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An updated assessment of *Symbiodinium* that associate with common scleractinian corals from Moorea (French Polynesia) reveals high diversity among background symbionts and a novel finding of clade B.

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The adaptative bleaching hypothesis (ABH) states that depending on the symbiotic flexibility of coral hosts (i.e., the ability of corals to “switch” or “shuffle” their algal symbionts), coral bleaching can lead to a change in the composition of their associated *Symbiodinium* community, and, thus, contribute to the coral’s overall survival. In order to determine the ~~capacity by which corals may be flexible~~, molecular tools are required that can provide precise systematic inferences, and detect low levels of coral-associated *Symbiodinium*. Here, we used highly sensitive quantitative (real-time) PCR (qPCR) technology to analyse five common coral species from Moorea (French Polynesia), that were previously only screened using conventional molecular methods, to assess the presence of low-abundance (background) *Symbiodinium*. Similar to other studies, each coral species exhibited a strong specificity to a particular clade, irrespective of the environment. In addition, however, each of the five species harboured at least one additional *Symbiodinium* clade, among clades A-D, at background levels. Unexpectedly, and for the first time in French Polynesia, clade B was detected as a coral symbiont. These results increase the number of known coral-*Symbiodinium* associations from corals found in French Polynesia, and likely indicate an underestimation of the ability of the corals in this region to associate with and/or “shuffle” different *Symbiodinium* clades. Altogether our data suggest that corals from French Polynesia may manage a trade-off between optimizing symbioses with a specific *Symbiodinium* clade(s), and maintaining associations with particular background clades that may play a role in the ability of corals to respond to environmental change.

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ABSTRACT

The adaptative bleaching hypothesis (ABH) states that depending on the symbiotic flexibility of coral hosts (i.e., the ability of corals to "switch" or "shuffle" their algal symbionts), coral bleaching can lead to a change in the composition of their associated *Symbiodinium* community, and, thus, contribute to the coral's overall survival. In order to determine the capacity by which corals may be flexible, molecular tools are required that can provide precise systematic inferences, and detect low levels of coral-associated *Symbiodinium*. Here, we used highly sensitive quantitative (real-time) PCR (qPCR) technology to analyse five common coral species from Moorea (French Polynesia), that were previously only screened using conventional molecular methods, to assess the presence of low-abundance (background) *Symbiodinium*. Similar to other studies, each coral species exhibited a strong specificity to a particular clade, irrespective of the environment. In addition, however, each of the five species harboured at least one additional *Symbiodinium* clade, among clades A-D, at background levels. Unexpectedly, and for the first time in French Polynesia, clade B was detected as a coral symbiont. These results increase the number of known coral-*Symbiodinium* associations from corals found in French Polynesia, and likely indicate an underestimation of the ability of the corals in this region to associate with and/or "shuffle" different *Symbiodinium* clades. Altogether our data suggest that corals from French Polynesia may manage a trade-off between optimizing symbioses with a specific *Symbiodinium* clade(s), and maintaining associations with particular background clades that may play a role in the ability of corals to respond to environmental change.

INTRODUCTION

The foundation of coral reefs is based on the symbiotic association between scleractinian corals and dinoflagellates in the genus *Symbiodinium*. Molecular phylogenetic analyses currently subdivide *Symbiodinium* into nine clades (A-I), each divided further into sub-clades or types based on various molecular techniques (reviewed in Pochon, Putnam & Gates, 2014). However, corals most commonly associate with *Symbiodinium* in clades A-D (reviewed in Baker, 2003), and, in ~~few~~ cases, with members of clades F and G (LaJeunesse et al., 2010; Putnam et al., 2012; Lee et al., 2016). *Symbiodinium* is assumed to provide up to 95% of the energy required for coral metabolic activities (Muscatine & Porter, 1977; Davy, Allemand & Weis, 2012), mostly due to their photosynthetic activity (i.e., production of carbohydrates). In return, the algae benefit by receiving a protected habitat from predation, and a source of inorganic nutrients derived from the host's metabolism. However, this symbiosis can break down, depending on the degree of ~~resistance~~ of either partner, in response to various stressors that may include natural and/or anthropogenic sources [e.g., increasing seasurface temperatures, ocean acidification, and sedimentation; (Pandolfi et al., 2011)].

The overall fitness of a coral colony depends on the biological and functional traits of the various organisms that comprise the coral holobiont, [i.e., the coral host, its *Symbiodinium* assemblages (Mieog et al., 2009b), and other associated microorganisms (e.g. bacteria) (Neave et al., 2016)]. Moreover, some ~~coral-*Symbiodinium* partnerships~~ are characterized as having different sensitivities to environmental conditions, which can ~~be correlated to~~ specific biological characteristics such as morphology (van Woesik et al., 2011). For example, the massive coral *Porites* predominately associates with a thermally tolerant *Symbiodinium*, type C15 (Fitt et al., 2009; Fabricius et al., 2011), and has been shown to exhibit increased resistance to environmental stressors such as temperature anomalies (Penin, Vidal-Dupiol & Adjeroud, 2012), and experience lower mortality and/or bleaching rates compared to those observed for branching corals such as *Acropora* and *Pocillopora* (Penin, Vidal-Dupiol & Adjeroud, 2012). To date, both *in situ* (e.g. Rowan et al., 1997; Baker, 2003; Berkelmans & Van Oppen, 2006; Sampayo et al., 2008) and *in vitro* physiological studies (e.g. Banaszak, 2000; Kinzie et al., 2001; Hennige et al., 2009) suggest that *Symbiodinium* ~~clades~~ are characterized by intrinsic physiological properties that enable them to be differentially suited for various environmental conditions.

Spatial partitioning of different *Symbiodinium* clades may occur within a single coral colony depending on the depth of the colony and/or solar irradiance (Rowan et al., 1997). In addition, coral-*Symbiodinium* associations may be diverse, and can include either mono or multi-clade associations (Fabina et al., 2012; Silverstein, Correa & Baker, 2012). Moreover, these assorted *Symbiodinium* assemblages have been described in different coral colonies from the same species (Cunning, Glynn & Baker, 2013), during coral ontogeny (Abrego, Van Oppen & Willis, 2009), and/or in 'normal' *vs.* 'stressful' environmental conditions (e.g. seawater temperature anomalies) (Berkelmans & Van Oppen, 2006). *Symbiodinium* in clade D have been identified as the predominant algal symbiont in resistant coral colonies during and after massive bleaching events, and/or, more generally, in reefs exposed to local stressors such as sedimentation and eutrophication (Van Oppen et al., 2001; Ulstrup & Van Oppen, 2003; LaJeunesse et al., 2010, 2014; Cooper et al., 2011). These observations highlight the importance of coral-*Symbiodinium* associations with respect to ~~thermo-tolerant capacity~~ (Berkelmans & Van Oppen, 2006; Stat, Carter & Hoegh-Guldberg, 2006; LaJeunesse et al., 2009). Consequently, it has been proposed that corals with flexible associations among various *Symbiodinium* clades (or types), those that result in a range of host-*Symbiodinium* associations, may contain an ecological advantage in the context of environmental change, and is the foundation of the 'Adaptive Bleaching Hypothesis' (ABH) (Buddemeier & Fautin, 1993).

The ABH asserts that there is potential for rapid 'adaptation' of corals facing stressful conditions by a dynamic modification of their *Symbiodinium* community composition either by i) the acquisition of resistant *Symbiodinium* clades from free algae present in the environment (i.e., 'switching') or ii) repopulation by background pre-existing resistant *Symbiodinium* clades (i.e., 'shuffling'). Therefore, in the context of the ABH, coral flexibility (the ability of a coral species to associate with multiple *Symbiodinium* types) is of the utmost importance, and has led to the characterization of coral hosts as either 'specialists' (associating with a specific *Symbiodinium* clade) or 'generalists' (associating with multiple *Symbiodinium* clades) (Fabina et al., 2012; Putnam et al., 2012; Silverstein, Correa & Baker, 2012).

The development of molecular tools with highly sensitive detection capacities such as real-time quantitative PCR (qPCR), which is up to 1000 times more sensitive than conventional methods (e.g. cloning, DGGEs, RFLP) (Mieog et al., 2007), allows for the detection of background symbionts (in addition to the dominant symbionts), and provides a measurable degree of host flexibility among corals (Silverstein, Correa & Baker, 2012). As a result, some studies have suggested that corals may be more flexible than previously thought (Mieog et al., 2007, 2009a; Silverstein, Correa & Baker, 2012). The goal of this study was to investigate the degree of flexibility in host-symbiont partnerships among particular coral species from the under-explored Moorea island in French Polynesia using qPCR. Coral flexibility, considered here as the ability of a coral species to associate with multiple *Symbiodinium* clades or types in different proportions, and which represents one of the main conditions of the ABH, was tested. The presence of *Symbiodinium* clades A-F was quantified among five coral species, *Acropora cytherea*, *Acropora pulchra*, *Pocillopora damicornis*, *Porites rus* and *Pavona cactus.* Although each coral species displayed a high degree of specificity to a dominant *Symbiodinium* clade, all of the coral species hosted multiple *Symbiodinium* clades in low abundance, including partnerships never recorded in French Polynesia.

MATERIALS & METHODS

Choice of coral species

Five coral species, chosen among the most common scleractinian coral genera from the Pacific: *Pocillopora* (*P. damicornis* type β *sensu* Schmidt-Roach et al., 2014; Genbank reference xx-xx), *Acropora* (*A. cytherea* and *A. pulchra*), *Porites* (*P. rus*) and *Pavona* (*P. cactus*), were collected from a fringing reef with a depth 0.5-2.0 m off Moorea island in French Polynesia (17°30'9S, 149°50'9W) (Fig. 1). These five coral species display different biological traits, and were among corals characterized as having varying resistance during severe local bleaching events in 2002 and 2007 (Penin, Vidal-Dupiol & Adjeroud, 2012). *Acropora* is considered the "sentinel" coral genus, described as having high sensitivity to environmental stressors (e.g. McClanahan et al., 2007; Penin et al., 2007; Penin, Vidal-Dupiol & Adjeroud, 2012). Conversely, the genus *Porites* was chosen for its high resistance to stress (e.g. Kayal et al., 2012; Penin, Vidal-Dupiol & Adjeroud, 2012), living in a wide range of various habitats on the island (e.g., sedimentary bays). Finally, the last two genera, *Pocillopora* and *Pavona*, were chosen because they are considered to have intermediate degrees of sensitivity (Penin, Vidal-Dupiol & Adjeroud, 2012).

All of the coral species were sampled during the dry season between August and October 2012, *P. damicornis* (N=27), *P. rus* (N=21) and *A. cytherea* (N=16) were sampled in greater proportions compared to *A. pulchra* (N=6) and *P. cactus* (N=7). Sampling was performed ~~among~~ five contrasting fringing reefs from the lagoon of Moorea island: Mahareapa (Ma) and Vaiare (Va) are exposed to anthropogenic influence, and Teavaro (Te), Linareva (Li) and Tiahura (Ti) are more isolated from human activities (Nahon et al. 2013; Rouzé et al., 2015).

DNA extraction

Small coral fragments (0.5-1 cm^3) were sampled from several areas of the top of each coral colony, ~~were directly placed~~ into a tube underwater, and immediately transferred at the surface into a new 1.5 mL centrifuge tube containing 80% ethanol. All samples were stored at -20°C until DNA extraction. Prior to extraction, all of the ethanol was discarded and the sample was gently rinsed with sterile freshwater to eliminate all traces of mucus. This allows for better targeting of *Symbiodinium* present in the host tissues.

Total coral DNA (i.e., *Symbiodinium*, polyps, and associated micro-organisms) was extracted using a CTAB-based extraction protocol adapted from Mieog *et al.* (Mieog et al., 2009a). To increase the efficiency of DNA extraction, coral samples were incubated in 600 µL of extraction buffer CTAB 2% (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8, 100 mM Tris-HCl pH 8 and 20 µg/mL proteinase K). They were then exposed to 3 cryo-shock cycles (5 min in nitrogen liquid following by 10 min at ambient temperature), and incubated at 60°C overnight while rotating. Next, the CTAB buffer was recovered and placed into new tube in which 600 µL of chloroform/iso-amyl alcohol (24:1 vol/vol) was added. The resulting solution was mixed thoroughly and centrifuged for 15 min at 12000 g (4°C). The aqueous phase was then transferred to a new tube and mixed with 600 µL of isopropanol at 0°C and incubated for 20 min at -20°C. After a new round of centrifugation, the supernatant was discarded and the pellet rinsed with

500 µL of 70% ethanol. After a final centrifugation of 10 min at 12000 g, the ethanol was removed and the DNA pellet air-dried before dilution in 100 µL sterile water (Sigma). All DNA samples were then stored at -40°C.

qPCR assay

Primer set assessment

Six primer sets optimized for the amplification of nuclear ribosomal 28S in *Symbiodinium* clades A-F (Yamashita et al., 2011), and one coral-specific 18S primer set for the coral host (i.e., polyps) were used. The 18S coral host primers (univPolyp-18SF: 5'-ATCGATGAAGAACGCCAGCCA-3' and univPolyp-18SR: 5'CAAGAGCGCCATTTGCGTTC-3') were designed with Primer 3 (Untergasser et al., 2012) from the 18S rDNA sequence alignment (276 sequences) of 18 coral species that are among the most abundant genera found in French Polynesia (*Porites* spp., *Pocillopora* spp., *Acropora* spp., *Montipora* spp., and *Povona* spp.) as well as *Symbiodinium* clades as negative controls.

The quality of the different primer sets for qPCR was confirmed using the evaluation of indicators of specificity and efficiency. Firstly, the specificity of the symbiont and host primer sets were verified with DNA from cultured *Symbiodinium* strains [available from the BURR Collection (http://www.nsm.buffalo.edu/Bio/burr/); clade A: CasskB8 and Flap1, B: Pe and Flap2, C: Mp, D: A001 and A014, E: RT383 and F: Sin and Pdiv44b], and with coral DNA from various species (*Acropora*: *A. pulchra, A. cytherea*, *A. hyacynthus*; *Pocillopora*: *P. damicornis*, *P. verrucosa*, *P. meandrina*; *Porites rus*; *Pavona cactus*; *Montipora* spp. and *Fungia* spp.). A percentage of specificity (Sp: expected with target / unexpected with non-target region) of the symbiont-specific primer sets was calculated according to the formula: Sp= 1- $\sum (100/2^{(Cti-Ctx)})$, where Cti and Ctx are Ct obtained from a specific primer set (Cti) and from other primer sets (Ctx) on the same targeted DNA sample. Secondly, the efficiency of the different primer sets was estimated from the standard curve method based on the log-linear regression of the Ct values with 10-fold serial dilutions of the DNA over 7 concentrations. For both *Symbiodinium* and the coral host, the matrix for dilution was based on a series of known DNA concentrations. In order to mimic multiclade associations and/or the DNA complexity, the matrix was performed by a mixture of several DNA extracts equally concentrated for *Symbiodinium* (70 ng of each clades A-F; one reference strain~~s~~ per clade; clade A: CasskB8, B: Pe, C: Mp, D: A001, E: RT383 and F: Sin), and the coral host (on 1/ mixture multi-specific: 50 ng of the ten coral species mentioned above or 2/ mixture mono-specific: 50 ng of five DNAs from the same coral species for *P. damicornis*, *P. rus* or *A. cytherea*). Additionally, for *Symbiodinium* the standard curve method was applied on a series of known 28S rDNA copy numbers (amplified DNA for clades A-F; Supplementary method), or a series of known cell densities of clade A, C and D isolated from the coral hosts (clade B was not available; Supplementary method). Percentage of efficiency (100% of efficiency indicates that the amount of PCR product doubles during each cycle) was the ratio of the observed slope and the expected slope (-3.322) of the log-linear regression. In addition, the standard curves of efficiency for each corresponding primer set denoted sensitivity, which corresponded to the threshold of Ct ranges to insure an accurate amplification (i.e., the limits of the detectable log-linear range of the PCR).

Quantification of Symbiodinium in coral hosts

Once validated, the absolute quantification of each *Symbiodinium* clade (from A to F) was normalized within coral hosts allowing the comparison between different coral DNA samples and the evaluation of the *Symbiodinium* densities. For each coral DNA sample, a value of polyp unit was estimated by the 18S copy quantification using the standard curve equation (Fig. S1b) in order to normalize the quantification of the *Symbiodinium* clades in 28S copy number, or in cell number per unit of 18S polyp.

All qPCR assays were conducted on a MX3000 Thermocycler (Stratagene) using SYBR-Green. Each reaction was performed, in a final volume of 25 µL containing: 12.5 µL of Brillant® SYBR Green Master Mix reagent, 2.5 µL of both reverse and forward primers diluted at the concentration of 4 µM, and 10 µL of DNA at various concentrations for standard curve analysis or at 1 ng.µL^{-}1 for field sample analysis. The following run protocol was performed: 1 cycle of pre-incubation of 10 min at 95°C; 40 cycles of amplification: 30s at 95°C, 1 min at 60°C or 64°C for *Symbiodinium* and coral host respectively, and 1 min at 72°C; and a final step, for melting temperature curve analysis, of 1 min at 95°C, 30s at 60°C and 30s at 95°C. Each sample was analysed twice on the same plate, as one technical replicate, and averaged when the variation between both Ct values was not exceeding 1 (if not, samples were re-processed until $\Delta Ct \leq 1$). An interplate calibrator (i.e., positive control with known concentrations and Ct values: mixture of DNA from *Symbiodinium* clades A-F), tested in triplicate (one technical replicate), was added to each plate to calibrate Ct values (performed manually on the MxPro software to set the fluorescent threshold to a fixed Ct value) among different plates of coral DNA samples. Positive amplifications were taken into account only when both technical replicates produced Ct values inferior to the estimated threshold ranges (i.e., limit sensitivity to insure an accurate quantification; Table S1) after correction with the interplate calibrator. In addition, all melting curve analyses ensured the specificity of the amplifications (Table S1). For new partnerships between *Symbiodinium* clade(s) and coral species, we further purified the qPCR products (~100 bp) using QiaEx II Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions [GATC Biotech (Cologne, Germany)].

Statistical analysis

For each *Symbiodinium* clade, positively quantified in coral DNA, the symbiont/host ratio (i.e., S/H ratio) were log+1 transformed for further analyses. Slopes, intercepts, and the Pearson correlation coefficient (R^2) were evaluated and compared by pairwise comparisons with Student's t-tests.

Discriminant analysis of principal components (DAPC) on S/H ratios, available for the 5 coral species, was performed in R (package ade4) in order to characterize their preferential endosymbiotic assemblages and densities. Therefore, the discrimination represented by ellipses was applied through the coral species as factor.

RESULTS

Validation and optimization of qPCR assay

For all clade-specific primer sets, the specificity of each qPCR assay was greater than 98%, and was characterized by a unique melting temperature (Table S1), confirming the high accuracy of each primer set to its targeted sequence. All clade-specific primers yielded a good fit linear regression with similar efficiencies close to the desired efficiency of 100% (95-101%; Table S1), strong linear correlations ($R^2 > 0.985$; Fig. S1) between Ct and concentrations of DNA template, and no significant differences among slopes. This indicates that the increase in clade-specific *Symbiodinium* quantity is directly proportional to the number of amplification cycles regardless of whether the tests were performed on DNA from either *Symbiodinium* culture strains (Table S1 and see Yamashita et al. 2011), purified PCR products (Fig. S1a), or from counted *Symbiodinium* cells (Fig. S2). The sensitivity of the clade-specific primers allowed two groups of primer sets to be distinguished. Pairwise comparisons of the intercepts (Student's t-test, $p<0.05$) between the standard regression lines of 28S amplicons (Fig. S1a) showed earlier detection of the primers specific to clades A, B, E and F ($i = 16.36 \pm 0.39$; Fig. S1a) when compared with the clade-specific primers to clades C and D ($i = 19.83 \pm 0.27$; Fig. S1a). From the *Symbiodinium* cell extraction, clade D sensitivity was significantly different from clades A and C (Student's t-tests, pairwise comparisons of slopes: D/A $P < 0.005$ and D/C $P = 0.104$; intercepts: D/A $P < 0.001$ and D/C $P = 0.015$; Fig. S2). The threshold of 28S copy number estimation for each clade A-F, evaluated by the absolute quantification of *Symbiodinium* clades, was effective under 200 copies of the gene (Table S1 and Fig. S1a).

Similarly, the specificity of the coral-specific primer set was confirmed with positive amplifications from 10 coral species and no amplifications with *Symbiodinium* DNA. In addition, the amplification of multi (mixture of 10 coral species) *vs*. mono-specific (mixture of *P. rus*, *P. damicornis* or *A. cytherea*) mixes with the coral-specific primer set yielded a good fit linear regression with similar efficiencies that were close to the desired efficiency of 100% (101%; Table S1b), contained strong linear correlations ($R^2 > 0.99$; Fig. S1b) between Ct and concentration of DNA template, and demonstrated no significant differences among linear correlation slopes and intercepts (Student's t-tests, pairwise comparisons among the 4 DNA mixes: $P > 0.05$). In order to consider the higher complexity of multi-partner coral DNA, we used for subsequent analyses relationships performed on multi-specific mixes of *Symbiodinium* and coral hosts to quantify the different *Symbiodinium* clades in coral DNA samples.

Diversity and flexibility of dominant vs. background *Symbiodinium* clades

Symbiodinium clades A, C and D (among the tested clades A-F) were detected at least once in association with each of the five coral species studied, except for *P. cactus* which was never found associated with clade A (Fig. 2). The ~~expression~~ of these clades either by 28S copy number or by cell density displayed similar order of magnitudes when present, whatever the species (Fig. 2A, B). For some coral species, this represents novel associations for corals from ~~for~~ Moorea: clade C for both *Acropora* species, *A. cytherea* and *A. pulchra*, clade D for *P. cactus*, and clades A and D for *P. rus* (Table 1). The corresponding 28S sequences for these novel coral-*Symbiodinium* partnerships revealed the presence of lineages within sub-clades: A13, C15, C1, and D1 (Table 1; Fig. S3). In addition, *Symbiodinium* clade B was detected in *P. damicornis* (N=2; Fig. 2), albeit in low abundances equivalent to 26 and 183 copies of 28S (4.25 and 6.21 in log+1, respectively Fig. 2A). However, no relationship was available to estimate this clade's cell number. The presence of clade B was confirmed by a match to a sequence within the sub-clade B1 (Genbank reference: XX). Two slight profiles in temperature melting curves were obtained with clade C amplification for *P. rus*. Their sequences showed that each profile corresponded to two distinct lineages within sub-clades (Fig. S3): C1 (Tm~82.95°C; Genbank reference: XX-XX) and C15 (Tm~83.5°C; Genbank reference: XX-XX). In subsequent analyses of the *Symbiodinium* community composition, each clade was expressed by 28S copy number per unit of coral 18S in order to cover clades A-F. The S/H ratio calculation displayed intra and inter-specific variation of the total *Symbiodinium* densities harbored within the host (Fig. 2A), either for a specific clade or from the total *Symbiodinium* density (all clade(s) included).

The occurrence of clades A, B, C, and D led to fifteen possible theoretical patterns among which nine have been observed previously, including assemblages of three clades together (Fig. 2): ACD (*A. cytherea* and *A. pulchra*), BCD (*P. damicornis*) or ABC (*P. damicornis*). However, *Symbiodinium* patterns that include clade B as either a unique clade (B) or as an additional clade (BA, BC, BD, BAD and ABCD) have never been recorded. The absolute quantification of each clade through the S/H ratio led to an estimation of their relative proportion within the coral host (Fig. 2C), and allowed for the their classification as either dominant (>5 %) or background clade(s) (≤5 %; Table 2). *Symbiodinium* clade B, only detected in *P. damicornis*, was always characterized as background regardless of the clade pattern (0.0002-0.0009% of the *Symbiodinium* communities; Table 2), and was systematically associated with at least clade C. All of the other three clades (A, C and D) were observed at least once as background clades, depending on the species and on the clade pattern. For example, clade A was occasionally background in *P. rus* with an AC-pattern (0.0001 % within Li-05 and 0.002 % within Va-03), and was frequently observed as background in *A. cytherea* (<2 %; Table 2). Clade D was background in *P. rus* (0.026 % within Va-05) or *P. cactus* (0.003 % within Ti-05) with a CD-pattern. Clade C was observed as a background clade only once in *P. damicornis* with a CD-pattern (0.04 % within Li-01). In some corals, different *Symbiodinium* clades occurred in ~~closed~~ proportions. For example, clades C (51.07 %) and D (48.93 %) within *P. damicornis* (Li-02; Fig. 2C) exhibited a BCD-pattern, and clades A (57.13 %) and D (42.87 %) showed AD-pattern within *A. cytherea* (Va-03; Fig. 2C).

Selective coral-*Symbiodinium* partnerships

The discriminant analysis of principal components (DAPC; Fig. 3) on the five coral species showed compositional differences among associated communities of *Symbiodinum* according to clade identity and to their density in the host. The first axis (43.9 % of total variance) of the DAPC opposed *Symbiodnium* communities characterized with higher clade D density (Pearson's corelation: $P < 0.001$, $t = 15.7$) from communities composed ~~with~~ higher clade C ~~density~~ (Pearson's correlation: $P < 0.001$, $t = -21.5$) and/or clade B (Pearson's correlation: $P = 0.01$, $t = -2.5$) densities. Clade D was strongly representative of *P. damicornis Symbiodinium* communities (100 % of coral colonies ~~sampling~~), nearly always appearing as a unique clade (24/27 = 89 %; Fig. 2). In contrast, *P. rus* (18/21 = 85.7 %; Fig. 2) and *P. cactus* (6/7 = 85.7 %; Fig. 2) colonies were nearly exclusively composed of mono-clade C communities. However, one *P. cactus* colony also associated with clade D (Fig. 2), underlying a larger ~~variation range of~~ the density of the associated symbiotic communities (wide size of discriminant ellipse, Fig. 3). The second axis (24.9 % of total variance) of the DAPC differentiated *Symbiodinium* communities was composed of clade A (Pearson's correlation: $P < 0.001$, $t = 11.4$), and was comprised of both *Acropora* species. These two species mainly associated with multi-clade communitities (*A. cytherea*: 81% and *A. pulchra*: 67 %) and were distinguished by a second preferential clade in addition to clade A (Figs. 2, 3): D for *A. cytherea* (AD and ACD patterns 11/16 = 68.8 %) and C for *A. pulchra* (AC and ACD patterns 4/6 = 66.7 %).

DISCUSSION

This study analysed the *Symbiodinium* communities of five abundant coral species from Moorea (*A. cytherea*, *A. pulchra*, *P. damicornis*, *P. cactus* and *P. rus*), and found *Symbiodinium* clades A, C and D (from the six clades tested, A-F) in all of the species except *P. cactus*, which was never observed in association with clade A. This is congruent with previous observations that have described these 3 *Symbiodinium* clades as the principal clades inhabiting scleractinian corals (Van Oppen et al., 2005). In contrast, while *Symbiodinium* clade B ~~was~~ commonly reported in Caribbean corals (Rowan et al., 1997; Diekmann et al., 2003; Pettay & Lajeunesse, 2007; Cunning, Silverstein & Baker, 2015) it ~~was~~ rarely reported in corals from the Central Pacific (e.g., LaJeunesse, 2001). This study is the first record of clade B found ~~among~~ corals from French Polynesia (see previous studies by Magalon, Flot & Baudry, 2007; Putnam et al., 2012). Clade B was detected exclusively as a background population in *P. damicornis*, and genotyped as belonging to sub-clade B1. Coincidentally, among the few detections of *Symbiodinium* clade B in corals to date (e.g. LaJeunesse, 2001; Silverstein, Correa & Baker, 2012; Lee et al., 2016), lineages of B1 were found in association with *P damicornis* in Hawaii (LaJeunesse, 2001). In addition, clade B has also been found in Moorea, but as a symbiont with the nudibranch *Aeolidiella alba* (Wecker, Fournier & Bosserelle, 2015).

The rarity and low abundance of B1 lineages in corals from Moorea may be consistent with a previous report in which a B1 type was found to opportunistically associate with bleached *Siderea siderastrea* following a coral bleaching event (LaJeunesse et al., 2009). However, a recent study by Lee et al. (2016) found clade B (type B2) to commonly reside in the host tissues of *Alveopora japonica*. Alternatively, although corals rarely associate with clade B, the function of this symbiosis may represent an, as of yet, unknown ecological niche. However, given the rarity of this association, the significance of this partnership it likely to have minor physiological consequences on the host's survival (e.g. sensitivity to thermal stress; Loram et al., 2007).

The qPCR assays revealed that each of the four clades A-D could be detected at least once at a background level (i.e., ≤5%), a finding that is consistent with previous studies (e.g.

Mieog et al., 2007; Silverstein, Correa & Baker, 2012). In addition, this study increases the number of known background clades, and presents novel partnerships between corals and *Symbiodinium* (e.g. *P. rus* with clades A or D). However, some coral-*Symbiodinium* pairs were not recovered. For example, *P. cactus* was not found to associate with clade A, and *P. rus, P. cactus* and the *Acropora* spp. ~~were not detected~~ with clade B. ~~Alternatively,~~ this could be due to a limited sampling effort among some of the corals (e.g., 6 *A. pulchra* sampled), rather than a selective exclusion by the host species ~~with~~ particular *Symbiodinium* by cellular recognition mechanisms (Silverstein, Correa & Baker, 2012; Davy, Allemand & Weis, 2012). While a majority of background clades were only occasionally detected within some coral species (e.g., clades A and D in *P. rus* or clade B in *P. damicornis*) the presence of clade A in low abundance in *A. cytherea* was nearly exclusive. Consequently, and in this case, the ability of corals to harbour multi-clade *Symbiodinium* communities at background levels may represent a meaningful ecological function that could influence holobiont resistance (Berkelmans & Van Oppen, 2006; Mieog et al., 2007). Indeed, background clades support the potential for dynamic ecological strategies (e.g., switching *vs*. shuffling), as described in the ABH, that could lead to a rapid selective mechanism of tolerant coral-*Symbiodinium* partnerships in response to environmental change (Buddemeier & Fautin, 1993; Baker, 2003).

Despite the observed increase in variation among *Symbiodinium* clade associations for the ~~six~~ coral species studied, each species was restricted to a particular dominant or background *Symbiodinium* clade(s). For example, Clade A was exclusively observed (>95 %; Fig. 1) in the *Symbiodinium* assemblages associated with both *Acropora* species, either as a background clade or as dominant clade. Similarly, other coral species exhibited a principal association ~~to~~ a particular clade: either exclusively, as with clade C in *P. rus* and clade D in *P damicornis*, or dominant, as with clade C in *P. cactus*. Similar to the study by Putnam et al. (2012), which investigated a wide range of various fringing reefs during different seasons (i.e., dry season in this study *vs*. April: wet season), our findings also support *Symbiodinium* specialization to particular clades (LaJeunesse et al., 2008; Stat et al., 2009). The few exceptions of the multi-clade associations found for *P. damicornis* or *P. cactus*, could be explained by a punctual acquisition of *Symbiodinium* clades (Muscatine, 1973; Yamashita et al., 2011), but is unlikely due to the spatial ~~variation~~ of *Symbiodinium* within host colonies (e.g. Rowan & Knowlton, 1995) given our standardized sampling method. Overall, our findings are consistent with corals as ‘specialists’ (low flexibility: specific to particular symbiont(s)) or ‘generalists’ (high flexibility: associated ~~to~~ various symbionts). To further explore symbiont diversity in corals, similar fine-scale molecular approaches (e.g., qPCR, next generation sequencing; see Barbrook, Voolstra & Howe, 2014) should be performed on a wide range of coral species throughout a large geographic range.

The association of coral species with specific clade(s) in this study is consistent with previous reports of stable partnerships between coral hosts and subsets of *Symbiodinium* (e.g. Thornhill et al., 2006, 2009; Suwa, Hirose & Hidaka, 2008; Rouzé et al., 2016). Such symbiotic specificity ~~is~~ derived from the ‘winnowing’ of multiple symbiont types initially present in the host (Nyholm & Mcfall-Ngai, 2004). This process occurs in many mutualisms (e.g. legume-zhizobial bacteria: Hirsch, Lum & Downie, 2001; squid-luminous bacteria: Nyholm & Mcfall-Ngai, 2004), and consists of a complex series of molecular recognition interactions between the host and the symbionts. It is likely that the specific partnerships observed between corals and *Symbiodinium* are derived from various biological traits (Yost et al., 2013), as well as different physiological and ecological attributes among *Symbiodinium* clades (Kinzie et al., 2001;

Berkelmans & Van Oppen, 2006; Hennige et al., 2009; Baker et al., 2013) that ultimately lead to the ecological success of distinct holobionts. For example, the high resistance of *Porites* to a variety of stressors could be explained, in part, by its stable association with *Symbiodinium* type C15 (Putnam et al. 2012). This symbiont has been characterized as thermally tolerant (LaJeunesse et al., 2003; Fitt et al., 2009) and more resilient to extreme environmental conditions compared to other clade C types (LaJeunesse et al., 2003), which may have contributed to an ecological radiation of this *Symbiodinium* type throughout the Indo-Pacific (LaJeunesse, 2005; Pochon et al., 2007). In this study, and similar to a previous report by Putnam et al. (2012), *P. cactus* always associated with *Symbiodinium* in clade C. However, it is likely belonging to type C1 (GenBank reference: XX-XX; Putnam et al. 2012) which is described as thermo-sensitive (Deschaseaux et al., 2014), and that could explain the lower resistance to environmental conditions of the species. Similarly, the ecological sensitivity of branching corals from the genera *Acropora* and *Pocillopora* could be explained, in part, by their specialization with *Symbiodinium* clade A, type A1 (Putnam et al. 2012) and A13, and clade D, type D1/D1a (Putnam et al. 2012) respectively. In some cases, *Symbiodinium* belonging to these clades have been reported as nominal contributors to host metabolism [e.g., growth and reproduction (Little, Van Oppen & Willis, 2004; Jones & Berkelmans, 2010) and/or nutrition (Stat, Morris & Gates, 2008; Cantin et al., 2009; Baker et al., 2013)].

The specialization of coral hosts to particular *Symbiodinium* clades likely represents a driver resulting in stable mutualisms, initiated from selective pressure, that enhances the benefits of specific symbiosis by co-evolution (Douglas, 2008; Thornhill et al. 2014). However, this specificity is contrasted with the detection of additional clades, at trace levels, within the five coral species examined. Lee et al. (2014) suggest that low abundance 'background'

Symbiodinium populations are not necessarily mutualistic but can reflect a transient relative abundance in the surrounding environment, such as non-directional ingestion by polyps leading to ephemeral symbiont shifts (LaJeunesse et al., 2009; Stat et al., 2009; Coffroth et al., 2010). However, the frequent association of *Acropora* corals with clade A in low abundance in this study may represent an ecological advantage for the coral. For example, *Symbiodinium* in clade F were never found in the host tissues of the five coral species examined, despite F being detected in the surrounding environment (Supplementary data: Fig.S4), and described as a dominant symbiont within several coral hosts (e.g., *Alveopora japonica*: Lee et al., 2016). This suggests a combination of physiological controlled process(es) among the coral host and its background *Symbiodinium* communities. Therefore, two opposite selection pressures may be co-occuring: i) the optimization of a symbiosis with a specific clade(s) and/or ii) the maintenance of the ability to integrate several different (but not all) clades in low abundance that could yield an overall benefit to the coral holobiont.

Altogether, these findings emphasize the need to better understand whether those *Symbiodinium* present in low abundance play an ecological role for the holobiont over time, and to further explore the processes that may govern the maintenance of *Symbiodinium* in low abundance in addition to the dominant symbioses that occur with particular clades.

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Figure 1 Map of Moorea island (Archipelago of society, French Polynesia) and the locations of the fringing reefs studied (black circles). Locations with an "x" indicate the sites investigated previously by Putnam et al. (2012). Vaiare (Va), Teavaro (Te), Maharepa (Ma), Tiahura (Ti) and Linereva (Li).

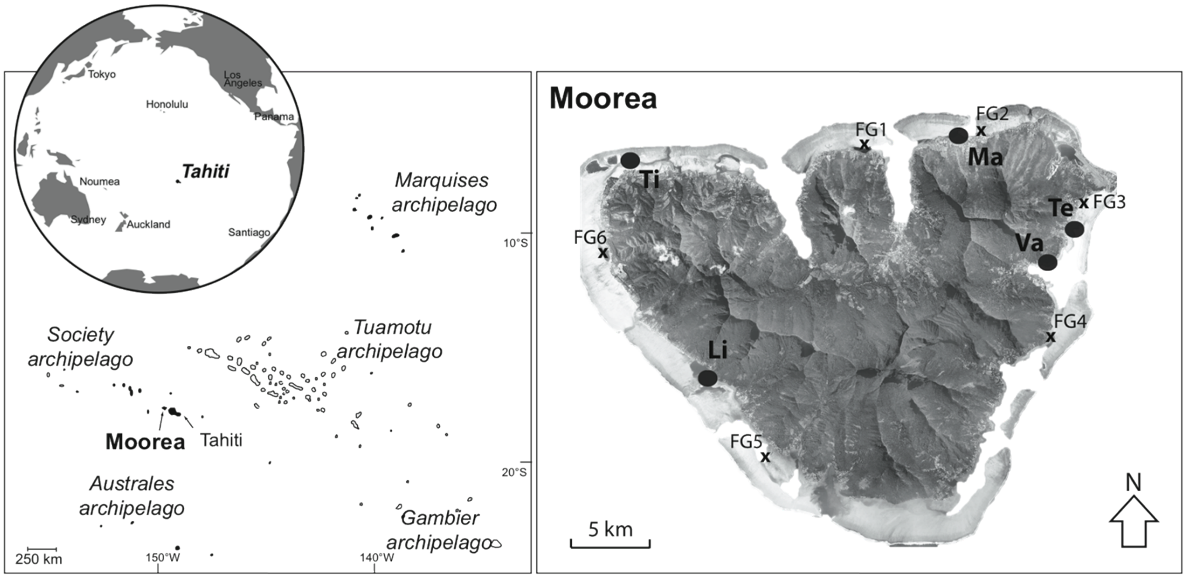

Figure 2 Quantitative composition of different *Symbiodinium* clades observed in association with ACYT: *A. cytherea*, APUL: *A. pulchra*, PCAC: *P. cactus*, PDAM: *P. damicornis* and PRUS: *P. rus* based on: (A) 28S copy number estimation (B) cell number estimation and (C) clade proportions within coral hosts: the grey circles represent the presence of background clades under a 5% threshold (dashed line).

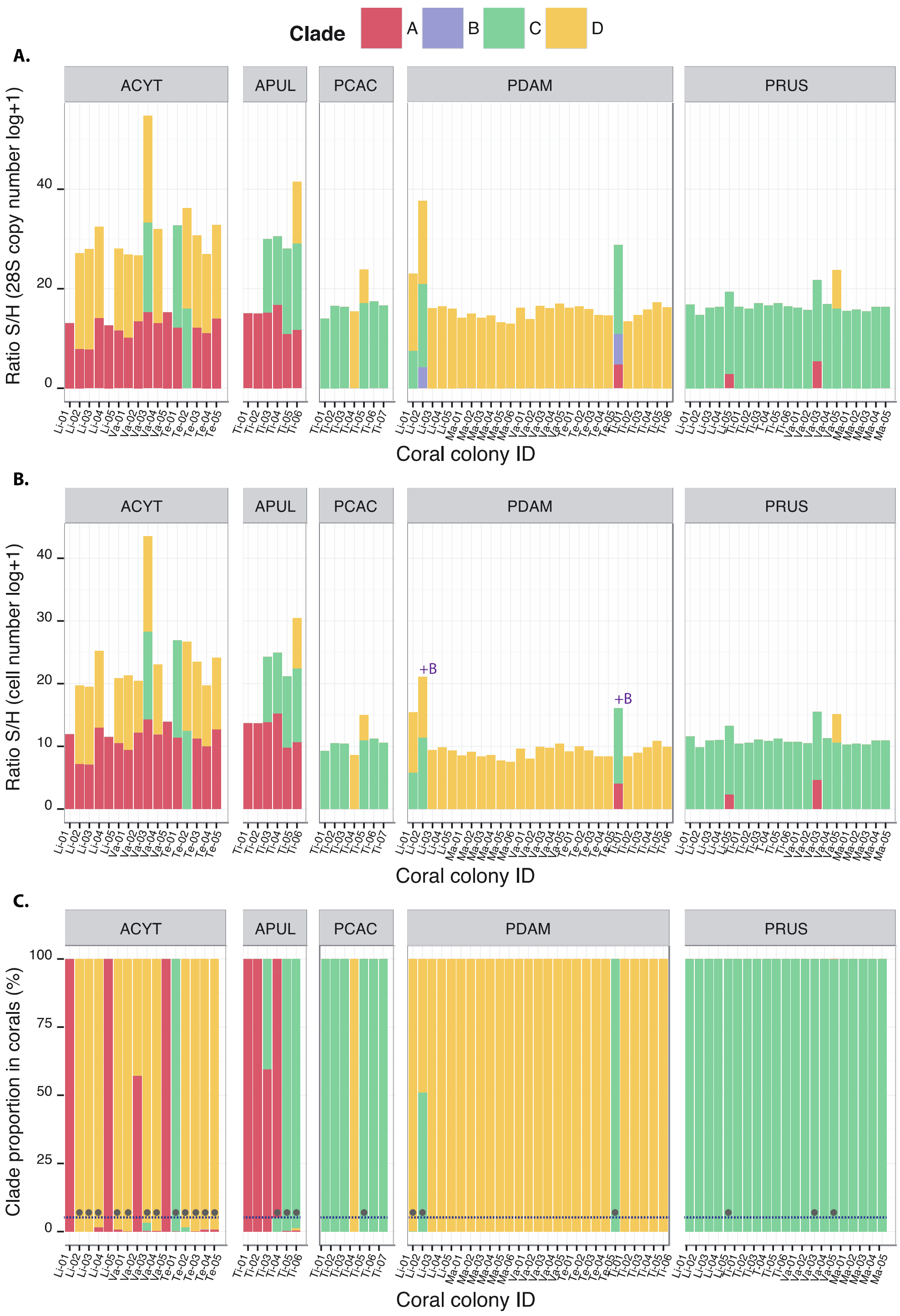
Clade
🟥 A
🟪 B
🟩 C
🟨 D
A.
ACYT
APUL
PCAC
PDAM
PRUS
Ratio S/H (28S copy number log+1)
0
20
40
Coral colony ID
B.
Ratio S/H (cell number log+1)
0
10
20
30
40
+B
+B
Coral colony ID
C.
Clade proportion in corals (%)
0
25
50
75
100
Coral colony ID
Li-01
Li-02
Li-03
Li-04
Li-05
Va-01
Va-02
Va-03
Va-04
Va-05
Te-01
Te-02
Te-03
Te-04
Te-05
Ti-01
Ti-02
Ti-03
Ti-04
Ti-05
Ti-06
Ti-07
Ma-01
Ma-02
Ma-03
Ma-04
Ma-05
Ma-06

Figure 3 Spatio-temporal multivariate analysis of clade A-D quantifications converted in 28S copy number. Axis 1 and 2 of the discriminant analysis of principal component (DAPC) according to the five coral species: *A. cytherea*, *A. pulchra*, *P. cactus*, *P. damicornis* and *P. rus*.

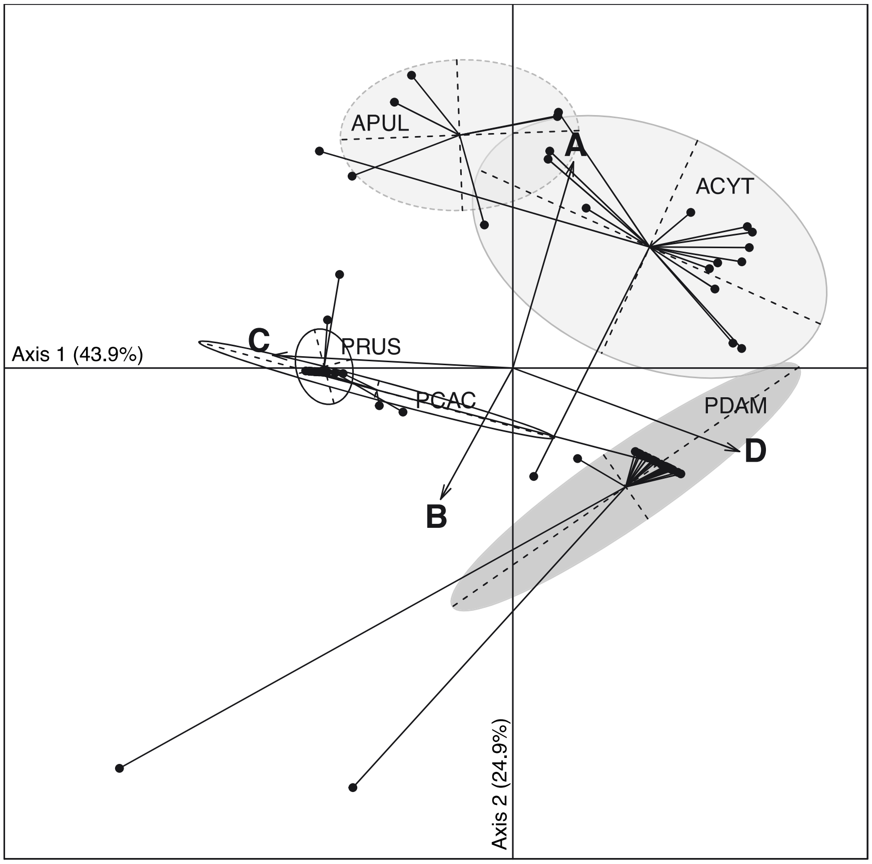

Table 1 Comparative census of *Symbiodinium* clades and types associated with common coral species from Moorea (*A. cytherea*, *A. pulchra*, *P. damicornis*, *P. cactus,* and *P. rus*) detected in a previous report by Putnam et al. 2012 [1] *vs*. the present study.

Coral species	**Previous report** [1]		**Present study**		
	clade(s)	type	clades	* type	* accession nos
A. cytherea	A, D	A1, D1	A, C*, D	* C1	XX
A. pulchra	A, D	A1, D1	A, C*, D		
P. damicornis	A, C, D	DA, A1, C15	A, B*, C, D	* B1	B: XX
P. rus	C	C15	A*, C**, D*	*: A13, D1; C15, **:C1	A: XX, C: XX; D: XX
P. cactus	C	C1, C3, C45	D*	*: D1, **C1	C: XX-XX, D: XX

** novel detected clade from this study*

*** new type of previously reported clade*

Table 2 Proportion of background clades identified within the coral hosts *A. cytherea*, *A. pulchra*, *P. cactus*, *P. damicornis* and *P. rus*.

Species	**Coral ID**	**Background clade proportion**	
A. cytherea	Li-02	A = 0.0012%	
	Li-03	A = 0.0005%	
	Li-04	A = 1.5718%	
	Va-01	A = 0.7750%	
	Va-02	A = 0.1496%	
	Va-04	C = 3.0797%	A = 0.2089%
	Va-05	A = 0.3314%	
	Te-02	A = 0.0242%	
	Te-03	C = 1.5921%	
	Te-04	A = 0.1931%	
	Te-05	A = 0.8460%	
	Te-06	A = 0.7958%	
A. pulchra	Ti-04	C = 5.0116%	
	Ti-05	A = 0.2073%	
	Ti-06	D = 0.7418%	A = 0.3984%
P. cactus	Ti-05	D = 0.0029%	
P. damicornis	Li-01	C = 0.0380%	
	Li-02	B = 0.0002%	
	Ti-01	B = 0.0009%	A = 0.0002%
P. rus	Li-05	A = 0.0001%	
	Va-03	A = 0.0020%	
	Va-05	D = 0.0259%	