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Development of a quantitative PCR-High Resolution Melting assay for absolute measurement of coral-Symbiodiniaceae associations and its application to investigating variability at three spatial scales

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Running title

Absolute quantification of endosymbionts in corals

Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

Absolute quantification assay development

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

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

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

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

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

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

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

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

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

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

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

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

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

Similarly, the family primer pair ‘Symb’ was used to amplify total DNA copies of Symbiodiniaceae in 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)

and

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

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

Validation test of host quantification using other coral species

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

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

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

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

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

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

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

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

Results

Quantification of symbiont and host cells

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

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

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

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

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

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

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

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

Validation tests of host quantification using other coral species

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

Field survey of Acropora pulchra at micro and macro-scales

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

Discussion

Quantitative PCR-High Resolution Melt assay

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

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

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

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

Limitations

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

s⁻¹ m²). Coral bleaching is often observed when high temperature and solar irradiance levels occur in combination (Brown and Dunne 2008). It has also been shown that, in addition to the symbiont identity, the abundance of Symbiodiniaceae cells within a coral may play a key role in influencing the function of coral-algal symbiosis, with clade C abundant corals being more prone to bleaching than those with abundant clade D or background C in branched corals (Cunning and Baker 2012; Cunning et al. 2016). As such, the lower total Symbiodiniaceae density observed in the clade C dominated *A. pulchra* colonies from Teavaro (i.e., with higher light levels), could be the result of a mild bleaching event at that location, contrasting with the colonies at Mahana where the putative ‘thermo-tolerant’ clade D and ‘opportunistic’ clade A co-dominated and were present at higher densities.

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

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

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

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

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Conflict of Interest:

The authors declare that they have no conflict of interest.

Ethical approval:

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

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Author contribution

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

Additional Information

The authors declare no conflict of interest.

Figure legend

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

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

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

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

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

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

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

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

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

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

Figure 2

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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_F	TTCCAACAAGTCATCGATCGC	TGGCGCATGGGCCA	65	1,88	This study
Clade C	Actin	Cact_F / Cact_R	CCAGGTGCGATGTCGATATTC	TGGTCATTGCTCACCAATG	96	2,02	Cunning and Baker (2012)
Clade D	Actin	Dact_F / Dact_R	GGCATGGGGTAAGCACTTCTT	GATCCTTGAAGTAGCCTTGGAAC	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	CCTGCTTTTTCGTTCCATATG	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