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Genetic variability of the symbiotic dinoflagellates from the wide ranging coral species *Seriatopora hystrix* and *Acropora longicyathus* in the Indo-West Pacific

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ABSTRACT: The scleractinian coral species, *Seriatopora hystrix* and *Acropora longicyathus*, are widely distributed throughout the latitudinal range of the tropical west Pacific. These 2 coral species live in a mutually beneficial relation with symbiotic dinoflagellates (zooxanthellae), which are passed to their progeny by vertical transmission (zooxanthellate eggs or larvae) and horizontal transmission (eggs or larvae that acquire symbionts from the environment), respectively. For *S. hystrix*, vertical transmission might create biogeographically isolated and genetically differentiated symbiont populations because the extent of its larval migration is known to be limited. On the other hand, horizontal transmission in corals such as *A. longicyathus* may result in genetically connected symbiont populations, especially if its zooxanthellae taxa are widely distributed. To examine these hypotheses, symbionts were collected from colonies of *S. hystrix* and *A. longicyathus* living in the Great Barrier Reef (Australia), South China Sea (Malaysia) and East China Sea (Ryukyus Archipelago, Japan), and were examined using restriction fragment length polymorphism and sequence analysis of large and small subunit rRNA genes. Phylogenetic analysis assigned the symbionts to 1 of 3 taxonomically distinct groups, known as clades. Symbionts from Australian and Japanese *S. hystrix* were placed in Clade C, and Malaysian *S. hystrix* symbionts in the newly described Clade D. Seven of 11 Australian and all Japanese and Malaysian colonies of *A. longicyathus* had symbiotic dinoflagellates that also grouped with Clade C, but symbionts from the remaining Australian colonies of *A. longicyathus* grouped with Clade A. Analysis of molecular variance of Clade C symbionts found significant genetic variation in 1 or more geographic groups (69.8%) and to a lesser extent among populations within geographic regions (13.6%). All populations of Clade C symbionts from *S. hystrix* were genetically differentiated according to geographic region. Although Clade C symbionts of *A. longicyathus* from Japan resolved into a distinct geographic group, those from Australia and Malaysia did not and were genetically connected. We propose that these patterns of genetic connectivity correlate with differences in the dispersal range of the coral or symbiont propagules and are associated with their respective modes of symbiont transmission.

KEY WORDS: *Seriatopora hystrix* · *Acropora longicyathus* · Symbiotic dinoflagellates · Geographic population variation

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INTRODUCTION

All hermatypic (reef-building) corals harbor endosymbiotic dinoflagellates (zooxanthellae) in their tissues that form a mutualistic symbiosis in which the host benefits from the transfer of photosynthetic products and the symbiotic dinoflagellate benefits from the provision of otherwise rare inorganic nutrients (Trench 1979). Other advantages are also thought to accrue from the association of the coral host and dinoflagel-

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late, such as the enhancement of calcification through the removal of carbon dioxide or phosphate or both (Goreau & Goreau 1959, Simkiss 1964a,b).

Symbiotic dinoflagellates of cnidaria are mostly classified under the genus *Symbiodinium* (Dinophyceae), although at least 1 species has been placed in genus *Gymnodinium* (Trench & Thinh 1995, Wilcox 1998). They are a taxonomically diverse group (Schoenberg & Trench 1980a,b,c, Blank & Trench 1985, Trench & Blank 1987, Trench & Thinh 1995) and generally host specific (Rowan & Powers 1991b). Among those that have been taxonomically defined are *S. kawaguti* (Trench & Blank 1987) and *S. meandrinae* (Trench 1997, isolated by Trench) from the scleractinian corals *Montipora verrucosa* and *Meandrina meandrites*, respectively; *S. goreauii* (Trench & Blank 1987), *S. pulchorum*, *S. bermudense*, *S. cariborum* and *S. californium* (Trench 1997, isolated by Trench) from the actinarians *Ragactis lucida*, *Aiptasia pulchella*, *A. tagetes*, *Condylactis gigantea* and *Anthopleura elegantissima*, respectively; *S. corculorum* that was isolated by Trench from the bivalve *Corculum cardissa*; *S. pilosum* (Trench & Blank 1987) from the zoanthid *Zoanthus sociatus*; and S. microadriaticum (Freudenthal emend. Trench) and *Gymnodinium linucheae* (Trench & Thinh 1995) from the medussae *Cassiopeia xamachana* and *Linuche unguilata*, respectively (Trench & Blank 1987, Trench & Thinh 1995, Trench 1997).

However, it must be pointed out that these symbionts have been cultured, and cultivation-dependent methods may not accurately represent the *in situ* diversity that may exist. This is because culturing might be enhancing or inhibiting the growth of particular microorganisms (Ward et al. 1990, Amann et al. 1995). In addition, Wilcox (1998) showed that morphological similarities do not always delineate symbiotic dinoflagellates into evolutionarily cohesive groups.

Analysis of large and small subunit ribosomal genes of several of these symbiotic dinoflagellate species has revealed that the symbionts belong to 1 of 3 diverse genotypic clades that have been nominally denoted clades ‘A’, ‘B’ and ‘C’ (Rowan & Powers 1991a,b). Although evidence suggests that all 3 clades are distributed evenly among Caribbean coral species, Clade C appears to be predominant among the relatively few Pacific coral species that have been surveyed (Baker & Rowan 1997, Loh et al. 1998). More recently, a fourth clade, ‘D’, of dinoflagellates has been identified (Baker 1999).

Many cnidarians, including some corals, transfer their symbionts maternally (vertical transmission) into larvae or into planulae that are initially brooded (Richmond 1981, Benayahu & Schleyer 1998, Shlesinger et al. 1998, Sier & Olive 1998, Titlyanov et al. 1998). Symbionts may also be transferred to pre-released eggs in other coral species (Szmant et al. 1980, Arai et al. 1993, Schwarz et al. 1999). Many other coral species release planulae or eggs that do not contain symbiotic dinoflagellates (Kojis 1986, Harrison & Wallace 1990). In this case, symbioses are initiated by capture of dinoflagellates from the water column or other environmental source (horizontal transmission). Conceivably, these transmission strategies may influence the diversity of symbionts that are established within coral colonies.

Many coral species occur across a wide range of tropical Indo-Pacific locations. Under these situations, the host and associated symbiont experience widely varying environmental conditions. On high latitude reef systems, corals may experience cold-water temperatures and very low light levels in the winter, and greatly contrasting higher water temperatures and light levels in the summer. The symbiotic dinoflagellates of reef-building corals are sensitive to changes in light and temperature (Jones et al. 1998, Hoegh-Guldberg & Jones 1999). Consequently, the question as to whether the identity of the symbiotic dinoflagellate remains the same in its coral host at different parts of their often wide-ranging distribution is of great interest. This is complicated further by the symbiont acquisition strategies of corals that rely on vertical or horizontal transmission. We have addressed this question by investigating the molecular diversity of symbiotic dinoflagellates from 2 widely distributed coral species, *Seriatopora hystrix* and *Acropora longicyathus*, at 3 disparate locations in the Indo-Pacific. *S. hystrix* is a brooding species (Ayre 1994) and transfers its symbionts vertically to its planulae from the maternal phase (Atoda 1951, D. Ayre, University of Wollongong, pers. comm.). *A. longicyathus* is a broadcasting species (Wallace 1985, Shlesinger et al. 1998) and eggs that are released by adult colonies do not have symbiotic dinoflagellates (Harrison & Wallace 1990, S. Ward, University of Queensland, pers. comm.). The locations chosen were the Great Barrier Reef, Australia in the south Pacific, and South China Sea, Malaysia and East China Sea, Japan in the northern Pacific.

MATERIALS AND METHODS

Collection and identification of coral samples. The 2 coral species used for this study were *Seriatopora hystrix* Dana (Scleractinia: Pocilloporidae) and *Acropora longicyathus* Edwards and Haime (Scleractinia: Acroporidae). Corals were sampled on snorkel at a depth of 1 to 6 m. The sites were coral reefs off the following islands: One Tree Island (23° 152′ S, 152° 06′ E), southern Great Barrier Reef, Australia; Pulau Redang and Pulau Gaya (6° N, 103° E, 6° N, 116° E, respectively, South China Sea), Malaysia; and Sesokojima

and Akajima (27° N, 128° E, 26° N, 127° E, respectively, East China Sea), Japan. For each species, colonies that were at least 10 m apart were chosen for sampling. The samples were identified using monographs (Veron & Pichon 1976, Veron & Wallace 1984) and confirmed by reference to the collection at the Queensland Tropical Museum, Townsville, Australia. Colony designations and their sampling locations are summarized in Table 1.

Extraction of DNA. Live fragments of coral colonies (approximately 4 cm^2) were collected, placed in sterile plastic bags and blown with directed jets of high-pressure air to remove tissues from the skeletons. The ensuing tissue slurry was collected from the bottom of the bag and an approximately equal volume of DNA preservative (20 % DMSO in 0.25 M EDTA, pH 8.0 and saturated with NaCl; Seutin et al. 1991) was added. The samples were stored and transported at temperatures that ranged from 0°C to ambient, or processed immediately.

Tissue slurries were incubated in 1 % sodium dodecyl-sulfate (Boehringer Mannheim, Mannheim, Germany) at 65°C for 1 h. This was followed by digestion with Proteinase K (Boehringer Manheim), which was added to a final concentration 0.5 mg ml^{-1} and incubated at 37°C for 8 to 12 h. An equal volume of phenol was added to the digested slurry, which was mixed and centrifuged (10 000 × *g*, 10 min, 21°C). The aqueous phase was removed and subjected to a second extraction using phenol-chloroform (25:24) and a third using chloroform-isoamyl alcohol (25:1). DNA was precipitated by the addition of $^1/_{10}$ volume of 3 M sodium acetate (pH 5.2) and an equal volume of cold isopropanol (0°C). The precipitate was washed with 70 % ethanol, dried, dissolved in 50 µl of sterile Milli-Q water and stored at –70°C.

PCR amplification and restriction fragment length polymorphism analysis. The 18S rRNA gene was amplified from symbiotic dinoflagellates using the primer set designed by Rowan & Powers (1991a). These were an equimolar mixture of 2 forward primers, 5′-GCA GTT ATA ATT TAT TTG ATG GTC ACT GCT AC-3′ and 5′-GCA GTT ATA ATT TAT TTG ATG GTT GCT GCT AC-3′, with the reverse primer 5′-AGC ACT GCG TCA GTC CGA ATA ATT CAC CGG-3′.

The variable domains D1 and D2 of the 28S rRNA gene were amplified from coral and their symbiotic dinoflagellates using a primer set designed by Zardoya et al. (1995). These were forward 5′-CCC GCT GAA TTT AAG CAT ATA AGT AAG CGG-3′ and reverse 5′-GTT AGA CTC CTT GGT CCG TGT TTC AAG A-3′ (position 26 and 741, respectively, in the 28S rDNA sequence of the free-living dinoflagellate *Prorocentrum micans*, Genbank accession no. X16108). Primers specific to symbiotic dinoflagellates were also designed based on subsequent sequencing and comparison of the D1 and D2 region of coral and symbiotic dinoflagellate 28S rRNA genes (see below for size differentiation of coral and symbiont amplicon). These primers were forward 5′-CCT CAG TAA TGG CGA ATG AAC A-3′ and reverse 5′-CCT TGG TCC GTG TTT CAA GA-3′.

All PCR reactions contained 0.4 µg of template DNA, 10 µl of 10 × PCR buffer (1 M Tris-HCl [pH 8.3], 0.5 M KCl, 15 mM $MgCl_2$), 1 µl of 10 mM total deoxyribonucleoside triphosphates, 40 µg DNAase-free bovine serum albumin (Amersham Biosciences, Uppsala, Sweden), 20 pmol of each primer and 5 units of *Taq* polymerase (Ampli-*Taq*, Perkin Elmer, Wellesley, MA) in a total volume of 100 µl. Amplifications were performed using a DNA thermal cycler (Cetus 2400, Perkin Elmer) with the following conditions: 94°C for 1 min, 55°C (18S rRNA gene primers) or 65°C (28S rRNA gene primers) for 2 min, and 72°C for 3 min (35 cycles).

The amplicons were analyzed by electrophoresis in 2 % agarose gels (10 V cm^{-1}; 40 mA), stained with ethidium bromide and visualized with UV transillumination. Where they were co-ampified, coral and dinoflagellate 28S rDNA amplicons were identified in the gel by their sizes of approximately 885 and 650 bp, respectively. A gel plug containing each separate amplicon was removed by plunging a truncated yellow pipette tip into the amplified band, which was then expunged into 50 µl of sterile Milli-Q water and melted at 95°C. Reamplification PCR reactions were set up as before except for the use of 1 µl melted gel solution as the template DNA.

Table 1. Coral host species, symbiont/colony codes used and sampling locations

Sceriatopora hystrix	*Acropora longicyathus*	Location
Sh1A, Sh2A, Sh3A, Sh4A, Sh5A, Sh6A, Sh7A, Sh8A, Sh9A, Sh10A	Al1A, Al2A, Al3A, Al5A, Al6A, Al13A, Al25A, Al26A, Al43A, Al45A, Al53A	Great Barrier Reef, Australia
Sh1M, Sh2M, Sh3M, Sh4M, Sh5M, Sh6M, Sh7M, Sh8M, Sh9M	Al1M, Al2M, Al3M, Al4M, Al5M, Al6M, Al7M, Al8M, Al9M, Al10M, Al11M, Al12M	South China Sea, Malaysia
Sh1J, Sh2J, Sh3J, Sh4J, Sh5J, Sh6J, Sh7J, Sh8J, Sh9J, Sh10J, Sh11J, Sh12J	Al1J, Al2J, Al3J, Al4J, Al5J, Al6J, Al7J, Al8J, Al9J, Al10J, Al11J	East China Sea, Japan

Restriction fragment length polymorphism (RFLP) analysis of the 18S or 28S rDNA amplicons was done by restriction digestion with *Taq* 1 restriction enzyme (Progen Industries, Darra, QLD, Australia) for 3 h at 65°C. The digested amplicons were then analyzed by electrophoresis in 2% agarose gels (10 V cm^{-1}; 40 mA), and visualization by ethidium bromide staining and UV transillumination.

DNA sequencing. PCR amplifications (or reamplifications) that produced a single amplicon were purified for direct sequencing by polyethylene glycol precipitation (Rosenthal et al. 1993). The sequence was determined from 1 or both ends of the rRNA gene using dye-terminators and a 373A DNA automated sequencer (Perkin-Elmer). Sequencing was done at the Sydney University and Prince Alfred Molecular Analysis Centre, and the Australian Genome Research Facility at the University of Queensland.

DNA sequences of symbiont genotypes derived in this study are available in GenBank Accession numbers for symbionts Al2A, Al3A, Al5A, Al6A, Al13A, Al25A, Al26A, Al45A, Al53A, Al1M, Al3M, Al11M, Al1J, Al2J, Al7J and Al11J are AF279914, AF282676, AF349553, AF349556, AF353170, AF349554, AF349555, AF353171, AF349557, AF353176, AF353178, AF353177, AF-353172, AF353174, AF353175 and AF353173 respectively. Accession numbers for symbionts Sh2A, Sh6A, Sh8A, Sh4M, Sh5M, Sh6M, Sh7M, Sh5J, Sh6J, Sh9J and Sh10J are AF349552, AF349551, AF349550, AF-349546, AF349547, AF349548, AF349549, AF349542, AF349543, AF349545 and AF349544 respectively.

Sequence and phylogenetic analyses. A search of GenBank using the derived sequences as search queries was conducted using FastA (Pearson & Lipman 1988). Seventeen sequences from closely related symbiotic dinoflagellates were found (Table 2). These were used as reference sequences for subsequent alignments and analyses. *Gymnodinium simplex* was used as an outgroup (accession no. AF060901; Wilcox 1998).

Sequences were aligned using CLUSTAL W (Thompson et al. 1994). Trees were constructed using the neighbor-joining and maximum likelihood analyses options within the PHYLIP package (Felsenstein 1993). Distance matrices of pairwise divergence values were generated in PHYLIP using Kimura's 2-parameter model. Bootstrap analysis (1000 replicates for neighbor-joining and 100 replicates for maximum likelihood) was performed. The nodes were considered significantly robust if bootstrap values >95% were obtained (Felsenstein 1985).

Data analysis. Analysis of molecular variance (AMOVA) was undertaken with the program Arlequin version 2.000 (Schneider et al. 2000) according to the procedure for DNA sequences. This procedure calculates standard variance components and an array of correlation measures or test statistics (ϕ_{ST}). The test statistic ϕ_{ST} is equivalent to Wright's F_{ST} (Wright 1951, Weir & Cockerham 1984). The significance of observed variance components and ϕ statistics were tested using a random permutation procedure available in the Arlequin program. Symbionts were grouped according to host species and isolation region (namely, the Australian, Malaysian and Japanese groups). Pairwise distances using Kimura's 2-parameter model (measured by ϕ_{ST}) among host species and regions were calculated. Negative ϕ_{ST} values were indicative of an

Table 2. Reference symbiotic dinoflagellate species and the accession numbers of their 28S rDNA sequences data stored in GenBank. Source 1: Trench & Thinh (1995); 2: Wilcox (1998); 3: Baker et al. (1997); 4: Baker (1999)

Dinoflagellate species / abbreviation	Host	Location of origin	Accession number	Clade	Source
Gymnodinium linucheae/G. lin	*Linucheae unguiculata*	Caribbean	AF060893	A	1, 2
Symbiodinium pilosum/S. pil	*Zoanthus sociatus*	Caribbean	AF060894	A	2
Symbiodinium sp./Sym1	*Cassiopea xamachana*	Caribbean	AF060895	A	2
S.microadriaticum/S. mic	*Cassiopea xamachana*	Caribbean	AF060896	A	2
Symbiodinium sp./Sym2	*Tridacna gigas*	IndoPacific	AF060897	A	2
Symbiodinium sp./Sym3	*Hippopus hippopus*	IndoPacific	AF060898	A	2
Symbiodinium sp./Sym4	*Acropora cervicornis*	Caribbean	U63480	A	3
S. bermudense/S. ber	*Aiptasia tagetes*	Caribbean	AF060891	B	2
S. pulchrorum/S. pul	*Aiptasia pulchella*	IndoPacific	AF060892	B	2
Symbiodinium sp./Sym5	*Aiptasis pallida*	Caribbean	U63484	B	3
Symbiodinium sp./Sym6	*Agaricia fragilis*	Caribbean	AF060889	C	2
Symbiodinium sp./Sym7	*Montastrea franksii*	Caribbean	AF069890	C	2
Symbiodinium sp./Sym8	*Acropora cervicornis*	Caribbean	U63481	C	3
Symbiodinium sp./Sym9	*Acropora cervicornis*	Caribbean	U63482	C	3
Symbiodinium sp./Sym10	*Pavona duerdeni*	IndoPacific	U63485	C	3
Symbiodinium sp./Sym11	*Pocillopora elegans*	IndoPacific	AF170148	D	4
Symbiodinium sp./Sym12	*Pocillopora elegans*	IndoPacific	AF170149	D	4

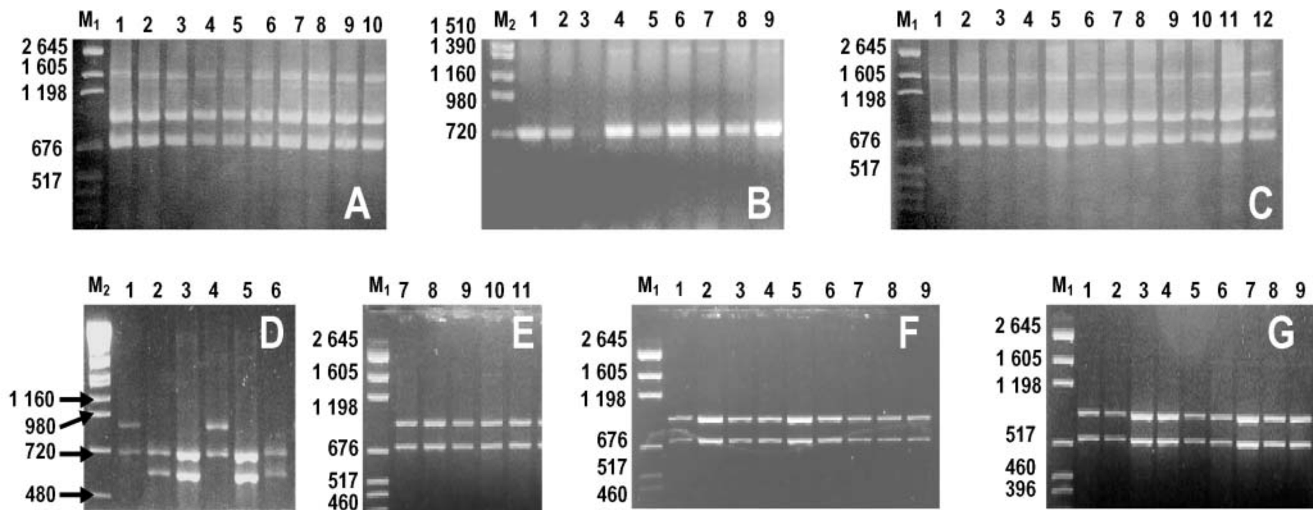

Fig. 1. Restriction fragment length polymorphism patterns derived from *Taq* 1 digestion of the amplified symbiotic dinoflagellate 18S rRNA gene. (A) Australian *Seriatopora hystrix* colonies; Lanes 1 to 10: Sh1A-Sh10A, respectively. (B) Malaysian *S. hystrix* colonies; Lanes 1 to 9: Sh1M-Sh9M, respectively. (C) Japanese *S. hystrix* colonies; Lanes 1 to 12: Sh1J-Sh12J, respectively. (D) & (E) Australian *Acropora longicyathus* colonies; Lanes 1 to 11: Al26A, Al6A, Al13A, Al43A, Al45A, Al53A, Al1A, Al2A, Al3A, Al5A and Al23A, respectively. (F) Malaysian *A. longicyathus* colonies; Lanes 1 to 9: Al1M, Al2M, Al3M, Al4M, Al7M, Al8M, Al10M, Al11M and Al12M, respectively. (G) Japanese *A. longicyathus* colonies; Lanes 1 to 9: Al1J, Al2J, Al3J, Al4J, Al5J, Al6J, Al7J, Al8J and Al9J. Lanes M_1 and M_2 contain pGem DNA markers (Promega) and SPP1/*Eco* R1 DNA markers (Progen), respectively

absence of genetic structure among the populations compared (Schneider et al. 2000). $p < 0.05$ was considered significant.

RESULTS

PCR amplification of symbiotic dinoflagellate 18S and 28S rRNA genes from all colonies produced amplicons of approximately 1500 and 600 bp, respectively. *Taq* 1 digestion of the amplified *Seriatopora hystrix* symbiont DNA produced 2 distinct RFLP patterns (Fig. 1 A to C). Symbionts from Australian and Japanese colonies produced an RFLP pattern typical for Clade C symbiotic dinoflagellates (Fig. 1 A,C, 2 discernible fragments, approximately 890 bp and 700 bp) (Rowan & Powers 1991a,b). Symbionts from Malaysian colonies produced a second RFLP pattern consisting of a single fragment of approximately 720 bp. Presumably, this consists of 2 equally sized fragments. This pattern was distinct from those previously described for Clades A, B or C, but resembled the pattern produced by the symbiont from *Montipora patula* from Hawaiian waters, which was not resolved into any clade (Rowan & Powers 1991b).

Taq 1 digestion of the DNA amplified from 7 Australian, all Malaysian and all Japanese *Acropora longicyathus* symbionts produced an RFLP pattern typical for Clade C (Fig. 1D to G). However, amplicons of symbionts from the 4 remaining Australian colonies, Al6A, Al13A, Al45A and Al53A, produced a distinct RFLP pattern (Fig. 1D, approximately 720 and 600 bp), which resembled that produced from Clade A symbiotic dinoflagellates (Rowan & Powers 1991a,b)

28S rDNA D1/D2 sequence analysis

We sequenced 400 to 550 nucleotides of the 28S D1/D2 rDNA from 14 *Seriatopora hystrix* and 16 *Acropora longicyathus* colonies, representing each putative Clade and the unique RFLP pattern. The sequences derived from 1 or both strands of DNA were identical and unambiguous. Hence, sequencing of both strands was not undertaken for all amplicons and cloning was not required to separate potentially paralogous rDNA (Buckler et al. 1997) known to exist in Dinophyceae (Scholin & Anderson 1996). A FastA search of Genbank using these sequences confirmed the clade identities suggested by the RFLP patterns. Furthermore, the FastA search with sequences from the Malaysian *S. hystrix* symbionts showed that they shared highest identity with Clade D symbionts (accession no. AF17-0148 and AF170149; Baker 1999). These sequences were aligned with each other and variable sites for each host species, clade and regional groupings were identified (Fig. 2).

Among Clade C symbionts there were 21 fixed differences observed between Australian and Japanese *Seriatopora hystrix*; 8 between Australian *S. hystrix*

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      111111111111111111111222222222222222222222222333333333333
      667777778999012233333477788888999013334555666666778881233444555556
      891247891312852602456224903478034142361567135678080198459168345 75

Sh2A  GCGGAGTGTTTCTCTGGGCCGGTTTTCCACATTGCAATACTAAGGCTCGATGCG.TT.CTAGG.G
Sh6A  ---------------------------------AN----N--N--T--AT--------------
Sh7A  ----------------------------------------------------------------
Sh8A  -------------------------------------------------AT-------------
Sh10A ----------------------------------------------------------------
Sh5J  -------------------------.---------------C----------A------C--A--
Sh6J  -------------------------.---------------C----------A------C--A--
Sh9J  -------------------------.---------------C----------A------C--A--
Sh10J -------------------------.---------------C----------A------C--A--
Al2A  --C---C------------------.-----------------A--------A-----TC-----
Al3A  -------------------------.-----------------A--------A-----TC-----
Al5A  -------------------------.-----------------A--------A-----TC-----
Al25A -------------------------.-----------------A--------A-N---TC-----
Al26A -------------------------.-----------------A--------A-N---TC-----
Al1M  -------------------------.-----------------A--------A-----TC-----
Al3M  -------------------------.-----------------A--------A-----TC-----
Al11M -------------------------.-----------------A--------A-----TC-----
Al1J  -------------------------.--------------------------A------C-----
Al2J  -------------------------.--------------------------A---CG-C-----
Al7J  -------------------------.--------------------------A------C-----
Al11J -------------------------.--------------------------A------C-----
Al6A  TT-A-C-ACAG---C-CAGT---CC.G-GT--C--TG------A------CAAA-----CCT--T
Al13A TT-A-C-ACAG---C-.A-----CC.G-GT--C--TG------A------CAAAA----CCT--T
Al45A TT-A-C-ACAG---C-CAGT---CC.G-GT--C--TG------A------CAAA-----CCT--T
Al53A TT-A-C-ACAG---C-CAGT---CC.G-GT--C--TG------A------CAAA-----CCT--T
Sh4M  ---AG-C----TATC-----A-A-C.GTGTGCCA-C--G-CT-AA-CT----A------C----T
Sh5M  ---AG-C----TA-C-----A-A-C.GTGTGCCA-C--G-CT-AA-CT----A------C----T
Sh6M  ---AG-C----TA-C-----A-A-C.GTGTGCCA-C--G-CT-AA-CTN---A------C----T
Sh7M  ---AG-C----TA-C-----A-A-C.GTGTGCCA-C--G-CT-AA-CT----A------C----T
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      33333333333333333333344444444444444444444444444444444444444444444
      67777777778888899999000000111111122222223333333344444444555555556666
      6134567892456756789012589013568901245912345689123456780245678 1234

Sh2A  ACCACCTCTCGAG.TAGGCTGCTGCCTG.GACATCGCACTGTGTCCAAGC.GTGAATTGTCCTGT
Sh6A  ----------------------------------------------------------------
Sh7A  ----------------------------------------------------------------
Sh8A  ----------------------------------------------------------------
Sh10A ----------------------------------------------------------------
Sh5J  ----TG------------------C-C-C-T-A-TG---G---T--C---------G----G-G
Sh6J  ----TG------------------C-C-G---A-GG---G-G-T--C---------G-C--G-G
Sh9J  ----TG------------------CTCCC---A-T----G---T--C---------G-C--G-G
Sh10J ----TG------------------CTCCC-T-A-TG---G---T--C---------G----G-G
Al2A  -----G-G--------------------------------TC-----------------
Al3A  -----G-G--------------------------------TC-----------------
Al5A  -----G-G-----G-.-----------------------A---TC----------------A
Al25A -----G-G--------------------------------T------------------
Al26A -----G-G--------------------------------TC-----------------
Al1M  -----G-G--------------------------------TC-----------------
Al3M  -----G-G--------------------------------TC-----------------
Al11M -----G-G--------------------------------TC-----------------
Al1J  -----G----------------------------------T----------G--------
Al2J  -----G-A--------------------------T--------T----------G--------
Al7J  -----G----------------------------------T--A-C-----G--------
Al11J -----G----------------------------------T----------G--------
Al6A  GT.GTGCTCGTGAG--AATG--CCATC---T-TGGA--TCC-T-T-GGCT-TGAG--C-CT-AA-
Al13A GTNGTGCTCGTGAG--AATG--CCATC---T-TGGA--TCC-T-T-GGCT-TGAG--C-CT-AA-
Al45A GT.GTGCTCGTGAG--AATG--CCATC---T-TGGA--TCC-T-T-GGCT-TGAG--C-CT-AA-
Al53A GT.GTGCTCGTGAG--AATG--CCATC---T-TGGA--TCC-T-T-GGCT-TGAG--C-CT-AA-
Sh4M  G---TG.-------CCAATGCT----C--T-TTAA-----TCT-T--G-A----GTGCA-TTA--
Sh5M  G---TG.-------CCAATGCT----C--T-TTAA-----TCT-T--G-A----GTGCA-TTA--
Sh6M  G---TG.-------CCAATGCT----C--T-TTAA-----TCT-T--G-A----GTGCA-TTA--
Sh7M  G---TG.-------CCAATGCT----C--T-TTAA-----TCT-T--G-A----GTGCA-TTA--
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      4444444444444
      6667777788888
      6791346891348 6

Sh2A  TTCTGCTCGGCACT
Sh6A  -------------
Sh7A  -------------
Sh8A  -------------
Sh10A -------------
Sh5J  --TACTG-C-A-T-
Sh6J  --TAC-G-C-A-T-
Sh9J  --TAC-G-C-A-T-
Sh10J --T--TG-C-A-T-
Al2A  -------------
Al3A  -------------
Al5A  ------------A-
Al25A -------------
Al26A -------------
Al1M  -------------
Al3M  -------------
Al11M -------------
Al1J  -------------
Al2J  -------------
Al7J  ----A---------
Al11J -------------
Al6A  -G-C---AAA--AC
Al13A -GTC---AAA-NAC
Al45J -G-C---AAA--AC
Al53J -G-A---AAA--AC
Sh4M  C------------C
Sh5M  C------------C
Sh6M  C------------C
Sh7M  C------------C
```

Fig. 2. Sequence alignments of variable positions of the *Symbiodinium* spp. 28S rRNA gene (D1/D2 domains) from *Seriatopora hystrix* and *Acropora longicyathus* (see Table 1 for species codes). Normal, italic and underscored font styles denote sequences from Clade C, A and D, respectively. Only variable positions are shown and deletions are indicated by a dot. Identity with the first sequence is indicated by dashes. Ambiguous or polymorphic nucleotide positions are shown as 'N'

and Australian/Malaysian *Acropora longicyathus*, 6 between Australian *S. hystrix* and Japanese *A. longicyathus*, 19 between Japanese *S. hystrix* and Australian/Malaysian *A. longicyathus*, 12 between Japanese *S. hystrix* and Australian/Malaysian *A. longicyathus*, none between Australian and Malaysian *A. longicyathus*, and 2 between Australian/Malaysian and Japanese *A. longicyathus*. Forty-eight fixed differences were observed between Clade A of Australian *A. longicyathus* and all the Clade C symbionts; 32 fixed differences were observed between Clade D symbionts and all Clade C symbionts; and 78 fixed differences observed between Clade A and Clade D symbionts.

The mean sequence identity among Clade C symbionts from Australian and Japanese *Seriatopora hystrix*; Australian, Malaysian and Japanese *Acropora longicyathus*, and a reference Clade C symbiont (U63485) was 96.5 ± 0.4%. Mean sequence identity among the Clade A Australian *A. longicyathus* symbionts and reference Clade A symbiont (accession no.U63480) was 98.3 ± 0.4%. The mean sequence identity among the Clade D Malaysian symbionts and the reference Clade D symbiont (AF170148) was 94.8 ± 0.1%.

Phylogenetic analysis

Neighbor-joining and maximum likelihood analysis of aligned 28S rDNA sequences produced trees that were congruent in topology. Only neighbor-joining trees are shown (Fig. 3). All of the Australian, Malaysian and Japanese symbionts from *Seriatopora hystrix* and *Acropora longicyathus* grouped with the appropriate reference clade symbionts.

Symbionts from Australian, Malaysian and Japanese *Seriatopora hystrix* formed distinct regional subgroups that were strongly supported by bootstrap analysis (Groups 1, 2 and 3, respectively. Fig. 3A). In contrast, all of the Clade C symbionts from *Acropora longicyathus* were genetically very similar to one another, regardless of region, and grouped in 1 strongly supported cluster (Group 4, Fig. 3B). Within this group, symbionts from Japan

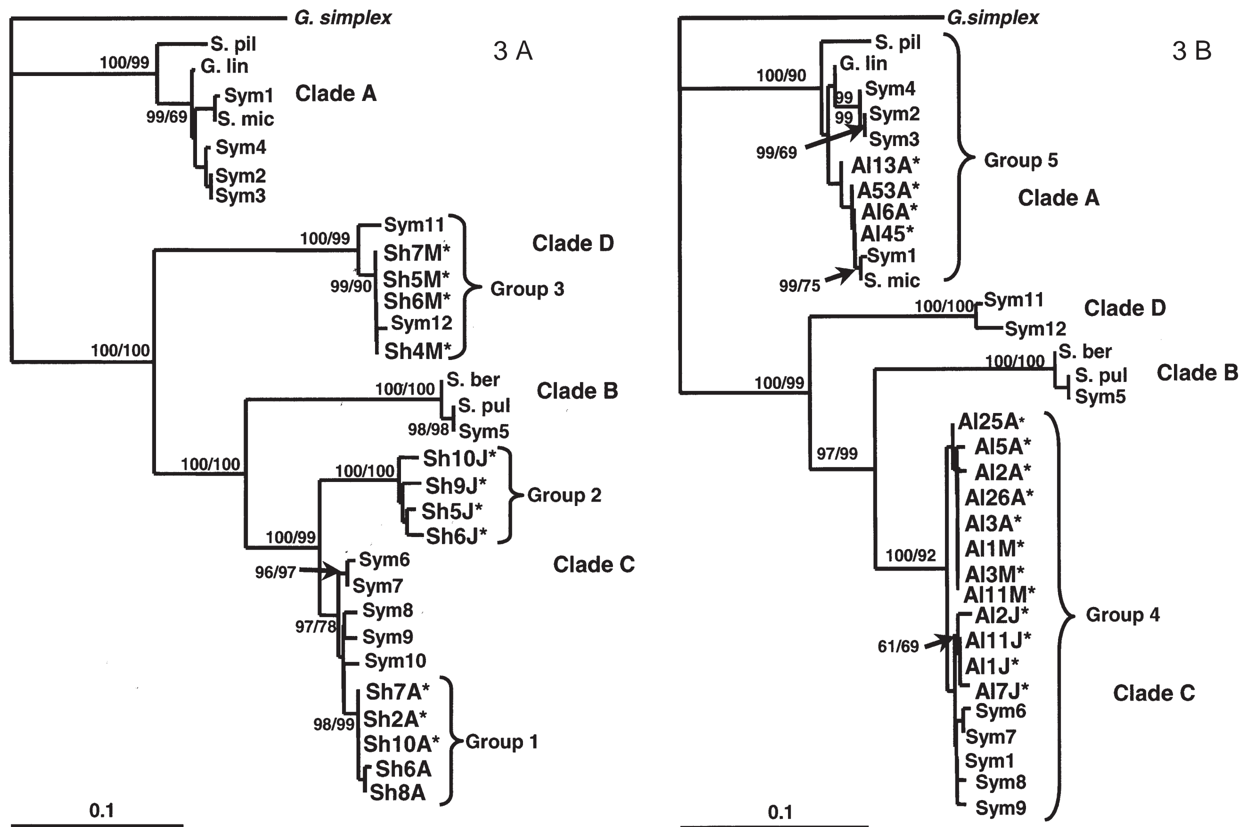

Fig. 3. Neighbor-joining trees derived from D1/D2 domains of the large subunit rRNA gene sequences of symbiotic dinoflagellates from (A) *Seriatopora hystrix* and (B) *Acropora longicyathus*, and other *Symbiodinium* spp. from several host species. *Symbionts from our study. Distances are number of substitutions per 100 bases. Unless indicated otherwise, only bootstrap values (neighbor-joining/maximum likelihood) greater than 95% are shown at the nodes. The sequence of the outgroup free-living dinoflagellate *Gymnodinium simplex* was obtained from GenBank (accession no.AF060901; Wilcox 1998). See Table 2 for species abbreviations

Table 3. Analysis of molecular variance. Variance of Clade C symbionts among host species, regions and between individuals within populations. Regions are defined in text. *After 1023 random permutations

Source of variation	df	Sum of the squares	Variance component	%Total	p*
Among host coral species	1	38.14	1.29	16.53	0.190
Among regions	3	69.89	5.43	69.83	0.000
Among individuals within regions	16	16.98	1.06	13.64	0.000
Total	20	125.01	7.78		

Table 4. Pairwise comparisons between populations of Clade C symbionts. Numbers are ϕ_{ST} values performed through 10100 permutations. *$p < 0.05$; **$p < 0.01$

		1	2	3	4	5
1	Australian *Seriatopora hystrix* Clade C	–				
2	Japanese *Seriatopora hystrix* Clade C	0.988**	–			
3	Australian *Acropora longicyathus* Clade C	0.862**	0.994**	–		
4	Malaysian *Acropora longicyathus* Clade C	0.866*	1.000*	–0.132	–	
5	Japanese *Acropora longicyathus* Clade C	0.7205**	0.982*	0.760**	0.756*	–

formed a subgroup; however, the branch separating this group was not supported by bootstrap analysis (61% and 69% for neighbor-joining and maximum likelihood analysis, respectively). Clade C symbionts of *A. longicyathus* from Australia and Malaysia could not be distinguished from each other. Clade A symbionts from *A. longicyathus* formed a closely related group (Group 5, Fig. 3B). When the phylogenetic relations among symbionts from both coral species were examined together, all 5 groups were maintained (Fig. 4).

Analysis of molecular variance

AMOVA was performed to evaluate the genetic differences between Clade C symbionts among the 2 coral species, among Australian, Japanese and Malaysian regions, and within populations from these regions. The results are shown in Table 3. This analysis was not performed on Clade A or D symbionts because they were found in 1 coral species and region only.

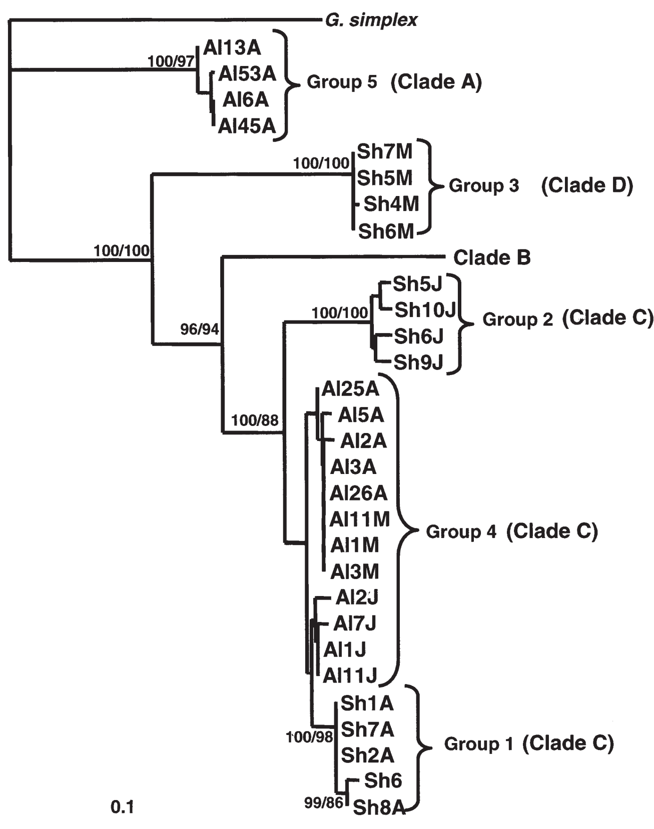

Fig. 4. Neighbor-joining tree derived from D1/D2 domains of the large subunit rRNA gene sequence alignments of symbiotic dinoflagellates from *Seriatopora hystrix* and *Acropora longicyathus*, with the free-living dinoflagellate *Gymnodinium simplex* as an outgroup. Distances are number of substitutions per 100 bases. Unless otherwise indicated, only bootstrap values (neighbor-joining/maximum likelihood) greater than 95% are shown

The greatest amount of variation was found among regional groups (69.8%, $p < 0.01$). A lower but non-significant level of variation was found between the 2 coral host species (16.5%, $p = 0.19$). Variation within populations was the lowest but was nevertheless significant (13.6%, $p < 0.01$).

Pairwise comparisons between Clade C regional populations, measured by ϕ_{ST} values, are shown in Table 4. Nine out of 10 pairwise comparisons showed significant differentiation ($p < 0.05$). Only Australian and Malaysian *Acropora longicyathus* symbiont populations could not be differentiated ($p > 0.05$).

DISCUSSION

Despite the importance of questions concerning the biogeography of symbiotic dinoflagellates inhabiting invertebrates such as reef-building corals and clams, an understanding of this area is relatively undeveloped. This study has attempted to investigate the biogeography of symbiotic dinoflagellates at a large scale in the western Pacific. A major finding is that the identities of the symbionts from distantly located colonies can vary greatly despite being taken from the same host species. This phenomenon has also been observed in the coral species *Plesiastrea versipora* (Loh et al. 1998, Baker 1999, Rodriguez-Lanetty et al. 2001). The presence of these various *Symbiodinium* may reflect an adaptation to environments experienced at respective locations. Previously, Rowan et al. (1997) found evidence to suggest that some corals can adapt to changed environmental conditions by altering symbiont genotype composition and distribution along large coral colonies. Baker et al. (1997) showed that the symbiont genotype in colonies of Caribbean acroporids varied depending on depth.

However, the identical distribution of Clades A and C in adjacent colonies of *Acropora longicyathus* is difficult to explain. Why a coral species should have 2 different types of symbiotic dinoflagellates in colonies that are growing side by side is unresolved. This flexibility in partnership may represent undetected local scale differences or may simply be a result of serendipity and evolution of 2 separate but suitable physiological interactions.

Symbiont diversity at broad scales

All Clade C symbionts in our study consistently formed part of a larger group that also included sym-

bionts of the corals *Pavona duerdeni* from Hawaii, and *Acropora cervicornis*, *Agaricia fragilis* and *Montastrea franksii* from the Caribbean. Similarly, all Clade A symbionts of our study formed part of a larger group that included symbionts of Indo-Pacific clams *Hippopus hippopus* and *Tridacna gigas*; Caribbean coral *A. cervicornis*; zoanthid *A. tagetes;* and the medussae *Linucheae unguiculata* and *Cassiopea xamachana*. Our Clade D Malaysian symbionts formed a group with symbionts of the eastern Pacific coral *Pocillopora elegans* from the reefs off Panama (Baker 1999). This pattern is consistent with the original observations of Rowan & Powers (1991a,b), who used RFLP methods to distinguish 3 major clades of symbiotic dinoflagellates.

Examination of the variability of symbiotic dinoflagellates within individual coral species revealed some interesting properties. Clade C symbionts from *Seriatopora hystrix* growing at Australia and Japan were significantly different from each other and yet highly similar within each location. AMOVA, pairwise comparison of population differences and phylogenetic analyses of the rRNA sequence data supported these differences. This indicates that the Clade C symbionts of *S. hystrix* are genetically differentiated among their respective regions although further sampling needs to be done to confirm this.

Clade C symbionts from *Acropora longicyathus* were distinct from those of *Seriatopora hystrix* but similar to each other, regardless of their region of origin. Phylogenetic analyses of the rRNA sequence data could not resolve these symbionts into distinct regional groups; however, more sensitive analysis using pairwise comparisons found significant ϕ_{ST} values between the Japanese symbionts and those from Australia and Malaysia. Nevertheless, these Australian and Malaysian Clade C symbionts appear to belong to a single group that is widespread in its distribution.

Since Clade A or D symbionts within our study were observed only in single locations, discussion of genetic connectivity (or lack of) is not possible for those groups.

Host-symbiont lifecycle and symbiont diversity

We hypothesize that genetically differentiated symbiont groups may be the result of events that led to periods of strong evolution of genetically isolated corals and symbionts. Conversely, genetically connected symbiont groups found over vast distances may result from the extent to which the holosymbionts or symbionts themselves are dispersed by ocean currents. In this case, some corals may propagate thousands of kilometers away from where the parent generation was located (Jokiel 1984, Richmond 1987, Jokiel 1990, Glynn et al. 1996). Symbionts may be propagated within drifting larvae or rafting corals, or may occur in a free-living phase and be acquired by hosts downstream. The mode of transmission of symbionts between host generations may also have a strong influence on the dispersal and extent to which symbiont and host are likely to co-evolve. The stronger intra-species divergence between populations of symbiotic dinoflagellates of *Seriatopora hystrix* from Australia, Japan and Malaysia suggests that symbiont populations have been isolated — perhaps by the fact that vertical transmission occurs in this species.

These observations are supported by the biology of this coral species, particularly the short time it spends in its larval phase. The short-lived larval stage in turn drives an isolated and genetically divided distribution that is strongly restricted by oceanic circulation and reef structure (Ayre 1994, Ayre et al. 1997). The symbiosis between *Seriatopora hystrix* and its dinoflagellates, once established with a particular genotype, might never change over successive generations since vertical transmission ensures a faithful transmission of symbiont genotypes from parent to offspring generations (Yamamura 1996, Douglas 1998, Law & Dieckmann 1998, Genkai-Kato & Yamamura 1999). This has potentially led to the evolution of genetically divergent symbiont genotypes common to particular regions.

In the 'closed' system of *Seriatopora hystrix*, distantly separated coral populations have also adopted different symbionts. Interestingly, Clade D symbionts of Malaysian *S. hystrix* appear identical to those of *Pocillopora elegans* (Fig. 3A) from the Pacific reefs off Panama (Baker 1999). This suggests that symbiosis with this genotype was established early in ancestral forms and perhaps in multiple coral species and implies that sometime in evolutionary history, *S. hystrix* did not have such a closed system. It is less likely that the symbiont has changed so much in symbiosis with *S. hystrix* that it belongs in a different clade since it also forms a symbiosis with another species of coral.

In contrast, *Acropora longicyathus* acquires symbionts from the water column (horizontal transmission). The weaker intra-species divergence observed between populations of Clade C symbiotic dinoflagellates from this coral growing in Australia, Japan and Malaysia is likely to result from this 'open' mode of acquisition. Selecting symbionts from the water column each generation effectively 'dilutes' the extent to which these may differ, especially if the symbiont taxon is ubiquitous, and the evolution of a suitable physiological interaction between *A. longicyathus* and symbiont has ensured preferential uptake of the widespread zooxanthella genotype at all locations. Nevertheless, horizontally transmitted zooxanthellae may yet show patterns of geographic variation that mirror their dispersal in free-living form, which may explain

the distinctiveness of Japanese *A. longicyathus* symbionts. The occurrence of Clades A or C in Australian colonies is perhaps a case in point. The hosts, occurring side by side, have adopted 2 very diverse symbionts that might provide respective physiological advantages. Although colonies of *Seriatopora hystrix* have also adopted diverse symbionts (either 1 of 2 Clade C or D genotypes), this variation is strongly linked with geographical separation.

To date, all species found to form symbioses with symbionts of more than 1 clade (*Montastrea annularis* and *M. faveolata* [Rowan & Knowlton 1995, Rowan et al. 1997], *Acropora cervicornis* [Baker et al 1997], *Diploria labyrinthiformis* and *Stephanocoenia michelinii* [Baker & Rowan 1997], *Acropora longicyathus* and *Pavona decussata* [Loh et al. 1998]) are species or belong to genera that have never been observed to release gametes or progeny equipped with symbiotic dinoflagellates (Harrison & Wallace 1990, Richmond & Hunter 1990), and hence probably have open symbioses.

These data show that the symbiotic dinoflagellates of widely distributed coral species are variable at the level of biogeographical regions. *Acropora longicyathus* symbionts appear to be genetically connected and widely distributed, perhaps because reinfection by the same zooxanthellae taxon occurs anew each generation. *Seriatopora hystrix*, perhaps because of an isolating effect of maternal transmission and limited larval distribution, have symbionts that are genetically differentiated according to geographic distribution. The different modes of symbiont transmission and resulting variation of symbionts within coral hosts may have major implications for evolution and versatility of dinoflagellate symbionts under the pressure of changing environmental conditions.

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