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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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 flexibility of corals, molecular tools are required to provide accurate species delineations, and to 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), previously screened using only traditional ~~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

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

60 The overall fitness of a coral colony depends on the biological and functional traits of the
61 various organisms that comprise the coral holobiont, [i.e., the coral host, its *Symbiodinium*
62 assemblages (Mieog et al., 2009b), and other associated microorganisms (e.g. bacteria) (Neave et
63 al., 2016)]. Moreover, some coral holobionts ~~are characterized as having~~ different sensitivities to
64 environmental conditions, which can correlate with specific biological characteristics such as
65 morphology (van Woesik et al., 2011). For example, the massive coral *Porites* predominately
66 associates with a thermally tolerant *Symbiodinium*, type C15 (Fitt et al., 2009; Fabricius et al.,
67 2011), and has been shown to exhibit increased resistance to environmental stressors such as
68 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* species 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 at micro-scales within a single coral colony depending on solar irradiance, or among individual colonies across different depths (Rowan et al., 1997; Kemp et al. 2015). 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; Little, van Oppen & Willis, 2004), 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-tolerance (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 references KY110998-KY111024), *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 at several areas ~~of~~ the top of each coral colony, placed directly 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 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

In order to compare *Symbiodinium* clade amounts between different coral samples, the raw number of 28S copies of each *Symbiodinium* clade (from A to F) was normalized within

coral hosts to evaluate the *Symbiodinium* densities *per* sample. 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

All statistical analyses were performed using R version 3.3.1 software. 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 using R package RVAideMemoire version 0.9-27 (Hervé, 2013).

Discriminant analysis of principal components (DAPC) on S/H ratios, available for the 5 coral species, was performed in R package ade4 version 1.7-4 (Dray & Dufour, 2007) 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 performed and used analyses on multi-specific mixes of *Symbiodinium* and coral hosts to quantify the different *Symbiodinium* clades in coral DNA samples.

276

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

278 *Symbiodinium* clades A, C and D (among the tested clades A-F) were detected at least
279 once in association with each of the five coral species studied, except for *P. cactus* which was
280 never found associated with clade A (Fig. 2). The quantification of these clades either by 28S
281 copy number or by cell density displayed similar orders of magnitudes when present, whatever
282 the species (Fig. 2A, B). For some coral species, this represents novel associations for corals
283 from Moorea: clade C for both *Acropora* species, *A. cytherea* and *A. pulchra*, clade D for
284 *P. cactus*, and clades A and D for *P. rus* (Table 1). The corresponding 28S sequences for these
285 novel coral-*Symbiodinium* partnerships revealed the presence of lineages within sub-clades: A13,
286 C15, C1, and D1 (Table 1; Fig. S3). In addition, *Symbiodinium* clade B was detected in
287 *P. damicornis* (N=2; Fig. 2), albeit in low abundances equivalent to 26 and 183 copies of 28S
288 (4.25 and 6.21 in log+1, respectively Fig. 2A). However, no relationship was available to
289 estimate this clade's cell number. The presence of clade B was confirmed by a match to a
290 sequence within the sub-clade B1 (Fig. S3: PDAM2_Moo). Two slightly different profiles in
291 temperature melting curves were obtained with clade C amplification for *P. rus*. Their sequences
292 showed that each profile corresponded to two distinct lineages within sub-clades (Fig. S3): C1
293 (Tm~82.95°C; Fig. S3: PRUS5_Moo and PRUS6_Moo) and C15 (Tm~83.5°C; Fig. S3:
294 PRUS3_Moo and PRUS4_Moo). In subsequent analyses of the *Symbiodinium* community
295 composition, each clade was expressed by 28S copy number per unit of coral 18S in order to
296 cover clades A-F. The S/H ratio calculation displayed intra and inter-specific variation of the
297 total *Symbiodinium* densities harbored within the host (Fig. 2A), either for a specific clade or
298 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. Using the *Symbiodinium* densities (S/H ratio) within the coral host (Fig. 2C), relative proportions were determined, and allowed for 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 more even 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*~~

322 according to clade identity and to their density in the host. The first axis (43.9 % of total
323 variance) of the DAPC opposed *Symbiodnium* communities characterized with higher clade D
324 density (Pearson's corelation: $P < 0.001$, t = 15.7) from communities composed of higher clade
325 C (Pearson's correlation: $P < 0.001$, t = -21.5) and/or clade B (Pearson's correlation: $P = 0.01$,
326 t = -2.5) densities. Clade D was strongly representative of *P. damicornis Symbiodinium*
327 communities (100 % of coral colonies sampled), nearly always appearing as a unique clade
328 (24/27 = 89 %; Fig. 2). In contrast, *P. rus* (18/21 = 85.7 %; Fig. 2) and *P. cactus* (6/7 = 85.7 %;
329 Fig. 2) colonies were nearly exclusively composed of mono-clade C communities. However, one
330 *P. cactus* colony also associated with clade D (Fig. 2), underlying a larger range of variation in
331 the density of the associated symbiotic communities (wide size of discriminant ellipse, Fig. 3).
332 The second axis (24.9 % of total variance) of the DAPC differentiated *Symbiodinium*
333 communities was composed of clade A (Pearson's correlation: $P < 0.001$, t = 11.4), and was
334 comprised of both *Acropora* species. These two species mainly associated with multi-clade
335 communitities (*A. cytherea*: 81% and *A. pulchra*: 67 %) and were distinguished by a second
336 preferential clade in addition to clade A (Figs. 2, 3): D for *A. cytherea* (AD and ACD patterns
337 11/16 = 68.8 %) and C for *A. pulchra* (AC and ACD patterns 4/6 = 66.7 %).
338
339 **DISCUSSION**
340 This study ~~analysed~~ the *Symbiodinium* communities of five abundant coral species from
341 Moorea (*A. cytherea*, *A. pulchra*, *P. damicornis*, *P. cactus* and *P. rus*), and found *Symbiodinium*
342 clades A, C and D (from the six clades tested, A-F) in all of the species except *P. cactus*, which
343 was never observed in association with clade A. This is congruent with previous observations
344 that have described these three *Symbiodinium* clades as the ~~principal~~ clades inhabiting

scleractinian corals (van Oppen et al., 2005). In contrast, while *Symbiodinium* clade B is commonly reported in Caribbean corals (Rowan et al., 1997; Diekmann et al., 2003; Pettay & Lajeunesse, 2007; Cunning, Silverstein & Baker, 2015) it is rarely reported in corals from the Central Pacific (e.g., LaJeunesse, 2001). This study is the first record of clade B found associated with 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 Pacific corals to date (e.g. LaJeunesse, 2001; Silverstein, Correa & Baker, 2012; Parkinson, Coffroth & Lajeunesse, 2015; 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 low abundances of clade B may also have come from an exogenous source (e.g., surface environmental cells), and, therefore, represents a non-symbiotic interaction with the host. However, the strict conditioning of the samples during DNA extraction (e.g., eliminating traces of mucus; described in Rouzé et al., 2016), and the absence of any detection of clade B in the other coral species from the same sampling site largely reduce this hypothesis. Instead, 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 cold-bleached *Pocillopora* colonies following a coral bleaching event (LaJeunesse et al., 2010). 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 Pacific 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. did not associate with clade B. 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 or symbiont to a particular partner 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, the ability of corals to harbour multi-clade *Symbiodinium* communities at background levels may be due to the environmental history of Moorea island, which has experienced a variety of massive bleaching events (Penin, Vidal-Dupiol & Adjeroud, 2012), and~~,~~ therefore~~,~~ represents 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 five 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 a dominant clade. Similarly, other coral species exhibited a ~~principal~~ association with 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 consistent associations of coral species to particular *Symbiodinium* clades (LaJeunesse et al., 2008; Stat et al., 2009). T~~he~~ few exceptions of the multi-clade associations found for *P. damicornis* or *P. cactus* could be preferentially attributed to a transient acquisition (Muscatine, 1973; Yamashita et al., 2011; Lee et al. 2016) ~~of *Symbiodinium* clades~~, rather than ~~to a~~ spatial partitioning 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 with 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) observed among Moorea's reefs is consistent with previous reports of stable partnerships between coral hosts and subsets of

413 *Symbiodinium* (e.g. Thornhill et al., 2006, 2009; Suwa, Hirose & Hidaka, 2008; Rouzé et al.,
414 2016). Such symbiotic specificity can be derived from the 'winnowing' of multiple symbiont
415 types initially present in the host (Nyholm & Mcfall-Ngai, 2004). This process occurs in many
416 ~~mutualisms~~, (e.g. legume-zhizobial bacteria: Hirsch, Lum & Downie, 2001; squid-luminous
417 bacteria: Nyholm & Mcfall-Ngai, 2004; cnidarian-*Symbiodinium*: Wolfowicz et al., 2016), and
418 consists of a complex series of molecular recognition interactions between the host and the
419 symbionts. It is likely that the specific partnerships observed between corals and *Symbiodinium*
420 are derived from various biological traits (Yost et al., 2013), as well as different physiological
421 and ecological attributes among *Symbiodinium* clades (Kinzie et al., 2001; Berkelmans & van
422 Oppen, 2006; Hennige et al., 2009; Baker et al., 2013) that ultimately lead to the ecological
423 success of distinct holobionts. For example, the high resistance of *Porites* to a variety of stressors
424 could be explained, in part, by its stable association with *Symbiodinium* type C15 (Putnam et al.,
425 2012). This symbiont has been characterized as thermally tolerant (LaJeunesse et al., 2003; Fitt
426 et al., 2009) and more resilient to extreme environmental conditions compared to other clade C
427 types (LaJeunesse et al., 2003), which may have ~~contributed to an~~ ecological radiation ~~of this~~
428 ~~*Symbiodinium* type~~ throughout the Indo-Pacific (LaJeunesse, 2005; Pochon et al., 2007). In this
429 study, and similar to a previous report by Putnam et al. (2012), *P. cactus* always associated with
430 *Symbiodinium* in clade C. However, it likely belongs to type C1 (Fig. S3; Putnam et al., 2012)
431 which is described as thermo-sensitive (Deschaseaux et al., 2014), and that could explain the
432 lower resistance to environmental conditions of the species. Similarly, the ecological sensitivity
433 of branching corals from the genera *Acropora* and *Pocillopora* could be explained, in part, by
434 their specialization with *Symbiodinium* clade A, ~~type A1 (Putnam et al., 2012~~) and A13, and
435 clade D, type D1/D1a (Putnam et al., 2012) respectively. Indeed, while both clades A and D are

436 ~~characterized~~, with eco-physiological benefits for the holobiont, including photo-protective and
437 thermo-tolerance abilities, respectively, ~~it has been mainly~~, described in stressful vs. non-stressful
438 conditions. This duality likely corresponds to trade-offs between coral host resistance and low
439 energetic budget contributions (reviewed in Lesser, Stat & Gates, 2013). In some cases,
440 *Symbiodinium* belonging to these clades have been reported as nominal contributors to host
441 metabolism [e.g., growth and reproduction (Little, van Oppen & Willis, 2004; Jones &
442 Berkelmans, 2010) and/or nutrition (Stat, Morris & Gates, 2008; Cantin et al., 2009; Baker et al.,
443 2013)].

444 The specialization of coral hosts to particular *Symbiodinium* clades likely represents a
445 driver resulting in stable mutualisms, initiated from selective pressure, that enhances the benefits
446 of specific symbiosis ~~by~~, co-evolution (Douglas, 2008; Thornhill et al., 2014). However, this
447 specialization is contrasted with the maintenance of the horizontal transmission of symbionts in
448 the majority of coral species as well as the detection of additional clades, at trace levels, within
449 the five coral species examined. Lee et al. (2016) suggest that low abundance 'background'
450 *Symbiodinium* populations are not necessarily mutualistic but can reflect a transient relative
451 abundance in the surrounding environment, such as non-directional ingestion by polyps leading
452 to ephemeral symbiont shifts (LaJeunesse et al., 2009; Stat et al., 2009; Coffroth et al., 2010).
453 Nevertheless, every *Symbiodinium* species may not be transiently ingested. For example,
454 *Symbiodinium* clade F was never found in the host tissues of the five coral species examined,
455 despite clade F being detected in the surrounding environment (Supplementary data: Fig.S4),
456 and, although in a temperate environment, described as a dominant symbiont within
457 *Alveopora japonica* (Lee et al., 2016). This suggests a combination of ~~physiological~~ controlled
458 processes among the coral host and its background *Symbiodinium* communities. Therefore, two

459 opposite selection pressures may be co-occuring in the context of Moorea's reef environment
460 (which has been exposed to consecutive massive bleaching events in the past): i) the
461 optimization of a symbiosis with a specific clade(s) and/or ii) the maintenance of the ability to
462 integrate several different (but not all) clades in low abundance that could yield an overall
463 benefit to the coral holobiont.

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

468

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701

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).

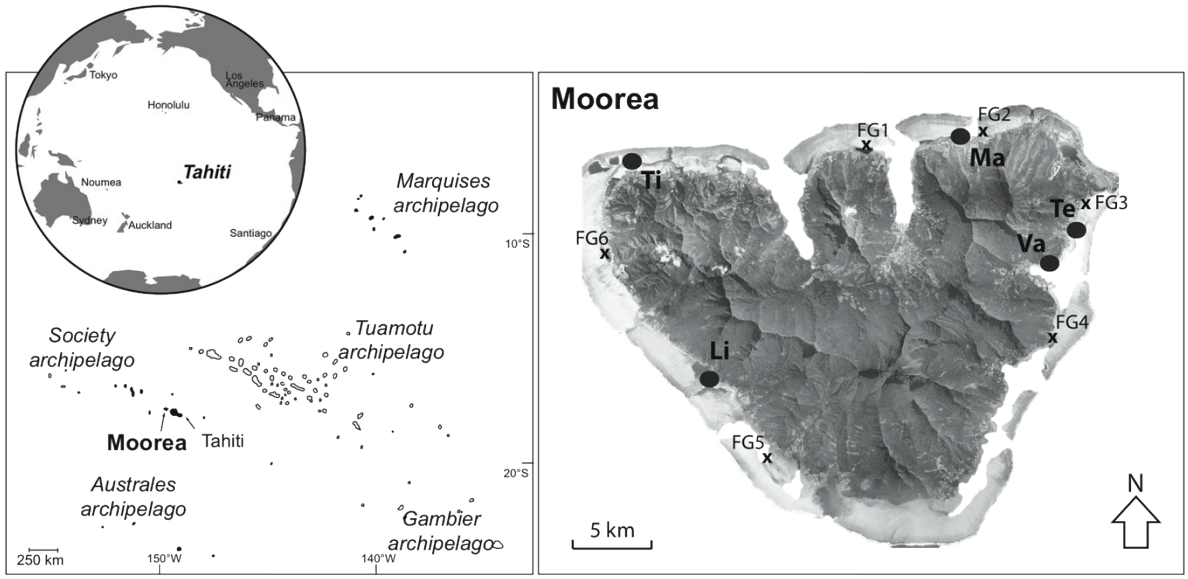
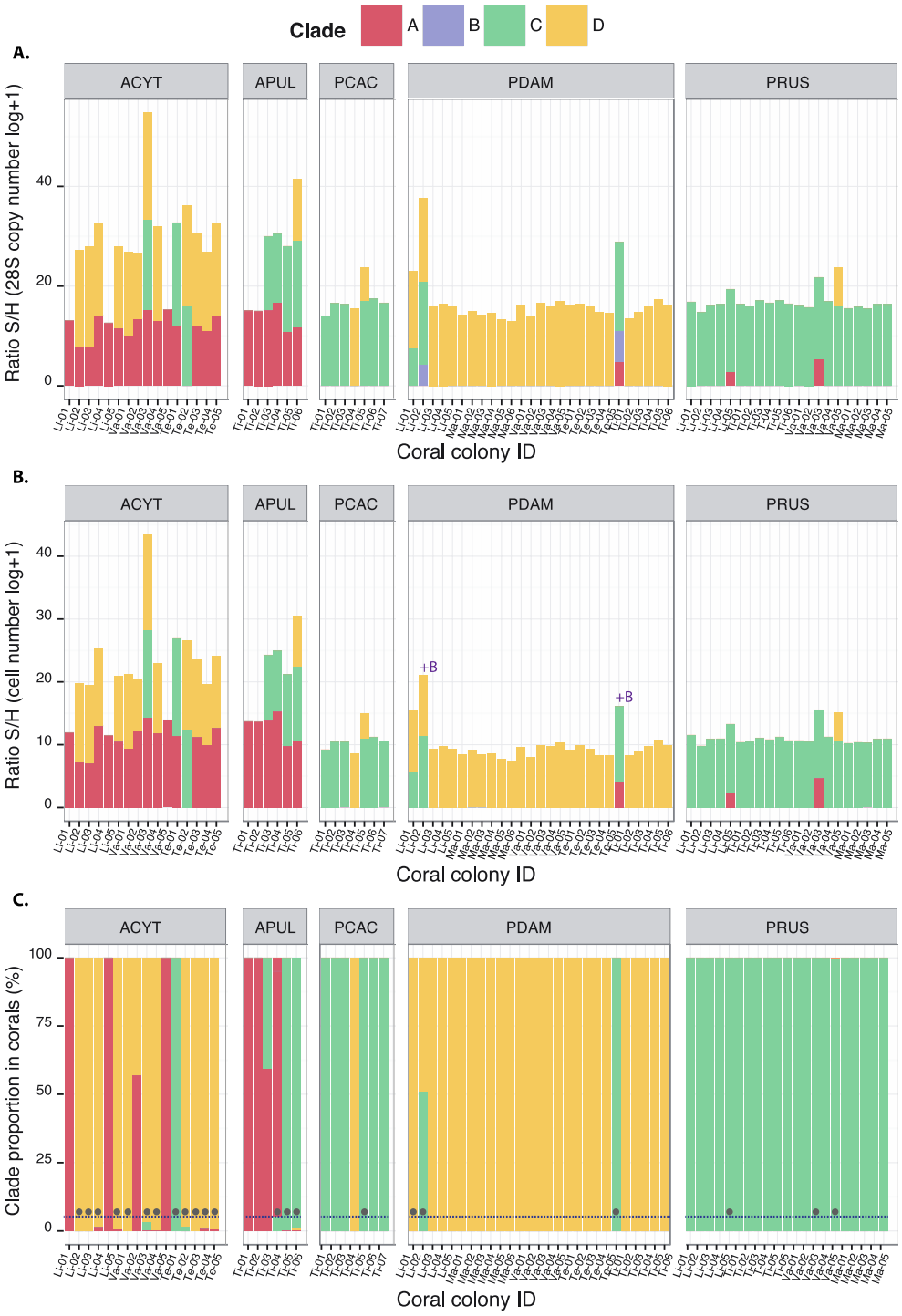
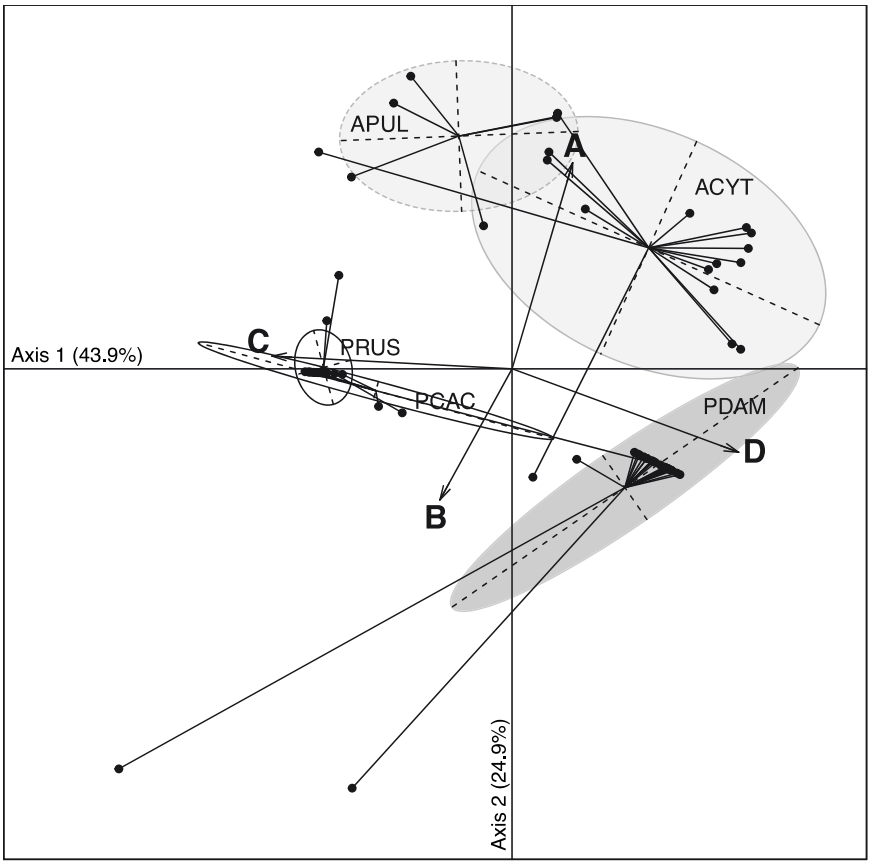


Figure 2 Quantitative composition of different *Symbiodinium* clades observed in association with ACYT: *A. cytherea*, APUL: *A. pulchra*, PCAC: *P. cactus*, PDAM: *P. damicornis* and PRUS: *P. rus* based on: (A) 28S copy number estimation (B) cell number estimation and (C) clade proportions within coral 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)
Coral colony ID
B.
Ratio S/H (cell number log+1)
+B
+B
Coral colony ID
C.
Clade proportion in corals (%)
Coral colony ID

714

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



718

719

720 **Table 1** Comparative census of *Symbiodinium* clades and types associated with common coral
721 species from Moorea (*A. cytherea*, *A. pulchra*, *P. damicornis*, *P. cactus,* and *P. rus*) detected in a
722 previous report by Putnam et al. 2012 [1] *vs*. the present study.
723

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

724 ** novel detected clade from this study*
725 *** new type of previously reported clade*

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

729

Species	Coral ID	Background clade proportion	
A. cytherea	Li-02	A = 0.0012%	A = 0.2089%
	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%	
	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%	A = 0.3984%
	Ti-05	A = 0.2073%	
	Ti-06	D = 0.7418%	
P. cactus	Ti-05	D = 0.0029%	
P. damicornis	Li-01	C = 0.0380%	A = 0.0002%
	Li-02	B = 0.0002%	
	Ti-01	B = 0.0009%	
P. rus	Li-05	A = 0.0001%	
	Va-03	A = 0.0020%	
	Va-05	D = 0.0259%	

730