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Patterns of coral–dinoflagellate associations in *Acropora*: significance of local availability and physiology of *Symbiodinium* strains and host–symbiont selectivity

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Like other reef-building corals, members of the genus *Acropora* form obligate endosymbioses with dinoflagellates (zooxanthellae) belonging to the genus *Symbiodinium*. Both *Symbiodinium* and its hosts are diverse assemblages, and the relationships between host and algal genotypes are unclear. In this study, we determined phylogenetic relationships between *Symbiodinium* isolates from a wide range of *Acropora* species and plotted the algal genotypes onto a molecular phylogeny of 28 *Acropora* species, using the same samples for the host and symbiont genotyping. In addition, we performed a preliminary survey of zooxanthella distribution in *Acropora* species from the central Great Barrier Reef. Three of the four known major zooxanthellae clades were represented in the 168 samples examined, and within the major clade C, three distinct subclades were identified. No evidence was found for coevolution, but several clear patterns of specificity were identified. Moreover, composition of the zooxanthella pool varied among locales and in one host species we found light-related patterns of zooxanthella distribution

Keywords: *Symbiodinium*; *Acropora*; SSCP; rDNA ITS; endosymbiosis; coevolution

1. INTRODUCTION

One of the dominant features of today's coral reefs is animal–algal endosymbiosis: a wide variety of microalgae live as endosymbionts with marine protists or invertebrate animals (reviewed in Trench 1993, 1997). All reef-building corals live in symbiosis with endosymbiotic dinoflagellates (zooxanthellae) belonging to a single genus, *Symbiodinium* (Trench 1993). Originally, it was believed that the genus contained one pandemic species, *S. microadriaticum* Freudenthal. More recent morphological, biochemical, physiological and karyotypic (e.g. Blank & Trench 1985, 1986; Schoenberg & Trench 1980) as well as molecular genetic (e.g. Rowan 1991; Rowan & Powers 1991*a*) studies, however, have shown that zooxanthellae are an extraordinarily diverse group exhibiting much higher sequence diversity than that observed between genera of non-symbiotic dinoflagellates.

Based on sequence divergence at nuclear small subunit ribosomal RNA (ssu rRNA) genes, three phylogenetic clades (A, B and C) were originally identified within *Symbiodinium* (Rowan & Powers 1991*a*, 1992). Later, a fourth clade (designated clade D, see Zooxanthella Diversity and Systematics email discussion list at http://www.symbiodinium.listbot.com) was identified in corals (Rowan & Knowlton 1995) and sponges (Carlos *et al.* 1999). *Symbiodinium* strains belonging to these main clades have also been found in a wide taxonomic range of non-scleractinian hosts, including bivalves, soft corals and foraminifers (e.g. Carlos *et al.* 1999; Pochon *et al.* 2000). Although four clades were observed in Caribbean corals, it was initially believed that Pacific Scleractinia harboured only clade C (Baker & Rowan 1997). Preliminary studies (Loh *et al.* 1998), however, indicate that members of clades A and B are also present in Indo-Pacific corals.

The general notion regarding the evolution of invertebrate–dinoflagellate symbioses is that, with the possible exception of those taxa that have maternal inheritance of zooxanthellae (Hunter *et al.* 1997), a lack of correlation between host and symbiont genotype is to be expected (Trench 1993; McNally *et al.* 1994). There is some support for this hypothesis in the literature (Rowan & Knowlton 1995; Baker *et al.* 1997; Rowan *et al.* 1997; Loh *et al.* 1998), but it has not been formally tested because appropriate host phylogenies have not been available. We have recently constructed a molecular phylogeny of 28 *Acropora* species (Scleractinia, Cnidaria) using a nuclear and a mitochondrial marker (van Oppen *et al.* 2001). In order to test the hypothesis of host–symbiont coevolution in the subgenus *Acropora Acropora*, we have now determined the genotypes of the *Symbiodinium* strains hosted by the same individuals as were used in the host phylogenetic study and we have plotted the zooxanthellae genotypes onto the host phylogeny. Matching between phylogenies of the host and symbiont lineages is expected if speciation in one partner of the symbiosis is accompanied by speciation in the other partner. Since no other thorough intra-generic molecular phylogenies are available for Scleractinia, this appears to be the first study to address the issue of host–symbiont co-speciation at a fine taxonomic level. We used sequences of the nuclear ribosomal DNA internal transcribed spacer 1 (rDNA ITS1) region to construct the symbiont phylogeny. Unlike the rDNA genes, which have been strongly conserved through evolutionary time due to functional constraints,

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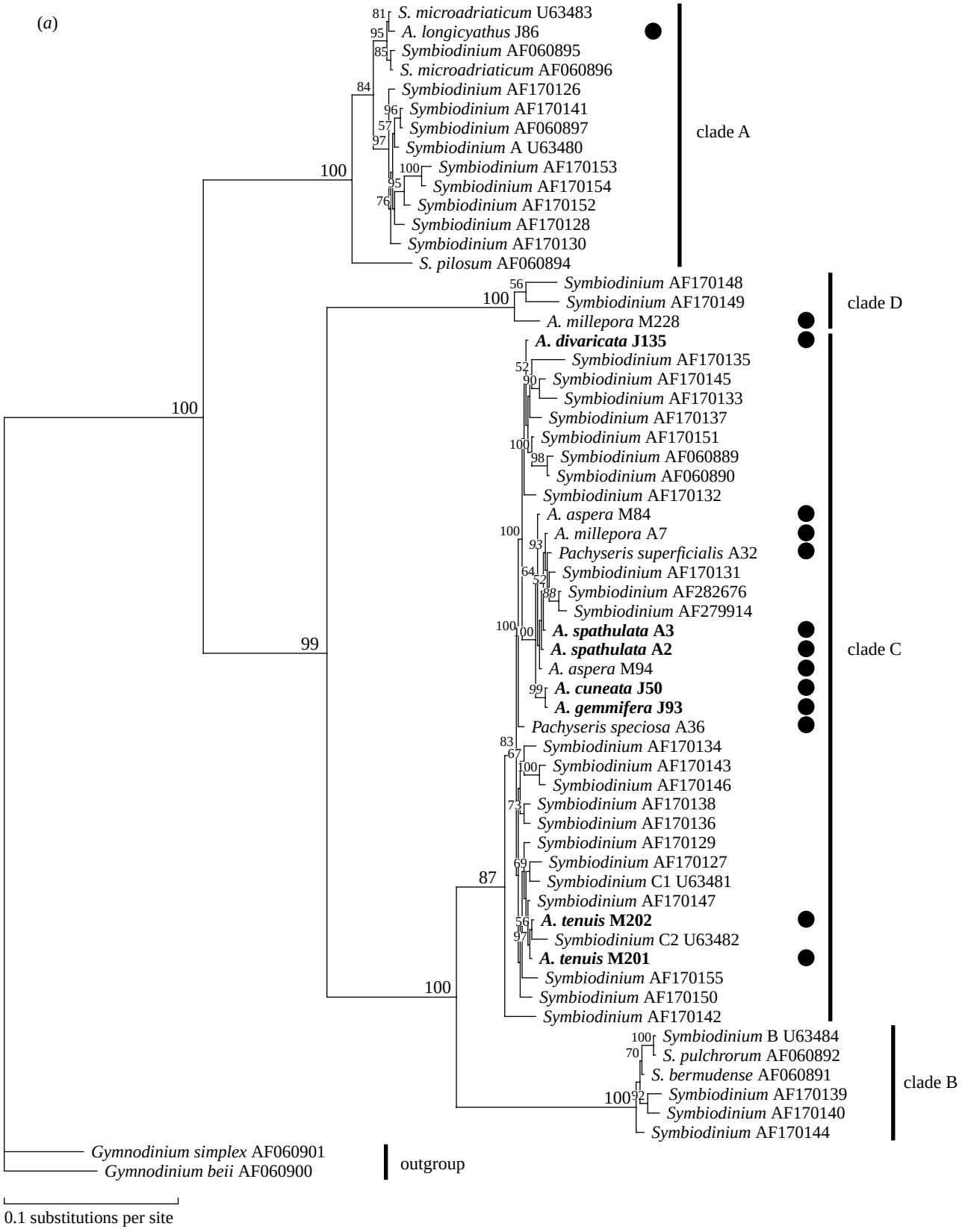

Figure 1(*a*)

the ITS regions are free to undergo much faster substitution rates and provide an amount of sequence variation generally suitable for comparisons at the inter- and intra-species level in algae (e.g. Kooistra *et al*. 1992; Peters *et al*. 1997).

In addition to the issue of co-speciation, we performed a preliminary survey of the distribution of zooxanthella strains (based on sequence variation in ITS1) in a range of *Acropora* species on the central Great Barrier Reef (GBR), to examine what the main factors are that

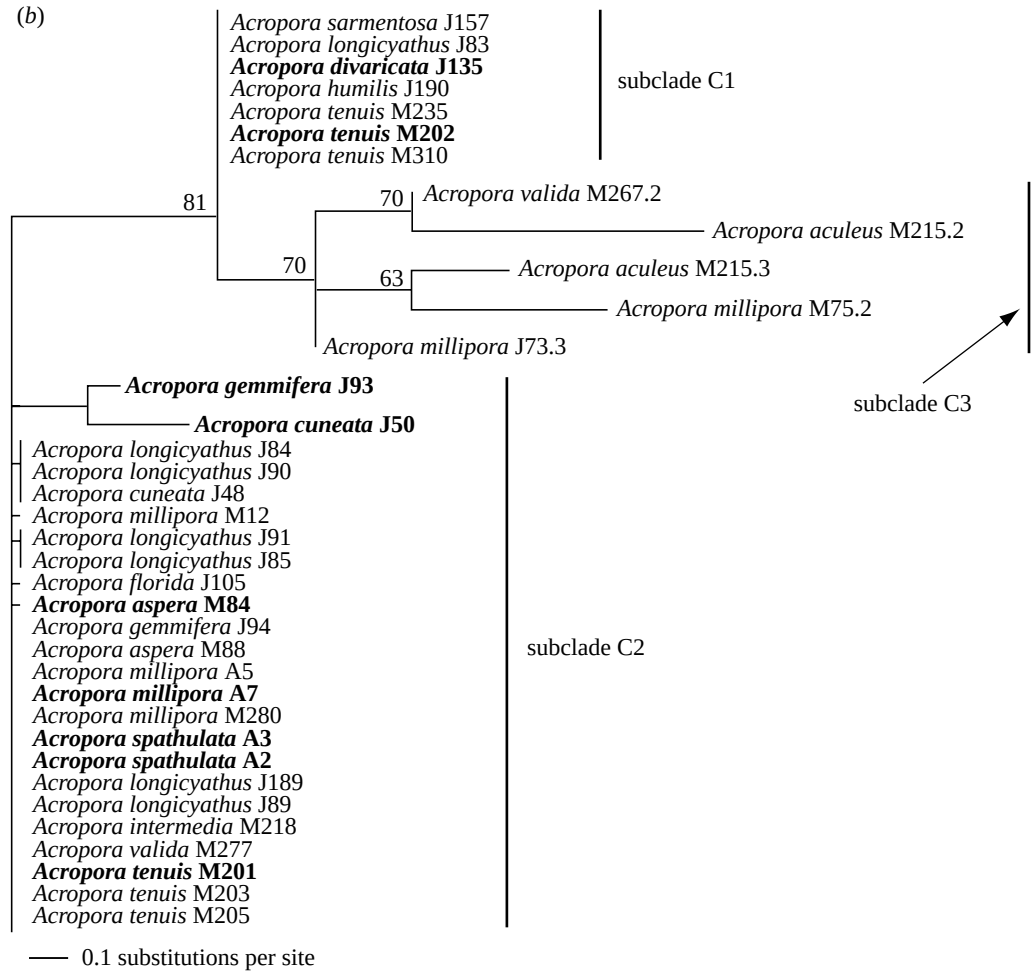

Figure 1. Phylogenetic analyses of zooxanthella rDNA lsu and ITS1 sequences. Names in bold font indicate taxa that occur in both the lsu and ITS1 tree. (*a*) Rooted maximum-likelihood tree based on partial rDNA lsu sequences. Bootstrap values are given above the branches. The black circles indicate the sequences that were obtained in this study. (*b*) Unrooted maximum-likelihood tree based on ITS1 sequences. Samples of which the sample code is followed by a dot and a number (e.g. *Acropora valida* M267.2) have been obtained via cloning of PCR products.

determine zooxanthella distribution. Our results suggest that some specificity in the *Acropora–Symbiodinium* symbiosis exists, but that local availability of zooxanthellae and environmental conditions also play roles in the establishment of these associations.

2. MATERIAL AND METHODS

(a) *Sampling*

The majority of the samples used for the phylogenetic analyses originated from a mid-shelf reef in the central GBR (Trunk Reef, 18°24′ S, 146°49′ E). The outgroup samples from the subgenus *Acropora Isopora* (*A. cuneata*) were collected from a nearby mid-shelf reef (Brittomart, 18°16′ S, 146°42′ E). Other collection localities were Heron Island (23°28′ S, 151°57′ E), Lizard Island (14°40′ S, 145°28′ E), Orpheus (18°34′ S, 146°30′ E), Pelorus Island (18°34′ S, 146°30′ E), and Coral Bay (23°09′ S, 113°45′ E) and Bundegi Reef (21°51′ S, 114°11′ E) in Western Australia (WA). Two *Acropora* samples from the Caribbean were also included in the analysis. To examine finer-scale distribution patterns of the various zooxanthella strains, additional samples were collected from Magnetic Island (Geoffrey Bay and Nelly Bay; 19°20′ S, 146°50′ E), Orpheus Island and Pelorus Island. Details of samples are given in the electronic appendix (available at http://www.pubs.royalsoc. ac.uk/proc_bio/proc_bio.html). Small pieces of branches were snapped off and preserved in 90–100% ethanol (EtOH).

(b) *DNA extraction, PCR, cloning and sequencing*

Methods for DNA extraction, cloning, sequencing and the coral *PaxC* intron PCR are given in van Oppen *et al.* (2001). To PCR-amplify the 5′-end (D1 and D2) of the zooxanthella rDNA lsu, we used the primer pair lsu-UFP1 (5′-CCCGCTAATT-TAAGCATATAAGTA-3′) and lsu-URP1 (5′-GTTAGACTCC TTGGTCGTGTTTCA-3′) (Zardoya *et al.* 1995) and the following PCR profile: one cycle of 3 min at 95 °C, 30 cycles of 20 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C, followed by 5 min at 72 °C. To amplify the zooxanthella ITS1 region we designed dinoflagellate-specific primers based on sequences available in the database: symITSFP (5′-CTCAGCTCTGGACGTTGYGTT GG-3′) and symITSRP (5′-GCTGCGTTCTTCATCGATGC-3′). The PCR profile was: one cycle of 3 min at 94 °C, 30 cycles of 30 s at 95 °C, 30 s at 59 °C and 30 s at 72 °C, followed by 5 min at 72 °C. PCR was carried out in 25 µl volumes and consisted of 1 µl of each primer (10 µM), 2.5 µl 10x buffer (Fisher Biotec, West Perth, WA 6005, Australia), 2.5 µl dNTP (2 µM), 2 µl $MgCl_2$

(25 μM), 0.13 μl Taq polymerase (5.5 units μl^{-1}; Fisher Biotec), 14.87 μl H_2O and 1 μl DNA (1–100 × diluted from the original stock).

(c) *SSCP*

Single-stranded conformation polymorphism (SSCP) of the ITS1 plus flanking regions was performed in 10 μl volumes following Sunnucks *et al.* (2000). Products were denatured and snap-cooled on ice, to allow the two DNA strands to fold back onto themselves, and 3 μl were loaded onto 20 cm long 8% non-denaturing polyacrylamide gels (Sunnucks *et al.* 2000). Along with the denatured PCR products, a double-stranded DNA sample was run to aid in interpretation of the gel. Recurring banding patterns were run on consecutive gels as references. Gels were run on constant power (6 W) and temperature (10 °C) for 10 h, and visualized by autoradiography.

(d) *Data analysis*

Sequences were aligned manually using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Maximum-likelihood analyses were performed in Molphy 2.3 (Adachi & Hasegawa 1996) under the HKY85 model of sequence evolution. Neighbour-joining distance and maximum-parsimony (heuristic search) analyses were conducted in Paup 4.0b3 (Swofford 1999). Gaps were either excluded or treated as a fifth base in parsimony analyses. Bootstrap analysis was performed with 1000 replicates.

3. RESULTS

(a) *Phylogenetic analyses*

Because ITS sequences could not be aligned between the major *Symbiodinium* clades (i.e. A, B, C and D), sequences of the more slowly evolving 5′-end of the lsu rDNA were obtained for the algal symbionts of 12 *Acropora* samples and one sample of each *Pachyseris superficialis* and *P. speciosa* from Taiwan (GenBank accession numbers AF380513–AF380529, alignment also available) and compared with sequences from the database (figure 1*a*). For nine of these samples ITS1 sequences were also obtained (taxa in bold font in figure 1*a*,*b*), and they were used as references to determine to which clade samples belonged. The lsu rDNA alignment comprised 66 taxa and 610 positions. There were 80 variable and 263 phylogenetically informative positions (gaps included). The phylogenetic analysis showed that most symbiotic dinoflagellates in our samples belong to clade C, one belongs to clade A and some to clade D (figure 1*a* and the electronic appendix). Clade B zooxanthellae were not encountered in our samples.

In total, 1 clade A, 37 clade C and 6 clade D ITS1 plus flanking region sequences (18S, 77 positions; ITS1, 225 positions; 5.8S, 38 positions) were obtained from the algal symbionts of a range of *Acropora* species (GenBank accession numbers AF380530–AF380565, alignment also available). Gene boundaries were determined by a comparison with *Symbiodinium* strain HH1A (accession number AF183575; Baillie *et al.* 2000). The sequences could not be aligned between clades, while within-clade variability was low. Only one variable alignment position (of a total of 343 positions) was observed among the six *Symbiodinium* D sequences. This occurred in a cloned PCR fragment, suggesting that it may represent a PCR error. The clade C alignment consisted of 340 positions, of which 13 were variable and 5 were phylogenetically informative (with gaps treated as a fifth base). Most sequences were obtained by direct sequencing of PCR products, and occasionally double peaks were observed (sequences in which this was observed are marked in the electronic appendix), indicating that either two or more zooxanthellae strains were present in a single host or that rDNA is not fully homogenized within individuals. In most instances, ITS types that were found in combination with other types were also found separately. This suggests that in general ITS has been homogenized within individuals in *Symbiodinium* and that the simultaneous occurrence of two or more ITS types represents intra-colony symbiont polymorphism. Only in cases where divergent ITS types were present in a single host colony (e.g. *A. aculeus* M215 and *A. valida* M267) did we clone the PCR products prior to sequencing, as described in van Oppen *et al.* (2001). Phylogenetic analysis showed that three main clusters could be distinguished (figure 1*b*). These three subclades were consistently found with maximum-likelihood, maximum-parsimony and neighbour-joining distance analysis. Despite having the smallest sample size, the highest sequence diversity was observed in subclade C3. All coral colonies that hosted this type of zooxanthella contained a mix of two subclade C3 sequences (see figure 1*b* and figure 2). Seven of the nine samples for which both ITS1 and lsu sequences were obtained fell in the same clusters in both analyses (taxa in italic font in figure 1*a*,*b*). The zooxanthella strains of *A. divaricata* J135 and *A. tenuis* M201, however, occurred at different positions in the two trees. This may be due to the small number of substitutions that have occurred among clade C taxa in the lsu rDNA and the stochasticity of nucleotide substitutions.

(b) *SSCP profiles*

After initial identification of the symbiont genetic diversity in a subset of samples by sequence analysis of the ITS1 region, SSCP was used to survey the distribution of zooxanthella genotypes across a larger number of samples. Figure 2 shows an autoradiogram of an SSCP gel on which ITS samples from all three C subclades (see figure 1*b*), as well as from clades A and D, were run (see figure 1*a*). Two high-intensity bands plus occasional fainter bands were generally seen when only one ITS type was present (lanes 1–18, 23–25). In the case of mixed, more divergent ITS types (e.g. the C3 subclade), four main bands were visible. SSCP analysis of cloned PCR products showed that the top two bands belong to one ITS type and the bottom two bands to the other (not shown). The data indicate that the SSCP technique is suitable for comparing products that differ by only a small number of base changes. For example, the product run in lane 5 differs by two substitutions from the product run in lane 2 and by two substitutions and one indel from that in lane 4. The two products co-amplified in lanes 20–22 differ by eight base substitutions. The clade A and D products have a distinct SSCP profile from clade C products, but still show similar mobility. Hence the resolution of this technique is reduced with increased genetic distances.

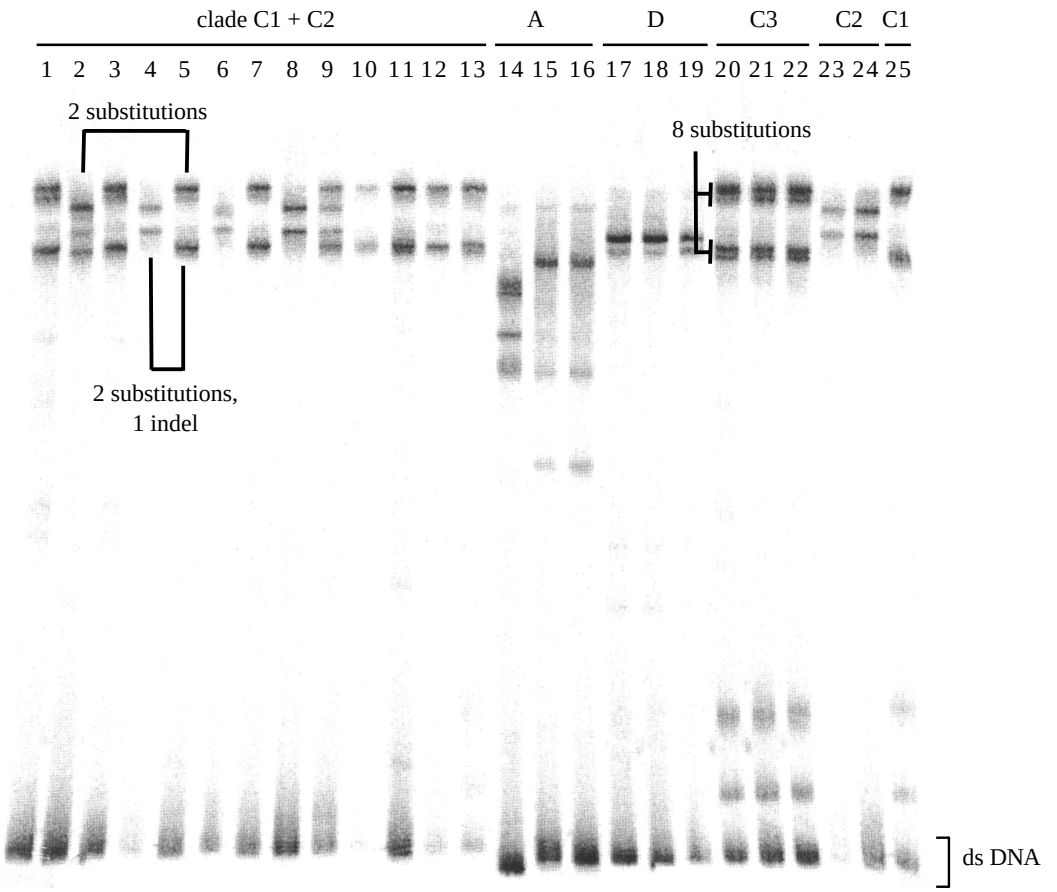

Figure 2. Autoradiogram of an SSCP gel showing the different *Symbiodinium* clades and subclades. MI, Magnetic Island; PB, Pioneer Bay; PI, Pelorus Island; WA, Western Australia. Lane 1, *A. florida* J103 Trunk; lane 2, *A. humilis* J189 Trunk; lane 3, *A. humilis* J190 Trunk; lane 4, *A. tenuis* (upper surface) M203 PB; lane 5, *A. tenuis* (under surface) M204 PB; lane 6, *A. tenuis* (upper-surface) M207 PB; lane 7, *A. tenuis* (under surface) M208 PB; lane 8, *A. tenuis* (upper-surface) M205 PB; lane 9, *A. tenuis* (side) M206 PB; lane 10, *A. tenuis* M240 MI; lane 11, *A. tenuis* M241 MI; lane 12, *A. longicyathus* J83 Trunk; lane 13, *A. longicyathus* M222 PI; lane 14, *A. longicyathus* J86 Trunk; lane 15, *A. palmata* M39 Caribbean; lane 16, *A. cervicornis* M40 Caribbean; lane 17, *A. millepora* M228 MI; lane 18, *A. millepora* M230 MI; lane 19, *A. millepora* M231 MI; lane 20: *A. millepora* M73 WA; lane 21, *A. millepora* M75 WA; lane 22, *A. millepora* M76 WA; lane 23, *A. aspera* M85 WA; lane 24, *A. aspera* M86 WA; lane 25, *A. aculeus* M215 PI.

(c) *Comparison of host and symbiont phylogenies*

The sequencing and SSCP results were pooled in order to compare the evolutionary histories of the coral hosts and their symbionts (figure 3). The different colours in the two trees indicate which symbionts are harboured by the coral hosts. The maximum-likelihood tree of the host (species of the subgenus *Acropora Acropora*) was rooted with members of the subgenus *Acropora Isopora* (i.e. *Acropora cuneata*) (van Oppen *et al.* 2001). The position of the root in the symbiont tree was based on the rDNA lsu phylogeny shown in figure 1*a*. This tree is a 'summary' tree between figure 1*a*,*b* and was drawn manually. The branch lengths were not drawn to scale and are therefore not informative. A statistical test (e.g. Page 1995; Peek *et al.* 1998) of phylogenetic congruence between coral and dinoflagellate phylogenies was not possible because it is currently unknown which of the C subclades is most ancestral, since ITS1 sequences could not be aligned between *Symbiodinium* clades A, C and D. In other words, we do not know where to connect the internode leading to clade C (broken line in figure 3) to the three subclades C1–C3. A second problem was that the coral hosts exhibit much higher levels of taxon diversity as compared with the symbionts (although the divergences were deeper for the symbionts), at least for the markers that were used here. Nevertheless, visual inspection of the trees clearly showed that no correlation exists between the two phylogenies. For example, the most ancestral *Symbiodinium* A was never observed in the basal *A. Acropora* clade, but only in one of the two most derived main clades. In contrast, subclade C1 and C2 symbionts were found in all host clades, also supporting phylogenetic incongruence.

(d) *Distribution of algal genotypes*

Clade D zooxanthellae were only found in the Magnetic Island and Pioneer Bay samples, while only one sample (*A. longicyathus* from Trunk Reef) contained clade A zooxanthellae (figure 4). Subclade C2 *Symbiodinium* was absent in our samples from Magnetic Island, while this was otherwise the most common strain. Although several coral colonies harboured more than one zooxanthella taxon, in only one species (*A. tenuis*) on one reef (Pioneer Bay) were these taxa found in either exposed or shaded parts of the same colony. *A. valida* colonies that occurred at the same

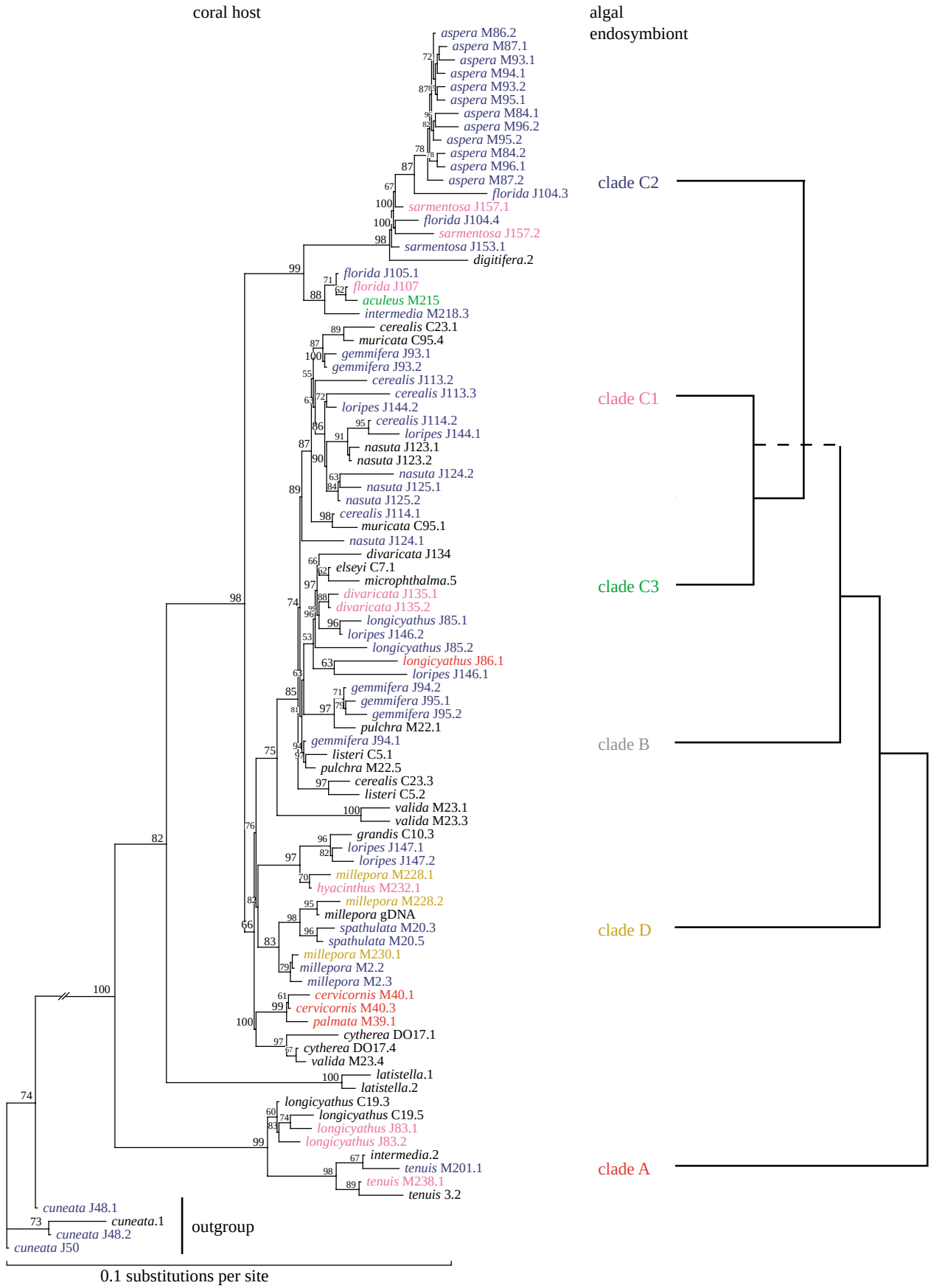

Figure 3. Comparison of the *PaxC* intron phylogeny of 29 *Acropora* species (van Oppen *et al*. 2001) with that of the endosymbionts from the same specimens. Note that many *Acropora* species are non-monophyletic (van Oppen *et al*. 2001). Colours in the two trees match the host with its symbiont. For coral taxa in black letters, the endosymbionts were not characterized (most of those are sequences derived from sperm or eggs, which lack zooxanthellae). The branch leading to *Symbiodinium* clade C is shown as a broken line, as it is unknown which of the three subclades (C1–C3) is ancestral. Bootstrap values are given above the branches.

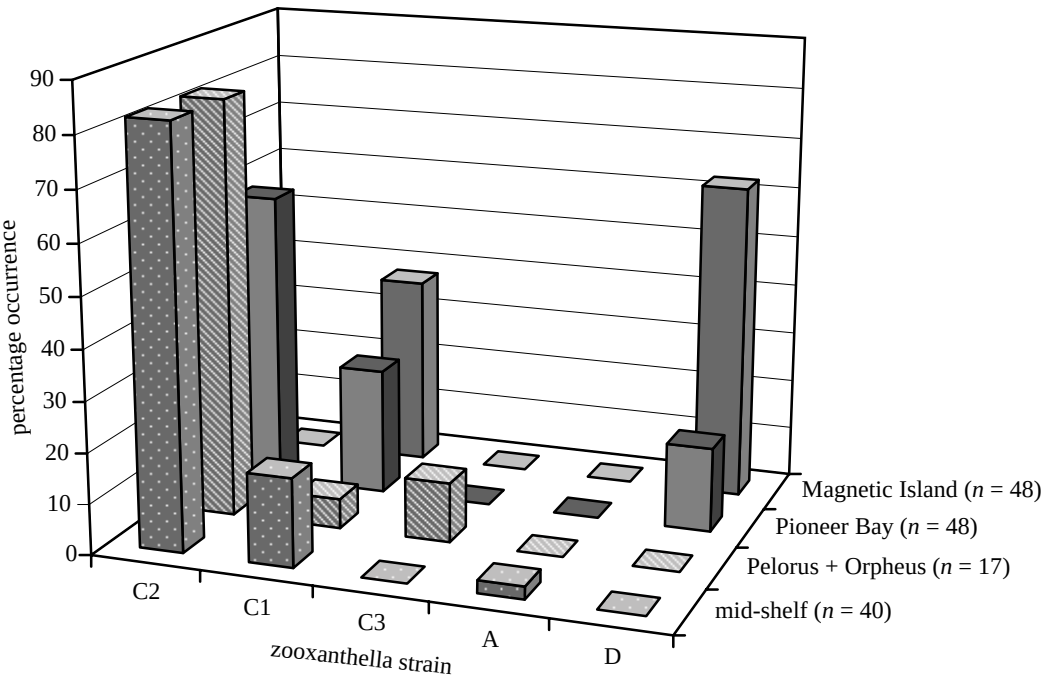

Figure 4. Relative abundance of the different *Symbiodinium* (sub)clades at four sites on the central GBR. *n*, number of samples taken.

depth and next to *A. tenuis* colonies in Pioneer Bay did not show this irradiance-correlated zooxanthella distribution, but hosted the same clade C and D strains simultaneously throughout the colony. Moreover, small *A. tenuis* colonies (possibly new recruits since the 1998 mass bleaching event) sampled from the reef flat did not show this light-related pattern either. Symbiont genotypes of all samples used in this study are given in the electronic appendix.

4. DISCUSSION

(a) *No evidence for coevolution between* A. Acropora *spp. and their algal symbionts*

The phylogenies of *A. Acropora* host species and their symbionts of the genus *Symbiodinium* are clearly incongruent (figure 3), and indicate that corals in the subgenus *A. Acropora* have not coevolved with their algal symbionts. *A. Acropora* species produce azooxanthellate eggs and newly settled polyps have to acquire endosymbionts from the environment every generation. In symbioses with horizontal transmission, hosts have generally been found to form associations with a broad range of symbiotic genotypes (Douglas 1998). Hence, it would be expected that corals with vertical symbiont transmission show higher levels of phylogenetic congruence with their algal endosymbionts. A preliminary study of *Porites* (which produce zooxanthellate eggs) is consistent with this (Hunter *et al.* 1997). Whether the same pattern exists in acroporid corals with vertical transmission of endosymbionts, such as *Montipora* spp., remains to be tested.

(b) *Specificity of the* Acropora*–dinoflagellate symbioses?*

Despite the absence of any convincing patterns of co-speciation, there are several clear examples of non-random associations between *Symbiodinium* strains and *Acropora* hosts. One complication with the interpretation of these data is that a number of factors appear to be involved, and it will be difficult to dissect genetic from biogeographical and physiological factors. Biogeographical variation in symbiont profiles can be clearly seen in the case of clade C2 zooxanthellae, which were not detected at Magnetic Island (figure 4), and clade C3 zooxanthellae, which present only in the Pelorus and Orpheus samples. These biogeographical trends may be due to environmental factors and lead to general patterns of association between hosts and algae. For example, at Magnetic Island, all of the *A. millepora* colonies sampled ($n = 15$) harboured clade D zooxanthellae, but in Pioneer Bay (*ca.* 70 km away) none of the *A. millepora* colonies hosts *Symbiodinium* D. Instead, 89% of *A. millepora* colonies ($n = 9$) harbour subclade C2, while the remaining 11% have C1 symbionts. The observed patterns might be explained by assuming that C2 is the preferred zooxanthella strain for *A. millepora*. In its absence (around Magnetic Island), associations are formed with *Symbiodinium* D. It is not clear, however, why some of the *A. millepora* colonies in Pioneer Bay harbour clade C1 symbionts and those at Magnetic Island harbour symbionts of clade D, while both strains are present at Pioneer Bay and Magnetic Island. The data suggest that *A. millepora* is capable of establishing a successful symbiosis with both *Symbiodinium* clade C and clade D.

The restricted distribution of *Symbiodinium* A may represent a case of real specificity. Although we detected this strain in only a single isolate of *A. longicyathus* (figure 1*a*; electronic appendix), there are precedents for the restriction of this symbiont genotype to this host species (Loh *et al.* 1998) and for the rareness of clade A *Symbiodinium* in coral hosts in the Indo-Pacific (e.g. Loh *et al.* 1998; Baker 1999). Note that, in the Caribbean, clades A and B are common in scleractinian corals (Rowan & Powers

1991*a*,*b*; Baker 1999). Either a symbiont recognition mechanism (e.g. McAuley 1988; Reynolds *et al.* 2000), environmental conditions or symbiont competition inside the host tissues (or a combination of these factors) must be operating to account for these observed patterns of distribution of the different *Symbiodinium* clades.

(c) *Irradiance-related patterns of intra-colony symbiont distribution in* A. tenuis

In only one out of eight host species from which both irradiated and shaded sides were sampled—all *A. tenuis* colonies on the reef crest in Pioneer Bay ($n=10$)—light-related patterns of symbiont distribution were found. The side of the colony that was directly exposed to light (i.e. the upper surface) contained zooxanthellae of clade C2, while the shaded side (i.e. the under surface) contained Cl zooxanthellae. Interestingly, a sample from the side of an *A. tenuis* colony (i.e. with intermediate light levels) contained both C2 and Cl zooxanthellae (figure 2, lane 9). In a similar environment off Magnetic Island and at the same depths, none of the *A. tenuis* ($n=6$) or other six species showed this irradiance-related pattern of intra-colony symbiont distribution. A similar (light-related) pattern of distribution has previously been documented in the case of the Caribbean corals *Montastrea annularis* and *M. faveolata* (Rowan & Knowlton 1995; Rowan *et al.* 1997), individual colonies of which may simultaneously harbour up to three *Symbiodinium* strains from clades A, B and C. Experimental work is required to determine the exact cause of these patterns, such as light intensity or UV-levels.

(d) *Conclusion*

The results presented here suggest that the *A. Acropora–Symbiodinium* symbiosis is an open system with no evidence for co-speciation, possibly related to the horizontal mode of symbiont transmission. The occurrence of a particular *Symbiodinium* taxon in *A. Acropora* species appears to be determined by a number of factors, including the local availability of zooxanthellae, ambient light levels, genetic identity of the host and possibly competition between physiologically different zooxanthella taxa. If biogeographical differences in zooxanthella pools (as those observed here) are common, then it may be difficult to distinguish biogeographical effects from true specificity of coral–algal symbioses by examination of their distribution patterns alone.

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