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1 Development of a quantitative PCR-High Resolution Melting assay for absolute measurement of
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4

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14

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17

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23

24 ***Running title***

25 Absolute quantification of endosymbionts in corals

26 **Abstract**

27 Previous molecular studies have shown that many corals host a dynamic consortium of dominant and
28 background populations of Symbiodiniaceae genotypes with putatively distinct physiological traits. In
29 the present study, we developed a quantitative PCR assay combined with High Resolution Melting
30 analysis (qPCR-HRM) to distinguish which Symbiodiniaceae clades are present in a sample. Because
31 the qPCR-HRM used in isolation yielded identical melt profiles for both clades A and C, this analysis
32 was then coupled with further specific qPCR assays to enable the absolute quantification of all
33 Symbiodiniaceae clades and host cells. When the assays were applied to *in hospite* samples, they had
34 an absolute quantification level corresponding to one coral embryo of two cells and 1,000 symbiont
35 cells. The assays were successful on coral fragments from twelve species (eight families). We then used
36 the qPCR-HRM assay in an ecological survey of *Acropora pulchra* at different spatial scales (within
37 colony, between colonies and between sites). Differences in abundance and composition of
38 Symbiodiniaceae clades in *A. pulchra* were observed at all spatial scales, suggesting that various
39 environmental factors drove changes in Symbiodiniaceae assemblages among and within coral colonies.
40 The qPCR-HRM assay developed in this study is a relatively simple, cost-effective and reproducible
41 tool that can be used to accurately differentiate and quantify endosymbiont Symbiodiniaceae clades in
42 coral in the field. This will provide new insights into coral-symbiont shuffling mechanisms and the
43 resilience of coral colonies to environmental stressors.

44

45 **Introduction**

46 Coral reefs provide niches for 25% of all marine species but occupy only 0.2% of the world's ocean
47 surface area, and are therefore important hot spots of biodiversity (Reaka-Kudla 1997; Spalding and
48 Grenfell 1997; Spalding et al. 2001). In nutrient poor environments, coral reefs depend on obligate
49 symbiotic associations between corals and photosynthetic unicellular dinoflagellates in the family
50 Symbiodiniaceae. This family is currently classified into nine divergent lineages, hereafter referred to
51 as clades A to I (sensu Pochon and Gates 2010), of which seven have recently been elevated to genus
52 level (Lajeunesse et al. 2018), and six are found in symbiosis with scleractinian corals (A–D, F and G;

53 Stat et al. 2008; Cooper et al. 2011a). Each Symbiodiniaceae clade encompasses significant within-clade
54 genetic diversity (Pettay and Lajeunesse, 2013; Thornhill et al., 2017) corresponding to multiples species
55 or types (Coffroth and Santos 2005). Experimental and field-based studies of coral bleaching events
56 have shown that the physiology of the coral host is influenced by the genotypes of associated
57 Symbiodiniaceae (Rowan 2004; Berkelmans and van Oppen 2006; Stat et al. 2006; Howells et al. 2012,
58 2013). For example, corals dominated by clade A symbionts were described as more prone to parasitism
59 (Stat et al. 2008), whereas associations with clade C improved overall growth (Little et al. 2004; Cantin
60 et al. 2009), and with type D1a (*Durusdinium trenchii* sensu LaJeunesse et al. 2018) thermo-tolerance
61 was enhanced (Baker 2004; Stat and Gates 2011).

62

63 The association between a coral and several Symbiodiniaceae clades, often results in the presence of
64 one dominant clade together with several background clades. The formation of partnerships between a
65 coral and dominant Symbiodiniaceae clades often appears stable (Thornhill et al. 2009; LaJeunesse et
66 al. 2010), and possibly driven by environmental conditions (Bongaerts et al. 2013; Tonk et al. 2014;
67 Kennedy et al. 2016). However, the ability of corals to associate with multiple symbiont assemblages
68 has been shown to be more widespread than previously reported (Silverstein et al. 2012).

69

70 The fragile symbiosis between corals and zooxanthellae is undergoing drastic changes due to shifting
71 environmental conditions (Hughes et al. 2003; Hoegh-Guldberg et al. 2007). Rising seawater
72 temperatures has led to an increase in stress-induced symbiosis breakdowns, also known as coral
73 bleaching. Several massive coral bleaching events have been reported, resulting in a worldwide coral
74 loss of 16% in 1998 (Wilkinson 2000). The most recent event was related to intense drought linked to
75 El Niño (2015/2016), which resulted in the third worst worldwide mass bleaching event (National
76 Oceanic and Atmospheric Administration [NOAA] Coral ReefWatch program 2016). The impact of this
77 event is still incomplete but it is believed that it was the most severe coral bleaching event on record in
78 the Great Barrier Reef (Hughes et al. 2017; NOAA Coral ReefWatch program 2017). During abnormally
79 high seawater temperatures, certain coral species are more resistant to bleaching than others, and some
80 bleached corals can recover (e.g. Jones et al. 2008). This observed resilience may be due to the capability

81 of some corals to 'switch' their symbionts (the uptake of more tolerant symbionts from the surrounding
82 environment) or 'shuffle' their existing assemblage composition (background taxa may become abundant
83 in higher temperatures; Baker 2003, 2004; LaJeunesse et al. 2009).

84
85 Few studies have investigated the impact of changes in background symbiont composition and density
86 on coral stress resistance (e.g. Cunning and Baker 2012, 2014), and how this composition shifts after
87 bleaching events (Cunning and Baker 2012; McGinley et al. 2012; Cunning et al. 2015a, 2016; Bay et
88 al. 2016). It has been shown that the Symbiodiniaceae taxonomic composition may change within a
89 coral colony (Ulstrup et al. 2007; Kemp et al. 2008, 2014). However, variation in absolute abundances
90 (symbionts normalized to host parameters, such as units of DNA or cell numbers) of the nine
91 Symbiodiniaceae clades remains unexplored. Development of a method to accurately measure absolute
92 abundances of all nine Symbiodiniaceae clades would enable significant advancements in our
93 understanding of the functional role of symbiont assemblages in coral symbiosis (Cunning and Baker
94 2014).

95
96 Quantitative PCR (qPCR) allows the description of the relative abundance of different symbiont clades,
97 based on the number of copies of specific DNA loci within a sample (Ulstrup and Van Oppen 2003;
98 Loram et al. 2007; Mieog et al. 2007). It has previously enabled the effective tracking of symbiont
99 communities dynamics from five symbiont clades (A, B, C, D and F), that were standardized to host
100 cells (Cunning and Baker 2012, Cunning et al., 2015a, Mieog et al. 2009). It has also enabled detection
101 of low-abundance Symbiodiniaceae genotypes associated with corals (Silverstein et al. 2012; Rouzé et
102 al. 2016) that were previously thought to harbor only a single dominant clade (Goulet 2007; but see
103 Baker and Romanski 2007 and Mieog et al. 2007). However, absolute quantification of all
104 Symbiodiniaceae clades had not yet been achieved because of; *i*) the lack of a robust and efficient DNA
105 extraction method applicable to a wide range of sample types (cells/polyps, colonies, and replicate), *ii*)
106 the inability of qPCR assays to target all clades (i.e. including clades G-I) and the absence of an internal-
107 control for identifying missing clades (i.e., a family-Symbiodiniaceae target), and *iii*) the lack of qPCR
108 amplification efficiency measurements amongst clades and single host cells.

109

110 A further benefit of qPCR is the ability to include High Resolution Melt curve to differentiate PCR
111 products with different sequences. Two sequences containing at least one mutation generate fragments
112 that rapidly denature and rejoin after PCR to form two (high-melting) homoduplexes and two (low-
113 melting) heteroduplexes (base-pairing mismatches). The melting properties of these double-stranded
114 PCR products are monitored through the release of a saturating fluorescent double stranded DNA
115 binding dye as temperatures are increased, thus forming different melting curve shapes (Wittwer et al.
116 2003). Homozygous variants are therefore distinguishable from each other through differences in their
117 melting temperature. We previously demonstrated that the identification of unknown specimens is
118 undertaken by comparing these specimens to a set of reference melting curves (Meistertzheim et al.
119 2012).

120

121 The aim of this study was to develop an accurate, fast and highly reliable qPCR-High Resolution Melting
122 (qPCR-HRM) assay that could be coupled with further qPCR assays to distinguish and quantify the nine
123 Symbiodiniaceae clades in a wide range of coral taxa. For this purpose, we designed seven new clade-
124 specific primer sets and one family-Symbiodiniaceae primer set, which were tested on a range of cultures
125 and pure DNA samples from each of the nine existing Symbiodiniaceae clades. Additionally, two coral-
126 specific (actin and nuclear ribosomal) primer sets were designed and used on Symbiodiniaceae-free
127 embryos of *Acropora pulchra* to produce host standard curves for the quantification of coral cells. We
128 validated our assay on colony fragments from twelve different species representing eight different
129 families, and undertook an ecological survey of *A. pulchra* at different micro- and macro-scales (within
130 colonies, between colonies and among sites).

131

132 **Material and methods**

133 *Primer development*

134 Alignment of 135 sequences of the nuclear large sub-unit ribosomal (28S) rRNA gene, including the 5'
135 regions of the 5.8S and Internal Transcribed Spacer 2 (ITS2), were performed using MEGA6 (Tamura

136 et al. 2013). Seven clade-specific forward/reverse primer sets were designed using Primer Express v2.0
137 software (Applied Biosystems). The primers targeted an approximately 70-150 nucleotides [nt] region,
138 one of the forward or reverse primer being located near the 3' end of the 5.8S rRNA gene or the 5' end
139 of the 28S rRNA gene, and the corresponding reverse or forward primer being located in the ITS2 region
140 (see Table 1). We also used previously published primers, including SYM_VAR_5.8S2 (Hume et al,
141 2015), and two actin primer sets specific to clades C and D (Cunning and Baker 2012). Host-specific
142 (coral) primers were designed using alignments of the actin and nuclear 5.8S rDNA genes from
143 GenBank (Table S1).

144 HRM analysis was used to quantify the number of clades present in each sample. This requires the
145 amplification of a variable 80-nt partial sequence of the 5.8S gene using the forward SYM_VAR_5.8S2
146 and reverse Symb_R primer, hereafter referred to as the family primer pair 'Symb', which targets
147 conserved flanked areas amplifying the nine different Symbiodiniaceae clades (Table 1; Figure S1).
148 There are between one to ten mutations among clades in this gene (Table S2), which allows the
149 distinction between them based on melting curve shape.

150

151 *Absolute quantification assay development*

152 Primer specificity was verified using DNA isolated from Symbiodiniaceae cell cultures in their
153 vegetative state, from DNA collections of clades G-I (Table S3), and embryos of *A. pulchra*. Cells from
154 Symbiodiniaceae-free embryos at division 2 and 4 cells were obtained following the procedure described
155 in Hédouin et al. (2015), using *A. pulchra* colonies that were collected in September 2012 on the fringing
156 reefs of Moorea Island (17° 30' S, 149° 50' W, French Polynesia, Society Archipelago, Figure 1 A-B).

157

158 We tested a new and automated extraction method, applicable to both coral embryos and polyps, which
159 yields consistent DNA quantity between replicates and allowed successful DNA extraction from 2 cells
160 for corals embryos. Symbiodiniaceae cultures, embryos and samples from coral colonies were lysed by
161 mechanic action using the Y Matrix on a FastPrep Instrument (MP Biomedical, USA). Samples were

162 incubated with proteinase K (57°C, 1 hr). DNA was then isolated using a Maxwell® Blood DNA
163 Purification Kit LEV (Promega, USA) on a Maxwell 16 MDx Instrument (Promega). DNA quality was
164 assessed by electrophoresis on a 1% agarose gel. Total genomic DNA concentration were measured
165 using spectrophotometry (Nanodrop ND-1000, Thermo Fisher scientific Inc., MA, USA).

166

167 Standard curves of Symbiodiniaceae and host cells were constructed to allow absolute quantification
168 (Table S2). Living Symbiodiniaceae cells were quantified using a Scepter Handheld Automated Cell
169 Counter system (Millipore, USA) combined with Scepter Pro software (Millipore). Automated cell
170 counts were compared to cell counts obtained using a hemacytometer under a light microscope. DNA
171 was extracted for each concentration as described previously. Standard curves were run in triplicate with
172 cell concentrations of Symbiodiniaceae clades of 1×10^3 to 3×10^6 cells per DNA extraction (clades A-
173 F), and using DNA concentrations ranging between 0.5 and about 40 ng DNA μL^{-1} (clades G, H and I).
174 Two standard curves for the quantification of host-cells were run in triplicate using Symbiodiniaceae-
175 free embryos with an increasing concentration of 2 to 400 cells per DNA extraction. *Acropora pulchra*
176 were in their first and second division stages, and concentrations were determined by binocular
177 microscopy.

178 Quantitative PCR reactions were performed in duplicate in a 10 μL volume containing genomic DNA,
179 $1 \times$ High Resolution Melting master mix (Roche Diagnostics, USA) and 0.3 μM of each primer. The
180 amplification protocol consisted of 10 min pre-incubation at 95°C followed by 40 cycles of 95°C for 15
181 s (ramp rate, 4.4 °C. s^{-1}), touchdown annealing from 65 to 53 °C for 15 s (ramp rate, 2.2 °C s^{-1}), and
182 extension at 72°C for 20 s (s^{-1} ramp rate, 4.4 °C. s^{-1}). The qPCR reactions were performed in 384-well
183 plates using a LightCycler®480 Instrument (Roche Diagnostics, USA). After qPCR amplification,
184 genotyping using HRM was undertaken. This consisted of a denaturing step at 95 °C for 1 min, followed
185 by a hybridization step at 40 °C for 1 min. Melting curves were then constructed by ramping from 65 to
186 95°C at 0.02°C s^{-1} , taking 25 acquisitions of fluorescence measure per each degree centigrade. A negative
187 (no-template) control was included in the PCR to detect potential DNA contamination or any formation

188 of primer dimers. Primer specificity was evaluated using qPCR analysis, gel electrophoresis and bi-
 189 directional sequencing of each positive qPCR product.

190 For each qPCR reaction, the crossing point (C_p ; corresponding to the cycle threshold [Ct]) was
 191 determined using the “second derivative max method” implemented in the LightCycler®480 v.1.5.0
 192 Software (Roche Diagnostics). The threshold for Ct calculation and normalization regions (leading and
 193 trailing ranges) for qPCR-HRM analysis were defined for each Symbiodiniaceae clade and host locus
 194 using serial dilutions of DNA and standard curves. Only successfully amplified samples with a C_p
 195 value within the linear part of the standard curves were considered for further analysis (Bustin et al.
 196 2009). Individual qPCR efficiencies (E) of the Symbiodiniaceae clades and host reference genes were
 197 calculated using the formula: $E=10^{(-1/\text{slope})}$, as described by Yuan et al. (2006). All primers had a qPCR
 198 efficiency between 1.84 and 2.02 (Table 1).

199 Conversion of quantification cycle values C_p into relative quantities Q_{clade_i} was undertaken for each
 200 DNA concentrations of the standard curves i using clade-specific primer pair using the modified
 201 equation described by Pfaffl (2001) without normalization by a reference gene:

$$202 \quad Q_{clade_i} = E_{clade_i}^{\Delta Ct, clade_i}, \text{ (arbitrary unit)}$$

203 where E_{clade_i} is the amplification efficiency of the clade-specific primer couple i and $\Delta Ct, clade_i =$
 204 $Ct_{clade_i}(\text{calibrator}) - Ct_{clade_i}(\text{sample})$.

205 The calibrator consisted of the maximal concentration of each Symbiodiniaceae clade included in the
 206 linear part of the standard curves.

207 The Q_{clade_i} was calculated for each DNA concentrations in nanograms (ng) from the linear part of each
 208 standard curve i , allowing the derivation of the following equation:

$$209 \quad DNA_{clade_i} = f(Q_{clade_i}) \text{ (ng)}$$

210 Similarly, the family primer pair ‘Symb’ was used to amplify total DNA copies of Symbiodiniaceae in
 211 one reaction such that:

212 $Q_{TotSymb} = E_{Symb}^{\Delta Ct, Symb}$, (arbitrary unit)

213 The $Q_{TotSymb}$ was then calculated for each DNA concentrations from the Symbiodiniaceae standard
 214 curves, subsequently allowing for the derivation of the following equation:

215 $DNA_{TotSymb} = f(Q_{TotSymb})$ (ng)

216 These standard curves allowed for the concentrations of each clade i and total Symbiodiniaceae (per
 217 cell or DNA unit) of the unknown samples to be determined (Pfaffl, 2004). We considered this method
 218 as sufficiently accurate when $DNA_{Symb} = \sum_{i=1}^9 DNA_{clade_i}$ (ng).

219 The quantity of host cells was calculated using the two Q_{ref_i} calculated as described above from the
 220 two host reference genes. The calculated values were plotted against the linear regression of each
 221 corresponding standard curve, where:

222 $Q_{ref_i} = E_{ref_i}^{\Delta Ct, ref_i}$, (arbitrary unit)

223 Finally, the concentration of host cells was assessed by the geometric mean of the Q_{ref_i} such as
 224 described by Hellemans et al., (2007) to normalize relative quantities with multiple reference genes:

225
$$Q_{Host} = \sqrt{\prod_i^2 Q_{ref_i}}$$

226 The Q_{Host} was then calculated for each DNA concentrations from the host standard curves,
 227 subsequently allowing for the derivation of the following equation:

228 $DNA_{Host} = f(Q_{Host})$ (ng).

229 Finally, the linear part of each standard curve allowed for the quantification of both Symbiodiniaceae
 230 and its host using specific and family primers starting from a known quantity of DNA, such that

231 $DNA_{Reaction} = DNA_{Host} + DNA_{Symb}$ (ng)

232 and

$$233 \quad DNA_{Reaction} = DNA_{Host} + \sum_{i=1}^9 DNA_{clade_i} \text{ (ng)}$$

234

235 The HRM data obtained with the family-Symbiodiniaceae primers ‘Symb’ resulted in differences in
236 curve shapes for each Symbiodiniaceae clade which were discriminated using the Gene Scanning
237 module of the LightCycler 480 v.1.5.0 Software (Roche Diagnostics, USA). Melting curve data was
238 adjusted manually as previously described (Meistertzheim et al. 2012), and normalized fluorescence
239 conditions were established and adjusted, with the threshold set to 0 and the sensitivity at 0.26 (Lavergne
240 et al. 2014). Curve shapes and T_m were compared between samples of the same clade and between
241 clades. Shape similarities or differences in the melting curves between samples were assessed visually
242 and mathematically by calculating the root-mean square error (RMSE) values. The latter were calculated
243 by pairwise comparison of the normalized fluorescence data of melting curves obtained for each
244 Symbiodiniaceae clade as recommended by Naue et al. (2014). The intra- and inter-run accuracy of
245 HRM results were estimated by calculating the RMSE values of three runs of triplicate amplifications
246 of all standard curves. The influence of template DNA was also investigated for each Symbiodiniaceae
247 clade and embryos of *A. pulchra* at a DNA concentration range between 100 pg and 500 ng. Finally, to
248 confirm the number of clades present in one sample by HRM, DNA from various clades was mixed in
249 different proportions to obtain reference melting curves of DNA mixtures. DNA from clades A-D, F
250 and G were cross-mixed in pairs using three different proportions of DNA (25:75, 50:50, 75:25) and by
251 using three to five clades combined at equimolar proportion, and at a final DNA concentration of 15 ng
252 DNA μL^{-1} .

253

254 *Validation test of host quantification using other coral species*

255 We then tested the applicability of only the two coral reference genes to be applied to species other than
256 *A. pulchra* because the other primer sets will target Symbiodiniaceae clades whatever the host species.

257 Of the 67 scleractinian species from nine different families previously described in Moorea Island
258 (Bosserelle et al. 2014), twelve species representing eight different families were chosen, including
259 Acroporidae (*Acropora pulchra*, *Astreopora* sp. and *Montipora capitata*), Agariciidae (*Pachyseris*
260 *speciosa* and *Pavona cactus*), Faviidae (*Dipsastraea [Favia] stelligera*), Fungiidae (*Danafungia* sp.),
261 Merulinidae (*Cyphastrea serailia*), Pocilloporidae (*Pocillopora damicornis*), Poritidae (*Porites rus* and
262 *Napopora irregularis*) and Psammocoridae (*Psammocora contigua*).

263 Two or three colony fragments (ca. 0.5-1 cm²) of the coral species were sampled by diving in several
264 reef sites of Moorea and Tahiti Islands. The coral fragments were collected using wire cutters and clamps
265 previously cleaned with sodium hydroxide and ethanol to avoid cross-contamination between samples.
266 Each fragment was collected from the tip of each coral and fixed separately in 70% ethanol.

267
268 Coral samples were washed in DNase-free water before performing DNA extraction, as described
269 above. DNA was diluted to 15 ng μL^{-1} and 30 ng was used to conduct qPCR-HRM assays. To ensure
270 replicability and homogeneity, each plate contained DNA from a representative of the nine
271 Symbiodiniaceae clades in duplicate, as well as Symbiodiniaceae-free coral embryos and a negative
272 control.

273

274 *Comparison of Symbiodiniaceae assemblages in Acropora pulchra at micro and macro-scale*

275 Twenty colonies of *A. pulchra* were collected in September 2012 from Mahana (17°29'18"S;
276 149°53'7"W) and Teavaro (17°30'23"S; 149°45'53"W), two fringing reef sites in Moorea Island (Figure
277 1). These two sites were chosen because *A. pulchra* colonies were found in high abundances at both
278 sites despite contrasting turbidity (0.12 vs 0.04 NTU in Mahana and Teavaro sites, respectively).
279 Turbidity, salinity, temperature, and pH were recorded using the multiparameter HORIBA U50 probe.
280 To examine the spatial distribution of symbiont clades within a host colony, branch fragments
281 (approximately 0.5-1 cm²) were sampled using individual sterilized wire cutters and clamps. Coral
282 samples were collected on both the upward and downward facing sides of coral branches, exhibiting

283 two distinct color morphs (normal [N] and slightly bleached [sB]), representing different micro-variation
284 of the environment; Figure 1C-E). These were preserved in 70% ethanol and stored at -20°C until DNA
285 extraction. DNA extractions were undertaken as described above. Absolute quantification by qPCR
286 assay and HRM was undertaken in duplicate using standard curves of Symbiodiniaceae clades and *A.*
287 *pulchra* embryos with a no-template control in each plate.

288
289 Tests for normality and homoscedasticity on obtained DNA concentrations were performed using the
290 Statistica software v10 (Statsoft) using Shapiro-Wilk and Levene tests. Concentration differences
291 between coral-color fragments and sites for the number of host-cells, total Symbiodiniaceae cells, and
292 cell counts for each specific Symbiodiniaceae clade were compared using non-parametric Kruskal-
293 Wallis and Mann-Whitney *U*-tests.

294

295 **Results**

296 *Quantification of symbiont and host cells*

297 Standard curves generated from increasing Symbiodiniaceae cells ranging from 1×10^3 to 3×10^6 cells per
298 extraction resulted in DNA ranging concentrations of 1 to 200 ng DNA μL^{-1} . For all clades, there was a
299 strong correlation between the DNA concentrations and the cell numbers with correlation coefficient R^2
300 higher than 0.95 (e.g., for clade C $R^2=0.96 \pm 0.03$ SD; Figure S2A). Linear curves were obtained for
301 host cell, with DNA concentrations gradually increasing (9 to 670 ng DNA μL^{-1}) as the number of host
302 cells per extraction rose from 2 to 400 ($R^2=0.996 \pm 0.004$, Figure S2B). Using qPCR analysis,
303 quantification of the total number of Symbiodiniaceae cells was performed using the family primer pair
304 ‘Symb’ with DNA extracted from 1×10^3 to 3×10^6 cells per extraction of all individual clades (Table S4).
305 The resulting standard curves obtained for each clade ($DNA_{TotSymb} = f(Q_{TotSymb})$) indicated high
306 correlation coefficients (mean $R^2=0.96 \pm 0.04$; Figure 2A for clade C). The clade-specific
307 Symbiodiniaceae primers, also had standard curves ($DNA_{clade_i} = f(Q_{clade_i})$) with high coefficient
308 correlation values (mean $R^2=0.96 \pm 0.04$; Figure 2A for clade C and Table S4). We obtained similar

309 specificity and efficiency (E_{Symb}) for qPCR amplifications tested on representative samples from each of
310 the nine clades using the family primers ‘Symb’ (Table S5), suggesting that these loci are conserved
311 within Symbiodiniaceae lineages. Host standard curves, which allowed for the quantification of poly
312 cells, were constructed (2 to 200 cells; Figure 2B) with the highest accuracy obtained between 2 and 50
313 cells ($R^2=0.98 \pm 0.02$), corresponding to DNA concentrations ranging between 10 and 90 ng DNA μL^{-1}
314 (Figure S2B). No DNA amplification was obtained from embryos cells when using primers designed
315 for Symbiodiniaceae, or from dinoflagellates cells when using primers designed for host genes.
316 Duplicates produced gap values between both Ct lower than 0.5.

317

318 *Reproducibility, accuracy and specificity of the High Resolution Melt curve genotyping*

319 The HRM assay using the family locus of 5.8S rDNA for the entire genus Symbiodiniaceae allowed for
320 correct discrimination between all clades except clades A and C when compared to the selected cell
321 cultures and DNA collection (Table S2). Discrimination was explored by visual inspection of the
322 melting curves (Figure 3A and 3B) and by calculating RMSE values (Figure 3C). The visual and
323 mathematical distinction between clades was clearly visible for all Symbiodiniaceae lineages explored
324 in this study, except for clades A and C. However, when different clades are co-occurring, the method
325 effectively detected the presence of heteroduplexes (e.g. AC; see below). Symbiodiniaceae
326 identification by HRM using the family-specific primer pair (Symb) on material derived from cell
327 cultures always corroborated the results from clade-specific primers. The T_m of all analyzed samples
328 ranged between 82°C and 84°C (Figure 3A). Seven clades were easily identifiable and the mean RMSE
329 values for comparisons of samples from the nine clades (Figure 3C) ranged from 2.70 (± 0.16) to 32.56
330 (± 0.03), corresponding to the number of mutations (1-13, Table S3). The lowest RMSE values were
331 obtained when comparing clades A and C (mean 0.84 ± 0.09), whose sequences differed by five
332 mutations (Table 2). Although up to two mutations were present within the reverse (*SymbR*) primer
333 region (Figure S1), similar amplification efficiencies were obtained using the family primer in the clade-
334 specific standard curves (Figure 2), highlighting similar amplification efficiencies between clades

335 (Table S5). No DNA amplification was obtained from our negative controls. Nine replicate reactions
336 per clade and per experimental run, including standard curves of cell cultures and triplicate DNA
337 samples, yielded similar Ct values ($\Delta C_t < 0.5$) and similar melting curves corresponding to low RMSE
338 for intra- and inter-run comparisons (mean values of 1.0 ± 0.3 and 2.2 ± 0.6 , respectively). Similar
339 melting curve shapes were obtained among sub-clade genotypes (Table S2) within five clades (A, B, F,
340 G and H), with RMSE values intra-clades ranging from 0.09 to 2.12 (mean value of 1.25 ± 0.46) (Table
341 S5).

342

343 *Limits of the High Resolution Melt curve method and application to coral tissue samples*

344 We obtained similar melting curve shapes for the nine Symbiodiniaceae clades, with DNA
345 concentrations ranging from 100 pg to 500 ng and a mean RMSE value of 0.68 ± 0.37 . It indicated that
346 low template DNA concentration did not significantly change the melting curve shapes. Melting curves
347 obtained using experimental mixtures of pairwise clade DNA comparisons (using mixed combinations
348 among clades A, B, C, D and F) always displayed more than two peaks, including the mixture of A and
349 C, thus confirming the presence of two different DNA amplifications and their corresponding
350 homoduplexes and heteroduplexes in the reaction (Figure 4A). DNA mixed in the same proportions
351 using three, four and/or five clades always yielded melting curve shapes with three peaks regardless of
352 the proportions used (Figure 4B), confirming the presence of three or more clades in a reaction which
353 was further validated by three or more positive amplifications using the specific primers. However, the
354 intensity of both peaks changed when applying variable proportions of two clades (Figure 4C).

355 The method assumes that the copy number of the locus targeted by the qPCR primers is the same within
356 each clade. However, further investigations are required whether this assumption is valid and can be
357 applied to the absolute quantification of all clades in coral samples.

358 *Validation tests of host quantification using other coral species*

359 For the twelve coral species, amplifications of the host DNA using the host-specific primer sets were
360 positive regardless of the coral species investigated in this study. The Cp values of the host primer set
361 (host genes) always fell within the constructed standard curves using *A. pulchra* embryos, i.e. less than
362 30 PCR cycles (data not shown).

363

364 *Field survey of Acropora pulchra at micro and macro-scales*

365 We analyzed a total of 40 fragments of *A. pulchra* collected from two sites (Mahana and Teavaro) and
366 including two distinct color morphs. Out of the nine existing Symbiodiniaceae clades, only three (A, C,
367 and D) were detected using the qPCR-HRM assay (Figures 5 and 6). Colonies showed significantly
368 higher concentrations of total Symbiodiniaceae DNA per total DNA in Mahana compared to Teavaro
369 (mean of $DNA_{TotSymb} \pm SD = 12.1\% \pm 3.0\%$, and $7.1\% \pm 1.3\%$, respectively, Mann-Whitney *U*-test,
370 $p < 0.001$). The values were similar between the normal and slightly bleached coral fragments within a
371 site (Figure 5). Concentrations of clade A cells were 2-fold higher in colonies from Mahana compared
372 to Teavaro (mean $6.5\% \pm 1.6\%$ and $2.7\% \pm 0.4\%$, respectively; Mann-Whitney *U*-test, $p < 0.001$). Clade
373 C cells were identified almost exclusively in colonies from Teavaro at a mean concentration of $7\% \pm$
374 1% , except for two colonies in Mahana with medium color loss (Figure 6). For these two colonies,
375 fragments collected from a branch (above vs below) harbored different proportions of Symbiodiniaceae
376 (8.2% vs 5.4% , 0% vs 3.0% and 5.6% vs 2.6% , for clades A, C and D, respectively, Figure 6). In contrast,
377 clade D cells were detected exclusively from Mahana colonies (mean $4.8\% \pm 2.6\%$).

378

379 **Discussion**

380 *Quantitative PCR-High Resolution Melt assay*

381 This study developed a new qPCR-HRM assay to explore Symbiodiniaceae clades present in a coral
382 fragment smaller than 1 cm^2 . This assay was coupled with further clade and host specific assays and
383 allowed, for the first time, the absolute quantification of the proportions of each Symbiodiniaceae clade

384 in coral tissues normalized by the number of host cells. This quantification was based on standard curves
385 generated using Symbiodiniaceae-free coral cells and representative DNA or cultures from each of the
386 nine existing clades, the latter being haploid in vegetative stage in both cultured and *in hospite* (Santos
387 and Coffroth 2003). The method assumes that copy number of the locus targeted by the qPCR primers
388 is the same in the cells used as the standard curve template and the cells present in the unknown sample
389 (within each clade). This allows for the establishment of a correlative relationship between the number
390 of living cells in each clade and qPCR values. However, additional research is required to confirm this
391 assumption.

392 The qPCR-HRM assay developed in the present study provides a simple way to identify which
393 clades are present in mixed environmental samples. Granados-Cifuentes and Rodriguez-Lanetty (2011)
394 showed that HRM is a more effective option, than relatively time-consuming molecular fingerprinting
395 methods such as Denaturing Gradient Gel Electrophoresis for identifying mixed-clade combinations.
396 Our results demonstrated that HRM is able to identify each Symbiodiniaceae clade in isolation but failed
397 to differentiate clades A and C due to their identical melting curves. Two amplicons can have the same
398 melting behavior despite sequences not being exactly the same, e.g., due to an erasing effect caused by
399 two mutations (e.g. T to G and G to T) (Druml and Cichna-Markl 2014). However, the present study
400 also demonstrated that when different clades are mixed together, including the mixing of A and C,
401 heteroduplexes are always produced which are easily detectable by HRM because they alter the shape
402 of the melting curves (Wittwer et al. 2003). Our HRM results were also corroborated by clade-specific
403 qPCR amplifications. By combining HRM with qPCR, we gained insights into the absolute
404 quantification of the coral polyp and its symbiont cladal content. Quantitative PCR analyses has
405 previously been described as a powerful method for quantifying Symbiodiniaceae clades A-F (Loram et
406 al. 2007; Mieog et al. 2007; Bay et al. 2016; Rouzé et al. 2016, 2017). These studies quantified the
407 relative proportion of PCR amplicons from each symbiotic clade, standardizing their assays using a
408 single host gene. However, there was no in-depth assessment of the potential effect additional clades
409 (i.e., G, H, and I) may have on the overall assay. Furthermore, differential efficiencies of PCR
410 amplification among clades and host cells were not taken into account possibly leading to an

411 underestimation of clade proportions. These assays also lacked total Symbiodiniaceae cell internal
412 controls. The assay described in the present study quantifies host cells through amplification of two
413 conserved reference host genes, and we showed that this could be applied to twelve coral species
414 representing eight different coral families. Using specific and family primers of the nine
415 Symbiodiniaceae clades and two host genes to construct standard curves of symbiotic and host cells, our
416 method quantifies the total abundance of Symbiodiniaceae and each specific clade composition,
417 normalized per host parameters, in terms of DNA values (in nanograms) or cell numbers, as previously
418 recommended (Cunning and Baker 2014).

419

420 *Limitations*

421 Using high concentrations of DNA can lead to misidentification of clades when using qPCR-HRM
422 assays (Granados-Cifuentes and Rodriguez-Lanetty 2011). In the present study, the upper limit of DNA
423 templates for the assay was not reached with maximal values of DNA for embryos at $670 \text{ ng} \cdot \mu\text{L}^{-1}$ and
424 Symbiodiniaceae at $200 \text{ ng} \cdot \mu\text{L}^{-1}$. We also obtained accurate and reproducible results with 30 ng of total
425 DNA extracted from each coral fragment. The lower limit of the method corresponded to one embryo
426 of two cells, and to 10^3 cells of Symbiodiniaceae. This limit is lower than population sizes measured
427 within planulae ($7.4 \cdot 10^4$ in 1 mm long; Marshall 1932) or within individual fragments of coral colony
428 ($\sim 10^6$ cells cm^{-2} ; Fitt et al. 2000). This method is therefore particularly useful for accurately detecting
429 background Symbiodiniaceae clades within a sample. Unlike High-Throughput Sequencing (HTS)
430 methods (Arif et al. 2014; Edmunds et al. 2014; Quigley et al. 2014; Thomas et al. 2014; Boulotte et al.
431 2016; Hume et al. 2018), our assay is unable to provide information on the total genetic diversity of
432 within-clade assemblages. Nevertheless, HTS methods are relatively costly and time-consuming
433 compared to the qPCR-HRM method presented here, which requires fewer processing steps and
434 produces results within hours and at relatively low cost. This method may effectively complement HTS
435 studies when dealing with large numbers of small individual samples (Cousins et al. 2012; Cristescu
436 2014).

437

438 *Endosymbiotic Symbiodiniaceae genotypes composition of Acropora pulchra at different spatial scales*

439 The ecological role of dominant Symbiodiniaceae genotypes in coral host physiology and stress
440 tolerance has been extensively studied (Rowan and Knowlton 1995; Baker 2003; Coffroth and Santos
441 2005; Stat et al. 2012). However, there is very limited understanding of the natural variability in
442 Symbiodiniaceae cladal abundance distribution *in hospite*, due to micro-environmental conditions
443 (Wangpraseurt et al. 2014, 2015; Cunning et al. 2015b). By applying the qPCR-HRM assay developed
444 in this study we explored the abundance and repartitioning of Symbiodiniaceae clades at different spatial
445 scales in *A. pulchra*. Our work revealed that the composition of Symbiodiniaceae clade assemblages in
446 this coral species varied at all spatial scales investigated, including geographical (between two sites),
447 inter-colony (within each site), and intra-colony (upward versus downward facing surface of the branch),
448 confirming the high degree of spatial variation in endosymbiotic associations with *Acropora* species
449 (Ulstrup and Van Oppen 2003; Ulstrup et al. 2007; Rouzé et al. 2017).

450 At the geographical scale, significantly higher abundance of total Symbiodiniaceae were
451 observed in *A. pulchra* originating from Mahana compared to Teavaro. The abundance of symbiont
452 types can be enhanced by various environment factors, such as nutrient levels or turbidity (Fabricius
453 2005; Cooper et al. 2011a). The higher total Symbiodiniaceae abundance at the Mahana site, which was
454 characterized by higher turbidity corroborates the findings of Bay et al. (2016) that higher
455 Symbiodiniaceae abundance in corals transplanted to inshore locations which are commonly associated
456 with higher dissolved nutrients. As previously observed in Moorea (Rouzé et al. 2017), up to three
457 distinct clades were detected in a single *A. pulchra* colony, which contrasts with findings of Thomas et
458 al. (2014) who reported exclusive clade C dominance in *A. pulchra* colonies from Western Australia. In
459 the present study, significant differences in the proportion of Symbiodiniaceae clades between sites were
460 observed with a co-dominance of clades A and C in Teavaro compared to multi-clades A and D and
461 clades A, C and D in Mahana. Despite the similar temperatures measured at both sites, (ranging from
462 28.7 to 28.9°C), a higher solar irradiance was recorded at Teavaro (average value: 1767 vs 2040 μmol

463 $s^{-1} m^2$). Coral bleaching is often observed when high temperature and solar irradiance levels occur in
464 combination (Brown and Dunne 2008). It has also been shown that, in addition to the symbiont identity,
465 the abundance of Symbiodiniaceae cells within a coral may play a key role in influencing the function
466 of coral-algal symbiosis, with clade C abundant corals being more prone to bleaching than those with
467 abundant clade D or background C in branched corals (Cunning and Baker 2012; Cunning et al. 2016).
468 As such, the lower total Symbiodiniaceae density observed in the clade C dominated *A. pulchra* colonies
469 from Teavaro (i.e., with higher light levels), could be the result of a mild bleaching event at that location,
470 contrasting with the colonies at Mahana where the putative ‘thermo-tolerant’ clade D and ‘opportunistic’
471 clade A co-dominated and were present at higher densities.

472 Although the broad biological flexibility of *Acropora* sp. to interact with a wide range of
473 Symbiodiniaceae genotypes may be advantageous under stable conditions (see Putnam et al. 2012), the
474 high incidence of clade A reported here in both studied sites may represent a lower resilience potential
475 of coral species from Moorea lagoons. It has been suggested that a higher proportion of clade A in corals
476 could increase their susceptibility to disease and *Vibrio* spp. colonization (Rouzé et al. 2016). The
477 omnipresence of clade A symbionts in *A. pulchra* from both sites is of concern for the health and future
478 of corals in Moorea. Understanding the factors that trigger shifts in Symbiodiniaceae clades abundance
479 and composition in corals around Moorea, through long-term analysis at different spatio-temporal
480 scales, is an interesting avenue for future research.

481 Previous studies (Ulstrup et al. 2007; Kemp et al. 2008, 2014) have described the differential
482 distribution of *in hospite* Symbiodiniaceae clades at the micro-scale (i.e., within a colony), but without
483 taking into account the absolute abundances of each clade and without measuring background symbiont
484 genotypes. Interestingly, 22% of fragments collected from a branch (above vs below) of the same colony
485 from Mahana harbored different proportions of Symbiodiniaceae. In this study, micro-scale variations
486 revealed the presence of C-type populations in coral fragments originating from Mahana with lighter
487 colors located below (shaded) compared to the ones located above branches. Although research efforts
488 have focused on revealing the flexibility of coral symbiosis to changing environments and the existence
489 of inter-colony variation among Symbiodiniaceae communities, comparatively little attention has been

490 given to intra-colony variation. Rowan et al. (1997) emphasized intra-colony zonation of
491 Symbiodiniaceae communities in response to environmental irradiance, suggesting micro-scale
492 physiological acclimatization at the level of colony. Skeletal and tissue properties of corals alter *in*
493 *hospite* light and oxygen availability to Symbiodiniaceae cells (Wangpraseurt et al. 2014, 2015), creating
494 different niches at the micro-scale and favoring the establishment of different symbiotic clade
495 partnerships that may help corals adapt to the prevailing conditions (Kemp et al. 2008; Suggett et al.
496 2015). Our study reinforces earlier researches emphasizing the importance of intra-colony variation of
497 Symbiodiniaceae and provides insights into the partitioning of Symbiodiniaceae clades within colonies
498 of *A. pulchra*. Our work highlights the importance of considering micro-niches in studies linking
499 symbiont diversity and coral response to environmental stress to assist in accurately predicting the
500 impact of rising seawater temperature on coral populations and community responses.

501 In the present study, we developed and validated a qPCR-HRM assay. When coupled with clade
502 and host specific qPCRs, it enabled the absolute quantification of endosymbiotic Symbiodiniaceae from
503 all the nine clades from small coral samples, using the combination of family-Symbiodiniaceae and
504 clades-specific primer sets. The method has the potential to provide useful data on the abundance and
505 repartition of the endosymbiont clades at different biological scales (cells, polyp, colony, population and
506 species), as well as different temporal and spatial scales, which may help understand the drivers behind
507 symbiont community changes (Chen et al. 2005), or those following bleaching events (Thornhill et al.
508 2006). The method provides a new tool to describe the complexity of coral-Symbiodiniaceae symbiosis
509 in a rapidly changing world. For example, it may contribute to elucidating the mechanisms governing
510 symbiont shuffling as a result of frequent cumulative thermal stress and be useful to quantify horizontal
511 and vertical transmission of symbionts from parental colonies to larvae, thus providing crucial
512 information for restoration applications.

513

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522

523 **Conflict of Interest:**

524 The authors declare that they have no conflict of interest.

525

526 **Ethical approval:**

527 All applicable international, national, and/or institutional guidelines for the care and use of
 528 animals were followed. Coral collection was performed according to the French Polynesia
 529 regulation.

530

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- 768

769 **Author contribution**

770 A.L.M., and L.H. designed research; A.L.M. performed research; A.L.M. and X.P. analyzed data;
771 A.L.M. and X.P. wrote the paper; and J.F.G., S.A.W. and L.H. revised the paper.

772 **Additional Information**

773 The authors declare no conflict of interest.

774

775 **Figure legend**

776 **Figure S1** Alignment of 5.8S rDNA partial sequences corresponding to the nine existing
777 Symbiodiniaceae clades extracted from Pochon et al., (2014) used for HRM-primer set design.

778 **Figure S2** Linear regressions derived from DNAs extracted from 1×10^3 to 3×10^6 Symbiodiniaceae cells
779 per extraction (A), and for 2 to 400 cells per extraction of *Acropora pulchra* embryos (B).

780 **Figure 1** Sampling locations and the host sampling strategy used to investigate the fine-scale assemblage
781 of Symbiodiniaceae phylogenetic clades in different colonies of *Acropora pulchra* from: (A) Moorea
782 Island (French Polynesia, South-Pacific), and (B) at the two sampling sites of Mahana and Teavaro.
783 *Acropora pulchra* type colony at Mahana (C), and (D) the pictorial description of the design used to
784 sample the normal (N) and slightly bleached (sB) fragments of colonies.

785 **Figure 2** Linear section of the standard curves obtained for the quantification of Symbiodiniaceae cells
786 using the clade-specific (blue) and genus-generalist Symbiodiniaceae primers (green) for a range of
787 1×10^3 to 3×10^6 cells (A). Standard curve obtained for the quantification of host cells corresponding to a
788 range of 2 to 400 *Acropora pulchra* cells (B).

789 **Figure 3** Melt curves and root-mean square error (RMSE) values for the Symbiodiniaceae clade-specific
790 qPCR-High Resolution Melt (HRM) assay. (a) Normalized HRM results using the 5.8S rRNA gene and
791 DNA samples belonging to Symbiodiniaceae clades A to I. (b) Melting peaks derived from the
792 normalized HRM curves presented in A. (c) Mean RMSE value (\pm SD) for the pairwise comparisons of

793 normalized HRM curves between clades presented in a). Dotted line represented the mean RMSE value
794 obtained for pairwise comparisons within clades.

795 **Figure 4** Melting peaks using the 5.8S rRNA gene from mixtures observed in samples (solid line) and
796 *in vitro* mixtures (dotted line) combining two clades (*a*) or three clades (*b*), and clade A and D at different
797 proportions (*c*). Reference melting curves of each clade are shown in grey.

798 **Figure 5** Cellular proportions of specific Symbiodiniaceae clades per total DNA (Symbiodiniaceae +
799 host) measured *via* qPCR- High Resolution Melt for both normal (N) and slightly bleached (sB)
800 fragments of *Acropora pulchra* colonies sampled at the Mahana and Teavaro sites.

801 **Figure 6** Means proportions and standard deviations (SD) of specific Symbiodiniaceae clades per total
802 DNA (Symbiodiniaceae + host) for normal (N) and slightly bleached (sB) fragments of *Acropora*
803 *pulchra* colonies sampled at the Mahana and Teavaro sites. *significant differences at $p < 0.05$,
804 **significant differences at $p < 0.01$, ***significant differences at $p < 0.001$.

805

806 **Table 1.** Combinations of forward and reverse primers used in real-time PCR and High
807 Resolution Melting analyses.

808

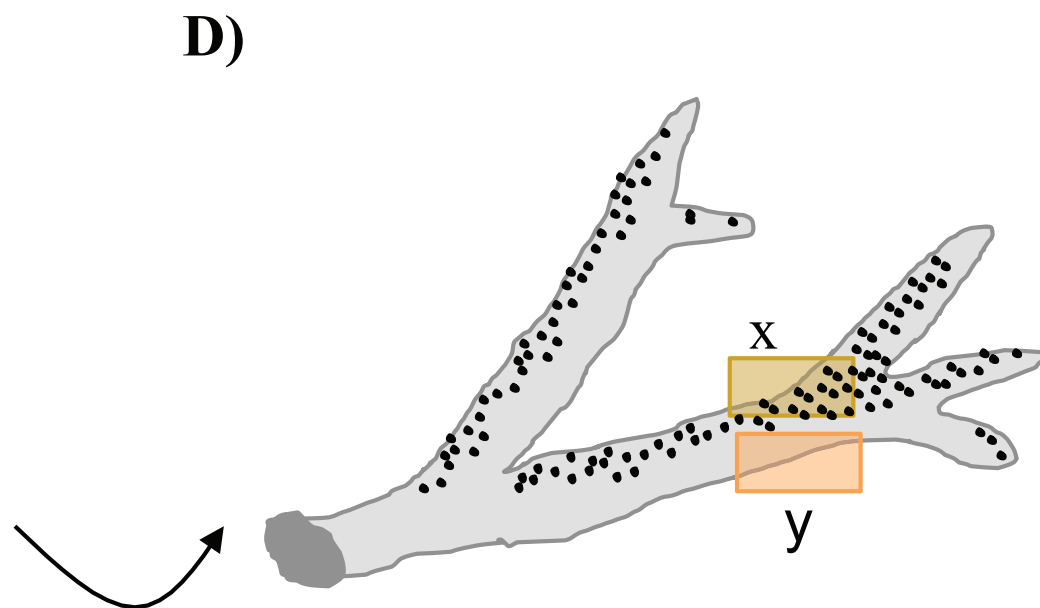
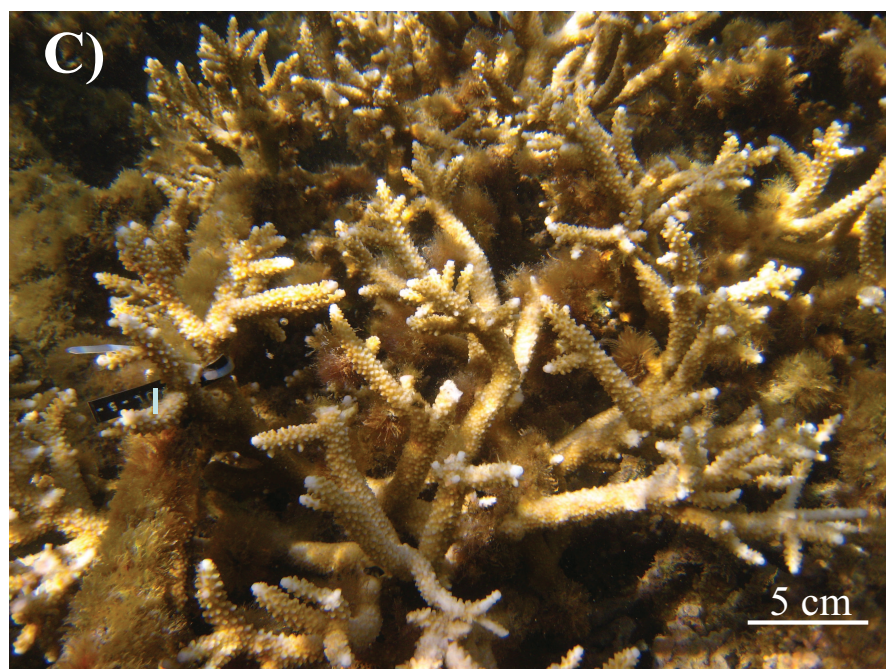
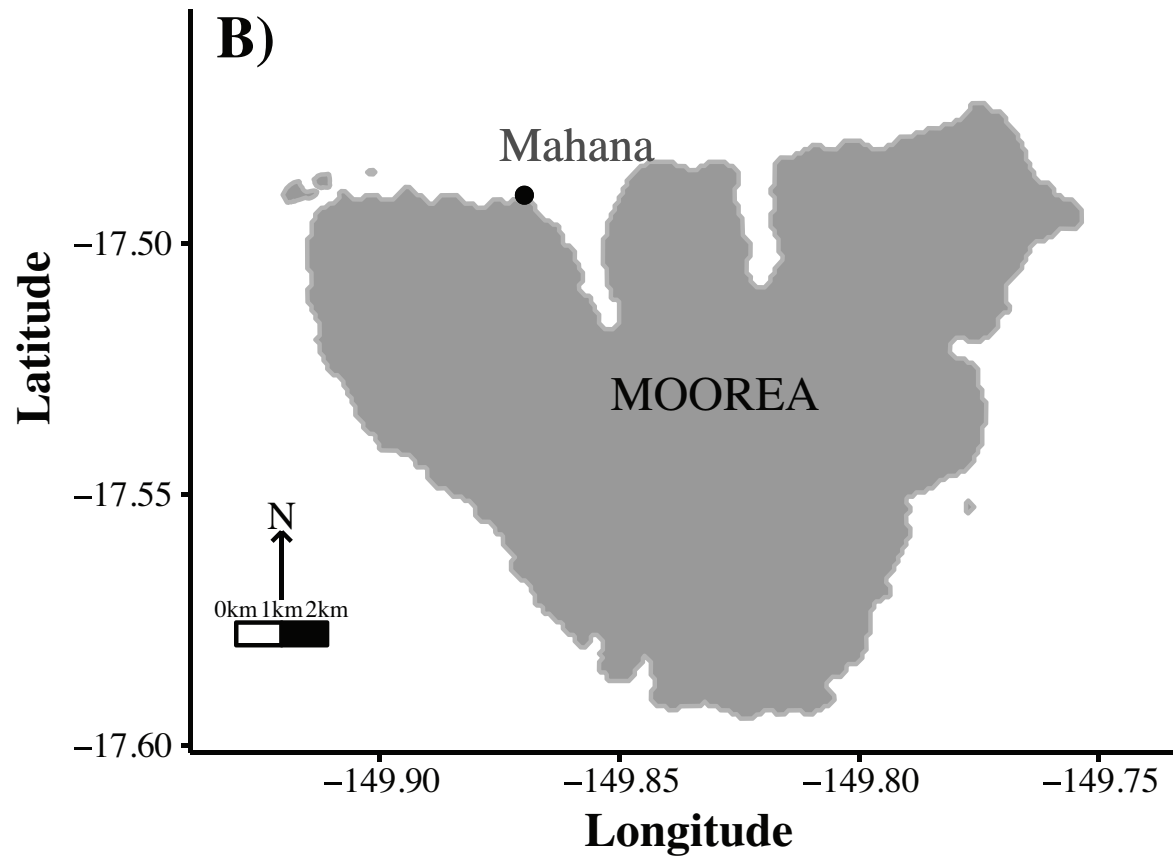
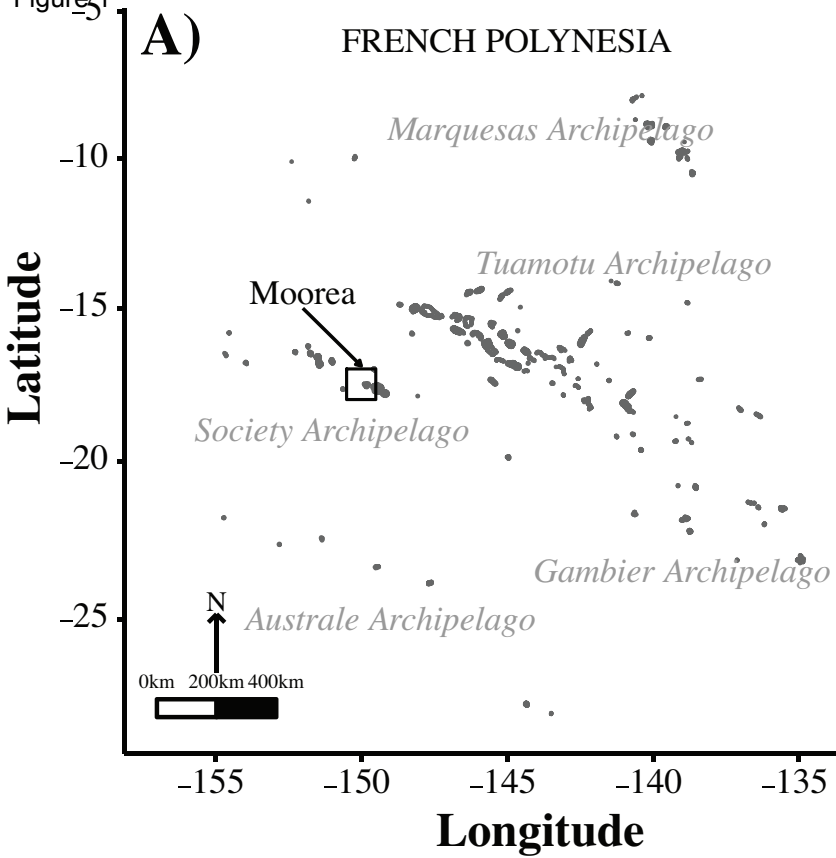


Figure 2

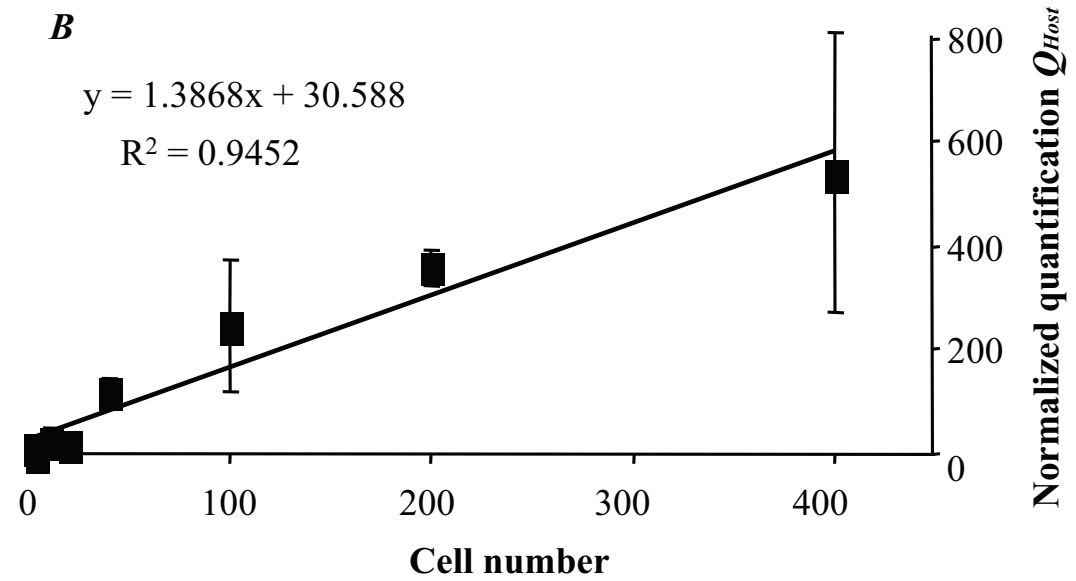
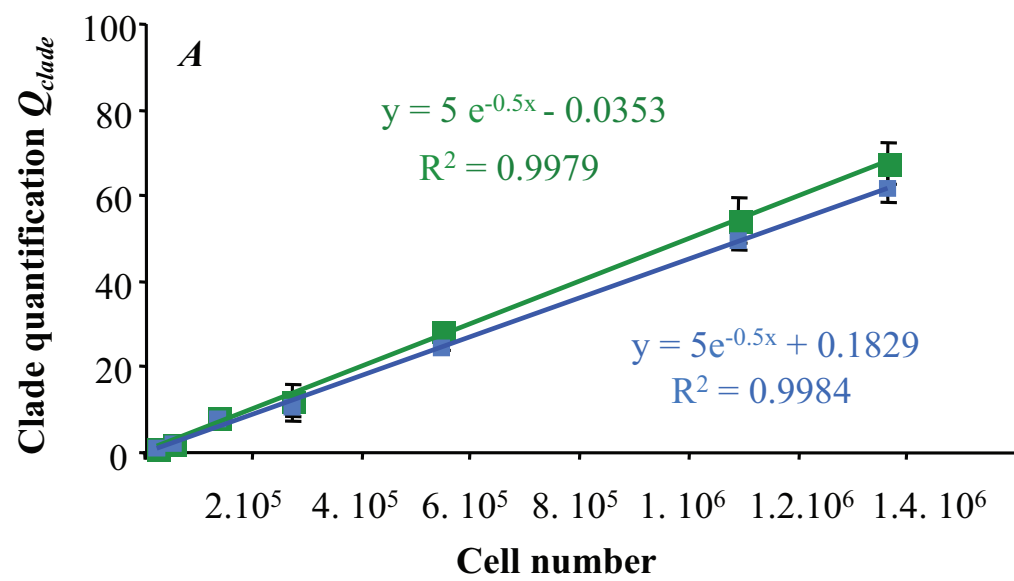


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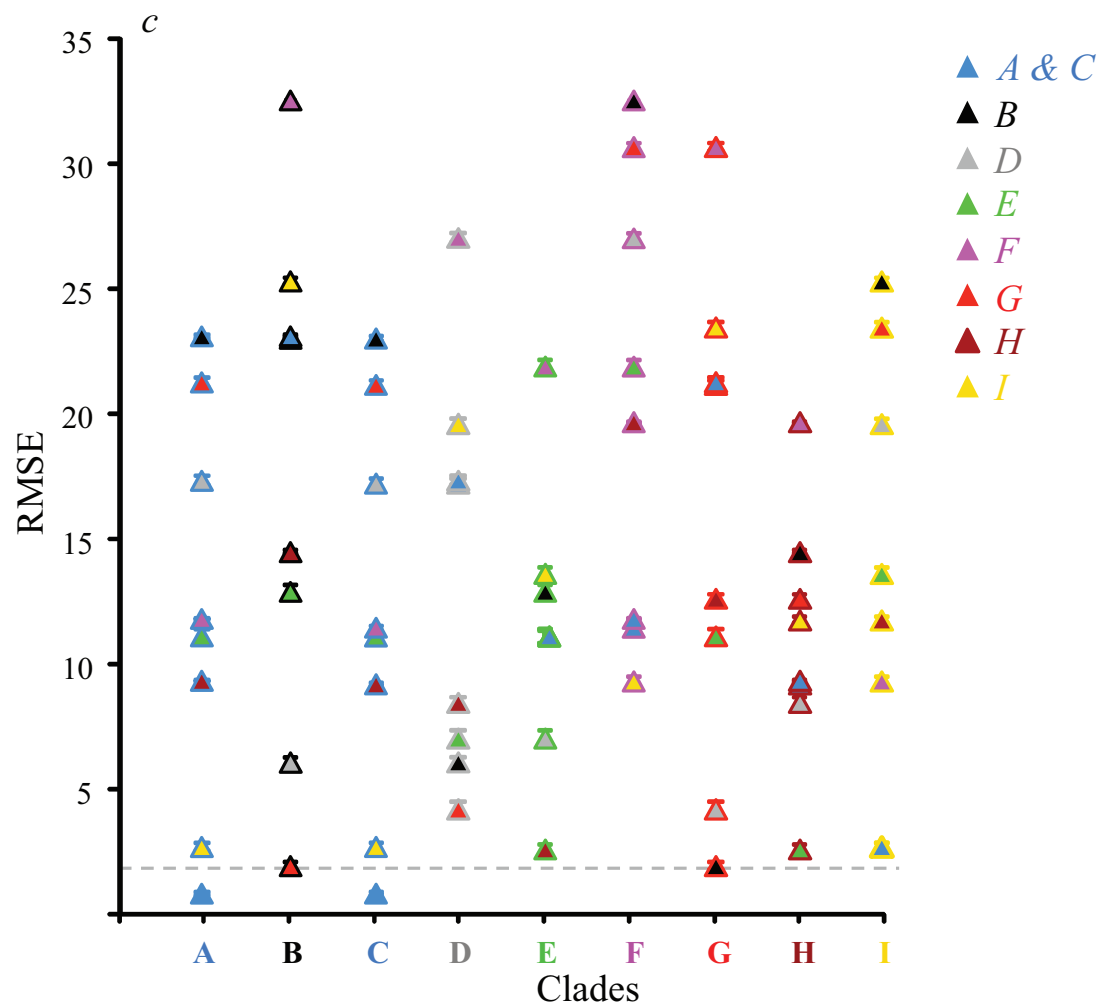
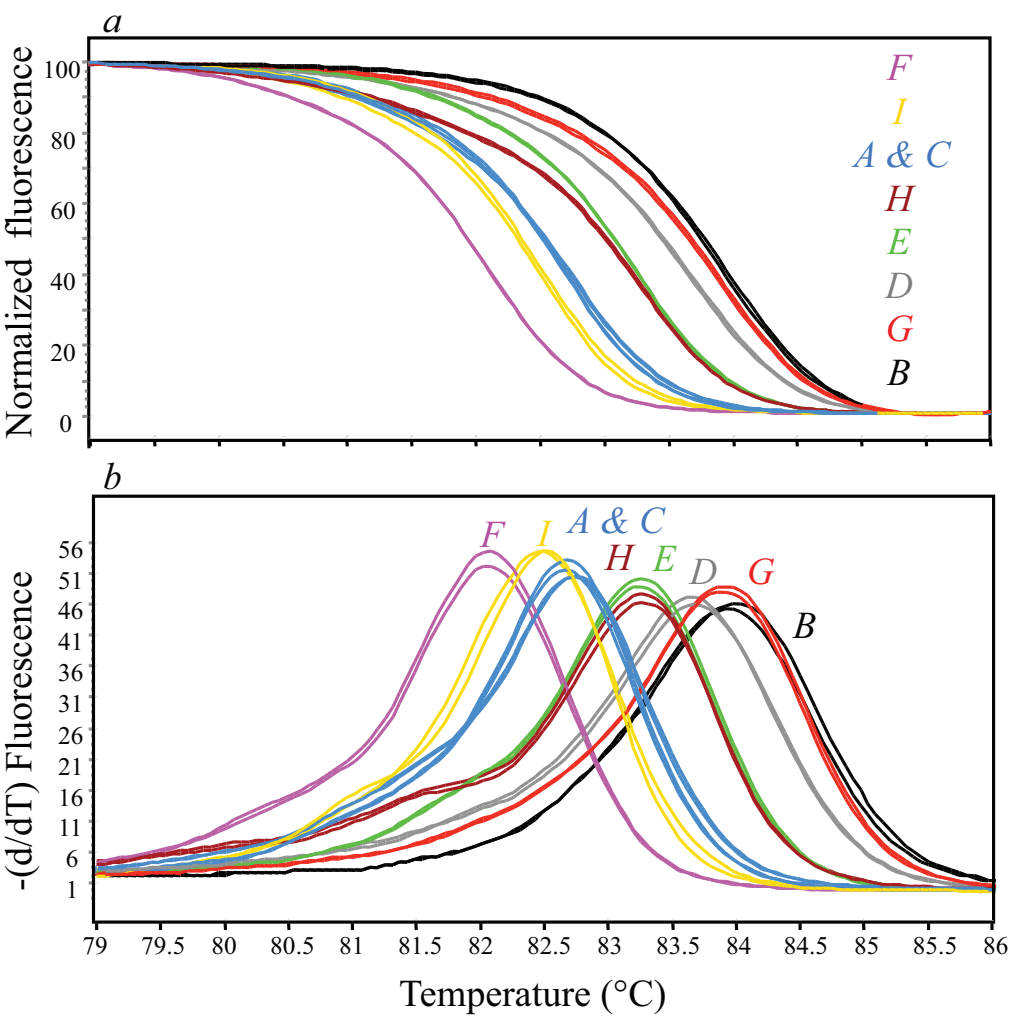


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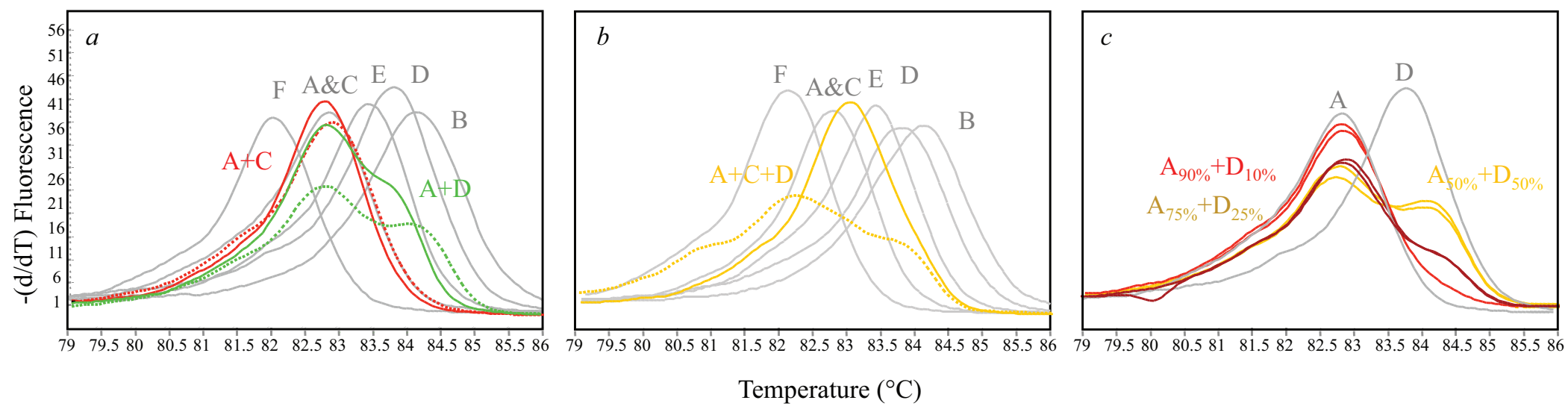


Figure 5

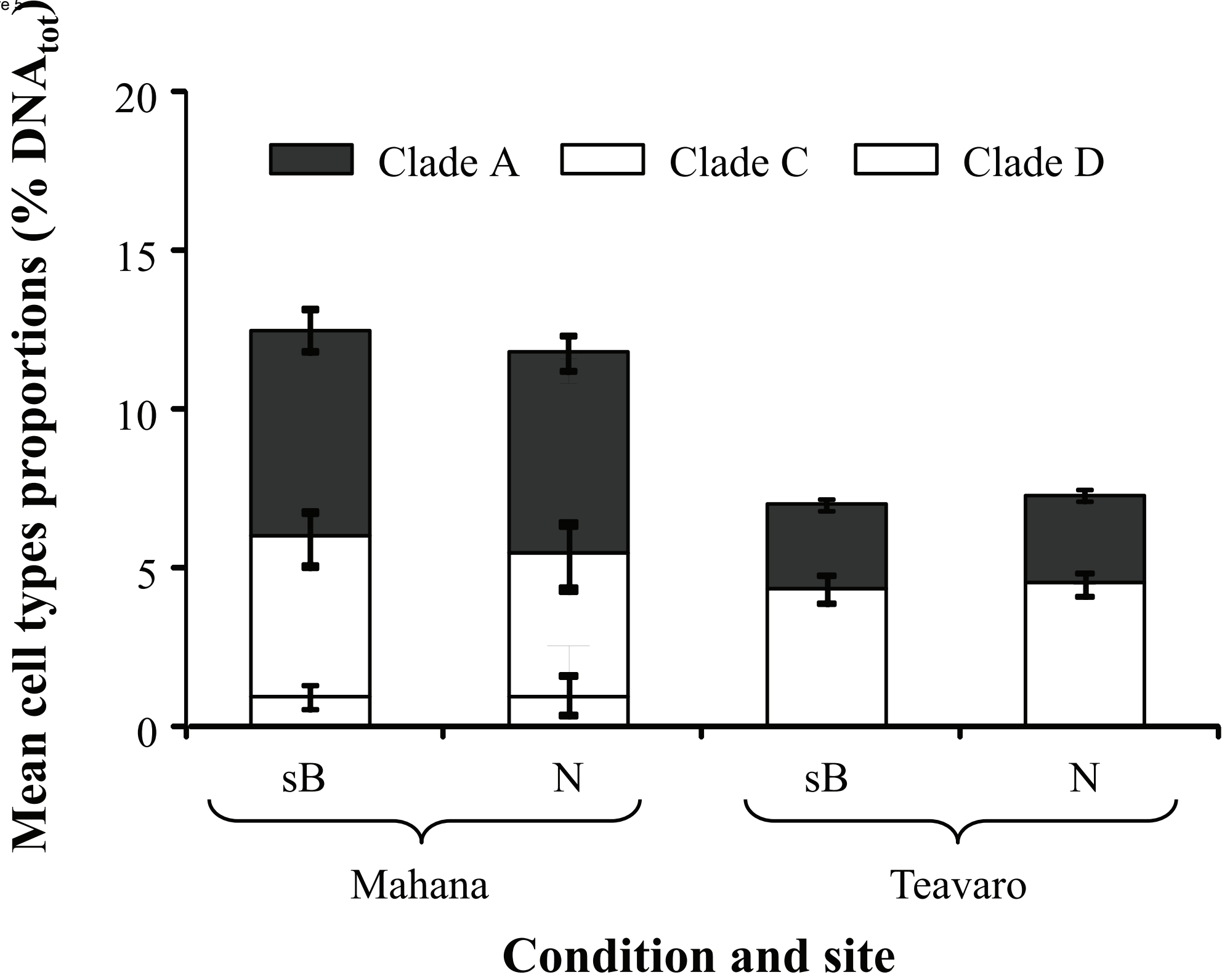


Figure 6

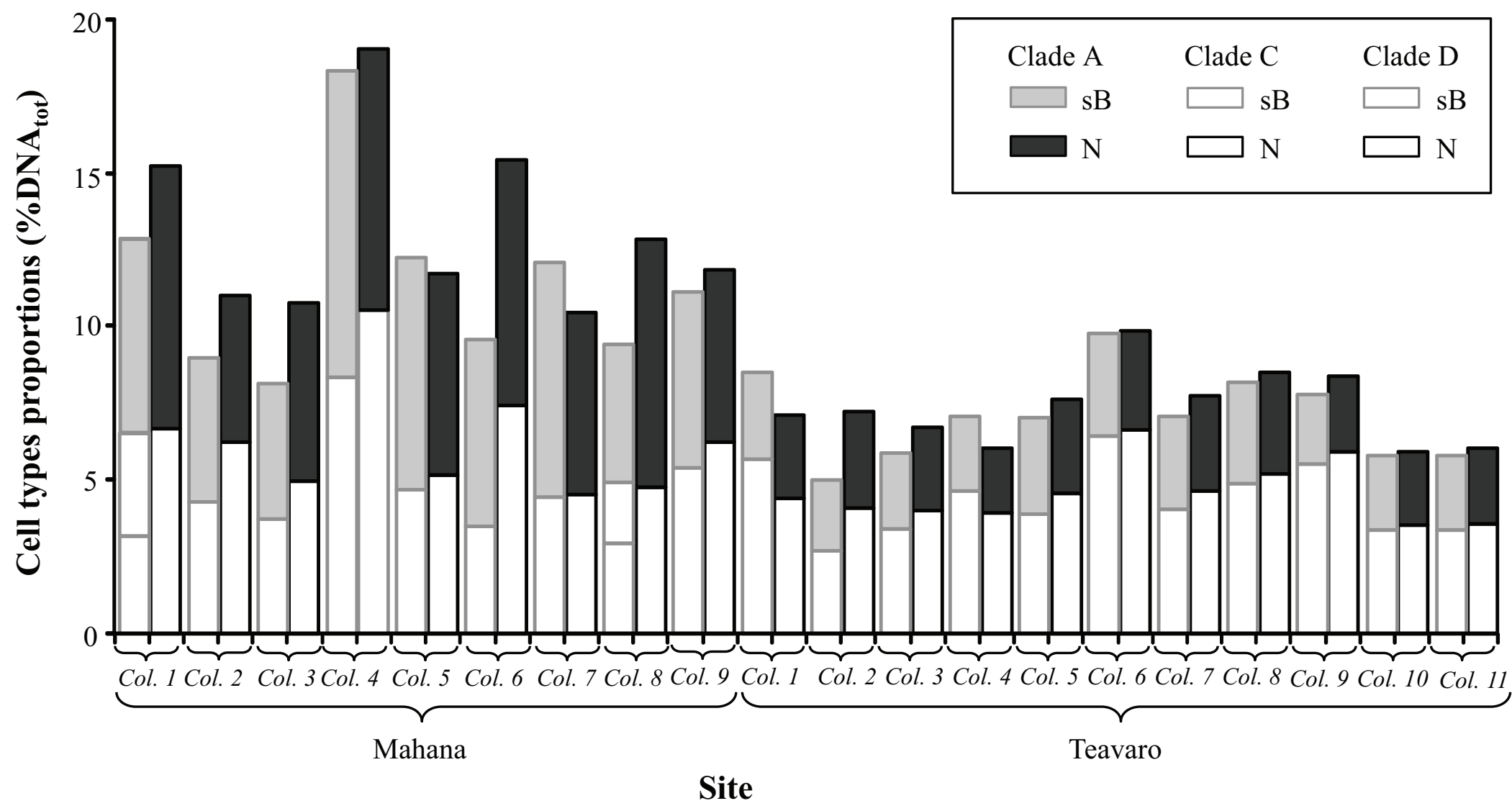


Table 1. Combinations of forward and reverse primers used in real-time PCR and High Resolution Melting analyses.

| Specificity | Target gene | Primer Names Forward / Reverse | Forward (5'-3') | Reverse (5'-3') | Amplicon size (bp) | Efficiency | Reference |
|-------------------------|-------------|--------------------------------|------------------------|----------------------------|--------------------|------------|---------------------------------|
| <i>Symbiont</i> | | | | | | | |
| Symbiodiniacea | 5.8S | SYM_VAR_5.8S2 / Symb_R | GAATTGCAGAACTCCGTGAACC | AGCACTGAAGCAGACATACTCTCAG | 80 | 2 | Hume et al. (2015) / This study |
| Clade A | ITS2 | Aits2_F / Aits2_R | AGCAGTGCTGCTGCATGCT | TGTCTGACTTCATGCTAGGAAGTGTT | 148 | 1,93 | This study |
| Clade B | ITS2 | Bits2_F / Bits2_R | TTCCAACAAGTCATCGATCGC | TGGCGCATGGGCCA | 65 | 1,88 | This study |
| Clade C | Actin | Cact_F / Cact_R | CCAGGTGCGATGTCGATATTC | TGGTCATTCGCTCACCAATG | 96 | 2,02 | Cunning and Baker (2012) |
| Clade D | Actin | Dact_F / Dact_R | GGCATGGGGTAAGCACTTCTT | GATCCTTGAAGTAGCCTTGAAAC | 106 | 1,96 | Cunning and Baker (2012) |
| Clade E | ITS2 | Eits2_F / Eits2_R | TTCCTGGAGAAGCCTTGAG | AGCTTACCTCCCGGTTTGTGTT | 83 | 1,84 | This study |
| Clade F | ITS2 | Fits2_F / Fits2_R | GCCCCTGTGAGCCATTGA | AAGGTGGAATCTTGAATAGAAGCG | 91 | 1,85 | This study |
| Clade G | ITS2 | Gits2_F / Gits2_R | GCCTCGGCGTGTTGTTG | AGCACGTGCATGCTTGCA | 72 | 2 | This study |
| Clade H | ITS2 | Hits2_F / Hits2_R | CCTGCTTTTGGCTTCCTATG | ACTCAAGCAAAGCCGTGCA | 122 | 2 | This study |
| Clade I | ITS2 | Iits2_F / Iits2_R | ACTCCTGCAAGCCATCGCT | TTGCTATGAATCACAAAGCGCT | 90 | 2 | This study |
| <i>Host</i> | | | | | | | |
| <i>Acropora pulchra</i> | Actin | Actin_F / Actin_R | ATCATGAAGTGCGATGTGGACA | CTGCATTCTGTCAGCGATTCC | 99 | 2,01 | This study |
| <i>Acropora pulchra</i> | 5.8S | 5.8S_F / 5.8S_R | CCGGTGGATCTCTTGGCTC | CGCCATTGCGTTCAAAGA | 107 | 1,99 | This study |



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Supplemental data.pdf