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Symbiosis in a giant protist (*Marginopora vertebralis*, Soritinae): flexibility in symbiotic partnerships along a natural temperature gradient

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ABSTRACT: Benthic foraminifera of the family Soritinae are important members of coral reef communities, contributing to carbonate deposition on coral reefs. These giant protists form photosymbiotic associations with microalgae of the genus *Symbiodinium*. The extent of flexibility in foraminefera–*Symbiodinium* partnerships is not well understood. While some studies suggest foraminifera exhibit strong specificity with regard to symbiont choice, recent work illustrated that at least a few taxa are able to host >1 symbiont type. We explored the symbiont diversity of a widely distributed soritid foraminifera (*Marginopora vertebralis*), sampling 369 individuals from 16 populations distributed across a wide latitudinal gradient (31 to 9° S) in the western Pacific Ocean using the internal transcribed spacer region 2 (ITS2) of rDNA. We discovered that *M. vertebralis* forms symbiotic associations with a high diversity of *Symbiodinium* types, which encompassed 27 unique ITS2 rDNA haplotypes from 4 major *Symbiodinium* clades. Distance-based redundancy analysis revealed that the observed geographic variation in symbiont community composition was correlated with several sea surface temperature parameters. Symbiont diversity was highest at the inshore Great Barrier Reef, in marginal habitats characterized by high seasonal fluctuations in environmental parameters. In those areas we found evidence of mixed infections, with individual hosts harboring multiple symbiont lineages. These findings suggest a high degree of flexibility in foraminifera–*Symbiodinium* partnerships and highlight the importance of environmental variables in shaping symbiotic associations. We discuss the results in light of the hypothesis that within-population symbiont polymorphism and mixed infections may be a mechanism to cope with temporal environmental fluctuations.

KEY WORDS: *Symbiodinium* · Temperature · Foraminifera · Symbiosis · Diversity

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INTRODUCTION

Large benthic foraminifera are important members of tropical coral reef communities, contributing to ca. 5% of a coral reefs' carbonate deposition (Hallock 1981, Langer et al. 1997). Approximately 80% of this contribution can be attributed to large symbiont-bearing taxa (Langer et al. 1997). A small group of benthic discoidal soritid foraminifera (Soritinae), including the genera *Amphisorus*, *Sorites* and *Marginopora*, forms symbiotic partnerships with a diversity of dinoflagellate types belonging to the genus *Symbiodinium* (Pawlowski et al. 2001, Pochon & Pawlowski 2006, Pochon et al. 2007). Members of this genus form symbiotic associations with many diverse taxa, including corals, sponges, foraminifera and mollusks (Rowan 1998), and, through their association with hermatypic corals, play a fundamental role in coral-reef formation processes (Muscatine & Porter 1977).

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The genus *Symbiodinium* was long thought to be monospecific, but molecular phylogenetic studies based on nuclear (18S, 28S, ITS1 and ITS2 regions) and chloroplast (23S) ribosomal DNA markers revealed that this genus is extraordinarily diverse (LaJeunesse 2001, Baker 2003, Pochon et al. 2006, Pochon & Gates 2010). *Symbiodinium* spp. are hierarchically classified in clades (A to I), subclades (e.g. F1 to F5), and types, the latter being usually identified by a single ITS1 or ITS2 haplotype (LaJeunesse 2001, Coffroth & Santos 2005, Pochon & Gates 2010). While there is evidence of physiological and ecological differentiation among subclade types of *Symbiodinium* (Ulstrup & van Oppen 2003, Sampayo et al. 2007), determining what constitutes distinct ecological groups (ecotypes) based solely on DNA sequence is a challenging task since mutations in fast-evolving, non-coding regions are not always indicative of distinct physiologies. Correa & Baker (2009) described a promising approach to classify *Symbiodinium* diversity in a meaningful way at the ecological and physiological levels based on genetic data. The authors showed that ecotypes of symbionts usually can be defined as clusters of closely related haplotypes within parsimony networks based on ITS2 sequences (Correa & Baker 2009). This genetic cluster approach, based on population theory, provides a useful framework to investigate specificity and flexibility in symbiosis.

Soritid formanifera are known to host a high diversity of *Symbiodinium*, including members of 6 of the 9 known clades (C, D, F, G, H, I), 4 of which (F, G, H, I, with the exception of Subclade F2) occur exclusively in Soritinae (Garcia-Cuetos et al. 2005, Pochon et al. 2007, Pochon & Gates 2010). The first extensive investigation of *Symbiodinium* diversity in foraminifera suggested a high degree of specificity in terms of symbiotic partnerships, whereby a large number of host phylotypes exhibit strict specificity with respect to the symbiont types harbored (Garcia-Cuetos et al. 2005). More recent studies provide additional evidence of host-specificity, but also hint that some host phylotypes are flexible with regards to symbiont choice (Pochon et al. 2007) and that individual foraminifera may host mixed and dynamic symbiont communities (Fay et al. 2009). The sampling strategy of these studies (Garcia-Cuetos et al. 2005, Pochon et al. 2007), while covering a large number of host phylogenetic lineages, was geographically restricted to a few very close locations, and often all members of a host phylotype were sampled from the same site (Garcia-Cuetos et al. 2005, Pochon et al. 2007). As a result these studies may have largely underestimated symbiont diversity and flexibility in symbiont choice, as plasticity in symbiotic associations due to local selective pressure and biogeography may have been overlooked.

Studies on anthozoan symbioses show that, while there is a certain degree of specificity at the clade level (Goulet 2006, 2007), flexibility in symbiotic partnerships both at the clade and subclade level is common and might be overlooked if the sampling design and detection techniques are not appropriate (Baird et al. 2007, Baker & Romanski 2007, Silverstein et al. 2012). When sampling is carried out in different geographic regions (Ulstrup & van Oppen 2003, Macdonald et al. 2008), along environmental gradients (Rodriguez-Lanetty et al. 2001, Ulstrup & van Oppen 2003, LaJeunesse et al. 2004a, Cooper et al. 2011) or before, during and after an environmental impact occurs (Little et al. 2004, Jones et al. 2008), flexibility in host–*Symbiodinium* associations seems to be common. Some coral species can harbor many unrelated symbiont types simultaneously (mixed infections) (Fay & Weber 2012, Silverstein et al. 2012), and shuffling low-level background symbionts may be an acclimatization strategy to deal with environmental stress (Rowan et al. 1997, Berkelmans & van Oppen 2006).

Based on recent observations of a few foraminiferal hosts (Pochon et al. 2007, Fay et al. 2009) and of anthozoan–*Symbiodinium* associations (for a review, see Fay & Weber 2012), we hypothesized that flexibility is an important factor in explaining symbiotic partnerships in foraminifera and that foraminifera–*Symbiodinium* associations are shaped by environmental factors. To test this hypothesis, we investigated symbiont community composition in a widely distributed soritid (*Marginopora vertebralis*) across a gradient spanning >20° of latitude in the western Pacific Ocean. We demonstrated that *Marginopra vertebralis* harbors a high diversity of *Symbiodinium* lineages. Most of the symbiont types are not specific to this host lineage; spatial differences in community composition are likely being shaped by variation in temperature, and diversity is highest in marginal habitats subject to high environmental fluctuations.

MATERIALS AND METHODS

Sample collection and microscopy

Individuals of *Marginopora vertebralis* were collected on a latitudinal gradient from Lord Howe Island (31° 33′ S), Australia to Milne Bay, Papua

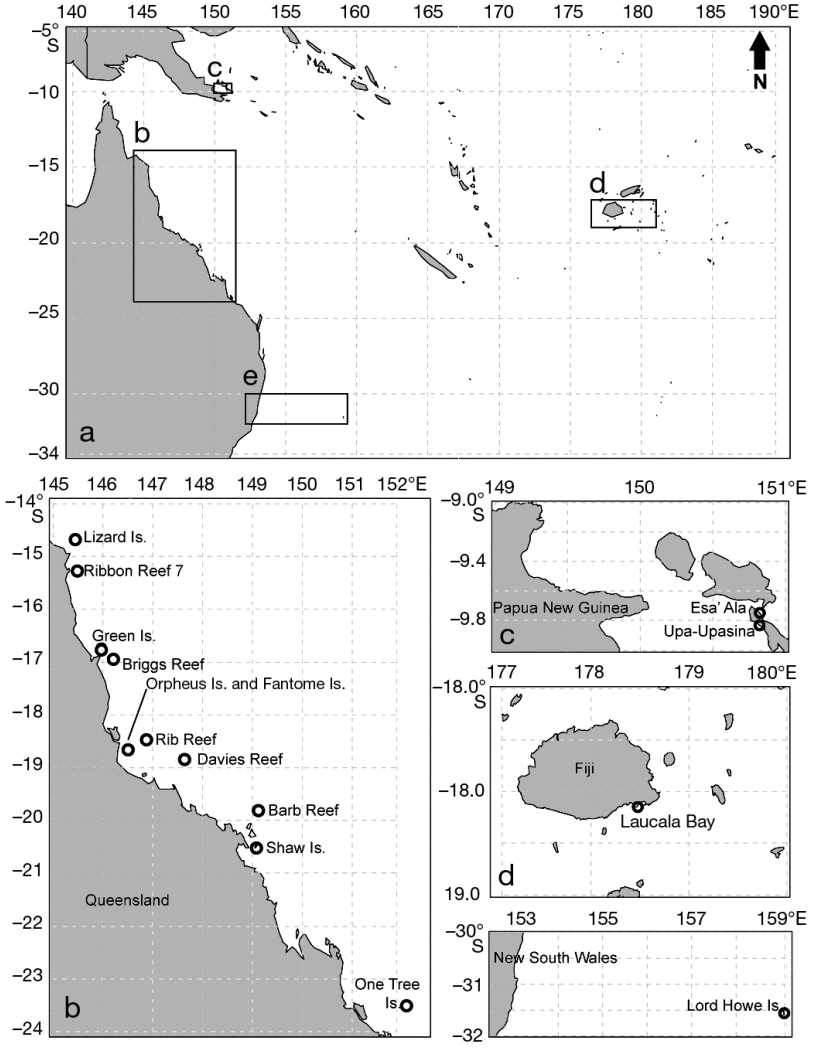

Fig. 1. Sampling areas within (a) the western Pacific Ocean, and detailed maps of collection sites (b) in the Great Barrier Reef, Australia, (c) Papua New Guinea, (d) Fiji, and (e) New South Wales, Australia

Table 1. Sampling locations. Date is given as dd/mm/yy. PNG: Papua New Guinea; GBR: Great Barrier Reef; NSW: New South Wales; N: no. of samples

Location	Region	Latitude (S)	Longitude (E)	Date	N
Upa-Upasina	PNG	9°49.69′	150°49.13′	23/04/11	24
Esa′Ala	PNG	9°44.29′	150°49.27′	25/0411	23
Lizard Is.	GBR	14°40.94	145°26.79′	06/10/04	22
Ribbon Reef No. 7	GBR	15°16.50	145°45.85′	16/07/11	24
Green Is.	GBR	16°45.71	145°58.33′	0/01/12	24
Briggs Reef	GBR	16°56.33	146°12.68′	04/01/12	24
Laucala Bay	Fiji	18°10.56	178°28.40′	27/07/11	18
Davies Reef	GBR	18°50.47	147°38.39′	17/09/08	24
Barb Reef	GBR	19°48.806	149°07.20′	20/02/07	18
Rib Reef	GBR	18°28.32	146°52.59′	26/01/11	24
North Bay, Fantome Is.	GBR	18°39.617	146°30.51′	25/01/11	24
Hazard Bay, Orpheus Is.	GBR	18°38.93	146°29.25′	24/01/11	22
Yank's Jetty, Orpheus Is.	GBR	18°39.08	146°29.20′	24/01/11	24
Shaw Is.	GBR	20°31.05	149°4.81′	06/08/09	47
One Tree Is.	GBR	23°30.33	152°5.61′	17/01/11	19
Lord Howe Is.	NSW	31°31.55	159°3.60′	24/02/05	8

New Guinea (PNG) (9° 44′ S, Fig. 1, Table 1). This species is epiphytic, and all samples were collected on either seagrass or macroalgal substrata adjacent to coral reefs, at a depth of 1 to 3 m. *M. vertebralis* were identified by light microscopy and phylogenetic analysis (see next subsections), and several representative samples were visualized by scanning electron microscopy (SEM; Fig. 2). Samples to be visualized by SEM were bleached for 1 h at 60C°, dehydrated in 100% ethanol, mounted on stubs and sputter-coated with gold. Samples were visualized on a JEOL JSM-5410LV scanning electron microscope at the Advanced Analytical Centre at James Cook University (Australia).

DNA extraction, amplification, sequencing and DGGE profiling

Total DNA was extracted from individual foraminiferal samples using a modified Chelex protocol (Walsh et al. 1991). As different symbiont types have been found to occupy different areas along the radius of foraminiferal tests (Fay et al. 2009), DNA extractions were performed in such a way to avoid bias. For smaller samples, DNA was extracted from half of the test. For larger samples a fragment extending from the center to outer edge of the test was used. Each fragment of foraminiferal test was placed in 100 µl Chelex solution (5% Chelex100®, 10 mM Tris-HCl) to which 5 µl of 20 mg ml^{-1} Proteinase K was added. Samples were digested at 55°C for 2 h, and following digestion proteinase K was denatured by heat-shocking at 95°C for 15 min. Samples were spun at 9200 × *g* for 5 min, and 0.5 µl of the supernatant was used as

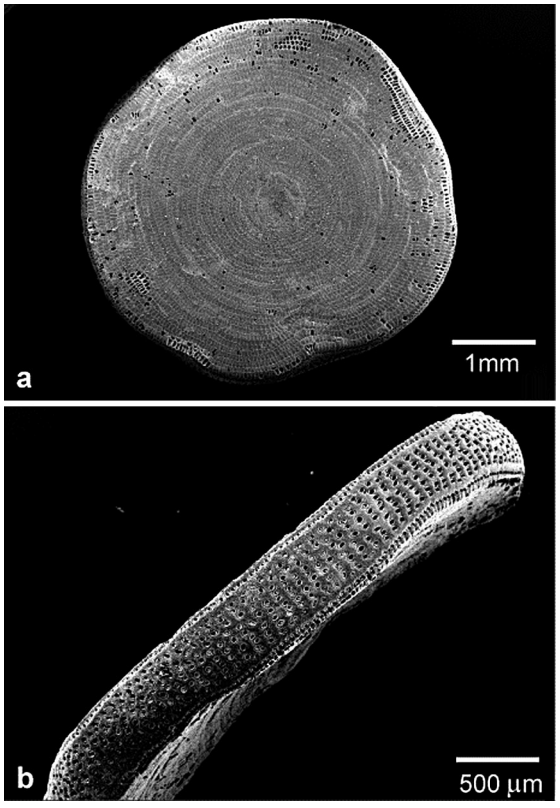

Fig. 2. *Marginopora vertebralis*. Scanning electron micrographs: (a) top view, (b) details of the lateral pores

template for PCR. All PCR reactions were prepared using the Qiagen Multiplex Kit, following manufacturer instructions.

For foraminiferal species confirmation, approximately 1700 bp of the 18S rRNA gene was amplified from representative samples of *Marginopora vertebralis* from each location using the primer pairs Sa10/LyS1 (Pawlowski 2000, Holzmann et al. 2001) and s6r/s17 (Pawlowski 2000) (Table 2). PCR cycling conditions were as follows: 15 min initial denaturation at 94°C, followed by 40 cycles of 40 s denaturation at 94°C, 40 s annealing at 56°C (Sa10/LyS1) or 50°C (s6r/s17), and 40 s elongation at 72°C and a final elongation step of 5 min at 72°C. PCR products were direct-sequenced with the amplification primers (Macrogen).

PCR and denaturing gradient gel electrophoresis (DGGE) of the ITS2 region of the *Symbiodinium* ribosomal RNA operon was conducted as per LaJeunesse & Trench (2000). Multiple representative bands of each DGGE profile were excised and reamplified using the primer pair ITSintFor2/ITSReverse (see Table 2) (Coleman et al. 1994, LaJeunesse & Trench 2000). PCR products were direct-sequenced using the primer ITSintFor2. Additionally, the D1 to D3 region of the 28S rRNA gene was amplified and sequenced for representatives of each ITS2 haplotype cluster. The approximately 900 bp long fragment of the 28S rRNA gene was amplified using the primer pair ITS2intFor/D3B (Scholin et al. 1994) (Table 2). PCR conditions were as for ITS2 amplification, with the difference that elongation time was 75 s, and the final elongation step was 10 min instead of 30 min.

Sequencing was carried out using a commercial service (Macrogen).

Sequence alignment, parsimony networks and phylogenetic analysis

Nucleotide sequence chromatograms were visually checked and assembled using the software ChromasPro (Technelysium). Sequence alignments were performed in ClustalW (Thompson et al. 1994) and visually refined using BioEdit (Hall 1999). All host sequences and representative sequences from each

Table 2. Primers used for amplification and sequencing. gc clamp underlined

Primer	Sequence	Organism	Region	Direction	Source
Sa10	CTCAAAGATTAAGCCATGCAAGTT	Foraminifera	18S	Forward	Holzmann et al. (2001)
LyS1	CTCCAACTATCTCCATCGA	Foraminifera	18S	Reverse	Pawlowski (2000)
S6r	GGGCAAGTCTGGTGC	Foraminifera	18S	Forward	Pawlowski (2000)
S17	CGGTCACGTTCGTTGC	Foraminifera	18S	Reverse	Pawlowski (2000)
ITSintFor2	GAATTGCAGAACTCCGTG	*Symbiodinium*	ITS/28S	Forward	LaJeunesse & Trench (2000)
ITSReverse	GGGATCCATATGCTTAAGTT CAGCGGGT	*Symbiodinium*	ITS	Reverse	LaJeunesse & Trench (2000)
ITS2Clamp	CGCC....GCCCGGGATCCATATGC TTAAGTTCAGCGGGT	*Symbiodinium*	ITS	Reverse	LaJeunesse & Trench (2000)
D3B	TCGGAGGGAACCAGCTACTA	*Symbiodinium*	28S	Reverse	Scholin et al. (1994)
D1R	ACCCGCTGAATTTAAGCATA	*Symbiodinium*	28S	Forward	Nunn et al. (1996)

Symbiodinium type were deposited in GenBank (Accession Numbers: KC802023 to KC802083).

Marginopora vertebralis

Near-complete 18S host sequences were aligned with *Marginopora* sp. sequences retrieved from GenBank (AJ842190, AJ842188 to AJ842190) and 2 *Sorites* sp. sequences (AJ842193, AJ404311) to be used as outgroups in the phylogenetic analysis. Substitution models were tested in Modeltest (Posada & Crandall 1998). A phylogeny based on 18S gene sequences was estimated by Bayesian inference (BI) and by analyzing 100 bootstrap datasets by maximum likelihood (ML). The ML phylogeny was estimated in PHYML 3.0 (Guindon et al. 2005, 2010) by ML using the GTR+I+G substitution model, 4 gamma categories, tree improvement by using the best of the nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) using 5 random starting trees. The Bayesian analysis was performed using the same substitution model in MrBayes 3.1 (Ronquist & Huelsenbeck 2003). The number of Monte Carlo Markov chain generations was set to 4 000 000, and trees were sampled every 100 generations. Convergence of 2 independent runs was tested by checking that standard deviation of split frequencies (as estimated for the last 75% of sampled trees) fell below 0.01, and by visually analyzing the stability of each parameter estimate in the software Tracer (Rambaut & Drummond 2003). The first 25% of sampled trees were discarded as burn-in.

Symbiodinium

ITS2 haplotypes obtained were aligned with sequences retrieved from GenBank, and separate ITS2 alignments were produced for each *Symbiodinium* clade represented in our samples (C, D, F, H). Sequences were trimmed to the length of the shortest sequence. Parsimony networks were produced using the software TCS (Clement et al. 2000), and genetic clusters were identified following the method outlined by Correa & Baker (2009). Clusters are here defined as groups of sequences of minimal sequence divergence within a parsimony network, where average within-cluster pairwise sequence divergence is <50% of the average between-cluster divergence (Palys et al. 1997, Correa & Baker 2009). Between- and within-cluster divergence were calculated using the 'DNA divergence between populations' function in DNAsp v.5.10 (Librado & Rozas 2009). The 28S partial sequences obtained from representatives of each genetic cluster were aligned with *Symbiodinium* sp. sequences representative of each known clade and subclade retrieved from GenBank. Model selection and 28S phylogenetic reconstruction followed the same procedure outlined above for *marginopora vertebralis* phylogenetic analysis, with the difference that the substitution model selected was GTR+G (instead of GTR+I+G).

Statistical analysis of *Symbiodinium* communities

We investigated the influence of various temperature metrics on the composition of symbiont communities hosted by *Marginopora vertebralis*. This analysis was restricted to the *M. vertebralis* Phylotype Mar II to avoid possible confounding effects of *Symbiodinium*–host specificity among distinct host phylotypes. Temperature series data from the last 11 yr for each of the sampled locations were obtained from the NOAA Comprehensive Large Array-Data Stewardship System (CLASS, www.class.noaa.gov). We used the SST50 surface temperature dataset, which consists of sea-surface temperature (SST) data at a 50 km resolution, obtained from a composite gridded-image derived from 8 km resolution global SST observations, and is generated twice weekly (every Tuesday and Saturday). From temperature series data, a number of metrics were derived: mean 11 yr temperature, maximum recorded temperature, minimum recorded temperature, mean winter temperature, mean summer temperature and mean yearly range (see Table A1 in Appendix 1). Because samples over a large geographic scale could not be sampled during the same season, we also statistically tested (see below) whether mean temperature in the month preceding sampling influenced the *Symbiodinium* type(s) present within samples.

The relationship between *Symbiodinium* type and temperature metrics was investigated with constrained analysis of principal coordinates (also known as distance-based redundancy analysis, db-RDA), which is an ordination method that tests the effects of individual or combined environmental variables on a community dataset using non-Euclidean distance matrices (Legendre & Anderson 1999). *Symbiodinium* community abundance data for each population were obtained by scoring symbionts (classified at the cluster level) for each individual host. Abundance data were row-standardized to account for different sample sizes. To test the effect of the

obtained temperature metrics, db-RDA was applied on Bray-Curtis distance matrices obtained from *Symbiodinium* community abundance data of all *Marginopora vertebralis* populations belonging to Phylotype Mar II from all survey sites (i.e. all samples with the exception of the Fiji population, see 'Results'). As different temperature metrics are likely to be strongly correlated, variables were assessed for collinearity, and only metrics with correlation coefficients of <0.9 were used. The significance of individual metrics was tested by permutation tests (10000 permutations). Only variables significant in the individual tests were included in the final model.

The effect of the experimental design on estimates of symbiont diversity was explored by creating a rarefaction curve showing the effect of the number of locations sampled on the number of genetic clustered detected (Fig. A1 in Appendix 1). These analyses were conducted using the Vegan package (Oksanen et al. 2011) of the R statistical environment (R Development Core Team, http://R-project.org).

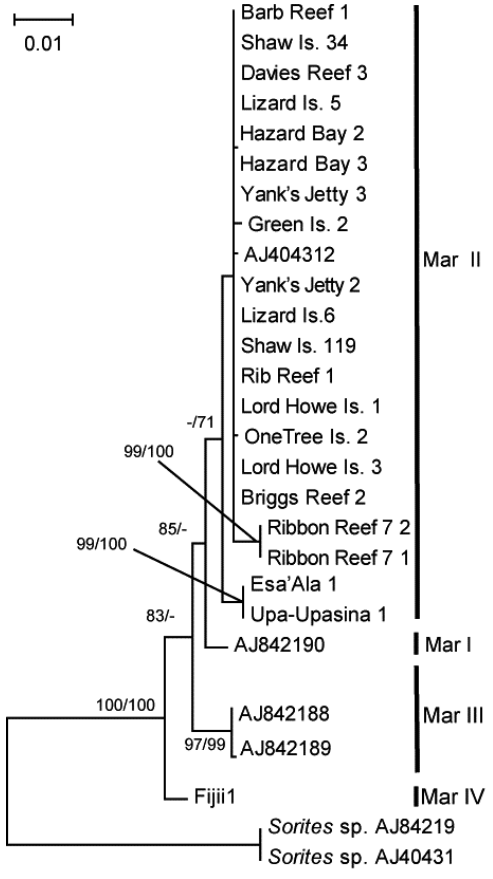

Fig. 3. Phylogeny of *Marginopora vertebralis* phylotypes based on near-complete 18S rRNA gene sequences. Branch support values (ML/BI) represent 100 bootstrap datasets by maximum likelihood (ML) and Bayesian clade credibility values (BI), respectively. Clades are named following Garcia Cuetos et al (2005)

RESULTS

Marginopora vertebralis genetic analysis and morphology

The final alignment of the near-complete 18S rRNA gene sequences included 27 individuals, 6 of which were obtained from GenBank. The alignment consisted of 1716 unambiguously aligned positions, 122 variable sites and 115 parsimony informative sites. The phylogenetic analysis (Fig. 3) supports the classification of *M. vertebralis* in distinct phylotypes (Mar I, Mar II and Mar III) (Garcia-Cuetos et al. 2005). However, while the distinction between Mar III and the other phylotypes is very clear and well supported, in our analysis the distinction between the Phylotypes Mar I and Mar II was not as obvious as that described by Garcia-Cuetos et al. (2005), despite the fact that we used a larger fragment of the 18S gene. Furthermore, we report a fourth phylotype (Mar IV), represented by an individual from Fiji (Fig. 2). The Phylotype Mar II shows the morphological features of *M. vertebralis* as described in Holzmann et al. (2001) by SEM (Fig. 2). With the exclusion of the individuals collected from Fiji, all our samples belonged to Phylotype Mar II and all representatives of Phylotypes Mar I and III represented sequences from GenBank.

Symbiodinium genetic analysis

Twenty-seven unique *Symbiodinium* ITS2 haplotypes were identified from 369 *Marginopora vertebralis* samples from the southwestern Pacific Ocean. Of these 27 haplotypes, some differed by a single base pair mutation or by a single insertion or deletion (Fig. 4). If 2 or more haplotypes were found to always co-occur in the same individuals, they were assumed to be intragenomic variants (circled by dotted lines in Fig. 4). Haplotype networks identified 23 haplotypes, 19 of which were novel (i.e. not represented in GenBank; Fig. 4). The reason for the observed incongruence is that sequences were trimmed to the length of the shortest sequence and gaps were treated as missing data; some variable sites were excluded as a result. The 23 unique haplotypes identified by the TCS analysis spanned 4 clades (C, D, F, H) and 10 genetic clusters. Intragenomic variants were always assigned to the same genetic cluster. Of the 10 gen-

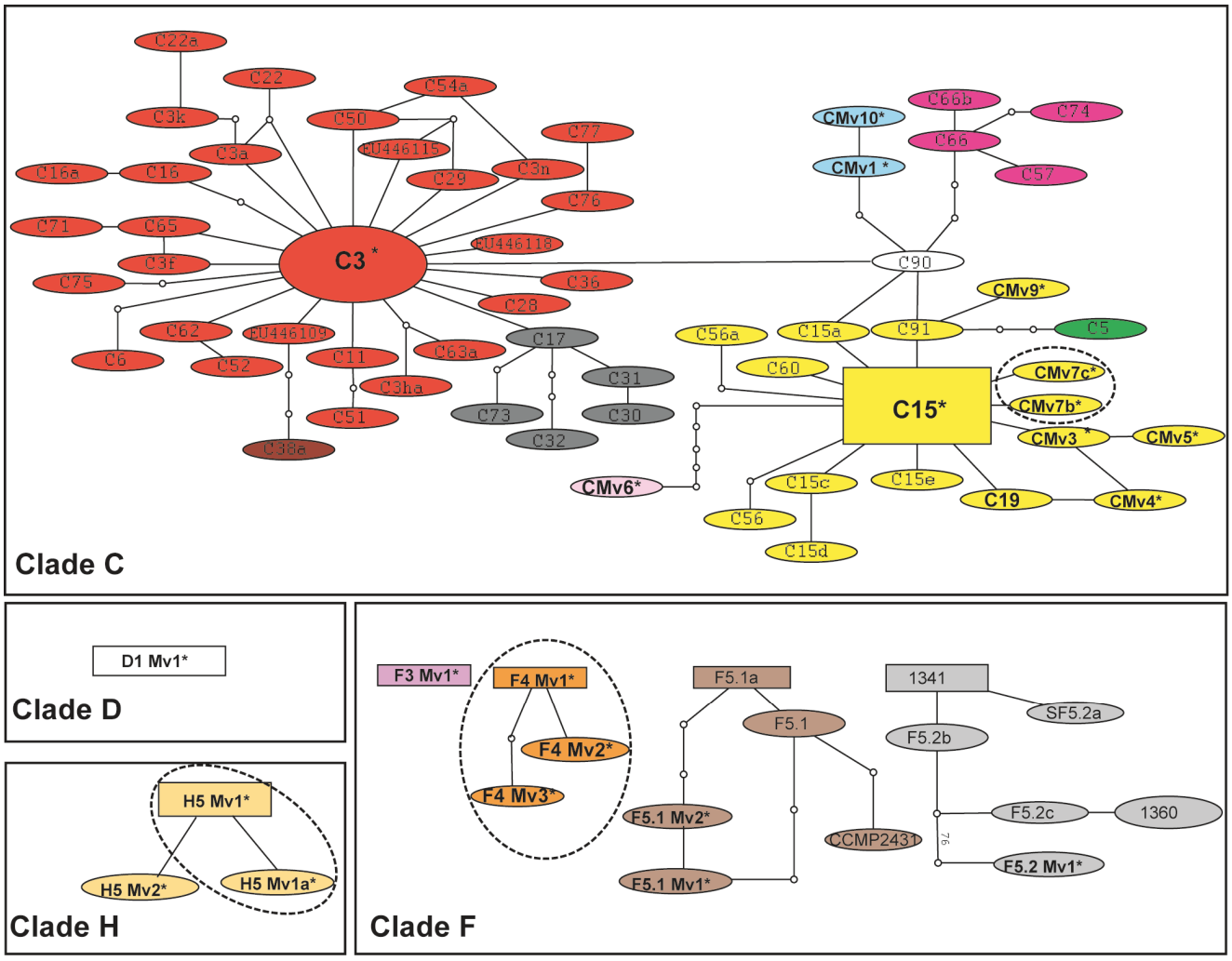

Fig. 4. Parsimony networks of *Symbiodinium* ITS2 haplotypes. Genetic clusters within each clade are shown in different colors. Haplotypes isolated in this study are shown in bold and marked with an asterisk. Intragenomic variants are circled in dotted lines

etic clusters, 6 represented novel clusters and 4 belonged to novel ITS2 networks (Fig. 4). The 4 known genetic clusters included symbiont groups previously identified in both coral (C3, C15, F5.1) and foraminiferal lineages (C3, C15, F5.1, F5.2).

A subset of *Symbiodinium* samples (76 sequences) was also run using 28S rRNA to confirm taxonomic conclusions derived by ITS2. The 28S rRNA gene alignment included 782 unambiguously aligned sites, 373 of which were variable and 336 parsimony informative. The phylogeny based on 28S sequences (Fig. 5) provided adequate identification of representative genetic clusters to the clade and subclade level, including the genetic clusters that belong to novel ITS2 networks. The phylogenetic tree based on 28S rRNA gene sequences was consistent with phylogenies produced in previous studies (Pochon et al. 2006, Pochon & Gates 2010) using both 28S rRNA and 23S cpRNA gene sequences, identifying 9 well-supported major clades (A to I), 2 subclades within Clade D and 4 within Clade F (Fig. 5). While each subclade in Clade F was well supported in both the ML and BI analyses, Clade F as a whole was not; thus, it is likely that this clade is polyphyletic and in need of taxonomic revision. Symbionts isolated from *Marginopra vertebralis* (Phylotype Mar II) spanned 4 major clades (C, D, F and H) (Fig. 5). Within Clade F symbionts isolated from our samples were well grouped within Subclades F4 and F5. Within Clade D symbionts from *M. vertebralis* grouped most closely with the symbionts isolated from the sponge *Haliclona koremella* (Carlos et al. 1999). The length of the ITS2 of this subclade was longer (>400 bp) than that of other *Symbiodinium* types.

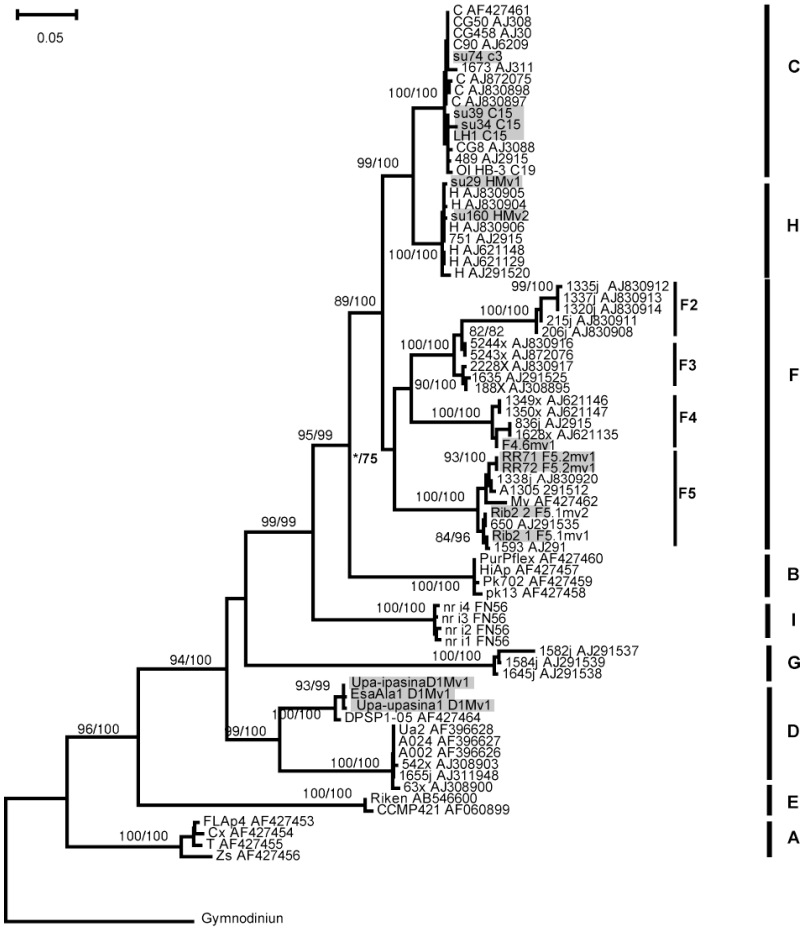

Fig. 5. *Symbiodinium* phylogeny based on partial 28S rRNA gene sequences (D1 to D3 region). Sequences obtained in this study are highlighted. Branch support values (ML/BI) represent 100 bootstrap datasets by maximum likelihood (ML) and Bayesian clade credibility values (BI), respectively

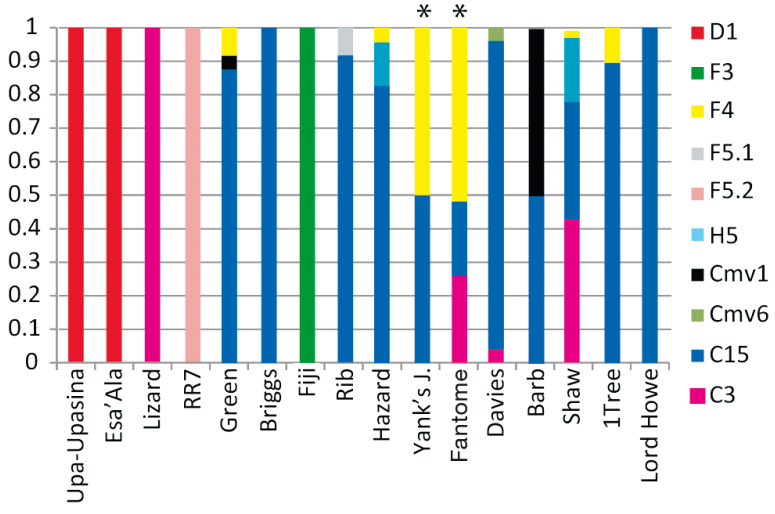

Fig. 6. *Marginopora vertebralis*. Frequency distribution of symbiont genetic clusters across symbiont communities in *M. vertebralis* populations. Populations are in order of decreasing latitude (north to south). Populations in which individual hosts were found to harbor multiple symbiont clusters are marked with an asterisk, and details on the occurrence of multiple infections are given in the 'Results'

Statistical analysis of *Symbiodinium* communities

Symbiont community composition varied among *Marginopora vertebralis* populations (Fig. 6). In the central and southern Great Barrier Reef (GBR) region and off Lord Howe Island, symbiont communities were largely dominated by the genetic Cluster C15, with some contributions from Clusters C3 and F4 in inshore reefs of the central GBR (Yank's Jetty, Fantome Island and Shaw Island) (Fig. 6). All populations in the central and southern GBR (with the exception of Briggs Reef) harbored >1 group of symbionts, with the highest diversity reported at Shaw Island (where 4 groups representing 3 major clades were present). In contrast, populations in the northern GBR (Lizard Island and Ribbon Reef No. 7) and outside of the GBR (PNG and Lord Howe populations) were dominated by a single genetic cluster of symbionts. The symbiont Clusters C15 and F4, common in central GBR, were entirely absent on reefs further north than Green Island (16°45S). Symbiont communities in the PNG populations were dominated by a single *Symbiodinium* cluster belonging to Clade D (Fig. 6). Only a few host individuals harbored multiple symbiont clusters. Multiple symbiont clades (C and F) were harbored by a small number of individuals in the inshore reefs of Orpheus Island (2 out of 22 at Yank's Jetty) and Fantome Island (3 out 24 individuals collected in North Bay).

Db-RDA was used to assess the relationship between *Symbiodinium* clusters and locations and several temperature-related metrics as environmental variables. As expected, most temperature-related parameters were highly correlated, and we chose 4 parameters which were less correlated ($R < 0.9$) for further analysis: mean winter temperature, mean summer temperature, mean temperature range and average temperature in the

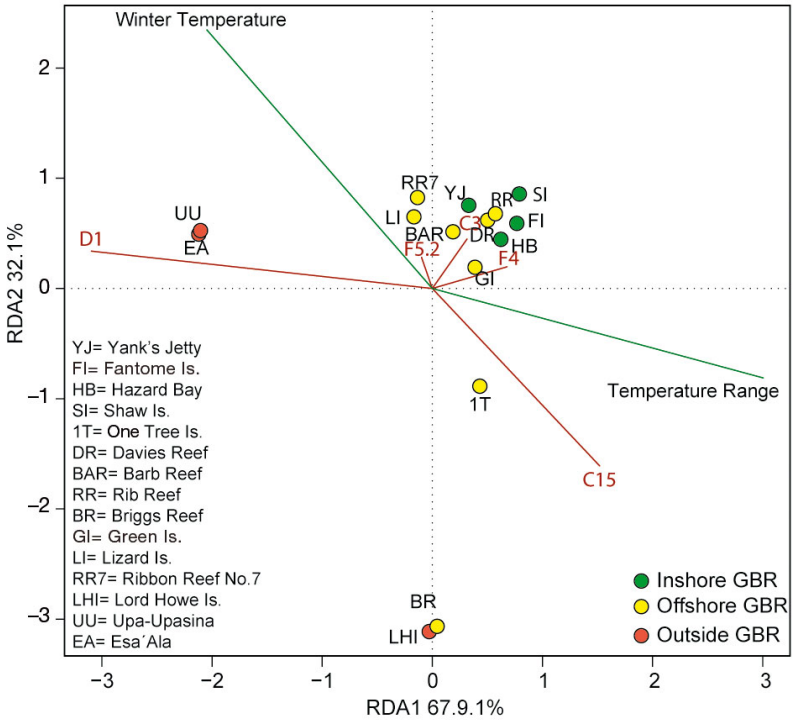

Fig. 7. *Marginopora vertebralis*. Distance-based redundancy (RDA) analysis of symbiont community composition among populations of *M. vertebralis*. To be noted: the strong negative correlation of *Symbiodinium* Cluster C15 with winter temperature, the positive correlation between C15 and temperature range and the positive correlation of Cluster D1 with low temperature range and high winter temperature. GBR: Great Barrier Reef

month before sampling). Permutation tests indicated that the winter temperature (pseudo-F = 3.49, p < 0.0026) and temperature range (pseudo-F = 6.73, p = 0.0001) explained a significant amount of the variation; results for the other 2 variables were not significant. In a db-RDA the 2 variables combined explained 39.7% of the distances observed between sample populations. The individual variables mean winter temperature and mean temperature range explained 22.8 and 35.2% of the distance, respectively.

The presence of *Symbiodinium* Cluster C15 was strongly negatively correlated with high winter temperature minima and positively correlated to a higher temperature range (Fig. 7). In contrast, Cluster D1 was highly correlated with a low temperature range and high winter temperatures. Thus, this analysis confirmed findings from the frequency analysis.

The rarefaction curve (Fig. A1) suggests that more intensive sampling would likely yield additional symbiont genetic clusters and that previous studies, which included only 1 or a few locations, may have undersampled clusters.

DISCUSSION

Symbiodinium-bearing foraminifera are subject to similar stresses as corals: they are susceptible to bleaching under high temperatures (Uthicke et al. 2012) and other stressors including reduced pH through ocean acidification (Uthicke & Fabricius 2012, Reymond et al. 2013, Uthicke et al. 2013). Nevertheless *Marginopora vertebralis* has a wide latitudinal distribution, being present in habitats with very different temperature regimes (such at Lord Howe Island and PNG). It is therefore possible that the type of symbionts they associate with plays a significant role in local acclimatization. We sampled *M. vertebralis* across a wide (>20°) latitudinal gradient and investigated symbiont community composition in each population. Our data suggest that *M. vertebralis* exhibits high diversity and flexibility in symbiotic associations, which are at least partially explained by environmental factors. The diversity of symbionts harbored by *M. vertebralis*—Phylotype Mar II sensu Garcia-Cuetos et al. (2005)—is remarkable, with 27 unique haplotypes belonging to 9 genetic clusters representing 4 clades, and more intensive sampling is likely to yield even higher symbiont diversity estimates (Fig. A1). While some of the detected genetic clusters are relatively rare, 6 (C3, C15, Cmv1, F4.6mv1, F5.2, DMv1) contribute to at least 50% of symbiont communities in local populations of *M. vertebralis* (Mar II).

This level of diversity is very high when compared with other *Symbiodinium*-bearing taxa. While symbiont polymorphism is a common phenomenon, most symbiont-bearing taxa associate only with a limited number of *Symbiodinium* types, and just a few are able to harbor many distinct lineages (Fay & Weber 2012). Many symbionts associated with *Marginopora vertebralis* (C3, C15, C19, F5.1, F5.2) in our study have been previously isolated from various species of corals and foraminifera, suggesting they are to some extent generalists (van Oppen et al. 2001, LaJeunesse et al. 2004b, 2009, Pochon et al. 2007, Bongaerts et al. 2010, Venera-Ponton et al. 2010). The Cluster D1Mv1 was novel, but closely related to symbionts previously isolated from another phylotype of *M. vertebralis*

(Phylotype Mar I) (Pochon et al. 2007) and from a sponge (Carlos et al. 1999).

Flexibility in symbiotic partnerships

The findings presented here suggest that foraminifera–*Symbiodinium* partnerships can be flexible. *Marginopora vertebralis* (Phylotype Mar II) can associate with a variety of symbiont types, and different symbionts may dominate depending on the location sampled. Because the marker used to explore hosts genetic diversity (18S) may not carry enough genetic variation to distinguish between subspecies, it cannot be excluded that some degree of specificity may exist at a lower host taxonomic level. However, of the 16 populations sampled, half of them harbored >1 distinct symbiont cluster, suggesting that even at the population level flexibility seems to be common.

The fact that low sampling effort may affect the perceived specificity of symbiotic partnerships has sparked debates in the past (Baker 2003, Goulet 2006, Baker & Romanski 2007). Previous studies of foraminifera–*Symbiodinium* associations considered a large number of foraminiferal phylotypes, but were limited to either a few individuals or a few locations per phylotype (Garcia-Cuetos et al. 2005, Pochon et al. 2007), and, therefore, most likely underestimated the level of diversity and flexibility in foraminifera–*Symbiodinium* partnerships. At single points in space and time, symbiont communities appear to be dominated in several populations by a single type (Garcia-Cuetos et al. 2005, Pochon & Gates 2010). Symbiont lineages may differ in physiological traits (van Oppen et al. 2001, Little et al. 2004), and, therefore, some symbiont–host associations may be positively selected in specific environmental conditions. Under stable environmental conditions a host species may show local preference for a particular symbiont lineage, at least until environmental conditions are perturbed. Since preferences may be host specific, local selective pressure could create localized patterns of host–symbiont associations that may be misinterpreted as evidence of host specificity. Garcia-Cuetos et al. (2005) interpreted such patterns as evidence of strict specificity in foraminifera–*Symbiodinium* partnerships. However, we argue that this is not evidence of fidelity, as it does not provide insight into whether a host has the flexibility to form symbiotic associations with multiple symbiont lineages under different environmental conditions (Baker 2003). This process may account for both the high degree of specificity previously reported in soritid–*Symbiodinium* symbioses at a local scale (Garcia-Cuetos et al. 2005), and the high flexibility in symbiotic partnerships exhibited by *Marginopora vertebralis* across a wide latitudinal range.

Spatial differences in symbiont community composition

The changes observed in symbiont community composition were not random, and symbiont types varied predictably with temperature regimes. No effect of temperature in the month prior to sampling was detected, suggesting that differences in sampling times in different regions were unlikely to be a confounding factor in our analysis. These results should however be interpreted with caution; since the effect of temperature was not investigated in controlled conditions, the interpretation of the results is necessarily made on the basis of the correlation that exists between temperature parameters and latitude.

Type C15 was negatively correlated with high winter temperature and positively correlated with a high average temperature range. This type is the dominant type in most populations in the central and southern GBR (mean winter temperatures: 19 to 22°C; mean yearly temperature range: 4.6 to 5°C), as well as off Lord Howe Island (mean winter temperature: 19.2°C; mean yearly range: 5°C), but is completely absent from the warmer populations. This pattern cannot be explained in terms of symbiont biogeographic distribution, as C15 is widespread and found in many other hosts across the Indo-Pacific (LaJeunesse et al. 2003). This symbiont type may confer a higher tolerance to lower temperatures, or offer a physiological advantage over the wider temperature range experienced by populations at higher latitudes. Pochon et al. (2007) reanalyzed the dataset from Pawlowski et al. (2001) and reported C15 in *Marginopora vertebralis* from Lizard Island, several hundred kilometers north of where we detected C15 on the GBR. However, it is unclear whether the population sampled by Pawlowski et al. (2001) belonged to the Phylotype Mar II. Both Phylotypes Mar II and Mar III have been reported from Lizard Island (Garcia-Cuetos et al. 2005), the latter being found in deeper waters and being associated with Clade C *Symbiodinium*. In light of the results of the present study, it is likely that the C15 *Symbiodinium* reported by Pochon et al. (2007) might have been associated with the deeper-water Mar III phylotype.

The low-latitude D1Mv1, on the other hand, is found exclusively in PNG populations, where winter

temperature is highest (27.4°C) and mean yearly temperature fluctuation lowest (2.3°C). A warm-water population of another phylotype of *Marginopora vertebralis* (Mar I from Guam; latitude: 13.5N; mean winter temperature: 27.7°C; mean yearly range: 1.75°C) was found to associate with a symbiont type closely related to D1Mv1 (Pochon et al. 2007), suggesting that temperature tolerance may be a shared character of this D subclade. Clade D symbionts have been associated with thermal tolerance in a range of coral species (Rowan et al. 1997, Rowan 2004, Berkelmans & van Oppen 2006, Ulstrup et al. 2006, LaJeunesse et al. 2009).

Symbiont diversity in *Marginopora vertebralis* decreases towards the latitudinal edges of the study area (latitudes: 9 to 10°S at PNG and 31 to 32°S at Lord Howe Island); communities at those locations consisted of a single symbiont type. In the central region of the GBR, symbiont communities were more heterogeneous and diversity was highest in inshore reefs. In those inshore reefs we also found evidence of multiple symbiont lineages in single *M. vertebralis* individuals. These inshore populations experienced yearly temperature fluctuations similar to higher latitude locations (see Table A1). For example, Shaw Island has a mean yearly temperature range of 5.3°C, a range comparable to the higher latitude populations of One Tree Island (5.3°C) and Lord Howe Island (5°C). In addition, inshore reefs are subject to much higher seasonal fluctuations in salinity, nutrient levels and water clarity when compared to offshore reefs and can experience elevated sea surface temperature, low salinity and increased nutrients levels in the summer months (Schaffelke et al. 2012). These reefs are marginal habitats for *M. vertebralis*, which is not found any closer inshore in the GBR lagoon (S. Uthicke pers. obs.). On inshore reefs *M. vertebralis* grows more slowly (Reymond et al. 2011), and experimental studies have confirmed that elevated nutrients and temperatures inhibit growth and photosynthesis in this species (Uthicke et al. 2012). It is possible that both the higher within-population and within-individual diversity observed in inshore reefs is related to the instability of environmental conditions which characterizes these marginal habitats. These results are consistent with the hypothesis proposed by Cooper et al. (2011) that, while SST parameters are important predictors of *Symbiodinium* types at a broad geographical scale, water quality parameters play an important role in shaping symbiotic partnerships at a local scale.

The hypothesis that temporal variability in environmental conditions can favor heterogeneous symbiont communities, both in host populations and in host individuals, is not new (see review by Fay & Weber 2012). *Acropora millipora* populations were found to harbor mixed symbiont (C2, C1 and D1) communities in the inshore reefs of the Whitsunday Islands (central GBR), characterized by higher fluctuations in water quality parameters, while corals in the outer zone of the Whitsundays harbored exclusively C2 symbiont types (Cooper et al. 2011). Rowan et al. (1997) showed that corals that harbor multiple symbiont types may be more resistant to bleaching, and concluded that temporal variability in environmental conditions may favor the coexistence of multiple symbiont types in a single host. Rowan et al (1997) investigated temperature stress, but other stressors (changes in salinity, light availability and nutrient levels due to run-off) may act in the same way.

Flexibility in symbiotic partnerships may provide a mechanism to cope with environmental stress, but this hypothesis has been challenged by Putnam et al. (2012). These authors found that coral species exhibiting higher flexibility in symbiont choice are often susceptible to environmental stress, while corals that are often classified as short-term 'ecological winners' show greater specificity in symbiotic partnerships (van Woesik 2001). Putnam et al. (2012) propose that flexible symbioses are disadvantageous for coping with environmental stress. However, the data presented are correlative, and the authors do not show any causality between flexibility specificity and susceptibility to environmental stress. The taxa compared have different life-history traits, and these differences are likely to play a key role in determining susceptibility to environmental stress and bleaching (van Woesik 2001). We argue that there is an alternative, equally parsimonious, hypothesis to explain the data by Putnam et al. (2012): hosts that are physiologically more susceptible to stress evolve greater flexibility in symbiotic partnerships to cope with environmental fluctuations.

It is important to keep in mind that DGGE is not able to reliably detect the presence of symbionts at background levels (<10% of symbiont community). More powerful molecular techniques, such as real-time PCR, are needed to assess the occurrence of background communities (Mieog et al. 2007). Therefore, the results presented in this study likely underestimate symbiont diversity within individual formanifera. Furthermore, while DGGE and the clustering method we adopted perform well in delineating symbiont ecotypes (Correa & Baker 2009), symbiont adaptation at the population level may occur before mutations in the ITS region arise and sym-

bionts of the same ITS2 type may be adapted to different thermal regimes (Howells et al. 2012). Finer scale genetic methods, such as the use of microsatellite markers, may reveal higher symbiont diversity at lower taxonomic levels that could be physiologically relevant.

In conclusion, *Marginopora vertebralis* harbors a high diversity of symbiont lineages, representing 4 distinct *Symbiodinium* clades. Symbiont community composition varies in a non-random fashion along a wide latitudinal gradient, and the occurrence of several symbiont types is correlated with temperature. These results highlight the importance of environmental factors in shaping symbiotic associations, and suggest that at a broad geographical scale foraminifera–*Symbiodinium* partnerships are more flexible than previously thought.

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Appendix 1.

Table A1. Sea-surface temperature (*T*) parameters for each sampling location

Location	Mean *T* (°C)	Mean summer *T* (°C)	Mean winter *T* (°C)	Min. *T* (°C)	Max. *T* (°C)	Mean *T* in month before sampling (°C)	Mean range (°C)	Latitude
Upa-Upasina	28.7	29.7	27.4	24.6	31.8	30.0	2.3	9.83
Esa'Ala	28.7	29.7	27.4	24.6	31.8	30.0	2.3	9.74
Laucala Bay	27.5	28.8	26.9	24.5	30.5	26.9	2.0	18.18
Ribbon Reef 7	27.0	29.0	24.9	22.7	30.9	23.9	4.1	15.28
Lizard Is.	26.9	29.0	24.9	22.8	31.3	24.7	4.1	14.68
Briggs Reef	26.8	28.9	24.7	22.2	31.1	29.3	4.3	16.94
Green Is.	26.8	28.9	24.7	22.2	31.1	29.3	4.3	16.76
Rib Reef	26.4	28.7	24.1	19.3	32.0	29.0	4.7	18.47
Fantome Is.	26.4	28.7	24.1	19.3	32.0	29.0	4.7	18.66
Hazard Bay	26.4	28.7	24.1	19.3	32.0	29.0	4.7	18.65
Yank's Jetty	26.4	28.7	24.1	19.3	32.0	29.0	4.7	18.65
Davies Reef	26.2	28.6	23.7	19.4	31.7	23.1	4.9	18.84
Barb Reef	26.0	28.3	23.6	21.9	30.1	28.7	4.8	19.81
Shaw Is.	25.7	28.3	23.1	21.4	30.3	23.1	5.3	20.52
One Tree Is.	24.7	27.2	22.0	20.3	29.7	27.6	5.3	23.51
Lord Howe Is.	21.6	24.2	19.2	17.8	26.5	24.3	5.0	31.53

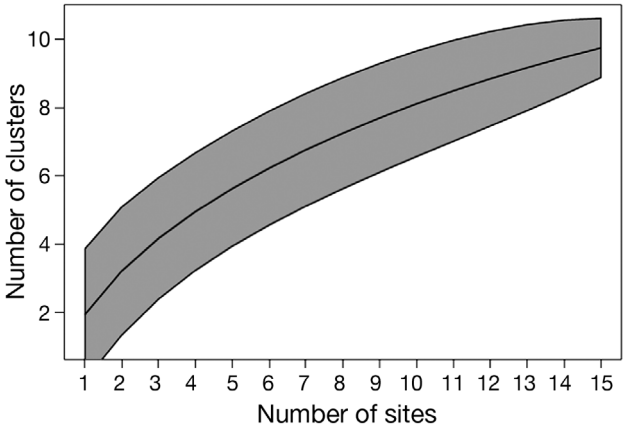

Fig. A1. Rarefaction curve showing the effect of the number of sites on the number of symbiont genetic clusters detected. Grey areas represent 95% confidence intervals. The slope of the line suggests that while most of the variation has been sampled, additional sites would likely result in the discovery of unsampled genetic clusters. This indicates that previous studies, which only included 1 or a few locations, may have been severely undersampled

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