

A peer-reviewed version of this preprint was published in PeerJ on 13 May 2019.

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Pochon X, Wecker P, Stat M, Berteaux-Lecellier V, Lecellier G. 2019. Towards an in-depth characterization of Symbiodiniaceae in tropical giant clams via metabarcoding of pooled multi-gene amplicons. PeerJ 7:e6898 <https://doi.org/10.7717/peerj.6898>

1 **Towards an in-depth characterization of Symbiodiniaceae in tropical giant clams via**
2 **metabarcoding of pooled multi-gene amplicons**

3

4 Xavier Pochon^{1,2*}, Patricia Wecker³, Michael Stat⁴, Véronique Berteaux-Lecellier⁵, Gaël
5 Lecellier^{5,6}

6

7 ¹ Coastal and Freshwater Group, Cawthron Institute, Nelson, New Zealand

8 ² Institute of Marine Science, University of Auckland, Auckland, New Zealand

9 ³ Consultant in marine Microbiology, 7 Av. des garennes, 62170 Montreuil sur mer, France

10 ⁴ Department of Biological Sciences, Macquarie University, North Ryde, NSW 2109, Australia

11 ⁵ UMR250/9220 ENTROPIE, IRD-CNRS-UR, LabEx CORAIL, 101, promenade Roger-Laroque,
12 BP A5 98848 Nouméa Cedex, New-Caledonia.

13 ⁶ Université Paris -Saclay, UVSQ, 55 Avenue de Paris 78035 Versailles Cedex, France

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15 **Competing Interests:** The authors have declared that no competing interests exist. Xavier Pochon
16 is an Academic Editor for PeerJ.

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18 * E-mail: xavier.pochon@cawthron.org.nz

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27 **ABSTRACT**

28 High-throughput sequencing is revolutionizing our ability to comprehensively characterize
29 free-living and symbiotic Symbiodiniaceae, a diverse dinoflagellate group that plays a
30 critical role in coral reef ecosystems. Most studies however, focus on a single marker for
31 metabarcoding Symbiodiniaceae, potentially missing important ecological traits that a
32 combination of markers may capture. In this proof-of-concept study, we used a small set
33 of symbiotic giant clam (*Tridacna maxima*) samples obtained from nine French Polynesian
34 locations and tested a dual-index sequence library preparation method that pools and
35 simultaneously sequences multiple Symbiodiniaceae gene amplicons per sample for in-
36 depth biodiversity assessments. The rationale for this approach was to allow the
37 metabarcoding of multiple genes without extra costs associated with additional single
38 amplicon dual indexing and library preparations. Our results showed that the technique
39 effectively recovered very similar proportions of sequence reads and dominant
40 Symbiodiniaceae clades among the three pooled gene amplicons investigated per sample,
41 and captured varying levels of phylogenetic resolution enabling a more comprehensive
42 assessment of the diversity present. The pooled Symbiodiniaceae multi-gene
43 metabarcoding approach described here is readily scalable, offering considerable analytical
44 cost savings while providing sufficient phylogenetic information and sequence coverage.

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47 **Keywords (6-10 words):** Biodiversity, Marine Ecology, Multi-gene Metabarcoding, High-
48 Throughput Sequencing, South Pacific Ocean, symbiosis, *Tridacna*.

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50

51 **INTRODUCTION**

52 Giant clams (Family Tridacnidae) play important roles in reef systems, acting as shelter
53 for a number of organisms (Mercier & Hamel 1996), contributing to primary production
54 through their symbiosis with dinoflagellates (Neo et al. 2015), and as effective filter feeders
55 (Klumpp & Griffiths 1994). Due to their large size, relative abundance and longevity, giant
56 clams can be considered as centennial barometers of reef health (Knop 1996).
57 Unfortunately, as a highly prized resource throughout much of their Indo-Pacific range,
58 giant clams also contain some of the most endangered species due to habitat degradation
59 and overfishing, i.e. wild stock depletion and local extinctions (IUCN Red List (List 2018).

60

61 Giant clams on shallow reefs allow for the establishment of a diverse *in-situ* reservoir of
62 interacting fungal, bacterial, and micro-algal communities (Baker 2003; Neo et al. 2015).
63 Importantly, they form obligatory symbioses with, and release living cells of,
64 Symbiodiniaceae *sensu* LaJeunesse et al. (2018), a group of dinoflagellates that are critical
65 for the survival of a myriad of tropical invertebrates, including corals. Despite these
66 dynamic interactions, very little is known about the extent of symbiont diversity within
67 giant clams and the potential exchange with other reef invertebrates engaged in similar
68 symbiotic associations (i.e. nudibranchs and corals; Wecker et al. 2015). Unlike traditional
69 molecular techniques (e.g. PCR-based fingerprinting methods and Sanger sequencing) that
70 have been extensively used to shed light on Symbiodiniaceae diversity in reef organisms
71 (reviewed in Coffroth & Santos 2005; Stat et al. 2006), recent advances in High-
72 Throughput Sequencing (HTS) technologies now enable unprecedented sequencing depth
73 for global biodiversity assessments of symbiotic and free-living communities of
74 Symbiodiniaceae (Boulotte et al. 2016; Cunning et al. 2015; Edmunds et al. 2014; Hume

75 et al. 2018; Shinzato et al. 2018; Thomas et al. 2014). Nevertheless, such studies usually
76 focus on metabarcoding analyses of single molecular markers in isolation, in particular the
77 *ITS2* marker (but see Smith et al. 2017; Thomas et al. 2014), potentially overlooking
78 intrinsic phylogenetic differences known to occur between distinct Symbiodiniaceae genes
79 (Pochon et al. 2012; Pochon et al. 2014).

80

81 A variety of HTS library preparation methods exist for metabarcoding biological samples
82 using Illumina™ sequencing platforms, including the use of fusion tag primers (Stat et al.
83 2017), the ligation of Illumina™ adapters using TruSeq™ PCR-free kits (Rhodes et al.
84 2014), and the addition of Illumina™ adapters via dual-index sequencing (Kozich et al.
85 2013). The latter technique requires two distinct rounds of PCR analyses. The first round
86 uses gene-specific primers modified to include Illumina™ adapter tails. Following
87 purification of the PCR products, a second short round of PCR is applied using Nextera™
88 library construction kits that involve individual primer sets containing the Illumina™
89 adapter and sequencing primer sequence. This second PCR step is usually performed on
90 individual PCR amplicon products before the pooling and sequencing of multiple samples
91 so that demultiplexing of sequence data results in appropriate identification of input
92 samples. For laboratories that use the services of external genomic facilities for the
93 preparation of their dual-index libraries, an increased sample set usually correlates
94 positively with the analytical cost due, in part, to the use of additional Nextera™ indexed
95 primers. Therefore, one solution for reducing costs when performing multi-gene analyses
96 of individual samples, is to pool the PCR amplicon products prior to the second PCR step,
97 followed by the sequencing and gene-specific demultiplexing per sample.

98

99 Here we conducted a preliminary assessment of a dual-index multi-gene metabarcoding
100 approach via the pooling and side-by-side HTS analysis of PCR amplicons from three
101 commonly employed nuclear and chloroplastic Symbiodiniaceae markers. The ability to
102 combine multiple gene amplicon targets per sample offers considerable analytical cost
103 savings while providing sufficient phylogenetic information and sequence coverage. This
104 study describes a multi-marker metabarcoding approach using giant clam *Tridacna maxima*
105 as a model and discusses future applications for improving analyses of coral reef
106 holobionts.

107

108 MATERIAL AND METHODS

109 *Sample collection and DNA extraction*

110 For this study, twelve DNA extracts from *Tridacna maxima* biopsies, previously
111 collected between February 1st 2011 and November 2nd 2013 from nine islands in the
112 French Polynesian Archipelagos (Figure 1, Table S1) were used (Dubousquet et al. 2018).

113

114 *Preparation of Multiplexed Amplicons High-Throughput Sequencing Libraries*

115 Three sets of Symbiodiniaceae-specific primers with Illumina™ adapter tails (Table S2)
116 were used to amplify each sample (S141-S152; Table 1) in separate Polymerase Chain
117 Reactions (PCR). Three markers were amplified: (i) the Internal Transcribed Spacer 2
118 (*ITS2*) of the nuclear ribosomal RNA array using primers ITSD_illu and ITS2rev2_illu ,
119 (ii) the D1-D2 region of the 28S large subunit (*LSU*) nuclear ribosomal RNA gene using
120 the newly designed primers LSU1F_illu and LSU1R_illu, and (iii) the hyper-variable
121 region of the chloroplast 23S (*23S*) ribosomal RNA gene using primers 23SHyperUP_illu
122 and 23SHyperDN_illu (Manning & Gates 2008; Pochon et al. 2010).

123

124 PCR was performed for each sample and for each gene separately in 50 μ L volumes, with
125 the reaction mixture containing 45 μ L of Platinum PCR SuperMix High Fidelity (Life
126 Technologies), 10 μ M of each primer, and 10-20 ng of template DNA. In order to maximize
127 specificity to Symbiodiniaceae, a touchdown PCR protocol was used for each reaction as
128 follows: (i) 95 $^{\circ}$ C for 10 min; (ii) 25 cycles of 94 $^{\circ}$ C for 30 s, 65 $^{\circ}$ C for 30 s (decreasing
129 the annealing temperature 0.5 $^{\circ}$ C for every cycle after cycle 1), and 72 $^{\circ}$ C for 1 min; (iii)
130 14 cycles of 94 $^{\circ}$ C for 30 s, 52 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 1 min; and (iv) a final extension
131 of 72 $^{\circ}$ C for 10 min. Amplicons of the correct size were purified using Agencourt AMPure
132 XP PCR Purification beads following the manufacturers' instructions. In order to sequence
133 the three gene amplicons per sample in multiplex using HTS, individual purified products
134 for each marker originating from the same giant clam were pooled together to enable the
135 attachment of the same NexteraTM index (i.e. 12 samples). This was achieved by
136 quantifying the amplicons using a Qubit Fluorometer 2.0 (Life Technologies), diluting to
137 1 ng/ μ L using Milli-Q water and mixing 5 μ L of each of gene amplicon from the same
138 giant clam together. To assess the levels of cross-contamination between samples
139 potentially arising during the library indexing step, nine unmixed amplicon products (i.e.
140 *ITS2*, *LSU* and *23S* amplicons from three haphazardly selected giant clams; samples S141-
141 S143; Table 1), each with their own unique index to be added, were also prepared.

142

143 The resulting 21 samples were sent to New Zealand Genomics Ltd. (University of
144 Auckland, New Zealand) for HTS library preparation which involved a second round of
145 PCR to attach the NexteraTM indexes on to the amplicons for MiSeq IlluminaTM sequencing.
146 PCR products were combined in equimolar concentrations and the final library paired-end

147 sequenced on an Illumina™ MiSeq using a 500 cycle (2 x 250) MiSeq® v2 Reagent Kit
148 and standard flow cell.

149

150

151 *Bioinformatics*

152 Illumina™ sequence datasets were prepared using the read preparation and dereplication
153 pipeline of USEARCH (Edgar 2010). Firstly, paired reads were merged (fastq_mergepairs
154 command) and filtered (fastq_filter command) with an expected number of error of 0.25.
155 More than 90% of the base pairs had a Q score > 40. Next, samples were demultiplexed in
156 three groups, primers were trimmed and a global trimming was operated according to the
157 recommendations for *ITS* amplicon reads (Edgar 2013). The sequence data were
158 dereplicated and unique singletons found across the complete dataset were discarded.

159

160 For phylogenetic assignments of Symbiodiniaceae, three distinct annotated reference
161 databases (*ITS2*, *LSU* and *23S*) were generated in fasta format, including sequence
162 representatives from each of nine Symbiodiniaceae clades (A to I), with (i) 409
163 representative *ITS2* phlotypes from GeoSymbio (Franklin et al. 2012), (ii) 37
164 representative *LSU* sequences from Pochon et al. (2012), and (iii) 104 sequences of *23S*
165 from Takabayashi et al. (2011). Symbiodiniaceae assignments were performed using the
166 software ‘Kallisto’ (Bray et al. 2016) which provides unprecedented speed and accuracy
167 for optimal analysis of large-scale datasets (e.g. large RNA-Seq data) without the need for
168 time-consuming alignment steps.

169

170 Because the main goal of the present pilot study was to investigate the sequencing depth

171 and potential inter-marker biases of the multi-marker metabarcoding approach using giant
172 clam samples as a proof-of-concept, as opposed to describing potentially novel
173 Symbiodiniaceae diversity in these samples, we modified the Kallisto pipeline to only
174 retain HTS reads yielding exact matches (i.e. without ambiguity amongst k-mers) to
175 individual referenced genotypes in each gene. This approach transforms each sequence
176 from reference databases into pseudo-alignments of k base-pairs (bp) k-mers which slide
177 along the sequence of reference one bp at a time. Individual sequences generated via HTS
178 were then blasted against all pseudo-alignments and exact matches against the entire
179 population of k-mers are recorded. To reduce mis-assignments, all merged reads with
180 ambiguities between k-mers of different reference genotypes were determined as chimeric
181 and removed from the dataset. The sequences that did not result in exact matches could
182 correspond to non-Symbiodiniacea sequences or to sequences not comprised in our custom
183 database. Therefore, a second comparison using BLASTn against the National Center for
184 Biotechnology Information (NCBI) nucleotide databases was performed and the accession
185 numbers yielding exact matches were retained for downstream analyses. The number of
186 unique sequences matching genotypes in the reference databases and GenBank was
187 recorded (Table S3). Raw sequence data were submitted to the BioProject Archive under
188 accession PRJNA471926 (SRR7181922-SRR7181942).

189

190 *Sequence Diversity Analyses*

191 Unique sequence genotypes found at or above a 0.05% threshold from the total
192 sequence abundance per sample were scored (Table S3) and the specific genotypes of
193 reference (i.e. from in-house reference databases and GenBank) were retained for sequence
194 diversity and phylogenetic analyses. Global sequence diversity from each of the three

195 datasets (*23S*, *ITS2*, and *LSU*) were visualized using the plug-in DataBurst implemented in
196 Excel (Microsoft Office version 2013 or later).

197

198 One sequence alignment was generated for each of the three investigated gene datasets
199 using the sequence alignment software BioEdit v7.2.5 (Hall 1999). Owing to the difficulty
200 in aligning sequences from *Symbiodinium* (clade A) and *Cladocopium* (clade C) genera
201 when using the *23S* and *ITS2* genes, and between Symbiodiniaceae and non-
202 Symbiodiniaceae (i.e. clams, fungi, and plants) sequences, phylogenetic reconstructions
203 only aimed at depicting pair-wise relationships between retained sequence genotypes.
204 Therefore, unrooted phylogenetic inferences were generated using the neighbor-joining
205 method implemented in the program MEGA v. 7.0 (Kumar et al. 2016), with the p-distance
206 model and gaps treated as pairwise deletions. Internal nodes support was tested using the
207 bootstrap method (Felsenstein 1985) and 500 replicates.

208

209 RESULTS

210 A total of 1,590,047 sequences were obtained from the 21 samples (75,716 +/- 41,576
211 sequences per sample), which included 12 amplicon samples (S141-S152) each containing
212 three pooled gene products (*23S*, *ITS2*, and *LSU*) and nine amplicon samples from three
213 selected giant clam isolates (S141, S142, and S143) which only contained a single gene
214 amplicon as internal controls (Table 1; Table S3). One sample (internal control S143 for
215 *ITS2*) failed the sequencing step with only 130 raw reads produced. After read cleaning,
216 the total number of high-quality sequences was 1,104,687 (52,604 +/- 29,250 sequences
217 per sample). The proportion of total reads (Table 1) between the three investigated genes
218 was well-balanced with 398,442 reads (*23S*), 339,780 reads (*ITS2*), and 359,768 reads

219 (*LSU*). In contrast, unique reads varied between 23,779 sequences for the *23S* gene and
220 71,776 sequences for the *LSU* gene (Table S3). The inclusion of nine positive controls,
221 representing three amplicon products per gene sequenced in isolation, revealed the
222 presence of low levels of sequence cross-contamination between samples (mean of 4.5
223 sequences \pm 4.6 SD) (Table 1). This low-level of background contamination (1 to 23
224 sequences per sample) represented <0.003% of the total reads per sample (Table S3).
225 Therefore, as a conservative measure, we chose to remove sequences that represented <
226 0.05% of the total sequence abundance per sample.

227

228 Our bioinformatics pipeline identified 43 Symbiodiniaceae *23S* chloroplast genotypes,
229 including 16 that matched the *23S* reference database and another 27 that matched
230 sequences in GenBank. After exclusion of genotypes represented by less than 0.05% of the
231 sequence abundance in each sample (Table S3), the number of unique *23S*
232 Symbiodiniaceae sequences retained for phylogenetic analysis was eleven (Figure S1).
233 Similarly, blasting *ITS2* and *LSU* datasets against both types of databases led to the
234 identification of 117 and 93 unique sequences when using the original datasets, and to 46
235 and 51 unique sequences following the 0.05% filtering threshold, respectively.

236

237 Diversity diagrams were generated to visualize the sequence abundance of
238 Symbiodiniaceae generic and sub-generic sequences recovered from the twelve giant clam
239 samples and among the three investigated genes (Figure 2). The pooled multi-gene
240 approach yielded similar proportions of dominant genera, but with some notable
241 differences. The genus *Symbiodinium* (previously Clade A) dominated in all three markers,
242 particularly in *23S* (91.8%; dominant sub-generic sequence *chvA2*), with lower but similar

243 proportions between *ITS2* (81.7%; dominant sub-generic sequences A3/A6) and *LSU*
244 (83.9%; dominant sub-generic sequences A3/A13). The genus *Cladocopium* (previously
245 Clade C) represented 7.9% (dominant sub-generic sequence chvC1), 18.2% (dominant
246 sequence C1), and 15.0% (dominant sequence C1) of reads for the *23S*, *ITS2*, and *LSU*
247 markers, respectively. *Geracladium* (previously clade G) was only detected using the
248 chloroplast *23S* gene (0.2% of reads), whereas the nuclear *LSU* gene displayed reduced
249 specificity for Symbiodiniaceae as indicated by ~1% of sequence reads matching other
250 organisms such as streptophytes (*Mitchella repens* and *Asclepias verticillata*), and the host
251 giant clam *T. maxima*. Overall, the proportion of dominant Symbiodiniaceae generic and
252 sub-generic sequences recovered between the multiplexed samples and the positive (single
253 gene) controls were very similar (Table S4).

254

255 **DISCUSSION**

256 *Multi-gene metabarcoding: more for less*

257 The concept of pooled multi-gene amplicons for dual-indexed metabarcoding, i.e. the
258 tagging and pooling of distinct gene amplicons before Illumina™ adapter indexing and
259 simultaneous sequencing of samples, has been used extensively in other research fields
260 (e.g., Keeley et al. 2018; Marcelino & Verbruggen 2016; von Ammon et al. 2018; Zhang
261 et al. 2018), but has never been applied to Symbiodiniaceae dinoflagellates. In this proof-
262 of-concept study, we show that the technique effectively recovered similar proportions of
263 sequence reads and Symbiodiniaceae genera among the three multiplexed genes
264 investigated per sample, providing more confidence that single gene primer biases did not
265 occur during Nextera™ indexing. Another advantage is the ability to simultaneously
266 visualize varying levels of phylogenetic resolution, enabling a more comprehensive

267 assessment of the diversity present. For example, while the traditional ‘species-level’ *ITS2*
268 marker (LaJeunesse 2001) enabled characterization of 46 Symbiodiniaceae sub-generic
269 sequences, the *LSU* marker, interestingly, offered both a similarly high resolution for
270 Symbiodiniaceae (46 sub-generic sequences) and a reduced specificity by also enabling
271 identification of other host-associated organisms such as streptophytes, as well as the host
272 *Tridacna*. The hyper-variable region of the chloroplast *23S* marker used here is more
273 conserved, but has been successfully used for specifically targeting low abundance free-
274 living Symbiodiniaceae cells from environmental samples (Decelle et al. 2018; Manning
275 & Gates 2008; Pochon et al. 2010; Takabayashi et al. 2011). The unique detection of
276 *Gerakladium* (clade G) using the *23S* marker highlights the added value of the multi-gene
277 approach for broader Symbiodiniaceae screening efficiency. Analytical cost is an important
278 consideration for any research group aiming to monitor coral reef ecosystems, and the
279 budget needed to include HTS for biodiversity assessments is highly variable. The cost
280 ranges between AU \$40-\$100 per sample (Stat et al. 2018) and depends on the number of
281 gene regions investigated, method of library preparation, sequencing depth, and whether
282 amplicon multiplexing is employed as shown here. In this context, our approach is readily
283 scalable and has the potential to offer substantial savings in terms of both time and cost,
284 for example, by enabling coral reefs researchers to generate multi-gene Symbiodiniaceae
285 data in a 96-well format for the price of a single dual-indexed Illumina™ MiSeq run.
286 Additional studies are required to investigate whether ‘true multiplexing’, i.e. the mixing
287 of multiple primer sets in the original PCR to produce multi-gene amplicons (De Barba et
288 al. 2014; Fiore-Donno et al. 2018) would result in similar proportions of Symbiodiniaceae
289 genotypes between markers such as shown in the present study. Such approach, if
290 validated, would allow significant additional cost savings.

291

292 ***Paving the way for comprehensive biodiversity assessment of giant clams***

293 Giant clams on shallow reefs allow for the establishment of a diverse *in-situ* reservoir
294 of interacting fungal, bacterial, and micro-algal communities (Baker 2003; Neo et al. 2015).
295 For example, they commonly harbor Symbiodiniaceae from at least three distinct genera
296 (*Symbiodinium* [clade A], *Cladocopium* [clade C], and/or *Durusdinium* [clade D])
297 simultaneously or in isolation within one host, with *Symbiodinium* being the dominant
298 symbiont genus in most clams (Baillie et al. 2000; DeBoer et al. 2012; Ikeda et al. 2017;
299 Ikeda et al. 2016; Pappas et al. 2017; Trench et al. 1981). Similar to coral symbiosis, it is
300 assumed that the genotypic composition of Symbiodiniaceae in giant clams is influenced
301 by environmental or physical parameters (e.g. temperature, irradiance), or by life stages
302 and taxonomic affiliation (Ikeda et al. 2017; Pappas et al. 2017). Giant clam larvae (veliger)
303 acquire free-living Symbiodiniaceae cells ‘horizontally’ from their surrounding
304 environment (Fitt & Trench 1981). When mature, giant clams (e.g. *Tridacna derasa*) expel
305 high numbers of intact symbionts in their faeces at rates of 4.9×10^5 cells d⁻¹ (Buck et al.
306 2002; Maruyama & Heslinga 1997). Despite the dynamic interaction of symbionts between
307 Tridacnidae and the environment, very little is known about the extent of symbiont
308 diversity within giant clams and the potential exchange with other reef invertebrates
309 engaged in similar symbiotic associations

310

311 In this preliminary study, we found that genera *Symbiodinium* and *Cladocopium* dominated
312 in adult giant clams in French Polynesia (Figure S2). *Symbiodinium* was the major genus
313 in our samples and in particular the closely related sub-generic ITS2 genotypes A3 and A6,
314 previously described as *Symbiodinium tridacniadorum*, and therefore associated

315 with *Tridacna* clams (Lee et al. 2015). A3 is the most dominant genotype in *T. Maxima*
316 around the world and both A3/A6 are more likely to be sampled in giant clams from
317 shallow reefs (Weber 2009).

318

319 Furthermore, for *Cladocopium* we found that the generalist ITS2 genotype C1 (LaJeunesse
320 et al. 2003) co-dominated in our samples, which is consistent with a previous study
321 showing C1 as a common genotype in *T. Maxima* from around the world (Weber 2009).
322 Noteworthy, we also found a smaller percentage of C3z, Cspd and C50 ITS2 genotypes,
323 which to our knowledge have not yet been found in *T. maxima* before, but are usually
324 restricted to corals (LaJeunesse et al. 2004; LaJeunesse et al. 2010; Macdonald et al. 2008;
325 Shinzato et al. 2018). Finally, we did not detect any symbiont from the genus *Durusdinium*
326 (Clade D) despite the in-depth sequencing afforded by our multiplexed amplicons method.
327 However, *Durusdinium* has never been detected in *T. maxima* from French Polynesia
328 compared to other regions such as the Indian Ocean (DeBoer et al. 2012; Weber 2009). As
329 we only worked with adult clams from shallow water, it would be interesting to confirm
330 the hypotheses of Ikeda et al. (2017) and Weber (2009) who argued that *Durusdinium*
331 symbionts might be restricted to ‘young’ *T. squamosa* clams (less than 11 cm) or that giant
332 clams harbored this dinoflagellate genus only when sampled from deeper reefs,
333 respectively. Nevertheless, the small dataset used in the present study precludes us from
334 making any relevant assumptions about potentially novel symbiont diversity in giant clams.
335 In particular, the use of the Kallisto bioinformatics pipeline which restricted the analysis to
336 100% sequence similarity hits is likely not suitable for the many studies where a high
337 degree of sequence novelty is found. It is our hope, however, that our multi-gene approach
338 will be investigated further using a more comprehensive giant clam dataset along with the

339 development of an alternative bioinformatics method guiding users on the assignment of
340 genus to species-level taxon ID to novel multi-gene sequences for deposition to GenBank.

341

342 CONCLUSIONS

343 This pilot project explored the use of multiplexed amplicons metabarcoding for rapid, cost-
344 effective and in-depth characterization of Symbiodiniaceae dinoflagellates using the giant
345 clam *T. maxima* as a model. Our results showed that the technique effectively recovered
346 similar proportions of sequence reads and Symbiodiniaceae diversity among the three
347 multiplexed gene amplicons investigated per sample enabling a more comprehensive
348 assessment of the diversity present, while also offering appreciable analytical cost savings.

349 We also found that *Symbiodinium* (clades A) and *Cladocopium* (clade C) were the
350 dominant genera in adult giant clams in French Polynesia, with similar sub-generic
351 genotypes (*ITS2* A3, A6, and C1) previously described as commonly associated with giant
352 clams from around the world. Our approach paves the way for more comprehensive surveys
353 of this important yet endangered group of reef invertebrates and its potential role as an
354 important *Symbiodinium* reservoir for declining coral reefs. Future investigations may also
355 expand on this method to clarify species-level differentiation among *Symbiodinium* taxa
356 using other markers (e.g. nuclear Actin, chloroplast *psbA*, mitochondrial *COI* and *16S*), or
357 simultaneously characterize all organisms (viruses, bacteria, fungi, and other eukaryotes)
358 associated with a more diverse host range. Such holistic diversity assessments will improve
359 our knowledge on the ecology and evolution of tropical holobionts and better predict the
360 adaptation of coral reefs in a rapidly changing environment.

361

362 ACKNOWLEDGMENTS

363 We thank Jonathan Drew for his support in the laboratory, and Charley Waters, Sam
 364 Murray and Chris Cornelisen for valuable discussions during manuscript preparation.

365

366 **Funding Statement**

367 This work was supported by Cawthron Institute Internal Investment Fund (IIF) #BST16931
 368 and by the French National Research Center (CNRS). The funders have no role in study
 369 design, data collection and analysis, decision to publish, or preparation of the manuscript.

370

371

372 **Table 1** Number of DNA sequences recovered from each sample (S141-S152), before and after
 373 quality filtration, and after demultiplexing into each gene. Samples S141 to S143 were used as
 374 control samples, each targeting only one of three PCR amplicons. Columns highlighted in grey
 375 show a low background contamination.

Sample	Source	Filtered	23S	ITS2	LSU
ID	reads	reads	reads	reads	reads
Multiplexed					
S141	75731	53654	22072	17813	13435
S142	89975	65312	26504	24395	14040
S143	78009	48881	21061	10256	17321
S144	172319	126860	48941	39131	38128
S145	147293	104743	31048	34457	38662
S146	72548	51886	23268	16817	11537
S147	118815	79339	29870	32449	16332
S148	50176	34810	12577	11695	10264

S149	4728	3381	2400	366	599
S150	88926	59387	20788	22068	16216
S151	53016	38314	15964	12882	9298
S152	60107	42239	17075	13108	11707

Controls
ITS2 only

S141	85824	52588	8	52335	1
S142	81924	52270	10	51988	6
S143*	130	13	5	6	2

LSU only

S141	56565	31134	8	7	30758
S142	92110	62629	23	0	62129
S143	114431	69823	9	0	69318

23S only

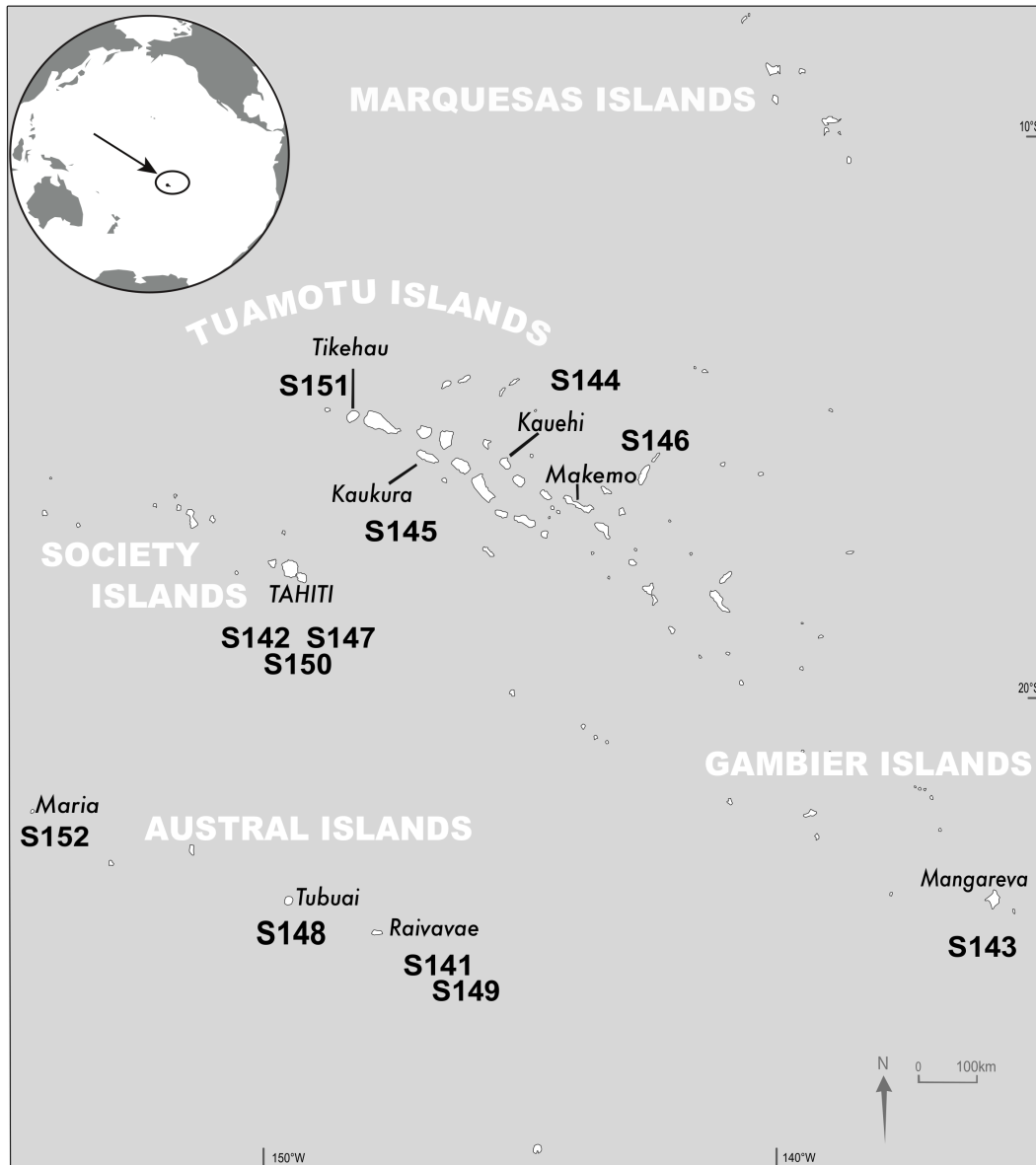
S141	77522	66763	66399	3	3
S142	42004	36422	36263	3	9
S143	27894	24239	24149	1	3

Total reads 1590047 1104687 398442 339780 359768

376 *One control sample (S143 ITS2) failed at sequencing, resulting in only 130 raw reads.

377

378



379

380 **Figure 1** Location and sample identification for the twelve *Tridacna maxima* samples investigated

381 in this study (credit to R. Canavesio).

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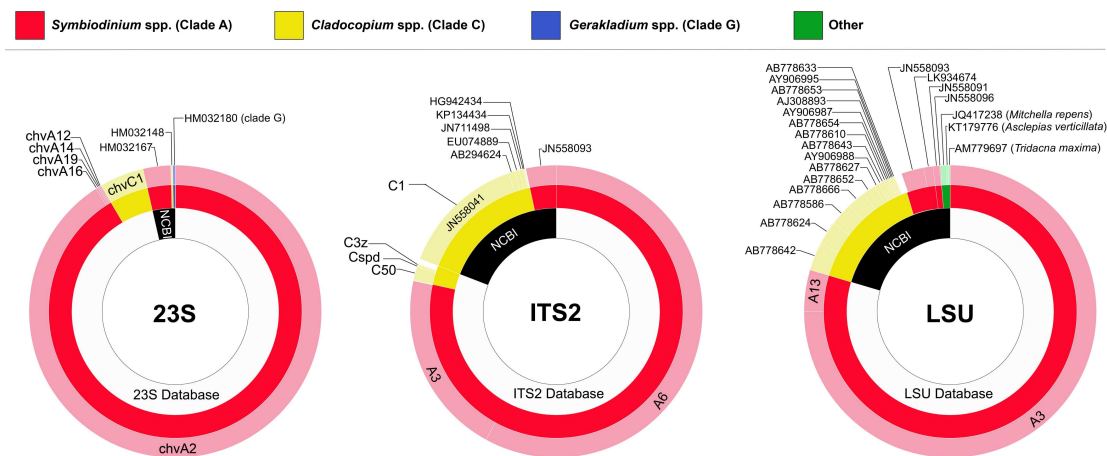
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391 **Figure 2** Global Symbiodiniaceae diversity charts obtained from each of the three datasets (left to
 392 right: *23S*, *ITS2*, and *LSU*). The proportion of sequences matching one of the three in-house
 393 reference databases or NCBI (inner circles) and their corresponding phylogenetic affiliation at
 394 genus (i.e. clade; middle circles) and sub-generic (i.e. subclade; outer circles) levels. Sequence reads
 395 representing <0.1% of total read abundance are not included.

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404 **Supplementary Information**

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406 **Table S1** Identification numbers, collection localities and date collected for the twelve samples of
407 *Tridacna maxima* investigated in this study.

408

409 **Table S2** List of primers used for generating PCR amplicons. Illumina adaptors are shown in bold.

410

411 **Table S3** Sequence counts and blast annotations for the 21 PCR amplicon samples analysed in
412 multiplex and individually (controls) over three distinct genes (*23S*, *ITS2*, *LSU*). Sheet 1 includes
413 the merged counts and dereplicated data; Sheet 2 includes exact *23S* sequence matches against the
414 Takabayashi et al. (2012) database and NCBI; Sheet 3 includes retained *23S* genotypes following
415 the 0.05% abundance threshold; Sheet 4 includes exact *ITS2* sequence matches against the
416 GeoSymbio database and NCBI; Sheet 5 includes retained *ITS2* genotypes following the 0.05%
417 abundance threshold; Sheet 6 includes exact *LSU* sequence matches against the Pochon et al. (2012)
418 database and NCBI; and Sheet 7 includes retained *LSU* genotypes following the 0.05% abundance
419 threshold.

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421 **Table S4** Percentage comparison of each Symbiodiniaceae sub-generic genotype recovered using
422 the three amplicon markers in ‘Multiplex’ versus single ‘Control’ markers (see Table 1). The
423 proportion of each sub-generic type between ‘Multiplex’ and ‘Control’ is almost identical for the
424 *23S* marker, but shows some minor differences for the *ITS2* and *LSU* markers. For example, four
425 *ITS2* types were detected in the ‘Multiplex’ but not in the ‘Control’ samples, and there were five
426 instances where *LSU* types were detected in the ‘Control’ but not in the ‘Multiplex’ samples. These
427 minor differences are likely attributable to PCR or sequencing biases.

428

429 **Figure S1** Unrooted circled trees of Symbiodiniaceae genotypes inferred using the Neighbor-
430 Joining method, with (A) 11 *23S* sequences, (B) 46 *ITS2* sequences, and (C) 51 *LSU* sequences.

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432 **Figure S2** Distribution of Symbiodiniaceae genera (i.e. clades) in *Tridacna maxima* obtained from
433 each of the three datasets (left to right: *23S*, *ITS2*, and *LSU*) per sample identification (S141-152).

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