

## *Exaiptasia diaphana* from the Great Barrier Reef: a valuable resource for coral symbiosis research

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## 23 Symbiodiniaceae

24 Abstract

25 The sea anemone, *Exaiptasia diaphana*, commonly known as *Exaiptasia pallida* or *Aiptasia*  
26 *pallida*, has become increasingly popular as a model for cnidarian-microbiome symbiosis  
27 studies due to its relatively rapid growth, ability to reproduce sexually and asexually, and  
28 symbiosis with diverse prokaryotes and the same microalgal symbionts (family  
29 Symbiodiniaceae) as its coral relatives. Clonal *E. diaphana* strains from Hawaii, the Atlantic  
30 Ocean, and Red Sea are now established for use in research. Here, we introduce Great Barrier  
31 Reef (GBR)-sourced *E. diaphana* strains as additions to the model repertoire. Sequencing of  
32 the 18S rRNA gene confirmed the anemones to be *E. diaphana* while genome-wide single  
33 nucleotide polymorphism analysis revealed four distinct genotypes. Based on *Exaiptasia*-  
34 specific inter-simple sequence repeat (ISSR)-derived sequence characterized amplified region  
35 (SCAR) marker and gene loci data, these four *E. diaphana* genotypes are distributed across  
36 several divergent phylogenetic clades with no clear phylogeographical pattern. The GBR *E.*  
37 *diaphana* genotypes comprised three females and one male, which all host *Breviolum*  
38 *minutum* as their homologous Symbiodiniaceae endosymbiont. When acclimating to an  
39 increase in light levels from 12 to 28  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , the genotypes exhibited  
40 significant variation in maximum quantum yield of Symbiodiniaceae photosystem II and  
41 Symbiodiniaceae cell density. The comparatively high levels of physiological and genetic  
42 variability among GBR anemone genotypes makes these animals representative of global *E.*  
43 *diaphana* diversity and thus excellent model organisms. The addition of these GBR strains to  
44 the worldwide *E. diaphana* collection will contribute to cnidarian symbiosis research,  
45 particularly in relation to the climate resilience of coral reefs.

46    1 Introduction

47    1.1 Coral Reefs

48    The Great Barrier Reef (GBR) contains abundant and diverse biota, including more than 300  
49    species of stony corals (Fabricius et al. 2005), making it one of the most unique and complex  
50    ecosystems in the world. In addition to its tremendous environmental importance, the GBR's  
51    social and economic value is estimated at \$A56 billion, supporting 64,000 jobs and injecting  
52    \$A6.4 billion into the Australian economy annually (O'Mahony et al. 2017).

53    Coral reef waters are typically oligotrophic, but stony corals thrive in this environment and  
54    secrete the calcium carbonate skeletons that create the physical structure of the reef. Corals  
55    achieve this through their symbiosis with single-celled algae of the family Symbiodiniaceae  
56    that reside within the animal cells and provide the host with most of its energy (Muscatine  
57    and Porter 1977). Additional support is provided by communities of prokaryotes, and  
58    possibly by other microbes such as viruses, fungi and endolithic algae living in close  
59    association with coral. This entity, comprising the host and its microbial partners, is termed  
60    the holobiont (Rohwer et al. 2002). During periods of extreme thermal stress, the coral-  
61    Symbiodiniaceae relationship breaks down. Stress-induced cellular damage creates a state of  
62    physiological dysfunction, which leads to separation of the partners and, potentially, death of  
63    the coral animal. This process, 'coral bleaching', is a substantial contributor to coral cover  
64    loss globally (Baird et al. 2009; De'ath et al. 2012; Eakin et al. 2016; Hughes et al. 2017;  
65    Hughes et al. 2018).

66    1.2 *Exaiptasia diaphana*

67    Model systems are widely used to explore research questions where experimentation on the  
68    system of interest has limited feasibility or ethical constraints. Established model systems,  
69    such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have played crucial roles in  
70    the progress of understanding organismal function and evolution in the past 50 years (Davis  
71    2004). The coral model *Exaiptasia diaphana* was formally proposed by Weis et al. (2008) as  
72    a useful system to study cnidarian endosymbiosis and has since achieved widespread and  
73    successful use.

74    *E. diaphana* is a small sea anemone ( $\leq 60$  mm long) found globally within temperate and  
75    tropical marine shallow-water environments (Grajales and Rodriguez 2014). Originally  
76    positioned taxonomically within the genus *Aiptasia*, *E. diaphana* and twelve other *Aiptasia*

77 species were combined into a new genus, *Exaiptasia* (Grajales and Rodriguez 2014).  
78 Although *Exaiptasia pallida* was proposed as the taxonomic name for the twelve  
79 synonymized species, the International Commission on Zoological Nomenclature (ICZN)  
80 ruled against this because the species epithet *diaphana* (Rapp 1829) predated *pallida* (Verrill  
81 1864) and therefore had precedence according to the Principle of Priority (ICZN 2017).

82 *E. diaphana* was first used to study cellular regeneration (Blanquet and Lenhoff 1966), with  
83 its systematic use in the study of cnidarian-algal symbioses dating back to 1976 when the  
84 regulation of *in hospite* Symbiodiniaceae density was explored (Steele 1976). Since then,  
85 studies using *E. diaphana* have focused on the onset, maintenance, and disruption of  
86 symbiosis with Symbiodiniaceae (Belda-Baillie et al. 2002; Fransolet et al. 2014; Bucher et  
87 al. 2016; Hillyer et al. 2017; Cziesielski et al. 2018). *E. diaphana* has also been used in  
88 studies of toxicity (Duckworth et al. 2017; Howe et al. 2017), ocean acidification (Hoadley et  
89 al. 2015), disease and probiotics (Alagely et al. 2011), and cnidarian development (Chen et  
90 al. 2008; Grawunder et al. 2015; Carlisle et al. 2017). Key differences between corals and *E.*  
91 *diaphana* are the absence of a calcium carbonate skeleton, the constant production of asexual  
92 propagates, and the greater ability to survive bleaching events in the latter. These features  
93 allow researchers to use *E. diaphana* to investigate cellular processes that would otherwise be  
94 difficult with corals, such as those that require survival post-bleaching to track re-  
95 establishment of eukaryotic and prokaryotic symbionts. Further, adult anemones can be fully  
96 bleached without eliciting mortality, which is often difficult for corals, thus providing a  
97 system for algal reinfection studies independent of sexual reproduction and aposymbiotic  
98 larvae.

99 Three strains of *E. diaphana* currently dominate the research field as models for coral  
100 research. H2 (female) was originally collected from Coconut Island, Hawaii, USA (Xiang et  
101 al. 2013), CC7 (male) from the South Atlantic Ocean off North Carolina, USA (Sunagawa et  
102 al. 2009) and RS (female, pers.comm., but see (Schlesinger et al. 2010)) collected from the  
103 Red Sea at Al Lith, Saudi Arabia (Cziesielski et al. 2018). The majority of *E. diaphana*  
104 resources have been developed from the clonal line, CC7, including reproductive studies  
105 (Grawunder et al. 2015), transcriptomes (Sunagawa et al. 2009; Lehnert et al. 2012; Lehnert  
106 et al. 2014) and an anemone genome sequence (Baumgarten et al. 2015). Prior work  
107 evaluating multiple *E. diaphana* strains has shown variation in sexual reproduction  
108 (Grawunder et al. 2015), Symbiodiniaceae specificity (Thornhill et al. 2013; Grawunder et al.

109 2015), and resistance to thermal stress (Bellis and Denver 2017; Cziesielski et al. 2018).  
110 However, Australian contributions to these efforts have been hampered as researchers have  
111 not had access to the established strains as import permits for alien species are difficult to  
112 secure.

113 The aim of the present work is to add GBR representatives to the existing suite of *E.*  
114 *diaphana* model strains. We describe the establishment and maintenance of four genotypes,  
115 including their provenance, taxonomy, maintenance, histology, physiology, and  
116 Symbiodiniaceae symbiont type. The results highlight characteristics that make GBR-sourced  
117 *E. diaphana* a valuable extension to this coral model, such as the physiological and genetic  
118 variability between genotypes, which is more representative of a global population. This  
119 foundation information will be useful to international researchers interested in using the  
120 GBR-sourced animals in their research and will give Australian researchers access to this  
121 valuable model.

122 2 Methods

123 2.1 Anemone acquisition and husbandry

124 Several pieces of coral rubble bearing anemones were obtained from holding tanks in the  
125 National Sea Simulator (SeaSim) at the Australian Institute of Marine Science and sent to  
126 Swinburne University of Technology, Melbourne (SUT) in late 2014. Additional anemones  
127 from the SeaSim were sent to the Marine Microbial Symbiont Facility (MMSF) at the  
128 University of Melbourne (UoM) in early 2016. Most material in the SeaSim tanks originates  
129 from the central GBR; therefore, this is the likely origin of the anemones. The SUT and UoM  
130 populations were consolidated at MMSF in early 2017 where they were segregated according  
131 to single polynucleotide polymorphism (SNP) analysis groupings (see below). Anemones  
132 were grown in 4 L polycarbonate tanks in reverse osmosis (RO) water reconstituted Red Sea  
133 Salt™ (RSS) at ~34 parts per thousand (ppt), and incubated without aeration or water flow at  
134 26°C under lighting of 12-20 µmol photons m<sup>-2</sup> s<sup>-1</sup> (light emitting diode - LED white light  
135 array) on a 12h:12h light:dark cycle in a walk-in incubator. Anemones were fed *ad libitum*  
136 with freshly hatched *Artemia salina* (brine shrimp, Salt Creek, UT, USA) nauplii twice  
137 weekly. Tanks were cleaned each week after feeding by loosening algal debris with water  
138 pressure applied through disposable plastic pipettes, removing algal biomass, and complete  
139 water changes. When cleaning, ~25% of the anemones were cut into 2-6 fragments to  
140 promote population expansion through regeneration of the tissue fragments into whole  
141 anemones. Every third week, all anemones were transferred to clean tanks.

142 2.2 Anemone identity and genotyping

143 Anemone identity was determined by Sanger sequencing of the 18S rRNA gene. Genomic  
144 DNA (gDNA) was extracted from six whole anemones following the protocol described by  
145 Wilson et al. (2002), modified with a 15 min incubation in 180 µM lysozyme, and 30 s bead  
146 beating at 30 Hz (Qiagen Tissue-Lyser II) with 100 mg of sterile glass beads (Sigma G8772).  
147 The 18S rRNA genes were PCR amplified from all anemone samples using external  
148 Actiniaria-specific 18S rRNA gene primers 18S\_NA, 5'-  
149 TAAGCACTTGTCTGTGAAACTGCGA-3' and 18S\_NB, 5'-TAAGCACTTGT  
150 CTGTGAAACTGCGA-3' (Grajales and Rodriguez 2016) with 0.5 U 2x Mango Mix  
151 (Bioline), 2 µL of DNA template, 0.2 µM of each primer, and nuclease-free water up to 25  
152 µL. PCR conditions consisted of initial denaturation at 94°C for 5 min, 35 cycles of 94°C for  
153 45 s, 55°C for 45 s, and 72°C for 45 s followed by a final extension at 72°C for 5 min. PCR

154 products were purified with an ISOLATE II PCR and Gel Kit (Bioline, BIO-52059)  
155 according to the manufacturers guidelines, and supplied to the Australian Genome Research  
156 Facility (AGRF) for Sanger sequencing with the external primers and four internal primers:  
157 18S\_NL, 5'-AACAGCCCAGTCAGTAACACG-3', 18S\_NC 5'-  
158 AATAACAATACAGGGCTTTCTAAGTC-3', 18S\_NY 5'-  
159 GCCTTCCTGACTTGTTGAA-3', and 18S\_NO 5'-  
160 AGTGTATTGGATGACCTCTTGGC-3'. The raw 18S reads were aligned in Geneious (v  
161 10.0.4) (Kearse et al. 2012) to produce the near-complete 18S rRNA gene sequence, which  
162 was evaluated by BLASTn (Altschul et al. 1990) to identify the anemones.

163 For genotyping, DNA was extracted as described above from 23 whole anemones or their  
164 tentacles and sent to Diversity Arrays Technology Pty Ltd (Canberra, Australia) for DArT  
165 next-generation sequencing (DArTseq). DArTseq combines complexity reduction and next  
166 generation sequencing to generate genomic data with a balance of genome-wide  
167 representation and coverage (Cruz et al. 2013). Complexity reduction was achieved by using  
168 restriction endonucleases to target low-copy DNA regions. These regions were then  
169 sequenced using Illumina HiSeq2500 (Illumina, USA) with an average read depth exceeding  
170 20x. The data were processed by DArT Pty Ltd to remove poor quality sequences and to  
171 ensure reliable assignment of sequences to samples. DArTsoft14 was then used to identify  
172 SNPs at each locus as homozygous reference, homozygous alternate or heterozygous for each  
173 individual (Melville et al. 2017). Monomorphic loci, loci with <100% reproducibility or  
174 missing values were removed in the R package, dartR, to improve the quality of and reduce  
175 linkage within the dataset; this reduced the dataset from 8288 loci to 1743 loci (R Core Team  
176 2013; Gruber et al. 2017).

177 Euclidean distances between individual anemones, based on differences in the allele  
178 frequencies at each of the SNP loci, were calculated from the reduced SNP dataset in dartR,  
179 then viewed as a histogram and printed into a matrix in RStudio. Although the genetic distance  
180 between individuals of the same genotype should be zero, small differences may occur due to  
181 sequencing errors and somatic mutations. The genetic distance between individuals of different  
182 genotypes will be larger than that within individuals. Therefore, the genetic distances among  
183 individuals from several genotypes should form a bi- or multi-modal distribution; one peak  
184 with a relatively small mean represents genetic distances between pairs of individuals of the  
185 same genotype, another represents inter-genotypic distances with a larger mean. The inter-

186 genotypic distribution can be multi-modal because different pairs of genotypes can differ by  
187 different amounts. Note that two samples from the same individual were genotyped to  
188 determine methodological error rates and verify the baseline for clonality. A principal  
189 coordinates analysis was performed and plotted in dartR to visualize the genotype assignments.

190 To compare the phylogenetic relationship of the GBR-sourced anemones with the previously  
191 described clonal lines, we used a set of four *Exaiptasia*-specific inter-simple sequence repeat  
192 (ISSR)-derived sequence characterized amplified region (SCAR) markers developed by  
193 Thornhill et al. (2013) and an additional six *Exaiptasia*-specific gene loci (Bellis and Denver  
194 2017). gDNA was extracted from five animals of each genotype, as described above, and  
195 amplified with each of the four SCAR marker (3, 4, 5, and 7) and gene primer pairs  
196 (Thornhill et al. 2013; Bellis and Denver 2017). PCR solutions for each marker contained  
197 0.5U MyTaq HS Mix polymerase (Bioline), 1 µL of DNA template, 0.4 µM of each primer,  
198 and nuclease-free water up to 25 µL. Thermocycling consisted of an initial denaturation at  
199 94°C for 1.5 min, 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72 °C for 1.5 min  
200 followed by a final extension at 72°C for 5 min. Amplified products were purified and  
201 sequenced in forward and reverse directions at AGRF. Sequences were aligned and edited in  
202 Geneious version 2019.2. Substantial non-specific binding of the SCAR marker 7 primers  
203 generated unusable sequence data. Further, the forward and reverse sequences from two  
204 anemone genotypes that were heterozygous for two or more indels at different points in the  
205 sequences of SCAR markers 3 (anemone AIMS1) and 4 (anemones AIMS2-4) could not be  
206 aligned and this prohibited the inclusion of those loci from analyses. Therefore, only  
207 sequence data from SCAR marker 5 was used for phylogenetic analyses. For the six  
208 *Exaiptasia*-specific gene loci, only AIPGENE19577, Atrophin-1-interacting protein 1 (AIP1)  
209 contained heterozygous indels or non-specific binding of the forward primer.

210 For each genotype, the SCAR marker 5 sequences and each of the six *Exaiptasia*-specific  
211 gene loci of the clonal replicates were aligned and a consensus sequence was generated. For  
212 the SCAR marker, the consensus sequences were aligned with twelve reference sequences  
213 from Thornhill et al. (2013) and three experimental anemone sequences (Grawunder et al.  
214 2015), while the *Exaiptasia*-specific gene sequences were aligned with five reference  
215 sequences from Bellis and Denver (2017). Each alignment was used to create a phylogenetic  
216 tree using the Maximum Likelihood method and General Time Reversible model (Nei and  
217 Kumar 2000) in MEGA X (Kumar et al. 2018). Topology, branch length, and substitution

218 rate were optimized, and branch support was estimated by bootstrap analysis of 1000  
219 iterations.

220 2.3 GBR anemone gender determination

221 Over a period of two months, several anemone individuals from each genotype were reared to  
222 a pedal disk diameter of ~7 mm. One animal per genotype was anesthetized in a 1:1 solution  
223 of 0.37 M magnesium chloride and filter-sterilized RSS (fRSS) for 30 min, dissected and  
224 viewed under a stereo microscope (Leica MZ8) to confirm the presence of gonads. When  
225 gonads were observed, one animal per genotype was anesthetized as described above, fixed  
226 in 4% paraformaldehyde in 1x PBS (pH 7.4) overnight, washed three times in 1x PBS and  
227 processed by the Biomedical Histology Facility of The University of Melbourne. Samples  
228 were embedded in paraffin and 7 µm transverse sections were cut (see supplemental  
229 information Online Resource 1 for histological processing). Slides were stained with  
230 hematoxylin and eosin (H&E) and viewed under a compound microscope (Leica DM6000B).

231 2.4 Symbiodiniaceae

232 The ITS2 region of Symbiodiniaceae gDNA was analyzed by metabarcoding to determine the  
233 Symbiodiniaceae species associated with the GBR-sourced anemones. gDNA extracted from  
234 three animals of each genotype, as described above, was amplified in triplicate with the ITS-  
235 Dino (forward; 5'-

236 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGAATTGCAGAACTCCGTG-  
237 3') (Pochon et al. 2001) and its2rev2 (reverse; 5'-

238 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTCCGCTTACTTATATGCTT-  
239 3') (Stat et al. 2009) primers modified with Illumina adapters. PCRs included 0.5 U MyTaq  
240 HS Mix polymerase (Bioline), 1 µL of DNA template, 0.25 µM of each primer, and nuclease-  
241 free water up to 20 µL. Thermocycling consisted of an initial denaturation at 95.0 °C for 3  
242 min, 35 cycles at 95.0 °C, 55.0 °C and 72.0 °C for 15 sec each, and 1 cycle at 72 °C for 3  
243 min. Library preparation on pooled triplicates and Illumina MiSeq sequencing (2x250 bp)  
244 was performed by the Ramaciotti Centre for Genomics at the University of New South  
245 Wales, Sydney. Raw, demultiplexed MiSeq read-pairs were joined in QIIME2 v2018.4.0  
246 (Bolyen et al. 2018). Denoising, chimera checking, and trimming was performed in DADA2  
247 (Callahan et al. 2016). The remaining sequences were clustered into operational taxonomic  
248 units (OTUs) at 99% sequence similarity using closed-reference OTU picking in vsearch

249 (Rognes et al. 2016). A taxonomic database adapted from Arif et al. (2014) was used to seed  
250 the OTU clusters and for taxonomic classification.

251 2.5 Anemone and Symbiodiniaceae physiological properties

252 Physiological assessments were performed on anemones maintained in a healthy, unbleached  
253 state for over two years. Three of the four anemone genotypes named AIMS2, AIMS3, and  
254 AIMS4, with oral disk diameters of 3-4 mm were transferred from the original holding tanks  
255 in the walk-in incubator and randomly distributed between three replicate (by genotype) 250  
256 mL glass culture containers containing RSS. Anemones of this size were chosen as they were  
257 considered sexually immature (Muller-Parker et al. 1990; Grawunder et al. 2015) and  
258 therefore variability in sexual development or gonadal reserves were unlikely to influence the  
259 results. AIMS1 was excluded from long-term assessment as it had poor survival at densities  
260 required for the analyses.

261 Glass culture containers were transferred from the walk-in incubator to experimental growth  
262 chambers (Taiwan HiPoint Corporation model 740FHC) fitted with red, white, and infrared  
263 LED lights. Initial light levels were set at  $12 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (HiPoint HR-350 LED  
264 meter) to correspond with the walk-in incubator conditions and ramped up to  $28 \mu\text{mol}$   
265  $\text{photons m}^{-2} \text{s}^{-1}$  over a period of 72 h on a 12 h:12 h light:dark cycle to approximate  
266 experimental conditions reported in *E. diaphana* literature (Online Resource 2; Fransolet et  
267 al. 2014; Hoadley and Warner 2017). While the commonly used *E. diaphana* strains (CC7,  
268 H2, RS) are often maintained at  $>50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (pers. comm.), the GBR strains  
269 appeared healthier, with extended bodies and open tentacles, at lower light intensities. The  
270 anemones were maintained in RSS, fed *A. salina* nauplii and cleaned as described above. The  
271 pH, salinity and temperature of the water and applied light levels were monitored thrice  
272 weekly and were stable over time:  $8.14 \pm 0.02$ ,  $35.0 \pm 0.04$  ppt,  $25.77 \pm 0.07^\circ\text{C}$  and  $28.08 \pm 0.12$   
273  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , respectively. Culture containers were placed on a single incubator shelf  
274 and randomly rearranged after each clean to minimize confounding by position.

275 The maximum dark adapted quantum yield ( $F_v/F_m$ ) of photosystem II (PSII) of *in hospite*  
276 Symbiodiniaceae provides an indication of photosynthetic performance and is useful as a bio-  
277 monitoring tool (Howe et al. 2017).  $F_v/F_m$  is measured as the difference in fluorescence  
278 produced by PSII reaction centers when either saturated by intense light ( $F_m$ ) or in the  
279 absence of light ( $F_o$ ), versus  $F_m$  (i.e.  $(F_m - F_o)/F_m$ , or  $F_v/F_m$ ). Symbiodiniaceae  $F_v/F_m$  was

280 measured weekly over nine weeks for each culture container after 4 hr into the incubator light  
281 cycle and 30 mins dark adaption using imaging pulse amplitude modulated (iPAM)  
282 fluorometry (IMAG-MAX/L, Waltz, Germany). Settings for all measurements were:  
283 saturating pulse intensity 8, measuring light intensity 2 with frequency 1, actinic light  
284 intensity 3, damping 2, gain 2. Average Fv/Fm values for each dish were calculated from  
285 readings taken on three to five anemones (Online Resource 3).

286 Anemones from each culture container were individually homogenized in a sterile glass  
287 homogenizer in 1 mL fRSS, and 100  $\mu$ L of homogenate was collected and stored at -20 °C  
288 for total protein measurement. The remaining 900  $\mu$ L of homogenate was centrifuged at 5000  
289 g for 5 min at 4°C to pellet the Symbiodiniaceae while leaving the anemone cells in  
290 suspension. A volume of 100  $\mu$ L of supernatant was collected and stored at -20 °C for host  
291 protein measurement. The pelleted Symbiodiniaceae were twice washed with 500  $\mu$ L fRSS  
292 and centrifuged at 5000 g for 5 min at 4 °C, and the final pellet resuspended in 500  $\mu$ L fRSS  
293 and stored at -20 °C for Symbiodiniaceae counts. Triplicate cell counts ( $\text{cells mL}^{-1}$ ) were  
294 completed within 48 hrs of sample collection on a Countess II FL automated cell counter  
295 (Life Technologies) and normalized to host protein ( $\text{mg mL}^{-1}$ ). This process was repeated 15  
296 times over the course of nine weeks for a total of 45 replicates per genotype.

297 Samples for protein analysis stored at -20 °C (see above) were used within one month of  
298 collection to determine total and host protein ( $\text{mg mL}^{-1}$ ) by the Bradford assay (Bradford  
299 1976) against bovine serum albumin standards (Bio-Rad 500-0207). Readings were taken at  
300 595 nm (EnSpire microplate reader MLD2300).

301 Fv/Fm values and Symbiodiniaceae cell densities were assessed for genotype-specific  
302 responses. All variables were tested for normality and homoscedasticity prior to parametric  
303 analyses, which were completed in R (v 3.6.0). Genotypic responses of Fv/Fm were  
304 compared using a linear model in the R package nlme (Pinheiro et al. 2017) to evaluate  
305 independent and interaction relationships between the factors of genotype and time. F-  
306 statistics were obtained using the analysis of variance (ANOVA) function, nlme, and  
307 pairwise *post hoc* analyses were performed using the glht function in the R package  
308 multcomp (Hothorn et al. 2016) with Tukey's correction for multiple comparisons.  
309 Symbiodiniaceae cell densities were pooled across time by genotype and analyzed with a  
310 one-way ANOVA with a *post hoc* Tukey test to determine pairwise significance.

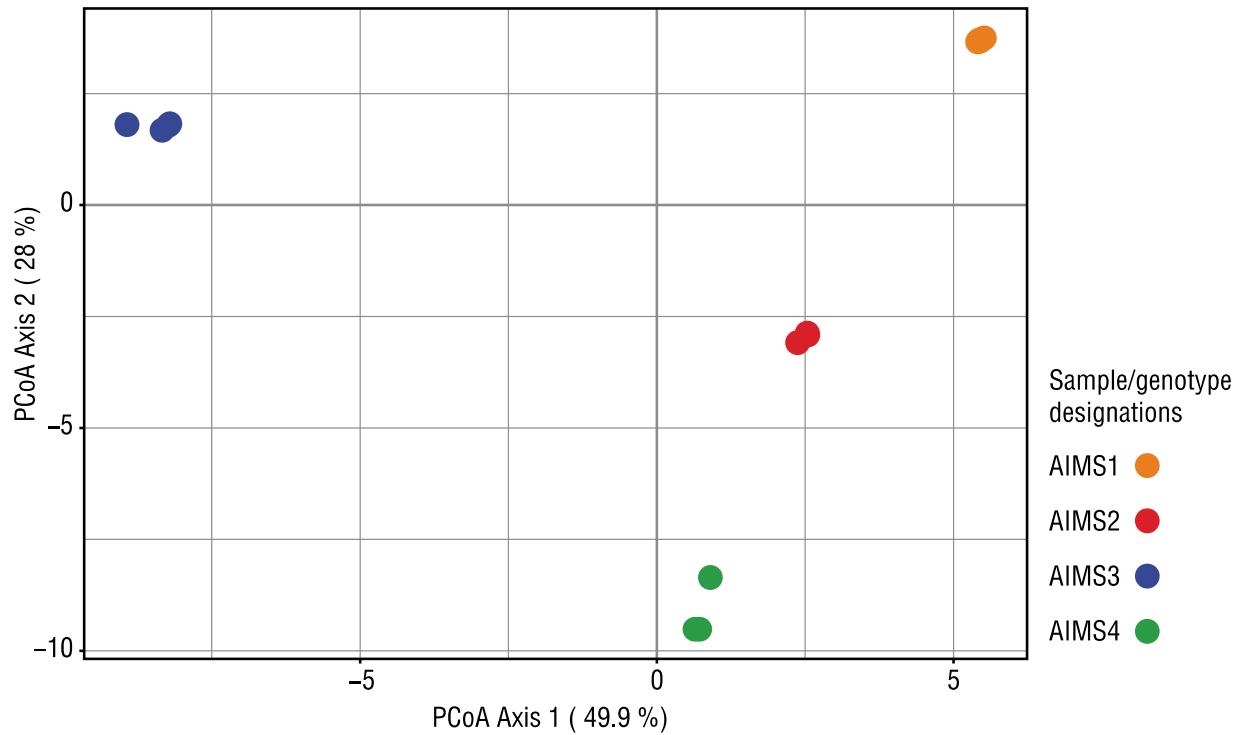
311 **Results and Discussion**

312 **3.1 Anemones**

313 The assembled 18S rRNA gene sequences of two genotypes (AIMS2 and AIMS4) covered  
314 1591 and 1594 bp, respectively, with two mismatches between the sequences. BLASTn  
315 against the NCBI database identified the samples as either *Aiptasia pulchella* or *Exaiptasia*  
316 *pallida*. *A. pulchella* has been synonymised with *E. pallida* (Grajales and Rodríguez, 2014).  
317 However, since *E. diaphana* has precedence (ICZN 2017), all samples were designated *E.*  
318 *diaphana*.

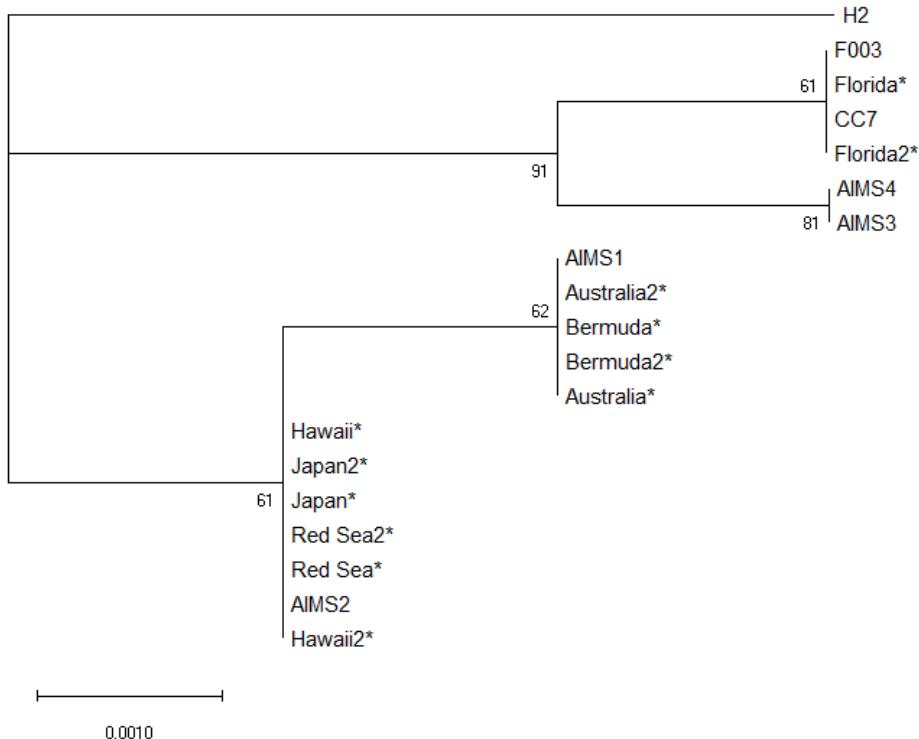
319 Four SNP genotypes of *E. diaphana*, which were of GBR-origin but AIMS-sourced, were  
320 identified and designated AIMS1, AIMS2, AIMS3 and AIMS4 (Fig. 1). The clonal  
321 distribution of Euclidean distances was identified (range of 0-9.61) and pairs of individuals  
322 with Euclidean distances within this distribution were inferred to have the same genotype  
323 (Online Resource 3-4). The Euclidean distance between the originally named Ed.11a and  
324 Ed.11b (replicates from the same individual; assigned AIMS3) is in the upper percentiles of  
325 the defined clonal distribution, confirming this cut-off is valid.

326 Using phylogenetic analysis with our data, previously described SCAR marker 5, and the  
327 *Exaiptasia*-specific gene sequence data (Thornhill et al. 2013; Grawunder et al. 2015; Bellis  
328 and Denver 2017), we placed the GBR anemones into a phylogeographical context. The  
329 alignment of SCAR5 sequences was 706 bp long and contained 19 variable nucleotide  
330 positions (Fig. 2), while the concatenated *Exaiptasia*-specific gene sequences were 3276 bp  
331 long with 92 variable nucleotide positions (Fig. 3).



332

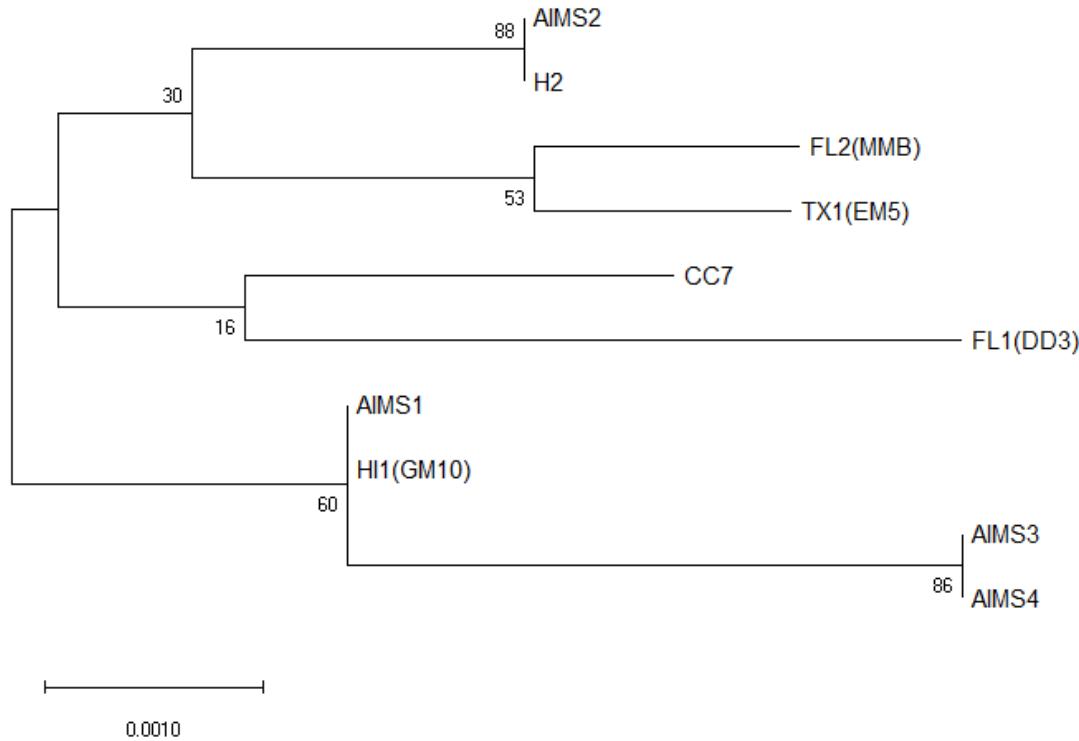
333 **Figure 1:** PCoA ordination of *Exaiphtasia diaphana* genotypes based on Euclidean genetic distance measurements  
334 of SNP data using allele frequencies within individuals to calculate genetic distance between them (AIMS1, n=8;  
335 AIMS2, n=5, AIMS3, n=7; AIMS4, n=3). Individuals of the same genotype may overlap in the plot due to their  
336 high similarity.



337

338 **Figure 2:** The phylogenetic relationships of the four GBR *E. diaphana* compared to other conspecific anemones  
339 sampled across the globe inferred from SCAR marker 5 using the Maximum Likelihood method and General  
340 Time Reversible model (Nei and Kumar 2000). The tree with the highest log likelihood is shown with bootstrap  
341 values next to the nodes. Initial trees for the heuristic search were obtained automatically by applying the  
342 Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of  
343 substitutions per site. This analysis involved 19 *E. diaphana* sequences and 706 nucleotide positions were  
344 included. Sequences were from our *E. diaphana* genotypes (AIMS1-4), globally distributed *E. diaphana* from

345 Thornhill et al. (2013), indicated with an asterisk, and from experimental genotypes CC7, H2, and F003  
346 (Grawunder et al. 2015).



347

348 **Figure 3:** The phylogenetic relationships of the four GBR *E. diaphana* (AIMS1-4) compared to other  
349 experimental anemones using the Maximum Likelihood method and General Time Reversible model (Nei and  
350 Kumar 2000) on six concatenated *Exaiptasia*-specific gene sequences (Bellis and Denver 2017). Data from Bellis  
351 and Denver (2017) was downloaded from GenBank (accession numbers KU847812-KU847847); tree branches  
352 are named as strain name followed by the alternative strain name in parentheses. Bootstrap values are shown next  
353 to the nodes. Initial trees for the heuristic search were obtained automatically by applying the Maximum  
354 Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per  
355 site. This analysis involved 10 *E. diaphana* sequences and 3267 nucleotide positions were included.

356 Based on SCAR marker data, *E. diaphana* is regarded as a single pan-global species with two  
357 distinct genetic lineages: one from the USA Atlantic coast, and a second consisting of all other  
358 *E. diaphana* worldwide (Thornhill et al. 2013). The SCAR5 allele sequenced for genotype  
359 AIMS1 is identical with that from anemones originally collected off Heron Island, Australia  
360 and Bermuda, while that of AIMS2 is nearly identical to (two base pair differences, both with  
361 an ambiguous base) samples from Hawaii, Japan and the Red Sea. AIMS3 and AIMS4 are  
362 most closely related to anemones from Florida and North Carolina, USA and are identical to  
363 one another in this region.

364 Similar to Bellis and Denver (2017), our data from the *Exaiptasia*-specific gene sequences  
365 (Fig. 3) show that, while anemone strains are genetically distinct, there is not a strong

366 phylogenetic separation between individuals collected from distant geographic locations.  
367 Again, AIMS1-4 show genetic variation, with AIMS1 and AIMS2 clustering with anemone  
368 strains originating from Coconut Island, Hawaii, USA which were collected independently in  
369 1979 and early 2000's, respectively. Corresponding with the SCAR5 loci data, AIMS3-4 have  
370 near identical sequences in the sequenced regions (differences at two heterozygous sites).  
371 Because the available data for SCAR5 and the *Exaiptasia*-specific gene targets are not from all  
372 the same individuals, with the exception of CC7 and H2, comparing between the two is not  
373 feasible. However, given the larger number of alignment positions and variable sites in the  
374 gene regions with better PCR results, we suggest that researchers use the primers presented in  
375 Bellis and Denver (2017) for future comparisons between *E. diaphana* used in experiments.  
376 The diversity that is revealed by whole genome SNP analysis (Fig. 1) is hidden with these six  
377 *Exaiptasia*-specific gene sequences, suggesting that there are more informative loci not yet  
378 published for *E. diaphana* genotyping.

379 There are several possible explanations for the AIMS1-4 anemones to be spread throughout  
380 the phylogenetic trees. First, due to small sample sizes at all sampling locations, only a subset  
381 of the alleles have been sampled and location-specific alleles may have been missed. Second,  
382 it is conceivable that the GBR is the source of all other *E. diaphana* populations and founder  
383 effects mean that other geographic locations have *E. diaphana* that represent only some of the  
384 diversity. Third, it is possible that the GBR *E. diaphana* was a distinct lineage and the GBR  
385 has since been invaded by *E. diaphana* from other lineages, or the GBR lineage has been  
386 introduced elsewhere. Introductions over such vast spatial scales may have occurred via ship  
387 ballast water or attached to ships hulls in fouling biomass, which is notorious for transporting  
388 marine life and introducing invasive animals and plants, or via the aquarium trade or marine  
389 farms. Irrespective of the cause, the genetic variation across the four GBR genotypes is more  
390 representative of global diversity than a single localized population. Because strain-specific  
391 responses to environmental variables have been observed among *E. diaphana* strains (Bellis  
392 and Denver 2017; Cziesielski et al. 2018) it is critical to conduct experiments, such as those  
393 regarding climate change and symbiosis, with a diverse set of individuals, much like the  
394 diversity presented by the GBR-sourced *E. diaphana*.

395 3.2 Symbiosis with Symbiodiniaceae

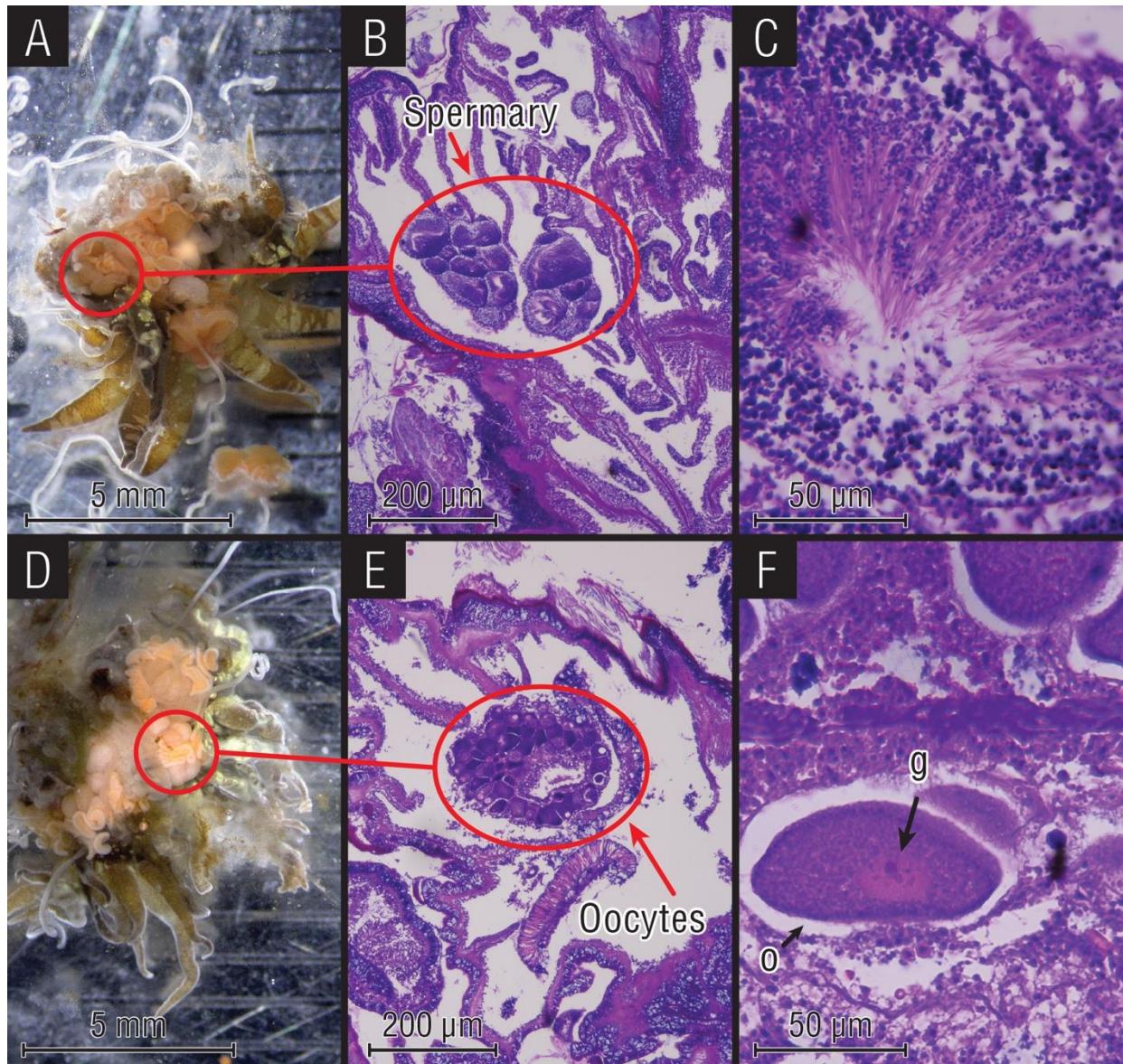
396 According to a global survey by Thornhill et al. (2013), Pacific Ocean *E. diaphana* (e.g., H2)  
397 associate exclusively with the Symbiodiniaceae species *Breviolum minutum* (formerly ITS2

398 type *Symbiodinium* Clade B, sub-clade B1 (LaJeunesse et al. 2018)). Symbiodiniaceae  
399 sequences from the four GBR *E. diaphana* genotypes were almost exclusively *Breviolum*  
400 *minutum* (>99.6%), thus concurring with Thornhill et al. (2013). Two as-yet unnamed species  
401 of *Breviolum* (previously known as *Symbiodinium* sub-clades B1i and B1L (LaJeunesse et al.  
402 2018)) were also identified in *E. diaphana*. Their very low relative abundance suggests they  
403 are either intragenomic variants or rare strains.

404 Stable endosymbiotic relationships between corals and Symbiodiniaceae are vital for  
405 sustaining coral reef ecosystems; this symbiotic relationship is the focus of much coral reef  
406 research. Interestingly, while the GBR anemones are genetically diverse, all host *B. minutum*  
407 as their sole Symbiodiniaceae. Given this, we may be able to investigate the symbiotic  
408 mechanisms of the host-algal relationship and answer questions about this symbiosis that are  
409 not restricted by anemone strain.

410 3.3 Sex of *E. diaphana*

411 *E. diaphana* lacks obvious gender defining morphological features, but gonad development is  
412 related to size (Chen et al. 2008). Partially developed oocytes with germinal vesicles (*i.e.*, the  
413 nucleus of an oocyte arrested in prophase of meiosis I) were observed in histological slides in  
414 animals of AIMS1, AIMS3 and AIMS4 and Stage V spermaries were observed in AIMS2 (Fig.  
415 4). In Stage V, the spermary is made up of a mass of spermatozoa with their tails facing in the  
416 same direction. In this advanced stage the spermatozoa are capable of fertilization (Fadlallah  
417 and Pearse 1982; Goffredo et al. 2012). While most AIMS1 anemones grew to only 5 mm in  
418 pedal disk diameter, gonad development was still observed. Differences between male and  
419 female gonads were present but were macroscopically cryptic. Male gonads have been  
420 observed to be smaller and lighter colored than female gonads in *E. diaphana* (Grawunder et  
421 al. 2015), and this was also the case in the GBR-sourced females (AIMS1, AIMS3 and AIMS4)  
422 and male (AIMS2) *E. diaphana*.

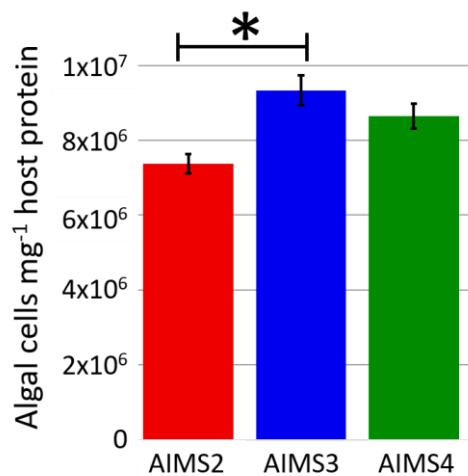


423

424 **Figure 4:** (A) Dissected male anemone with developing gonads (AIMS2, ~1 cm pedal disk diameter); (B) H&E  
425 stained tissue section, with stage 5 spermary; (C) Increased magnification of stage 5 spermary;  
426 (D) dissected female anemone with developed gonads (AIMS4, ~1 cm pedal disk diameter); (E) H&E stained tissue section  
427 with oocytes; (F) increased magnification of female gonad with developing oocyte (o) and germinal vesicle (g).

428 3.4 *E. diaphana* and Symbiodiniaceae Physiology

429 Throughout the nine-week evaluation period all *E. diaphana* maintained a healthy  
430 appearance, including *in hospite* Symbiodiniaceae, tentacle extension, active feeding of *A.*  
431 *salina* nauplii and asexual propagation. Average Symbiodiniaceae cell densities (normalized  
432 to host protein) were significantly different between genotypes (one-way ANOVA  
433 ( $F_{(3,200)}=3.985$ ,  $p=0.00872$ ; Fig. 5; Online Resource 6). All anemones hosted  $\sim 10^6$   
434 Symbiodiniaceae cells  $\text{mg}^{-1}$  host protein, which is comparable to densities reported for other  
435 lab cultured model *E. diaphana* systems (Hoadley et al. 2015; Hawkins et al. 2016b;  
436 Rädecker et al. 2018) and is similar to Symbiodiniaceae cell densities found in scleractinian  
437 corals (Cunning and Baker 2014; Ziegler et al. 2015; Kenkel and Bay 2018). The only  
438 statistically significant difference observed was the lower Symbiodiniaceae cell density of  
439 AIMS2 (mean  $\pm$  SE;  $7.17 \times 10^6 \pm 2.73 \times 10^5$  per mg host protein,  $n=66$ ) compared to AIMS3  
440 (mean  $\pm$  SE;  $8.46 \times 10^6 \pm 2.96 \times 10^5$  per mg host protein,  $n=66$ ) (Fig. 5).



441

442 **Figure 5:** Mean  $\pm$  1SEM Symbiodiniaceae cells  $\text{mg}^{-1}$  host protein for GBR *E. diaphana* genotypes AIMS2-4.  
443 Seventy-five AIMS2-4 anemones were collected over a period of nine weeks. Asterisk indicates significant  
444 difference,  $p<0.05$ .

445 All genotypes experienced a nearly identical drop in Fv/Fm after the light intensity was  
446 increased from 12 to 28  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{s}^{-1}$ , from an average of 0.53 on day 0, to an  
447 average of 0.40 by day 21 (Online Resource 7; Fig. 6). However, by day 36 the Fv/Fm values  
448 had returned to initial levels.

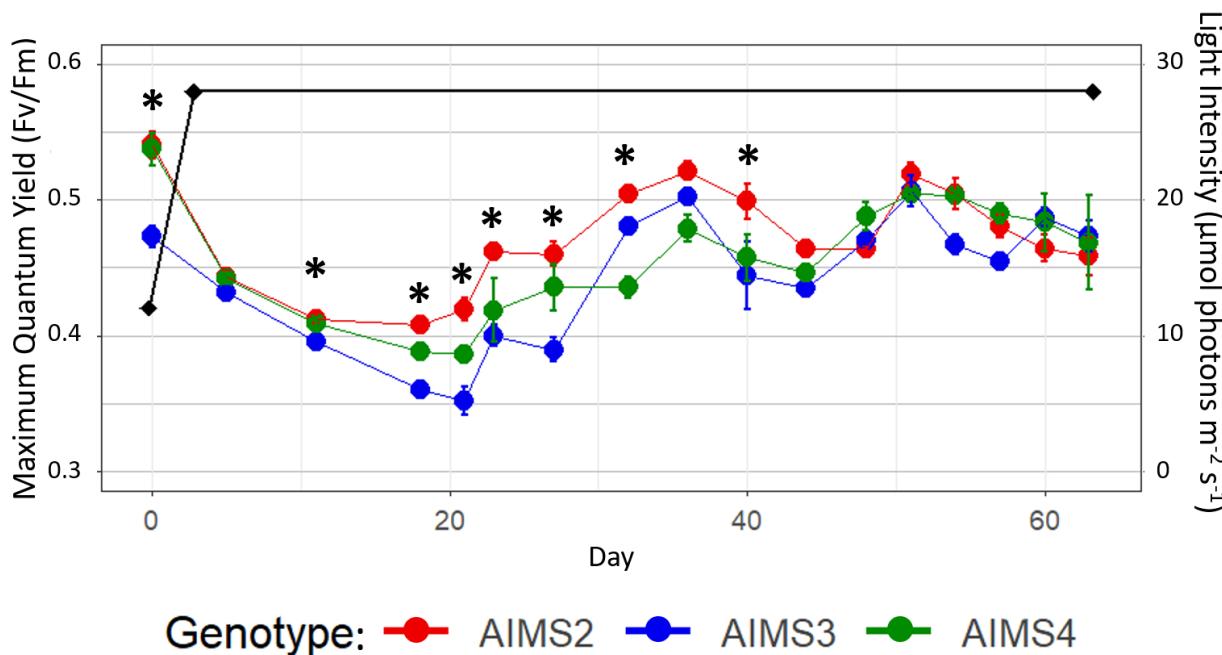
449 Changes in environmental variables, such as light intensity, are known to influence  
450 photobiological behavior of Symbiodiniaceae (Wangpraseurt et al. 2014; Hoadley and

451 Warner 2017). A change in light intensity can alter photosynthetic efficiency as measured by  
452 Fv/Fm (Hoadley and Warner 2017). GBR anemone genotypes AIMS2, AIMS3 and AIMS4  
453 took 36 d to recover from this environmental change and return to photosynthetic efficiencies  
454 recorded at 12  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Such information is critical for planning and conducting  
455 symbiosis studies. Most of the statistical differences between the genotypes occurred between  
456 days 19–28 (Online Resource 8) when Fv/Fm was lowest. Once acclimated at day 36, average  
457 Fv/Fm values for all genotypes were not significantly different (mean  $\pm$  SE;  $0.479 \pm 0.003$ ,  
458 n=80), except for day 40 where the Fv/Fm of AIMS2 was significantly higher than AIMS3  
459 ( $p=0.0068$ ).

460 As all the GBR-sourced anemones harbour *Breviolum minutum* as their homologous  
461 symbiont type, it is not unexpected that the different genotypes would have similar maximum  
462 Fv/Fm values. Furthermore, similar Fv/Fm values have been reported for other anemones  
463 hosting homologous *B. minutum* (Hawkins et al. 2016b; Hillyer et al. 2017). However, it is  
464 noteworthy that AIMS2 is not only able to recover its photosynthetic efficiency quicker from  
465 changing light levels with a milder dip in Fv/Fm values (Fig. 6), but also hosts significantly  
466 fewer Symbiodiniaceae cells  $\text{mg}^{-1}$  host protein compared to AIMS3 (Fig. 5). Reductions in  
467 the maximum quantum yield of photosystem II (PSII) (Fv/Fm) is observed in the early phases  
468 of natural bleaching events (Gates et al. 1992; Franklin et al. 2004) and the ability of AIMS2  
469 to maintain a higher efficiency of PSII photochemistry during changing environmental  
470 conditions could translate into higher thermal tolerance (Suggett et al. 2008; Ragni et al.  
471 2010; Goyen et al. 2017).

472 These individuals are genetically diverse based on SNP genotyping (Fig. 1) and phylogenetic  
473 analysis (Fig. 2–3); the phenotypic feature of AIMS2 being more robust to increasing light  
474 levels compared to AIMS3 and AIMS4 could be a host genotypic effect. There is evidence  
475 that genetic variation of *E. diaphana* may influence holobiont response to heat stress, though  
476 this hypothesis has only been tested on anemone strains hosting different Symbiodiniaceae  
477 species (Bellis and Denver 2017; Cziesielski et al. 2018) or after experimentally bleaching  
478 anemones and inoculating with new heterologous algal cells (Perez et al. 2001). As we have  
479 four GBR-sourced *E. diaphana* genotypes with inherent genetic variability and all contain *B.*  
480 *minutum* as their homologous symbiont, we will be able to explore the roles of host and  
481 symbiont in the bleaching response.

482 An alternative possibility is that the GBR anemones' Symbiodiniaceae communities  
483 comprise diversity that may be hidden under the resolution of the ITS2 sequences we used in  
484 this experiment, which are driving the differences in photosynthetic efficiency. Distinct  
485 strains of a given Symbiodiniaceae species can have different susceptibilities to thermal  
486 stress (Ragni et al. 2010; Howells et al. 2012; Hawkins et al. 2016a), with evidence that these  
487 variations in thermal optima can drive host-Symbiodiniaceae interactions (Hawkins et al.  
488 2016a). Thus, varying rates of recovery of Fv/Fm among Symbiodiniaceae strains (Fig. 6)  
489 could provide a mechanism for the emergence of novel and potentially resilient cnidarian-  
490 Symbiodiniaceae associations in a rapidly warming environment.  
  
491 Another explanation for the physiological differences between AIMS2 and AIMS3 (Fig. 5) is  
492 through algal cell density moderation by the host. It is thought that the coral host controls  
493 Symbiodiniaceae densities through nitrogen limitation (Falkowski et al. 1993), although the  
494 mechanisms are not well understood (Davy et al. 2012). During temperature stress, higher  
495 densities of Symbiodiniaceae have been implicated in increasing the susceptibility of corals  
496 to bleaching, potentially as a result of the higher reactive oxygen species production relative  
497 to corals' antioxidant capacity (Cunning and Baker 2012). Altogether, our data suggest that  
498 AIMS2, which hosted fewer algal symbionts and recovered from increased light conditions  
499 faster than AIMS3, may be more resilient to thermal stress, while AIMS3 could be more  
500 susceptible to bleaching with AIMS4 as an intermediate.



501

502 **Figure 6:** Fv/Fm measurements for anemones AIMS2, AIMS3 and AIMS4 over a 63-day period. Anemones  
503 were initially exposed to light levels (black line) of  $12 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (12:12 light:dark cycle), which were  
504 then increased to  $28 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  over a 72 h period. Day 0 marks the start of light ramping. The  
505 anemones took  $\sim 35$  d to recover their maximum quantum yield due to the increase in light exposure although  
506 Symbiodiniaceae densities remained largely constant. Asterisks indicate significant differences in pairwise  
507 comparisons between genotypes at given time points (Online Resource 8)

#### 508 4 Conclusions and Future Directions

509 The study of *E. diaphana* anemones of GBR origin described here provide further  
510 information on phenotypic and genetic variation within this species and complements data on  
511 the more widely used *E. diaphana* strains CC7 and H2. The four genotypes in our collections  
512 capture a level of genetic diversity previously observed in animals from different oceans and  
513 are therefore a hugely valuable addition to the model collections. Knowledge of their  
514 characteristics enhances and broadens the potential of this model system for climate change  
515 research in corals, particularly, but not exclusively, for Australian researchers. We propose  
516 future research on this collection should focus on characterization of associated prokaryotes  
517 to explore the value of these animals as models for coral-prokaryote symbiotic interactions.  
518 Future research in cnidarian-prokaryotic interactions would be enhanced by the development  
519 of axenic (germ-free) or gnotobiotic (with a known microbial community) *E. diaphana*  
520 cultures. They could be used to test the influence of native and non-native microbiota on  
521 holobiont performance, and the ability of probiotic inocula to support animal health during  
522 stress (Alagely et al. 2011; Damjanovic et al. 2017; Rosado et al. 2018).

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