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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1   **ORIGINAL PAPER**

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5   **and Microscopic Methods**

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22   **Running title:** *Hyalosphenia papilio* and Microalgae

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32 **Symbiotic relationships between heterotrophic and phototrophic partners are common in**

33 **microbial eukaryotes. Among Arcellinida (Amoebozoa) several species are associated**

34 **with microalgae of the genus *Chlorella* (Archaeplastida). So far, these symbioses were**

35 **assumed to be stable and mutualistic, yet details of the interactions are limited. Here, we**

36 **analyzed 22 single-cell transcriptomes and 36 partially-sequenced genomes of the**

37 **Arcellinida morphospecies *Hyalosphenia papilio*, which contains *Chlorella* algae, to shed**

38 **light on the amoeba-algae association. By characterizing the genetic diversity of**

39 **associated *Chlorella*, we detected two distinct clades that can be linked to host genetic**

40 **diversity, yet at the same time show a biogeographic signal across sampling sites.**

41 **Fluorescence and transmission electron microscopy showed the presence of intact algae**

42 **cells within the amoeba cell. Yet analysis of transcriptome data suggested that the algal**

43 **nuclei are inactive, implying that instead of a stable, mutualistic relationship, the algae**

44 **may be temporarily exploited for photosynthetic activity before being digested.**

45 **Differences in gene expression of *H. papilio* and *Hyalosphenia elegans* demonstrated**

46 **increased expression of genes related to oxidative stress. Together, our analyses**

47 **increase knowledge of this host-symbiont association and reveal 1) higher diversity of**

48 **associated algae than previously characterized, 2) a transient association between *H.***

49 ***papilio* and *Chlorella* with unclear benefits for the algae, 3) algal-induced gene**

50 **expression changes in the host.**

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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* (e.g., Takahashi 2016) and multiple symbionts from across the tree of life in diverse Foraminifera species (e.g., *Minutocellus polymorphus, Navicula* sp., e.g. 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 (e.g., 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 (e.g., Lara and Gomaa 2017).

82   Associations with microalgae are commonly found among shell-building amoebae of the

83   order Arcellinida (Amoebozoa), that are the focus of this study. At least three morphospecies of

84   Arcellinida are known to harbor photosynthetic organisms inside their cytoplasm (e.g., Gomaa et

85   al. 2014; Lara and Gomaa 2017). These associations are assumed to be stable and mutualistic,

86   as the amoeba species containing these symbionts are usually not found without them (e.g.,

87   Lara and Gomaa 2017). Genotyping of the microalgae in Arcellinida revealed a surprisingly low

88   diversity among the *Chlorella* strains associated with these amoebae and identified them as

89   strains that are common within a range of other microbial eukaryotes, including ciliates

90   (Flemming et al. 2020; Gomaa et al. 2014; Zagata et al. 2016). Based on analysis of the plastid

91   *rbcL* gene, Gomaa et al. (2014) even argued that a single clade of *Chlorella* is the dominant

92   symbiont across diverse species of Arcellinida, including *Hyalosphenia papilio* and *Heleopera*

93   *sphagni,* and Rhizaria: including *Archerella flavum* and *Placocista spinosa.* In addition, no

94   variability among the algae was found to be associated with the high amounts of cryptic diversity

95   observed in some of the host species (e.g., Gomaa et al. 2014; Singer et al. 2019). The lack of

96   diversity in *Chlorella* strains within these diverse species has led to the postulation that while

97   vertical acquisition of symbionts is possible, a great portion of these symbionts are acquired

98   horizontally from the environment, though symbiont acquisition has never been directly

99   observed in these uncultivable organisms (Lara and Gomaa 2017).

100   In this study, we examine the relationship between the Arcellinida morphospecies

101   *Hyalosphenia papilio* and its associated algae *Chlorella* sp. (Archaeplastida) in detail by using

102   sequencing and imaging approaches. *H. papilio* is one of the most abundant species of

103   Arcellinida in low pH bogs, where it is found on *Sphagnum* moss (Gomaa et al. 2014; Heal

104   1962; Lahr et al. 2019; Ruggiero et al. 2020). This species appears bright green under the light

105   microscope due to the high number of *Chlorella* cells contained within its cytoplasm. We

106   investigate the diversity of *Chlorella* algae living within *H. papilio* samples from locations across

107   New England, USA, to assess whether extensive sampling in one area would reveal additional

4

108   algal diversity than previously observed, and to assess whether symbiont diversity is related to

109   host diversity and/or shows biogeographic signal across sampling sites. Further, we explore the

110   nature of the relationship between host and algae to gain a better understanding of where it falls

111   along the spectra regarding stability and degree of benefit to the partners. If, for example, the

112   organisms have a stable, mutualistic relationship, we would expect evidence of a

113   transcriptionally active *Chlorella* cell living inside *H. papilio*, whereas if the relationship is

114   transient, we may see signs of degradation and/or transcriptional inactivity of the algal nucleus.

115   To explore these relationships, we take advantage of the "bycatch" from single-cell whole

116   genome and transcriptome amplifications targeting the Arcellinida, as symbiont nucleic acids

117   are co-amplified. Consequently, these samples represent the microbiome present within the

118   Arcellinida test (i.e. shell) at the time of DNA/RNA amplification. Here we focus on the presence

119   of algal chloroplast and nuclear DNA/RNA in both whole genome amplifications (WGAs) and

120   whole transcriptome amplifications (WTAs). We also carried out fluorescence microscopy on

121   sections of resin embedded *H. papilio* harboring *Chlorella* cells as well as transmission electron

122   microscopy (TEM) to shed further light on the state of the algae within its host.

123   In addition, we sought to understand the effect of symbiont-induced oxidative stress on

124   host gene expression. Free oxygen radicals that are produced during photosynthesis by the

125   symbiont can have detrimental effects on the host if not enough antioxidants are present (e.g.,

126   Betteridge 2000). This stress can lead to changes in gene expression in the host to maintain the

127   integrity of cellular functioning (e.g., Johnson et al. 2007). To investigate the impact of

128   photosynthesis by the algal symbionts in *H. papilio*, we analyzed differential gene expression

129   between *H. papilio* and its congener *Hyalosphenia elegans*, which lacks photosynthetic

130   symbionts. Together, our analyses contribute to a better understanding of the association

131   between mixotrophic microbial eukaryotes and their photosynthetic symbionts.

132

133   **Results**

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134

Generation of Single-cell Genomes and Transcriptomes

For this study we analyzed a total of 36 single-cell whole genomes and 22 transcriptomes of the

Arcellinida morphospecies *H. papilio*. In addition, we generated 12 transcriptomes for the

congener species *H. elegans*. (Supplementary Material Table S1). All of the sequenced data are

available on GenBank (BioProject number PRJNA761372).

140

Symbiont and Host Diversity and Phylogeny

We assessed the diversity of algal symbionts associated with *H. papilio* using the *rbcL* marker

gene characterized from 17 partially-sequenced WGAs from which we recovered full-length *rbcL*

sequences (Supplementary Material Table S1, File S1). We did not detect a full-length *rbcL*

contig in the remaining 19 WGAs, and were unable to capture chloroplast transcripts from

WTAs due to their lack of poly-A tails. The resulting multiple sequence alignment and phylogeny

(Fig. 1; Supplementary Material Files S1 and S2) contained the 17 sequences from our samples

as well as 208 sequences from GenBank and the maximum likelihood tree recovered the testate

amoebae *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 amoebae from across the northeastern USA (Fig. 1;

Supplementary Material File S2). By applying a read mapping approach (see Methods and

Supplementary Material Fig. S1) we were able to identify three additional *H. papilio* samples as

containing TACS I despite their lack of full-length *rbcL* sequences. Overall, we found five

samples that contain symbionts of the TACS I clade and 15 that contain TACS II symbionts

(Supplementary Material Table S1).

In addition to the symbiont diversity, we also assessed the host genetic diversity by

reconstructing a phylogenetic tree based on COI sequences extracted from the WGA samples

for which we had *rbcL* sequences (Fig. 2; Supplementary Material Table S1, Files S3 and S4).

6

160   We identified three genetic clades within our samples (clade M (n=3), clade K (n=2), clade F

161   (n=12); Fig. 2), all of which had been described before (Gooma et al. 2014; Heger et al. 2013;

162   Singer et al. 2019). In addition, there were three additional samples that did not have enough

163   reads for assembly, but the reads were long enough to be compared to the alignment and to

164   identify genotypes: two grouped with clade M and one with clade F. Linking host diversity to the

165   encountered symbiont clades we observed that TACS I is exclusively associated with clade M

166   hosts, whereas symbionts of TACS II are found within *H. papilio* clades K and F (Supplementary

167   Material Table S1; Fig. 2). We did not find evidence for the occurrence of multiple symbionts of

168   different clades within one host cell as the read mapping-based analysis did not detect

169   polymorphisms within individual reference alignments as would be expected if multiple *Chlorella*

170   clades were present in each amoeba (Fig. S1).

171   Examining the distribution of our samples, we found a biogeographic pattern in the

172   occurrence of both the host clades as well as the two symbiont clades. The five *H. papilio* clade

173   M samples that all contain TACS I algae only occurred in Harvard Forest, while *H. papilio* clade

174   K containing TACS II was found in Acadia Bog and clade F with TACS II occurred in the open

175   bogs at Hawley Bog and Orono Bog (Supplementary Material Table S1; Figs 1, 2).

176   Because sequences from three free-living *Chlorella* (GenBank accession numbers:

177   KM514889, KM514890, KM514866), isolated from lakes in Jiangsu Province, China (Zou et al.

178   2016), are sister to the testate amoebae-associated clades (Fig. 1), we assessed the potential

179   monophyly of TACSI and TACS II. Both AU and SH tests of alternative topologies reject the

180   hypothesis of a single origin of *Chlorella* symbiont clades, either when we constrain the three

181   clades (TACS I, TACSII, *Paramecium bursaria* symbiont clade; p-SH 0.0267, p-AU 0.0109) or

182   only the *Chlorella* strains from testate amoebae (clades TACSI and TACSII; p-SH 0.0054, p-AU

183   0.0036), suggesting two independent origins of the symbiotic association between *Chlorella* and

184   testate amoebae.

185

Assessing the Presence and Expression of *Chlorella* Nuclei

186   To determine where the *H. papilio – Chlorella* relationship falls on the spectrum spanning stable

187

188   to transient symbioses, we first assessed whether the *Chlorella* cells in *H. papilio* are intact cells

189   including nuclei or if, in the extreme case of kleptoplasty, only their chloroplasts are retained

190   inside the amoebae. A substantial part of the granuloplasmic mass of *Hyalosphenia* cytoplasm

191   was occupied by autofluorescence emitting (in a wide range of wavelengths, from blue to far-

192   red) *Chlorella* cells (Fig. 3A, B). Both DNA staining on semi-thin sections and TEM analysis

193   revealed the presence of seemingly intact *Chlorella* cells within *H. papilio*, at least some of

194   which contain a nucleus (Fig. 3). The *Chlorella* cells measure on average 4.18 +/- 0.32 µm in

195   diameter and their nucleus was on average 1.16 +/- 0.20 µm in diameter. While the sectioning

196   plane did not always cut through a nucleus, the observation of six consecutive sections (0.5 µm

197   each) stained with DAPI did not reveal a single anucleate algae. In addition to the algae cells

198   found in the *H. papilio* cytoplasm, we also detected *Chlorella* cells in food vacuoles

199   (Supplementary Material Fig. S2). Poorly preserved ultrastructure details (e.g., indistinguishable

200   chloroplasts or nuclei; Supplementary Material Fig. S2) along with entirely missing

201   autofluorescence signal (Fig. 3A, B, D) in these cells indicated that digestion was underway.

202   We then tested whether or not the *Chlorella* nuclei are actively transcribing by using a

203   phylogenomic approach including a wide diversity of eukaryotic taxa to identify if genes in the

204   transcriptome samples came from the *Chlorella* nucleus. Since we chose conserved eukaryotic

205   genes with general housekeeping functions (Supplementary Material Table S2), they can be

206   expected to be expressed in the amoebae and the algae cells, assuming the algae are complete

207   and actively expressing their genes. If, on the other hand, the algae are deprived of their nuclei

208   or the nuclei are in an inactive state, we should only obtain transcripts from the host and not the

209   algae.

210   Analyzing a total of 150 gene trees, we observed only six cases in which sequences

211   from the transcriptome data fell among Archaeplastida clades (Fig. 4; Supplementary Material

212   Table S2). These six sequences came from five different samples, whereas the remaining 17 *H.*

213   *papilio* transcriptome samples never had sequences fall among Archaeplastida. This suggests

214   that the algae may not be actively expressing their housekeeping genes, either because the

215   nuclei – and over time the entire algae cells – are being degraded or that their metabolism is

216   closely linked to the amoeba and general housekeeping functions are fulfilled by the host. For

217   further investigation of the transcriptional activity of the *Chlorella* nuclei, we also searched for

218   the presence of *Chlorella* genes related to photosynthesis. These genes would be expected to

219   be present in a long-term, stable symbiosis, as nuclear products are necessary to maintain

220   chloroplast function. However, this analysis revealed a similarly low level of nuclear signal from

221   the algae as we only found a signal in seven out of 22 transcriptome samples and the highest

222   number of genes expressed was four out of 27 in one sample (Fig. 4; Supplementary Material

223   Table S2).

224   To further assess the stability of the relationship between the *Chlorella* and their

225   amoebae hosts, we diluted 10 Arcellinida cells in filtered bog water, essentially removing free-

226   living *Chlorella* as a food source. At the start of this experiment (day 0), and after 3, 5, 7, and 12

227   days, we assessed the presence of *Chlorella* plastid genome by partial sequencing of single-cell

228   whole genome amplifications. Using average coverage of the chloroplast genome, we found

229   highest coverage on day 0 with a rapid decline over time (Fig. 5), suggesting a gradual

230   degradation of the *Chlorella* cells and chloroplasts. This is consistent with light micrographs

231   taken during the starvation experiment in which the algae cells and chloroplasts appear

232   increasingly degraded as time progresses (Fig. 5; Supplementary Material Fig. S3).

233

234   **Differential Gene Expression**

235   To measure the effect of the presence of photosynthetic *Chlorella* within the amoeba cell, we

236   characterized genes that were expressed in *H. papilio* but absent from *H. elegans,* a species

237   that does not have an association with algal cells*.* Using PhyloToL (Cerón-Romero et al. 2019)

238   as a tool for homology assessment, we found a total of 132 genes from 92 gene families

239   expressed in *H. papilio* that were not expressed in the majority of the congener *H. elegans*. We

240   used Blast2GO (Götz et al. 2008) to identify the functions of these gene families. The majority of

241   gene families (86) fulfilled housekeeping functions, e.g. they contribute to metabolic processes

242   and biosynthesis. However, we also found six gene families that are related to oxidation-

243   reduction processes (Supplementary Material Table S3, File S5). When comparing the

244   functional categories of these 92 differentially expressed gene families to a random set of 92

245   gene families that are shared between the two species, we found the same "housekeeping

246   functions". However, oxidation-reduction processes as functional category is missing from the

247   shared dataset, making it a unique category in *H. papilio* that could indicate a response to the

248   presence of symbionts.

249

250   **Discussion**

251

252   The three main insights from this study are: 1) two distinct clades of *Chlorella* are associated

253   with the Arcellinida morphospecies *H. papilio* in our New England samples, a finding that

254   contrasts with previous claims of a single world-wide partnership; 2) our analyses suggest that

255   the relationship may be transient as we find no evidence of gene expression from the green

256   algal nucleus, despite the presence of a nucleus as shown by TEM and DAPI; and 3) analyses

257   of the host – *H. papilio* – transcriptomes suggest changes in gene expression consistent with

258   oxidative stress.

259

260   **Two Distinct Clades of *Chlorella* are Associated with Testate Amoebae in New England**

261   **Bogs and Fens**

262 Analysis of *Chlorella rbcL* sequences from a single morphospecies of testate amoebae sampled

263 in New England refutes the "one alga to rule them all" hypothesis of a single *Chlorella* strain

264 found across multiple morphospecies of testate amoebae (Gomaa et al. 2014). Instead, we find

265 evidence for at least two *Chlorella* clades existing within *Hyalosphenia papilio* in New England.

266 Phylogenetic analyses show that these two clades are non-monophyletic and are separated by

267 free living strains (e.g. *Chlorella* sp. sens*u* Zou et al. 2016 clade) and symbiotic strains (e.g.,

268 *Chlorella variabilis-Paramecium bursaria* symbiont clade; Fig. 1). The observation of multiple

269 clades of *Chlorella* is consistent with other systems that show a wide diversity of symbionts: a

270 study of green algal symbionts of *Paramecium bursaria* found *Chlorella variablis*, *Chlorella*

271 *vulgaris*, *Micractinium conductrix*, and *Choricystis parasitica* as endosymbionts (Flemming et al.

272 2020; Fujishima et al. 2012; Pröschold et al. 2011; Zagata et al. 2016).

273 　　　　The morphospecies *H. papilio* is known to contain a high amount of genetic diversity that

274 has been assigned to ~12 clades (e.g., Heger et al. 2013; Singer et al. 2019), three of which are

275 represented in our samples (Fig. 2). While we observe a link between symbiont and host

276 diversity, with the TACS I clade exclusively being associated with clade M amoebae, this pattern

277 is overlain by a biogeographic distribution pattern and can therefore not be considered as

278 evidence for host-symbiont coevolution. Only once amoebae of different genotypes containing

279 symbionts of different clades are found at the same sampling station can a case for genotype

280 specific associations be made. To date, it appears more likely that testate amoebae pick up

281 their symbionts from the environment instead of inheriting them vertically, as has already been

282 argued by Gomaa et al. (2014). Future studies could test whether cross-infection experiments

283 allowed *H. papilio* of different genetic types to accept symbiont clades from different geographic

284 areas.

285 　　　　Considering the proximity of our sampling locations, encountering a biogeographic

286 distribution pattern was rather surprising. We sampled amoebae from Harvard Forest and

287 Hawley Bog, sites which are separated by only ~80 km with no major dispersal barriers; given

288   the small size of *Chlorella* we expected high dispersal rates and distances at the scale of the

289   Northeastern USA (Foissner 2006). However, none of the *Chlorella* genotypes found at Harvard

290   Forest (TACS I) were found at Hawley Bog or either of the Maine sampling sites (Figs 1, 2).

291   Indeed, only five of our *H. papilio* samples contained TACS I, with most amoebae (15) harboring

292   TACS II *Chlorella* symbionts. This is in contrast to the study of Gomaa et al. (2014), who

293   sampled testate amoebae from diverse Arcellinida clades across the world and predominantly

294   detected green algae from the TACS I clade with only a few samples harboring *Chlorella* from

295   other clades (e.g. 18/LC/10-KJ446811). The sampling of Gomaa et al. (2014) was limited to

296   nutrient rich and poor fens and bogs at high latitudes (>46° N) and it is possible that the TACS I

297   clade has a northern distribution that made it less likely for our study to detect. Alternatively, the

298   ecology of these sites could explain the distribution patterns of *Chlorella* clades. Hawley Bog,

299   Orono Bog, and Big Heath all represent low-nutrient bog habitats (Davis and Anderson 2001;

300   Kearsley 1999) while our sampling site at Harvard Forest is a rich fen impacted by damming in

301   the late 19th century (Swan and Gill 1970). Interestingly, the worldwide Holarctic sampling of

302   Gomaa et al. (2014) did not detect amoeba from clades F, K, or M. Clades K and M are only

303   known from Eastern North America (Singer et al. 2019) where Gomaa et al. (2014) did not

304   sample, while clade F has been collected in Alaska where Gomaa et al. (2014) collected only

305   samples from clades C and D. There remain three lineages (B, I, and L) from Northwest North

306   America from which no *Chlorella* have been genotyped, leaving the possibility that further

307   symbiont diversity exists. Additional sampling of *H. papilio* from diverse ecologies and latitudes

308   will help to elucidate this pattern.

309   Despite finding a higher symbiont diversity than previously reported, we still only

310   detected a single clade of symbiont within each amoeba individual. This is in agreement with the

311   results of cloning experiments carried out by Gomaa et al. (2014) and is either simply due to the

312   biogeographic distribution of the algae or it may suggest some type of preferential feeding by

313 the host and/or competition between *Chlorella* genotypes preventing colonization of *H. papilio*

314 by multiple genotypes.

315

316 **Relationship Between Host and Symbiont May be Transient**

317 In contrast to the expectation of a mutualistic symbiosis between Arcellinida and *Chlorella*, our

318 analyses suggest that only the chloroplast genome, and not the *Chlorella* nucleus, are active

319 within *H. papilio* (Fig. 4). Support for this includes our observation of no *Chlorella* nuclear

320 transcripts (either housekeeping or photosynthesis related) in the transcriptome samples, the

321 degradation of chloroplast DNA in genome samples as the amoebae are starved (Fig. 5), and

322 the presence of *Chlorella* cells in food vacuoles in TEM images (Supplementary Material Fig.

323 S2). It is possible that the algae are taken up by the host from the environment, their

324 photosynthetic activity is exploited while they are maintained inside the host cell and later they

325 are digested as additional food source, consistent with our starvation experiment. Considering

326 our observations, the *Chlorella – H. papilio* association may therefore fall closer to a transient

327 relationship on the spectrum of symbioses. This relationship resembles an "intermediate

328 mixotrophic mechanism" as defined by Esteban et al. (2010) as organisms that retain

329 photosynthetic cells for a period of time followed by digestion. Though the long-term functioning

330 of chloroplasts relies on nuclear signaling and nuclear encoded proteins (Pogson et al. 2008), in

331 several systems chloroplasts remain viable for days to weeks without nuclear products or

332 signals: in ciliate-algae relationships the kleptoplast survival period can last up to a month

333 (Johnson et al. 2007) while in Foraminifera-algae relationships the survival period is up to a

334 maximum of around 10 weeks (Correia and Lee 2002). The molecular and/or physiological

335 mechanisms limiting nuclear activity in the *H. papilio – Chlorella* association remain to be

336 elucidated, and coupling cultivation with techniques such as fluorescence in-situ hybridization

337 (FISH) will allow for fine grained analysis of host and symbiont identities (McManus and Katz

338 2009).

13

339

Oxidative Stress from *Chlorella* Influences Host Expression

The presence of a photosynthetic symbiont affecting the gene expression of a host 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

(Supplementary Material 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 (Supplementary Material 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). 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 Arcellinida morphospecies *H. papilio* harbors

diverse *Chlorella* strains that influence host gene expression, and that the relationship between

the amoebae and the microalgae *Chlorella* may not be as advantageous to both partners as

was previously assumed. Further studies of the microbiomes of microbes will surely reveal

additional nuances to symbiotic relationships.

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

14

365   Massachusetts and "Orono Bog" (44.870752, -68.723785) and "Big Heath" in Acadia National Park

366   (44.335780, -68.274809) in Maine (Supplementary Material Table S1). At each site we collected a

367   handful of *Sphagnum* moss from different sampling points. Back in the lab we washed off the Arcellinida

368   from the moss by putting about 10 strands of moss into 50 ml conical tubes with 20 ml filtered (2 µm filter)

369   water from the sampling sites and shaking the tubes. The moss and water were then filtered over a 300

370   µm mesh into a Petri dish to eliminate large particles. We then hand-picked individual healthy-looking

371   Arcellinida cells from the filtrate under the microscope. We only chose *H. papilio* cells that were bright

372   green, indicating the presence of healthy, undigested microalgae. Each individual was photo-documented

373   and cleaned in filtered (2 µm filter) bog water before being transferred in 1 µl bog water to a sterile 0.2 ml

374   tube and stored at -80 °C before either genome or transcriptome amplification, which took place within 1-

375   3 days of collection.

376   **Single-cell transcriptomics and genomics:** For the amplification of the transcriptomes of

377   individual *H. papilio* and *H. elegans* cells we first added 1.4 µl nuclease-free water to the tubes with the

378   isolated amoebae and then 0.25 µl of the lysis buffer contained within the SMART-Seq® v4 Ultra® Low

379   Input RNA Kit for Sequencing (TaKaRa Bio USA, Inc., Mountain View, CA, USA). The subsequent

380   transcriptome amplification and reverse transcription we conducted according to the manufacturer's

381   protocol, yet in quarter reactions. The SMART-Seq® v4 Ultra® Low Input RNA Kit was selected for this

382   study as it has been applied successfully across diverse microbial eukaryotes, including the difficult to

383   lyse Foraminifera (e.g., Weiner et al. 2020).

384   For the generation of single-cell genomes we used the REPLI-g Single-Cell Kit (Qiagen,

385   Germantown, MD, USA). We added 1 µl of single cell water and 1.5 µl DLB buffer (both are part of the kit)

386   to the picked cell and then followed the protocol according to the manufacturer's instructions.

387   After transcriptome/genome amplification we purified all samples using the AMPure XP PCR

388   Purification system (Beckman Coulter Life Sciences, Indianapolis, IN, USA) and quantified the amplified

389   nucleic acid content using a Qubit 3.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA).

390   Barcoded sequencing libraries were prepared using the Nextera XT DNA Library Preparation Kit

391   (Illumina, San Diego, CA, USA) and the samples were sequenced on a HiSeq 4000 platform at the

392   Institute for Genome Sciences at the University of Maryland.

393   **Transcriptome/genome assembly and post-assembly processing:** We used FastQC

394   (Andrews, 2010) for quality checking of the raw sequencing reads, trimmed adapters using the BBMap

395   toolkit (Bushnell 2014) and then assembled the reads using SPAdes (for genomes) or rnaSPAdes (for

396   transcriptomes; Bankevich et al. 2012). After assembly, we processed the transcriptomes using our

397   phylogenomic pipeline PhyloToL (Cerón-Romero et al. 2019). This post-assembly processing includes

398   the removal of prokaryotic sequences and ribosomal DNA, assignment of the assembled sequences to

399   gene families as defined by OrthoMCL (Li et al. 2003) using USearch (Edgar 2010), translation into amino

400   acid sequences and removal of short, highly identical sequences.

401   **Algal diversity and phylogeny:** In each of the 36 trimmed genome assemblies (Supplementary

402   Material Table S1) we searched for contigs containing the chloroplast-encoded *rbcL* sequence using

403   BLASTn version 2.9.0 (Boratyn et al. 2012) with a database from the *Chlorella rbcL* reference sequence

404   (KJ446796.1) from Gomaa et al. (2014). We added 208 representative *Chlorella rbcL* sequences (limited

405   in length to 700-5,000bp) from GenBank (including sequences from Gomaa et al. (2014)) to our dataset

406   and aligned all sequences using Mafft version 7.419 (Katoh and Standley 2013). We trimmed the

407   alignment to match the length of the *rbcL* sequences from Gomaa et al. (2014) and used jModelTest2

408   version 2.1.10 to select the best substitution model using the CIPRES Science Gateway (Miller et al.

409   2010). We then built a maximum likelihood phylogeny using RAxML version 8.2.12 (Stamatakis 2014),

410   with GTRGAMMAI specified as the substitution model and 100 bootstraps.

411   To test for evidence of multiple engulfing events of *Chlorella* algae by distinct protist lineages (i.e.,

412   amoebae, ciliates) we performed AU and SH tests with 10,000 re-samplings using the RELL method in

413   IQ-TREE version 1.6.12 (Nguyen et al. 2015). We tested three hypotheses: 1) the best tree from the

414   RAxML analysis above, 2) a constrained tree requiring monophyletic clades of amoebae- and ciliate-

415   associated *Chlorella*, and 3) a constrained tree requiring a monophyletic clade of amoebae-associated

416   *Chlorella*.

417   To evaluate the results of the *rbcL* phylogeny, and to assess evidence for both multiple *Chlorella*

418   strains per amoeba cell and low coverage samples (samples without enough reads for successful *de-*

419   *novo* assembly, but enough reads to determine *rbcL* type) we assembled the trimmed reads to the

420 *Chlorella rbcL* reference sequence KJ446796.1 using BBMap version 37.56 (Bushnell 2014). Reference

421 alignments were checked by eye using Geneious version 2019.0.4 (Kearse et al. 2012).

422 **Host diversity and phylogeny:** To assess the genetic diversity of the hosts and the potential link

423 with algal diversity we extracted mitochondrial cytochrome oxidase (COI) gene sequences from the *H.*

424 *papilio* genome samples from which we obtained *rbcL* sequence data. We applied a read mapping

425 approach using the BBMap toolkit (Bushnell 2014) to identify reads from the genome datasets that match

426 *H. papilio* COI sequences from GenBank representing the diversity of genetic lineages encountered

427 within this morphospecies to date (e.g., Gomaa et al. 2014; Heger et al. 2013; Singer et al. 2019). We

428 then assembled the mapped reads using SPAdes (Bankevich et al. 2012) and selected the longest

429 contigs for phylogenetic analysis. We produced a multiple sequence alignment with the inferred COI

430 sequences, the GenBank *H. papilio* sequences and two sequences of *Nebela* sp. as outgroups using

431 MAFFT version 7.419 (Katoh and Standley 2013). We trimmed the MSA to adjust the sequences to equal

432 length and built a phylogenetic tree using RAxML version 8.2.12 (Stamatakis 2014) with rapid

433 bootstrapping using a GTRCAT model and automatic halting of bootstrapping under the autoMRE

434 criterion. All phylogenetic programs were accessed *via* the CIPRES Science Gateway (Miller et al. 2010).

435 We visualized the tree in FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and rooted it with

436 the outgroup sequences.

437 **Fluorescence and transmission electron microscopy:** To assess the state of the algae cells

438 within *H. papilio*, we conducted fluorescence and transmission electron microscopy. For fluorescence

439 microscopy, hand-picked *H. papilio* cells were preserved in 100% ethanol and subsequently infiltrated

440 and embedded in medium grade LR-White resin. Semithin sections (500 nm) were mounted on glass

441 slides and stained with 4′,6-diamidino-2-phenylindole (DAPI) to reveal the presence of nuclei. The stained

442 sections were observed using an inverted epifluorescence microscope (Axio Observer.D1, Carl Zeiss,

443 Jena, Germany) equipped with a monochrome high-resolution camera (AxioCam MRm, Carl Zeiss, Jena,

444 Germany). The green autofluorescence was imaged and subtracted to the blue fluorescence in order to

445 reveal the DNA signal. Autofluorescence subtraction and overlays were done using the GIMP® software.

446 For transmission electron microscopy (TEM) we again hand-picked individual *H. papilio* cells from

447 environmental samples. We transferred the cells from the original petri dish to a drop of freshly-filtered (2

17

448 µm filter) in situ water and repeated this washing step until all obvious contamination on the outside of the

449 cells was removed. We then fixed the cells overnight in 3% glutaraldehyde (GTA) in 0.1 M PHEM buffer

450 (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9).

451   After washing off the fixative, the cells were post-fixed for 1 hour using 1% aqueous osmium

452 tetroxide solution and subsequently washed three times in water before dehydration through an acetone

453 series. Cells were finally infiltrated and flat-embedded in Epon-Araldite resin as described in Müller-

454 Reichert et al. (2003). Thin sections (100 nm) were mounted on formvar coated slot grids and stained for

455 7 min in 2% uranyl acetate followed by 10 min in Reynolds Lead Citrate. The cells were observed and

456 imaged with a Jeol® 1400 TEM.

457   **Assessing algal nuclear activity in transcriptome samples:** If the algae residing within *H.*

458 *papilio* cells are actively transcribing their nuclear DNA, we expect their transcriptomes to be present in

459 our sequencing reads, as the nucleic acids of organisms living within the shell or cytoplasm of the

460 amoeba will be co-amplified. In order to search for an algae signal in our transcriptome data, we used

461 PhyloToL (Cerón-Romero et al. 2019) to produce multiple sequence alignments and gene trees for 150

462 conserved eukaryotic gene families (Supplementary Material Table S2). These gene families were

463 selected based on their presence in at least four of the five major eukaryotic clades (SAR, Amoebozoa,

464 Archaeplastida, Excavata and Opisthokonta). We included a total of 278 species of all major eukaryotic

465 clades, bacteria and archaea in the analysis in addition to our 22 *H. papilio* and 12 *H. elegans*

466 transcriptomes. If both the amoeba and algae transcriptomes were present in a sample, we would expect

467 to find sequences of one sample appearing in Amoebozoa and Archaeplastida in the gene trees. We

468 used custom Python scripts to determine the sister branch of each sequence of our transcriptome

469 samples in each gene tree (Supplementary Material Table S2). In addition, we calculated the branch

470 length to avoid long-branch artifacts. All identified cases were additionally screened by eye.

471   To further investigate active gene expression by the algae we also assessed the number of times

472 that *H. papilio* samples appeared among green algae in the gene trees of 27 photosynthesis related gene

473 families (Supplementary Material Table S2). We used the KEGG (Kanehisa and Goto 2000) pathway for

474 *Chlorella* photosynthesis to identify nuclear encoded genes associated with photosynthesis. These genes

475 were then analyzed using PhyloToL following the methods described above.

476   **Starvation experiment:** In order to investigate the stability of the amoeba/algae relationship over

477   time, we maintained living *H. papilio* in the lab for two weeks. These cells were obtained from the

478   environment as described above, isolated from the surrounding substrate by hand-picking from the petri-

479   dish and placed in a new dish with freshly-filtered (0.2 µm filter) in situ water, which did not contain free-

480   living *Chlorella*, *Sphagnum* or any other food sources. We kept the dishes in an incubator at 20 °C under

481   a 12h light/dark cycle. We then removed two cells each on day 0, 3, 5, 7, and 12, photo-documented

482   them and froze them in RLT buffer for subsequent single-cell genome analysis. The amplified genomes

483   were sequenced and processed and the assemblies then scanned for chloroplast contigs using BLASTn.

484   All procedures were conducted as described above. We used GeSeq (Tillich et al. 2017) for annotation of

485   the contigs and to determine if they derive from chloroplasts and then mapped reads to the longest

486   identified chloroplast contig using BBMap version 37.56 (Bushnell 2014) with default settings. The

487   average coverage of each alignment was determined using bedtools genomecov (Quinlan and Hall

488   2010).

489   **Differential gene expression:** To assess whether the presence of the algae within the *H. papilio*

490   cells has an influence on the gene expression of the amoeba we compared the composition of the

491   transcriptomes of 16 of our *H. papilio* samples to our 12 samples of *H. elegans* (Supplementary Material

492   Table S1), a closely related species without associated algae. After counting all gene families present in

493   each transcriptome sample using custom Python scripts, we identified gene families differentially present

494   in the two morphospecies. We then used PhyloToL (Cerón-Romero et al. 2019) to construct multiple

495   sequence alignments and gene trees to assess homology prior to functional analysis in Blast2GO (Götz

496   et al. 2008). To compare the functional categories of the differentially expressed genes, we analyzed a

497   set of equivalent size that was made up of randomly chosen gene families shared among both amoeba

498   species.

499

500   **Acknowledgements**

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516   **Data Availability**

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518   All sequenced transcriptomes are available on GenBank under the SRA BioProject

519   PRJNA761372.

520
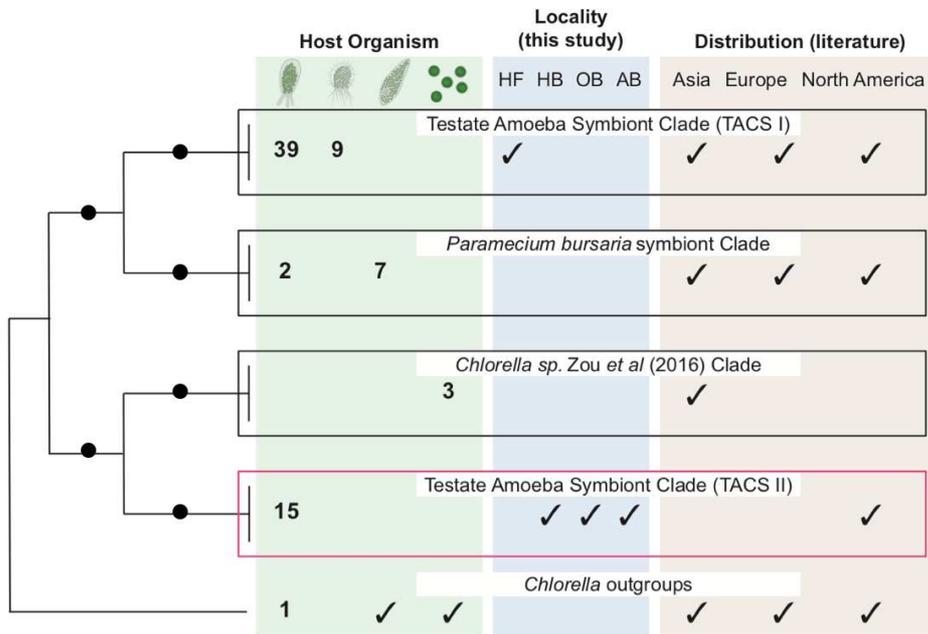
Figures

Figure 1. Genetic diversity and phylogeny 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.
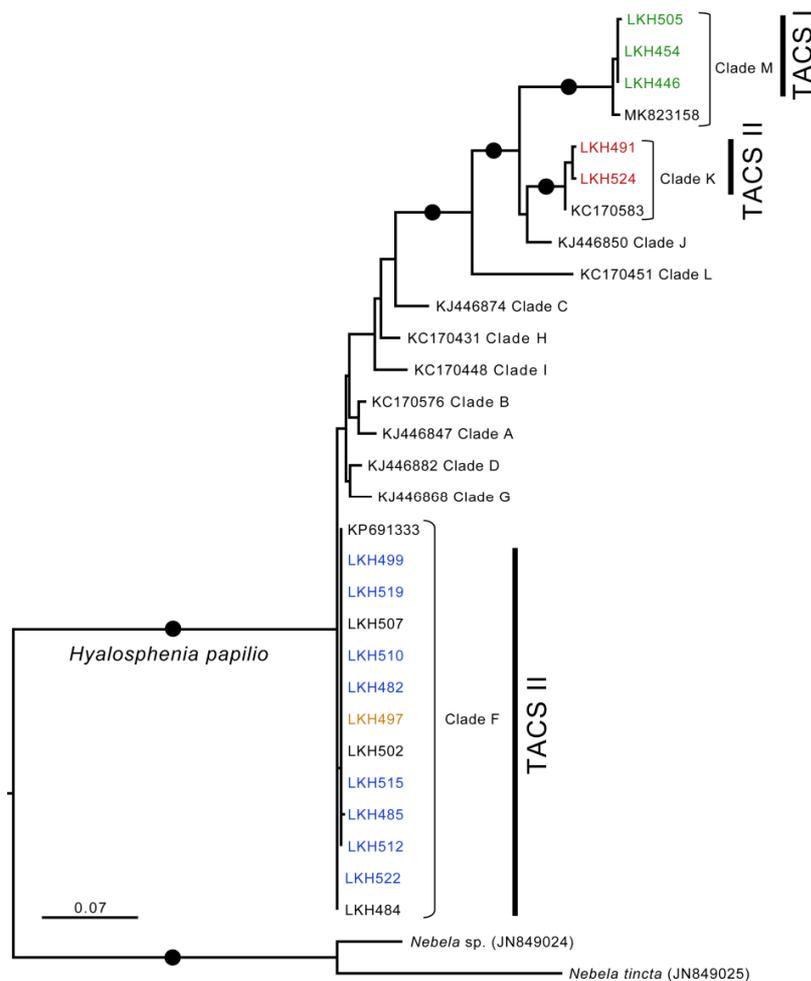
Figure 2. Genetic diversity within *Hyalosphenia papilio*. A maximum likelihood phylogeny of the genetic diversity within the morphospecies *H. papilio* based on a ~430 bp fragment of the mitochondrial cytochrome oxidase (COI) gene. Included in the tree are 17 COI sequences extracted from our genome samples, *H. papilio* sequences from GenBank representative for all known clades (Gomaa et al. 2014; Heger et al. 2013; Singer et al. 2019) and *Nebela* sp. sequences as outgroups. Our samples group among clades M, K and F. Samples in clade M are associated with symbionts of the TACS I clade and clades K and F with symbionts of TACS II. Colored in green are samples collected at Harvard Forest, in blue are samples from Hawley Bog, red from Acadia National Park and orange from Orono Bog. Bootstrap support values greater than 70 are indicated with filled circles.
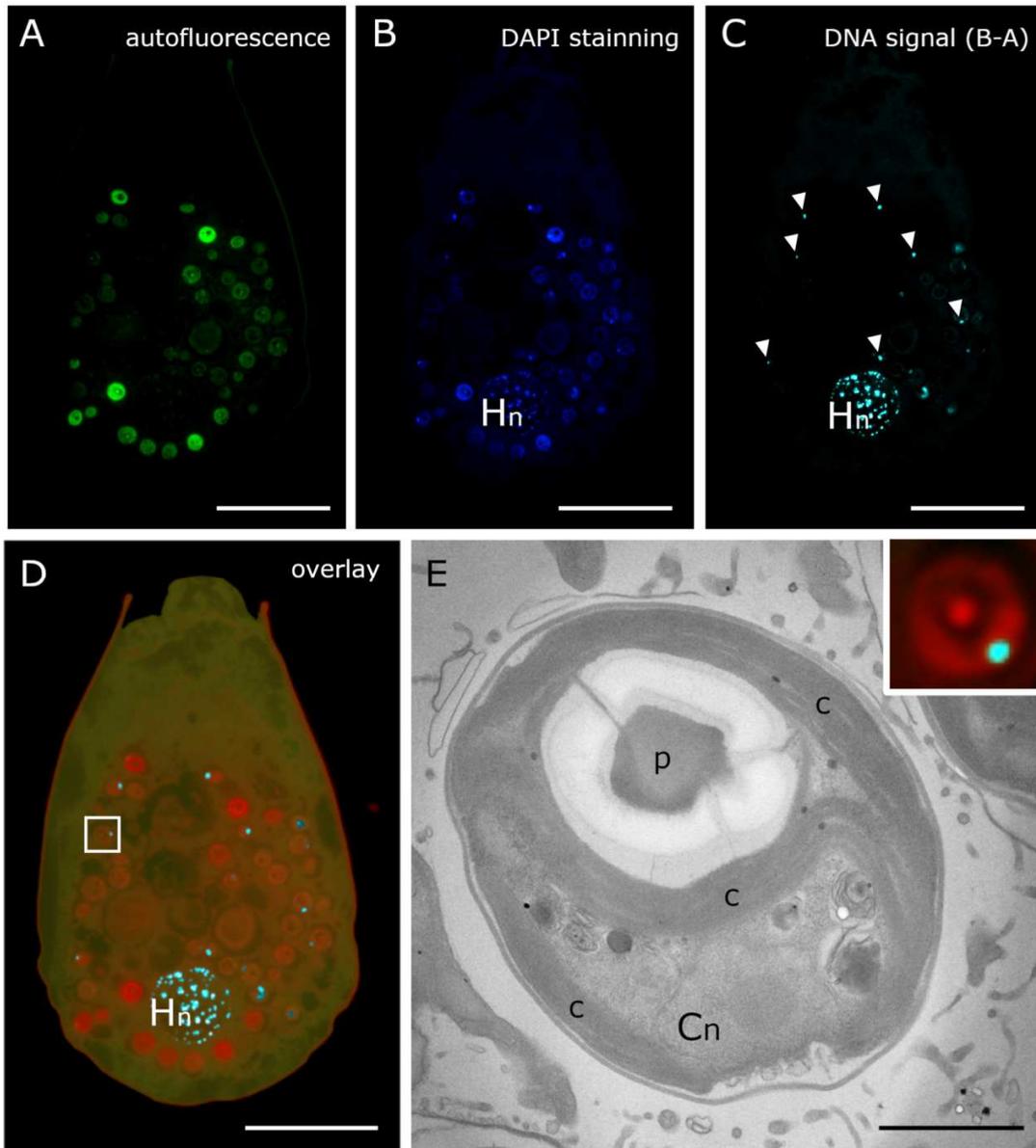
555
556
Figure 3. *Hyalosphenia papilio* harboring *Chlorella* symbionts. **A-D.** Semi-thin sections of a
single *Hyalosphenia papilio* cell inspected by fluorescence microscopy, Hn *Hyalosphenia*
nucleus. **A.** Green autofluorescence of the *Chlorella* chloroplasts; **B.** Blue autofluorescence
along 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

565   fluorescence overlay (D, area delimited by white square; nucleus = teal color, chloroplast and

566   pyrenoid autofluorescence = red). Scale bar A-D = 30 µm; scale bar E = 1 µm (insert = ×5.3).

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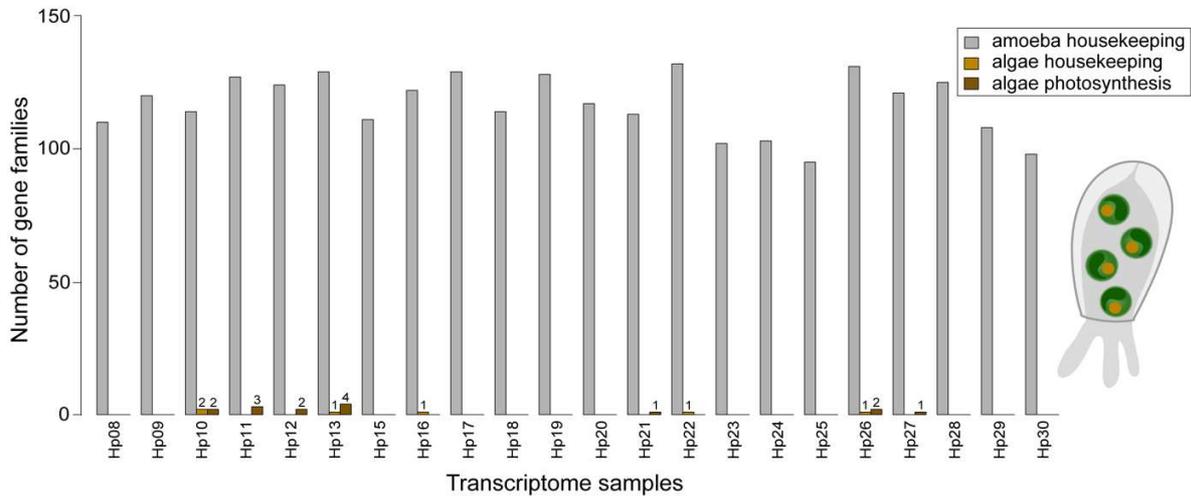
568
569
570   **Figure 4.** Presence of expressed algal nuclear genes (housekeeping and photosynthesis

571   related) in *Hyalosphenia papilio* transcriptome samples. The bar chart indicates the number of

572   gene families – out of 150 conserved housekeeping gene families and 27 photosynthesis

573   related gene families – that are expressed by either *H. papilio* (grey) and/or the *Chlorella*

574   symbionts (yellow, brown) in each of the samples from our transcriptome dataset. Exact

575   numbers of gene families expressed by the algae are shown above the bars. PhyloToL was

576   used to produce gene trees and assess the position of sequences among either Amoebozoa or

577   Viridiplantae. In the housekeeping gene families very few contained sequences were classified

578   as Viridiplantae, which suggests that *Chlorella* housekeeping genes are not being actively

579   transcribed. There were also very few nuclear encoded photosynthesis genes recovered from

580   the transcriptomes (Supplementary Material Table S2).

581
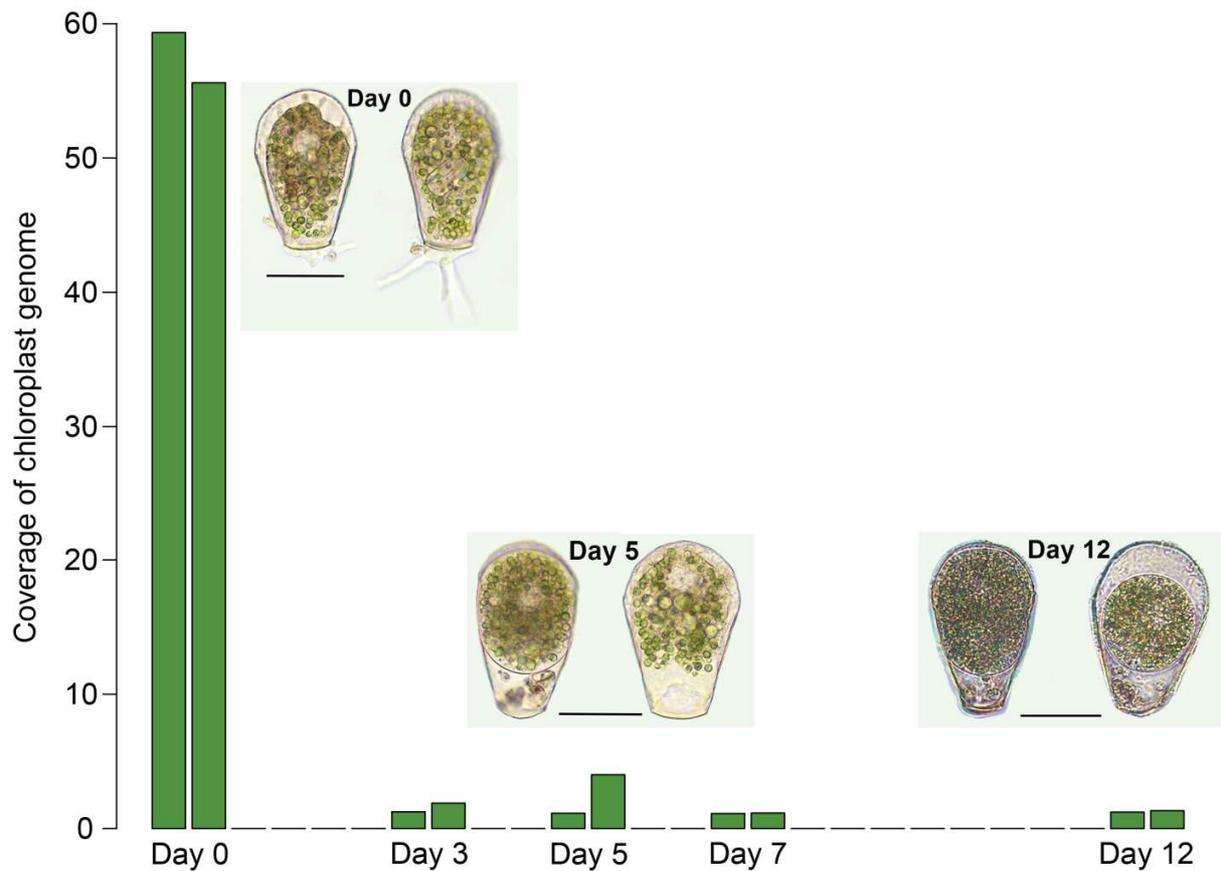
Figure 5. Starvation experiment. Evidence of a transient relationship seen in the reduction of *Chlorella* chloroplast genome during a starvation experiment, indicated as average coverage (average depth of reads per reference base). High genome coverage on Day 0 indicates healthy chloroplasts, however, 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.

590 **Supplementary Material**
591
592
593 **Table S1.** Sampling table indicating details of all single-cell genomes and transcriptomes
594 obtained for this study.
595
596 **Table S2.** Presence/absence of *H. papilio* sequences in the gene trees of 150 selected
597 housekeeping gene families and 27 photosynthesis related gene families generated using
598 PhyloToL. Further indicated are the sister branches to each sequence and the number of times
599 we detect *Chlorella* signal in the *H. papilio* samples.
600
601 **Table S3.** Functional characterization using Blast2GO on genes expressed in *H. papilio*, but not
602 *H. elegans* cells suggest that the algal symbiont is causing oxidative stress. We find eight genes
603 involved in oxidation-reduction processes from six gene families.
604
605 **Figure S1.** Reads from two samples mapped to the TACSI *rbcL* reference from Gomaa et al.
606 (2014; KJ446796.1). Differences from the reference are colored in the alignment. **A.** LKH454
607 from TACSI has no fixed differences to reference, and no consistent polymorphisms, though a
608 few reads that differ at the 3' end. **B.** LKH484 from the TACSII lineage has many fixed
609 differences from the reference but few polymorphisms.
610
611 **Figure S2.** Food vacuole containing *Chlorella* cells. In contrast with the intact *Chlorella* cells
612 located in the *Hyalosphenia* cytoplasm (Fig. 2E), the partially digested cells present in the food
613 vacuole lack a distinguishable nucleus, chloroplast, or pyrenoid.
614
615 **Figure S3.** Light microscope images of *H. papilio* cells from the starvation experiment sampled
616 after 0, 3, 5, 7 and 12 days showing the gradual digestion of the *Chlorella* algae. Scale bars
617 indicate 50 µm.
618
619 **File S1.** FASTA file of a MAFFT alignment of the rbcL sequences used for phylogenetic
620 inference of *Chlorella* diversity.
621
622 **File S2.** RAxML tree showing *Chlorella* diversity. This tree formed the basis for the cartoon tree
623 shown in Figure 1.
624

625   **File S3.** FASTA file of an alignment of the COI sequences used for inference of the genetic

626   diversity within the *H. papilio* samples.

627

628   **File S4.** RAxML tree showing the *H. papilio* genetic diversity and separation into different

629   genetic lineages. This tree includes sequences from all currently known genetic lineages of *H.*

630   *papilio* as published in Gomaa et al. (2014) and Heger et al. (2013).

631

632   **File S5.** FASTA file of representative sequences that are differentially expressed in *H. papilio*

633   compared to *H. elegans* and that may play a role in oxidative processes invoked by

634   photosynthesis (Supplementary Material Table S3).

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