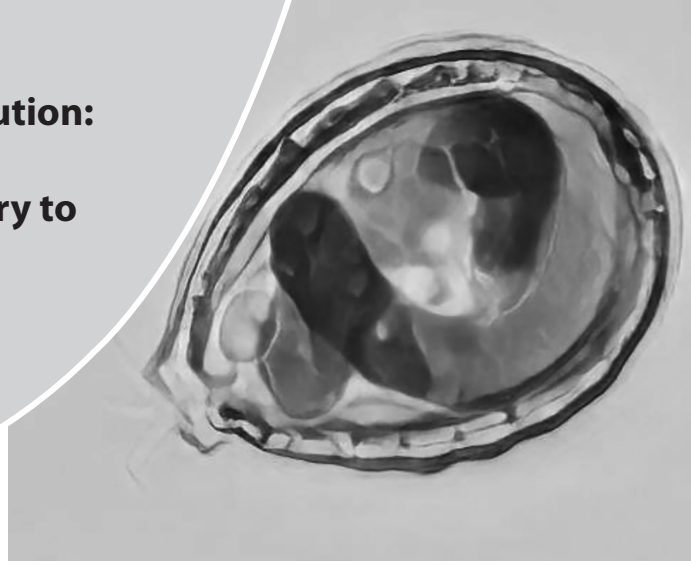
INQUIRY & INVESTIGATION

Evidence for Macroevolution: Using a Microbial Phylogenetics Laboratory to Teach Endosymbiosis



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Abstract

The teaching and learning of macroevolutionary processes have received limited attention in the evolution education literature despite their importance in evolution acceptance and evolution understanding. This necessitates the development of pedagogical content knowledge, including best practices in curriculum and instruction, on macroevolutionary processes that support student understanding and acceptance of macroevolution. One promising approach is to consider macroevolution at the microbiological level by teaching endosymbiotic theory while capitalizing on pre-existing pedagogical content knowledge of tree thinking and bioinformatics. Here, we present a computational laboratory activity that guides students through the construction of a phylogeny based on the universal small subunit ribosomal RNA gene. The resulting phylogenetic tree demonstrates that the photosynthetic organelles of the protist Paulinella chromatophora *evolved independently of the chloroplasts of plants and algae. This not only addresses the need for pedagogical content knowledge in macroevolution in an interdisciplinary and integrative fashion, but also serves as a foundation for future research into the teaching of endosymbiosis. This activity is designed for a 15–20 student introductory/intermediate biology laboratory.*

Key Words: *macroevolution; pedagogical content knowledge; endosymbiosis; molecular phylogenetics.*

○ Introduction

Research on the teaching and learning of macroevolutionary processes has received more attention since initial calls for its inclusion (Catley, 2006; Padian, 2010). Educational research has included topics such as fossils and the fossil record (Borgerding & Raven, 2018; Dodick & Orion, 2003), transitional forms (Mead, 2009; Platt, 2009), deep time (Delgado, 2014; Stenlund et al., 2022; Stenlund & Tibell, 2019), speciation (Evans, 2000; Samarapungavan & Wiers, 1997), the evolution of multicellularity (Pentz et al., 2015; Ratcliff et al., 2014), and evolutionary transitions in individuality (Michod et al., 2022). This has facilitated the development of pedagogical content knowledge concerning best practices for teaching macroevolution (Ziadie & Andrews, 2018, 2019). This is especially important since explicit instruction on macroevolution has been shown to increase evolution acceptance (Cotner et al., 2010; Gibson & Hoefnagels, 2015; Nadelson & Southerland, 2010; Walter et al., 2013), which is a major goal of evolution education (Dunk et al., 2019; Smith & Siegel, 2016). Not surprisingly, macroevolution has been identified as a top priority of evolution education research (Harms & Reiss, 2019; Ziadie & Andrews, 2018, 2019). Because students tend to accept microevolution (within-species evolution) but not necessarily macroevolution (evolution of new species or higher-level clades) (Barnes et al., 2021, 2022), it is necessary to further develop macroevolutionary curriculum and instruction strategies.

An interdisciplinary approach is necessary to bridge the gaps between the fields of microbiology, cell biology, and evolutionary biology to present macroevolutionary accounts of microbes to undergraduate students.

Evolution education has largely focused on examples of large multicellular organisms, likely because we are large multicellular organisms and our own species' evolution is important to us. However, given the political and intellectual milieu of our time, human evolution may not be the best nor most convincing starting point (Grunspan et al., 2021; Miller et al., 2006, 2022; Pobiner, 2016). Teaching macroevolutionary processes of microbes may more effectively increase evolution acceptance. Problematically, content experts of both evolutionary biology and microbiology/cell biology are usually not the same person. Evolutionary concepts are not the focus of microbiology or cell biology courses; and similarly, cell biology and microbiology, if even mentioned, are not the focus of evolution courses. An interdisciplinary approach is necessary to bridge the gaps between the fields of microbiology, cell biology, and evolutionary biology to present macroevolutionary accounts

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of microbes to undergraduate students (Burmeister & Smith, 2016; Fraga, 2018). Therefore, any tools created to facilitate this interdisciplinary approach to macroevolution education will likely start at the microbiological level. Here, we outline an introductory-level computational lab to develop a framework for introducing the evidence for endosymbiosis, the macroevolutionary process that led to the evolution of mitochondria and chloroplasts. This activity is grounded in tree thinking, which has a rich history in evolution pedagogy (Gregory, 2008; Meisel, 2010; Novick & Catley, 2016; Phillips et al., 2012), and can be used as an exemplar for teaching macroevolution at the microbiological level. This activity is designed for an introductory biology laboratory with between 15 and 20 students. In our experience, students can follow the instructions and troubleshoot the workflow with little intervention. We highly recommend instructors deliver a short introductory lecture on endosymbiosis and the hypothesis being tested in the activity (slides are provided as Supplemental Material with the online version of this article).

○ Endosymbiosis and Tree Thinking: Overview and Core Concepts

Endosymbiosis as a Microbiological Macroevolutionary Process

What is endosymbiosis? Endosymbiosis is when one cell lives inside another cell. The relationships of endosymbioses can run the gamut of other symbioses; parasites, commensals, or mutualists can live within host cells. In longstanding endosymbioses, the symbiont loses its autonomy and becomes an organelle with only a remnant of its past freedom (McCutcheon, 2021). The two most famous organellar endosymbioses resulted in the mitochondria and the chloroplast. Mitochondria evolved from an alphaproteobacterium over 2 billion years ago (Roger et al., 2017), whereas chloroplasts evolved from a cyanobacterium about 1 billion years ago (Falcón et al., 2010). There are younger endosymbioses that have resulted in relationships that blur the boundaries between organelle and endosymbiont (McCutcheon, 2021), one of which is the focus of the activity presented below (Delaye et al., 2016; Stephens et al., 2021). While this activity focuses on primary endosymbioses of bacteria, more complicated secondary and tertiary endosymbioses have resulted in the many different kinds of unicellular algae spread across the tree of eukaryotes (Burki, 2017; Cavalier-Smith, 1999; Dorrell et al., 2017; Miller & Delwiche, 2015; Petersen et al., 2014; Ševcíková et al., 2015; Stiller et al., 2014). In this activity, we are exclusively looking at a relatively recently discovered young primary endosymbiosis that evolved independent of the plant primary endosymbiosis (Bodył et al., 2007; Kepner, 1905; Macorano & Nowack, 2021; Nowack & Grossman, 2012).

How do longstanding endosymbioses evolve? Any event that ends up with one cell inside of another can result in an endosymbiotic relationship. Cells eat other cells, and sometimes delaying the digestion of photosynthetic food can lead to some extra sugar. Sometimes, digestion can be delayed permanently (McCutcheon, 2021). At some point, the symbiont begins to lose redundant genes and even transfer some to the host nucleus in a process called horizontal gene transfer. Transferred genes are transcribed in the nucleus and translated on cytosolic ribosomes and transported into the symbiont. The process is unidirectional and leads to ever-shrinking gene complement such as those found in the genomes of mitochondria and chloroplasts.

How do we KNOW that mitochondria and chloroplasts are derived from other cells? Endosymbiotic theory is over a century old starting with Merezhkovsky and a few other Russian scientists (Sapp, 1994), but popularized by Lynn Margulis in the latter half of the twentieth century (Sagan, 1967). Mitochondria and chloroplasts were known to contain their own genomes and ribosomes for some time, but the first genetic evidence for the relationship between organelles and specific bacterial groups emerged from Dalhousie University in Canada in the 1970s (Bonen et al., 1977; Bonen & Doolittle, 1975). Researchers were able to show that mitochondrial and chloroplast ribosomes were most similar to particular bacterial groups. Since then, the evidence has only grown and no evidence to the contrary has been presented to our knowledge. Both organelles have two membranes, just like gram-negative bacteria. The inner membranes house electron transport chains, just like the inner membranes of gram-negative bacteria. When phylogenetic trees are constructed using mitochondria and chloroplast genes, the organelles branch near alphaproteobacteria and cyanobacteria, respectively. Since this discovery, several more recent endosymbioses have been identified.

Tree thinking as an approach to study endosymbiosis

One of the most active areas of evolution education research regarding macroevolutionary processes centers around "tree thinking" (Baum et al., 2005; O'Hara, 1988, 1997), or the knowledge and skills necessary to accurately read, interpret, and construct phylogenetic trees (Gibson & Hoefnagels, 2015; Gregory, 2008; Meisel, 2010; Novick & Catley, 2013). Education research thus far has focused on several aspects of tree thinking and student understanding of phylogenetic trees, such as instructional practice (Eddy et al., 2013; Halverson, 2011; Halverson et al., 2011; Marcelos & Nagem, 2012; Novick & Catley, 2016, 2018; Perry et al., 2008; Phillips et al., 2012), assessment (Blacquiere et al., 2020; Blacquiere & Hoese, 2016; Smith et al., 2013), tree style and topology (Catley et al., 2010; Dees et al., 2017, 2018; Podani, 2013), skills for reading trees (Schramm et al., 2019, 2021), common misconceptions (Dees et al., 2014; Gregory, 2008; Kummer et al., 2016; Meir et al., 2007; Meisel, 2010), and teleological issues (Omland et al., 2008; Schramm & Schmiemann, 2019). In addition to studies on various aspects of tree thinking, there has been development of several measures to analyze student understanding of phylogenetic trees, such as the concept inventories (Kummer et al., 2019; Naegle, 2009) and a diagnostic instrument (Jenkins et al., 2022). Therefore, the body of pedagogical content knowledge concerning a tree thinking approach to understanding macroevolutionary patterns has been well-developed.

In the present laboratory activity, we leverage this well-developed body of pedagogical content knowledge on tree thinking, as well as previous activities that use bioinformatic and computational approaches, to construct phylogenetic trees (Campo & Garcia-Vazquez, 2008; Zhang, 2012), to facilitate learning about endosymbiosis as a microbiological macroevolutionary process. This will concern a recent (~ 60 million years ago) endosymbiotic event that resembles the original chloroplast endosymbiosis within the shelled amoeba *Paulinella chromatophora*. Within the *P. chromatophora* cell reside two chromatophores that derive from a symbiosis with cyanobacteria. A simple ribosomal DNA phylogeny can be constructed to demonstrate the close relationship between these chromatophores and cyanobacteria that are distinct from the cyanobacteria that gave rise to primary chloroplasts in other

Protist – a eukaryote that is not a fungus, plant, or animal. Examples include amoebae, ciliates, flagellates, euglenids, algae, and kelp.

Rhizaria – a very large group of eukaryotes that include many heterotrophic flagellated protists as well as shelled amoebae such as *Paulinella chromatophora*. Other examples include forams (Foraminifera) and radiolarians made famous by Haeckel's extravagant drawings.

Paulinella chromatophora – a shelled amoeboid rhizarian protist with photosynthetic organelles called chromatophores.

Microevolution – within-species evolution: changes in gene frequency over generational time.

Macroevolution – evolution beyond the species level. This includes speciation.

Endosymbiosis – a symbiosis in which one organism lives inside the cells of another. Mitochondria and chloroplasts evolved from ancient endosymbioses with alphaproteobacteria and cyanobacteria, respectively.

Primary endosymbiosis – an endosymbiosis in which the endosymbiont is a prokaryote.

Secondary endosymbiosis – an endosymbiosis in which the endosymbiont is a eukaryote that already has a prokaryotic endosymbiont (e.g., green algae living inside an amoeba).

Mitochondria – eukaryotic organelles derived from an ancient endosymbiosis with an alphaproteobacterium, the powerhouse of the eukaryotic cell.

Chloroplasts – photosynthetic eukaryotic organelles. All chloroplasts derive from a single primary endosymbiotic event.

Chromatophores – photosynthetic endosymbionts of *Paulinella chromatophora*.

Primary chloroplasts – chloroplasts in the archaeplastid lineage, which includes red algae, green algae (and all plants), and glaucophyte algae.

Secondary chloroplasts – chloroplasts resulting from a secondary endosymbiosis of red or green algae.

Homology – the case when two traits have a shared ancestry. Bird wings, bat wings, and whale fore-fins have homology to one another as tetrapod fore-limbs.

Homologue – genes that evolved from a common ancestral gene.

Homologous site – nucleotide or amino acid sequences that derive from an ancestral site in an ancestral homologous gene/protein.

16S SSU ribosomal sequence – the small subunit ribosomal gene of a prokaryote.

18S SSU ribosomal sequence – the small subunit ribosomal gene of a eukaryote.

Clade – a monophyletic group.

Monophyletic Group – a group of organisms that include ALL the descendants of a common ancestor.

Paraphyletic Group – a group of organisms that include an ancestor and some of its descendants.

Horizontal gene transfer – in contrast to vertical gene transfer, horizontal gene transfer occurs between lineages rather than within a lineage.

Bootstrapping – in simplified terms, bootstrapping is a kind of replication test that provides confidence estimates of the branches in a phylogenetic tree.

Figure 1. Glossary of important vocabulary.

photosynthetic eukaryotes. The following laboratory activity walks students through the construction of this ribosomal DNA phylogeny to understand the recent endosymbiosis of the chromatophores of *Paulinella chromatophora*. Figure 1 contains important vocabulary terms referenced throughout the laboratory activity to support the teaching and learning of key ideas in endosymbiotic theory.

○ Laboratory Activity

Computational Requirements

Participants will require a computer with an Internet connection and a web browser. A text editor is also required. While any text editor can be used (e.g., Wordpad), we recommend something such as Atom (https://atom.en.softonic.com/) or BBEdit (https://www.barebones.com/products/bbedit/download.html). Finally, to visualize and manipulate the final tree, a tree viewing program is also required. We recommend FigTree (https://github.com/rambaut/figtree/releases), as it is an excellent open-source viewer that is rather intuitive and simple to use. An alternative is to use iToL (https://itol.embl.de/) (Letunic & Bork, 2021), but this interface is less intuitive and has several complicating features, though it is easily accessible online and therefore does not require any software downloads.

Overview

Phylogenetics is the study of evolutionary relationships between taxa. Phylogenies are evidence-based hypotheses about evolutionary relationships depicted as evolutionary trees or cladograms. One way to infer phylogenies is to compare molecular data, or DNA and protein sequences, using a variety of computer programs. These molecular sequences from various taxa are analyzed by identifying differences between the sequences through the process of alignment, and it is from the alignment of these molecular sequences that a molecular phylogeny is developed.

The first step is to align homologous sequences (i.e., sequences with a common evolutionary origin). Figure 2 depicts a small section of a DNA alignment from three different genera: *Chromera* (an alga related to the malaria parasite), and *Clostridium* and *Sporacetigenium* (two bacteria). This alignment shows the similarity between the DNA sequences of even taxa from very different lineages. Similarities between the DNA sequences are indicated in three ways in Figure 2: (1) nucleotides highlighted in light blue indicate that all three sequences have the same nucleotide in the same position, (2) nucleotides highlighted in gray indicate that two of the three sequences have the same nucleotide in the same position, and (3) nucleotides highlighted in white indicate where there is a different nucleotide in that specific position relative to the other DNA sequences in the alignment. The high degree of identity and lack of gaps in the alignment indicate that these sites are likely homologous and appropriate for molecular phylogenetic analysis. Once the sequences are aligned, phylogenetic trees can be generated using bioinformatics programs. Different programs use different algorithms to compute likely phylogenies; some programs will use maximum likelihood (ML) methods to generate these trees while others will use Bayesian (Posterior Probability) analyses (Douady et al., 2003; Svennblad et al., 2006). For the purposes of this laboratory activity, the phylogenetic tree will be constructed using a program called PhyML (Guindon et al., 2010), which uses ML methods.

When inferring deep-branching phylogenies, which compare DNA or protein sequences to analyze evolutionary relationships at high levels of biological classification such as supergroups or domains, it is important to use very slow-evolving sequences (Moreira & Philippe, 2000). Slow-evolving sequences allow for analysis of divergence between sequences that occurred deep in evolutionary time. While there are many examples of slow-evolving sequences, some of the most commonly used are ribosomal sequences, the small ribosomal subunit (SSU, also called 16S for bacteria and 18S for eukaryotes) in particular. Ribosomal sequences are extremely slow-evolving due to their conserved essential functions across all domains of life, but not so slow that no changes accrue, and thus will be useful in analyzing evolutionary relationships with respect to endosymbiosis.

This activity will determine whether the chromatophore, a chloroplast-like organelle, in the cercozoan rhizarian *Paulinella chromatophora* is related to the chloroplasts found in other eukaryotes such as archaeplastids (e.g., plants, red algae, green algae), haptophytes, cryptophytes, stramenopiles (e.g., diatoms), alveolates (e.g., dinoflagellates, apicomplexans), and a closely related cercozoan rhizarian, the chlorarachniophyte *Bigelowiella natans*. Current understanding of the tree of eukaryotes can be found in recent papers

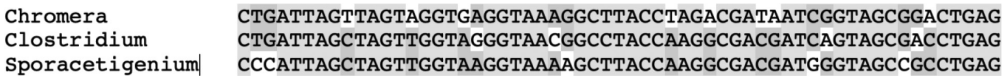

Figure 2. Ribosomal DNA sequence alignment between genera: *Chromera, Clostridium,* and *Sporacetigenium.*

Table 1. List of all prokaryotic and eukaryotic organisms included in the laboratory activity. The activity uses the 16S ribosomal sequences to create a molecular phylogeny based on the plastids, although the 18S ribosomal sequences from the nuclear DNA of some species are included for the assessment activity as indicated by the asterisks. Prokaryotic species include the gram-positive and chloroflexi bacteria (the outgroup for the final tree) and the cyanobacteria (to differentiate the chromatophore in *P. chromatophora* from chloroplasts). Eukaryotic species include the Archaeplastida (plants, green algae, red algae) which have plastids related to the cyanobacteria, and an assortment of eukaryotic groups that have plastids from secondary and tertiary endosymbioses (Stramenopila, Alveolata, Rhizaria, Excavata, Haptophyta, Cryptophyta) to confirm that the chromatophore is not related to these plastids from higher level endosymbioses.

Prokaryota		**Eukaryota**	
Gram-positive bacteria	*Clostridium sp.*	Archaeplastida	
	Sporacetigenium mesophilum	Streptophyta	*Arabadopsis thaliana**
	Thermotalea metallivorans	Chlorophyta	*Micromonas sp.**
	Geosporobacter sp.		*Chlamydomonas reinhardtii**
Chloroflexi	*Kouleothrix aurantiaca*	Rhodophyta	*Cyanidium caldarium**
	Nitrolancea hollandica		*Cyanidioschyzon merolae**
	Uncultured Chloroflexi	Other eukaryotes	
Cyanobacteria	*Aphanothece sp.*	Stramenopila	*Ectocarpus siliculosis**
	Synechococcus sp.	Alveolata	*Chromera velia*
	Cyanobium sp.		*Vitrella brassicaformus*
	Prochlorococcus marinus		*Dinophysis norvegica*
	Leptolyngbya sp.	Rhizaria	*Bigelowiella natans**
	Pseudanabaenaceae cyanobacterium		*Paulinella chromatophora**
	Oscillatoriales cyanobacterium	Excavata	*Euglena gracilis*
	Aphanothece minutissima	Haptophyta	*Emiliania huxleyi*
	Cyanodictyon sp.	Cryptophyta	*Guillardia theta*

(Adl et al., 2019; Burki et al., 2020). Ribosomal sequences from a representative species of each of these eukaryotic lineages, including that of *Paulinella chromatophora*, will be compared with ribosomal sequences of cyanobacteria and gram-positive (monoderm) bacteria to yield insights as to the evolutionary origin of the chromatophore. These species are listed in Table 1, and their SSU sequences from bacteria and chloroplasts are in the Supplementary Material II textfile (.txt) provided with the online version of this article.

This selection of prokaryotic and eukaryotic lineages has been chosen for the following reasons. Firmicutes and chloroflexi bacterial SSU sequences were chosen as outgroups to cyanobacteria (Hug et al., 2016). A selection of cyanobacterial SSU sequences, including the representatives closely related to primary plastids were selected because they are needed to differentiate chloroplasts, which are plastids derived from a singular primary endosymbiosis of a cyanobacterium ~1 billion years ago (Falcón et al., 2010), from chromatophores. A selection of green algal, red algal, and plant primary plastid SSUs were selected as representatives of the archaeplastid lineage, the only known group with plastids most closely related to cyanobacteria. A selection of SSUs from secondary/tertiary plastids were chosen to confirm that chromatophores are not related to any higher-level endosymbioses (Table 1). Finally, we include as Supplemental Materials III textfile (.txt) provided with the online version of this article the corresponding nuclear (18S) SSUs from most eukaryotes mentioned above that can be used in an assessment activity (see below).

The following steps are designed to walk both educators and students through the process of creating a molecular phylogenetic tree that includes three iterative steps: (1) obtaining DNA sequences from the National Center for Biotechnology Information (NCBI), (2) creating a multiple sequence alignment with MUSCLE (Madeira et al., 2022), and (3) constructing a molecular phylogenetic tree from the multiple sequence alignment using PhyML. The full assignment, with directions and visuals from NCBI, MUSCLE, and PhyML, along with assessment questions, are included in the Supplementary Material I document provided with the online version of this article. Here, we summarize the overarching goals and steps for each phase of the laboratory activity.

Step One: Obtain DNA Sequences

Ribosomal sequences from representative species can be obtained from the NCBI, which is a United States government-funded national resource for molecular biology information. It provides

access to many public databases, references, genomes, and several bioinformatic programs, and can be accessed here: www.ncbi.nlm.nih.gov. The representative sequences, from which specifically the SSU ribosomal sequences are included in the provided text file, are listed in Table 1. The *Paulinella chromatophora* is in bold as it is not provided and must be retrieved from an online as repository described below.

To obtain the *Paulinella chromatophora* SSU DNA sequence, students will navigate to the NCBI website (www.ncbi.nlm.nih.gov) and use the "Nucleotide" search with "Paulinella chromatophora 16S" as a query and choose the longest sequence that contains only the 16S ~1400bp. The best sequence has the GenBank accession: FJ456919.1 (https://www.ncbi.nlm.nih.gov/nuccore/FJ456919.1?report=fasta). This sequence will be added to the provided text file in FASTA format (Pearson & Lipman, 1988), which is a standard format for protein and nucleotide sequences in bioinformatic analyses. These FASTA formatted sequences are easily collated to be used in the multiple sequence alignment, the next step in the laboratory activity.

Step Two: ONE-CLICK Phylogeny

The collated FASTA sequences can be pasted as a program input for a phylogenetic pipeline at phylogeny.fr (Dereeper et al., 2008) that includes the multiple sequence alignment program MUSCLE (Edgar, 2004), which aligns SSU sequences. The alignment is then fed into the program GBLOCKS (Castresana, 2000), which trims

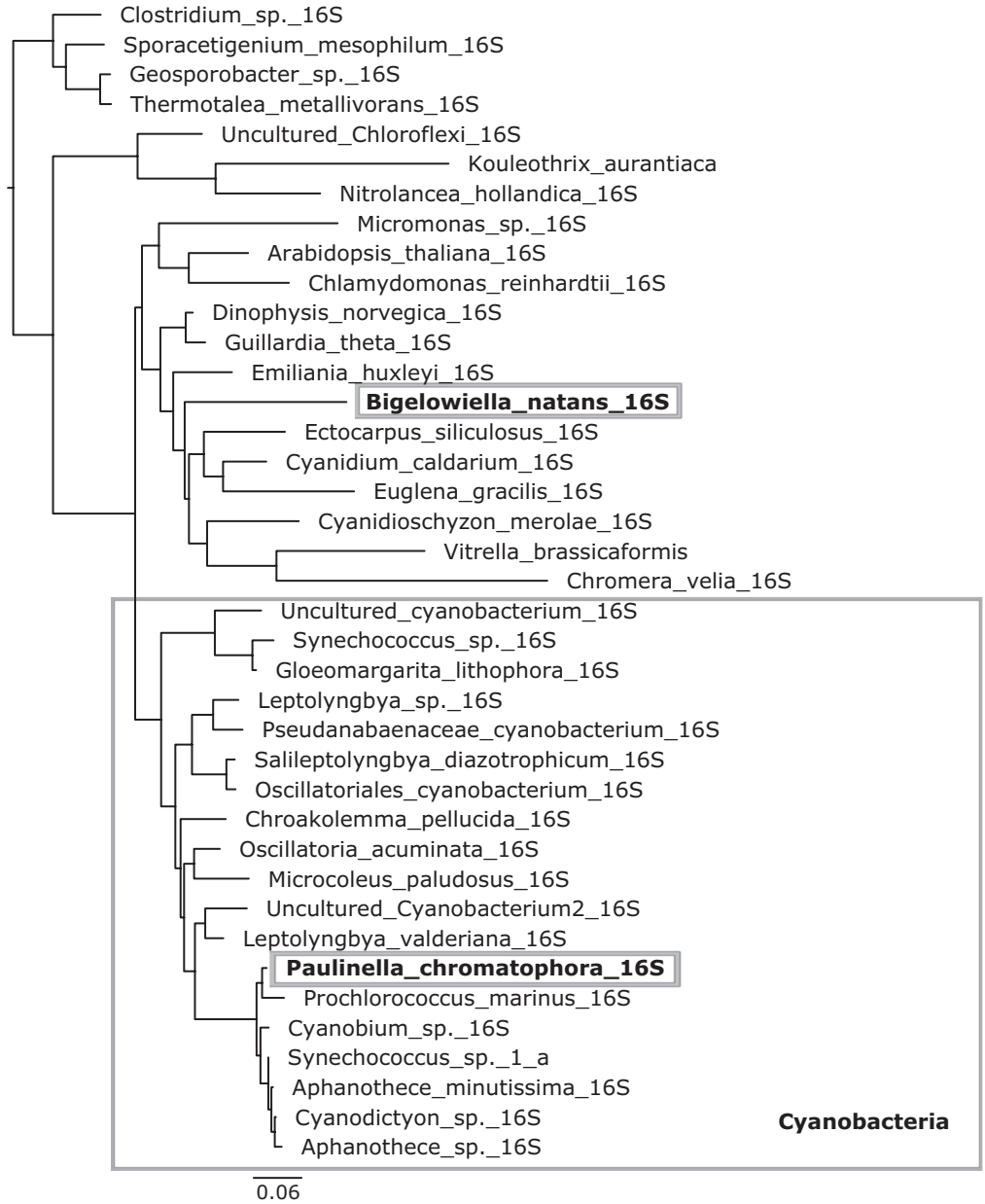

Figure 3. Final molecular phylogenetic tree that is produced by FigTree at the end of the lab activity. Observe the positions of *Paulinella chromatophora* and *Bigelowiella natans*, both cercozoan rhizarians, and the cyanobacteria in this final tree. The cercozoan rhizarians are highlighted in light blue boxes and the cyanobacteria are highlighted in a green box labeled "Cyanobacteria." The gram-positive bacteria (i.e., *Clostridium* sp., *Sporacetigenium mesophilum*, *Geosporobacter* sp., and *Thermotalea metallivorans*) are the appropriate outgroup for this tree.

poorly aligned regions. Finally, the GBLOCKS output is used to construct a phylogeny using PhyML (Guindon & Gascuel, 2003). The resulting phylogeny can be downloaded in Newick format and viewed and manipulated in FigTree (http://tree.bio.ed.ac.uk/software/figtree/) (Figure 3) or, with practice, iToL (https://itol.embl.de/) (Letunic & Bork, 2021). To answer some of the research questions, the tree will need to be rooted with the appropriate organisms as an outgroup.

Using Molecular Phylogenetic Trees to Understand Endosymbiosis

This final molecular phylogenetic tree will provide an evidence-based hypothesis for the relationship between the chromatophore of *Paulinella chromatophora* and chloroplasts found within multiple eukaryotic lineages compared with cyanobacteria and gram-positive bacteria. Once the final molecular phylogenetic tree has been constructed, students can answer the following questions:

1. Why did you choose to root the tree where you did? (Hint: what is the outgroup for this experiment?). **A: I chose to root the tree with the non-cyanobacterial bacteria (or with one of these groups) because this represents the outgroup for this experiment. The important part of the tree is within the cyanobacterial derived sequences (including plastids).**
2. In this tree what kind of group do the cyanobacteria form? What about the Archaeplastida? Explain. **A: Cyanobacteria**

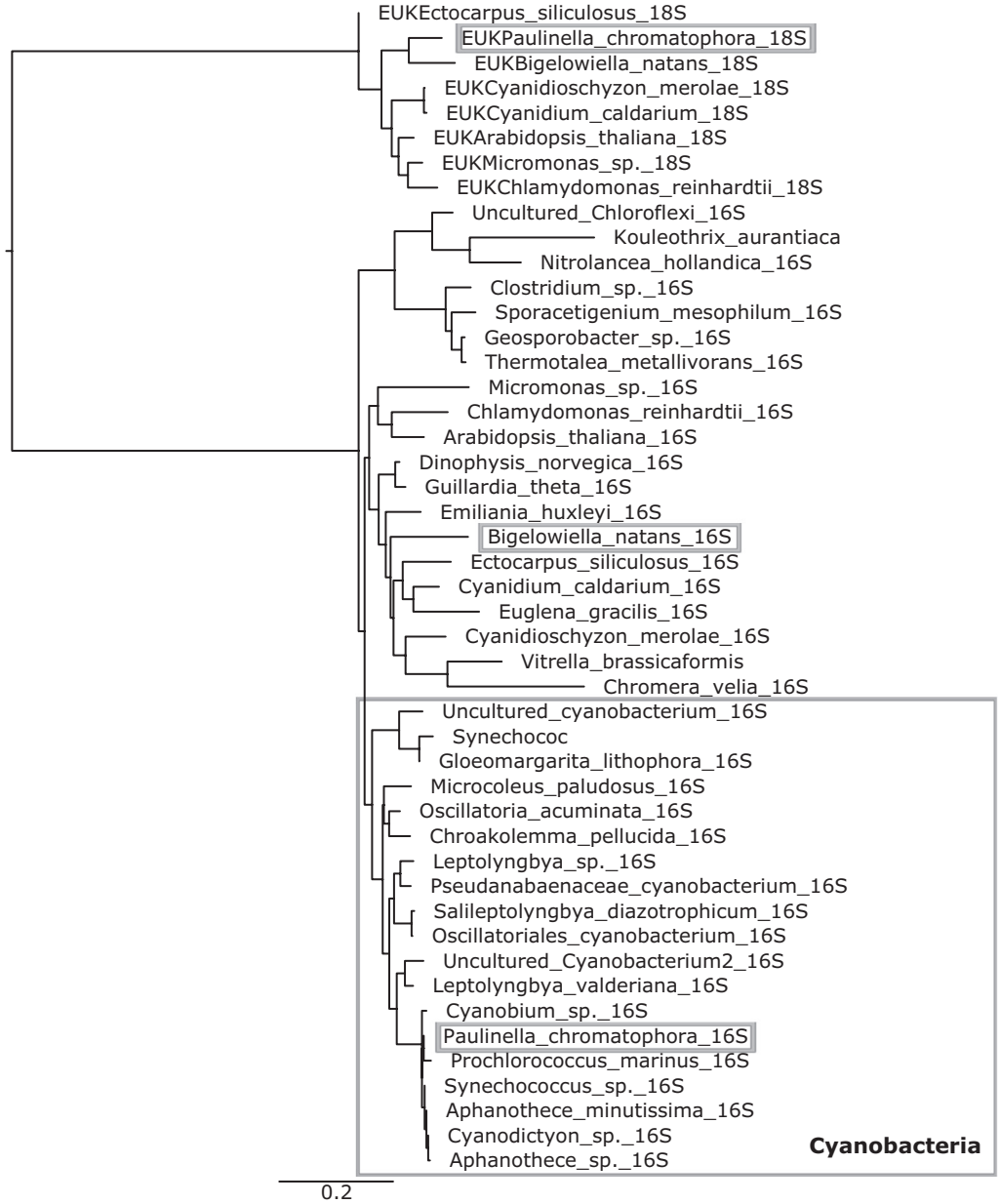

Figure 4. Molecular phylogenetic tree that is produced by FigTree with the 18S ribosomal sequences. Observe how the 16S (prokaryotic) sequences and 18S (eukaryotic) sequences form two different clades in this tree. Once again, observe the positions of *Paulinella chromatophora* and *Bigelowiella natans*, both cercozoan rhizarians, which are highlighted in blue. Make sure to observe the positions with respect to both the 16S and 18S sequences of both rhizarian species. In addition, once again observe the position of the cyanobacteria, highlighted in the green box labeled "Cyanobacteria."

form a paraphyletic group because the *Paulinella* chromatophore intervenes. Cyanobacteria and *Paulinella* forms a monophyletic group. Archaeplastida also form a polyphyletic group since the secondary/tertiary plastid 16S sequences (i.e., cryptophytes, haptophytes, stramenopiles, alveolates) branch close to rhodophyte sequences (i.e., *Cyanidioschyzon* and *Cyanidium*).

3. What organisms are most closely related to the *Paulinella* chromatophore? What conclusions can you make about the origins of the *Paulinella* chromatophore versus the chloroplasts found in other eukaryotic groups? **A: The chromatophore 16S branches within a particular group of cyanobacteria near *Prochlorococcus*. This is strongly supported. The chloroplasts appear clustering together, but not with any particular cyanobacterial group, likely due to how divergent they are after 1 billion years of evolution. We can conclude that the *Paulinella* chromatophore arose more recently than chloroplasts and derive from a cyanobacterium closely related to *Prochlorococcus*.**
4. Draw the tree (rooted appropriately) that you would expect to see if we added all of the eukaryotic cytoplasmic ribosomal sequences (i.e., the 18S ribosomal sequences) from the organisms in this study (collapse the prokaryotic groups if this makes the tree easier to draw). Use the provided 18S eukaryotic sequences to test your hypothesis. **A: This can be used as a summative assessment. Students can redo the activity and should get the tree shown in Figure 4.**

Discussion questions for students who complete the summative assessment could include: Why don't the eukaryotic cytosolic ribosomal sequences branch with their respective plastid ribosomal sequences? (A: because they are not as closely related [i.e., plastids are related to cyanobacteria]). Why is the branch leading to the cytosolic ribosomal sequences so long? (A: because eukaryotes have been evolving independently from bacteria for almost 4 billion years, so more changes have taken place). If you included archaeal 16S sequences, where do you predict they would branch? (A: eukaryotes evolved from archaea, so if archaeal 16S sequences were included, they would branch near the eukaryotic cytosolic ribosomal sequences). Does this tree change your conclusions about the origins of the *Paulinella* chromatophore? (A: No, the tree topology is almost exactly the same if the cytosolic ribosomal sequences are ignored. Therefore, conclusions about chromatophore origins would not change).

○ Discussion

Teaching Endosymbiosis as a Microbiological Macroevolutionary Process

Ziadie and Andrews (2018, 2019) identify the explicit teaching of macroevolutionary processes to be a top priority in evolution education research due to the lack of pedagogical content knowledge on the subject. Despite the calls for its inclusion in evolution education research (Catley, 2006; Padian, 2010), calls for teacher professional development materials on macroevolution (Friedrichsen et al., 2016), and its connection to evolution acceptance (Nadelson & Southerland, 2010), there has been little research on the teaching of macroevolution outside of phylogenetic biology. Endosymbiosis as a major macroevolutionary process remains untouched by evolution education research, despite research in analogous areas such as the evolution of multicellularity (Pentz et al., 2015; Ratcliff et al., 2014) and evolutionary transitions in individuality (Michod et al., 2022). Our efforts here leverage the pedagogical content knowledge on tree thinking (Baum et al., 2005; O'Hara, 1988, 1997) to teach the core principle of endosymbiosis from an evolutionary cell biological point of view. This starting point aims to make research on the teaching of endosymbiosis, and macroevolution, tractable.

Future Directions

Future research on the teaching and learning of modern endosymbiotic theory supports current efforts toward increasing attention on the role of student understanding of macroevolutionary processes on student knowledge, understanding, and acceptance of evolutionary biology. First, overarching conceptual frameworks of the core tenets of endosymbiotic theory, including current scientific research, needs to be translated into the evolution education literature for use in future evolution education studies regarding macroevolution. Second, in parallel to the development of conceptual frameworks, explicit laboratory activities centered around key concepts in endosymbiotic theory, such as endosymbiotic gene transfer, would provide instructors at the secondary and postsecondary levels of education specific activities for teaching these concepts (Friedrichsen et al., 2016). Third, future research should consider whether inclusion of endosymbiosis in the evolutionary biology curriculum leads to an increase in evolution acceptance, especially since students tend to accept microevolution rather than macroevolution (Barnes et al., 2021, 2022).

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