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Molecular genetic evidence for probable reticulate speciation in the coral genus *Madracis* from a Caribbean fringing reef slope

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Abstract For many corals, the existence of morphologically distinct yet sympatric populations/species implies reproductive isolation. Conversely, the presence of many intermediate and overlapping morphologies combined with synchronous, mass spawning suggests incomplete reproductive isolation. In *Madracis* (Scleractinia: Astrocoeniina: Pocilloporidae), high levels of morphological plasticity among the five most commonly recognized species (*M. mirabilis*, *M. senaria*, *M. decactis*, *M. formosa* and *M. pharensis*) on Caribbean reefs led us to question species boundaries. Phylogenetic relationships were investigated at the intra-individual, inter-individual and inter-specific levels using the ITS1-5.8S-ITS2 region (ca. 613 bp) of the ribosomal DNA cistron. Inter-specific divergence was ca. 6%, while intra-individual and intra-specific divergences ranged from 0% to 4.9% and 3.3% to 3.5%, respectively. *M. senaria* and *M. mirabilis* formed monophyletic groups. *M. formosa, M. decactis* and *M. pharensis* formed a paraphyletic complex. High levels of intra-individual and intra-specific ITS polymorphism in the *decactis-formosa-pharensis* cluster may be the result of very recent speciation within the clade (i.e. maintenance of ancestral polymorphism and incomplete lineage sorting), or the result of repeated introgressive hybridization among the three taxa. Polymorphism parsimony of 89 sites, including nine that showed additivity, revealed a phylogenetic topology more consistent with inter-taxal hybridization. Results are discussed in terms of weak reproductive barriers, and phylogenetic fission and fusion under Veron's model of reticulate speciation in corals. Ecological studies involving *Madracis* should consider *M. decactis*, *M. formosa* and *M. pharensis* as a complex.

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Introduction

Coral species are generally described according to differences in the morphology of the colony and micromorphological characters of the coral skeleton (Wells 1956; Lang 1984; Veron 1993). Morphological variation is known to be influenced by environmental conditions (Roos 1967; Barnes 1973; Van Veghel and Bak 1993) such as light, depth and water movement; as well as geographic variation (Wijsman-Best 1972; Veron 1995; Bruno and Edmunds 1997). Morphological variation may also be the result of various levels of inter-taxal hybridization (Grant 1981; Veron 1995). With the advent of various DNA analysis methods (Avis 1994; Ferraris and Palumbi 1996; Hillis et al. 1996), it has become possible to explore taxon boundaries from a molecular genetic perspective. Results repeatedly show that morphological boundaries are not necessarily related to genetic boundaries and that the existing "classical" taxonomies of corals are in need of critical reevaluation. For example, no clear genetic distinctions have been found among seven morphological species within the genus *Platygyra* (Miller and Benzie 1997), and there is still disagreement about the status of morphologically different but genetically equivalent forms within the *Montastraea annularis* complex (Knowlton et al. 1992; Van Veghel and Bak 1993; Szmant et al. 1997; Lopez et al. 1999).

The role of introgressive hybridization in the diversification of animals has generally been considered negligible, but new data and new discoveries, especially in marine organisms such as corals, are beginning to change this view (Gardner 1997). In their recent review

of introgressive hybridization in animals, Dowling and Secor (1997) stressed the importance of thinking about introgression and hybridization as processes rather than as results. In this more relaxed view, hybridization is defined as the interbreeding of individuals from two populations, or groups of populations, which are distinguishable on the basis of one or more heritable characters. Introgression is defined as the incorporation of genes from one set of differentiated populations into another. Determining the quantity and type of differences necessary for recognition of hybrid taxa then becomes a question of degree. In Veron's model of coral speciation by repeated fission and fusion (Veron 1995), species are considered as independently evolving populations or groups of populations that may or may not have achieved reproductive isolation.

Direct evidence for introgressive hybridization comes from direct cross-fertilization experiments [Miller and Babcock 1997; Szmant et al. 1997 (*Montastraea*); Willis et al. 1997 (*Platygyra*); Hatta et al. 1999 (*Acropora*)] and inferences based on chromosome counts [Kenyon 1997 (*Acropora, Montipora* and *Fungia*)]. Indirect assessments of hybridization based on shared intron sequences from the mini-collagen gene [Hatta et al. 1999 (*Acropora*)] and nuclear ribosomal ITS [Odorico and Miller 1997 (*Acropora*)] have been used to independently confirm introgressive events in some of the above studies.

Madracis (Scleractinia: Astrocoeniina: Pocilloporidae) Milne-Edwards and Haime, 1849 is distributed from the tropics to temperate waters in the Indo-West Pacific, Caribbean, Red Sea and Mediterranean regions (Zibrowius 1980; Veron 1993; Swedburg 1994). Circa 17 species have been described of which about 15 are currently recognized (Cairns 1999). Among these, eight are described for the Caribbean and five (those considered in the present study) are common reef-building species (Wells 1973a,b). These include *M. mirabilis* Duchassaing and Melotti, *M. decactis* Lyman, *M. formosa* (Wells 1973b), *M. senaria* (Wells 1973a) and *M. pharensis* Heller.

All five *Madracis* species occur sympatrically at a single reef site on Curaçao. *M. formosa* occurs only at depths below 30 m, whereas *M. mirabilis* is found in the shallower parts of the reef (2–15 m). In contrast, *M. decactis*, *M. pharensis* and *M. senaria* exhibit overlapping distributions across all depths. *M. mirabilis* forms large mono-specific beds of fragile, branching colonies. In contrast, *M. senaria* forms distinctive, vertically oriented crusts. *M. decactis* forms low-relief, clavate to bulbous knobs, but may also form thick, stubby columns. *M. pharensis* forms encrusting sheets that follow the contours of the substratum, whereas *M. formosa* is branched and sometimes reticulate. At the level of corallite micromorphology, the presence of overlapping characters provide only weak diagnostic differences. For example, all *Madracis* species have ten septa, except for *M. formosa* with eight and *M. senaria* with six proto- and four meta-septa. While overall colony morphology and septal arrangement makes identification of the five morphospecies relatively easy, the amount of morphological variation continues to hinder our understanding of the phylogeny of the genus including the phylogenetic significance (if any) of particular morphological characters.

The nuclear rDNA internal transcribed spacers (ITS1 and ITS2) are widely used for phylogenetic studies at the species and subspecies levels (Baldwin et al. 1995) in a wide variety of organisms including marine algae (Bakker et al. 1995a,b; Van Oppen et al. 1995; Olsen et al. 1998), corallimorpharians [Chen and Miller 1996 (*Rhodactis*)] and corals [Hunter et al. 1997 (*Porites*); Odorico and Miller 1997 (*Acropora*); Lopez et al. 1999 (*Montastraea*); Medina et al. 1999 (*Montastraea*); Van Oppen et al. 2000 (*Acropora*)]. As part of a multi-gene family, the many copies of the tandemly arranged cistrons are expected to become rapidly homogenized through mechanisms of concerted evolution (Dover 1982; Arnheim 1983; Schlötterer and Tautz 1994; Elder and Turner 1995). This process leads to greater similarity among members of a repeated family within a species than among species. However, a number of factors can affect the rate at which homogenization occurs, leading to varying degrees of intra- and inter-specific polymorphism of the ITS regions. These include loss of recombination through prolonged asexual reproduction, loci located on more than one chromosome, polyploidy, recent speciation, and introgressive hybridization (Quijada et al. 1997; Hugall et al. 1999). Evidence for hybridization in corals (sensu Veron 1995) has been demonstrated by Odorico and Miller (1997) and Van Oppen et al. (2001).

Here we use ITS sequences to explore phylogenetic relationships within the coral genus *Madracis*.

Materials and methods

Study site and specimen collection

The island of Curaçao is situated in the southern Caribbean (12°N; 69°W) about 80 km off the coast of Venezuela (South America) and is part of Netherlands Antilles (Fig. 1). It is surrounded by fringing reefs. Leeward reefs are characterized by a shallow terrace (50–100 m wide), a drop-off at a depth of 8–12 m, and a steep seaward slope extending to a depth of 50–60 m (Bak 1975, 1977). Our study

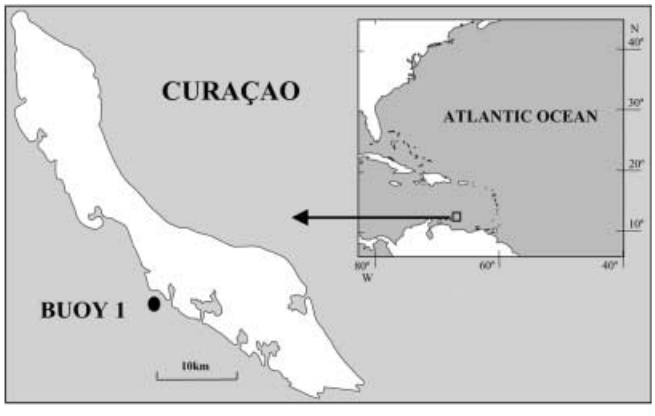

Fig. 1 Curaçao, Netherlands Antilles showing the Buoy-1 study site

site, the Buoy-I reef, is situated 500 m west of the Ecological Institute Carmabi and is a long-established site for coral reef research (e.g. Bak 1975; Bak and Engel 1979; Van Veghel 1994; Bak and Nieuwland 1995; Meesters 1995; Nagelkerken and Bak 1998). The area sampled in this study was ca. 4,000 m^2, i.e. 100 m parallel to shore and 40 m perpendicular to shore (extending to ca. 50 m in depth). Coral specimens were collected from depths of 2–45 m (Table 1). Care was taken to insure that samples for a given morphospecies were collected from all depths in which they occurred. Samples consisted of small fragments (ca. 50 cm^2) taken from the living surface of individual colonies. These were transported to the laboratory in seawater and transferred to a running-seawater table. Each sample fragment was divided into two pieces. One sub-sample was bleached and dried for further skeletal examination, while the second sub-sample was preserved in 70% EtOH for DNA extraction. Skeletal and EtOH-preserved specimens of *Madracis pharensis* (Marseille, Mediterranean), *Stylophora mordax* (Guam, W. Pacific) and *Pocillopora damicornis* (Guam, W. Pacific) were also included.

DNA extraction

DNA was extracted following a modified CTAB protocol (De Jong et al. 1998) as follows. The soft-tissue, surface layer was scraped from each coral fragment (3–4 cm^2) into a chilled mortar containing 900 µl DNA extraction buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0 and 2% cetyltrimethylammoniumbromide) and 0.2% β-mercaptoethanol. After grinding the slurry was transferred into a 2 ml Eppendorf tube and incubated at 65°C for 1 h followed by one phenol extraction and two CIA (chloroform:isoamylalcohol, 24:1 v/v) extractions. The crude DNA was recovered by overnight precipitation in an equal volume of 100% EtOH. After centrifugation the pellet was washed twice with 80% EtOH and dried under vacuum. The DNA was redissolved in 100 µl of 0.1× TE (Tris-EDTA, pH 8). Additional DNA purification steps were not necessary.

ITS amplification

The ITS1-5.8S-ITS2 rDNA region together with a partial sequence of the flanking small subunit rDNA and large subunit rDNA genes were amplified with the universal forward primer TW5 and universal reverse primer JO6 (Table 2). Zooxanthallate and azooxanthellate specimens of *M. pharensis* were used as a control in order to check that coral ITS was being amplified. A PCR reaction (100 µl) consisted of 10 µl of 10× reaction buffer (Promega, Madison, Wis., USA), 10 µl of 10× dNTP (200 µM), 6 µl $MgCl_2$ (25 mM), 4 µl of each primer (50 mM) (Table 2), 4 µl of template

Table 1 *Madracis* species and sampling information

Species	Number of individuals	Code	Depth (m)	Number of clones (sequenced per ind.)	Clone designation
M. mirabilis	4	mir1	6.3	5	a, b, c, d, e
		mir6	24.0	5	a, b, c, d, e
		mir55	2.2	3	b, c, d
		mir9	19.0	1	c
M. decactis	8	dec2	10.0	4	a, b, c, d
		dec3	4.7	2	a, e
		dec11	30.0	1	c
		dec12	32.3	3	a, b, d
		dec13	34.7	3	a, d, e
		dec100		5	a, b, c, d, e
		dec103	20.0	4	a, b, c, d
		dec107	29.0	5	a, b, c, d, e
M. formosa	7	for11	38.8	1	a
		for12	43.0	1	a
		for13	46.0	3	b, c, d
		for14	32.6	4	b, c, d, e
		for15	38.8	1	b
		for25	39.6	4	a, b, c, h
		for35	39.6	2	c, e
M. senaria	6	sen3	10.8	4	a, c, d, e
		sen4	7.0	1	a
		sen7	14.0	1	a
		sen8	13.4	1	e
		sen13	32.3	1	b
		sen14	32.9	4	b, c, d, e
M. pharensis	7	pha2	4.3	4	a, b, c, d
		pha4	7.0	2	b, c
		pha60	40.0	1	b
		pha61	16.6	4	b, c, d, e
		pha62	13.4	5	a, b, c, d, e
		pha111	27.0	2	b, c
		pha112	27.0	1	
Outgroup (*M. pharensis*)[a]	2	Med	5.0	1	
		Medzx	10.0	1	
Total	34			90	
Stylophora mordax[b]	1				
Pocillopora damicornis[b]	1				

[a] Specimens were collected by H. Zibrowius (Université de Marseille, Marseille, France). Medzx (zooxanthellated *M. pharensis* forma *luciphila*) was collected at Port-Miou (Marseille, France). Med (azooxanthellated *M. pharensis* forma *pharensis*) was collected at Jarre Island at 10 m depth from the ceiling of a dark cave

[b] Collected by R. Rowan (University of Guam)

Table 2 List of primers used for amplification and sequencing of the ITS1-5.8S-ITS2 region. Primers OED58F and OED58R are *Madracis* specific

Primer	Direction	Sequence	Position	Reference
TW5	Forward	5′-CTTAAAGGAATTGACGGAAG-3′	1129 in (18S)[a]	White et al. (1990)
JO6	Reverse	5′-ATATGCTTAAGTTCAGCGGGT-3′	48 in (28S)[a]	Our laboratory
C1	Forward	5′-GTACACACCGCCCGTCGCTCC-3′	1624 (18S)[a]	Our laboratory
OED58F	Forward	5′-GTCTGTCTGAGTGTCGGATAT-3′	In 5.8S	Our laboratory
OED58R	Reverse	5′-ATATCCGACACTCAGACAGAC-3′	Complement of primer OED58F	Our laboratory

[a]Nucleotide position from start of gene in *Chlamydomonas reinhardtii*

DNA (see below), 2.5 U *Taq* DNA polymerase (Promega). Amplification was carried out on a Perkin-Elmer 9700 (PE Applied Biosystems, Perkin Elmer, Calif., USA) thermocycler with the following profile: 1 cycle of 3 min 96°C; 24 cycles of 1 min 94°C, 2 min 50°C and 2 min 72°C; and 1 cycle of 1 min 93°C, 2 min 50°C and 5 min 72°C. Test PCR reactions were performed with undiluted, 10× and 100× diluted DNA in order to find the optimal DNA concentration for each sample. Amplifications were checked for length, purity and yield on ethidium-bromide-stained 1.5% TAE agarose gels according to standard methods. The PCR fragments were purified using the Qiaquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol.

Cloning

Fragments were ligated and transformed using the pGEM-T Easy Vector System, JM109 competent cells and standard blue/white colony screening (IPTG, X-gal/ampicillin), all from Promega, and following kit instructions. The ratio of insert to vector was 3:1. In order to check that the clones were of coral origin (and not zooxanthellar origin), a colony-PCR was done on the white colonies using the coral-specific primer OED58F in the 5.8S region and the universal primer JO6 in the LSU (Table 2). Positive white colonies were then grown overnight in 2× YT medium at 37°C on a shaker. Plasmid isolation was done using the FlexiPrep Kit (Amersham-Pharmacia, Biotech, Uppsala, Sweden). To check the concentration of the plasmid, 2 µl were loaded on a 1.5% TAE agarose gel along with a dilution series of DNA standards (25–200 ng μl^{-1}). Plasmid yield was quantified using Image-Quant (ver. 4.2) software from Molecular Dynamics (M.B.T. Benelux, Maarssen, The Netherlands).

Sequencing

Cycle sequencing was performed with 200 ng of double-stranded plasmid DNA template and ABI Prism dRhodamine Terminator Cycle Sequence Ready Reaction Kit and, later, the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Perkin-Elmer). The internal coral-specific primers OED58F, OED58R and the universal primer C1, as well as the amplification primer JO6 (Table 2), were used to sequence ITS1, 5.8S and ITS2 in both forward and reverse directions. Sequencing was carried out on either an ABI 310 or ABI 377 Automated Sequencer (PE Applied Biosystems, Perkin-Elmer). Inter-specific (5 morphospecies), intra-specific (34 isolates) and intra-individual variation (90 clones total) were analyzed.

Sequence alignment

Sequences were managed using Navigator and Factura software (ABI- Perkin-Elmer, Calif., USA). A complete sequence alignment was done using the Mega alignment program from the DNAstar Sequence Analysis Software package (DNAStar, Madison, Wis., USA) on a Macintosh Quadra. Secondary structure was assessed using the RNAstructure program version 3.5 (Mathews et al. 1999). In addition to improving the alignment more generally, we were also interested in checking whether or not polymorphic sites were located in stems or in loops. Sequences were easy to align and required few gaps.

From the complete sequence alignment, a second alignment was constructed that would allow for phylogenetic analyses based on polymorphic sites. This was accomplished by recoding sites following the IUPAC (International Union of Pure and Applied Chemistry) symbols coding (W = A/T, Y = C/T, R = A/G, K = G/T, M = A/C, S = C/G) and altering the PAUP input file from "Data = DNA" to "Data = symbol". This allows polymorphisms to be recognized as new character-states, not as ambiguities.

Individual sequences, as well as the alignment, have been deposited in GenBank (see "Appendix").

Outgroups

Madracis pharensis (Marseille, Mediterranean), *Stylophora mordax* (Guam, W. Pacific) and *Pocillopora damicornis* (Guam, W. Pacific) were chosen as potential outgroups. The latter two proved to be extremely divergent and unalignable. Therefore, they were dropped from the analysis.

Phylogenetic reconstruction

Phylogenetic reconstruction was done using PAUP 4.0 ver. Beta-2 (Swofford 1999). The full alignment of all 90 sequences, as well as sub-sets of the dataset (inclusion and exclusion of gaps; gaps as single events) were analyzed using maximum parsimony (MP) under the heuristic search setting with random addition of taxa. Bootstrap analyses (1,000 replicates in which "max trees" was set at 100) were performed on the complete dataset. Neighbor joining (NJ) analysis was performed under the Kimura-2-parameter model of nucleotide substitution. Stability of the resulting NJ phylogeny was assessed by 1,000 bootstrap replicates. Maximum likelihood (ML) could not be performed due to computational limitations.

For the reduced second alignment, IUPAC codes were used to code new polymorphic states. Only MP can be applied using the "Data = symbols" setting (ML and NJ recognize the codes only as ambiguities, not as new states). In this approach to the analysis, additivity on a site-per-site basis at the intra- and inter-specific levels can be incorporated. Additivity at a site occurs when two site-specific nucleotides are simultaneously present in one individual. Site-specific polymorphisms can occur by chance as a result of ancestral polymorphisms or as a result of hybridization between individuals from isolated populations or species. When additivity is found at several sites, however, the argument for possible hybridization is strengthened (Campbell et al. 1997; Dowling and Secor 1997).

Results

Data properties

Nucleotide composition was uniform among all isolates with a GC content ranging from 57.36% to 58.50%

(mean of 57.93%). Pairwise comparisons in Table 3 of transition:transversion ratios show no evidence of saturation (Holmquist 1983) and range from 0.91 to 2.62 (average of 1.96). Inter-taxal sequence divergences (measured as p-distances) are generally small, but show a significant overlap at the intra- and inter-individual levels. Intra-specific divergence ranged from 0% to 4.9%, inter-specific divergences from 2.6% to 8.4% and a maximum of 11% between all *Madracis* species and the Mediterranean sample of *M. pharensis* used as the biogeographic outgroup.

Total length of the ITS1-5.8S-ITS2 fragments ranged from 599 to 605 bp. Sequences were easy to align. The final alignment (available from GenBank) consisted of 615 positions (gaps included) of which 503 were invariant, 112 characters were variable and 37 (6%) were phylogenetically informative (Table 4). We note that the 3′-end of ITS2 contained a duplication of seven bases between positions 595 and 601 in 8 of 16 clones of *M. formosa*, and a CA-repeat of two to five repeat units from position 602 onwards in all taxa. The duplication is phylogenetically informative within the *M. formosa* group (Fig. 2, clade containing for11a), whereas the CA-repeat is not. Secondary structural analysis confirmed that the CA-repeat occurs in a large stem-loop, where slipped-strand mispairing is common during normal replication.

In *Madracis*, intra- and inter-individual nucleotide differences were found to be substantial and are summarized for individual clones in Table 5. Intra-individual variation is most pronounced in *M. decactis*. The distribution of the polymorphism and its significance are further explored using polymorphism parsimony.

Global phylogenetic analysis

MP analysis of the complete alignment, i.e. multiple clones from all individuals from the five morphospecies including an outgroup, resulted in 11,970 most-parsimonious trees (Table 4) of which one is shown in Fig. 2. Inclusion or exclusion of gaps did not change the topology except for a few tip-taxa. Despite the large number of MPTs, a majority-rule consensus tree yielded the same topology as shown by the bold-gray lines in Fig. 2. Bootstrap values (> 70%) are also shown in Fig. 2. Since a NJ analysis produced the same backbone topology of the tree, we were able to perform a bootstrap analysis under NJ. These bootstrap values were similar to the MP bootstrap values shown in Fig. 2. Values are generally low, which will be discussed below.

The key results from the global analysis are the following. *M. senaria* always forms a monophyletic group. *M. mirabilis* forms a monophyletic clade (Fig. 2) except for one clone of individual mir1 (Fig. 2). *M. decactis*, *M. pharensis* and *M. formosa* form a paraphyletic complex (blue-gray box in Fig. 2). Various attempts to force monophyly on morphospecies (shown in colors) always resulted in longer trees. This is because many of the individuals show significant intra-individual variation. Clones from a single individual that appear in different places in the tree in Fig. 2 are marked with symbols. Intra- individual variation is prominent in *M. formosa* and *M. decactis*, where clones of the two combine in a *M. formosa*/*M. decactis* clade. We note that *M. decactis* individuals (dec11, dec12, dec13, dec100, dec103, dec107 in Table 1) that have clones in different clades are morphologically different from *M. decactis* individuals (dec2 and dec3 in Table 1).

Table 3 Pairwise comparisons of transition:transversion ratios (*lower left matrix*) and sequence divergence as percent based on p-distances (*upper right matrix*). Values *in parentheses along the diagonal* represent intra-individual transition:transversion ratios and sequence divergence, respectively

	M. mirabilis	*M. decactis*	*M. formosa*	*M. senaria*	*M. pharensis*	Outgroup
M. mirabilis	(2.11)(0.0)	3.4	2.6	7.7	4.9	11.0
M. decactis	2.01	(1.65)(2.4)	3.5	7.8	4.2	11.0
M. formosa	2.01	2.11	(0.91)(2.2)	8.8	4.8	11.0
M. senaria	1.99	2.22	1.39	(2.62)(3.7)	7.9	11.0
M. pharensis	1.19	1.84	0.98	2.34	(2.43)(4.9)	11.0
Outgroup	2.62	2.03	1.97	2.36	2.37	(–)

Table 4 Data properties and parsimony analysis (*MPTs* number of most-parsimonious trees found; *CI* consistency index; *RC* rescaled consistency index)

	Full alignment of all clones under parsimony		Polymorphism parsimony (Fig. 3)
	Gaps excluded	Gaps included	
Total characters	615	606	112
Variable characters	112	120	–
Phylogenetically informative	37 (6%)	46 (8%)	30 (27%)
MPTs	11,970	4,128	4,960
Tree length	135	158	157
CI/RC	0.859/0.809	0.823/0.778	0.854/0.728

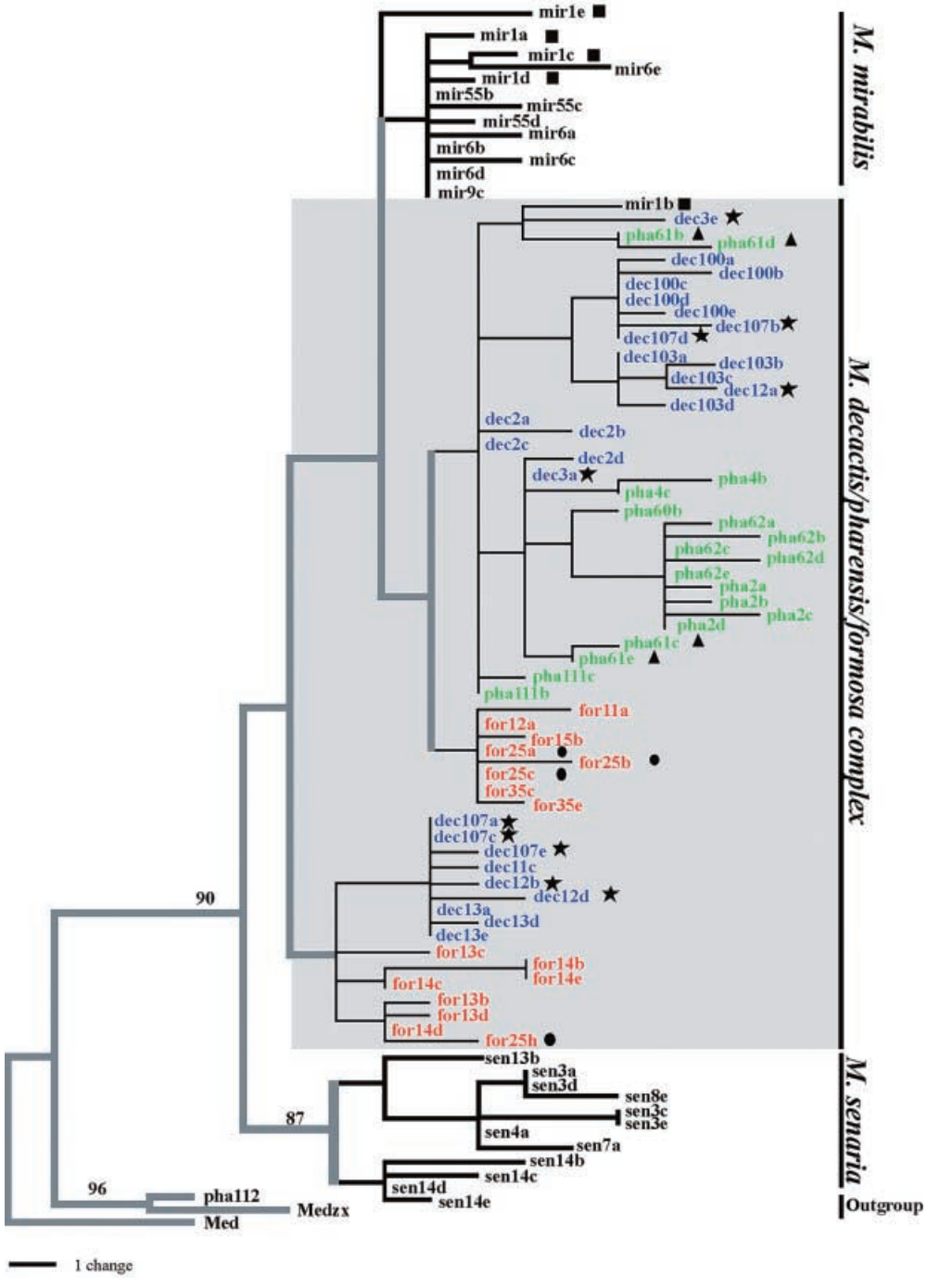

Fig. 2 One of 11,970 most-parsimonious trees based on all 90 clones (Table 1). Monophyletic morphospecies are shown in *bold-black*. Invariant backbone structure found in the majority-rule MP-consensus and NJ trees is shown in *bold-gray*. Clades within the *blue-gray box* belong to the paraphyletic *decactis-pharensis-formosa* complex. Colors correspond to traditionally recognized morphospecies. ITS clones derived from one individual that appear in different parts of the tree are marked by ■, ▲, ● and ★, respectively. Branch lengths are proportional to change. Bootstrap values (1,000 replicates, > 70%) were estimated under MP in which "max trees" was set at 100. Estimates based on a NJ bootstrap were similar; see "Results"

ITS polymorphism analysis

Among 112 variable sites extracted from the complete alignment, 86 sites were found to be polymorphic (Fig. 3). Among these, 30 were phylogenetically informative, 23 were autapomorphic, and 67 were variable but neither autapomorphic nor phylogenetically informative (under polymorphism). Nine sites showed additivity. An examination of Fig. 3 shows that most of the polymorphism is randomly distributed across the alignment with no concentrations associated in particular regions of the ITS or with secondary structure. All of the polymorphisms occur in loop regions. This polymorphism (i.e. non-homogenization of the ITS) may be due to ancestral polymorphism and incomplete lineage sorting associated with very recent speciation. This is the simplest and least contentious explanation. However, there are also nine additive sites in Fig. 3 (gray), six at the intra-specific level (positions 85, 161, 195, 213, 474, 607) and three at the inter-specific level (positions 224, 515, 592). Additivity is an important indicator of possible introgressive hybridization. Though not proof by itself, the more additive

Table 5 *Madracis* spp. Average number of nucleotide differences in the ITS1-5.8S-ITS2 region (*intra-individual* average number of differences between clones within one individual; *inter-specific* average number of differences between species, including the outgroup). Number of clones screened are in *parentheses*. Individuals that show high polymorphism are shown in *bold*

Species	Individual	Intra-individual	Inter-specific					Outgroup
			M. mirabilis	*M. decactis*	*M. formosa*	*M. senaria*	*M. pharensis*	
M. mirabilis								
	mir1	**5.4 (4)**						
	mir55	2.0 (3)	5.4 (12)					
	mir6	3.2 (4)						
	mir9	– (1)						
M. decactis			7.2					
	dec100	1.6 (5)						
	dec103	1.7 (4)						
	dec107	**5.4 (5)**		5.7 (27)				
	dec11	– (1)						
	dec12	**8.0 (3)**						
	dec13	0.7 (3)						
	dec2	2.0 (4)						
	dec3	5.0 (2)						
M. formosa			6.0	10.4				
	for11	– (1)						
	for12	– (1)						
	for13	3.3 (3)			5.0 (16)			
	for14	3.0 (4)						
	for15	– (1)						
	for25	**4.5 (4)**						
	for35	1.0 (2)						
M. senaria			10.2	10.6	10.4			
	sen13	– (1)						
	sen14	3.0 (4)						
	sen3	2.7 (4)				5.3 (12)		
	sen4	– (1)						
	sen7	– (1)						
	sen8	– (1)						
M. pharensis			7.8	6.4	7.3	10.0		
	pha4	2.0 (2)						
	pha60	– (1)						
	pha61	3.5 (4)					5.4 (19)	
	pha62	2.0 (5)						
	pha2	2.0 (4)						
	pha1111	1.0 (2)						
	pha1112	– (1)						
Outgroup	Med		13.7	14.4	13.3	12.5	13.2	10.0 (2)

sites there are, the stronger the support. Additivity at the *intra*-specific level means that clones from one individual have, for example, both C and T at a given site. If different individuals within that morphospecies also have this site-specific polymorphism, it suggests that these individuals (e.g. M.dec12 and M.dec107 for position 85) may have been derived from a combination of ''only-C'' and ''only-T'' donors/parents (e.g. M.dec2 and M.dec11). For additivity at the inter-morphospecies level, the reasoning is similar. For position 224, all individuals of *M. mirabilis*, *M. senaria* and *M. pharensis* have a C. *M. decactis* and *M. formosa* have A, C and A + C. For position 515, all individuals of *M. mirabilis* (except for 1 clone out of 14) and *M. senaria* have a C, whereas all individuals of *M. pharensis* (except for 1 clone out of out of 24) have a G. *M. decactis* and *M. formosa* have C, G and C + G. For position 592, all individuals of *M. mirabilis* have an A, whereas *M. senaria* and *M. pharensis* have a T. In contrast *M. decactis* and *M. formosa* have A, T and A + T. These results suggest that *M. decactis* and *M. formosa* have interbred at some point in the past. Likewise, interbreeding involving *M. decactis* and *M. pharensis* may have occurred.

Polymorphism parsimony analyses (Fig. 4) of the matrix in Fig. 3 support the global analysis while refining relationships within and among putative morphospecies. Despite there being nearly 5,000 MPTs (Table 4), both *M. senaria* and *M. mirabilis* form monophyletic groups in Fig. 4, whereas *M. decactis*, *M. pharensis* and *M. formosa* maintain a paraphyletic assemblage based on the recognized morphospecies. The strict consensus tree (Fig. 4B) again recovers the backbone topology with a continued lack of resolution within the *decactis-pharensis-formosa* complex. Bootstrap values remain low (< 70%) for most of the clades to the right of the asterisk in Fig. 4A. This is because the number of phylogenetically informative characters is low and bootstrapping involves only a sub-set of the

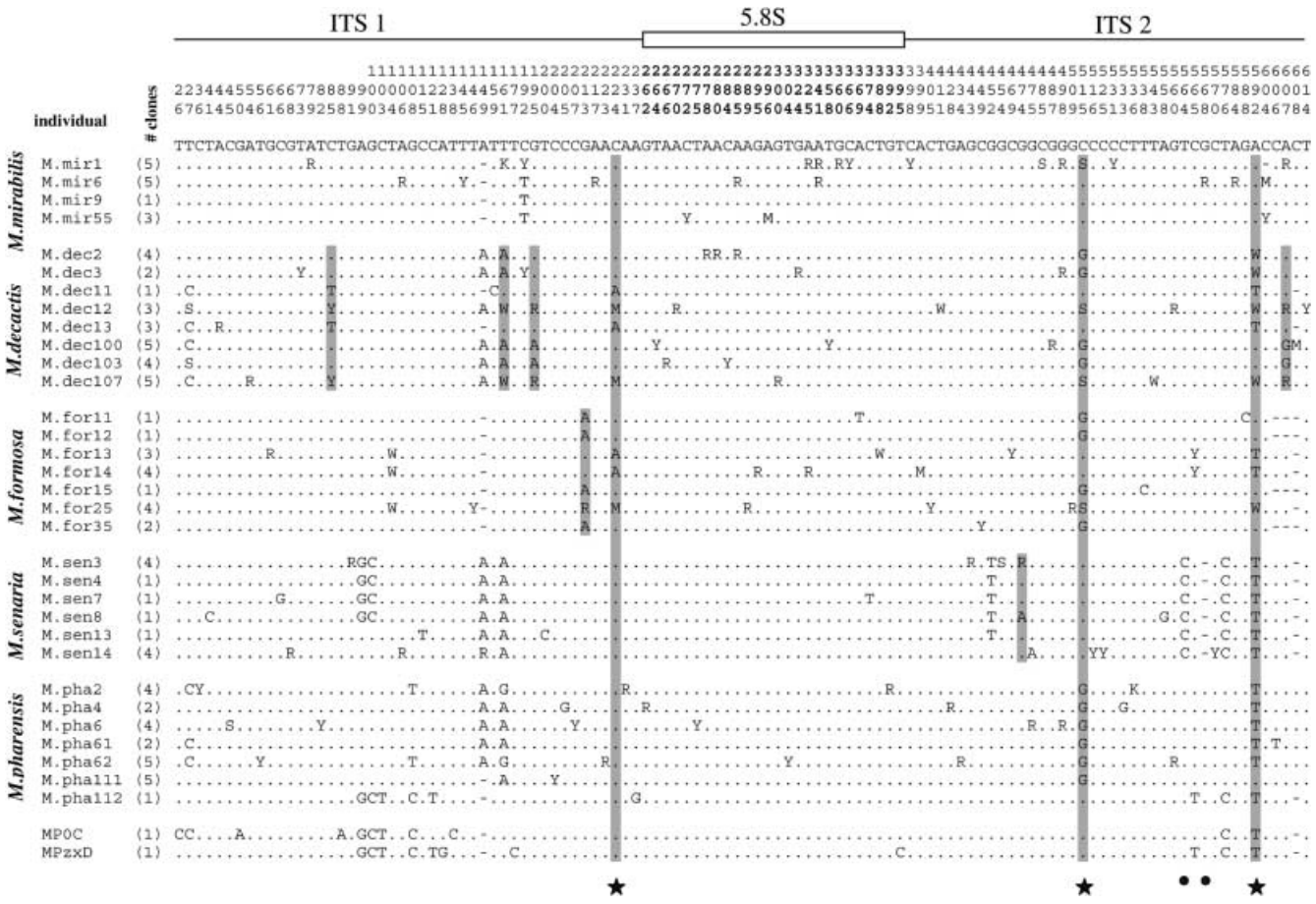

Fig. 3 Data matrix of polymorphic sites derived from the original 615-bp-alignment of ITS1-5.8S-ITS2. *Numbers at top* refer to positions in the original alignment (see "Appendix"). IUPAC symbols are used to represent polymorphic sites (*W* A/T; *Y* C/T; *R* A/G; *K* G/T; *M* A/C; *S* C/G). *Gray highlighted areas* indicate additive sites at the intra-specific and/or inter-specific levels; *star symbols* are indicative of possible hybridization; *filled circles* at positions 564 and 568 indicate species-specific for *Madracis senaria*. The complete alignment can be viewed in GenBank, see "Appendix" for accession numbers

phylogenetically informative characters in each round of the analysis. Even though consensus topologies are well supported and consistent, datasets with few informative characters are particularly sensitive to collapse under bootstrap resampling. This occurs in the *Madracis* dataset within the *decactis-pharensis-formosa* complex and especially when including all levels of clonal variation (Fig. 2). In Fig. 4A, for example, the bootstrap value at the asterisk is 54%. Thirty-four percent of the bootstrap values involve rearrangements of the *M. mirabilis* clade as a dichotomy or trichotomy with clades (Fig. 4B) and 12% other relationships.

Discussion

The ITS1-5.8S-ITS2 region in *Madracis* is not unusual with respect to length (ca. 613 bp) as compared with other scleractinians (Cullings and Vogler 1998). *Acropora* has a short ITS region (ca. 340 bp) (Odorico and Miller 1997) and *Stylophora* (800 bp) has a relatively long ITS region (Takabayashi et al. 1998). Intra- and inter-species variation within genera varies widely. In *Madracis* it is 1–8% but among *Acropora* species it is >60% (Van Oppen et al., unpublished data). On the one hand, widely differing intra- and inter-specific variability within and among genera can limit the utility of ITS. In our study, for example, the putative sister genera *Stylophora* and *Pocillopora* were found to be so widely divergent as to be unalignable. On the other hand, high variation within ITS, also provides an opportunity to use non-homogenization to investigate relationships among intra- and inter-specific complexes.

Recent speciation or introgressive hybridization?

Biogeographic separation for long periods of time is expected to lead to differentiation and eventual reproductive isolation, i.e. speciation. Yet there are cases in which taxa that have been diverged for millions of years are able to interbreed if brought into contact. This has been shown in *Mytilus* and leads to complex patterns of geographic variation (Hilbish 1996). Veron (1995) hypothesized that contemporary coral species have not evolved via continual, hierarchical splitting of lineages.

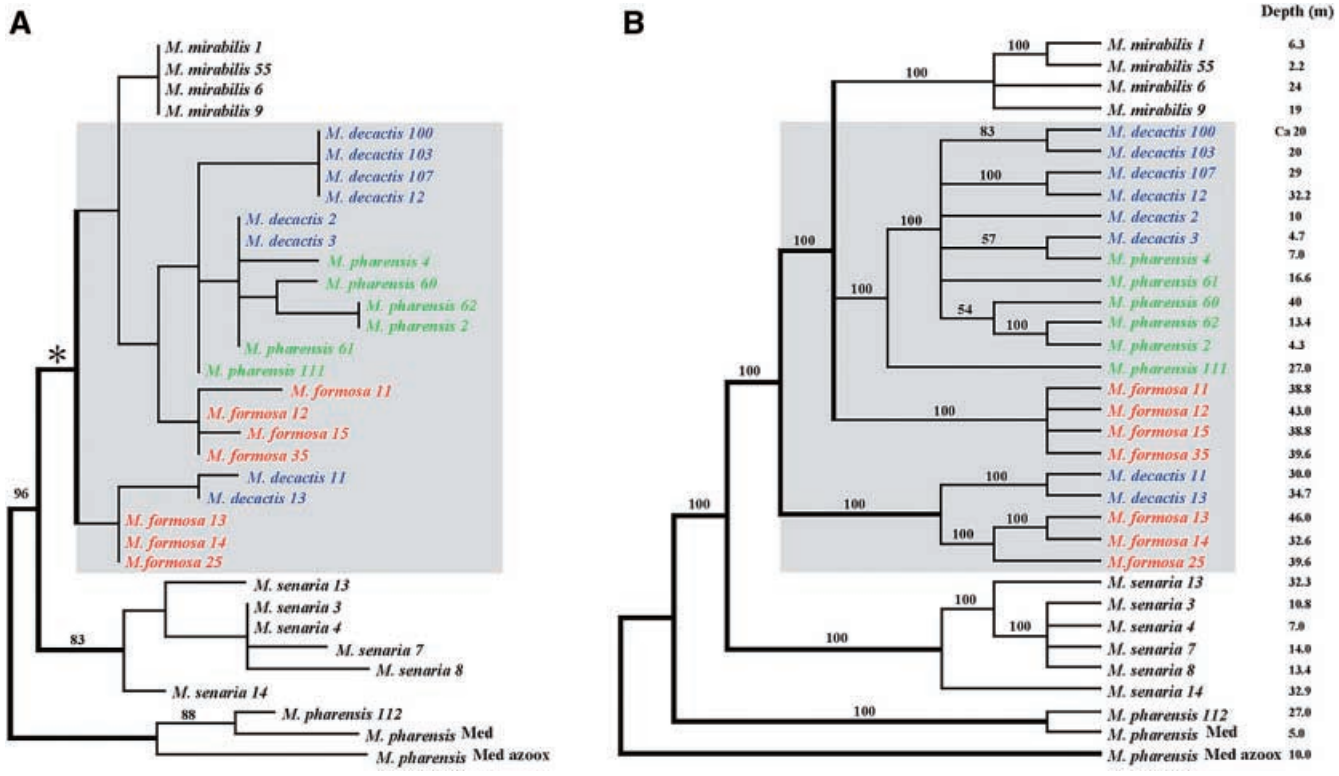

Fig. 4A, B Polymorphism parsimony analysis among morphospecies of *Madracis*. **A** One of 4,960 most-parsimonious trees based on analysis from alignment in Fig. 3. The invariant backbone topology is shown in *bold*. Clades within the *blue-gray box* belong to the paraphyletic *decactis-pharensis-formosa* complex. Colors correspond to traditionally recognized morphospecies. Bootstrap values (> 70%) based on 1,000 replicates are shown, see "Results" for explanation of the *asterisk*. **B** Consensus tree of 4,960 MPTs (majority-rule and strict topologies are the same) among morphospecies of *Madracis*. Consensus indices for the strict analysis are given as well as the sampling depth for each coral individual

Instead they remain interconnected by continual rounds of phylogenetic fission and fusion through "surface circulation vicariance" or what amounts to permanent disturbance/disequilibrium on a short geological time span of tens of thousands of years.

ITS sequence divergence across *Madracis* (Table 3) verifies the ability of ITS to resolve inter-specific relationships in the genus, which is reflected in the recovery of *M. senaria* and *M. mirabilis* in Fig. 4. Therefore, the discovery of an unresolved, paraphyletic complex and high intra- and inter-specific ITS polymorphism involving *M. decactis, M. pharensis* and *M. formosa* could not be attributed to a lack of resolving power per se. Non-homogenization of ITS can have several causes. These include polyploidy, loci located on more than one chromosome, recent speciation, or introgressive hybridization. Polyploidy has been reported in *Acropora* (Kenyon 1997), but there is no evidence for multiple chromosomal locations.

Recent speciation within the *M. decactis, M. pharensis, M. formosa* cluster is one possibility. In this interpretation, all of the polymorphisms in Fig. 3 are explained as ancestral polymorphisms that have not yet undergone lineage sorting, i.e. there has simply not been enough time to homogenize ITS repeats using the mechanisms of concerted evolution. However, if we consider the level of sequence divergence and the fossil history of *Madracis*, this explanation becomes somewhat less attractive.

The earliest described fossils of *Madracis* are of Cretaceous origin. However, fossils of *M. mirabilis* and *M. decactis* are known from the Caribbean from 15–11 million years ago (Budd et al. 1994, 1995; Swedberg 1994). There is only one record of *M. pharensis* dated at 1.5 million years ago (Budd and Johnson 1999), and no records for *M. formosa* or *M. senaria*. Whether their absence is an artifact is unknown. In any case, the modern species of *Madracis* are certainly not older than 12–10 million years and perhaps as young as 5 million years. These estimates correspond to the observed levels of ITS sequence divergence of ca. 6% among the five Caribbean taxa (Table 3), which correspond to a divergence time of 4.8–12 million years based on an ITS/18S molecular clock calibrated for green algae (Bakker et al. 1995a,b). Given the observed sequence divergence and approximate age of *Madracis* species (whether based on fossils or a molecular clock), homogenization of the ITS regions would normally be expected.

An alternative explanation for non-homogenization among *M. decactis*, *M. pharensis* and *M. formosa* is that they are part of a hybrid/reticulate complex in which the three taxa share a gene pool and in which *M. decactis* is more closely related to *M. pharensis* and *M. formosa* than *M. pharensis* is to *M. formosa*. The main evidence in support of this interpretation is the mixed paraphyly

of all three taxa and observed site-specific polymorphisms including the presence of additivity at nine sites – three at the inter-specific level (Fig. 3). We recognize that without additional supportive evidence from crosses among putative donors/parents, possible polyploidy and/or the presence of a large, shared insertion/deletion (Campbell et al. 1997), the interpretation remains provisional. Nevertheless, the origin of polymorphism through gene flow between divergent lineages needs to be considered because reproductive isolation among corals appears to be minimal.

Reproductive barriers in corals

Differential spawning times or subtle spatial factors may provide the only reproductive barriers for many corals. If these barriers break down fairly frequently, then sporadic cross-(sub)species reproductive interactions are likely to be permanently maintained. Incomplete reproductive isolation is now well documented for a number of coral species. In *Platygyra*, Miller and Babcock (1997) found that between-morphospecies fertilization rates were equivalent to within-morphospecies rates. Inter-specific hybridization has also been shown in *Montipora, Fungia* and *Montastraea* (Knowlton et al. 1997; Szmant et al. 1997; Willis et al. 1997). However, the most complete picture of the reticulate mode is known from *Acropora,* based on chromosome counts (Kenyon 1997), direct cross-fertilization studies (Willis et al. 1997; Hatta et al. 1999; Van Oppen et al., unpublished data), indirect analysis of mini-collagen gene intron (Hatta et al. 1999) plus the ITS region (Odorico and Miller 1997; Van Oppen et al. 2000, 2001, and unpublished data) and nuclear *Pax*-C intron plus the intergenic mtDNA region (Van Oppen et al., 2001).

There is still relatively little known about the details of reproduction in *Madracis*. Sexual reproduction is presumed to be dominant in all of the species, except *M. mirabilis* where asexual reproduction by fragmentation of the delicate branches is common (Bak and Engel 1979). Field observations indicate that all morphospecies of *Madracis* are brooders (M.J.A. Vermeij, R.P.M. Bak, O.E. Diekmann, unpublished data). Nothing is known about other possible reproductive barriers such as sperm-binding proteins or post-zygotic barriers in *Madracis*.

To date, all documented cases of hybridization in corals have involved broadcast spawners. The synchronous mass release of gametes, concentrated to a few hours on successive nights after full moon, is expected to result in a higher frequency of encounters per spawn and a higher probability of cross-species mating. However, brooders may compensate with low-level, continuous release of sperm and internally fertilized eggs (i.e. brooded larvae) over a period of several months (M.J.A. Vermeij, unpublished data). The absence of the temporal barrier associated with mass spawning would favor the potential for cross-fertilization among brooding species such as *Madracis,* while self-fertilization absolutely insures reproductive success. Brazeau et al. (1998) found that *Favia fragum* and *Porites astreoides* displayed a high level of self-fertilization (49% in *F. fragum* and 34% in *P. astroides*), whereas *Montastraea annularis* (a broadcaster) displays virtually zero (Szmant et al. 1997). Mixed-mode reproduction would allow brooders to maintain local adaptive advantages through selfing while promoting genetic variability by outcrossing.

Morphospecies

The phylogeny presented here is in disagreement with the characterization of classical morphospecies of *Madracis* according to Wells (1973a,b), yet this need not be regarded as negative. Morphospecies of corals will remain a mix of distinct and less distinct forms whose morphological variation can have several causes, ranging from independent (i.e. convergent) evolution under similar selection regimes (no hybridization required as explanation) to the control of skeletal features that are repeatedly transposed into successive genetic backgrounds through introgression. Van Oppen et al., (2001, and unpublished data) found that hybrids of *Acropora aspera* and *A. pulchra* often shared the attributes of only one donor/parent rather than being intermediate. They hypothesized that skeletal morphology is defined by a small number of closely linked genes with dominant and recessive characteristics.

The emerging, though still controversial, picture of reticulate speciation in many corals adds a new dimension to marine phylogeographic, ecological and population-level studies that may also apply to other invertebrates and algae. Veron's model of reticulate speciation was originally directed at the Indo-West Pacific region, where paleoclimatic and tectonic changes have generally been considered more dramatic. He originally suggested that the Caribbean and Gulf of Mexico regions were perhaps too small and too uniform for reticulate patterns to have developed (Veron 1995).

In the present study we have examined sympatric morphospecies at a single location. What might we expect if we expanded the sampling of *Madracis* species to other islands or to more distant geographic locations? Potts (1984) argued that the Pliocene/Pleistocene glaciations were a continual source of evolutionary disruptions for shelf corals, with minor changes in sea level having the most effect. He also emphasized the longevity of colonies (genets) in which the age of generations may actually approach the temporal scales of climate change. Colonies only a few generations removed from the last ice ages may still be contributing to the gametic pool of modern populations. This implies that if our study were expanded to include the entire Caribbean basin, the observed phylogenetic pattern would not change nor would it be likely to show any biogeographic correspondence.

In conclusion, the fact that reproductive barriers appear to be very weak in corals and the fact that interspecific hybridization among several species of corals

has been shown in the laboratory and in nature show that introgressive hybridization plays a role – possibly a very significant role – that has not been previously appreciated. Although our evidence for possible hybridization in *Madracis* is provisional, taken together with the more complete study on Caribbean *Acropora* (Van Oppen et al. 2000) and several Pacific genera, these studies show that both the Indo-West Pacific and Caribbean are behaving similarly – though perhaps to different degrees.

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Appendix

rDNA-ITS sequences of *Madracis* can be obtained from GenBank under the accession numbers given below. Sequences are linked to the alignment used in the global analysis. Codes correspond to Table 1.

Madracis mirabilis

mir1a AF251847, mir1b AF251848, mir1c AF251849, mir1d AF251850, mir1e AF251851, mir55b AF251852, mir55c AF251853, mir55d AF251854, mir6a AF251855, mir6b AF251856, mir6c AF251857, mir6d AF251858, mir6e AF251859, mir9c AF251860.

Madracis decactis

dec100a AF251861, dec100b AF251862, dec100c AF251863, dec100d AF251864, dec100e AF251865, dec103a AF251866, dec103b AF251867, dec103c AF251868, dec103d AF251869, dec107b AF251870, dec107a AF251871, dec107c AF251872, dec107d AF251873, dec107e AF251874, dec11c AF251875, dec12a AF251876, dec12b AF251877, dec12d AF251878, dec13a AF251879, dec13d AF251880, dec13e AF251881, dec2a AF251882, dec2b AF251883, dec2c AF251884, dec2d AF251885, dec3a AF251886, dec3e AF251887.

Madracis formosa

for11a AF251888, for12a AF251889, for13b AF251890, for13c AF251891, for13d AF251892, for14b AF251893, for14c AF251894, for14d AF251895, for14e AF251896, for15b AF251897, for25 h AF251898, for25a AF251899, for25b AF251900, for25c AF251901, for35c AF251902, for35e AF251903.

Madracis senaria

sen13b AF251904, sen14b AF251905, sen14c AF251906, sen14d AF251907, sen14e AF251908, sen3a AF251909, sen3c AF251910, sen3d AF251911, sen3e AF251912, sen4a AF251913, sen7a AF251914, sen8e AF251915.

Madracis pharensis

pha4b AF251916, pha60b AF251917, pha61b AF251918, pha61c AF251919, pha61d AF251920, pha61e AF251921, pha62a AF251922, pha62b AF251923, pha62c AF251924, pha62d AF251925, pha62e AF251926, pha2a AF251927, pha4c AF251928, pha2b AF251929, pha2c AF251930, pha2d AF251931, pha111c AF251932, pha111b AF251933, pha112f AF251934.

Outgroup (*Madracis pharensis* from the Mediterranean).

MedC AF251935, MedzxD AF251936.

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