Intra-host symbiont diversity and extended symbiont maintenance in photosymbiotic Acantharea (clade F)

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10 **Abstract**

11 Photosymbiotic protists contribute to surface primary production in low-nutrient, open-ocean
12 ecosystems and constitute model systems for studying plastid acquisition via endosymbiosis. Little is
13 known, however, about host-symbiont dynamics in these important relationships, and whether these
14 symbioses constitute mutualisms is debated. In this study, we applied single-cell sequencing methods
15 and advanced fluorescent microscopy to investigate host-symbiont dynamics in clade F acantharians,
16 a major group of photosymbiotic protists in oligotrophic subtropical gyres. We amplified the 18S
17 rRNA gene from single acantharian hosts and environmental samples to assess intra-host symbiont
18 diversity and to determine whether intra-host symbiont community composition directly reflects the
19 available symbiont community in the surrounding environment. Our results demonstrate that clade F
20 acantharians simultaneously host multiple species from the haptophyte genera *Phaeocystis* and
21 *Chrysochromulina*. The intra-host symbiont community composition was distinct from the external
22 free-living symbiont community, suggesting that these acantharians maintain symbionts for extended
23 periods of time. After selective staining of digestive organelles, fluorescent confocal microscopy
24 showed that symbionts were not being systematically digested, which is consistent with extended
25 symbiont maintenance within hosts. Extended maintenance within hosts may benefit symbionts
26 through protection from grazing or viral lysis, and therefore dispersal, provided that symbionts retain
27 reproductive capacity.

28 **1   Introduction**

29 Photosymbiosis, a nutritional symbiosis where a heterotroph hosts photosynthetic endosymbionts,
30 substantially increases surface primary production in oligotrophic marine ecosystems (Not et al.
31 2016). Photosymbiosis is also believed to have led to evolution of eukaryotic oxygenic
32 photosynthesis and eventual emergence of diverse photosynthetic eukaryotes, with many evolving
33 from eukaryote-eukaryote secondary and tertiary endosymbioses (Keeling 2004). Eukaryote-
34 eukaryote photosymbioses continue to be extremely common among marine protists and contribute
35 significantly to the productivity of oligotrophic open-ocean ecosystems (Decelle et al. 2015).
36 Nonetheless, little is known about host-symbiont dynamics, such as host-symbiont specificity or host
37 mechanisms for symbiont recognition, uptake, and maintenance. Whether photosymbioses are

38 mutualistic continues to foster debate, because the exact nature of these relationships may explain
39 how they evolve and persist (Keeling & McCutcheon 2017).
40
41 The Acantharea belong to the Rhizaria, a large group of amoeboid protists that includes many
42 photosymbiotic lineages (Burki & Keeling 2014). Photosymbiotic acantharians are often the most
43 abundant photosymbiotic Rhizaria in oligotrophic surface waters (Michaels et al. 1995), where they
44 contribute significantly to primary production (Caron et al. 1995). The majority of acantharian
45 species (clades E and F) host algal symbionts in the Haptophyte genus *Phaeocystis* (Decelle et al.
46 2012). *Phaeocystis* is a globally distributed genus with species that present multiple phenotypes—
47 solitary, flagellated, and colonial—and sometimes form harmful algal blooms (Schoemann et al.
48 2005). Despite the ecological significance of both partners, this symbiosis remains largely unstudied.
49 There is some evidence, however, suggesting that this relationship is not a case of mutualism and
50 symbionts are instead exploited (Decelle et al. 2012).
51
52 Traditionally, photosymbioses are considered mutualisms under the assumption that hosts provide
53 nitrogen to symbionts and symbionts provide organic carbon to hosts in return (Garcia & Gerardo
54 2014). Whether these relationships are truly mutualistic or are instead cases of symbiont exploitation
55 has been increasingly questioned in recent years and there is mounting evidence that exploitation is
56 the rule rather than the exception (Keeling & McCutcheon 2017). In high-light and low-prey
57 conditions, as found in oligotrophic surface waters, protistan photosymbiotic hosts benefit from an
58 increased growth-rate, but symbiont growth-rate is suppressed and photosynthetic efficiency is
59 decreased compared to free-living symbionts. These results indicate that algal symbionts may
60 actually experience restricted nitrogen availability *in hospite* and do not benefit from symbiosis
61 (Lowe et al. 2016). Estimated free-living populations of *Phaeocystis* in oligotrophic conditions
62 (Moon-van der Staay et al. 2000) are much larger than possible symbiotic populations estimated from
63 acantharian abundance and symbiont load (Michaels 1991). Symbiont growth rate may therefore be
64 decreased within acantharian hosts, potentially indicating that Acantharea-*Phaeocystis*
65 photosymbioses are also exploitative rather than mutualistic (Decelle 2013).
66
67 Increased growth rate is not the only means by which a symbiont may benefit from a host: decreased
68 predation, viral attack, or competition *in hospite* may allow symbionts to benefit from enhanced
69 dispersal and future reproduction, assuming reproductively viable symbionts are released from hosts
70 (Douglas 2010; Garcia & Gerardo 2014). Reproducing symbionts are known to be released from
71 some photosymbiotic protistan hosts: *Chlorella* escapes from *Paramecium* hosts and establishes free-
72 living populations when low-light inhibits host benefit (Lowe et al. 2016) and dinoflagellate
73 symbionts of colonial radiolarians establish free-living populations when isolated from hosts (Probert
74 et al. 2014). Some photosymbiotic forams, however, digest all of their symbionts prior to
75 gametogenesis (Takagi et al. 2016). It is currently unknown whether symbiotic *Phaeocystis* retains
76 reproductive capacity, but symbiotic cells have not yet been cultured from hosts (Decelle et al. 2012).
77 It is possible that phenotypic changes observed in symbiotic *Phaeocystis*—additional plastids and an
78 enlarged central vacuole (Febvre et al. 1979)—are evidence that symbionts are incapable of cell
79 division, which would make the relationship an ecological and evolutionary dead-end for *Phaeocystis*
80 and preclude the possibility for mutualistm (Decelle et al. 2012).
81
82 The number of symbionts observed in individual acantharians increases with host size (Michaels
83 1991), thus requiring that symbionts reproduce *in hospite*, that acantharians recruit new symbionts, or
84 possibly both. If acantharians recruit one or a few symbionts early in development and then support a
85 reproducing symbiont community, the intra-host symbiont community would exhibit low diversity
86 and may be divergent from the free-living environmental community. If symbionts divide within

87 hosts, hosts must exert an alternative form of population control, potentially by shedding (mutualism)
88 (Boettcher et al. 1996; Fishman et al. 2008) or digesting (exploitation) excess symbionts (Titlyanov
89 et al. 1996). Conversely, if acantharians recruit new symbionts, the intra-host symbiont community is
90 likely to be more diverse and representative of the available free-living symbiont community in the
91 surrounding waters. Low-diversity intra-host symbiont communities would therefore suggest that
92 symbionts maintain reproductive capacity and allows for possible symbiont benefit, whereas high
93 diversity communities may be interpreted as further evidence against mutualism. However, neither
94 intra-host symbiont diversity, nor the relationship between symbiont identity and environmental
95 availability of potential symbionts have been investigated in clade E or F acantharians.
96
97 In this study, we applied single-cell Next Generation Sequencing (NGS) to illuminate intra-host
98 symbiont diversity in individual clade F acantharians collected from 7 sampling sites along the
99 Ryukyu Archipelago, spanning more than 1,000 km in the East China Sea (ECS), and near Catalina
100 Island (California, USA). We further applied NGS to evaluate the environmental availability of
101 symbionts to acantharian hosts sampled from the ECS. We compared molecular symbiont diversity
102 with intra-host symbiont population size by enumerating symbionts with fluorescent confocal
103 microscopy in a subset of acantharians prior to nucleic acid extraction. Additional acantharians were
104 collected and imaged after selectively staining lysosomes and phagolysosomes in order to observe
105 their proximity to symbionts and to determine if symbionts are systematically digested. This study
106 provides new evidence to the mutualism-exploitation debate relative to Acantharea-*Phaeocystis*
107 symbioses by investigating intra-host symbiont diversity and by assessing host-symbiont specificity
108 in the context of environmental symbiont availability.

2 Materials & Methods

2.1 Individual acantharian sampling

111 Single acantharians were collected from coastal water near Catalina Island (California, U.S.A.),
112 Okinawa Island (Okinawa, Japan), and from cruise stations visited during the Japan Agency for
113 Marine-Earth Science and Technology (JAMSTEC) MR17-03C cruise to the ECS aboard the R/V
114 *Mirai* in May and June 2017 (Figure 1, Supplementary Table S1). Okinawa Island and Catalina
115 Island plankton samples were collected by pulling a Rigo Simple 20 cm diameter, 100-µm-mesh
116 plankton net or a SEA-GEAR 12" diameter, 163-µm-mesh plankton net, respectively, along the sea
117 surface approximately 5 m behind a small craft at its lowest speed. Aboard the R/V *Mirai*, plankton
118 samples were collected by passing unfiltered seawater pumped from the sea surface through a 100-
119 µm-mesh, hand-held plankton net (Rigo). Plankton samples were observed under a dissecting
120 microscope and individual acantharians were transferred by glass micropipette to clean petri-dishes.
121 Acantharians were rinsed with 0.2-µm-filtered seawater several times until all visible contaminants
122 were removed and were then incubated for 0.5–2 hr to allow additional self-cleaning. Acantharians
123 collected aboard the R/V *Mirai* and those from near Okinawa Island were imaged with inverted light
124 microscopy (Zeiss Primovert, Olympus CKX53, Supplementary Figures S1 and S2). Several
125 acantharians collected near Okinawa Island were also imaged with laser confocal microscopy
126 (described below). Each acantharian was transferred to a maximum recovery PCR tube (Axygen) and
127 successful transfer was confirmed by microscopy before adding 30 µL of RLT-plus cell-lysis buffer
128 to each tube (Qiagen). Immediately following buffer addition, samples were flash-frozen with liquid
129 nitrogen and stored at -80°C until later processing in the lab.
130

2.2 Environmental sampling

131
132 Seawater samples were collected at each ECS cruise station visited by the JAMSTEC MR17-03C
133 cruise where acantharians were also isolated. Two replicates of 4.5 L of seawater were collected from
134 the sea surface by bucket and sequentially filtered under a gentle vacuum through 10.0 μm and 0.2
135 μm pore-size Polytetrafluoroethylene (PTFE) filters (Millipore) to size-fractionate plankton and
136 separate free-living *Phaeocystis* (< 10 μm) from acantharian hosts (> 10 μm). Filters were flash-
137 frozen in liquid nitrogen onboard and stored at -80°C until processing onshore.

2.3 RNA extraction from individual acantharian hosts

139 RNA extractions from single acantharians (n = 42) were accomplished by modifying methods of
140 Trombetta et al. (2015). Samples were thawed over ice, vortexed twice (10 s, speed 7, Vortex-Genie
141 2), and then incubated at room temperature for 5 min to fully lyse cells. Agencourt RNAClean XP
142 magnetic beads (Beckman Coulter) were added to each sample at a 2.2 : 1 v:v ratio and fully mixed
143 by pipette prior to a 30-min incubation in order to bind all RNA to the magnetic beads. After two
144 80% ethanol washes, RNA was eluted from the beads in 11 μL of a custom elution buffer (10.72 μL
145 nuclease-free water, 0.28 μL RNAase inhibitor (Clonetech)) and 10.5 μL of eluted RNA was further
146 processed following the single-cell protocol for the SMART-seq v4 Ultra Low Input Kit (Clonetech)
147 with 18 cycles in the primary PCR. The resulting cDNA from each sample was quality checked with
148 the Bioanalyzer High Sensitivity DNA Assay (Agilent) and quantified with the Qubit dsDNA High
149 Sensitivity Assay (Qubit 3.0, ThermoFisher).

2.4 DNA extraction from environmental samples

151 Environmental DNA was extracted from the 0.2-μm pore-size PTFE filters (n = 12, 2 replicates from
152 6 stations) following manufacturer protocols for the Qiagen AllPrep DNA/RNA Mini Kit with
153 limited modifications. Half of each PTFE filter was submerged in RLT-plus cell-lysis buffer
154 (Qiagen) with garnet beads in a 2-mL tube (MoBio/Qiagen). Samples were heated for 10 min at 65°C
155 and then vortexed at maximum speed (Vortex-Genie 2) for 5 min with the MoBio/Qiagen vortex
156 adapter to fully lyse cells. After cell lysis, DNA extraction proceeded without further modifications.
157 Extracted DNA was quantified with the Qubit dsDNA High Sensitivity Assay on a Qubit 3.0
158 instrument (ThermoFisher).

2.5 Library preparation and sequencing

160 Library preparation for acantharian cDNA samples and environmental DNA samples followed
161 procedures described in the Illumina 16S Metagenomic Sequencing Library Preparation manual,
162 modified only to include universal eukaryotic primers for the v4 region of the eukaryotic 18S rRNA
163 gene (Stoeck et al. 2010) and amplicon PCR conditions most appropriate for these primers. The
164 forward primer, TAReuk454FWD1 (CCAGCASCYGCGGTAATTCC, Stoeck et al. 2010), was used
165 unmodified. The reverse primer, TAReuk454REV3 (ACTTTCGTTCTTGATYRA, Stoeck et al.,
166 2010) was reported not to amplify the *Phaeocystis* 18S gene in *in silico* PCRs (Tanabe et al. 2016).
167 Further investigation revealed a mismatch between the final 3' adenine in the primer sequence and
168 the *Phaeocystis* 18S gene sequence. Although we found that the original primers do amplify the v4
169 region of the *Phaeocystis* 18S gene in *de facto* PCRs with DNA extracted from *Phaeocystis* cultures,
170 the mismatch could create bias against *Phaeocystis* sequences in more diverse samples. We therefore
171 eliminated the 3' "A" in the TAReuk454REV3 sequence and used a new reverse primer
172 TAReuk454REV3.1 (ACTTTCGTTCTTGATYR). The optimum annealing temperature for the
173 Illumina-adapted primers was determined by performing temperature gradient PCRs (53–65°C, 0.5°C

4

174   steps) and the annealing step in the amplicon PCR was set at 58°C thereafter. Following the second,
175   indexing PCR and final product purification, amplicon libraries were quantified with the Qubit
176   dsDNA High Sensitivity Assay (Qubit 3.0, ThermoFisher) and the amplicon size was determined
177   with the Bioanalyzer High Sensitivity DNA Assay (Agilent). Amplicon libraries were then submitted
178   to the Okinawa Institute of Science and Technology (OIST) sequencing center for 300x300-bp
179   paired-end sequencing on the Illumina MiSeq sequencing platform with v3 chemistry.

180   ## 2.6   Amplicon sequence analysis and annotation

181   Demultiplexed paired-end sequences provided by the OIST sequencing center were imported to
182   Qiime2 (v2017.11) software (Caporaso et al. 2010, qiime2.org). The Divisive Amplicon Denoising
183   Algorithm (DADA) was implemented with the DADA2 plug-in for Qiime2 for quality filtering,
184   chimera removal, and feature table construction (Callahan et al. 2015). DADA2 models the amplicon
185   sequencing error in order to infer sample composition more accurately than traditional Operational
186   Taxonomic Unit (OTU) picking methods (Callahan et al. 2015). Taxonomy was assigned to the
187   feature table in Qiime2 by importing SILVA 18S representative sequences and consensus taxonomy
188   (release 128, Quast et al. 2013). The feature table and associated taxonomy were then extracted from
189   Qiime2 for further analysis in the R statistical environment (R Core Team 2013). The feature table
190   was split into a table of acantharian samples and a table of environmental samples and both were
191   converted to phyloseq objects for preprocessing and analysis (McMurdie & Holmes 2013).
192   Prevalence filtering was applied to the acantharian feature table to remove low-prevalence (< 5%)
193   SVs and decrease the chance of data artifacts affecting the analysis (Callahan et al. 2016). Prevalence
194   filtering effectively eliminated sequences from known Rhizaria prey (i.e. metazoans and diatoms
195   (Swanberg & Caron 1991)) and parasites (i.e. alveolates (Bråte et al. 2012)). The phyloseq object was
196   further filtered to eliminate SVs originating from hosts by retaining SVs classified as belonging to
197   the phylum Haptophyta, which includes *Phaeocystis,* leaving 21 symbiotic SVs. The environmental
198   feature table was then filtered to include only the 21 symbiotic SVs found in acantharian samples.
199
200   The 21 symbiotic SVs were further classified by building a phylogenetic tree. An initial BLAST
201   query (BLASTn 2.8.0+, 03/23/2018, Camacho et al. 2009) indicated that the symbiont SVs belong to
202   the Haptophyte genera *Phaeocystis* and *Chrysochromulina.* Likewise, a SILVA SSU sequence search
203   (03/23/2018, Quast et al. 2013) classified 18 of the sequences as class Prymnesiophycae, order
204   Phaeocystales or Prymnesiales, and genus *Phaeocystis* or *Chrysochromulina,* when classified to
205   genus level. The remaining 3 symbiotic SVs were designated "unclassified" in the SILVA sequence
206   search. Reference 18S rRNA gene sequences were downloaded from GenBank (Benson et al. 2012)
207   for the 5 Haptophyte orders (Pavlovales, Phaeocystales, Prymnesiales, Isochrysidales, and
208   Coccolithales (Edvardsen & Medlin 2007)) and included in a Multiple Sequence Comparison by Log
209   Expectation (MUSCLE) v3.8.31 (Edgar 2004) alignment along with the 21 symbiotic SVs. A
210   Bayesian phylogenetic tree was then built from the alignment using MrBayes v3.2.7 (Ronquist &
211   Huelsenbeck 2003) (Figure 3). The phylogenetic relationships between the Haptophyte orders in the
212   resulting tree match published phylogenies for Haptophyta (Edvardsen & Medlin 2007; Sáez et al.
213   2004). Similarly, the phylogenetic relationships between cultured clades of *Phaeocystis* matched
214   published phylogenetic trees (Andersen et al. 2015), and the placement of the uncultured clade,
215   Phaeo2, is similar to published single-gene phylogenetic reconstruction with the 18S rRNA gene
216   (Decelle et al. 2012). A phylogenetic tree was built for the 5 dominant acantharian host SVs
217   following the same methods (Figure 4) and the reconstruction matched published phylogenetic
218   relationships for clades E and F Acantharea (Decelle et al. 2012b).

219   ## 2.7   Statistical analyses

220 Bray-Curtis distances between symbiont community compositions in acantharian and environmental
221 samples were computed from relative abundances of symbiotic SVs in the filtered feature tables with
222 the R package phyloseq (McMurdie & Holmes 2013). Bray-Curtis distances were used to perform
223 Principal Coordinate Analyses (PCoA) within the phyloseq package, and PCoA plots were rendered
224 with the R package ggplot2 (Wickham 2009). Permutational Multivariate Analyses of Variance
225 (PERMANOVA) with 999 permutations were performed with the adonis function in the R package
226 vegan (Oksanen et al. 2013) to determine whether clustering observed in the ordination plots was
227 statistically significant and to discern which covariates were deterministic of symbiont community
228 composition. PERMANOVA were also performed with the beta-group-significance function in the
229 Qiime2 diversity plugin, which allows for pairwise comparisons. Differences were considered
230 statistically significant when the p-value was ≤ 0.05.

2.8 Fluorescent confocal microscopy

232 In order to observe and enumerate symbionts within hosts for which symbiont communities were also
233 evaluated, acantharians collected near Okinawa in April (Oki.3A, Oki.4A) and May (Oki.3, Oki.7,
234 Oki.10, Oki.11) were imaged without staining using an inverted laser scanning confocal microscope
235 (Zeiss LSM 780) prior to RNA extraction. A z-stack of chlorophyll autofluorescence (em670 nm)
236 and halogen light images was assembled for each host to compare the number of visually observable
237 symbionts to the number of symbiotic SVs identified. In order to evaluate possible host digestion of
238 symbionts, additional acantharian samples were collected in December 2017 (n = 3) and stained with
239 LysoTracker Green DND-26 (ThermoFisher), a fluorescent dye that selectively stains acidic
240 compartments (i.e. digestive organelles) within cells, including lysosomes and phagolysosomes. The
241 LysoTracker dye was diluted from the 1-mM stock solution to a 100-nM working solution in 0.2-μm-
242 filtered seawater and each sample was incubated in 100 μL working solution in the dark for 2 hr
243 before imaging. Z-stacks for these samples were assembled by imaging with 3 channels: red for
244 autofluorescence from symbiont chlorophyll (em670 nm), green for LysoTracker-stained host
245 lysosomes and phagolysosomes (em511 nm), and grey for halogen light imaging. FIJI Image-J
246 software (Schindelin et al. 2012) was used to adjust image brightness, merge color channels, and
247 create 3-D projections for all imaged host cells.

3 Results

3.1 Intra-host symbiont diversity in individual acantharians

250 To determine if and to what extent intracellular symbiont diversity exists in acantharians, we
251 performed single-cell RNA extractions and sequenced 18S rRNA gene amplicons from 42 individual
252 acantharians. A total of 6,154,808 sequences were generated from acantharian samples, with 28,828–
253 241,809 sequences per sample. After quality filtering and feature table construction, 3,294,093 total
254 sequences remained (5,579–136,339 per sample). Within each acantharian sample, 1–17% of
255 sequences derived from symbionts. We identified 21 symbiotic SVs in the acantharian samples, and
256 each acantharian host contained 4–12 unique symbiotic SVs (mean = 8, standard deviation = 2)
257 (Figure 2). Our phylogenetic analysis determined that symbiotic SVs belonged to four *Phaeocystis*
258 clades: the *globosa* clade, the *cordata* clade, the *jahnii* clade, which has not previously been reported
259 as a symbiont, and the uncultured Phaeo2 clade, which was discovered by Decelle et al. (2012) as an
260 acantharian symbiont near Okinawa (Figure 3). Additionally, four symbiotic SVs belonged to the
261 genus *Chrysochromulina*, which had not previously been identified as a symbiont in clade F
262 acantharians. The majority of symbiotic SVs in acantharians collected from the ECS belonged to the
263 *Phaeocystis* clades *cordata*, *jahnii* and Phaeo2, and only 3 of these samples contained SVs belonging

6

264 to *Chrysochromulina*. The opposite pattern was observed in samples collected near Catalina Island:
265 the majority of symbiotic SVs in these samples were *Chrysochromulina spp.* However, all three
266 samples from Catalina Island hosted symbionts from the Phaeo2 *Phaeocystis* clade, which had
267 previously only been found in hosts collected near Okinawa Island (Decelle et al. 2012). These
268 results demonstrate that acantharians simultaneously host multiple symbiont species and that
269 *Phaeocystis*-hosting acantharians can also host *Chrysochromulina spp*.

271 We also investigated the effect of host type and collection location on symbiont community
272 composition within individual acantharians. Acantharian samples were each dominated by 1 of 5
273 unique Rhizarian SVs, all of which belonged to Acantharea clade F and represented 3 genera:
274 *Amphibelone* (Host SVs 1 and 3), *Amphilonche* (Host SVs 2 and 4), and *Acanthometra* (Host SV5)
275 (Figure 4). Host SV did not appear to determine the symbiont community in a Principal Coordinate
276 Analysis (PCoA) plot based on Bray-Curtis distances between acantharian symbiont community
277 compositions, but collection location appeared to play a role and the Catalina Island samples formed
278 a distinct cluster in the PCoA plot (Figure 5). Statistical testing showed that collection location had a
279 significant effect on symbiont community ($p = 0.001$, $R^2 = 0.47$) and in pairwise comparisons, only
280 two comparisons—st. 17 to st. 12 and st. 13 to Okinawa—were not significantly different
281 (Supplementary Table S2). Host SV also significantly affected symbiont community ($p = 0.009$, $R^2 =$
282 0.19), but pairwise comparisons showed that the significance was driven solely by the symbiont
283 community associated with host SV 5, which was only found near Catalina Island, indicating that
284 host effect was confounded by location (Supplementary Table S3).

3.2 Comparison of intra-host and free-living symbiont communities

286 To determine whether relative abundance of symbionts within hosts is a reflection of relative
287 abundance of available symbionts in the surrounding water, we compared symbiont communities
288 within acantharians collected from cruise stations in the ECS to environmental samples taken at the
289 same time and place. 1,852,276 sequences were generated from environmental samples with 93,291–
290 246,564 reads per sample. After quality filtering and feature table construction, 645,325 sequences
291 remained, with 35,691–89,163 sequences per sample. A PCoA plot based on Bray-Curtis distances
292 between the entire community for each environmental sample confirmed that replicates from each
293 location were more similar to each other than to replicates from other locations (Figure 6). Symbiotic
294 SVs identified in acantharian samples comprised 1–3% of sequences in environmental samples and
295 only 11 of 21 symbiotic SVs were also found in environmental samples (SVs 2, 5, 7, 11, 13, 14, 15,
296 16, 18, 19 were missing) (Figure 7). Intra-host and environmental symbiont communities clustered
297 separately in a PCoA based on Bray-Curtis distances between samples (Figure 8) and the observed
298 difference between community compositions in the two sample types was statistically significant ($p =$
299 0.001, $R^2 = 0.33$). These results indicate that the intra-host community composition of symbiotic SVs
300 is distinct from the surrounding environmental community composition.

3.3 Visualization of host-associated symbionts and host digestive-organelles

302 By imaging symbiont chlorophyll autofluorescence with laser confocal microscopy, we were able to
303 clearly enumerate photosynthetic symbionts within hosts. This technique allowed us to discern that
304 there were more individual symbiont cells than observed SVs in each imaged acantharian collected
305 near Okinawa in April and May (Figure 9A–B, Supplementary Figure S3). Symbionts with the free-
306 living phenotype (twin parietal plastids, cell diameter < 5 μm) and symbionts with the symbiotic
307 phenotype (more than 2 plastids, enlarged central vacuole, cell diameter > 5 μm) were observed
308 inside the same host cells (Figure 9B, Supplementary Figure S3). By selectively staining host

309   digestive-organelles with a fluorescent dye, we were able to visualize host lysosomes and
310   phagolysosomes and their proximity to symbionts (Figure 9C–E). We observed lysosomes in both
311   host exoplasm and endoplasm, while symbionts were only observed in host endoplasm. No
312   symbionts were enclosed in phagolysosomes and lysosomes did not appear to be concentrated near
313   symbiont cells.

314   **4   Discussion**

315   Acantharians are abundant globally and are especially abundant in low-nutrient subtropical gyres
316   where they contribute to primary production by harboring intra-cellular algal symbionts (Michaels
317   1991; Michaels et al. 1995). Despite their ecological importance, host-symbiont dynamics in
318   acantharian photosymbioses remain largely unstudied. Decelle et al. (2012) discovered that
319   Acantharea-*Phaeocystis* symbioses are flexible in regard to symbiont species and hypothesized that
320   the relationship is more akin to enslavement than mutualism (Decelle 2013; Decelle et al. 2012). If
321   symbionts are exploited to the extent that they are unable to reproduce, hosts need to continuously
322   recruit symbionts and should host diverse symbiont communities reflecting the relative availability of
323   different symbionts. Our amplicon sequencing results show that acantharians do simultaneously host
324   multiple species of *Phaeocystis* as well as *Chrysochromulina spp.*, but we found that the host-
325   associated symbiont community does not simply mirror the free-living community. Our microscopy
326   results show that individual hosts harbor symbionts exhibiting the free-living phenotype as well as
327   the symbiotic phenotype and demonstrate that symbionts are not being systematically digested.
328   Together, these results suggest that hosts continue to recruit new symbionts, but they do not digest or
329   shed symbionts at the same rate that they acquire them.
330
331   As acantharians increase in size, the number of symbionts they host also increases (Michaels 1991).
332   To accomplish this, acantharians could recruit microalgal partners early in development and nurture
333   reproducing symbiont populations. Alternatively, acantharians could recruit symbionts continuously,
334   which would likely lead to multiple species of symbionts coexisting within individual hosts. In this
335   study, all 42 acantharians collected hosted multiple species of *Phaeocystis* symbionts and several also
336   hosted *Chrysochromulina spp.*, suggesting that acantharians recruit symbionts more than once. The
337   observation of symbionts with the free-living phenotype alongside symbionts with the symbiotic
338   phenotype within single hosts is further evidence that hosts continue to recruit new symbionts
339   (Febvre et al. 1979, Figure 9). However, these results do not exclude the possibility that symbionts
340   reproduce *in hospite,* and symbionts are known to reproduce within larger photosymbiotic Rhizarians
341   that can be maintained in culture (Takagi et al. 2016). Acantharians in this study contained more
342   individual symbiont cells than unique Sequence Variants, indicating that either hosts recruit multiple
343   symbiont cells from a clonal environmental population or that symbionts reproduce *in hospite*.
344   Therefore, it is possible that symbiont cells exhibiting the free-living phenotype within hosts are
345   recently divided cells rather than recently engulfed cells.
346
347   Since acantharians in this study simultaneously hosted multiple *Phaeocystis* and *Chrysochromulina*
348   species, we expected that the intra-host symbiont community would reflect environmental symbiont
349   availability. Our results, however, showed that the intra-host symbiont community was distinct from
350   the free-living community. Additionally, acantharians sometimes hosted *Phaeocystis* genotypes (e.g.
351   *P. jahnii* SV 5, *P. globosa* SVs 13 & 14) that were absent in environmental samples collected from
352   the same place. These results suggest acantharians may maintain symbionts for extended periods,
353   beyond that required for external populations to respond to changing environmental conditions. This
354   is feasible since *Phaeocystis* generation times can be as brief as 6.6 h and vary by species in different
355   light and temperature conditions (Jahnke 1989), while acantharians likely survive for at least a month

356   (Suzuki & Not 2015). Intra-host communities may therefore be cumulative representations of all
357   encountered environmental symbiont communities, rather than just a snapshot of the current
358   community.
359

360   The observed differences between acantharians symbiont communities and the availability of
361   symbionts could also indicate that hosts selectively uptake symbionts that are most beneficial in
362   prevailing environmental conditions. *Chrysochromulina* SVs were dominant in each acantharian
363   sample collected near Catalina Island, but were only found in one acantharian from an ECS cruise
364   station, despite being well-represented in all cruise station environmental samples. Perhaps
365   *Chrysochromulina spp.* make better partners in the Catalina Island environment compared to the
366   Ryukyu Archipelago. If acantharians select and concentrate environmentally rare symbionts, it would
367   also increase the likelihood that our environmental sampling missed those symbionts, especially
368   because symbiotic SVs constituted only 1-3% of the community. Additionally, it remains possible
369   that the differences observed between intra-host and environmental symbiont communities could
370   derive from different nucleic acid extraction methods used. However, we did not observe digestion of
371   symbionts by hosts, which provides additional support for the extended maintenance of symbionts *in*
372   *hospite*.
373

374   This study demonstrates for the first time that intra-cellular symbiont diversity exists in cade F
375   acantharian photosymbioses. Acantharians in the clade B genus, *Acanthochiasma*, also host multiple
376   symbiont types, including *Chrysochromulina spp.* and several dinoflagellate genera (Decelle et al.
377   2012c). Radiolarian and foraminiferan (Rhizaria) hosts also host dinoflagellates and prasinophytes or
378   prymnesiophytes (Gast & Caron 2001). Theoretically, simultaneously hosting multiple symbiont
379   species or genotypes is ineffective and should negatively impact host fitness since different
380   symbionts would compete for space and resources within hosts (Douglas 1998). Planktonic hosts can
381   be transported long distances and may therefore experience larger environmental gradients on shorter
382   time-scales than stationary photosymbiotic hosts. This could make hosting a diverse community of
383   symbionts more effective for planktonic hosts, especially if some symbionts perform better under
384   different conditions. Indeed, different *Phaeocystis* species have different light and temperature
385   optima (Jahnke 1989) and different strains of a single species have varying abilities to utilize
386   different nitrogen sources (Wang et al. 2011). Drifting buoys in the Global Drifter Program
387   (Lumpkin et al. 2013) passing our sampling sites in spring, the season we sampled, traveled an
388   average of 3.66° latitude in 30 days ($n = 42$), the estimated minimum survival time of acantharians
389   (Suzuki & Not 2015), suggesting they can travel at least this far in their lifetime. While the
390   associated mean temperature gradient of 1.93°C is smaller than experimental temperature gradients
391   shown to differentially influence *Phaeocystis spp.*, changes in day length and irradiance may affect
392   photosynthetic output of *Phaeocystis* strains differently (Jahnke 1989).
393

394   The possible fitness benefit for symbionts associated with acantharians remains enigmatic, but the
395   evidence we present for extended symbiont maintenance allows that *Phaeocystis* could glean some
396   advantage from the symbiosis. Acantharian symbionts were not enclosed in phagolysosomes in host
397   cells imaged in this study, which could be due to symbionts escaping phagosomes (Jamwal et al.
398   2016) or a failure of host lysosomes to fuse with symbiont-containing phagosomes (Hohman et al.
399   1982; Sibley et al. 1985). Electron microscopy suggests that there is a host membrane surrounding
400   acantharian symbionts (Febvre et al. 1979), so it is more probable that lysosomes do not fuse with
401   symbiont-containing phagosomes and they instead represent symbiosomes or long-term perialgal
402   vacuoles. Symbiont signaling prevents lysosomes from fusing with symbiosomes housing *Chlorella*
403   symbionts in *Paramecium* (Kodama et al. 2011) and *Hydra* (Hohman et al. 1982) hosts. Complex
404   signaling between hosts and symbionts leading to development of symbiosomes and symbiont

405 differentiation is suggestive of co-evolution (Hinde & Trautman 2001). If *Phaeocystis* also actively
406 prevents lysosomes from fusing with phagosomes, then *Phaeocystis* has adapted to avoid digestion
407 and perhaps to promote a stable symbiosis. Although our results suggest that acantharians maintain
408 symbionts, we cannot rule out that they digest symbionts when stressed or before gametogenesis,
409 which would rule out mutualism. Acantharian gametes do not contain symbionts, but it is not yet
410 clear whether symbionts are digested or released during reproduction (Decelle & Not 2015).
411 Additionally, it remains an open question whether released symbionts are reproductively competent
412 outside the host (Decelle et al. 2012).
413
414 Our results demonstrate that clade F acantharians simultaneously associate with multiple *Phaeocystis*
415 and *Chrysochromulina* species, providing further evidence that the Acantharea-*Phaeocystis*
416 photosymbiosis is highly flexible. Our results suggest that symbionts escape host digestion for
417 extended periods, but whether symbionts are capable of reproducing *in hospite* or after release, and
418 whether they benefit from the relationship is still undetermined. Until acantharians can be maintained
419 for prolonged periods under laboratory conditions, it will remain challenging to elucidate many
420 aspects of the host-symbiont dynamics in this system. LysoTracker dyes can be utilized to track
421 symbiosome conditions for as long as hosts survive and species- or genotype-specific fluorescent
422 probes may be used to investigate whether different species or genotypes are differentially
423 transformed into the symbiotic phenotype or are compartmentalized within the host. If symbionts
424 with a single 18S rRNA gene Sequence Variant (or a unique combination of SVs) are co-localized, it
425 would provide evidence for *in hospite* reproduction. Efforts to culture symbionts from *Phaeocystis*-
426 hosting acantharians should continue. If successful, it would demonstrate unequivocally that
427 symbionts maintain reproductive capacity and cultured symbionts would be an invaluable resource
428 for comparative genomics and transcriptomics. Differential gene expression analysis could then be
429 utilized to investigate physiological shifts in symbiotic *Phaeocystis* or *Chrysochromulina* compared
430 to cultured free-living cells and could illuminate mechanisms of host-symbiont interaction. Further
431 investigation into whether symbionts benefit from this relationship will be important to
432 understanding host-symbiont dynamics in this and other protistan photosymbioses.

433 ## 5   Author contribution

434 MB, MG, and SM planned the research. MB performed the research. LM optimized single-cell RNA
435 techniques and performed research. MB analyzed the data and wrote the manuscript. MG and SM
436 edited drafts.

437 ## 6   Funding

441 ## 7   Conflict of Interest

442 The authors declare that the research was conducted in the absence of any commercial or financial
443 relationships that could be construed as a potential conflict of interest.

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453 Unit, Steven D. Aird, and David Caron edited and provided comments on the manuscript.

454 ## 9   Data availability statement

455 Sequences generated and analyzed in this study have been submitted to the European Nucleotide
456 Archive under the study accession number PRJEB24538. Intermediate data files and data analysis
457 pipelines are available at https://github.com/maggimars/AcanthareaPhotosymbiosis and
458 https://maggimars.github.io/AcanthareaPhotosymbiosis/Analysis.html.

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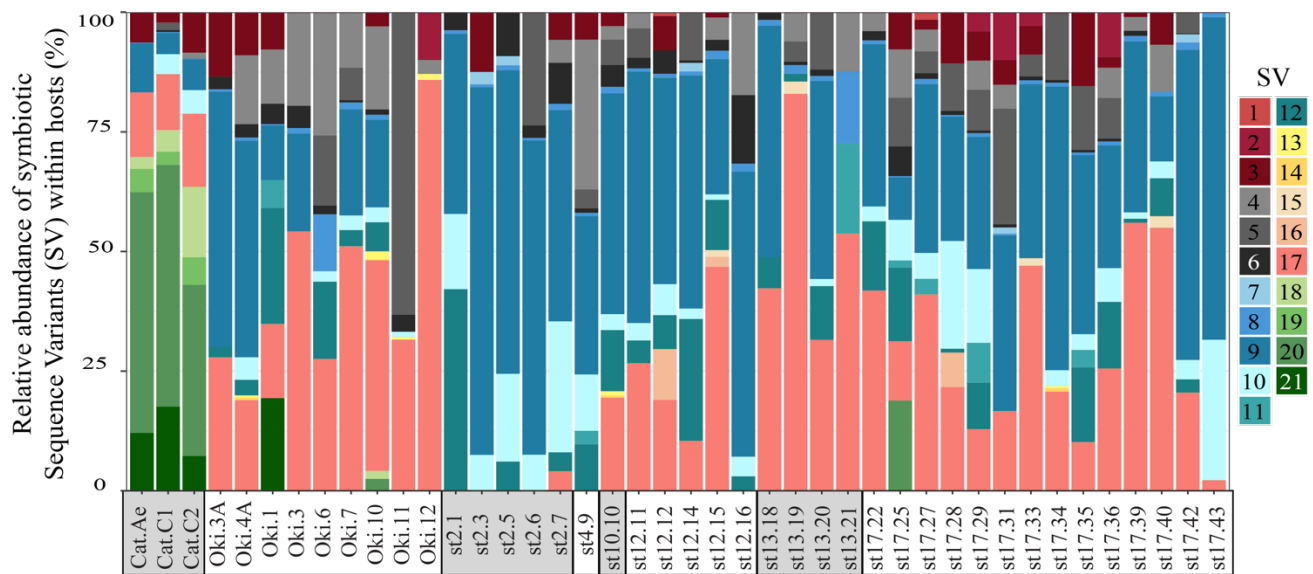589   Lancelot. 2005. "Phaeocystis Blooms in the Global Ocean and Their Controlling Mechanisms:

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631 **11 Figures**

632
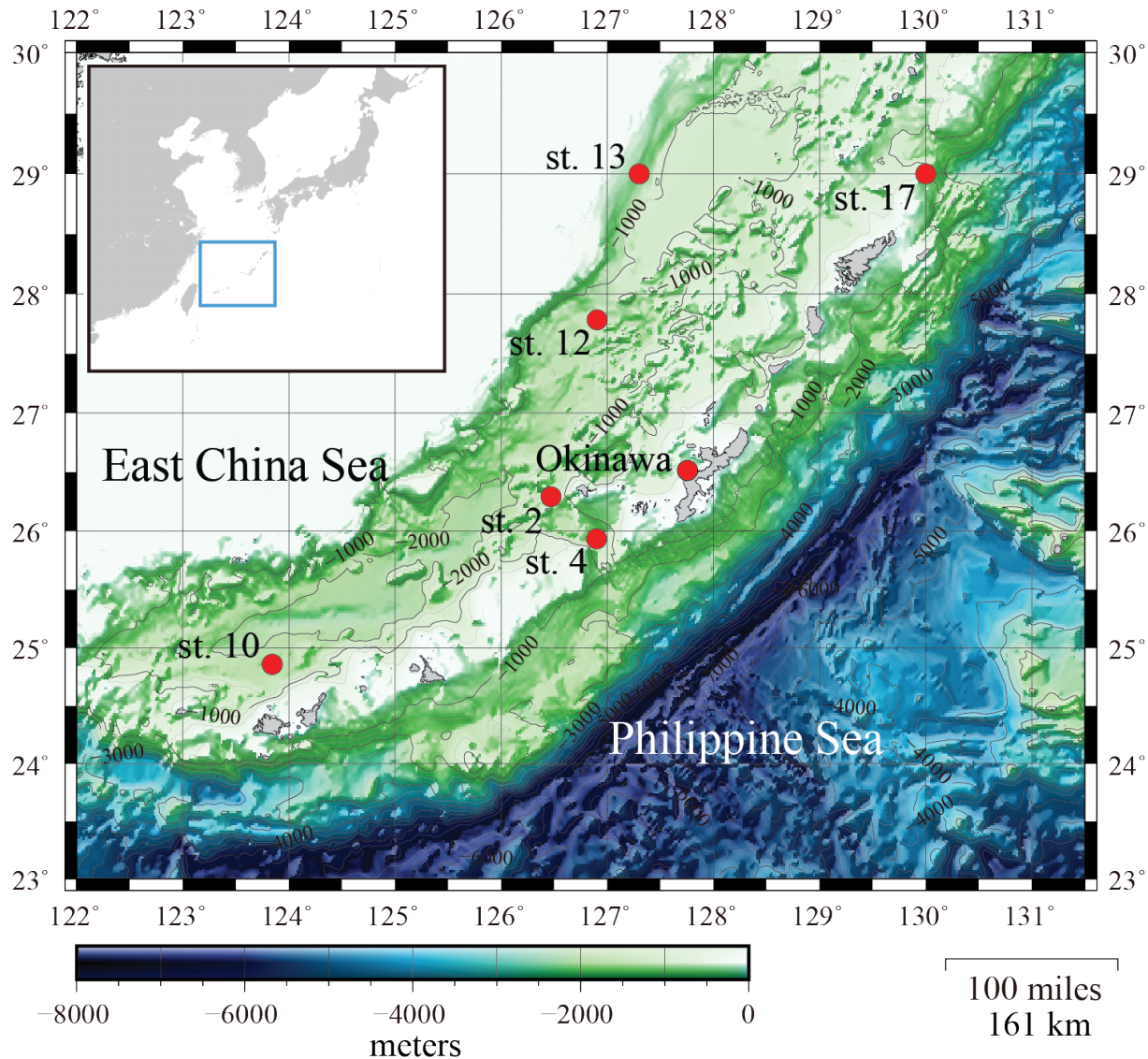
Figure 1. Acantharea and seawater samples were collected along the Ryukyu Archipelago, spanning 1,000 km in the East China Sea (ECS). Stations 2, 4, 10, 12, 13, and 17 were sampled during the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) MR17-03C cruise to the ECS aboard the R/V *Mirai* in May and June 2017. Samples were collected from the Okinawa Island (Okinawa, Japan) sampling site in April, May, and December 2017. Additional samples were collected near the University of Southern California's Wrigley Institute for Environmental Studies on Catalina Island (California, U.S.A., GPS: 33°26'59.12"N 118°29'17.17"W) in May 2017. Bathymetry map is courtesy of JAMSTEC.

15

Figure 2. Individual acantharian samples contain multiple Haptophyte Sequence Variants (SVs). The stacked bar graph represents the relative abundance of 21 Haptophyte SVs that were observed in individual acantharian samples. Each bar represents a single acantharian host and is labeled by collection location (Cat: Catalina Island, Oki: Okinawa Island, st#: ECS cruise station number) and sample ID. Individual acantharians contained 4-12 symbiotic SVs (mean = 8, SD = 2). The figure was created with R package ggplot2.

16

Figure 3. Symbiotic SVs from individual acantharians belong to Haptophyte genera *Phaeocystis* and *Chrysochromulina*. Phylogenetic tree showing the taxonomy of the 5 Haptophyte orders including the 21 symbiotic SVs identified in this study. The symbiotic SVs are highlighted to match the key in Figures 2 and 7. *Phaeocystis* clades are color coded and include the Phaeo2 clade, which is an uncultured clade identified from symbiotic sequences from acantharians collected near Okinawa (Decelle et al. 2012). The tree was built with MrBayes v3.2.7 using a MUSCLE v3.8.31 alignment of the 21 SVs and reference sequences downloaded from GenBank. *Pavlova gyrans* was designated as the outgroup. Values associated with nodes are posterior probabilities as a percent after 100,000 generations (average SD of split frequencies = 0.019). The scale bar indicates 0.02 changes expected per site.

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Acantharians maintain diverse intra-cellular symbiont communities
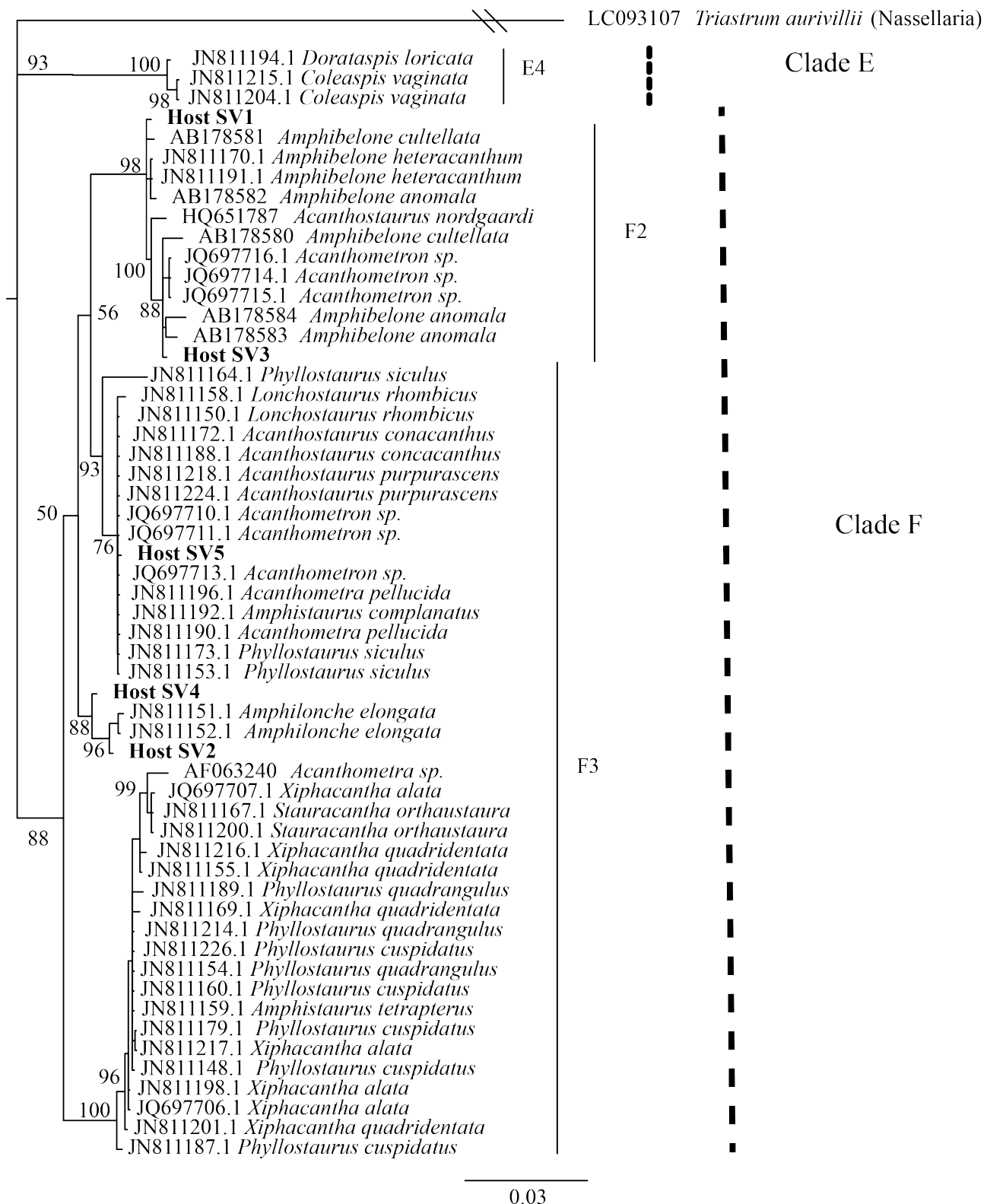
660
661 **Figure 4. Host SVs belong to 3 genera of clade F Acantharea.** Phylogenetic tree including
662 acantharian host SVs and acantharian reference sequences. The tree was built with MrBayes v3.2.7
663 using a MUSCLE v3.8.31 alignment of the 5 host SVs and reference sequences downloaded from
664 GenBank. The nasellarian radiolarian *Triastrum aurivillii* served as the outgroup. Values associated
665 with nodes are posterior probabilities as a percent after 40,000 generations (average SD of split
666 frequencies = 0.01). The scale bar indicates 0.03 changes expected per site.

18

Figure 5. Host collection location has larger effect than host type on the intra-host symbiont community. PCoA plot from Bray-Curtis distances between acantharian symbiont community compositions (n = 42). Point shape corresponds to host sequence variant (SV) and point color corresponds to the collection location of the host acantharians. The symbiont communities associated with acantharians collected near Catalina Island and from ECS station 2 form clusters while communities from the other locations do not cluster separately. A PERMANOVA by location (excluding st. 4 and st. 10 due to insufficient sample size) performed on the Bray-Curtis distances between samples confirmed that location has a significant effect on symbiont community (p = 0.001, R^2 = 0.47 after 999 permutations). Host SV also had a significant effect (p = 0.009, R^2 = 0.19), but only SV 5 was significantly different in pairwise comparisons. Analyses were performed with R packages phyloseq and vegan and plot was rendered with R package ggplot2.

Figure 6. Biological replicates of environmental microbial eukaryote communities in each location are more similar to each other than replicates from other locations. PCoA plot from Bray-Curtis distances between ECS cruise station samples calculated from entire microbial eukaryote communities (> 0.2 μm, < 10.0 μm) in environmental samples collected from ECS cruise stations in May and June 2017. PCoA was performed with the R package phyloseq and plotted with ggplot2.
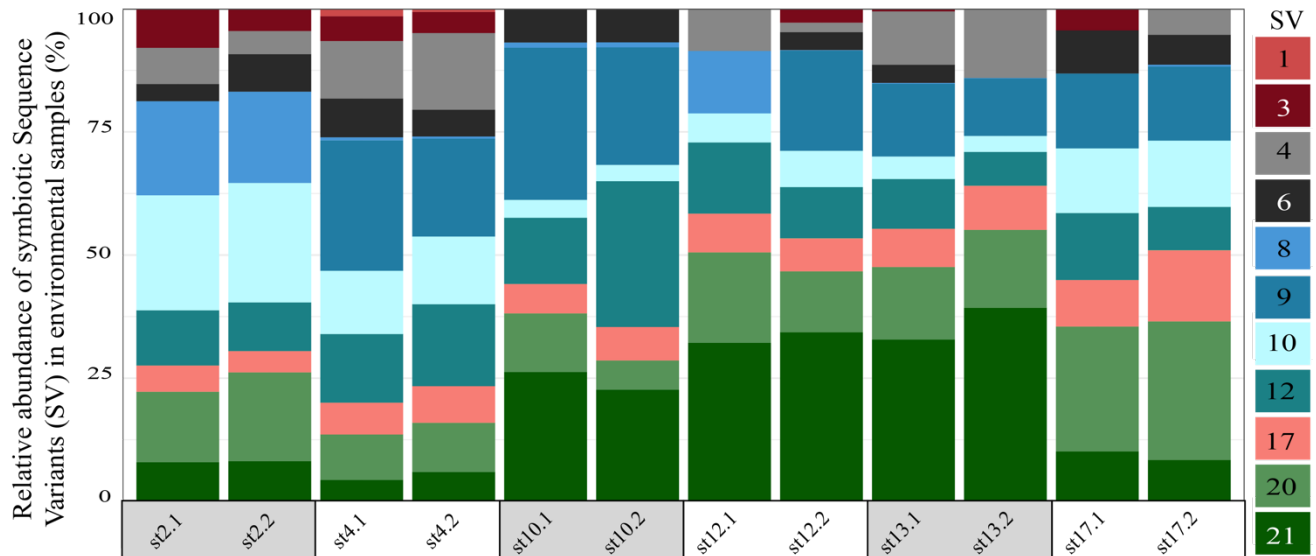
Figure 7. Relative abundances of symbiotic Haptophyte SVs in East China Sea (ECS) environmental samples are different than in ECS acantharian samples. The stacked bar graph displays the relative abundances of symbiotic SVs identified from acantharian samples that were also found in replicate environmental samples from each ECS cruise station. Symbiotic SVs are colored by clade: green is *Chrysochromulina*, blue is *Phaeocystis* clade Phaeo2, pink is *Phaeocystis cordata*, grey/black is *Phaeocystis jahnii*, and purple is *Phaeocystis*, but not placed with known clades. Only 11 of the symbiotic SVs were also found in environmental samples. *Chrysochromulina* SVs are present at all ECS cruise stations despite only being found in 1 acantharian sample from an ECS cruise station. The figure was created with R package ggplot2.
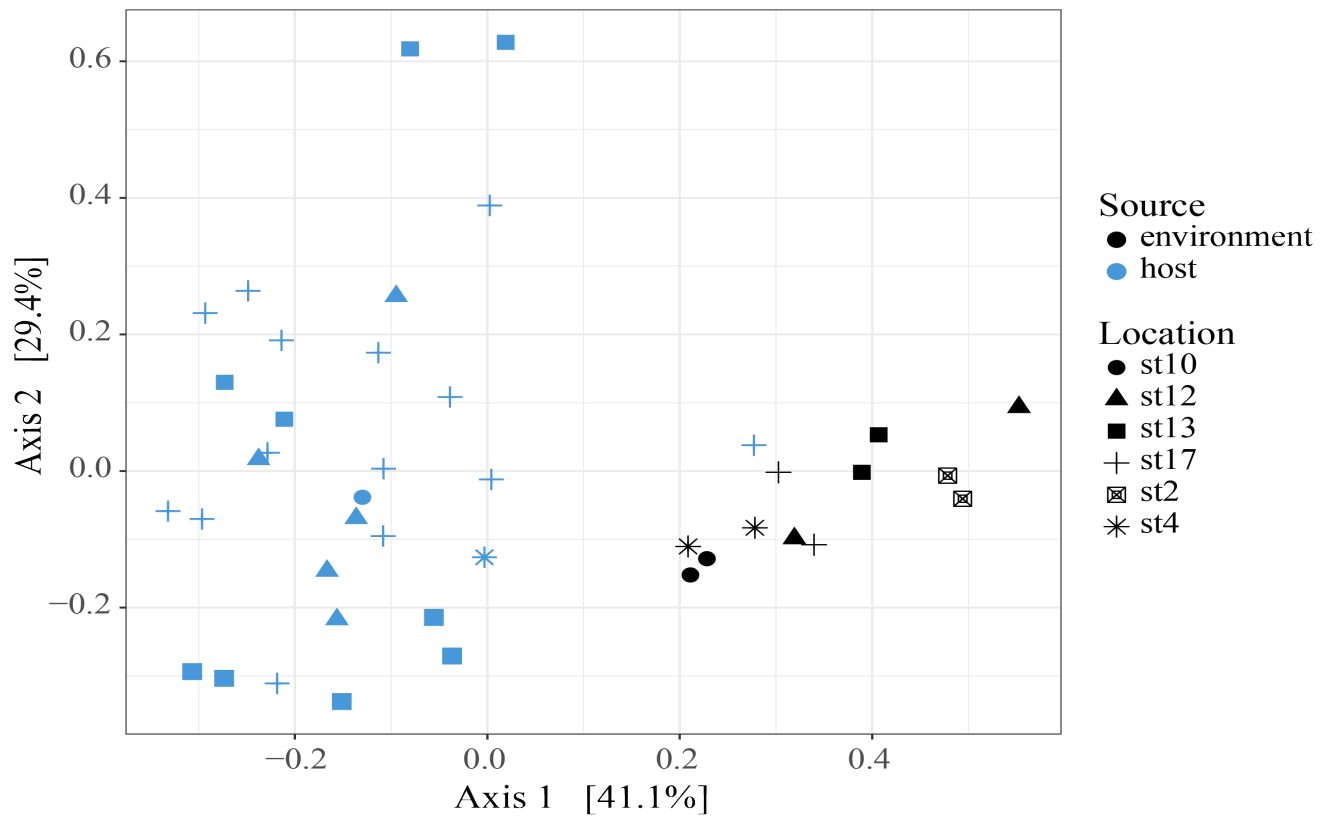
707
Figure 8. East China Sea (ECS) intra-host and free-living symbiont communities form distinct clusters in a Principal Coordinate Analysis (PCoA) plot. PCoA plot from Bray-Curtis distances between ECS environmental free-living and the host-associated symbiotic community compositions, including all symbiotic SVs identified in this study. The two sample types cluster separately and the difference in community composition is statistically significant (PERMANOVA, p = 0.001, R^2 = 0.326, 999 permutations). Analyses were performed with R packages phyloseq and vegan and plot was rendered with R package ggplot2.
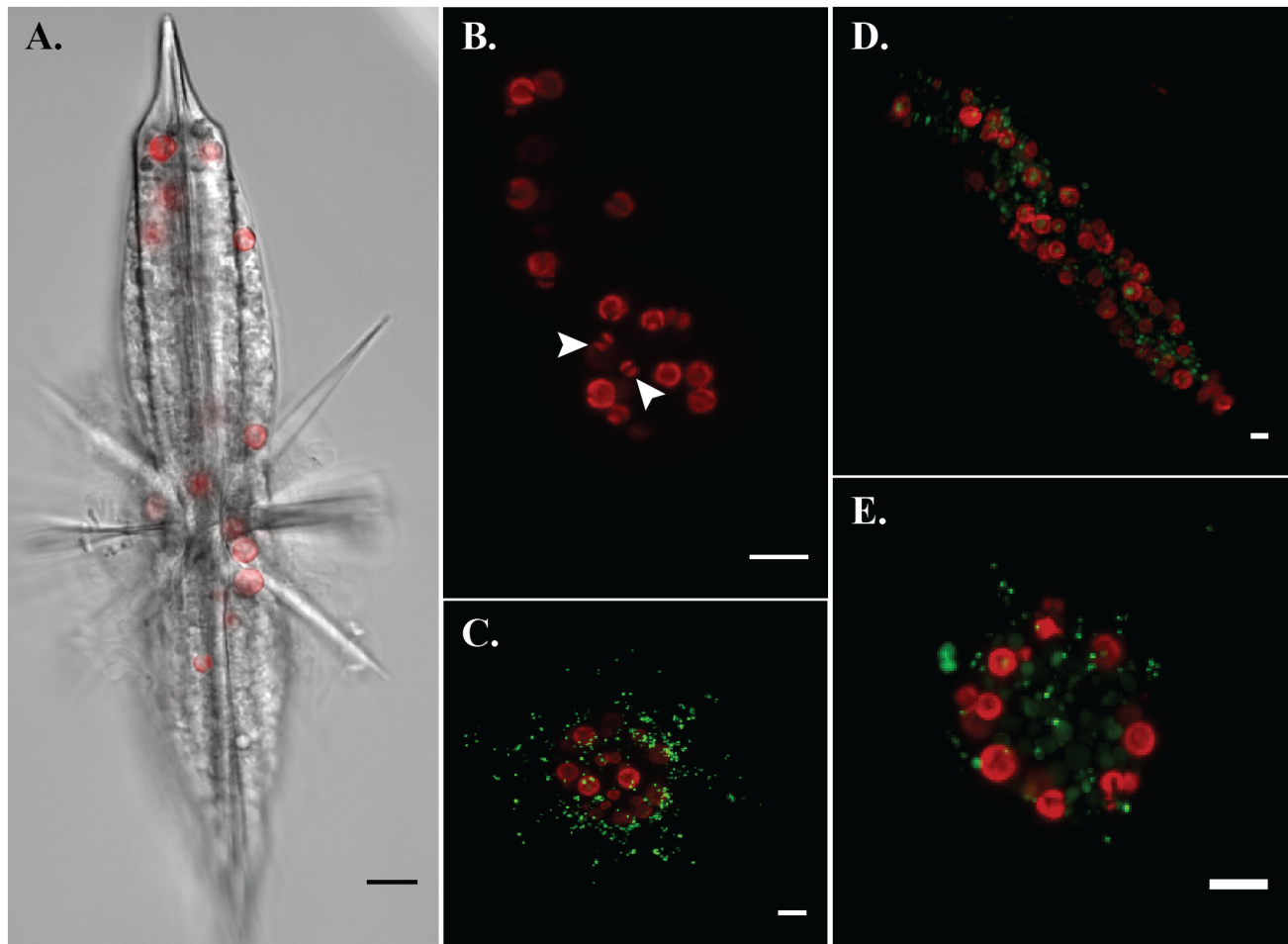
Figure 9. Fluorescent confocal microscopy of acantharians and their symbionts demonstrates more individual symbionts inside individual hosts than observed SVs and demonstrates that symbionts are not enclosed in digestive phagolysosomes. A. Single optical slice displaying autofluorescence of symbiont chlorophyll (red) and halogen light imaging of an entire host, sample Oki.3A. **B.** Maximum projection of a z-stack spanning the entire host sample Oki.4A, which contains 23 visible symbionts and 9 symbiotic sequence variants. Red fluorescence is symbiotic chlorophyll autofluorescence. Arrows indicate symbionts presenting the free-living phenotype: smaller cell diameter and two elongate, parietal chloroplasts. **C–E.** Maximum projections of z-stacks spanning entire hosts collected near Okinawa in December 2017. Red fluorescence is symbiotic chlorophyll autofluorescence. Green fluorescent staining is LysoTracker Green, which selectively binds to low-pH digestive organelles, including lysosomes and phagolysosomes. Symbionts are not held in phagolysosomes and lysosomes are not concentrated around symbionts, indicating that symbionts are not actively being digested. Scale bars are 10 μm in all panels.