Examining the relationship between the testate amoeba *Hyalosphenia papilio* (Arcellinida, Amoebozoa) and its associated intracellular microalgae using molecular and microscopic methods

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Abstract

Symbiotic relationships between heterotrophic and phototrophic partners are common in microbial eukaryotes. Among Arcellinida (Amoebozoa), several species are associated with microalgae of the genus *Chlorella* (Archaeplastida). So far, these symbioses were assumed to be stable and mutualistic, yet details of the interactions remained scarce. Here, we analyzed 22 single-cell transcriptomes and 36 genomes of the Arcellinida morphospecies *Hyalosphenia papilio*, which contains *Chlorella* algae, to shed light on the amoeba-algae association. By characterizing the genetic diversity of associated *Chlorella*, we detected two distinct clades with a biogeographic signal across sampling sites. Fluorescence and transmission electron microscopy showed the presence of intact algae cells within the amoeba cell. However, analysis of sequencing data suggested transcriptional inactivity of the algae nuclei, implying that instead of a stable, mutualistic relationship, the algae may be temporarily exploited for photosynthetic activity before being digested by the host. Differences in gene expression of *H. papilio* and *Hyalosphenia elegans* (a congener without algae) demonstrated increased expression of genes related to oxidative stress in *H. papilio*. Together, our analyses help increase knowledge of this host-symbiont association and reveal 1) higher diversity of associated algae than previously characterized, 2) a transient association between *H. papilio* and *Chlorella* with unclear benefits for the algae, and 3) symbiont-induced gene expression changes in the host.

Key words: protists, Arcellinida, *Hyalosphenia papilio*, *Chlorella*, symbiosis, microalgae, transcriptomics, genomics, fluorescence microscopy, TEM

Introduction

Many lineages of microbial eukaryotes live in close association with intra- or extracellular symbionts that can either be other microbial eukaryotes or prokaryotes (reviewed in: Gast et al., 2007; Nowack and Melkonian, 2010). The nature of these associations and their degree of closeness fall along a spectrum, reaching from obligate to facultative symbioses (e.g., Stoecker et al., 2009). While obligate symbionts are unable to survive without their host, facultative symbionts can also be found as free-living organisms (e.g., Fisher et al., 2017). Among the most common symbioses of microbial eukaryotes is the association between a heterotrophic organism and microalgae (Esteban et al. 2010; Nowack and Melkonian, 2010). This relationship provides benefits to the heterotroph in the form of organic compounds produced by the algae through photosynthesis, while the algae may benefit from a refuge from predation and a controlled cytoplasmic environment (e.g., Johnson et al., 2007). Examples from across microbial eukaryotes for these types of associations include the green algal symbionts of *Paramecium bursaria* (Takahashi, 2016) and multiple symbionts from across the tree of life in diverse Foraminifera species (e.g. *Minutocellus polymorphus, Navicula* sp., Schmidt et al., 2018).

Symbioses can be further classified based on the duration of the relationship between host and symbiont, from transient to stable, and eventually even organelle incorporation (Nowack and Melkonian, 2010, Stoecker et al., 2009). In addition, symbioses can provide a variable degree of benefit to the partners from being mutualistic and beneficial for each to being antagonistic and thus only beneficial to one partner at the expense of the other (e.g. Bronstein 1994). Studying examples of symbioses from along these spectra is a useful way to gain an understanding of the evolution of photosynthetic organisms and how photosynthetic organelles may have been acquired throughout the history of life (Lara and Gomaa, 2017).

Associations with microalgae are commonly found among shell-building amoebae of the order Arcellinida (Amoebozoa), that are the focus of this study. At least three morphospecies of Arcellinida are known to harbor photosynthetic organisms inside their cytoplasm (Gomaa et al.,

2014; Lara and Gomaa, 2017). These associations are assumed to be stable and mutualistic, as the amoeba species containing these symbionts are usually not found without them (Lara and Gomaa, 2017). Genotyping of the microalgae in Arcellinida and other protist lineages, including ciliates, revealed surprisingly low diversity among *Chlorella* strains associated with these microbial eukaryotes (Gomaa et al., 2014; Zagata et al 2016; Flemming et al 2020). Based on analysis of the plastid *rbcL* gene, Gomaa et al. (2014) argued that a single clade of *Chlorella* is the dominant symbiont across diverse species of Arcellinida, including *Hyalosphenia papilio* and *Heleopera sphagni,* and Rhizaria: including *Archerella flavum and Placocista spinosa.* In addition, no variability among the algae was found to be associated with the high amounts of cryptic diversity observed in some of the host species (e.g. Singer et al., 2019). The lack of diversity in *Chlorella* strains within these diverse species has led to the postulation that while vertical acquisition of symbionts is possible, a great portion of these symbionts are acquired horizontally from the environment, though symbiont acquisition has never been directly observed due to the difficulty of maintaining lab cultures of these organisms (Lara and Gomaa, 2017).

In this study, we examine the relationship between the Arcellinida morphospecies *Hyalosphenia papilio* and its associated algae *Chlorella* sp. (Archaeplastida) in more detail by using sequencing and imaging approaches. *Hyalosphenia papilio* is one of the most abundant species of Arcellinida in low pH bogs, where it is found on *Sphagnum* moss (Gomaa et al., 2014; Heal, 1962; Lahr et al., 2019; Ruggiero et al., 2020). This species appears bright green under the light microscope due to the high number of *Chlorella* cells contained within its cytoplasm. We investigate the diversity of *Chlorella* algae living within *H. papilio* samples from locations across New England, USA, to assess if extensive sampling in one area would reveal additional algal diversity than previously observed. Further, we explore the nature of the relationship between host and algae to gain a better understanding of where it falls along the spectra regarding stability and closeness of association. If, for example, the organisms have a

stable, mutualistic relationship, we would expect evidence of a fully functional and active *Chlorella* cell living inside *H. papilio*, whereas if the relationship is transient, we may see signs of degradation and/or inactivity of the algae. To explore these relationships, we take advantage of the "bycatch" from single-cell whole genome and transcriptome amplifications targeting the Arcellinida, as symbiont nucleic acids are co-amplified. Consequently, these samples represent the "community" of organisms present within the Arcellinida test at the time of DNA/RNA amplification. Here we focus on the presence of algal chloroplast and nuclear DNA/RNA in both whole genome amplifications (WGAs) and whole transcriptome amplifications (WTAs) of *H. papilio* samples. We also carried out fluorescence microscopy on sections of resin embedded *H. papilio* harboring *Chlorella* cells as well as transmission electron microscopy (TEM) to shed further light on the state of the algae within its host.

In addition, we sought to understand the effect of symbiont-induced oxidative stress on host gene expression. Free oxygen radicals that are produced during photosynthesis by the symbiont can have detrimental effects on the host if not enough antioxidants are present (Betteridge, 2000). This stress can lead to changes in gene expression in the host to maintain the integrity of cellular functioning (Johnson et al., 2007). To investigate the impact of photosynthesis by the algal symbionts in *H. papilio*, we analyzed differential gene expression between *H. papilio* and its congener *Hyalosphenia elegans*, which lacks photosynthetic symbionts. Together, our analyses contribute to a better understanding of the association between mixotrophic microbial eukaryotes and their photosynthetic symbionts.

Materials and Methods

Sample collection and preparation

We collected Arcellinida samples at four different bogs and fens in New England: "Hawley Bog" (42.576774, -72.890266) and "Harvard Forest "(42.531728, -72.189973) in Massachusetts and "Orono Bog" (44.870752, -68.723785) and "Big Heath" in Acadia National

Park (44.335780, -68.274809) in Maine (**Table S1**). At each site we collected a handful of *Sphagnum* moss from different sampling points. Back in the lab we washed off the Arcellinida from the moss by putting about 10 strands of moss into 50 ml conical tubes with 20 ml filtered (2 µm filter) water from the sampling sites and shaking the tubes. The moss and water were then filtered over a 300 µm mesh into a Petri dish to eliminate large particles. We then hand-picked individual healthy-looking Arcellinida cells from the filtrate under the microscope. We only chose *H. papilio* cells that were bright green, indicating the presence of healthy, undigested microalgae. Each individual was photo-documented and cleaned in filtered (2 µm filter) bog water before being transferred in 1 µl bog water to a sterile 0.2 ml tube for either genome or transcriptome amplification.

Single-cell transcriptomics and genomics

For the amplification of the transcriptomes of individual *H. papilio* and *H. elegans* cells we first added 1.4 µl nuclease-free water to the tubes with the isolated amoeba and then 0.25 µl of the lysis buffer contained within the SMART-Seq® v4 Ultra® Low Input RNA Kit for Sequencing (TaKaRa Bio USA, Inc., Mountain View, CA, USA). The subsequent transcriptome amplification and reverse transcription we conducted according to the manufacturer's protocol, yet in quarter reactions. The SMART-Seq® v4 Ultra® Low Input RNA Kit was selected for this study as it has been applied successfully across diverse microbial eukaryotes, including the difficult to lyse Foraminifera (e.g. Weiner et al. 2020).

For the generation of single-cell genomes we used the REPLI-g Single-Cell Kit (Qiagen, Germantown, MD, USA). We added 1 µl of single cell water and 1.5 µl DLB buffer (both are part of the kit) to the picked cell and then followed the protocol according to the manufacturer's instructions.

After transcriptome/genome amplification we purified all samples using the AMPure XP PCR Purification system (Beckman Coulter Life Sciences, Indianapolis, IN, USA) and quantified the amplified nucleic acid content using a Qubit 3.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). Barcoded sequencing libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and the samples were sequenced on a HiSeq 4000 platform at the Institute for Genome Sciences at the University of Maryland.

Transcriptome/genome assembly and post-assembly processing

We used FastQC (Andrews, 2010) for quality checking of the raw sequencing reads, trimmed adapters using the BBMap toolkit (Bushnell, 2014) and then assembled the reads using SPAdes (for genomes) or rnaSPAdes (for transcriptomes; Bankevich et al., 2012). After assembly, we processed the transcriptomes using our phylogenomic pipeline PhyloToL (Cerón-Romero et al., 2019). This post-assembly processing includes the removal of prokaryotic sequences and ribosomal DNA, assignment of the assembled sequences to gene families as defined by OrthoMCL (Li et al., 2003) using USearch (Edgar, 2010), translation into amino acid sequences and removal of short, highly identical sequences.

Algal diversity and phylogeny

In each of the trimmed 36 genome assemblies (**Table S1**) we searched for contigs containing the chloroplast-encoded *rbcL* sequence using BLASTn version 2.9.0 (Boratyn et al., 2012) with a database from the *Chlorella rbcL* reference sequence (KJ446796.1) from Gomaa et al. (2014). We added 208 representative *Chlorella rbcL* sequences (limited in length to 700-5,000bp) from GenBank (including sequences from Gomaa et al. (2014)) to our dataset and aligned all sequences using Mafft version 7.419 (Katoh and Standley, 2013). We trimmed the alignment to match the length of the *rbcL* sequences from Gomaa et al. (2014) and used jModelTest2 version 2.1.10 to select the best substitution model using the CIPRES Science

Gateway (Miller et al., 2010). We then built a maximum likelihood phylogeny using RAxML version 8.2.12 (Stamatakis, 2014), with GTRGAMMAI specified as the substitution model and 100 bootstraps.

To test for evidence of multiple engulfing events of *Chlorella* algae by distinct protist lineages (i.e., amoeba, ciliates) we performed AU and SH tests with 10,000 re-samplings using the RELL method in IQ-TREE version 1.6.12 (Nguyen et al., 2015). We tested three hypotheses: 1) the best tree from the RAxML analysis above, 2) a constrained tree requiring monophyletic clades of amoeba- and ciliate-associated *Chlorella*, and 3) a constrained tree requiring a monophyletic clade of amoeba-associated *Chlorella*.

To confirm the results of the *rbcL* phylogeny, assess evidence for multiple *Chlorella* strains per amoeba cell and assess low coverage samples (samples without enough reads for successful de-novo assembly, but enough reads to determine *rbcL* type) we assembled the trimmed reads to the *Chlorella rbcL* reference sequence KJ446796.1 using BBMap version 37.56 (Bushnell, 2014). Reference alignments were checked by eye using Geneious version 2019.0.4 (Kearse et al., 2012).

Fluorescence microscopy

To assess the state of the algae cells within *H. papilio*, we conducted fluorescence and transmission electron microscopy. For fluorescence microscopy, hand-picked *H. papilio* cells were preserved in 100% ethanol and subsequently infiltrated and embedded in medium grade LR-White resin. Semithin sections (500 nm) were mounted on glass slides and stained with 4′,6-diamidino-2-phenylindole (DAPI) to reveal the presence of nuclei. The stained sections were observed using an inverted epifluorescence microscope (Axio Observer.D1, Carl Zeiss, Jena, Germany) equipped with a monochrome high-resolution camera (AxioCam MRm, Carl Zeiss, Jena, Germany). The green autofluorescence was imaged and subtracted to the blue

fluorescence in order to reveal the DNA signal. Autofluorescence subtraction and overlays were done using the GIMP® software.

Transmission Electron Microscopy (TEM)

For TEM analysis we again hand-picked individual *H. papilio* cells from environmental samples. We transferred the cells from the original petri dish to a drop of freshly-filtered (2 µm filter) in situ water and repeated this washing step until all obvious contamination on the outside of the cells was removed. We then fixed the cells overnight in 3% glutaraldehyde (GTA) in 0.1 M PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9).
After washing off the fixative, the cells were post-fixed for 1 hour using 1% aqueous osmium tetroxide solution and subsequently washed three times in water before dehydration through an acetone series. Cells were finally infiltrated and flat-embedded in Epon-Araldite resin as described in Müller-Reichert et al. (2003). Thin sections (100 nm) were mounted on formvar coated slot grids and stained for 7 min in 2% uranyl acetate followed by 10 min in Reynolds Lead Citrate. The cells were observed and imaged with a Jeol® 1400 TEM.

Assessing algal nuclear activity in transcriptome samples

If the algae residing within *H. papilio* cells are actively transcribing their nuclear DNA, we expect their transcriptomes to be present in our sequencing reads, as the nucleic acids of organisms living within the shell or cytoplasm of the amoeba will be co-amplified. In order to search for an algae signal in our transcriptome data, we used PhyloToL (Cerón-Romero et al., 2019) to produce multiple sequence alignments and gene trees for 150 conserved eukaryotic gene families (**Table S2**). These gene families were selected based on their presence in at least four of the five major eukaryotic clades (SAR, Amoebozoa, Archaeplastida, Excavata and Opisthokonta). We included a total of 278 species of all major eukaryotic clades, bacteria and archaea in the analysis in addition to our 22 *H. papilio* and 12 *H. elegans* transcriptomes. If both

the amoeba and algae transcriptomes were present in a sample, we would expect to find sequences of one sample appearing in Amoebozoa and Archaeplastida in the gene trees. We used custom python scripts (https://github.com/Katzlab) to determine the sister branch of each sequence of our transcriptome samples in each gene tree (**Table S2**). In addition, we calculated the branch length to avoid long-branch artifacts. All identified cases were additionally screened by eye.

To further investigate active gene expression by the algae we also assessed the number of times that *H. papilio* samples appeared among green algae in the gene trees of 27 photosynthesis related gene families (**Table S2**). We used the KEGG (Kanehisa and Goto 2000) pathway for *Chlorella* photosynthesis to identify nuclear encoded genes associated with photosynthesis. These genes were then analyzed using PhyloToL, following the methods described above.

Starvation Experiment

In order to investigate the stability of the amoeba/algae relationship over time, we maintained living *H. papilio* in the lab for two weeks. These cells were obtained from the environment as described above, isolated from the surrounding substrate by hand-picking them from the petri-dish and placed in a new dish with freshly-filtered (0.2 µm filter) in situ water, which did not contain free-living *Chlorella*, *Sphagnum* or any other food sources. We kept the dishes in an incubator at 20°C under a 12h light/dark cycle. We then removed two cells each on day 0, 3, 5, 7, and 12, photo-documented them and froze them in RLT buffer for subsequent single-cell genome analysis. The amplified genomes were sequenced and processed and the assemblies then scanned for chloroplast contigs using BLASTn. All procedures were conducted as described above. We used GeSeq (Tillich et al., 2017) for annotation of the contigs and to determine if they derive from chloroplasts and then mapped reads to the longest identified chloroplast contig using BBMap version 37.56 (Bushnell, 2014) with default settings. The

253 average coverage of each alignment was determined using bedtools genomecov (Quinlan and
254 Hall, 2010).
255
256 **Differential gene expression**
257 To assess whether the presence of the algae within the *H. papilio* cells has an influence
258 on the gene expression of the amoeba we compared the composition of the transcriptomes of
259 16 of our *H. papilio* samples to our 12 samples of *H. elegans* (**Table S1**), a closely related
260 species without associated algae. After counting all gene families present in each transcriptome
261 sample using custom Python scripts (**https://github.com/Katzlab**), we identified gene families
262 differentially present in the two morphospecies. We then used PhyloToL (Cerón-Romero et al.,
263 2019) to construct multiple sequence alignments and gene trees to assess homology prior to
264 functional analysis in Blast2GO (Götz et al., 2008). To compare the functional categories of the
265 differentially expressed genes, we analyzed a set of equivalent size that was made up of
266 randomly chosen gene families shared among both amoeba species.
267
268 **Results**
269 **Generation of single-cell genomes and transcriptomes**
270 For this study we sequenced a total of 36 single-cell whole genomes and 22
271 transcriptomes of the Arcellinida morphospecies *H. papilio*. In addition, we generated 12
272 transcriptomes for the congener species *H. elegans*. (**Table S1**). All of the sequenced data are
273 available on GenBank (SRA accession #xxxxx).
274
275 **Algal diversity and phylogeny**
276 We assessed the diversity of algal symbionts associated with *H. papilio* using the *rbcL*
277 marker gene characterized from 17 WGAs, from which we recovered full-length rbcL sequences
278 (we did not detect a full-length *rbcL* contig in the remaining 19 WGAs, **Table S1, File S1**). The

resulting multiple sequence alignment and phylogeny contained 17 sequences from our samples as well as 208 sequences from GenBank and recovered the Testate Amoeba *Chlorella* Symbiont (TACS I) clade reported in Gomaa et al., (2014). In addition to this previously known clade, we discovered a novel clade, TACS II, that represents samples of *Chlorella* associated with testate amoeba from across the northeastern USA (**Figure 1, File S2**). We found a geographic pattern in the occurrence of these two clades: all five samples from Harvard Forest were included in TACS I (two samples had full length *rbcL* contigs; three samples were confirmed with read mapping), and all other samples from open bogs at Hawley Bog, Orono Bog, and Acadia Bog (15 samples with full length *rbcL* contigs) formed a distinct clade (TACS II, **Figure 1**). The read mapping-based analysis did not detect high levels of polymorphism in the individual reference alignments which could be interpreted as evidence of multiple *Chlorella* clades per amoeba cell (Figure S3).

Because sequences from three free-living *Chlorella* (GenBank accession numbers: KM514889, KM514890, KM514866), isolated from lakes in Jiangsu Province, China, are sister to the testate amoeba-associated clades (**Figure 1**), we assessed the potential monophyly of TACSI and TACS II. AU and SH tests of support for alternative topologies reject the hypothesis of a single origin of *Chlorella* symbiont clades, either when we constrain the three clades (TACS I, TACSII, *Paramecium bursaria* symbiont clade; p-SH 0.0267, p-AU 0.0109) or only the *Chlorella* strains from testate amoeba (clades TACSI and TACSII; p-SH 0.0054, p-AU 0.0036), suggesting two independent origins of the symbiotic association between *Chlorella* and testate amoebae.

Assessing the presence and expression of *Chlorella* nuclei

To determine where on the spectrum spanning stable to transient symbioses the *H. papilio* – *Chlorella* relationship falls we first assessed whether the *Chlorella* cells in *H. papilio* are intact cells including nuclei or if, in the extreme case of kleptoplasty, only their chloroplasts

305 are retained inside the amoebae. A substantial part of the granuloplasmic mass of *Hyalosphenia*
306 cytoplasm was occupied by autofluorescence emitting (in a wide range of wavelengths, from
307 blue to far-red) *Chlorella* cells (**Figures 2A, B**). Both DNA staining on semi-thin sections and
308 Transmission Electron Microscopy (TEM) revealed the presence of seemingly intact *Chlorella*
309 cells within *H. papilio*, that – as far as it could be assessed – appeared to contain a nucleus
310 (**Figure 2**). The *Chlorella* cells measure on average 4.18 +/- 0.32 µm in diameter and their
311 nucleus was on average 1.16 +/- 0.20 µm in diameter; consequently, the sectioning plane did
312 not always cut through a nucleus. However, the observation of six consecutive sections (0.5 µm
313 each) stained with DAPI did not reveal a single anucleate algae. In addition to the algae cells
314 found in the *H. papilio* cytoplasm, we also detected *Chlorella* cells in food vacuoles (**Figure S2**).
315 Poorly preserved ultrastructure details (e.g., indistinguishable chloroplasts or nuclei; **Figure S2**)
316 along with entirely missing autofluorescence signal (Fig. 2A, B, D) in these cells indicated that
317 digestion was already underway.
318 We then tested whether or not the *Chlorella* nuclei are actively transcribing by using a
319 phylogenomic approach including a wide diversity of eukaryotic taxa to identify if the genes
320 expressed and sequenced in the transcriptome samples stem from the *H. papilio* host or the
321 *Chlorella* algae. Since we chose conserved eukaryotic genes with general housekeeping
322 functions (**Table S2**), they can be expected to be expressed in the amoeba and the algae cells,
323 assuming the algae are complete and actively expressing their genes (**Figure 3)**. If, on the other
324 hand, the algae are deprived of their nuclei or the nuclei are in an inactive state, we should only
325 obtain transcripts from the host and not the algae.
326 Analyzing a total of 150 gene trees, we observed only six cases in which sequences
327 from the transcriptome data fell among Archaeplastida clades in the trees and did not represent
328 long branches (**Figure 3, Table S2**). These six sequences came from five different samples,
329 whereas the remaining 17 *H. papilio* samples never had sequences fall among Archaeplastida.
330 This suggests that the algae may not be actively expressing their housekeeping genes, either

because the nuclei – and over time the entire algae cells – are being degraded or that their metabolism is closely linked to the amoeba and general housekeeping functions are fulfilled by the host. In order to further assess the transcriptional activity of the *Chlorella* nuclei, we also searched for the presence of *Chlorella* genes related to photosynthesis. These genes would be expected to be present in a long-term, stable symbiosis, as nuclear products are necessary to maintain chloroplast function. However, this analysis revealed a similarly low level of nuclear signal from the algae (Table S2).

To further assess the stability of the relationship between the *Chlorella* and their amoeba hosts, we diluted 10 Arcellinida cells in filtered bog water, essentially removing free-living *Chlorella* as a food source. At the start of this experiment (day 0), and after 3, 5, 7, and 12 days, we assessed the presence of *Chlorella* plastid genome by single-cell whole genome amplification. Using average coverage of the chloroplast genome, we found highest coverage on day 0 with a rapid decline over time (**Figure 4**), suggesting a gradual degradation of the *Chlorella* cells and chloroplasts. This is consistent with light micrographs taken during the starvation experiment in which the algae cells and chloroplasts appear increasingly degraded as time progresses (**Figures 4**, **S1**).

Differential gene expression

To measure the effect of the presence of photosynthetic *Chlorella* within the amoeba cell, we characterized genes that were expressed in *H. papilio* but absent from *H. elegans,* a species that does not have an association with algal cells. Using PhyloToL (Cerón-Romero et al., 2019) as a tool for homology assessment, we found a total of 132 genes from 92 gene families expressed in *H. papilio* that were not expressed in the majority of the congener *H. elegans* (Table **S3**). We used Blast2GO (Götz et al., 2008) to identify the functions of these gene families. The majority of gene families (86) fulfilled housekeeping functions, e.g. they contribute to metabolic processes and biosynthesis. However, we also found six gene families

that are related to oxidation-reduction processes (**Table S3** and **File S3**). When comparing the functional categories of these 92 differentially expressed gene families to a random set of 92 gene families that are shared between the two species, we found the same “housekeeping functions”. However, oxidation-reduction processes as functional category is missing from the shared dataset, making it a unique category in *H. papilio* that could indicate a response to the presence of symbionts.

Discussion

The three main insights from this study are: 1) two distinct clades of *Chlorella* are associated with the Arcellinida morphospecies *H. papilio* in our New England samples, a finding that contrasts with previous claims of a single world-wide partnership; 2) our analyses suggest that the relationship may be transient as we find no evidence of gene expression from the green algal nucleus, despite the presence of a nucleus as shown by TEM and DAPI; and 3) analyses of the host – *H. papilio* – transcriptomes suggest changes in gene expression consistent with oxidative stress.

Two distinct clades of *Chlorella* are associated with testate amoebae in New England bogs and fens

Though we were not able to recover full length *rbcL* sequences from all samples, the 17 full length *Chlorella* sequences from a single morphospecies of testate amoeba sampled in New England refute the “one alga to rule them all” hypothesis of a single *Chlorella* strain found across multiple morphospecies of testate amoeba (Gomaa et al., 2014). Instead, we find evidence for at least two *Chlorella* clades existing within *Hyalosphenia papilio* in New England. Phylogenetic analyses show that these two clades are non-monophyletic and are separated by free living strains (e.g. *Chlorella* sp. sensu Zhou et al 2016 clade) and symbiotic strains (e.g. *Chlorella variabilis-Paramecium bursaria* symbiont clade; **Figure 2**). The observation of multiple

clades of *Chlorella* is consistent with other systems that show a wide diversity of symbionts: a study of green algal symbionts of *Paramecium bursaria* found *Chlorella variablis*, *Micractinium conductrix*, and *Choricystis parasitica* that were accepted as endosymbionts (Fleming et al. 2020, Zagata et al 2016). The ability to culture *H. papilio* without endosymbionts would allow cross-infection experiments to determine if TACS I and II would be accepted by *H. papilio* from opposing geographic areas.

Though we find evidence of two *Chlorella* clades from New England samples, each amoebae individual appears to host only members of a single clade, in agreement with the results of cloning experiments carried out by Gomaa et al. (2014). This suggests some type of preferential feeding, local scale distribution differences of the *Chlorella* genotypes, or competition between *Chlorella* genotypes preventing colonization of *H. papilio* by multiple genotypes. In our study, we sampled amoebae from Harvard Forest and Hawley Bog, sites which are separated by only ~80 km with no major dispersal barriers; given the small size of *Chlorella* we expect high dispersal rates and distances at the scale of the Northeastern USA (Foissner 2006). However, none of the *Chlorella* genotypes found at Harvard Forest (TACS I) were found at Hawley Bog or either of the Maine sampling sites. Indeed, only five of our *H. papilio* samples contained TACS I, with most amoebae (15) harboring TACS II *Chlorella* symbionts. Though it is possible the amoebae at Harvard Forest and Hawley bog represent cryptic species Gomaa et al. (2014) sampled testate amoeba from diverse clades across the world and predominantly detected green algae from the TACS I clade with only a few samples harboring *Chlorella* from other clades (e.g. 18/LC/10-KJ446811). The sampling of Gomaa et al., (2014) was limited to nutrient rich and poor fens and bogs at high latitudes (>46° N) and it is possible that the TACS I clade has a northern distribution that made it less likely for our study to detect. Alternatively, the ecology of these sites could explain the distribution patterns of *Chlorella* clades. Hawley, Orono, and Big Heath all contain ombrotrophic bog habitats (Davis and Anderson, 2001; Kearsley, 1999) while our sampling site at Harvard Forest is a rich fen

409 impacted by damming in the late 19th century (Swan and Gill, 1970). Further sampling of *H.*
410 *papilio* from diverse ecologies and latitudes may help to elucidate this pattern.
411
412 **Relationship between host and symbiont may be transient**
413 In contrast to the expectation of a mutualistic symbiosis between Arcellinida and
414 *Chlorella*, our analyses suggest that only the chloroplast genome, and not the *Chlorella* nucleus,
415 are active within *H. papilio* (**Figure 3**). Support for this includes our observation of no *Chlorella*
416 nuclear transcripts (either housekeeping or photosynthesis related) in the transcriptome
417 samples, the presence but subsequent degradation of chloroplast DNA in genome samples as
418 the amoebae are starved, and the microscopic observations of *Chlorella* cells being present in
419 food vacuoles in TEM images (**Figure S2**). It is possible that the algae are taken up by the host
420 from the environment, their photosynthetic activity is exploited while they are maintained inside
421 the host cell and later on they are degraded and digested as additional food source, as the
422 results of the starvation experiment demonstrate. Considering our observations, the *Chlorella* –
423 *H. papilio* association may therefore fall closer to a transient relationship on the spectrum of
424 symbioses. Esteban et al. (2010) describe organisms that retain photosynthetic cells for a
425 period of time followed by digestion as using an “intermediate mixotrophic mechanism.” Though
426 the long-term functioning of chloroplasts relies on nuclear signaling and nuclear encoded
427 proteins (Pogson et al. 2008), in several systems chloroplasts remain viable for days to weeks
428 without nuclear products or signals: in ciliate-algae relationships the kleptoplast survival period
429 can last up to a month (Johnson et al., 2007) while in foraminifera-algae relationships the
430 survival period is up to a maximum of around 10 weeks (Correia and Lee, 2002). The molecular
431 and/or physiological mechanisms limiting nuclear activity in the *H. papilio* – *Chlorella* association
432 remain to be elucidated. The development of culture techniques for mixotrophic testate
433 amoebae would represent a major and valuable step toward untangling these relationships.

Coupling cultivation with techniques such as fluorescence *in-situ* hybridization (FISH) allows for fine grained analysis of host and symbiont identities (McManus and Katz 2009).

Oxidative stress from *Chlorella* influences host expression

The presence of a photosynthetic symbiont can affect the gene expression of a host as has been documented in other lineages (Betteridge, 2000; Kodama et al., 2014). Of the 92 gene families that are expressed in *H. papilio* and not *H. elegans*, six are related to reducing oxidative stress (**Table S3**, with two gene families containing paralogs). As *H. papilio* has a photosynthetic symbiont, there is a likelihood that the increase of reactive oxygen species present can create changes within the host as oxidative stress leads to free radicals (reactive oxygen species (ROS)) building up in greater proportion to the production of antioxidants (Betteridge, 2000), which may be processed by these enzymes (**Table S3**). Similarly, in the case of the *Paramecium bursaria* - *Chlorella variabilis* relationship, differentially expressed genes in cells with symbionts include the down-regulation of oxidoreductase processes (Kodama et al., 2014). Similar changes in gene expression are also found in the sea anemone, *Anemonia viridis,* that has a photosynthetic protist living within it (Richier et al., 2005). As the protist photosynthesizes, the anemone upregulates the production of antioxidant enzymes to combat this increase of oxygen (Richier et al., 2005).

Taken together our results suggest that the relationship between the Arcellinida morphospecies *H. papilio* and the microalgae *Chlorella* may not be as advantageous to both partners as was previously assumed. Together with new information on *Chlorella* symbionts we demonstrate that these amoeba harbor diverse *Chlorella* strains that influence host gene expression.

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Data availability:

All sequenced transcriptomes are available on GenBank under the SRA BioProject xxxxxx. The scripts used for data analyses are available under github.com/Katzlab/xx.

Figures

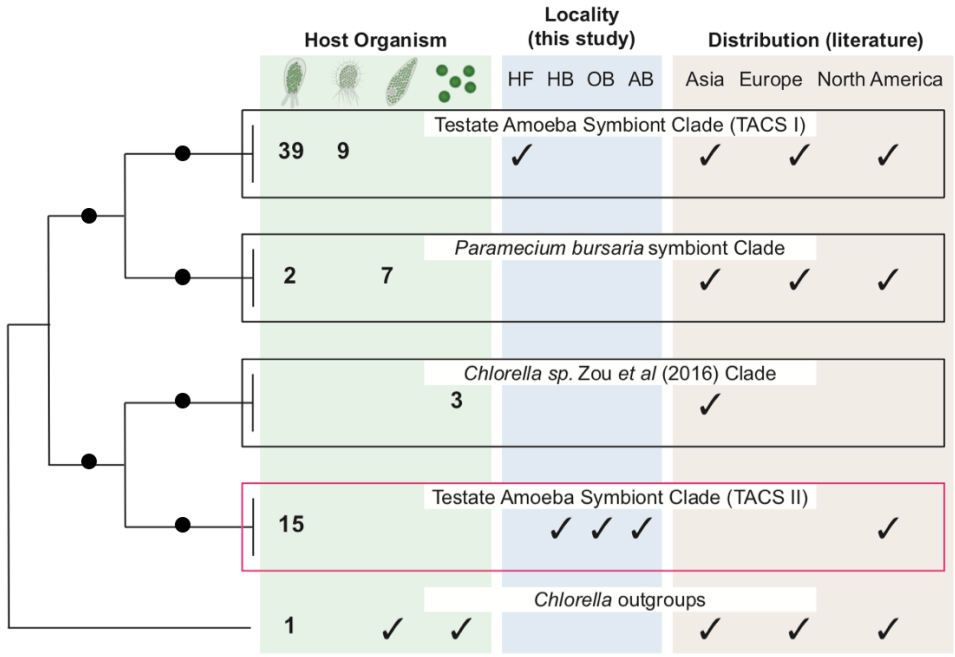

Figure 1: Phylogenetic diversity of *Chlorella* algae

A maximum likelihood phylogeny of a ~700 bp portion of the large subunit of the ribulose-bisphosphate carboxylase (rbcL) chloroplast gene indicates that *Hyalosphenia papilio* samples from New England harbor two non-monophyletic *Chlorella* lineages (TACS I and II). The host organisms depicted with line drawings are *H. papilio*, *Placocista spinosa* (representing all other testate amoebae from Gomaa et al, 2014), *Paramecium bursaria,* as well as free-living *Chlorella*. The two TACS clades are separated by *Chlorella* clades that are either primarily associated with *Paramecium* or free-living (Zou et al. 2016 clade). Our work across the northeastern USA located lineage TACS II at three sites (Hawley Bog (HB), Orono Bog (OB) and Big Heath (AB)) and TACS I at one site (Harvard Forest (HF)), while previous world-wide work in testate amoebae predominantly recovered TACS I (Gooma et al., 2014). Numbers indicate the number of sequences in the alignment corresponding to a given host, check marks indicate geographic distribution, except in the case of the outgroups where they represent the 149 outgroup sequences split between free living and *Paramecium* associated. Bootstrap support values greater than 70 are indicated with filled circles.

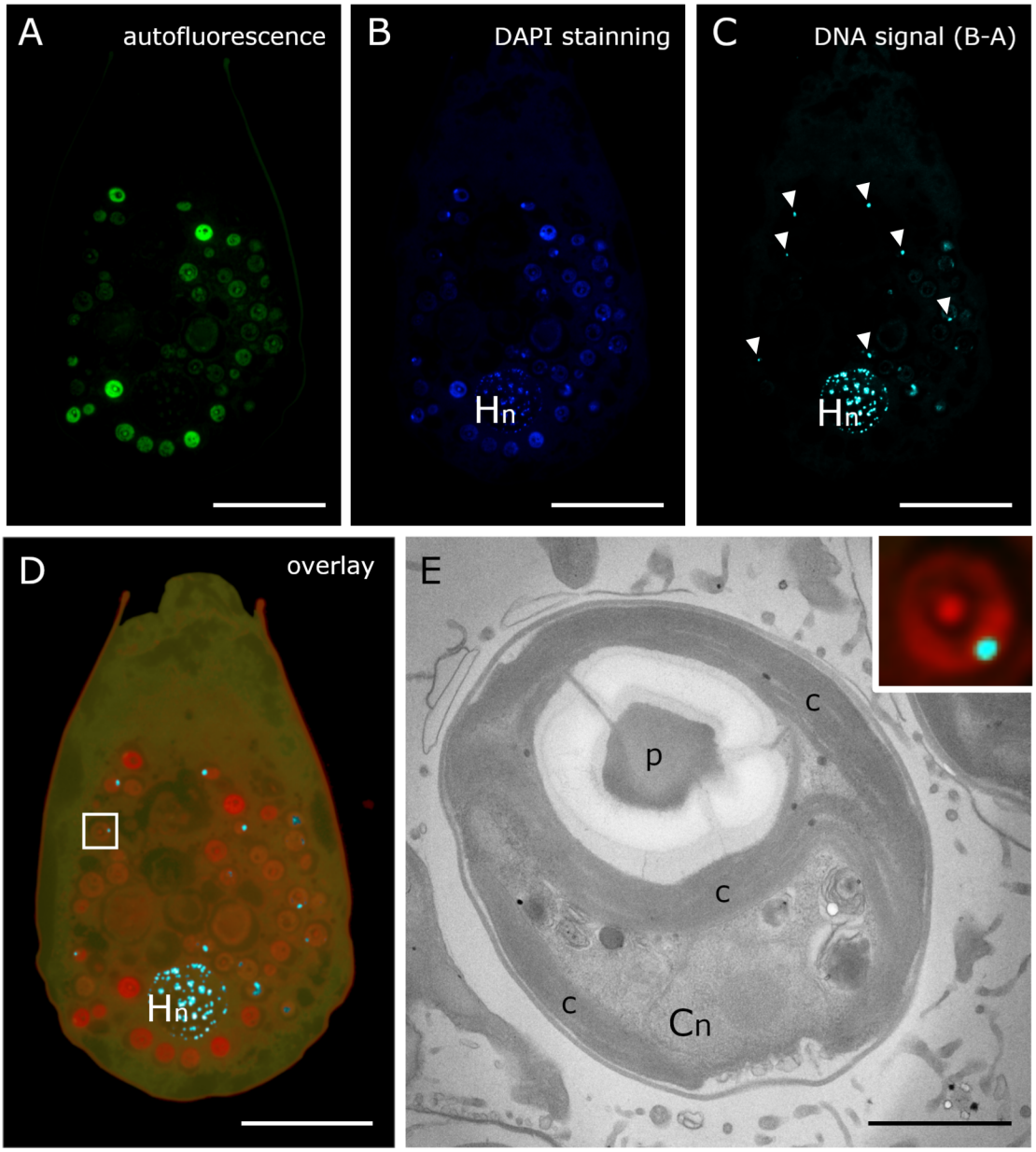

Figure 2: Hyalosphenia papilio harboring Chlorella symbionts. A-D. Semi-thin sections of a single Hyalosphenia papilio cell inspected by fluorescence microscopy, Hn Hyalosphenia nucleus. **A.** Green autofluorescence of the Chlorella chloroplasts; **B.** Blue autofluorescence along with DNA (DAPI staining) signal. **C.** DNA signal (teal color, the DAPI-stained structures yielded by subtraction of the green autofluorescence (A) from the blue fluorescence (B)), Chlorella nuclei (arrowheads); **D.** Overlay of fine structure, DAPI signal, and autofluorescence across blue, green, red and far-red channels. E. Fine structure of Chlorella symbiont showing its nucleus (Cn), chloroplast (c), and pyrenoid (p); insert displays detail of fine structure and fluorescence overlay (D, area delimited by white square; nucleus = teal color, chloroplast and pyrenoid autofluorescence = red). Scale bar A-D = 30 µm; scale bar E = 1 µm (insert = ×5.3).

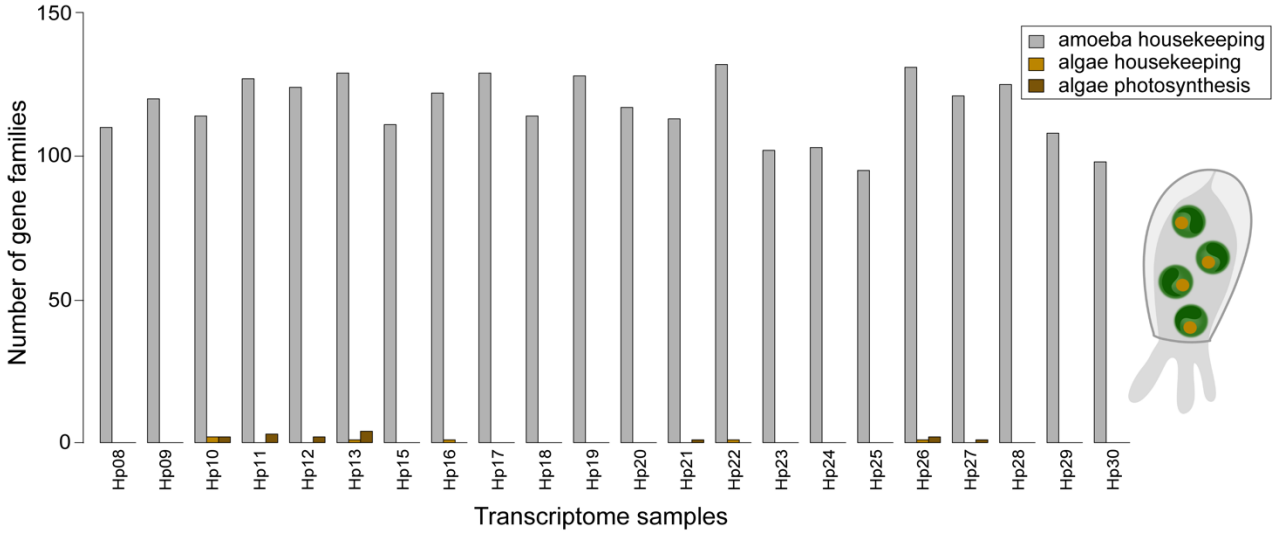

Figure 3: Presence of expressed algal nuclear genes (housekeeping and photosynthesis related) in *Hyalosphenia papilio* transcriptome samples

The bar chart indicates the number of gene families – out of 150 conserved housekeeping gene families and 27 photosynthesis related gene families– that are expressed by either *H. papilio* (grey) and/or the *Chlorella* symbionts (yellow, brown) in each of the samples from our transcriptome dataset. PhyloToL was used to produce gene trees and assess the position of sequences among either Amoebozoa or Viridiplantae. In the housekeeping gene families very few contained sequences classified as Viridiplantae, which suggests that *Chlorella* housekeeping genes are not being actively transcribed. There are also very few nuclear encoded photosynthesis genes recovered from the transcriptomes.

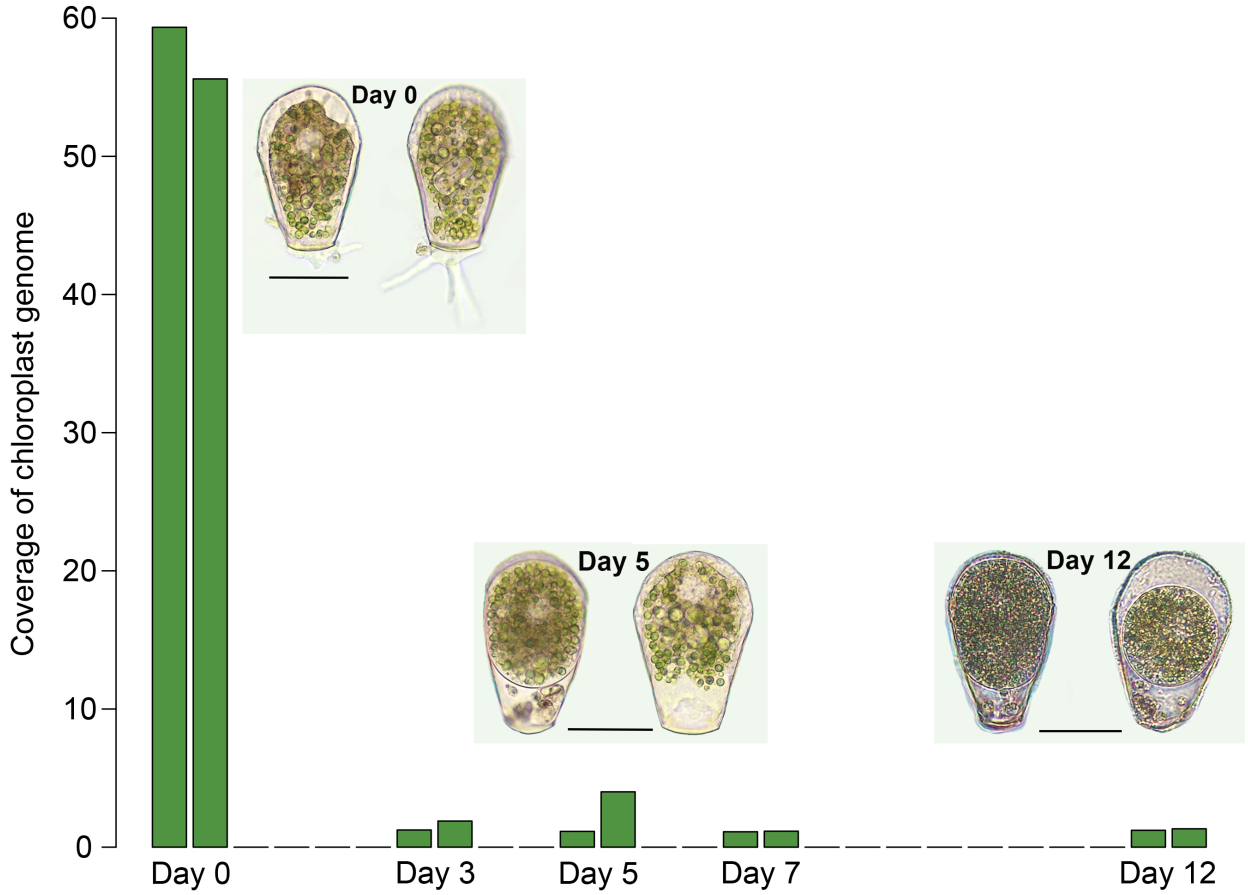

Figure 4: Starvation experiment
Evidence of a transient relationship seen in the reduction of *Chlorella* chloroplast genome during a starvation experiment, indicated as average coverage (average depth of reads per reference base). High genome coverage on Day 0 indicates healthy chloroplasts, however, they quickly degrade in micrographs and genome coverage as the amoeba is deprived of food over a period of 12 days. Scale bars in micrographs are 50 µm.

Supplementary Material

Table S1: Sampling table indicating details of all single-cell genomes and transcriptomes obtained for this study.

Table S2: Presence/absence of *H. papilio* sequences in the gene trees of 150 selected housekeeping gene families and 27 photosynthesis related gene families generated using PhyloToL. Further indicated are the sister branches to each sequence and the number of times we detect *Chlorella* signal in the *H. papilio* samples.

Table S3: Functional characterization using Blast2GO on genes expressed in *H. papilio*, but not *H. elegans* cells suggest that the algal symbiont is causing oxidative stress. We find eight genes involved in oxidation-reduction processes from six gene families.

Gene family (Pfam)	Function	Sequence name
Ofd1 (PF10637)	Oxygen-dependent sterol synthesis	LKH351_1100_Len1371
DAO (PF01266)	FAD dependent oxidoreductase family	LKH325_2841_Len1170
FA_hydroxylase (PF04116)	Fatty acid hydroxylase superfamily	LKH131_10248_Len777
FA_hydroxylase (PF04116)	Fatty acid hydroxylase superfamily	LKH106_10928_Len861
TauD (PF02668)	Taurine catabolism dioxygenases	LKH340_3865_Len957
Cu2_monooxygen* (PF01082)	Copper type II ascorbate-dependent monooxygenase	LKH106_2174_Len1812
Cu2_monooxygen* (PF01082)	Copper type II ascorbate-dependent monooxygenase	LKH363_582_Len1302
Thioredoxin (PF00085)	Redox signaling	LKH325_975_Len1590

*The pairs of sequences that match to the same gene families (FA_hydroxylase (PF04116) and Cu2_monooxygen (PF01082)) are paralogous.

Figure S1: Light microscope images of *H. papilio* cells from the starvation experiment sampled after 0, 3, 5, 7 and 12 days showing the gradual digestion of the *Chlorella* algae. Scale bars indicate 50 µm.

Figure S2: Food vacuole containing *Chlorella* cells. In contrast with the intact *Chlorella* cells located in the *Hyalosphenia* cytoplasm (Fig. 2E), the partially digested cells present in the food vacuole lack a distinguishable nucleus, chloroplast, or pyrenoid.

Figure S3: Reads from two samples mapped to the TACSI *rbcL* reference from Gomaa et al. (2014) (KJ446796.1). Differences from the reference are colored in the alignment. A. LKH454

from TACSI has no fixed differences to reference, and no consistent polymorphisms, though a few reads that differ at the 3' end. B. LKH484 from the TACSII lineage has many fixed differences from the reference but few polymorphisms.

File S1: FASTA file of a MAFFT alignment of the rbcL sequences used for phylogenetic inference of *Chlorella* diversity.

File S2: RAxML tree showing *Chlorella* diversity. This tree formed the basis for the cartoon tree shown in Figure 2.

File S3: FASTA file of representative sequences that are differentially expressed in *H. papilio* compared to *H. elegans* and that may play a role in oxidative processes invoked by photosynthesis (Table S3).

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