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Examination of the *Montastraea annularis* Species Complex (Cnidaria: Scleractinia) Using ITS and COI Sequences

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Abstract: The Caribbean coral *Montastraea annularis* has recently been proposed to be a complex of at least three sibling species. To test the validity of this proposal, we sequenced the ITS region of the nuclear ribosomal RNA gene family (ITS-1, 5.8S, and ITS-2), and a portion of the mitochondrial DNA gene cytochrome *c* oxidase subunit I (COI) from the three proposed species (*M. annularis, M. faveolata,* and *M. franksi*) from Florida reefs. The ITS fragment was 665 nucleotides long and had 19 variable sites, of which 6 were parsimony-informative sites. None of these sites was fixed within the proposed species. The COI fragment was 658 nucleotides long with only two sites variable in one individual. Thus, under both the biological species concept and the phylogenetic species concept, the molecular evidence gathered in this study indicates the *Montastraea annularis* species complex to be a single evolutionary entity as opposed to three distinct species. The three proposed *Montastraea* species can interbreed, ruling out prezygotic barriers to gene flow (biological species concept), and the criterion of monophyly is not satisfied if hybridization is occurring among taxa (phylogenetic species concept).

Key words: *Montastraea,* ITS, COI, speciation

Introduction

The increasingly frequent recognition of sibling species complexes in the marine realm is a major problem confronting ecologists, physiologists, and evolutionary biologists (Knowlton, 1993, Knowlton and Jackson, 1994). The *Montastraea annularis* species complex is a good example of this problem. These species are important reef-building corals on Caribbean reefs and have been intensively investigated. However, it is still unclear how many species this group actually comprises. Studies of a Panamanian population of these corals yielded low congruence between morphotypes of an array of characters (genetic [allozymes], ecologic, behavioral [aggressive behavior], and morphometric) (Knowlton et al., 1992), while temporal separation of spawning events and hybridization experiments yielded few larvae (Knowlton et al., 1997). These results were interpreted to support the designation of at least three sibling species (Weil and Knowlton, 1994).

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Similar studies (allozymes, behavior, and morphometrics) of Curaçao corals, however, were interpreted as indicating that all morphotypes belonged to the same species because no fixed allelic differences were found at nine loci (Van Veghel and Bak, 1993). Throughout the Caribbean, the three species are morphologically distinct, but many intermediate morphologies are common as well (Figure 1 in Szmant et al., 1997). Furthermore, the proposed species occur sympatrically, species spawn on the same nights at slightly different but overlapping times, and hybridization studies with Florida corals have shown that all possible crosses can yield high levels of fertilization under laboratory conditions (Szmant et al., 1997). For a more detailed summary of the differences between Caribbean *Montastraea* species, see Table 1 in Szmant et al. (1997).

The inability to unambiguously differentiate *Montastraea* species throughout their geographic and depth ranges leads to the need to develop molecular markers with greater resolution than protein electrophoresis (Knowlton et al., 1997). From a preliminary survey of different types of nuclear and mitochondrial markers by Lopez and Knowlton (1997), only two AFLP primers appeared as potential diagnostic markers for *M. faveolata* versus *M. franksi*.

One of the best-characterized regions of DNA for phylogenetic inference is the ribosomal DNA multigene family (Hillis and Dixon, 1991). In eukaryotes the nuclear ribosomal small subunit gene (18S) is separated from the 5.8S gene by one internal transcribed spacer (ITS-1), and a second internal transcribed spacer (ITS-2) separates the 5.8S gene from the large subunit gene (28S). Ribosomal genes and spacers evolve at different evolutionary rates (Hillis and Dixon, 1991), making this gene family a suitable candidate for phylogenetic analysis at many systematic levels. Both 18S genes (Bridge et al., 1994) and 28S genes (Chen et al., 1995; Odorico and Miller, 1997a) have been used for higher-level systematics of Cnidarians. The ITS regions have higher rates of evolution because they have fewer functional constraints than the ribosomal genes, making them useful for lower-level taxonomic comparisons. The ITS regions have been successfully used in Cnidarians (Beauchamp and Powers, 1996; Chen et al., 1996) and corals in particular (Odorico and Miller, 1997b), as well as in other taxa to study relationships at the population level (Vogler and DeSalle, 1994; Caporale et al., 1997) and species level (Fritz et al., 1994; Tang et al., 1996).

Mitochondrial DNA markers have also proved useful for questions in systematics and historical biogeography (Avise et al., 1987). A higher rate of evolution compared to with that of nuclear DNA and its maternal inheritance make this molecule suitable for population-level and species-level comparisons (Brown, 1983). Sequence analysis of the mitochondrial cytochrome *c* oxidase subunit I (COI) has revealed genetic structure in populations of marine invertebrates such as copepods (Burton and Lee, 1994) and sea urchins (Palumbi, 1996). COI sequences have also been used to resolve phylogenetic relationships among closely related species of sea urchins (Bermingham and Lessios, 1993; Palumbi, 1996) and gastropod mollusks (Harasewych et al., 1997).

Because processes such as lineage sorting result in differences between a gene genealogy and organismal phylogenies (Avise and Ball, 1990), we have employed sequences from two different genome regions that evolve at different rates to investigate the validity of the subdivision of the *Montastraea annularis* taxon into three separate species. One of the sequences we used is the ITS region of the nuclear ribosomal DNA gene family. The second marker is a portion of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene.

Materials and Methods

Sample Collection

Portions of colonies of the three proposed species of *Montastraea* were collected from Florida reefs (Key Largo), and placed in separate containers with filtered seawater to spawn. Additional samples of spawn were collected by suspending collecting nets over individual colonies in situ 2 to 3 hours before spawning (Szmant et al., 1997). Gamete bundles were selected with Pasteur pipettes from the spawn of individual colonies and placed into 1-ml microcentrifuge tubes (Table 1). Gametes were chosen as the source of DNA because they are not yet infected by the symbiotic algae (zooxanthellae) typical of adult coral tissues. Samples were frozen at −20°C until used for DNA extraction. In addition, tissue samples were collected from one colony from each of the three *Montastraea* species from Puerto Rico, and one colony of *M. cavernosa* from Florida.

DNA Isolation

Total DNA was isolated by standard sodium dodecyl sulfate (SDS)/proteinase K digestion (Sambrook et al., 1989). Egg masses were gently homogenized in a microfuge tube in 700 μl of 1× NET buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA) with 1% SDS and 100 μg/ml proteinase

Table 1. Samples Sequenced for the Three *Montastraea* Species, Collection Information, and GenBank Accession Numbers

Species	Sample ID*	Collection site/date†	GenBank accession no. ITS	COI
M. annularis	I4	KLDR/8-28-94	AF013728	AF013737‡
	I6	KLDR/8-28-94	AF013729	AF013737‡
	I7	KLDR/8-28-94	AF013730	NS
	I8 (C)	KLDR/8-28-94	U59907‡	NS
	I9	KLDR/8-28-94	AF013731	AF013737‡
	I195	AR/8-16-95	U59903	AF013737‡
	I295 (B)	AR/8-16-95	AF013727‡	AF013737‡
	I395	AR/8-16-95	U59904	AF013737‡
	IPR (B)	ER/3-20-92	AF013727‡	NS
M. faveolata	II195	AR/8-16-95	AF013732	AF013737‡
	II295 (B)	AR/8-16-95	AF013727‡	AF013738
	II1 (C)	LG/8-27-94	U59907‡	NS
	FII2	KLDR/8-28-94	U59905	AF013737‡
	FII5 (A)	KLDR/8-28-94	U59906‡	AF013737‡
	IIA (A)	ER/3-20-92	U59906‡	NS
	II8	KLDR/8-28-94	AF013733	NS
M. franksi	FIII5 (C)	KLDR/8-28-94	U59907‡	NS
	FIII6 (B)	KLDR/8-28-94	AF013727‡	AF013737‡
	III195 (C)	AR/8-16-95	U59907‡	AF013737‡
	III395 (C)	AR/8-16-95	U59907‡	AF013737‡
	IIIKL	KL/6-24-93	AF013734	NS
	IIIPR	ER/3-20-92	AF013735	NS
M. cavernosa	MCWL2	AR/6-11-92	NS	AF013736

*Samples that shared the same alleles (identical sequence) for the ITS region are indicated by letters (A,B,C). The only sample with nucleotide difference for the COI region was II295.

†Sample collection sites: AR indicates Alina's reef, Biscayne National Park, Fla.; ER, Enrique Reef, La Parguera, Puerto Rico; KL, unnamed reef, Key Largo, Fla.; LG, Little Grecian, Fla.; KLDR, Key Largo Dry Rocks, Fla.

‡Identical sequences with same accession number. NS indicates not sequenced.

K, and incubated at 65°C for 1 to 2 hours. Nucleic acids were isolated by standard phenol:chloroform (3:1) extractions until the interface was clear, followed by a chloroform:isoamyl alcohol (24:1) extraction and precipitation by the addition of Na-acetate to 300 mM and 2.5 to 3 vol of absolute ethanol. Total DNA was resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and RNase (50 μg/μl) treated at 37°C, followed by a chloroform:isoamyl (24:1) extraction and ethanol precipitation. The DNA samples were resuspended in TE.

PCR Amplification and Sequencing

Amplifications were performed in a 25-μl volume of a solution containing approximately 50 ng of DNA, 1× polymerase chain reaction (PCR) buffer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 1.25 units of *Taq* (Perkin-Elmer/Cetus). The conditions used were a denaturing step of 2 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C, with a final extension step of 5 minutes at 72°C. The primers used for the ITS fragment were the universal primers for the entire nuclear ribosomal ITS region (White et al., 1990): ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′), located on the 18S and 28S flanking regions, respectively. The primers used for the mitochondrial COI fragment were the universal primers: LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198: (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) developed by Folmer et al. (1994). Double-stranded PCR products were purified with the Gene Clean Kit (Bio 101 Company).

The COI primers were end-labeled with γ-^{33}P (NEN Dupont), and samples were cycle-sequenced with both primers using the Δ *Taq* sequencing kit (U.S. Biochemical). Internal primers were designed to obtain the full length of the sequence in both directions. Sequence samples were run on a 6% polyacrylamide gel.

Most of the ITS PCR products were sequenced manually following the same procedure as for the COI fragments, also using internal primers. Access to a LiCor automated sequencer was available in the final stages of the project. The remainder of the ITS samples were cloned into a TA plasmid vector and sequenced with dye-labeled M13 forward and reverse primers using the Sequitherm cycle-sequencing kit (Epicentre Technology). The latter sequencing products were run on a 4% acrylamide denaturing gel in the LiCor automated sequencer (see footnotes in Table 2).

Data Analysis

DNA sequences from both strands were proofread and aligned manually. The ITS aligned sequences were imported into PAUP release 3.1.1. (D. Swofford, 1993) for parsimony analysis. A heuristic search was performed with 10 random additions and TBR branch swapping using midpoint rooting. Consistency index (CI) was calculated as a measure of fit between the data and the reported topologies. Because more than one tree was found, a 50% majority rule strict consensus was reported (Figure 1).

Montastraea cavernosa COI sequences could be aligned with the rest of the *Montastraea* samples, but no further phylogenetic analysis was possible owing to the lack of polymorphisms within the *M. annularis* species complex.

Results

The two ITS primers used amplified a 665-bp fragment that included the ITS-1, the 5.8S gene, and the ITS-2. A total of 22 samples were sequenced (*M. annularis, n* = 9; *M. faveolata, n* = 7; *M. franksi, n* = 6; Table 1). Attempts to amplify this fragment for a specimen of *M. cavernosa* were not successful. The sequence analysis also included the ITS sequence for a specimen of *M. franksi* from Panama (accession number U60605; Lopez and Knowlton, 1997). The base composition for the ITS region was 21% A, 27% C, 29% G, and 23% T. This region exhibited 19 variable sites, of which 6 were parsimony-informative sites (Table 2). None of these six sites was diagnostic for any of the three

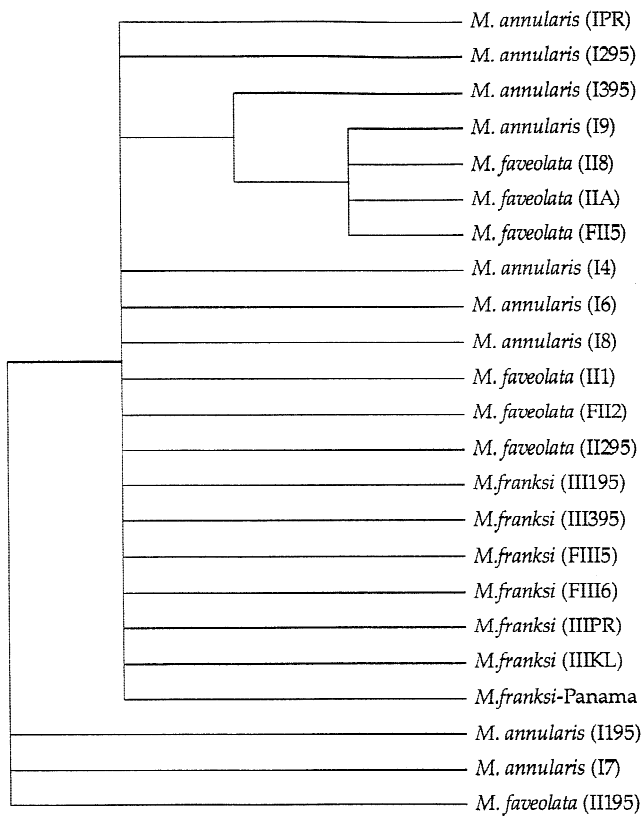

Figure 1. Strict consensus tree of six most parsimonious trees based on the ITS data (TL = 27 and CI = 0.815). This tree shows no sign of phylogenetic structure by species owing to the high homogeneity of the ITS data set.

Montastraea species. Three different sequences (identified as alleles A, B, and C) were each shared by more than one individual (Table 1). A maximum parsimony analysis yielded six shortest trees with tree length (TL) of 27 and a consistency index (CI) of 0.815. The strict consensus of these trees is presented in Figure 1. This tree shows that there is no clear genetic separation between these *Montastraea* taxa based on the combined ITS and 5.8S sequences.

The sequence of the mitochondrial COI fragment (658 bp) was identical for the 13 samples sequenced (*M. annularis, n* = 6; *M. faveolata, n* = 4; *M. franksi, n* = 3), except for one specimen of *M. faveolata* (II295) that varied at two nucleotide sites. Nucleotide sequences were translated into amino acids using the "invertebrate" mitochondrial genetic code in MacVector, version 4.1.4. The two nucleotide changes in II295 result in two amino acid differences. The *M. cavernosa* specimen differed at 16 nucleotide positions, 14 of them third positions, and 2 of them leading to amino acid changes. The COI nucleotide composition was 22% A,

Table 2. Variable Sites Within the ITS Regions for all Samples Sequenced.

		Nucleotide position*																						
		1	1	1	1	1	1	1	2	2	3	3	4	4	4	4	4	5	5	5	5	6	6
	Sample	0	2	2	3	3	6	7	0	4	1	7	0	2	3	3	5	3	6	6	9	0	2
Species	ID	3	6	9	3	4	9	5	6	4	2	1	1	5	3	5	4	1	3	6	1	1	1
M. annularis	IPR	G	T	A	G	C	T	C	T	G	C	A	T	T	A	G	G	T	G	G	G	C	T
	I195†	.	C	G	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	T	C
	I295	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	I395†	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	A	.	.	.
	I4†	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	.	.
	I6	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
	I7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	T	.
	I8	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.
	I9†	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	A	T	.	.
M. faveolata	II1	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.
	II8	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	A	T	.	.
	IIA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	T	.	.
	FII2	A	.	.	.	.	C	.	A	.	.	.	.	.	G	.	.	.	.	A	.	.	.
	FII5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	T	.	.
	II195	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	T	.
	II295	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M. franksi	III195†	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.
	III395	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.
	FIII5	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.
	FIII6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	IIIPR	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
	IIIKL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	Panama	.	.	.	.	.	.	.	.	A	G	.	.	.	.	C	.	C	.	A	.	.	.

*Numbers above each nucleotide correspond to the position in the complete fragment. Three sites were uniquely different in the *M. franksi* sequence from Panama.

†Samples cloned into a TA vector.

16% C, 21% G, and 41% T. The sequence divergence was 2.4% between *M. cavernosa* and all the other *Montastraea* specimens except the sample II295 of *M. faveolata* (2.7%). The sequence divergence was 0.02% between II295 and the rest of the *Montastraea* samples. Similar results were obtained for *Montastraea* sp. samples from other Caribbean localities (Tonya Snell, manuscript in preparation). In her study, a portion of the same region of the COI gene (616 bp) was sequenced from seven specimens of *M. cavernosa* from the Gulf of Mexico (East Flower Garden Bank, Tex.), Florida (Bahia Honda and Carysfort Reef), and Bermuda, one specimen of *M. faveolata* (East Flower Garden Bank), and one of *M. franksi* (Bermuda). The COI sequences for *M. cavernosa* and for the two *M. annularis* group species were identical to the sequences reported in this study for the respective species.

Discussion

Our results and those of previous studies (Knowlton et al., 1992; Van Veghel and Bak, 1993) are a good illustration of how different genetic markers (i.e., allozyme vs. DNA sequence) can yield incongruent phylogenetic results for phylogenetic relationships among species, especially when divergence may be recent and hybridization may persist after speciation.

The discovery that neither the ITS nor the COI regions exhibit any sign of genetic divergence among the three pro-

posed *Montastraea* taxa does not support the proposal of three sibling species based on allozyme, morphologic, and other markers (Knowlton et al., 1992; Weil and Knowlton, 1994). Our findings are also consistent with the results of fertilization studies (Knowlton et al., 1997; Szmant et al., 1997) that show no prezygotic barrier to hybridization. These DNA data together with the hybridization study can be interpreted as compelling evidence that the three proposed taxa all belong to the same morphologically variable *Montastraea* species.

Nuclear and mitochondrial genes have different coalescence times to a common ancestor because of differences in effective population size and other factors. Nuclear genes may maintain polymorphism for a longer period of time because of interbreeding, whereas mtDNA haplotypes can reach fixation sooner owing to lineage sorting events (Avise, 1994). The ancestral lineages to *M. cavernosa* and *M. annularis* appear to have diverged as early as the beginning of the Miocene about 24 million years ago (Budd, 1991). COI sequence divergence between species of sea hares from the genus *Aplysia* (which also appeared in the Miocene) is approximately 16% (Medina, 1998) as opposed to 2.4% between *M. cavernosa* and *M. annularis*. Even for species with more recent divergences such as sea urchins separated by the Isthmus of Panama (3.5 million years ago), the COI sequence divergence is greater, being approximately 6% (Bermingham and Lessios, 1993). Thus for the COI gene the small sequence divergence (2.4%) between the *M. annularis* species complex and *M. cavernosa* suggests that their shared ancestor had little or no haplotype diversity at the splitting time, and that the two substitutions observed in II295 occurred after divergence.

For the ITS region three identical sequences (alleles A, B, and C) were shared by more than one individual (Table 1). Unfortunately, we were unsuccessful in amplifying the ITS region for *M. cavernosa* for outgroup comparison. Alleles B and C were found in all three taxa and thus cannot be diagnostic characters for species under the criteria of cladistics. Allele A was found in only two *M. faveolata* samples, and thus could be species specific. However, given the small sample size and that five other specimens of *M. faveolata* did not have this allele, it is more likely that its absence from *M. annularis* and *M. franksi* is a sampling artifact.

A 1997 study of the ITS regions from closely related species of the genus *Acropora* from the Indo-Pacific revealed high levels of sequence divergence across taxa (up to 23%) that permitted distinction between closely related species (Odorico and Miller, 1997b). However, some ITS sequences were common to more than one species of *Acropora,* which was interpreted as recent interspecific hybridization and evidence for reticulate evolution. The pattern observed in *Montastraea* species contrasts with that of *Acropora* species, because in the former both intraspecific and interspecific sequence divergences are very low, which can be interpreted as robust evidence of no speciation events in this group.

The results of this study can also be interpreted as evidence that the two gene fragments utilized do not have the resolution to address the speciation events in *Montastraea*. If the three *Montastraea* taxa are indeed different species, then by inference the species divergence must have taken place very recently, or may be ongoing, there may be a geographic component to speciation in these taxa (i.e., greater genetic divergence in Panama populations), or the nuclear and mtDNA loci examined here evolve at rates too slow to detect speciation events in such closely related taxa.

Multiple Evidence Is Not Concordant

The longer two populations have been reproductively separated, the greater the chance for genetic divergence and accumulation of concordant phylogenetic relationships at multiple loci (Avise and Ball, 1990). Thus short separation times will not allow for genealogic concordance to develop, and the probability of inferring the correct organismal tree of such a group is small. The fossil record indicates that the *M. annularis* species complex probably diverged from its ancestor (likely *M. limbata*) about 1.7 million years ago (Budd, 1991; Weil and Knowlton, 1994). This is a relatively short time for nucleotide substitutions to accumulate, especially at protein coding or ribosomal RNA coding regions. However, one would expect to find changes at degenerate sites and introns if lineages have diverged into reproductively isolated taxa. Hybridization seems to be a common event in scleractinian corals (Wallace and Willis, 1994; Veron, 1995; Miller and Babcock, 1997; Szmant et al., 1997; Willis et al., 1997). If introgression due to hybridization has occurred after separation (a likely scenario for the *M. annularis* complex), most loci will not exhibit genetic separation or reflect the hierarchical order of divergence among taxa.

Species Concept: Does One Apply?

There has always been controversy surrounding the definition of a species; however, two definitions of species are

most popular in the literature: the biological species concept (BSC) based on reproductive isolation (see Templeton, 1989), and the phylogenetic species concept (PSC) based on phylogenetic relationships determined by monophyly (see Cracraft, 1989). The adoption of either one is difficult in defining species boundaries when reticulate evolution is suspected.

Under BSC, prezygotic and postzygotic isolating mechanisms play an important role in the definition of a species. The three proposed *Montastraea* species have a great potential to interbreed as shown in fertilization studies (Szmant et al., 1997, but see Knowlton et al., 1997), ruling out prezygotic barriers to gene flow (Mayr, 1963). In subsequent fertilization studies, hybrid larvae successfully settled and survived for some time (A.M. Szmant, unpublished results), but no hybrid adults have been reared, and therefore postzygotic isolation mechanisms still need to be evaluated.

For all versions of PSC, monophyly is a critical condition for delimiting species boundaries (de Queiroz and Donoghue, 1990). The concept of monophyly states that (1) a taxon must comprise an ancestor and all of its descendants and (2) all members should be more closely related to one another than to any entity outside the taxon (de Queiroz and Donoghue, 1990; Graybeal, 1995). These two aspects apply to hierarchical relationships above the species level. However, interbreeding sets a lower limit on the application of cladistic analysis due to reticulation (de Queiroz and Donoghue, 1990; Veron, 1995). Thus, in the case of a species complex such as *Montastraea* with hybridization potentially occurring among taxa, the criterion of monophyly is not satisfied.

Clearly there is conflict between the different types of evidence used in different areas of the Caribbean to elucidate associations within the *M. annularis* species complex. However, on the basis of the molecular evidence presented in this study and under both BSC and PSC, the *Montastraea annularis* species complex appears to be a single evolutionary entity.

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References

Avise, J.C. (1994). *Molecular Markers, Natural History and Evolution*. New York, N.Y.: Chapman & Hall.

Avise, J.C., and Ball, R.M. Jr. (1990). Principles of genealogical concordance in species concepts and biological taxonomy. *Oxf Surv Ecol Evol* 7:47–67.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J., Reeb, C.A., and Saunders, N.C. (1987). Intraspecific phylogeography: the bridge between population genetics and systematics. *Ann Rev Ecol Sys* 18:489–522.

Beauchamp, K.A., and Powers, D.A. (1996). Sequence variation of the first internal spacer (ITS-1) of ribosomal DNA in ahermatypic corals from California. *Mol Mar Biol Biotechnol* 5(4):357–362.

Berminhgam, E., and Lessios, H.A. (1993). Rate variation of protein and mitochondrial DNA evolution as revealed by sea urchins separated by the Isthmus of Panama. *Proc Natl Acad Sci USA* 90:2734–2738.

Bridge, D., Cunningham, C.W., DeSalle, R., and Buss, L.W. (1994). Class-level relationships in the phylum Cnidaria: molecular and morphological evidence. *Mol Biol Evol* 12(4):679–689.

Brown W.M. (1983). Evolution of animal mtDNA. In: *Evolution of Genes and Proteins,* Nei, M. and Kohen, R.K. (eds.). Sunderland, Mass.: Sinauer, 62–88.

Budd, A.F. (1991). Neogene paleontology in the Northern Dominican Republic, 11: the family Faviidae (Anthozoa: Scleractinia), Part I: the genera *Montastraea* and *Solenastrea. Bull Amer Paleon* 101(338):1–48.

Burton, R.S., and Lee, B.N. (1994). Nuclear and mitochondrial gene genealogies and allozyme polymorphism across a major phylogeographic break in the copepod *Tigriopus californicus. Proc Natl Acad Sci USA* 91:5197–5201.

Caporale, D.A., Beal, B.F., Roxby R., and Beneden, R.J. (1997). Population structure of *Mya arenaria* along the New England coastline. *Mol Mar Biol Biotechnol* 6(1):33–39.

Chen, C.A., Odorico, D.M., Lohuis, M.T., Vernon, J.E.N., and Miller, D.J. (1995). Systemic relationships within the Anthozoa (Cnidaria: Anthozoa) using the 5′-end of the 28S rDNA. *Mol Phyl Evol* 4(2):175–183.

Chen, C.A., Willis, B.L., and Miller, D.J. (1996). Systematic relationships between tropical corallimorpharians (Cnidaria: Anthozoa: Corallimorpharia): utility of the 5.8S and internal transcribed spacer (ITS) regions of the RNA transcription unit. *Bull Mar Sci* 59(1):196–208.

Cracraft, J. (1989). Speciation and its ontogeny: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. In: *Speciation and Its Consequences,* Otte, D., and Endler, J.A. (eds.). Sunderland, Mass.: Sinauer, 28–57.

De Queiroz, K., and Donaghue, M.J. (1990). Phylogenetic systematics and species revisited. *Cladistics* 6:83–90.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial *c* oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 3:294–299.

Fritz, G.N., Conn, J., Cockburn, A., and Seawright, J. (1994). Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Mol Biol Evol* 11(3):406–416.

Graybeal, A. (1995). Naming species. *Sys Bio* 44(2):237–250.

Harasewych, M.G., Adamkewicz, S.L., Blake, J.A., Saudek, D., Spriggs, T., and Bult, C.J. (1997). Phylogeny and relationships of pleurotomariid gastropods (Mollusca: Gastropoda): an assessment based on partial 18S rDNA and cytochrome *c* oxidase I sequences. *Mol Mar Biol Biotechnol* 6(1):1–20.

Hillis, D.M., and Dixon, M.T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol* 66:411–453.

Knowlton, N. (1993). Sibling species in the sea. *Annu Rev Ecol Syst* 24:189–216.

Knowlton, N., and Jackson, J.B.C. (1994). New taxonomy and niche partitioning on coral reefs: jack of all trades or master of some? *TREE* 9:7–9.

Knowlton, N., Weil, E., Weigt, L.A., and Guzmán, H.M. (1992). Sibling species in *Montastraea annularis,* coral bleaching, and the coral climate record. *Science* 255:330–333.

Knowlton, N., Mate, J.L., Guzman, H.M., Rowan, R. and Jara, J. (1997). Direct evidence for reproductive isolation among the three species of the *Montastraea annularis* complex in Central America (Panama and Honduras). *Mar Biol* 127:706–711.

Lopez, J.V., and Knowlton, N. (1997). Discrimination of species in the *Montastraea annularis* complex using multiple genetic loci. *Proc 8th Int Coral Reef Symp* 2:1613–1618.

Mayr, E. (1963). *Animal Species and Evolution.* Cambridge, Mass.: Harvard University Press.

Medina, M. (1998). Molecular systematics and population genetics of sea hares (Gastropoda: Opisthobranchia: Anaspidea). Ph.D. thesis, University of Miami, Florida.

Miller, K., and Babcock, R. (1997). Conflicting morphological and reproductive species boundaries in the coral genus *Platygyra. Biol Bull* 192:98–110.

Odorico, D.M., and Miller, D.J. (1997a). Internal and external relationships of the Cnidaria: implications or primary predicted secondary structure of the 5′-end of the 23S-like rDNA. *Proc R Soc Lond B* 264:77–82.

Odorico, D.M., and Miller, D.J. (1997b). Variation in the ribosomal internal transcribed spacers and 5.8S rDNA among five species of *Acropora* (Cnidaria; Scleractinia): patterns of variation consistent with reticulate evolution. *Mol Biol Evol* 14(5):465–473.

Palumbi, S.R. (1996). Microspatial genetic structure and speciation in marine taxa with high dispersal abilities. In: *Molecular Zoology, Advances, Strategies and Protocols,* Ferraris, J.D., and Palumbi, S.R. (eds.). New York, NY: Wiley-Liss, 101–117.

Sambrook, J, Fritsch, E., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Szmant, A.M., Weil, E., Miller, M.W., and Colon, D.E. (1997). Hybridization within the species complex of *Montastraea annularis. Mar Biol* 129:561–572.

Tang, J., Toe, L., Back, C., and Unnasch, T.R. (1996). Intra-specific heterogeneity of the rDNA internal transcribed spacer in the *Simulium damnosum* (Diptera: Simuliidae) complex. *Mol Biol Evol* 13:244–252.

Templeton, A.R. (1989). The meaning of species and speciation: a genetic perspective. In: *Speciation and Its Consequences,* Otte, D., and Endler, J.A. (eds.). Sunderland, Mass.: Sinauer, 3–27.

Van Veghel, M.L.J., and Bak, R.P.M. (1993). Intraspecific variation of a dominant Caribbean reef building coral, *Montastraea annularis:* genetic, behavioral and morphometric aspects. *Mar Ecol Prog Ser* 92:255–265.

Veron, J.E.N. (1995). *Corals in Space & Time.* Ithaca, N.Y.: Cornell University Press.

Vogler, A.P., and DeSalle, R. (1994). Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cincindela dorsalis. Mol Biol Evol* 11(3):393–405.

Wallace, C.C., and Willis, B.L. (1994). Systematics of the coral genus *Acropora:* implications of new biological findings for species concepts. *Annu Rev Ecol Syst* 25:237–262.

Weil, E., and Knowlton, N. (1994). A multi-character analysis of the Caribbean coral *Montastraea annularis* (Ellis & Solander, 1786) and its two sibling species, *M. faveolata* (Ellis & Solander, 1786) and *M. franksi* (Gregory, 1895). *Bull Mar Sci* 55:151–175.

White, T.J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications,* Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (eds.). New York, N.Y.: Academic Press, 315–322.

Willis, B.L., Babcock, R.C., Harrison, P.L., and Wallace, C.C. (1997). Hybridization and breeding incompatibilities within the mating systems of mass spawning reef corals. *Proc 8th Int Coral Reef Sym* 1:81–90.