequences focusing on the NAG01 group that includes two separate phylogenetic groups recovered from tadpole liver tissue samples. The phylogeny is estimated from a masked alignment consisting of 292 taxa and 776 characters. Bayesian posterior probability (6,000 samples from 2,000,000 MCMCMC generations), LogDet distance bootstrap (1,000 pseudoreplicates), and maximum likelihood bootstrap (1,000 pseudoreplicates) values are added to each node using the following convention: support values are summarized by black circles when all are equal to or greater than 0.9/80%/80%, and white circles when the topology support is less but equal to or greater than 0.6/50%/50%. Five sequences of *Amoebophrya* sp. were used as outgroup. Each square represents one environmental operational taxonomic unit (OTU), and the provenance of the OTUs is indicated by colored boxes (see key for the detail of sample provenance) (SI Appendix, Table S2 provides more details on the environments sampled). A red diamond indicates the individual clone sequences from the *L. sphenoccephalus* 2006 mass mortality event in Georgia (United States) (12). A subset of the published environmental sequences have been reduced to representative triangles (see SI Appendix, Table S10 for detail of each environmental clade). (B) Representation of the V4 hyper-variable region of the template SSU rDNA and the relative position of the different primers used in this study (not to scale). (C) Histogram representing the percentage of clones per clade A, B, and C within each host superfamily from infected tadpoles.  $n_1$  represents the number of total clones sequenced per host superfamily/number of infected tadpoles per host superfamily. (D) Geographical distribution of clade A, B, and C. Pie charts represent the proportion of clones per clade A, B, and C in each of the five geographical locations where NAG01 was detected (United Kingdom, French Guiana, São Tomé, Cameroon, and Tanzania).  $n_2$  represents the number of clones sequenced per geographical location/number of infected tadpoles per geographical location.

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analysis has considerably expanded the known diversity of Perkinsea-like organisms (21–25). The putative causal agent of the *L. sphenoccephalus* MME lies within a clade (monophyletic group) of this environmental sequence diversity (Fig. 1A) (12) named here, for convenience, Novel Alveolate Group 01 (NAG01).

In this study, we developed SSU rDNA primers that preferentially target the NAG01 group. We used these PCR primers for targeted screening of NAG01 diversity in both tropical and temperate freshwater environments, demonstrating the efficacy of this PCR protocol and expanding the diversity of Perkinsea-like sequences sampled from freshwater environments. Using the same protocol, we also detected NAG01 from livers of a wide taxonomic diversity of tadpoles from five countries across three continents. Within our study sample, we found evidence of infection by lineages different from the Perkinsea-like *L. sphenoccephalus* pathogen identified by Davis et al. (12), suggesting that multiple Perkinsea lineages infect tadpoles.

## Results and Discussion

**Development of a Targeted PCR Assay for Perkinsea-Like Infection of Frog Tissue.** Culture-independent environmental DNA methods can be useful for detecting microbial lineages from the environment, including from the tissues of plants and animals (e.g., refs. 26–28). To investigate the prevalence and diversity of Perkinsea-like parasites infecting tadpoles, we designed two independent sets of PCR primers targeting ~800 base pairs (bp) of the small subunit (SSU) rRNA-encoding gene, including the V4 variable region (29) (see Fig. 1B and *SI Appendix, Table S1* for primers used in this study). The primers were designed to amplify the rDNA sequences of the wider NAG01 group (Fig. 1A), including the previously sampled SSU rDNA sequence of the Perkinsea-like infectious agent of the Southern Leopard Frog (12). To investigate the specificity of these two pairs of primers, we performed PCR on 10 environmental DNA samples from three tropical (French Guiana) and seven temperate (United Kingdom) planktonic freshwater samples (*SI Appendix, Table S2*). A total of 248 clones were sampled from the UK temperate water masses and 60 clones from the French Guiana water masses (*SI Appendix, Table S2*). Among these 308 clones, sequences from 240 clustered together into 46 nonidentical sample-specific NAG01 sequences (*SI Appendix, Table S2 and S3*) that were included in the phylogenetic analysis (Fig. 1A). The remaining 68 clones were non-NAG01 sequences encompassing a mixed assemblage of Fungi, Cryptophyta, and nematode sequences. Based on these results, our PCR protocol was judged as adequate for preferentially targeted environmental clone library analyses of NAG01, including the previously identified Perkinsea-like *L. sphenoccephalus* infectious agent and seven environmental DNA sequences present in the GenBank database that were recovered from freshwater planktonic samples (30–32) (*SI Appendix, Table S4*).

**Investigating the Global Prevalence of NAG01 Infections in Tadpoles.** Using the same PCR protocol used to detect NAG01 from freshwater environmental samples, we screened for NAG01 sequences in DNA extractions from liver samples dissected from 182 ethanol-preserved tadpoles. We sampled tadpoles from French Guiana (80 individuals from 8 sampling localities), Cameroon (37 from 14), Tanzania (15 from 1), the island of São Tomé (4 from 1), the United Kingdom (40 from 5), and the Czech Republic (6 from 3), of which 38 (21%) were PCR-positive for NAG01. See *SI Appendix, Table S5* for more details, including the following: GPS location, sampling date, and description of environment where the tadpoles were found. For each positive sample, the PCR was repeated three times, and the amplicons were pooled. Each PCR product was checked on a 1% agarose gel for the presence of a single band of ~800 bp. The PCR product was then purified, cloned, and sequenced. We sequenced

six clones using M13F primers from each PCR-positive liver sample, resulting in 228 sequences. Based on preliminary sequence analysis of the forward reads, all NAG01 SSU sequences that were unique were double-strand sequenced. This sequencing effort encompassed a minimum of four clones per tadpole liver DNA clone library even though most libraries included fewer nonidentical clones (*SI Appendix, Table S6*).

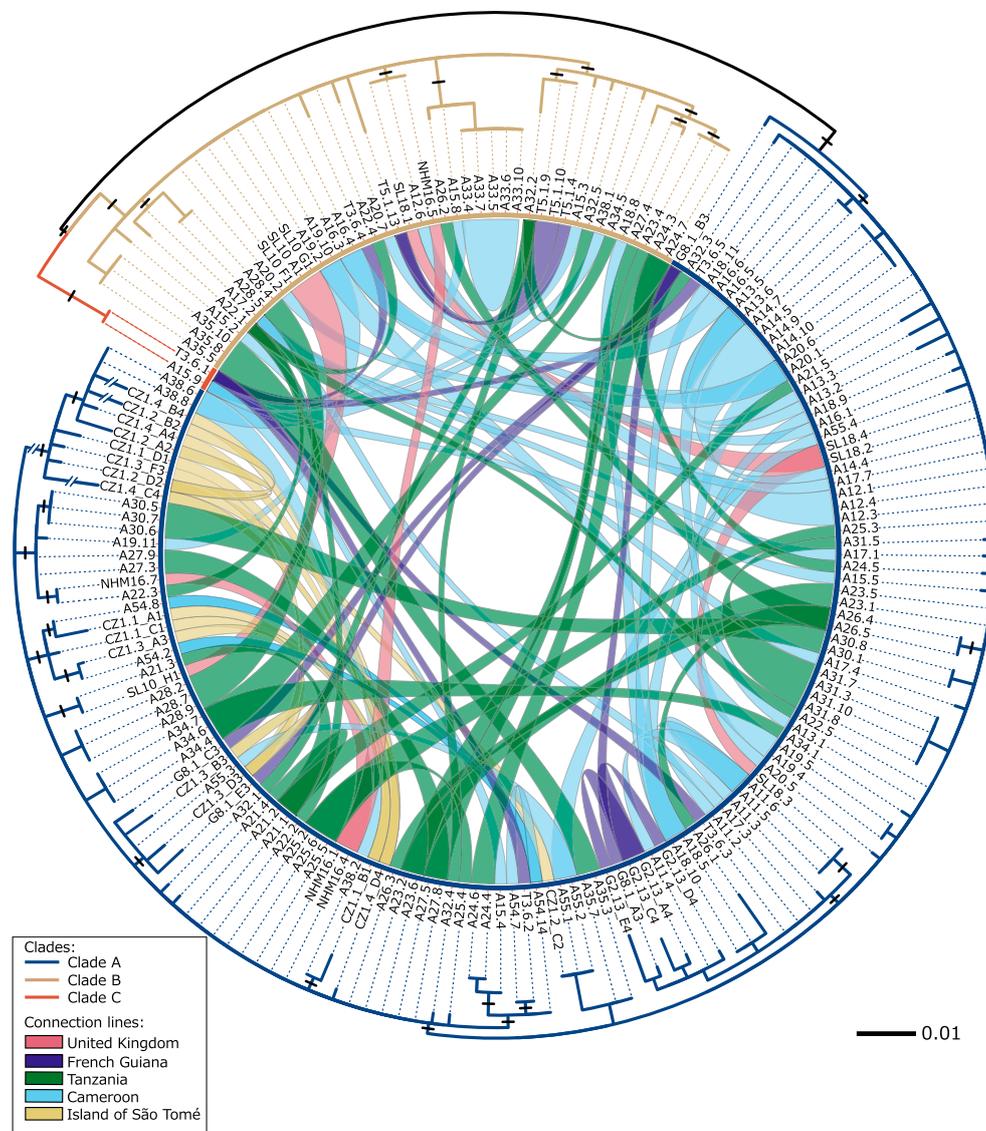
Phylogenetic analysis demonstrated that all 177 sample-specific, unique NAG01 sequences recovered from tadpole livers form three discrete strongly supported and closely related clades (labeled clades A, B, and C in Fig. 1A). Clades A, B, and C do not correspond directly with host taxon or geographic origin (Figs. 1C and D and 2), a similar result to that described for *Perkinsus* sp. (33) parasites of marine bivalves. Interestingly, 21 of the 38 (55%) NAG01-positive tadpole liver samples yielded sequences from both clade A and B (Fig. 2), suggesting that these livers harbored representatives from different nonclonal strains/species or that there is intranuclear SSU rDNA variation within NAG01 genomes sampled. Indeed, there is less than 3% nucleotide variation among clade A, B, and C sequences (*SI Appendix, Fig. S2*), consistent with intranuclear variation such as that observed in SSU-5.8S-LSU paralogues and pseudogenes. For example, variant copies of the rRNA gene are known to occur in alveolates (34, 35) with SSU rRNA gene paralogues, with 11% difference (36) transcribed at different stages of *Plasmodium* spp. life cycle (37).

It is possible that the NAG01 DNA detected could have arisen from contamination from the environment and/or gastrointestinal tissue in the liver samples. However, this possibility was judged unlikely because all of the NAG01 SSU sequences detected from tadpole liver sequences grouped into one discrete phylogenetic subgroup whereas the primers used were capable of detecting a wide diversity of Perkinsea-like sequences from environmental DNA samples (Fig. 1A). To further test for cases of environmental contamination, DNA was extracted from tail samples (muscle and fin) taken from the same 182 tadpoles as a control to identify possible sources of nontissue-specific PCR detection or environmental contamination of NAG01 sequences. Although infections of an unknown alveolate-like parasite have been identified in the muscles of adult frogs (38), the tadpoles sampled here showed no evidence of disease progression so this experimental approach was judged as an adequate control to identify cases of environmental and wider animal tissue contamination. All controls were negative for the two primer-paired NAG01-specific PCR protocols, suggesting that detection of NAG01 was not an artifact of environmental contamination but instead a tissue-specific signal consistent with infection of a Perkinsea-like protist associated with the liver of these tadpoles. Interestingly, these liver-derived NAG01 sequences are closely related to some of the sequences recovered from filtered plankton environmental DNA sample sequences, suggesting that this protist group is found both associated with tadpoles and either as free living stage or associated with additional microbial hosts. Indeed, experimental manipulations have shown that infection of putative members of the NAG01 clade likely occur through ingestion of spores and/or zoospores from the watercolumn (39).

**Diversity of Perkinsea Parasites Recovered from Liver Tissues from United States Mass Mortality Event.** Sequences generated from the tadpole livers sampled here are from a different NAG01 Perkinsea lineage from that detected in liver tissues of *L. sphenoccephalus* tadpoles sampled from an MME in Georgia (United States) in 2006 (Fig. 1A). Only formalin-fixed, paraffin-embedded liver samples were available from this event (12). We successfully performed two DNA extractions from a historical sample. The standard NAG01 primers failed to generate an amplicon from both DNA samples, most likely because the template DNA was highly fragmented. Consequently, we targeted a shorter template using

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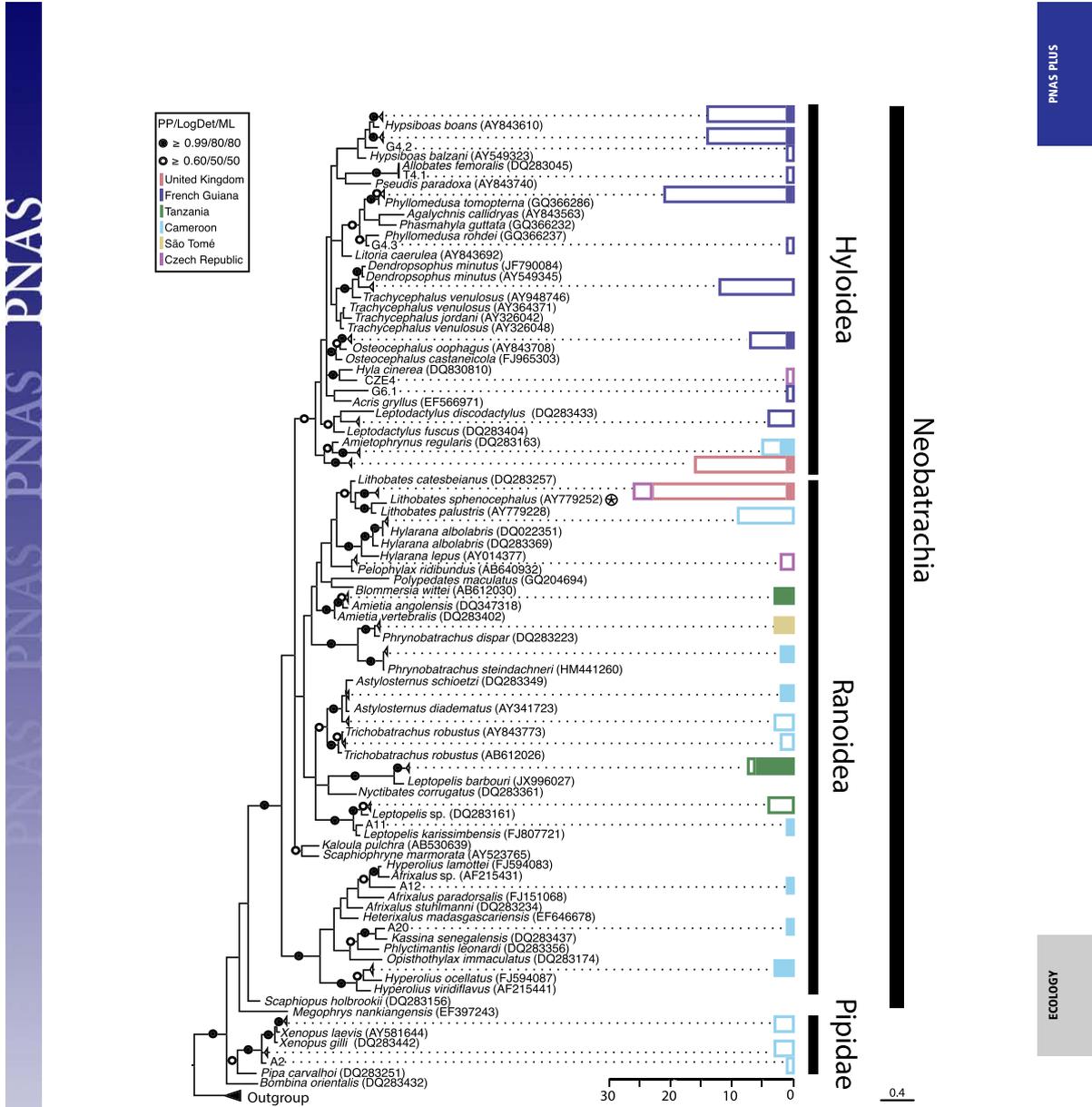


**Fig. 2.** A maximum likelihood phylogenetic circle tree (inverted) showing clades A, B, and C and demonstrating the provenance of NAG01 phlotypes detected in tadpole liver tissue. The RAxML phylogeny is estimated from a masked alignment consisting of 177 NAG01 sequences and 806 characters from infected tadpoles. Branches proportionally shortened by 1/2 are labeled with a double-slashed line. Each clone sequence detected from the same liver sample is connected across the central circle. The colors of the connected lines were defined by the geographical location of tadpoles sampled (see the key). Black lines on the branches mark sequence variation confirmed across multiple samples and therefore cannot be the product of PCR error during clone library construction.

the 300F-B forward primer in combination with a eukaryotic general reverse primer 600R (*SI Appendix, Table S1*), and we amplified 414 bp of the SSU rDNA. PCR reactions were repeated three times for both DNA samples, and amplicons were pooled

and cloned separately for the two DNA samples. In total, across the two DNA samples, we sequenced 45 clones in the forward and reverse direction. Phylogenetic analysis revealed that 44 clones represented four unique sequences that are closely related to

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**Fig. 3.** Bayesian 16S rDNA phylogenetic tree of tadpole diversity sampled in this study, with histograms showing prevalence of NAG01 detection. The phylogeny is inferred from a masked alignment consisting of 247 taxa and 440 characters. Bayesian posterior probability (6,000 samples from 2,000,000 MCMCMC generations), LogDet distance bootstrap (1,000 replicates), and maximum likelihood bootstrap (1,000 replicates). Support values are summarized by black circles when all are equal to or greater than 0.9/80%/80%, and a white circle when topology support is weaker but all values are equal to or greater than 0.6/50%/50%. Sequences of *Ambystoma* sp. and *Pleurodeles* sp. (salamanders) were used as outgroup. Some frog species with multiple nonidentical 16S rDNA sequences recorded in GenBank are retained. The color-coded histogram represents the number of NAG01-negative tadpole samples (uncolored bars) and the number of NAG01-positive samples (colored bars). Each color corresponds to the tadpole's country of origin as detailed in the key. The superfamily and suborder of the tadpoles tested is indicated on the histogram. The circled star indicates the host species described by Davis et al. (12) during the 2006 mortality event.

the published sequence of the *L. spheocephalus* infectious agent (EF675616) and one unique sequence closely related to NAG01 clade B (Fig. 1A).

**Barcode Sequencing Reveals a Wide Host-Taxon Diversity and Biogeography for NAG01.** Precise taxonomic identification of tadpoles using morphological characters can be difficult, especially in

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geographic regions with high species diversity. We amplified a mitochondrial 16S rDNA barcode, shown to be effective for higher taxonomic assignment of amphibians (40, 41), for all 182 sampled tadpoles. The majority ( $n = 175$ ) of the tadpoles sampled are members of the Neobatrachia (which comprises >95% of extant frogs) whereas the other 7 are tadpoles of the Pipidae: i.e., *Xenopus* (Fig. 3). We note that, although the pipid tadpoles sampled all tested negative, increased sampling is required to more confidently determine the presence/prevalence of this protist in this group of frogs.

NAG01 DNA was detected in tadpoles of two of the largest groups of neobatrachians, with 6% of sampled Hyloidea ( $n = 102$ ) infected and 42% of Ranoidea ( $n = 73$ ) infected (Fig. 3). In separate studies, a Perkinsia-like parasite linked to local mortality events was detected using histology in six species of Ranoidea (*L. sphenoccephalus*, *Lithobates capito*, *Lithobates sevosus*, *Lithobates catesbeianus*, *Lithobates heckscheri*, and *Lithobates sylvaticus*) and one species of Hyloidea (*Acris gryllus*) ([www.nwhc.usgs.gov/publications/quarterly\\_reports](http://www.nwhc.usgs.gov/publications/quarterly_reports); accessed October 29, 2014) (*SI Appendix*, Fig. S1). Similar dissection-based approaches have also shown related infectious agents present in a wide diversity of ranids, as well as *A. gryllus*, *Hyla femoralis*, *Hyla gratiosa*, *Pseudacris ornata*, and *Gastrophryne carolinensis* (39). In the absence of molecular data, it is not clear whether these infections were of protists from NAG01 clades A, B, or C reported here, the *L. sphenoccephalus* parasite detected by Davis et al. (12), or a lineage not yet sampled for DNA analysis. However, the NAG01 sequences from tadpole livers detected in this study were recovered in five of the six countries sampled, including both tropical and temperate environments and an oceanic island. Mean prevalence estimate per country and 95% confidence intervals (CIs) using the Jeffreys method were as follows: 3% (four of 80, 95% CI of 0.4–34%) in French Guiana (eight sampling events); 55.2% (13 of 37, 11–78% CI) in Cameroon (14 sampling events); 93.3% (14 of 15, 72–99% CI) in Tanzania (one sampling event); 100% (four of four, 73–100% CI) on the Island of São Tomé (one sampling event); and 9% (three of 40, 1–40% CI) in the United Kingdom (five sampling events). Taken together, these data suggest a high prevalence and broad spatial distribution of infection by a specific subclade of NAG01 Perkinsia-like protists in neobatrachian tadpole populations specifically of the superfamily Ranoidea. Information connecting the putative phylogeny inferred taxonomy of the host tadpole to the presence of the NAG01 sequence type is given in *SI Appendix*, Table S5 to supplement the results shown in Fig. 3.

**NAG01 Perkinsia-Like Protist and Disease.** All our tadpole field samples were preserved in ethanol, and, as such, we could not attempt to purify NAG01 clade A, B, or C into culture. Infection by Perkinsia-like parasites identified using histological and/or dissection microscopy techniques have been reported as visible from Gosner life cycle stages 24–42 (39, 42). In some cases, the disease phenotype of tadpoles infected by Perkinsia-like parasites has previously been described as bloated, lethargic, and showing cutaneous hemorrhages (12, 13, 42, 43) although we note that these symptoms are not diagnostic for Perkinsia disease of tadpoles, because other diseases can cause similar pathologies. The majority of the 38 infected tadpoles analyzed in this study were sampled from early Gosner stages, with the majority being of, or close to, Gosner stage 25, with one at Gosner stage 42 (*SI Appendix*, Table S5). The infected tadpoles showed no gross morphological symptoms of disease. A more precise definition of the disease in the cases of Perkinsia infection is infiltration of the liver and other visceral organs by large numbers of Perkinsia-like organisms (12, 38). Indications of tissue level disease in samples showing molecular evidence for the presence of NAG01 were sought through histology of liver with H&E staining of 5- $\mu$ m sections from representative samples

(two from French Guiana, three from Cameroon, and three from Tanzania; for examples, see *SI Appendix*, Fig. S3). These data showed no identifiable tissue damage consistent with disease and no cells attributable to the NAG01 microbes detected using molecular methods, demonstrating that these infectious agents are either a small cellular form and/or badly preserved in histological sections, or, alternatively, that the population of NAG01 is very low, suggesting a low infection intensity in these tadpoles. Thus, we cannot confirm that the infectious protists identified here are pathogenic, either because (i) the tadpoles were all sampled in an early phase of disease progression and/or the infectious protists are currently dormant, (ii) NAG01 clades A, B, or C detected in this study and that branch in a different part of the phylogenetic tree to previously reported disease-causing Perkinsia of tadpoles (Fig. 1A and ref. 12) have a limited or absent disease pathology, and/or (iii) disease is caused only in association with other infectious pathogens such as *Ranavirus* (43) or other forms of host stress (44). Other parasitic Perkinsia, such as *Perkinsus sensu stricto* (parasites of bivalves), are also widely geographically distributed, but infection and catastrophic host population MMEs are localized, with pathogenicity related in part to abiotic factors (33, 45–47).

These data demonstrate that the NAG01 protists detected in tadpole livers in this study (i) are not a ubiquitous agent or contaminant but instead are liver-associated, (ii) represent a closely related phylogenetically distinct subgroup within NAG01, (iii) lie within the phylum Perkinsia, for which all known taxa are potential parasites (e.g., refs. 18, 48, and 49), and (iv) are prevalent in a range of tadpole developmental stages (Gosner stages 25–42) (*SI Appendix*, Table S5). Although frog MMEs associated with a Perkinsia-like parasite have been recorded only in the United States, our results demonstrate that a greater diversity of Perkinsia-like protist infections of tadpoles are widespread. There are increasing efforts to monitor the health of wild amphibian populations (50–52), with justifiable focus on fungal chytrid pathogens and *Ranavirus*, both of which have been identified as causing disease in adult frogs (6–8, 10). Further studies on the etiology of tadpole (and other larval amphibian) infections are necessary to understand the impact of these protists on amphibian populations and to inform conservation planning.

## Materials and Methods

**Developing Group-Specific Primers.** Based on a multiple sequence alignment of available Perkinsia SSU rDNA sequences (*SI Appendix*, Tables S4 and S7) assembled using ARB (53), sets of “NAG01-specific primers” were designed to recover a central portion of the SSU rRNA-encoding gene, including the variable V4 region (Fig. 1B and *SI Appendix*, Table S1). Specificity of the PCR primers was checked first in silico by submitting sequences to the National Center for Biotechnology Information (NCBI) nonredundant (nr) DNA database (using Primer-BLAST–May 2014) and SILVA (TestProbe search–May 2014) databases (54) and, second, in situ using an environmental DNA clone library approach.

**Environmental DNA Sampling of Freshwater Environments.** Water samples from the surface of the watercolumn were collected from multiple freshwater environments in the United Kingdom and French Guiana (details of each sample are given in *SI Appendix*, Table S2). For the sampling in French Guiana, water samples were prefiltered through 10- $\mu$ m polycarbonate filters (Merck Millipore), and the filtrate was then serially filtered through 5- $\mu$ m and then 2- $\mu$ m polycarbonate filters to collect size-specific subsections of the microbial community. This process was conducted until each filter became saturated (the volume of water filtered for each sample is reported in *SI Appendix*, Table S2). Saturated filters were then submerged in RNAlifeGuard (MoBio) and then stored at  $-20^{\circ}\text{C}$  for 2 weeks before being transported back to the United Kingdom at ambient temperatures and then finally stored at  $-80^{\circ}\text{C}$ . The UK samples were processed in a similar manner but were serially passed through 20- $\mu$ m and then 2- $\mu$ m or 0.2- $\mu$ m filters and transformed directly to storage at  $-80^{\circ}\text{C}$ . DNA extraction was performed using the PowerWater DNA Isolation Kit (MoBio) using the protocol recommended by the manufacturer. Details of filtration size for positive detection of NAG01 are

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given in *SI Appendix, Table S2* and indicate the presence of this group in both the 0.2- to 20- $\mu\text{m}$  and 2- to 5- $\mu\text{m}$  size fraction samples.

**Sampling of Tadpole Tissue for NAG01 Molecular Screening.** A total of 182 ethanol-preserved tadpoles were examined: Details of the phylogenetic diversity sampled are given in Fig. 3, and the highest BLAST hit for each sample in the NCBI nr database (accessed October 2014) is given in *SI Appendix, Table S8*. *SI Appendix, Table S5* provides details of the environmental provenance of the tadpoles. Tadpoles preserved whole in ethanol were dissected using sterile tools. A piece of liver of each tadpole was removed (taking care not to pierce the gut) and placed in a fresh tube of ethanol. Concurrently, a similarly sized piece of tail was excised and placed in a separate tube of ethanol. Total DNA was extracted from all tadpole tissue samples using the Blood and Tissue DNeasy extraction kit (Qiagen) following the manufacturer's protocol, with an overnight lysis and incubation step.

**SSU rDNA Clone Libraries from Environmental DNA and Tadpole Liver DNA.** Environmental DNA and tadpole liver and tadpole tail DNA extractions were used as a source template to construct NAG01-specific SSU rDNA gene clone libraries. Two sets of primers were used, including two NAG01-specific forward primers paired with two general eukaryotic reverse primers (Fig. 1B). For every PCR, we included a negative control (distilled H<sub>2</sub>O). All PCR amplification reactions were performed in 25  $\mu\text{L}$  of total volume containing 8 ng of DNA and PCR MasterMix (Promega). Cycling reactions were as follows: 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 120 s at 72 °C, with an additional 10-min extension at 72 °C. For each template, three independent PCR reactions were performed, mixed together, purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega), and cloned using the Strataclone PCR cloning kit (Stratagene) according to the manufacturer's instructions. Clones were blue/white screened, and a subset was selected for PCR using M13F and M13R primers, which flank the vector insertion site. Clones showing the presence of an insert of the correct size were sequenced using M13F primers. For each environmental clone library, a minimum of 20 clones was sequenced. All of the tadpole tail samples were PCR-negative. For each tadpole liver sample with a positive PCR result, we sequenced six clones using the M13F primer. All M13F NAG01 SSU sequences that were unique in one or more position were double-strand sequenced.

**Mitochondrial Encoded 16S SSU rRNA Gene Barcoding of Tadpoles.** Tadpole liver DNA extracts were subject to PCR using the 16Sar-F and 16Sar-R primers (40), which amplify an ~600-bp region of the 16S SSU rRNA-encoding gene. PCR amplification reactions were performed in 50  $\mu\text{L}$  of total volume containing 8 ng of DNA and PCR MasterMix (Promega). Cycling reactions were as follows: 2 min at 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C, and 120 s at 72 °C, with an additional 10-min extension at 72 °C. PCR products were checked on 1% agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega).

**DNA Extraction and SSU rDNA Clone Library Construction from Formalin-Fixed, Paraffin-Embedded Liver Tissue from the United States Southern Leopard Frog MMEs.** Frozen and/or ethanol-preserved tadpole tissue from the 2006 mortality event (12) was unavailable due to theft of copper wiring from the freezers of the M.J.Y. laboratory, resulting in loss of these samples. Thus, only formalin-fixed and paraffin-embedded tissues used for microscopy sections were available for molecular analysis. Two liver sections were excised from the paraffin block using sterile scalpel blades. Each section was incubated with xylene at 50 °C for 3 min until the paraffin had dissolved. After this process, the liver section still constituted a compact tissue aggregate. The xylene solution was removed by pipetting, and the tissue sample was washed twice using pure ethanol and dried for 15 min at 37 °C. DNA was then extracted using the DNeasy Blood and Tissue kit (Qiagen) protocol with an overnight incubation in 100  $\mu\text{L}$  of lysis buffer at 55 °C. The DNA extractions were conducted twice on the two different formalin-fixed, paraffin-embedded liver samples.

The DNA extractions were checked using a 2100 Bio-analyzer (Agilent Technologies) demonstrating highly fragmented template DNA (similar to ancient DNA samples) with an average fragment size of ~160 bp. We thus amended our PCR protocol to target a shorter amplicon using the forward NAG01-specific 300F-B primer with the eukaryotic general reverse primer 600R (see *SI Appendix, Table S1* for details). The PCR amplification reaction was performed in 25  $\mu\text{L}$  of total volume of PCR MasterMix (Promega) again with an additional negative control reaction (distilled H<sub>2</sub>O). Cycling reactions were as follows: 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 59 °C, and 120 s at 72 °C, with an additional 10-min extension at 72 °C. For

each clone library, three independent PCRs were completed, mixed together, and cloned. Two clone libraries were constructed using the Strataclone cloning kit (Stratagene) according to the manufacturer's instructions. Forty-five independent clones with insertions of appropriate size were selected and sequenced in both directions using M13F and M13R primers.

**Sequencing and Assembly.** All sequencing was performed externally by Beckman Coulter Genomics. The final sets of sequences were trimmed to regions of high sequencing quality; vector sequences were removed, sequence reads were assembled into a contiguous sequence using Sequencher (Genecodes), and ambiguous sites were corrected.

**Multiple Sequence Alignment and Phylogenetic Analysis.** Our NAG01 clone library sequencing resulted in 177 sample-specific unique clones from the tadpole liver samples (*SI Appendix, Table S6*), 46 sample-specific unique clones from the environmental DNA samples, and five sample-specific unique clones from formalin-fixed, paraffin-embedded liver samples (see *SI Appendix, Table S3* for details). These sequences were assembled into a multiple sequence alignment with 59 *Perkinsea*-like sequences and *Amoebophrya* sp. SSU rDNA outgroup sequences (HQ658161, HM483395, HM483394, AY208894, and AF472555) recovered from the NCBI nr database (accessed May 2014; see *SI Appendix, Tables S4 and S7*). The sequences were aligned using MUSCLE (55), available via the graphical multiple sequence alignment viewer Seaview v4.2.12 (56), using default settings. The alignment was then checked and masked manually in Seaview, resulting in a data matrix of 292 sequences and 776 alignment positions. We note that the alignment included some partial database sequences to best sample the diversity of sequences sampled previously. However, *Perkinsea*-like sequences with the major central portion of the sequence absent were excluded from the final analysis (e.g., EUY162621, EUY162622, and EUY162623). This data matrix contained 487 variable alignment positions (excluding alignment positions with gaps) and 571 parsimony informative sites (including gaps).

All *Perkinsea* SSU rDNA sequences generated as a part of this study have been deposited in GenBank (see *SI Appendix, Tables S3 and S6* for details). The *Perkinsea* alignment is available in the Seaview (56) Mase format with the alignment mask information retained and is available at doi 10.5281/zenodo.12712.

The 182 tadpole 16S SSU rDNA sequences were aligned with a collection of frog 16S SSU rDNA sequences (*SI Appendix, Table S9*) using the same approach described for the NAG01 sequences and using MUSCLE (55) via Seaview v4.2.12 (56). All tadpole 16S SSU rDNA sequences generated in this study were BLASTn searched against the NCBI nr database (accessed May 2014), and the most similarly named frog sequence based on sequence similarity was noted (*SI Appendix, Table S8*). Preliminary phylogenies were compared with published trees (57), and additional frog 16S SSU rDNA sequences representing intermediate branches (arbitrarily selected) were added to the phylogeny. Sequences of Caudata (salamanders) were chosen as outgroup for the phylogeny: *Pleurodeles waltl* (DQ283445), *Pleurodeles nebulosus* (DQ092266), *Ambystoma mexicanum* (EF107170), and *Ambystoma tigrinum* (DQ283407). The alignment was then checked and masked manually in Seaview, resulting in a data matrix of 247 sequences and 440 alignment positions with 225 variable alignment positions (excluding alignment positions with gaps) and with 232 parsimony informative sites (including sites with gaps in the masked data matrix).

All frog SSU rDNA sequences generated as a part of this study have been deposited in GenBank (see *SI Appendix, Table S5* for details). The amphibian alignment is available in the Seaview (56) Mase format with the mask information retained and is available at doi 10.5281/zenodo.12712.

The best-fitting nucleotide substitution model for each alignment was determined using the information criterion and likelihood ratio tests implemented in Modelgenerator v0.85 (58). For *Perkinsea* and amphibian alignments, GTR+ $\Gamma$  and GTR+I+ $\Gamma$  models were selected, respectively. The  $\alpha$  parameters for the  $\Gamma$  distributions were 0.38 and 0.30, respectively, with eight discrete rate categories whereas the I parameter for the amphibian alignment was 0.28. These parameters, where possible, were input into a Bayesian analysis using MrBayes v3.1.2 (59): i.e., lset, nst = 6 rates = gamma (or invgamma in the case of the amphibian alignment), and, in both cases, we included the covariances parameter search. Two independent runs of four Metropolis-coupled (MC) Markov chain Monte Carlo (MCMC) chains (with a heat parameter of 2) were run for 2,000,000 generations. Trees were sampled every 250 generations. In both analyses, the MCMC searches had converged within the first 25% of the generations sampled; as such, the first quarter of the search results were discarded (as the burn-in). Convergence between the runs and burn-in were assessed using Tracer v1.6 ([tree.bio.ed.ac.uk/software/tracer/](http://tree.bio.ed.ac.uk/software/tracer/)). The consensus

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topologies and posterior probabilities of each node were then calculated from the remaining sampled trees.

Support for the Bayesian tree topology was evaluated using two bootstrap methods and the Bayesian posterior probabilities from the MrBayes runs. Bootstrap support values were estimated using (i) RAxML v8.0.3 (60) with 1,000 pseudoreplicates and (ii) LogDet distance analysis with 1,000 pseudoreplicates using a BioNJ search method (available through Seaview v4.2.1). This second bootstrap analysis was conducted for comparison because it uses a method that minimizes artifacts arising from biases in base composition across the alignment (61).

To further investigate the phylogenetic diversity of the NAG01 sequences recovered from the tadpole livers, we resampled the alignment mask specifically focusing on sequences recovered from clades A, B, and C with the aim of maximizing unambiguously aligned sites. This analysis excluded all environmental sequences and retained only tadpole-associated *Perkinsea* sequences from clades A, B, and C. This new alignment resulted in a data matrix of 177 sequences and 806 alignment positions. This data matrix was analyzed with ModelGenerator, selecting the GTR +  $\Gamma$  ( $\alpha$  parameter of 1.18) using the standard Akaike information criterion. The phylogeny was then estimated using RAxML v8.0.3 (60) using the GTR +  $\Gamma$  substitution model. For this analysis, we aimed to calculate a single tree that best displays the phylogenetic relationships of the NAG01 sequences sampled from tadpole livers. This alignment encompassed little sequence variation, and tree searches did not result in a consensus tree with consistently high/moderate bootstrap support values. This phylogenetic analysis therefore primarily serves to demonstrate the distribution of different SSU types across the different tissue samples and not to present a resolved phylogeny. To display codetection of NAG01 clades A, B, and C from specific liver samples, we used the Circos tool. Fig. 2 was created using Inkscape (<https://inkscape.org/en/>), Circos (62), and the Interactive Tree of Life (IToL) ([itol.embl.de](http://itol.embl.de)) (63). Connections highlighting phylogenetically disparate clones from the same frog were plotted and color-coded by geographic sampling location (see key in Fig. 2). This plot was then combined with an inverted circular tree generated by IToL using Inkscape.

**Histology of Representative Tadpole Tissue Samples.** For eight samples, half of the liver that was not used for DNA extraction was stored in 100% ethanol at 4 °C. Each liver was then embedded in paraffin wax (Sigma-Aldrich) and cut

into serial sections 5  $\mu$ m thick using a Shadon tissue processor (Thermo Electron Corporation). Sections were collected onto glass slides and stained with hematoxylin and eosin staining methods (Thermo Fisher). The sections of each slide were mounted using Histomount (National Diagnostics). Sections were examined by light microscopy (Microscope Olympus IX73) for the presence of putative parasites, and digital images were obtained using the Infinity 3 camera (Lumenera Corporation).

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# CRYPTIC INFECTION OF A BROAD TAXONOMIC AND GEOGRAPHIC DIVERSITY OF TADPOLES BY PERKINSEA PROTISTS

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ECOLOGY

## B.5 A BALLOON-BASED PAYLOAD FOR EXPOSING MICROORGANISMS IN THE STRATOSPHERE (E-MIST)

### *Methods Article*

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### **A Balloon-Based Payload for Exposing Microorganisms in the Stratosphere (E-MIST)**

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#### **ABSTRACT**

The survival and transit of microorganisms in Earth's upper atmosphere is relevant to terrestrial ecology and astrobiology, but the topic is understudied due to a scarcity of suitable flight systems. We designed, built, and flew a self-contained payload, Exposing Microorganisms in the Stratosphere (E-MIST), on a large scientific balloon launched from New Mexico on 24 August 2014. The payload carried *Bacillus pumilus* SAFR-032, a highly-resilient spore-forming bacterial strain originally isolated from a NASA spacecraft assembly facility. Our test flight evaluated E-MIST functionality in the stratosphere, including microbiological procedures and overall instrument performance. Herein, we summarize features of the E-MIST payload, protocols, and preliminary results that indicate it is possible to conduct a tightly-controlled microbiological experiment in the

stratosphere while collecting pertinent environmental data. Additional studies of this nature may permit survival models for microbes traveling through Earth's harsh upper atmosphere. Moreover, measuring the endurance of spacecraft-associated microbes at extreme altitudes may help predict their response on the surface of Mars.

#### **INTRODUCTION**

Microorganisms in the upper atmosphere emanate from surface and marine ecosystems (Burrows et al., 2009), and are capable of reaching high altitudes by strong uplifting forces or mixing between the troposphere and stratosphere (Homeyer et al., 2011; Randel and Jensen, 2013). In addition to microbes naturally lofted, landfills, wastewater treatment plants, slash-and-burn agriculture, air traffic, and desertification also contribute to the total amount of bioaerosols in the atmosphere (Smith, 2013). Prevailing winds can connect distant biomes on Earth (Creamean et al., 2013; Smith et al., 2013), and residence time in the upper atmosphere exerts a harsh combination of stresses on microbes outside the range of conditions normally encountered on the surface (e.g., lower pressure, higher irradiation, desiccation, and oxidation). Thus, airborne transport might provoke

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*Key words:* Balloon; Stratosphere; Microbes; Bacterial Spores; Survival; Mars; Planetary Protection

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# A BALLOON-BASED PAYLOAD FOR EXPOSING MICROORGANISMS IN THE STRATOSPHERE (E-MIST)

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exceptional types of cellular damage or mutation. Some bacteria found in previous atmospheric surveys (reviewed by Griffin (2007) and Polymenakou (2012)) have traits that would improve persistence aloft – including cell pigmentation, DNA repair, and the ability to form endospores (hereafter referred to as ‘spores’). While microbial survival has been examined using environmental simulation chambers (Smith *et al.*, 2011) and small meteorological balloons (Beck-Winchatz and Bramble, 2014), more comprehensive platforms for controlled, long-duration experiments in the upper atmosphere are needed. Earth’s middle stratosphere, about 25 to 40 km above sea level (ASL), resembles the surface conditions of Mars (Kaplan, 1988); thus, the planetary protection community could obtain an improved understanding of the survival of terrestrial microbes on Mars rovers and landers by flying stratospheric experiments. Furthermore, data from survival experiments could contribute to models for bioaerosols carried on globally-circulated winds (Smith *et al.*, 2013; Yu *et al.*, 2013), which is vitally important to global food security (Brown and Hovmøller, 2002). For example, aerially-dispersed wheat fungi threaten a staple calorie and protein source for 4.5 billion people across 94 developing nations (Singh *et al.*, 2011).

Studying microbial survival in the upper atmosphere presents two fundamental challenges: first, removing potential influences from pre-flight, ascent, descent, and landing so as to limit the experiment to targeted altitudes; second, maintaining aseptic conditions within a closed payload system to preserve the integrity of test samples. Large scientific balloons provide unique access to the stratosphere and careful control over exposure experiments. Our aim in this study was to design, construct, and fly a self-contained payload (autonomous avionics, power, environmental sensors) that could attach to the exterior of large balloon gondolas, permitting a survival-based microbiology experiment at desired altitudes. A secondary aim was to collect baseline data and establish microbiology procedures (including ground and negative controls) for enabling future science flights. We worked with *Bacillus pumilus* SAFR-032, originally isolated from a spacecraft assembly facility at the Jet Propulsion Laboratory (Kempf *et*

*al.*, 2005). The strain was a suitable model microorganism for our study because: (1) the resistance of its spore to environmental extremes is well documented (Gioia *et al.*, 2007; Vaishampayan *et al.*, 2012 and references therein); (2) the full genome is available for transcriptomic and proteomic analysis (Gioia *et al.*, 2007; Tirumalai *et al.*, 2013); (3) the species has no exosporium or extraneous layer associated with spores (Link *et al.*, 2004); and (4) *Bacillus* sp. are commonly found in the upper atmosphere (Smith *et al.*, 2012).

## MATERIALS AND METHODS

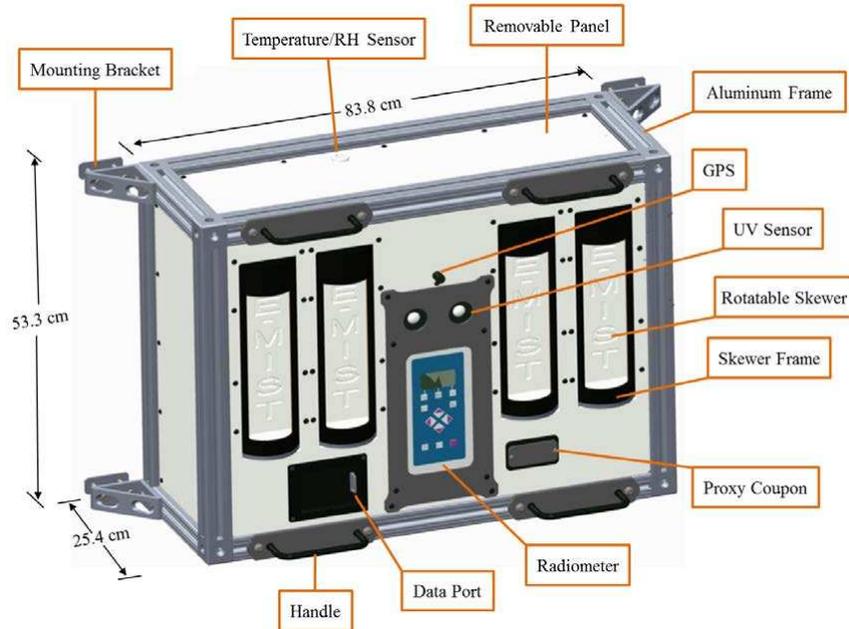
### Payload Description

Exposing Microorganisms in the Stratosphere (E-MIST) was built to mount onto the exterior of a high-altitude balloon gondola (Figures 1-2). The payload (83.3 cm x 53.3 cm x 25.4 cm; mass 36 kg) had four independent “skewers” (Figure 3) that rotated 180° for exposing samples to the stratosphere. During ascent or descent, the samples remained enclosed within dark cylinders at ~25°C. Each skewer had an aluminum base plate holding ten separate, rectangular aluminum coupons (M4985, Seton) with spore samples deposited on the surface. Coupon dimensions were 5.40 cm (w) x 1.75 cm (h) x 0.51 cm (thick), including 0.28 cm diameter holes on ends for mounting to skewer base plates. The inside of each cylindrical skewer was a frame laced with Nomex felt to prevent outside light from leaking into the system. A hex shaft through each skewer was attached to a gear system powered by a commercially-purchased motor (SPG30E-300K, Cytron) controlled by a four-channel FD04A motor controller (Brushed DC Moto Controller, Cytron). The motors, gears, and light shield were held together by a frame composed of aluminum cutouts and 3D-printed polycarbonate-ABS components.

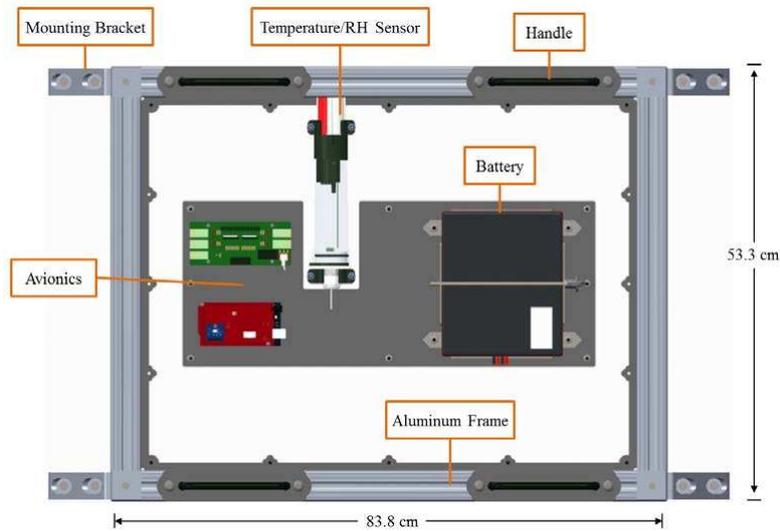
T-slotted 80/20 aluminum extrusions formed the framework of the E-MIST payload, with detachable, white powder-coated aluminum panels on each face of the box. Angle brackets on the back plate were used to mount the system onto the balloon gondola and four foldable handles (McMaster-Carr) were attached to the front-facing frame. Multiple sensors, instruments, and computers were embedded within the housing. In

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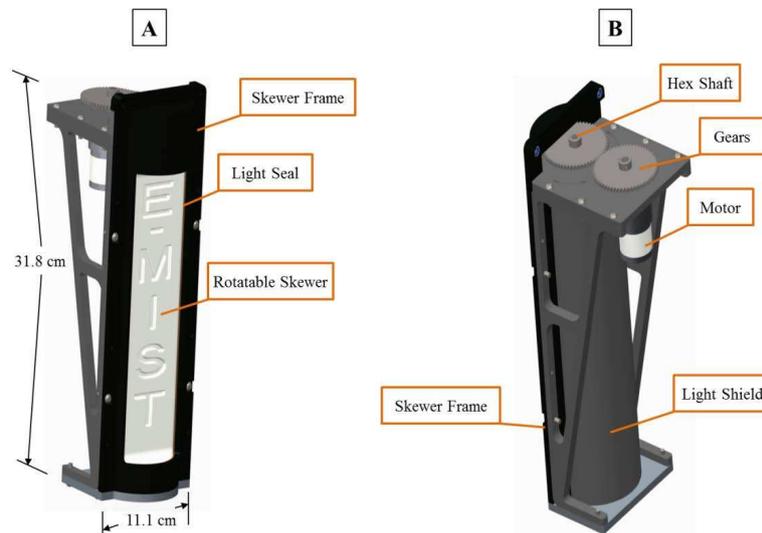
**Figure 1.** Labeled model of payload. Key design features of the Exposing Microorganisms in the Stratosphere (E-MIST) payload, which mounted onto the exterior of a large high-altitude balloon gondola. See text for details of labeled system and components. The mass of the payload was 36 kg.



**Figure 2.** Modeled front view of the E-MIST payload with front panel removed. The key flight computer components, power source, and temperature/relative humidity (RH) sensors are shown.

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**Figure 3. Model of the E-MIST skewers. Four independently-rotating skewers were embedded within the payload and controlled by the flight computer. A light seal and shield on each skewer ensured that the microbe sample exposure was restricted to the target altitudes. This design minimized the influence of the outside environment during other flight activities (e.g., launch, ascent, descent, and recovery). Panel A shows the front face, while Panel B depicts the motor and gear system on the backside of each skewer.**

the center of the system was a stand-alone radiometer (PMA2100, Solar Light) with two ultraviolet (UV) sensors (PMA2107 and PMA2180, Solar Light) that measured UV levels (400 to 230 nm) every five min. The front panel data port contained two Universal Serial Bus (USB) ports, two light emitting diodes (LEDs), a Secure Digital (SD) card module, and two key switches. One key switch was used to power on the system and the other was used to manually rotate the skewers (for loading and removing samples). A sliding door on the front port assembly was held in place by two small magnets. One of the USB ports was used to start, stop, and retrieve data from the HOBO, a stand-alone external humidity and temperature sensor (U23-001, Onset) that collected data every ten s. The other USB port was used to retrieve data from the radiometer. LEDs illuminated when the global positioning system (GPS) (SPK-GPS-GS407A, S.P.K. Electronics Co.) had a lock on location (altitude, time, latitude, and longitude) and when the flight computer was on. The SD card

(uDRIVE-uSD-G1, 4D Systems) module used a universal asynchronous receiver/transmitter to poll a microcontroller (Mega2560, Arduino) and the SD card.

Other major payload components included: an altimeter (MS5607, Parallax), three 8.5 W heaters, three resistance temperature detector (RTDs) (SA1-RTD-B, Omega), and a temperature and humidity sensor (RHT03/DHT22, Aosong Electronics Co.). Power was generated by a 14.8 v 25.2 Ah lithium-ion polymer battery (CU-J141, BatterySpace) fastened in place with a stainless steel battery holder. The power circuit used a DC-DC converter for stepping down 14.8v to 5v. Thermal performance for the payload during tropospheric ascent was modeled using Thermal Desktop (C&R Technologies). Heating pads (5V Heating Pad 5x10 cm, WireKinetics) were included in the payload so that in-flight heat pulses could keep sensors and instruments within desired operating temperature ranges. This regulation system was controlled by the flight computer and RTDs on the battery, radiometer,

# A BALLOON-BASED PAYLOAD FOR EXPOSING MICROORGANISMS IN THE STRATOSPHERE (E-MIST)

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and sample base plate. An additional RTD was placed on a proxy coupon located on the front E-MIST panel.

## Bacterial Preparation and Processing Techniques

*Bacillus pumilus* SAFR-032 was initially grown on tryptic soy agar and incubated at 32°C for 24 h. A nutrient broth sporulation medium induced sporulation, followed by a harvest and purification as previously described (Vaishampayan *et al.*, 2012). The spore stock was re-suspended in sterile deionized water, heat-shocked (80°C for 15 min), and stored at a concentration of  $10^8$  cfu (colony forming units)  $ml^{-1}$  in glass tubes at 4°C. To create a uniform

spore monolayer on the aluminum sample coupons, 100  $\mu l$  aliquots diluted from the stock were spotted onto the surfaces and allowed to dry for 4 h in a dark laminar flow hood (NuAIRE Biological Safety Cabinet, Class II Type A/B3, Model NU-602-400) at standard room temperature (25°C) and pressure (1,013 mb). All coupons had been sterilized overnight with a dry-heat oven at 130°C and cooled to 24°C before deposition of the stock solution. After drying, coupons were stored in sterile, dark containers. We examined a random coupon with a scanning electron microscope (SEM) (JSM-7500F, JEOL Ltd.) to assess the distribution of spores on the surface (Figure 4).

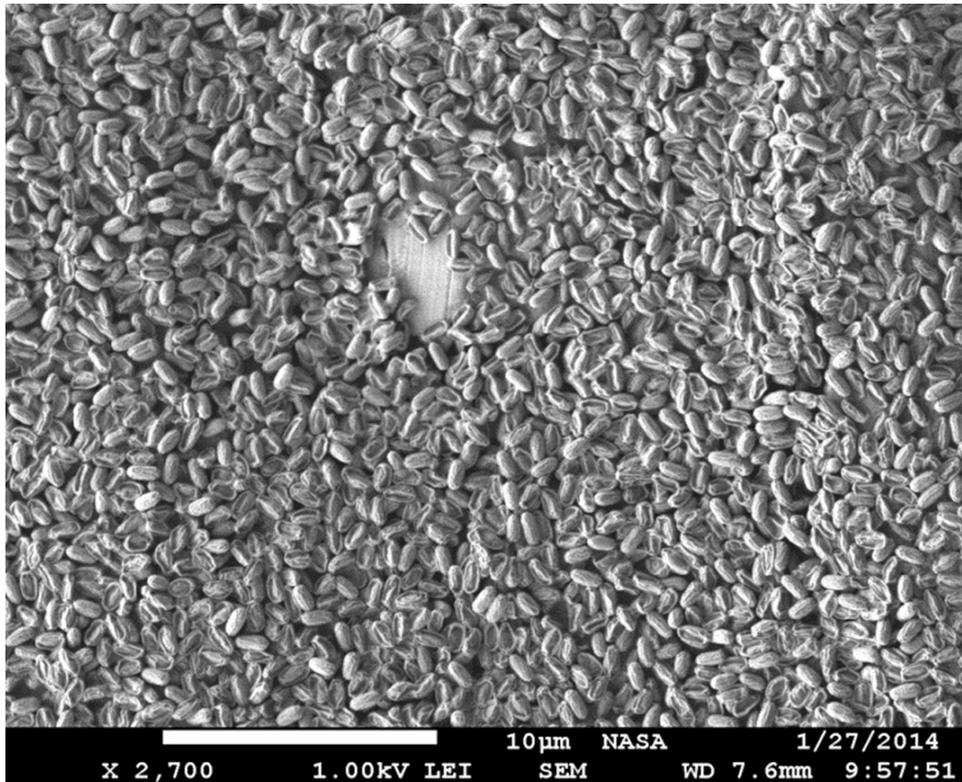


Figure 4. Distribution of *Bacillus pumilus* SAFR-032 spores on an E-MIST sample coupon. Each coupon contained approximately  $1 \times 10^6$  spores and the aluminum coupon surface is visible in the circular gap of spores. The scale bar on the scanning electron micrograph is 10  $\mu m$ .

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Spore viability was measured using the Most Probable Number (MPN) enumeration technique that has been described elsewhere (Mancinelli and Klovstad, 2000; Smith *et al.*, 2011). In brief, spores were dislodged from coupons by vortexing in sterile deionized water and sand for 2 min, and then processed through 6 consecutive serial dilutions. Using 96-well plates, 20  $\mu$ l from each serial dilution, and 180  $\mu$ l of sterile media (Per 1 L: 16 g Difco nutrient broth, 5 g KCl, 0.22 g CaCl<sub>2</sub>, 1.6 g FeCl<sub>3</sub>, 3.4 mg MnSO<sub>4</sub>, 12 mg MgSO<sub>4</sub>, 1 g D-glucose) were loaded into 16 wells per dilution and scored (presence or absence of bacterial growth, assessed by turbidity) after incubation for 36 h at 30°C.

We followed the ribonucleic acid (RNA) recovery procedure described by Moeller *et al.* (2012) to demonstrate the feasibility of this method with our flight coupons. Briefly, spores were lifted from coupons using a 1 ml polyvinyl alcohol solution. Air bubbles were removed by running a sterile glass slide coverslip across the polyvinyl alcohol aliquot, which was then dried overnight inside a petri dish and re-suspended in 1 ml of sterile molecular grade H<sub>2</sub>O. Next, spores were germinated using the protocol established by Nicholson *et al.* (2012), with each centrifugation step at 5,000 rpm for 10 min, except for the final culture volume that was spun down at 14,000 rpm for 5 min. Samples were processed with RNeasy® Protect Bacteria Kit (Cat. No. 74524, Qiagen Inc.) using protocols supplied by the manufacturer. Sample processing then continued at Step 3 Part 1 of the RNeasy® Mini Kit (Cat. No. 74104, Qiagen), followed by an on-column DNase digestion in Part 2 of the RNeasy® Mini Kit (Steps 1 to 4). Finally, the RNA cleanup in Part 2 of the RNeasy® Mini Kit (Steps 5 to 7) were followed, including the optional spin after Step 5 and elution in a volume of 30  $\mu$ l RNase-free H<sub>2</sub>O. RNA yield was measured using the Qubit® 2.0 Fluorometer and standards from the Qubit® RNA HS Assay Kit (Cat. No. Q32855, Life Technologies).

## Procedures for Ground and Balloon Flight Operations

A fully integrated test flight (all payload components powered on and *Bacillus pumilus* SAFR-032 samples loaded) was conducted on a NASA Balloon Program Office large scientific balloon, launched from Ft. Sumner, New Mexico,

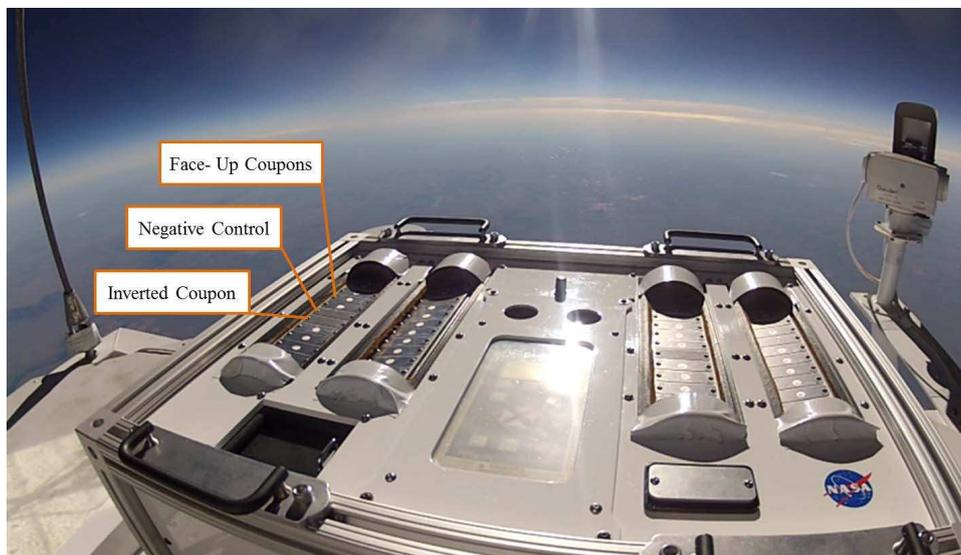
on 24 August 2014. E-MIST was one of several payloads on the Long Duration Balloon Tech Flight 651N. The gondola was carried into the stratosphere by an 8 x 10<sup>5</sup> m<sup>3</sup> helium balloon. Prior to flight (in a launch site hangar), sample coupons were installed onto skewer base plates working inside a portable hood with sterile screws, a screwdriver, and forceps. The hood established an 8-fold reduction in airborne particles (Model 229 Particle Counter, Met One). Loaded E-MIST base plates were sealed until mounting on the flight-line at 1230 Coordinated Universal Time (UTC), about 90 min prior to balloon lift-off. The installation procedure was done at dawn in 10 min, limiting the exposure of samples to light and open air. Sterile tools were carried inside 50 ml Falcon tubes and the entire payload surface was wiped down with isopropyl alcohol before installation. Prior to mounting E-MIST base plates, the inside of each skewer was sprayed with sterile air from a canister. Once installed, the samples were rotated back to the closed position and the other instruments within E-MIST were powered on. Thus, *Bacillus pumilus* SAFR-032 samples remained in the sealed position until the payload reached the lower stratosphere (~20 km ASL), at which point the flight computer rotated the skewers into the outside air (Figure 5). After a short rotation (2 s), all skewers reverted to the closed position for the remainder of the flight. The gondola stayed at a float altitude of 37.6 km for almost 4 h before beginning a 23 min descent on parachute at 1956 UTC. It landed 294 km southwest (33°55'42.6" N; 107°21'46.8" W) of the launch site (34°29'30.1" N; 104°13'36.1" W) and was recovered by Columbia Scientific Balloon Facility (CSBF) personnel. E-MIST was powered off, removed from the gondola, and kept at ambient conditions inside an air-conditioned vehicle. One week later, samples were shipped back to NASA Kennedy Space Center (KSC) still inside the payload.

## Experimental Design

Temperature, relative humidity (RH), atmospheric pressure, and UV levels were measured across the test flight by our team and additional CSBF instruments. Each E-MIST skewer base plate carried 10 *Bacillus pumilus* SAFR-032 coupons: 9 facing up and 1 inverted, since this will be the configuration for future

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**Figure 5.** Test flight deployment on 24 August 2014 in the lower stratosphere over New Mexico. E-MIST payload in flight, with the four skewers rotated open, briefly (2 s) exposing the *Bacillus pumilus* SAFR-032 samples and other control coupons to the stratosphere. On Face-Up Coupons, the spore aliquots are visible as white dots in the center of each sample. One Inverted Coupon (protecting spores from sunlight) was included on each skewer base plate as well. A Negative Control (blank, sterile coupon) was also flown to verify payload seals prevented outside contamination.

flight experiments. The upright coupons received the full stratospheric exposure, including irradiation from sunlight; whereas the inverted coupons were subjected to all other stratospheric effects, except for sunlight. Since the skewer rotation demonstration lasted only 2 s in our test flight, we anticipated no survival differences between the treatments – unless spores were dislodged on inverted coupons due to possible contact with the skewer base plate. Two sets of positive ground controls were prepared along with the flight coupons. One set was transported, but not flown, and another set remained at KSC. We expected the survivability of the ground controls to match flight coupons if the payload successfully prevented outside environmental influences. One (sterile) negative control coupon was also included to understand if the sample base plates were protected from exterior contamination sources.

## RESULTS AND DISCUSSION

Our test flight demonstrated the functionality and reliability of a new payload for exposing microbes in the stratosphere. Baseline data collected can better prepare future research teams using this system. Table 1 summarizes upper and lower limits of key environmental data from the balloon launch, ascent, and float. Internal payload heaters performed nominally, keeping hardware components and sensors within operating limits – particularly in the upper troposphere during the coldest part of the flight. For instance, at 16.4 km ASL when the free air temperature was  $-67.5^{\circ}\text{C}$  (measured by an independent, CSBF gondola sensor), the avionics board inside E-MIST remained at  $-4.10^{\circ}\text{C}$ . However, a few instruments and components did not function properly. First, the altimeter failed, possibly due to radio frequency interference with other gondola instruments. Second, UV measurements were lost

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because the stand-alone radiometer was not powered off during payload recovery (forcing the instrument to eventually overwrite the data stored during float). Obtaining UV data on future experiments will be critical since other survival studies have shown a relationship between irradiation and bacterial inactivation (Smith *et al.*, 2011). Looking forward, modifying the radiometer to store data on the payload flight computer will resolve this issue, and stratospheric UV measurements from other balloon missions (e.g., McPeters *et al.*, 1984) can be used to establish an expected range of irradiation. Finally,

the rotation of the sample skewers was suboptimal. In principle, end-to-end bacterial coupons will receive near-identical sunlight if the skewers open evenly (on the same plane) and no shadows cross the payload face – yet, neither condition was observed in flight. Intermittent gondola shadows passed over the skewers but were transitory since the balloon was constantly rotating. We will build a smart switch system that communicates with the motor controller to ensure skewers open evenly on the next flight opportunity.

**Table 1. Launch, Ascent, and Float Profile.**

	Max.	Min.	Remarks
Atmospheric Pressure (mb)	837	4.26	Altitude of Ft. Sumner, NM, 1.25 km ASL
Air Temp. (°C)	23.3	-67.5	CSBF Free Air Thermistor
Payload External Temp. (°C)	18.5	-13.7	HOBO measurements
Payload Internal Temp. (°C)			Internal heaters pulsed during ascent and descent
Avionics	22.3	-4.10	
Proxy Coupon	46.1	-28.0	
Battery	16.0	-4.65	
Radiometer	37.1	4.15	
Payload Internal RH (%)	65.0	< 3.5	Measurements below sensor sensitivity
Payload External RH (%)	60.5	< 3.5	Measurements below sensor sensitivity
UV (W m <sup>-2</sup> )	N/A	N/A	Data were lost; see text for details

Concerns about contamination (e.g., external microbes penetrating the payload) or inactivation (e.g., outside biocidal factors besides stratospheric conditions) motivated this methods-focused study. Including *Bacillus pumilus* SAFR-032 on the test flight allowed us to evaluate the success of payload containment, microbiological procedures, and protocols. A properly-controlled stratospheric microbiology survival experiment should only expose samples to the targeted region of the upper atmosphere. Our experimental design assessed unknowns associated with sample transportation, balloon gondola installation, balloon ascent/descent, and time lingering in the hot,

dusty New Mexico desert awaiting launch and recovery. We created a batch of experimental control coupons (each containing approximately 1 x 10<sup>6</sup> spores) used throughout the investigation for ground and flight test purposes. Several treatment categories were evaluated: Lab Ground Coupons (kept in the KSC laboratory); Transported Ground Coupons (traveled to New Mexico and back, but not installed in payload); Face-Up Flight Coupons (flown, experienced internal payload conditions and exposed to stratospheric sunlight for 2 s); Inverted Flight Coupons (flown, experienced internal payload conditions and exposed to stratospheric conditions other than sunlight for 2

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s). A subset of coupons from each treatment category was processed, resulting in MPN values reported in Table 2. Averages between coupon groups were compared using one-way permutations (Kruskal-Wallis rank-sum test) at a 95% confidence level, and the differences in the means were not statistically significant. Notably, the brief (2 s) skewer rotation in the lower stratosphere did not reduce the number of spores on the Face-Up Coupons. Also, time spent on the ground did not impact survival numbers.

Temperatures ranging from 80°C to 110°C are generally capable of inactivating spores (Ghosh *et al.*, 2009), but the highest temperatures recorded on the ground (32.4°C for Payload Internal Temperature; 40.1°C for Proxy Coupon) and in flight (46.1°C for Proxy Coupon) were below the threatening range. Taken together, nearly identical numbers from all coupons indicate that balloon flight operations and payload procedures did not influence spore survival.

**Table 2. Most Probable Number of Viable Spores.**

Sample Category	Treatment	MPN ( <i>N</i> , $\sigma$ )
Lab Ground Coupons	Kept in the laboratory	$1.84 \times 10^6$ (1, N/A)
Transported Ground Coupons	Transported to launch site but not installed	$1.93 \times 10^6$ (2, $3.74 \times 10^5$ )
Face-Up Flight Coupons	Flown, exposed for 2 s	$1.91 \times 10^6$ (4, $4.30 \times 10^5$ )
Inverted Flight Coupons	Flown, exposed for 2 s without sunlight	$1.66 \times 10^6$ (4, $5.96 \times 10^5$ )

If future flights deployed the same experimental design and exposed microbes for hours at float, we would expect to see a rapid inactivation. Smith *et al.* (2011) simulated stratospheric conditions and measured a 99.9% loss of viable *Bacillus subtilis* spores after only 6 hours of direct UV irradiation. Beyond investigating viability alone, transcriptome sequencing and differential expression analysis of RNA could provide valuable insight into the functional effects of stratospheric transport on surviving microorganisms. For instance, this approach could help identify genes associated with repairing damage to the cell envelope, genome, and/or core metabolic proteins. Even with complete survival, our coupons yielded only between 0.53 to 0.97  $\mu\text{g}$  total RNA. Generally, about 1  $\mu\text{g}$  RNA per sample is needed for preparing a transcript library from germinated survivors. Recent developments in library preparation may soon allow reproducible single-cell transcriptomics using as little as 10 pg of starting total RNA (Sasagawa *et al.*, 2013). Nevertheless, follow-on stratosphere experiments

may require an order of magnitude increase in the number of spores doped onto coupon surfaces since longer exposures will inactivate a greater portion of samples. Boosting biomass to harvest enough RNA may generate misleading survival data, resulting in a layer of spores that block irradiation effects. Our coupon preparation method using a 100  $\mu\text{l}$  aliquot generated a layer of 1 to 3 spores (Figure 4). Larger coupons (accommodating more spores across the surface) or flying more replicates (to pool samples) may yield enough recoverable RNA without creating unwanted spore layers. The simplicity of the skewer base plate lends itself to customization. Biological requirements can guide the optimal engineering of the skewer base plate. Future investigators can easily reconfigure the base plate to allow for the desired number of replicates and microbe concentrations, or accommodate other categories of microorganisms incompatible with the aluminum coupons.

Earth's stratosphere is extremely dry, cold, irradiated, and hypobaric, and it may be useful for the archive of microorganisms isolated from

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NASA spacecraft assembly facilities (e.g., Benardini III *et al.*, 2014) to be evaluated in an environment analogous to Mars. Survival-based studies were recently deployed outside the International Space Station (ISS) (Horneck *et al.*, 2012) and the same category of experiments can be conducted in the stratosphere. We hope the baseline data, procedures, and controls discussed herein can provide a pathway for future investigations using E-MIST. While spore-forming bacteria have remarkable resistance to atmospheric extremes, non-spore-forming bacteria, archaea, fungi, algae, and viruses should also be examined. A species-specific inactivation model that predicts the persistence of microbes in Earth's upper atmosphere (e.g., pathogenic cereal rusts), or even on the surface of Mars, is one of many possible outcomes from stratospheric microbiology research.

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## Molecular diversity and distribution of marine fungi across 130 European environmental samples

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Environmental DNA and culture-based analyses have suggested that fungi are present in low diversity and in low abundance in many marine environments, especially in the upper water column. Here, we use a dual approach involving high-throughput diversity tag sequencing from both DNA and RNA templates and fluorescent cell counts to evaluate the diversity and relative abundance of fungi across marine samples taken from six European near-shore sites. We removed very rare fungal operational taxonomic units (OTUs) selecting only OTUs recovered from multiple samples for a detailed analysis. This approach identified a set of 71 fungal ‘OTU clusters’ that account for 66% of all the sequences assigned to the Fungi. Phylogenetic analyses demonstrated that this diversity includes a significant number of chytrid-like lineages that had not been previously described, indicating that the marine environment encompasses a number of zoospore fungi that are new to taxonomic inventories. Using the sequence datasets, we identified cases where fungal OTUs were sampled across multiple geographical sites and between different sampling depths. This was especially clear in one relatively abundant and diverse phylogroup tentatively named Novel Chytrid-Like-Clade 1 (NCLC1). For comparison, a subset of the water column samples was also investigated using fluorescent microscopy to examine the abundance of eukaryotes with chitin cell walls. Comparisons of relative abundance of RNA-derived fungal tag sequences and chitin cell-wall counts demonstrate that fungi constitute a low fraction of the eukaryotic community in these water column samples. Taken together, these results demonstrate the phylogenetic position and environmental distribution of 71 lineages, improving our understanding of the diversity and abundance of fungi in marine environments.

### 1. Background

Fungi are osmotrophs and therefore feed by secreting enzymes into the environment to process nutrients externally before taking the resulting metabolites into the cell [1–3]. Using this strategy, Fungi have diversified into important parasitic, mutualistic and saprotrophic forms [2]. Fungi are particularly diverse and abundant in soils, plant-associated habitats [4–11] and freshwater environments

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# MOLECULAR DIVERSITY AND DISTRIBUTION OF MARINE FUNGI ACROSS 130 EUROPEAN ENVIRONMENTAL SAMPLES

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[12–14]. However, the diversity and abundance of fungal microbes in marine environments are unclear, although recent progress has documented 1112 putative marine fungi [15]. Culture/morphology-based analyses have recovered fungi from marine samples [16,17], yet the diversity recovered is much lower than that of terrestrial environments. For example, Kis-Papo [18] reported 467 marine species of fungi from 244 genera, while Hyde *et al.* [19] report 444 species, both results are equivalent to less than 1% of described fungal species at the time of these analyses.

Polymerase chain reactions (PCR) that target the eukaryotic small subunit ribosomal RNA (SSU rRNA) gene have shown a low recovery of fungal sequences from the upper marine water column of both coastal and open water environments [20,21]. Meta-analyses of marine water column samples including 23 coastal libraries (1349 clones) and 12 open-water libraries (826 clones) recovered 16 fungal clones (0.8%) [21], suggesting that fungi are present in low diversity and abundance in water column environments or the methodologies used are biased against recovery of fungal sequences. The low representation of fungi in marine water column clone library analyses is in contrast to equivalent freshwater analyses that demonstrate both a high diversity and relative abundance of fungal OTUs [12–14].

The PCR with primers that preferentially amplify fungal SSU rRNA genes has recovered additional diversity from marine sediment, anoxic and deep-water samples [22–24]. Many of the sequences recovered constitute closely related groups sampled across different environments [25]. Meta-analysis has also demonstrated that clone library sampling of marine fungi was in the most part dominated by Dikarya, yet contained a significant diversity of sequences that branch close to chytrids (fungi with a flagellated spore). Furthermore, this ‘chytrid-like’ diversity encompassed highly variant rDNA sequences when compared with sequences from described fungi [25]. This marine diversity of chytrid-like phylotypes also includes several SSU sequences that branched with the Cryptomycota (syn. Rozellida/Rozellomycota) [25,26], a diverse putative phylum that includes the intracellular myco-parasite *Rozella* and is thought to group with microsporidia as the deepest branch in the Fungi or sister to the Fungi [27–29].

In contrast to the surface marine water column studies, clone library studies using DNA recovered from deep-sea environments have identified a higher relative representation of fungal sequences [30–32]. Both second-generation SSU V4 rR/DNA diversity tag sequencing [33] and meta-transcriptome sequencing [34] suggest fungi dominate eukaryotic communities in deep-sea sediments. Edgcomb *et al.* targeted the eukaryotic community of sediment cores using both RNA and DNA templates and demonstrated that the diversity recovered was dominated by fungi, specifically basidiomycete yeasts branching close to *Cryptococcus* and *Malassezia* [31] and similar results have been recovered in additional studies [23,30–32,35]. Furthermore, fungi have also been recovered from marine animals, algae, muds and hydrothermal vents [16,18,19,36,37]. Here, we use BioMarKs V4 SSU rR/DNA derived Roche/454 sequence tag dataset [38] from 130 samples from six European marine sites combined with fluorescence microscopy for the detection of eukaryotic microbes with chitin cell walls to investigate the diversity and abundance of fungi in water column and surface sediment samples.

**Table 1.** Summary of sequencing results.

sequencing results	number of reads
454 reads included in the analysis ('cleaned')	1 431 325
SWARM-OTUs classified as fungi (before multi-occurrence threshold rule applied)	10 840
processed <sup>a</sup> fungal reads	7202
total reads from sediment samples ( <i>n</i> = 24)	216 013
processed <sup>a</sup> fungal reads from sediment samples	5249
total reads from water column samples ( <i>n</i> = 106)	1 215 312
processed <sup>a</sup> fungal reads from water column	1955

<sup>a</sup>After multi-occurrence threshold rule applied.

## 2. Results and discussion

### (a) Sampling of multi-provenance operational taxonomic unit-clusters

Initial clustering of the tag sequences identified 1752 fungal SWARM-OTUs encompassing 10 840 reads from European marine waters (table 1). Figure 1 summarizes the taxonomic assignment of these 1752 fungal SWARM-OTUs. Taxonomic assignment was accomplished by using the most numerous sequence read within each SWARM-OTU for VSEARCH against a copy of the PR2 V4 SSU rRNA database [39]. The fungal-assigned OTUs recovered were dominated by Chytridiomycota and unclassified fungi followed by Ascomycota and Basidiomycota.

These reads were filtered in two additional ways: first, representative sequences from each OTU were aligned and masked using the approaches described in the electronic supplement material. This allowed for manual checks to identify sequencing errors such as homo-polymer errors. Phylogenetic analyses demonstrated many OTUs were phylogenetically identical when placed in trees generated from masked alignments. Therefore, the masked OTUs were re-clustered using CD-HIT, allowing for 1 nt variation, to form ‘OTU clusters’.

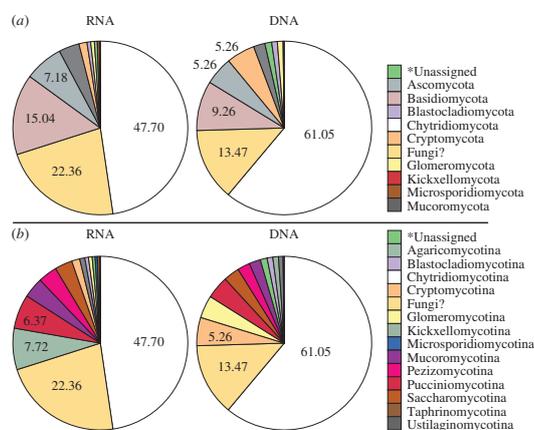
Many of the BioMarKs sampling sites were close to land, as such the diversity profile sampled is likely to be influenced by passive dispersal of spores and other propagules from terrestrial environments. To minimize this source of artefact, and to remove OTUs that were represented by a low number of sequences, we retained only those OTU clusters present in two or more samples if one sample was derived from an RNA template, or present in three or more samples if the OTU clusters were present only in DNA samples. This filtering process resulted in 71 OTU clusters encompassing 7202 reads (table 1) compared with OTUs initially identified (1752 OTUs, 10 840 reads). Although this strict filtering process removed 96% of the diversity of OTUs, it retained 66% of the reads initially assigned to fungi. Indeed, 1107 (77%) of the marine fungal OTUs removed because of low recovery across samples were single sequence-single sample OTU clusters. Furthermore, only 34 OTUs were excluded because they were present in two DNA samples. These 34 ‘DNA OTUs’ encompassed 170 sequences (0.01% of the total quality checked sequencing effort). It is possible that these criteria may lead

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**Figure 1.** Taxonomic composition of the fungal BioMarks sequences prior to multi-occurrence filtering. (a) Phylum-level groupings. (b) Subdivision level groupings. \*'Unassigned' when taxonomy could not be assigned using the higher support threshold used here. 'Fungi?' means the sequences can be assigned to fungi but not to a phylum or subdivision.

to erroneous exclusion of true marine fungal OTUs, but these low numbers suggest that this is a minor factor, and unimportant in comparison to other sources of artefact such as partial primer coverage of the Fungi and/or incomplete sequence sample saturation of the amplicon libraries. However, such processing allows us to identify a subset of fungi likely to be functional in these marine environments.

The 71 'OTU clusters' contained an average of 99.7% ( $\pm$  s.e.m. = 0.106) sequence similarity (comparison of unmasked sequences reads) with 99.3% being the lowest mean pair-wise level of similarity within a cluster ( $\pm$  s.e.m. = 0.106; electronic supplementary material, table S3). Nonetheless, each OTU cluster potentially encompasses considerable strain/species variation. This is because the V4 SSU rDNA, as with all regions of the SSU rRNA encoding gene, does not encompass enough variation to accurately track species diversity in the Fungi (as such ITS markers are often favoured [14,40]). Consequently, the 71 OTU clusters identified are likely to represent clusters of closely related strains/species.

## (b) Diversity of repeat-sampled operational taxonomic units

Seventy-one rDNA sequences, each one representing an OTU cluster, were aligned with sequences derived from known fungal taxa and environmental sequences. Phylogenetic analysis allowed us to assign these sequences to two separate alignments: Dikarya (31 OTU clusters; figure 2) or chytrid-like (40 OTU clusters; figure 3). Phylogenetic analyses were then conducted using both maximum-likelihood and Log-Det distance methods. These analyses placed all 31 Dikarya-like sequences and seven chytrid-like sequences with known fungal or Cryptomycota/Rozell[ida]-omycota/Aphelid CRA (CRA) species with greater than or equal to 50% bootstrap support according to one or both phylogenetic methods. Twenty-three of the chytrid-like SSU rDNA sequences branch with greater than or equal to 50% bootstrap support with published environmental SSU rDNA sequences that had previously been shown to branch within the Fungi/CRA

sequences ([12,13,23,41,42]; figure 3) using full-length SSU rDNA phylogenies. The 10 remaining sequences affiliated with chytrid-like sequences (eight specifically with CRA) but their phylogenetic placement was not supported by greater than or equal to 50% bootstrap support. Fifty per cent is a low level of bootstrap support for identifying phylogenetic affiliations; it was used here as the phylogenies are calculated from short sections of the SSU rRNA encoding gene with relatively few positions sampled for the alignment (i.e. 342 and 316). As such phylogenetic analysis of these datasets is unlikely to consistently identify strong bootstrap results for even established phylogenetic relationships.

### (i) Dikarya diversity

A diversity of Dikarya phylotypes was detected, such as *Rhodotorula*, *Rhodospiridium*, *Sporobolomyces*, *Kondoa* and *Cryptococcus* (Basidiomycota yeasts), and *Geotrichum*, *Debaryomyces*, *Saccharomyces*, *Candida* and *Pichia* (Ascomycota yeasts). The sequences sampled also include an OTU cluster representative of the marine *Malassezia*-like yeast [35]. These results are consistent with previous findings that the marine Dikarya is dominated by lineages capable of living as yeasts [23,25]. Possible alternative explanations for this result could be an experimental artefact arising from filtration and/or DNA/RNA extraction methods that do not adequately recover template from filamentous fungi with robust cell walls (i.e. Pezizomycotina), consistent with this hypothesis, very few Pezizomycotina (2.32 and 3.52%) were recovered in the 454 sequences prior to multi-occurrence filtering (figure 1b).

### (ii) Chytrid diversity

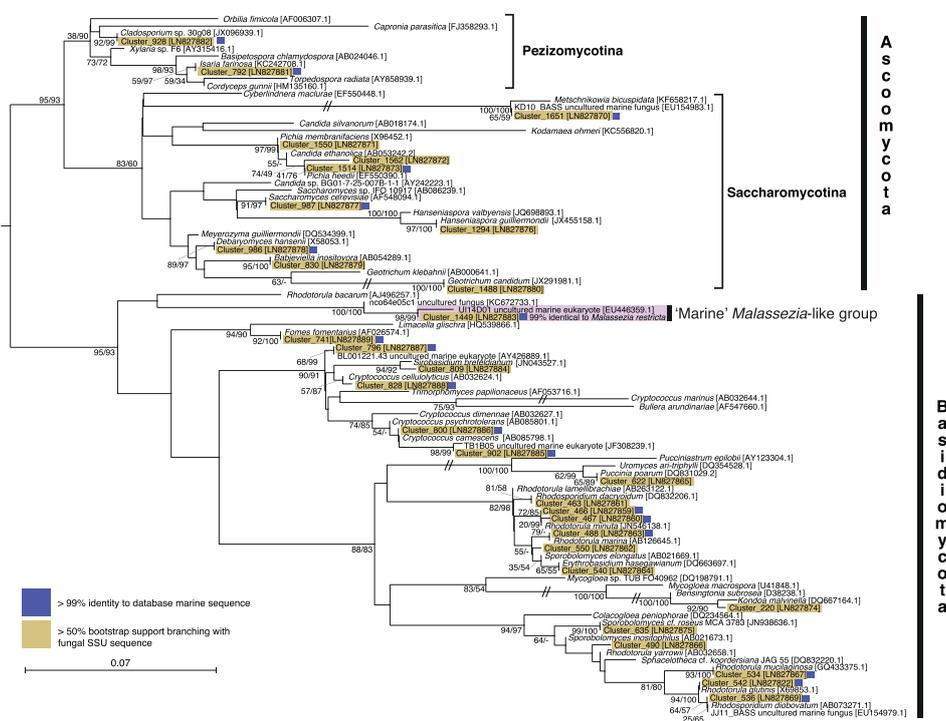
The tag sequencing recovered a diversity of chytrid-like sequences (figure 3). Six OTU clusters branched with known Chytridiomycota, e.g. *Lobulomyces*, *Chytridium* (a close relative of *C. polysiphonia* a parasite of algae [43]) and *Kappamyces*. These data also demonstrate 20 OTU clusters branching close to chytrid-like environmental DNA sequences. Seventeen of these OTU clusters branched in a clade defined by a long terminal branch and bootstrap support of 82/84%, and

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**Figure 2.** Phylogeny of the Dikarya marine OTU clusters. ML phylogeny calculated from an alignment of 94 sequences and 342 positions. Bootstrap values from both 1000 ML and 1000 Log-Det distance pseudo-replicates are shown when  $> 50\%$ . Branches with a double slash mark indicate a branch reduced in length by 1/2. Blue squares next to each sequence indicates OTU clusters which have  $>99\%$  identity to a database sequence from a marine environment.

encompassing a diversity of shorter branches, named here 'Novel Chytrid-Like-Clade 1' (NCLC1, figure 3). This phylogenetic grouping had previously been identified as a divergent marine clade representing a 'basal' branch of fungi [23–25,44]. Indeed, this clade was named Basal Clade 1 by Nagahama *et al.* [44]. Six NCLC1 OTU clusters (414, 778, 766, 445, 1012 and 521) showed a high relative representation in both DNA- and RNA-derived libraries (figure 4a). Furthermore, OTU cluster group 445 was recovered in all the filtration size fractions, suggesting it has a multimodal life cycle as both a small (e.g. spores/cysts) and large cellular form (e.g. multi-cellular [zoo]sporangia or forming saprotrophic or symbiotic interactions). The phylogenetic data presented in figure 3 demonstrate two additional clades labelled NCLC2 and NCLC3 that include chytrid-like environmental phylotypes recovered from aquatic environments.

The phylogenetic results also demonstrate a diversity of 12 OTU clusters that branch with the CRA group (figure 3). This is consistent with previous data suggesting that representatives of this group are present in marine environments [26], although the OTU clusters identified were recovered at a low relative proportion of the sequences (figure 4a). The phylogenetic analysis recovered four OTU clusters branching with the CRA group with greater than or equal to 50% bootstrap support (Clusters: 1542, 996, 1066, 1158). Of interest, Cluster 1066 is part of a putative marine/halotolerant branch [41,45–47]. Cluster 1542 is closely related to sequences sampled from marine and

freshwater environments [48–50] and Cluster 1158 is a divergent relative of *Amoebophilidium* [51]. These data show that the majority of the basally derived fungal lineages detected in these environments belong to Chytridiomycota lineages and not to the CRA group.

### (c) Biogeographic distribution of operational taxonomic unit clusters

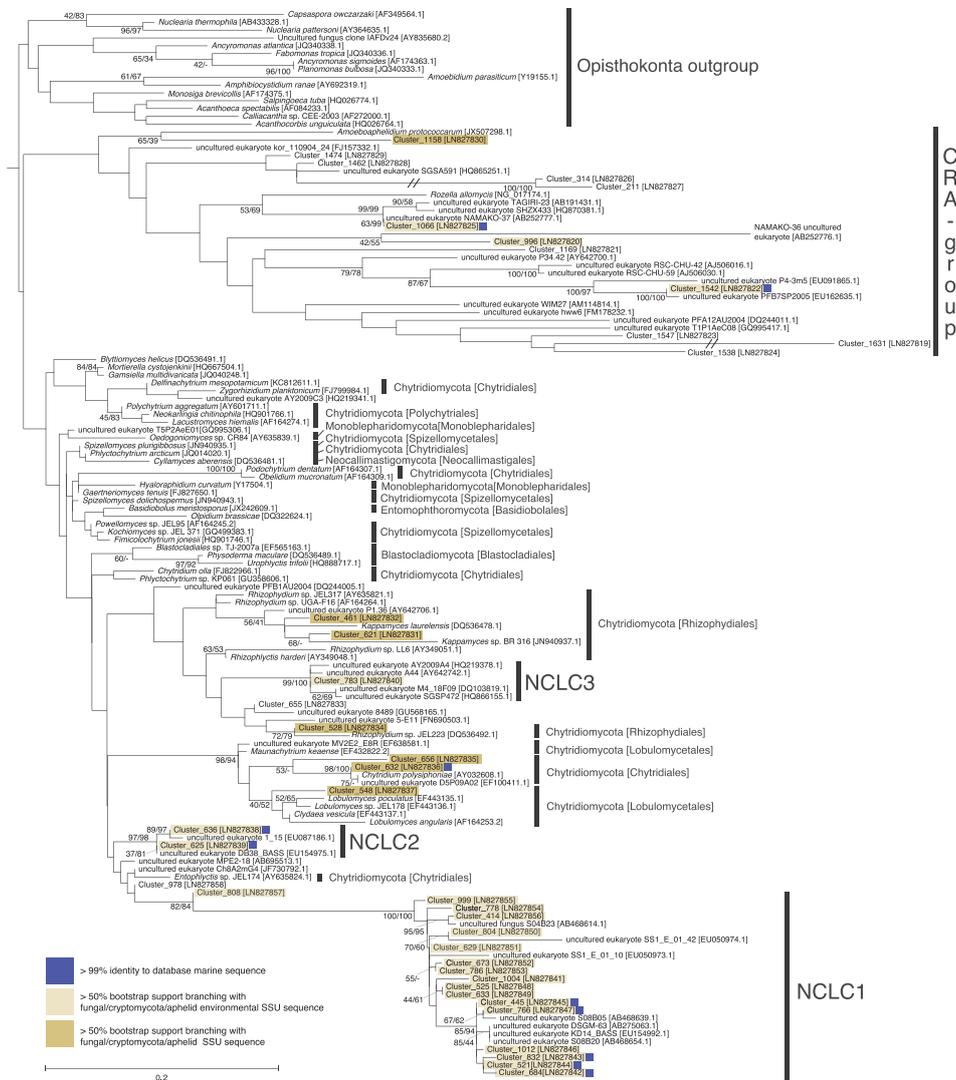
Five of the Dikarya OTU clusters show biogeographic distribution patterns across three or more geographical sampling sites (figure 4a, OTU clusters: 534 (*Rhodotorula mucilaginosa*), 986 (*Debaromyces hansenii*), 463 (*Rhodosporeidium dacryoidum*), 220 (similar to *Kondoa malvinella*) and 488 (*Rhodotorula marina*)). Notably, two of these OTUs (220 and 488) were highly represented in the Varna Black Sea anoxic environment while also showing distribution patterns across multiple geographical sites (figure 4a).

Eight of the chytrid-like sequences were recovered from three or more geographical sites (OTU clusters: 461, 1004, 804, 629, 673, 786, 414, 778), demonstrating a high degree of distribution for these lineages. Interestingly, seven of these OTUs branch within the NCLC1 group. This group has also been detected in previous marine environmental DNA clone library analyses including hydrothermal vent samples [23–25,44]. This pattern of sequence recovery is consistent with the conclusion that NCLC1 encompasses a significant marine radiation of

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**Figure 3.** Phylogeny of the chytid-like marine OTU clusters. ML phylogeny calculated from an alignment of 136 sequences and 316 positions. Bootstrap values from both 1000 ML and 1000 Log-Det distance pseudo-replicates are shown when the values are greater than or equal to 50%. Blue squares next to each sequence indicates OTU clusters that have greater than 99% identity to a database sequence from a marine environment. Branches with a double slash mark indicate a branch reduced in length by 1/2. 'CRA group' shortened name given to Cryptomycota/Rozell[id]a-omycota/Aphelid group. NCLC (Novel Chytid-Like-Clade) groups are labelled.

fungi. None of the CRA group OTU clusters were represented in three or more geographical sites.

Notably, 18 of the 31 Dikarya and 10 of the 41 chytid OTU clusters showed greater than 99% sequence similarity to an SSU rDNA phylotype (figures 2, 3 and 4a) previously sampled from the marine environment [25]. These results further demonstrate evidence of the biogeographic distribution patterns of the fungal OTU clusters identified here (figure 4a) and provide additional support for the hypothesis that these groups represent *bona fide* marine lineages.

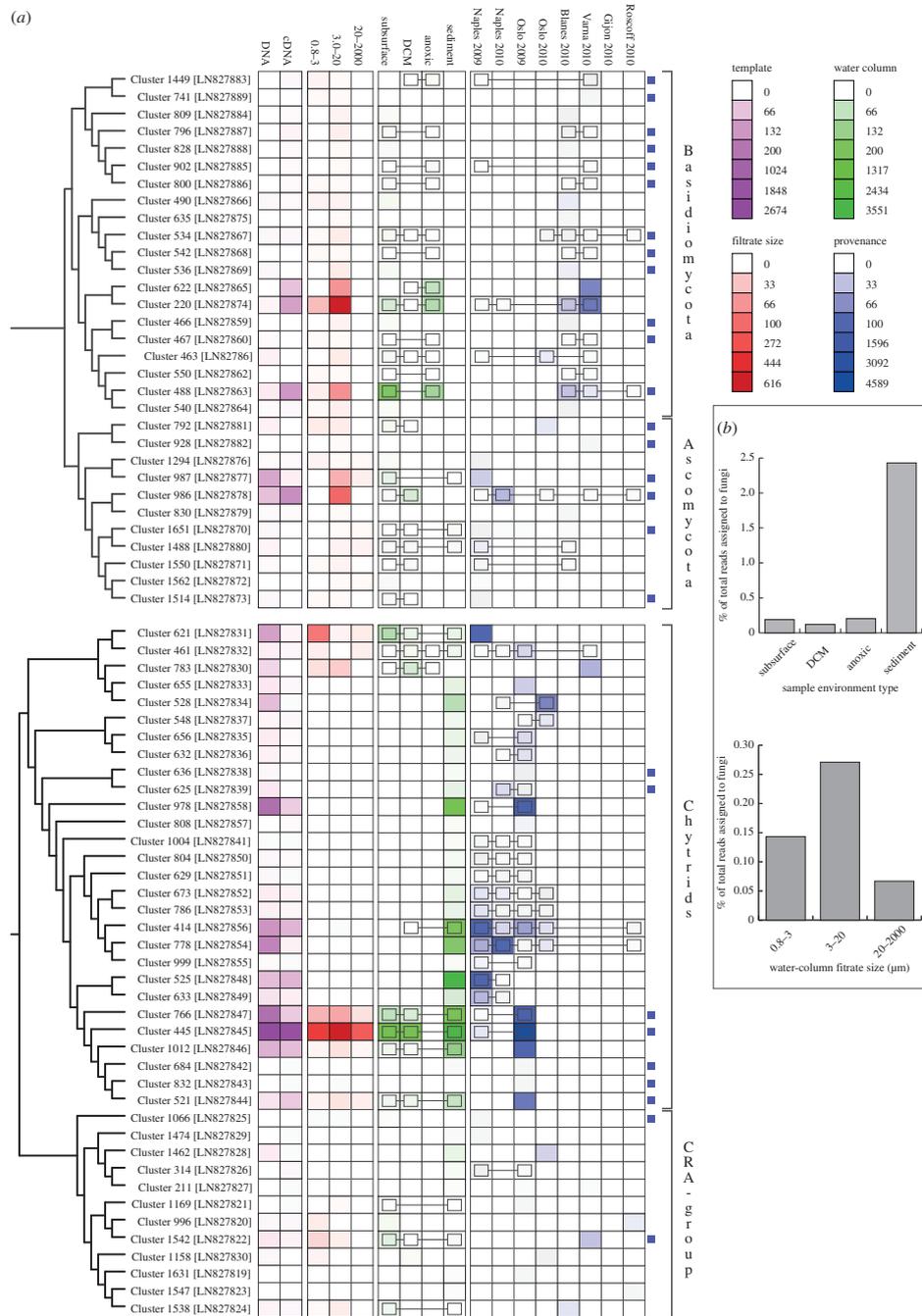
The sequence data demonstrated a higher recovery of fungi sequences from sediment compared with water column (figure 4b), suggesting fungal diversity/abundance is increased in the sediment. This is consistent with a high abundance and diversity of fungi generally found in solid substrate detrital environments, i.e. soils [8] and aquatic sediments [31,33]. However, this observation needs further experimental validation as comparisons between the water column and sediment samples are limited here because DNA and RNA recovery were not conducted using equivalent nucleotide extraction processes (see the

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**Figure 4.** (a) Heat maps showing the sampling provenance of the 71 OTU clusters. Blue squares at the end of a row indicate OTU clusters that have greater than 99% identity to a database sequence from a marine environment. Colour scales are detailed in the legend box (indicating number of reads in each case). The linked boxes on the heat map indicate connected OTU clusters across sampling sites. (b) Per cent of sequencing effort assigned to fungi from different environment types. These results are calculated after the multi-occurrence threshold rule is applied and show a clear increase in fungal representation in sediment environments, either because fungal diversity and abundance is increased in this environment type or because the nucleotide extraction protocol differed between water column and sediment samples.

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electronic supplementary material) and so it is possible that water column sampling failed to recover fungal species with robust cell walls. This could explain the reduced recovery of fungal diversity from water column samples generally (figure 4b) and specifically fungi from the 20 to 2000  $\mu\text{m}$  size fraction where the filamentous fungal forms, with robust and refractory cell-wall structures, are likely to be sampled. Indeed, taxonomic assignment analysis of the total BioMarKs fungal-assigned dataset showed that a very low proportion of the total sequences was assigned to fungal groups known to form filamentous structures in terrestrial environments, for example Pezizomycotina represented only 2.32% and 3.52% of the total DNA and RNA reads, respectively (figure 1b). It also could explain why Dikarya yeasts and chytrid-like sequences were preferentially recovered in these datasets, as both cellular forms are relatively fragile and therefore more readily sampled for RNA/DNA sequencing.

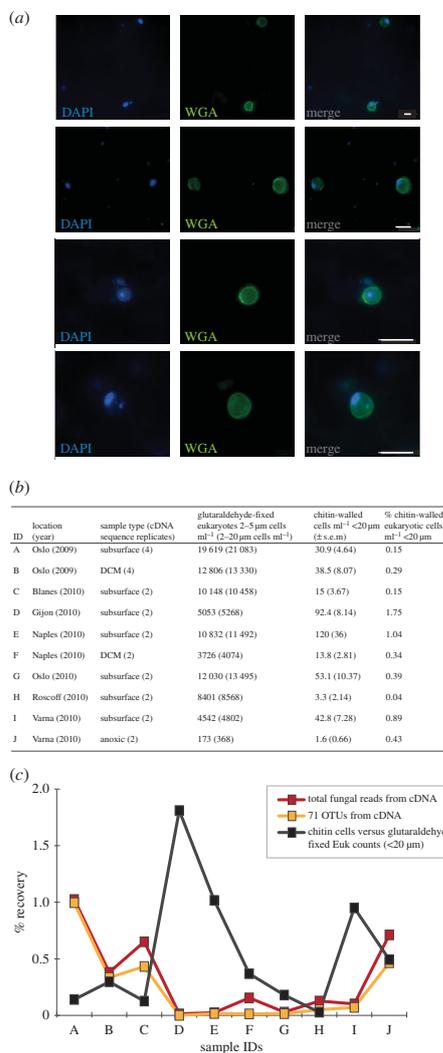
## (d) Chitin-walled cell counts in water column samples

Detection of cells with a chitin wall using the stain Calcofluor White (CFW) has been proposed as a method for assessment of abundance of fungi in water column samples [52]. This method is problematic as many non-fungal species have chitin on their cell surface [53–56], some fungal life cycle stages do not have chitin cell walls (e.g. zoospores) and furthermore, CFW binds to other cell surface polysaccharides such as cellulose [57,58]. We have adapted this approach replacing CFW with a fluorescent-labelled wheatgerm agglutinin (WGA) [27] lectin, which binds chitin. WGA can bind other polymers containing *n*-acetylglucosamine, specifically bacterial peptidoglycan in Gram-positive bacteria, as such it is important to co-stain with a second marker to confirm the target cell is a eukaryote. Here we used DAPI (4',6-diamidino-2-phenylindole) to confirm the target cell contained a distinct DNA containing nucleus-like structure [59,60].

Initially, to compare CFW and WGA approaches we used a separate sample, with a high abundance of chitin cell-walled microbes, to investigate the relative abundance of WGA-stained cells and of cells stained with both CFW and WGA. Counting three independent filters demonstrated a concentration of 1248 cells  $\text{ml}^{-1}$  (s.d.  $\pm 232$  cells) that had double cell-wall staining (WGA and CFW), while for single WGA staining we observed 1231 cells  $\text{ml}^{-1}$  ( $\pm 580$ ). These results suggest that WGA performs similar to the double-staining approach.

As part of the BioMarKs sampling strategy, microbial cells were collected and processed for fluorescent microscopy from the same environmental samples as used for the DNA/RNA samples. Using 10 representative samples, we used microscopy to identify microbes with chitin cell walls (figure 5a) and counted the total number of eukaryotic cells per millilitre recovered in the less than 20  $\mu\text{m}$  filtration fractions that had a WGA-labelled wall. The WGA microscopy confirmed the presence of spherical cells enclosed within chitin walls with a distinct DAPI-stained nucleus-like structure, i.e. putative yeast or encysted cells (figure 5a), but very few cells with filamentous structures indicative of hypha were identified (less than 50; electronic supplementary material figure S1). These results are consistent with the filtration size fraction (less than 20  $\mu\text{m}$ ), i.e. it is unlikely that we would sample fungal hyphal cells at this size fraction.

The 10 samples contained a mean of 1–120 eukaryotic cells with putative chitin walls per millilitre (figure 5b). These results were compared with the total eukaryotic cell counts per



**Figure 5.** The abundance of fungal cells in the marine water column samples using relative abundance in RNA-derived tag libraries and chitin cell-wall detection. (a) Examples of chitin-walled cells detected using wheatgerm agglutinin (WGA) and DAPI detection. All scale bars measure 5  $\mu\text{m}$ . (b) Provenance and abundance of eukaryotic cells and eukaryotic cells with a chitin cell wall. (c) Comparison of per cent recovery of fungal sequences from RNA sequencing to per cent chitin cells across 10 samples (for sample IDs, see figure 5b).

millilitre from the glutaraldehyde-fixed samples [61]. This demonstrated between 0.15 and 1.75% of the eukaryotic cells in the water column possessed a chitin cell wall (figure 5b). This low rate of recovery is consistent with the RNA relative abundance data from equivalent samples, which demonstrates fungal tag sequences represent a small proportion of the sequencing results recovered (figure 5c) and confirms that there is no abundant population of fungal cells (less than 20  $\mu\text{m}$ ) with chitin walls in the water column that were not detected as part of the molecular sampling. Although the RNA and cell counting

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results are similar in terms of low proportional representation of putative fungi, these data show a weak correlation between parallel samples (figure 5c,  $R^2 = 0.2186$ ,  $p = 0.12$ ). This weak correlation suggests that relative RNA tag abundance and/or chitin detection is a bad proxy for identifying low abundance populations of fungi in the water column.

## 3. Conclusion

Eukaryotic diversity tag sequencing from European water column and sediment samples processed to identify repeat-sampled OTUs demonstrates a low diversity of repeat-sampled putative marine fungi. Furthermore, the RNA-derived tag sequencing also suggests a low relative abundance of fungi (figure 5c). Cell-wall staining confirmed a low abundance of chitin-walled cells in representative water column samples including, but not exclusively, fungal cysts and yeast cells.

We applied a strict criterion for retaining OTU clusters present in multiple sample sets, a process that considerably reduced the number of OTU clusters by 96% but retained 66% of the sequence reads identified as fungi. We argue that this approach is valid as it allows us to identify OTUs that are likely to represent *bona fide* marine lineages and exclude OTUs with low representation across samples. Consistent with this approach, 28 of the 71 OTU clusters are greater than 99% identical to lineages previously sampled from marine environments (figures 2, 3 and 4a). Interestingly, these results demonstrate a substantial diversity of chytrid-like sequences that represent undescribed taxonomic groups, many of which occupy a distinct phylogenetic placement and encompass considerable diversity (e.g. NCLC1, figure 3).

The fungal OTU clusters identified were predominately chytrid-like and yeast Dikarya phylotypes. As discussed this profile may be a product of the sampling strategy. Alternatively, it may suggest that filamentous fungal forms such as Pezizomycotina are less suited for marine water column environments—instead preferentially colonizing solid substrates rich in organic matter such as soils and sediments [25]. As environmental DNA/RNA sampling increases, cross-comparisons will allow for an improved understanding of which OTU clusters represent true marine fungi. It is certain that increased sampling of different marine habitats, including, for example, animals and algae, would reveal further fungal diversity not captured in these samples. Future questions relating to the status of 'marine' fungi include: what are the ecological characteristics of the

marine fungi that allow them to survive in these habitats, how frequently has the marine/terrestrial transition occurred, what are the trophic strategies employed by marine fungi (e.g. parasitism, saprotrophy or mutualistic symbiosis) [25]? However, many of the fungi identified here are likely to be difficult to propagate in culture, either because they are outgrown by contaminating terrestrial fungi also present in the environmental samples, or alternatively their life cycle is dependent on a symbiotic interaction. As such, targeted single cell genomics/transcriptomics [62] represents a useful tool for sampling marine fungi.

**Data accessibility.** Electronic supplementary material that accompanies the online version of this article includes materials and methods, a description of the environments sampled (electronic supplementary material, tables S1 and S2), and a table showing the per cent similarity within each of the OTU clusters (electronic supplementary material, table S3). Electronic supplementary material, figure S1 eukaryotic cells with putative chitin cell walls and filamentous cell structures. Representative sequences of the 71 have been submitted to the European Nucleotide Archive: LN827819-LN827889. Additional supporting data are available at GitHub ([https://github.com/guyleonard/marine\\_fungi](https://github.com/guyleonard/marine_fungi) with doi:10.5281/zenodo.16817). These data include: (i) all the sequences grouped into the 71 OTU clusters (fasta files), (ii) a spread-sheet showing the recovery of tag sequences classified in the 71 OTU clusters from the 130 environmental samples, (iii) the water column cell counting data, (iv) the two V4 SSU rDNA alignments as MASE files with mask details available using SeaView [63], and (v) the tree files of the phylogenies shown in figures 1 and 2.

**Authors' contributions.** T.A.R., G.L., J.d.C., Fr.M., Fi.M. and M.D. contributed to the bioinformatic analysis. T.A.R., S.R., M.D., C.D.V., R.M. and A.C. contributed to sampling and molecular analysis. J.d.C. analysed the wider Opisthokonta dataset for misplaced fungal OTUs, A.C. and M.D.M.J. conducted the chitin cell-wall counts. R.M. conducted the eukaryotic cell counts. T.A.R., G.L. and A.C. analysed the data. T.A.R., R.M. and A.C. wrote the manuscript.

**Competing interests.** We have no competing interests.

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