Evolution, 58(2), 2004, pp. 324–337

GEOGRAPHIC DIFFERENCES IN SPECIES BOUNDARIES AMONG MEMBERS OF THE *MONTASTRAEA ANNULARIS* COMPLEX BASED ON MOLECULAR AND MORPHOLOGICAL MARKERS

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Abstract.—The three members of the *Montastraea annularis* complex (*M. annularis, M. franksi*, and *M. faveolata*) are dominant reef builders in the western Atlantic whose species status has been controversial for over a decade. Although differences in colony morphology and reproductive characteristics exist, interspecific fertilizations are possible in the laboratory and genetic differentiation is slight. Here we compare the three taxa genetically and morphologically in Panama and the Bahamas, widely separated locations spanning most of their geographic ranges. In Panama, analyses of three AFLP loci, a noncoding region of the mitochondrial genome, and ITS sequences reveal that *M. faveolata* is strongly differentiated genetically. Discriminant function analysis also indicates no overlap with the other two species in the fine structure of the corallites that comprise the colony. Genetic analyses of larvae from interspecific crosses between *M. faveolata* and the other two taxa confirmed the hybrid status of the larvae, but no examples of the most probable F_1 genotype were observed in the field. Although *M. annularis* and *M. franksi* were more similar, they also exhibited strong frequency differences at two AFLP loci and in the mitochondrial noncoding region, as well as distinct corallite structure. In the Bahamas, in contrast, the three taxa exhibited overlapping morphologies. *Montastraea franksi* and *M. annularis* were indistinguishable genetically, and *M. faveolata* was distinct at fewer genetic loci. Once again, however, the most probable F_1 genotype involving *M. faveolata* was not observed. Geographic differences between Panama and the Bahamas explain why past studies have come to different conclusions concerning the status of the three species. In general, the genetic and morphological data suggest a north to south hybridization gradient, with evidence for introgression strongest in the north. However, reproductive data show no such trend, with intrinsic barriers to gene flow comparable or stronger in the north.

Key words.—Caribbean, coral reef, hybridization, *Montastraea*, sibling species complex.

Received January 16, 2003. Accepted November 24, 2003.

Reef-building corals create the most diverse marine habitats on the planet. Despite their ecological importance, the nature of coral species is far from settled, particularly with regard to the possibility of reticulate evolution (e.g., Knowlton et al. 1992, 1997; van Veghel and Bak 1993; Wallace and Willis 1994; Veron 1995; Kenyon 1997; Szmant et al. 1997; Hatta et al. 1999; Lopez et al. 1999; van Oppen et al. 2000, 2001, 2002; Diekmann et al. 2001; Marquez et al. 2002a, 2002b; Vollmer and Palumbi 2002; Miller and van Oppen 2003). Widespread reticulate evolution is generally accepted for plants but appears to be more limited in animals (Arnold 1997; Dowling and Secor 1997); thus corals represent an important model in this regard.

The *Montastraea annularis* complex lies at the heart of this debate. Its three putative species (*M. annularis*, *M. faveolata*, and *M. franksi*) are ecologically dominant, broadly sympatric, and often in close proximity on many tropical western Atlantic reefs. For many years, it was thought that these three morphotypes represented intraspecific variation, but later studies revealed that differences in colony morphology are paralleled by additional differences in growth rate, stable isotope chemistry, aggressive behavior, allozymes, corallite structure, and life history (Tomascik 1990; Knowlton et al. 1992, 1997; van Veghel and Bak 1993, 1994; van Veghel and Kahmann 1994; Weil and Knowlton 1994; van Veghel and Bosscher 1995; van Veghel et al. 1996; Szmant et al. 1997; Hagmann et al. 1998a, b; Manica and Carter 2000; Knowlton and Budd 2001). The concordance of these presumably independent characters (sensu Avise and Ball 1990) supports Weil and Knowlton's (1994) resurrection of two species that were previously synonymized with *M. annularis* sensu stricto.

However, it has proved difficult to find genetic differences among these taxa using DNA sequence data. For example, analyses of the ITS regions of rDNA, an intron in the B-tubulin gene, and mitochondrial COI sequences showed no fixed DNA sequence differences among them (Lopez and Knowlton 1997; Medina et al. 1999). More recently, however, one amplified fragment length polymorphism (AFLP) locus was identified that distinguished *M. faveolata* from the other two taxa (Lopez et al. 1999), and sequence analysis confirmed that these were alternative alleles that differed by several small deletions.

Although these differences suggest that there is little genetic interchange, at least between *M. faveolata* and the other taxa, the potential for some hybridization among all members of the complex is supported by experimental fertilizations in separate studies in Panama (Knowlton et al. 1997; Levitan et al. 2004), the Bahamas (Levitan et al. 2004), Florida

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(Szmant et al. 1997), and the Gulf of Mexico (Hagman et al. 1998a, b). In Florida and Panama, complete fertility between *M. annularis* and *M. franksi* in laboratory crossings was observed, whereas in the Gulf of Mexico and the Bahamas there was some evidence for partial incompatibility. Low rates of interspecific fertilization (comparable to selfing, except in crosses with very old eggs) were obtained for pairings of *M. faveolata* with the other taxa in Panama, the Bahamas, and the Gulf of Mexico, although in Florida fertilization rates were more variable and generally higher in comparable pairings. At all four sites, *M. franksi* typically spawns 1.5 h earlier than *M. faveolata* and *M. annularis*, which spawn in approximate synchrony.

Our previous AFLP studies were confined to Panama, where colony morphologies are quite distinctive. In the Bahamas and Florida, however, colony morphologies can be more difficult to classify (Szmant et al. 1997; Manica and Carter 2000; see Materials and Methods—Colony Identification), suggesting that there may be genetic differences between Panama and these more northern sites. To address this possibility, we used the previously characterized GGAG 920/880 locus (Lopez et al. 1999) and additional AFLP loci, as well as sequence data from the mitochondrial noncoding region and the nuclear internal transcribed spacers (ITS1, ITS2) of nuclear ribosomal genes, to characterize colonies from Panama and the Bahamas. We also confirmed the hybrid status of larvae produced in interspecific laboratory pairings involving *M. faveolata*. In a companion paper (Levitan et al. 2004), we describe the fertilization biology of these taxa at the same sites.

Materials and Methods

Colony Identification

In Panama, colony identification was based on the criteria outlined in Weil and Knowlton (1994). In brief, columnar colonies with senescent basal borders and smooth surfaces were identified as *M. annularis*; massive/platy/sub-columnar colonies with nonsenescent, irregular growing edges and irregularly bumpy surfaces were identified as *M. franksi*; and massive/platy colonies with generally smooth surfaces, sometimes with ridges or regular cone-shaped protrusions, and smooth, nonsenescent growing edges were identified as *M. faveolata*. There were few ambiguous colonies (lumpy columns) observed, all of which spawned early and clustered micromorphologically with *M. franksi* and were assigned to that species on that basis.

In the Bahamas, we used the same criteria to assign colonies to species in the field, but these criteria were more difficult to apply in shallow to intermediate depths (3–15 m). Many colonies conformed to the classical phenotypes, but there were more ambiguous colonies than in Panama. Some were largely columnar (like *M. annularis*) but with platy extensions (like *M. faveolata*), others were columnar (like *M. annularis*) but with lumpy corallites (like *M. franksi*), and some had a conspicuous yellow-tan coloration with extremely large, irregular and protruding corallites (especially along growing edges) that did not resemble anything observed in Panama. We assigned members of the first two groups to *M. annularis* based on reproductive compatibility and timing studies, and the last group to *M. franksi* based on its early spawning time and highly aggressive behavior (five of 70 Bahamian colonies in total reassigned based on reproductive biology and aggression). In deep water (25–30 m), most colonies had typical *M. franksi* morphologies, and the occasional *M. faveolata* at these depths had typical colony morphologies.

Sampling and DNA Extraction

For genetic analyses, sperm DNA samples previously collected in Panama from the San Blas (1994–1997) and Bocas del Toro (1998–1999) were used (see Lopez et al. 1999). We also collected additional sperm (for methods, see Lopez et al. 1999) and tissue (9 cm^2) samples from reefs near Bocas del Toro, Panama, and Lee Stocking Island, Bahamas, in 2000 and 2001. Planula larvae were obtained from intra- and interspecific crosses performed in Bocas del Toro in 2000 (for details, see Levitan et al. 2004).

Sperm samples and individual larvae were digested in approximately twice their volume of CHAOS solution (4M guanidine thiocyanate, 0.1% N-lauroyl sarcosin sodium, 10 mM Tris pH8, 0.1M 2-mercaptoethanol [modified from Sargent et al. 1986]) for several days at room temperature (DNA can be stably stored in this way for at least three months). Samples of tissue with skeleton were broken into pieces less than 4 cm^2 prior to being so treated. An equal volume of phenol extraction buffer (PEB) (100 mM TrisCl pH8, 10 mM EDTA, 0.1% SDS) was added to the CHAOS solution just before DNA extraction. Total DNA was extracted from the CHAOS solution with PEB by phenol/chloroform extraction and ethanol precipitation, and resuspended in TE (10 mM Tris, pH 7.5, 1 mM EDTA) with RNaseA (l0 mg/ml).

Nuclear Markers

Amplified fragment length polymorphisms (AFLP)

The 920/880 locus previously identified using the GGAG AFLP primer (Lopez et al. 1999) was amplified by polymerase chain reaction (PCR) with Taq polymerase and the following primers specific for this locus: 5′CCC TGA TCA GTA TTT TGGG 3′ (880f), 5′GGA GGG CTC TGT TAT TCT ATC 3′ (880r). The PCR profile was 30 sec at 94°C, 45 sec at 65°C, and 60 sec at 72°C for 30 cycles. Band patterns of the PCR products were observed using 1.2% agarose/TBE gels. The PCR products were then separated from the 0.7% agarose/TAE gels and cloned in pGEM vectors (Promega Corp., Madison, WI). Five to ten clones for each colony were sequenced for both strands with the PCR primers using an automated ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA).

To identify additional nuclear markers, AFLP reactions were performed for five to ten colonies from each of three taxa using a variety of AFLP primers (for methods, see Lopez et al. 1999). We were able to identify two additional markers of interest, a second locus (or loci, see Results) using the GGAG primer, and a locus using the AGAA primer. Using the AGAA primer, one informative band was found that was isolated from the agarose gel, cloned, and sequenced as described above. The following specific primers were then designed based on these DNA sequences: 5′GCC TTG GTT

GAT TCT GAA ATT CG 3′ (500f) and 5′AGA TCG AAT TAT ACA CCC ACC CA 3′(500r). PCR using these primers was performed and bands were observed as described above. Another fragment was obtained using the GGAG primer when the samples were treated by a ''preamplification'' PCR (PCR performed with an extension primer possessing one additional base [G] in order to improve the targeted band signal and reduce background [Lopez et al. 1999]). This band was also isolated from the agarose gel, cloned and sequenced. The following specific primers were then designed: 5′GAT TCA TCC CGC TGT CAA AGA 3′ (410f), 5′GCC AAC CAC AGA CAG AGC GGA 3′ (410r). PCR was performed using the same conditions as that for the 920/880 locus, and the band patterns were observed in agarose/TBE gels. The bands were then separated from the agarose and cloned in pGEM vectors (Promega) for at least 10 colonies of each taxon, and five to 10 clones for each colony were sequenced for both strands using the PCR primers 410f,r.

Internal transcribed spacers

Ribosomal DNA ITS (internal transcribed spacers, ITS1, ITS2) fragments were obtained and sequenced using previously reported methods (Lopez and Knowlton 1997). The bands for ITS were separated from the agarose, cloned in pGEM vectors (Promega), and 5 to 10 clones for each colony were sequenced for both strands with ITS PCR primers (Lopez and Knowlton 1997).

Mitochondrial Markers—Noncoding Region and COI

We identified a noncoding region (891 bp) between the cytochrome oxidase subunit 1 (COI) and lrRNA genes. The region was amplified by the following specific primers: 5′GAG CTG GGC TTC TTT AGA GTG 3′ (MNC1f) and 5′GTG AGA CTC GAA CTC ACT TTTC 3′ (MNC1r). A large part of the mitochondrial COI gene (1239 bp) was also amplified using the following specific primers: 5′TCT CTA CAA ATC ATA AAG 3′ (MCOI1kf) and 5′CAC TCT AAA GAA GCC CAG CTC 3′ (MCOI 1kr). The PCR profile used for these genes was 94°C/45 sec, 65°C /45 sec, and 72°C / 90 sec for 30 cycles. Small aliquots (10–15 μl) of PCR products were treated with 10 U of Exonuclease I and 1–2 U of shrimp alkaline phosphatase, and incubated at 37°C for 2 h. The enzyme was inactivated by heating to 80°C for 20 min. The enzyme-treated PCR product was used directly as template in a cycle sequencing reaction using the dRhodamine terminator cycle sequencing kit (Perkin Elmer, Wellesley, MA). The internal primers MCOIseqf (5′TGT TAG CGG GTG CAA TTA CT 3′) and MCOIseqr (5′GAG AAA TTA TAC CAA AAC CAG 3′) for COI were used in addition to PCR primers. The DNA sequences were determined using an automated ABI 377 DNA sequencer.

Molecular Phylogenetic Analyses

DNA sequences from the nuclear ITS and the mitochondrial noncoding region were manually aligned. ITS sequences were analyzed with PAUP version 4.0b10 (Swofford 2002). Phylogenetic trees were constructed by neighbor-joining (NJ) (Saitou and Nei 1987) with Kimura's two parameter method, and by maximum-likelihood (ML) with HKYmodel (Hasegawa et al. 1985) with gamma parameter and proportion of invariable positions and a heuristic approach with tree bisection and reconnection branch swapping. The model used in ML analysis was evaluated by the program Modeltest 3.06 (Posada and Crandall 1998). The topology of the trees constructed by both methods was similar. Bootstrap analyses (500 replicates) were also performed for both methods. To estimate genetic subdivisions between taxa and between regions for AFLP data and for DNA sequence data (ITS and the mitochondrial noncoding region), we calculated F_{ST} (Wright 1951, 1965) using software ARLEQUIN vers. 2.0 (Schneider et al. 2000).

Morphological Analyses

To analyze differences in corallite morphology among species, we digitized 3D Cartesian coordinates (x-y-z) of 25 landmarks on calical surfaces using a Reflex microscope (Fig. 1). The landmarks consisted of spatially homologous points designed to reflect the shape of the septal margin (the uppermost growing edge) and costal extensions between corallites. Three adjacent costosepta were digitized on six mature calices on the top and side of each colony.

Size and shape coordinates (Bookstein 1991) were calculated from the data using the computer program GRF-ND (Generalized rotational fitting of n-dimensional landmark data, 1994, written by Dennis E. Slice, available at http://life.bio.sunysb.edu/morph/). Centroid size was calculated by summing the squared distances from each of the 25 landmarks to a common centroid. Shape coordinates were calculated using 23 triangles formed by triplets of the 25 points, in which the same pair of landmarks (#25, #10) served as a fixed x-y baseline positioned at coordinates (0, 0) and (1, 0) within a plane oriented relative to the highest point (#12) in the z-dimension (i.e., the z-coordinate of #12 = 0). The 23 triangles were translated, rotated, and rescaled relative to the baseline. Calculation of shape coordinates in three dimensions involves translation of the triangle so that one vertex lies on the origin, rigid rotation and scaling about the y and z axes so that a second point is coincident with (1, 0, 0), and rotation about the x axis to place the third point in the first quadrant of the x-y plane (for details see Budd et al. 1994a; Budd and Johnson 1996). The x-y-z coordinates of the 23 unfixed third points (three coordinates × 23 points = 69; 69 minus the z-dimension for #12 = 68 total), termed ''shape coordinates,'' served as variables in subsequent statistical analyses.

To test for differences among species, canonical discriminant analysis was performed using centroid size and 25 shape coordinates as variables. The 25 shape coordinates used (of the 68 that were calculated) consisted of all uncorrelated measures of the heights, lengths, or widths of individual septa or costae (Fig. 1). Location and the initial field identifications based on colony morphology defined the a priori groups except as noted above.

RESULTS

AFLP Markers

Panama field collections

At the GGAG-1 locus, *M. faveolata* was fixed for the 880 allele, while the 920 allele was nearly fixed in *M. franksi* and

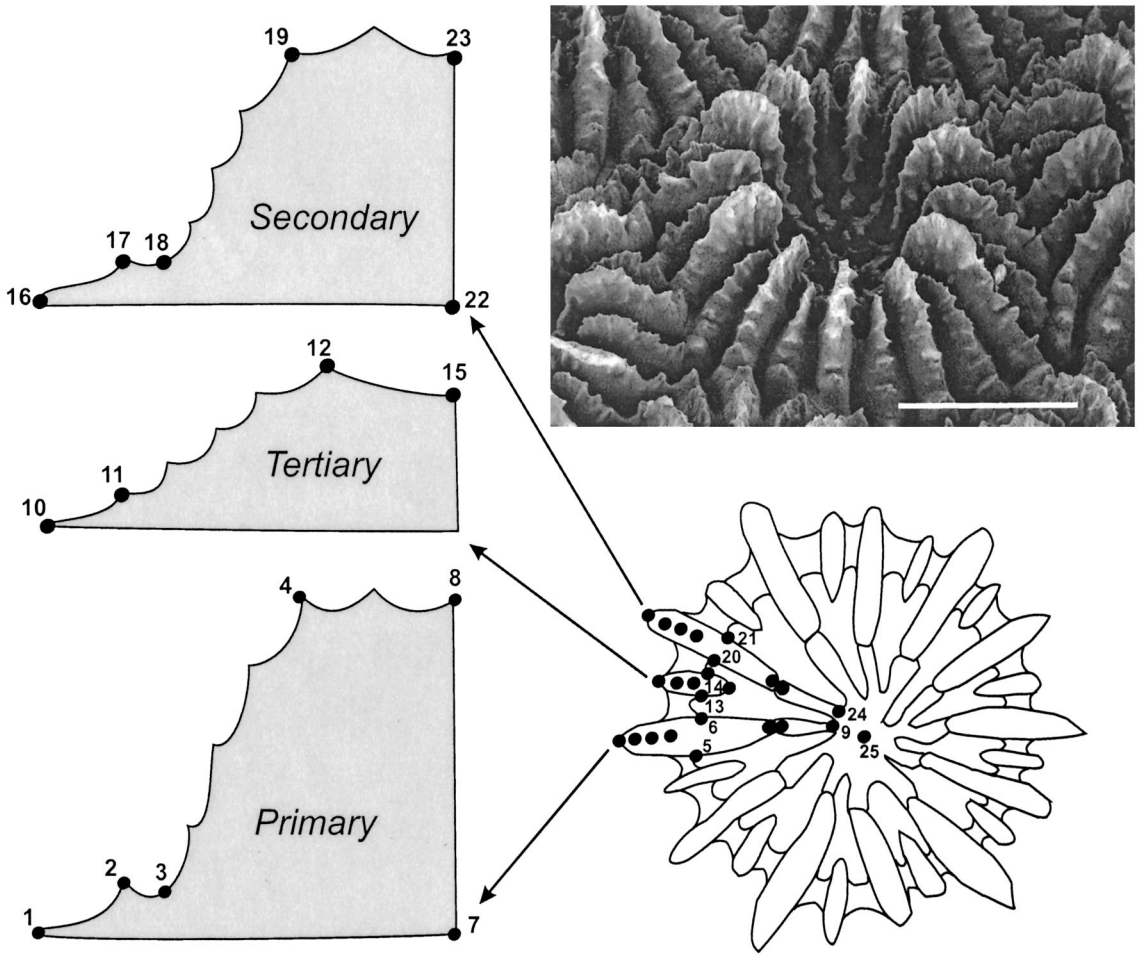

FIG. 1. Scanning electron micrograph of corallite and schematic diagrams (left, vertical profiles of costosepta; right, calical surface) showing landmark positions. On the primary (1st), secondary (2nd), and tertiary (3rd) costosepta, the landmarks (indicated by black dots on entire corallite diagram and with numbers on costoseptal diagram) are as follows, working from outside to center of corallite: outermost point: 1(1st), 16 (2nd), 10 (3rd); tip of tooth next to outermost point: 2 (1st), 17 (2nd), 11 (3rd); depression before next tooth: 3 (1st), 18 (2nd); outermost high point: 4 (1st), 19 (2nd), 12 (3rd), etc. Landmarks 5 and 6 (1st), 20 and 21 (2nd), and 13 and 14 (3rd) mark the junction of the costosepta with the wall; landmarks 8 (1st), 23 (2nd), and 15 (3rd) mark the innermost high point on the costoseptum; points 7 (1st) and 22 (2nd) mark the point of maximum curvature along the primary septal margin. Landmarks 9 (1st) and 24 (2nd) mark the junction of the septum with the columella, and landmark 25 is at the center of the columella. Scale bar is 1.5 mm.

TABLE 1. Frequency pattern of DNA band patterns at three AFLP loci. A, B, C, and * in parentheses refer to symbols for band patterns as used elsewhere in figures and tables. *K*, *A*, and *V* refer to *M. franksi*, *M. annularis*, and *M. faveolata*, respectively.

	M. franksi (*K*)		*M. annularis* (*A*)		*M. faveolata* (*V*)	
Loci/bp	Pan	Bah	Pan	Bah	Pan	Bah
GGAG-1						
920 (A)	51	18	42	37	0	1
880 (B)	1	0	4	0	51	13
920/880 (A/B)	0	0	8	1	0	0
GGAG-2						
450 (A)	51	18	50	37	2	2
410 (B)	1	0	0	0	25	4
430 (C)	0	0	3	0	0	0
450/410 (A/B)	0	0	0	0	24	8
AGAA						
500 (A)	9	15	50	30	19	9
null (*)	43	3	4	8	32	5

dominant in *M. annularis* (Table 1); these frequency differences were statistically significant (Table 2). Only *M. annularis* had individuals heterozygous at this locus; these colonies yielded both 880 and 920 type sequences (data not shown).

At the GGAG-2 locus (or loci, see breeding experiments below), bands representing 450, 430, and 410 bp were observed. Both *M. annularis* and *M. franksi* were nearly fixed for the 450 band; in contrast, most colonies of *M. faveolata* either had only the 410 band, or exhibited both the 450 and 410 bp bands (this difference between *M. faveolata* and the other two taxa was also statistically different, Table 2). Double-banded individuals had sequences associated with both the 450 and 410 alleles (data not shown), which differed from the 450 allele by a deletion of 39 bp. Three *M. annularis* individuals had a 430 bp band, which differed by a different 17 bp indel and three single bp substitutions.

At the AGAA locus, most *M. annularis* had a 500 bp band,

TABLE 2. Comparison of population pairwise F_{ST} statistics of each analysis. Kimura 2-parameter distance method was used for calculations for the mitochondrial noncoding region and internal transcribed spacers. The probability of obtaining these F_{ST} values was estimated from 1023 permutations.

	GGAG-1	GGAG-2	AGAA	Mt noncoding	ITS
Overall	0.8595**	0.6233**	0.4008**	0.2805**	0.1214**
Between regions					
*K*_Pan/*K*_Bah	−0.0002	−0.0252	0.5924**	0.2005*	0.1731**
*A*_Pan/*A*_Bah	0.0823**	0.0262	0.0577	−0.0027	0.2367**
*V*_Pan/*V*_Bah	0.0000	0.0102	0.0963	0.2297*	−0.0272
Within regions					
K/*A* in Panama	0.0943**	0.0143	0.7242**	0.2746**	0.0298
K/*V* in Panama	0.9806**	0.6942**	0.0778**	0.4090**	0.0836*
A/*V* in Panama	0.8468**	0.6623**	0.4976**	0.5739**	0.1301**
K/*A* in Bahamas	−0.0046	0.0000	−0.0363	0.0347	0.1109
K/*V* in Bahamas	1.0000**	0.6753**	0.0308	0.0562	−0.1212
A/*V* in Bahamas	0.9774**	0.7753**	0.0075	0.0373	−0.0412

* $P < 0.05$, ** $P < 0.01$.

whereas for *M. faveolata* and *M. franksi*, well over half of the individuals had a null band pattern (Table 1); frequency differences among the species were significant (Table 2). Several primers were designed at sites through the 500 bp DNA sequence, but no band was amplified; similarly, no band was amplified using low annealing temperatures. This suggests that the absence of a band represents a gap in this region. Therefore, individuals with the 500 bp band could be either homozygous or heterozygous.

Three-locus AFLP genotypes for Panama are summarized in Figure 2A. Genotypes of *M. faveolata* were highly distinct, showing almost no overlap with the *M. annularis* and *M. franksi* genotypes. Moreover, no individuals exhibited the most likely F_1 genotype for hybrids involving *M. faveolata* (920/880 and 450/410 double bands for the two GGAG loci). *Montastraea annularis* and *M. franksi* showed greater overlap, but frequencies of the two most common three-locus genotypes were again quite distinctive.

Bahamas field collections

Many aspects of the patterns in the Bahamas resemble those found in Panama (Table 1, Fig. 2). As in Panama, *M. faveolata* in the Bahamas was strongly differentiated from the other two taxa at the two GGAG loci, and no individuals had the most probable genotype for F_1 hybrids involving *M. faveolata*. For *M. franksi* and *M. annularis*, however, there were statistically significant differences in allele frequency distribution between Panama and the Bahamas (Table 2); in contrast to Panama, only a minority of *M. franksi* individuals from the Bahamas had the null genotype at the AGAA (500/null) locus, and only one *M. annularis* individual had the 880

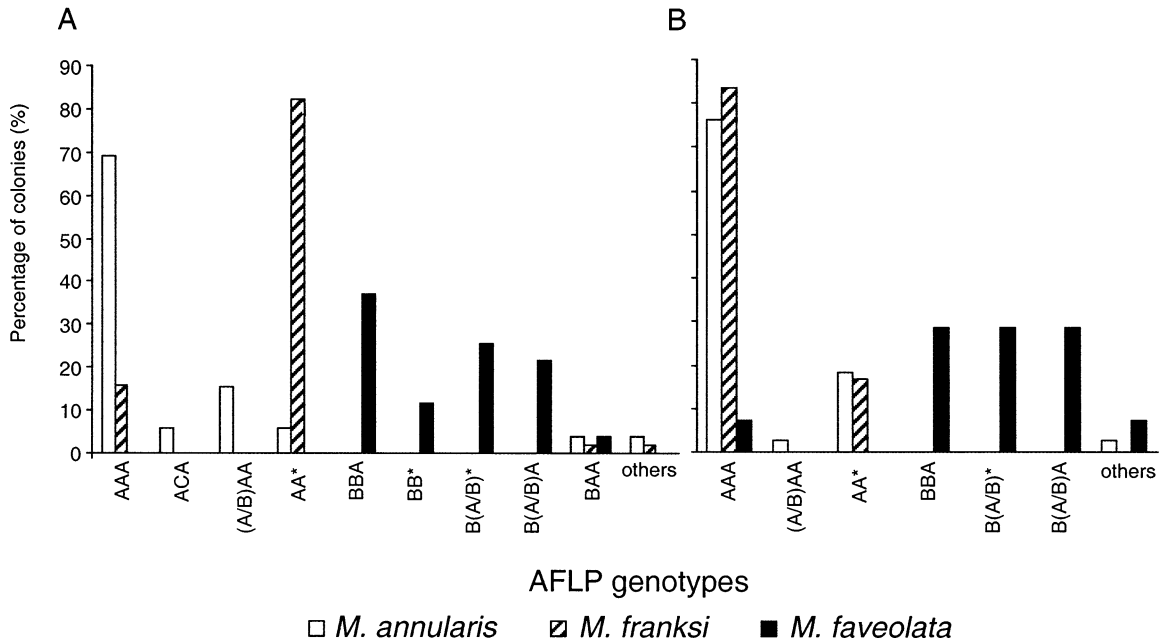

FIG. 2. Frequency distribution of three-locus AFLP genotypes in Panama (A) and the Bahamas (B). Order of the alleles is GGAG-1, GGAG-2, AGAA.

TABLE 3. Results of experimental interspecific crosses. Colony identification, AFLP genotypes, fertilization rates, larval genotypes and numbers of each larval genotype are shown. Crosses were performed in 2002 in Bocas del Toro (Levitan et al. 2004). See Table 1 for explanation of AFLP genotypes, which are listed from left to right GGAG-1, GGAG-2, and AGAA.

Parent no. 1		Parent no. 2		Fertilization rate	Hybrid larvae	
ID	AFLP	ID	AFLP		Genotype	Numbers
M. franksi		*M. faveolata*				
Pa00-39	A A *	Pa00-36	B (A/B) A	97%	(A/B) (A/B) A	10
					(A/B) (A/B) *	2
Pa00-1	A A *	Pa00-31	B (A/B) A	2%	(A/B) (A/B) A	12
Pa00-3	A A A	Pa00-31	B (A/B) A	14%	(A/B) (A/B) A	12
M. annularis		*M. faveolata*				
Pa00-14	A C A	Pa00-11	B (A/B) *	53%	(A/B) (A/C/B) A	8
					(A/B) (A/C/B) *	4
Pa00-37	A A A	Pa00-9	B (A/B) A	1%	(A/B) (A/B) A	12

allele at the GAGG-1 (920/880) locus. As a consequence, in the Bahamas *M. franksi* and *M. annularis* had very similar genotypes (Tables 1, 2; Fig. 2B).

The comparison of the DNA sequences between *M. annularis* and *M. franksi* at the GGAG-2 (450/430/410) locus revealed that some Bahamian corals (three of 12 corals sequenced for *M. annularis*, three of 13 corals sequenced for *M. franksi*) had one highly divergent 450 allele (with three or four times more nucleotide substitutions) in addition to the two typical alleles; these highly divergent alleles were not observed in Panama (data not shown). The presence of three sequences in single individuals suggests that multiple copies occur in tandem within this locus, or that more than one locus is present (see also following section).

Larvae

Hybrid larvae were obtained from interspecific crosses between *M. franksi* and *M. faveolata*, and between *M. annularis* and *M. faveolata* (Table 3), which occasionally (Levitan et al. 2004) yielded moderate to high numbers of viable larvae (particularly with aged eggs). DNA analyses of the three AFLP loci for each parent and larvae from each cross are consistent with Mendelian inheritance at the GGAG-1 (920/880) and the AGAA (500/null) loci. For the former, double bands were always apparent in the hybrid larvae (examples in Fig. 3A), whereas all larvae from intraspecific crosses had single bands matching those of their parents (Fig. 3B). DNA sequences for two crosses (both parents and one larva) revealed that the hybrids contained two alleles, one from each

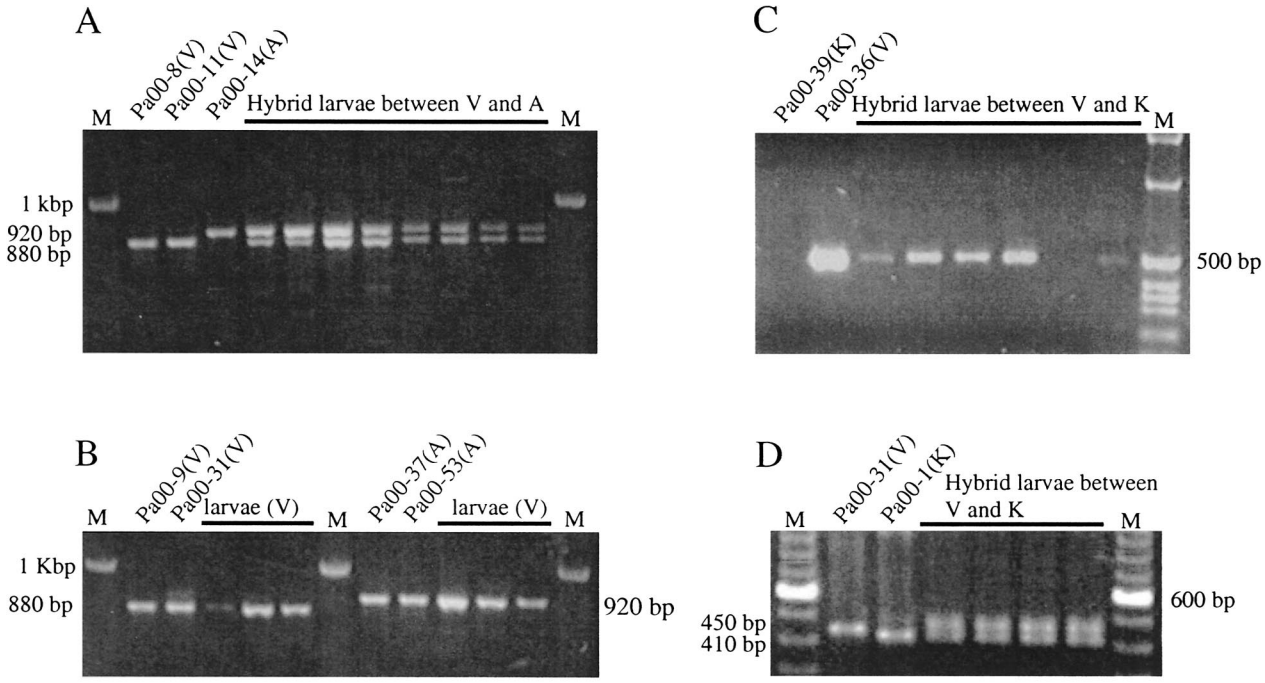

FIG. 3. Illustrative gels showing AFLP band patterns of parents and their offspring. (A) GGAG-1 (920/880) locus for an interspecific cross, (B) GGAG-1 (920/880) locus for two intraspecific crosses, (C) AGAA (500/null) locus for two interspecific crosses, (D) GGAG-2 (450/430/410) locus for an interspecific cross. Crosses are described further in Table 3.

parent (data not shown). For the AGAA locus, the band pattern of the hybrid larvae generally had the 500 bp band (example in Fig. 3C). Two crosses that yielded some larvae with the null pattern (Table 3) suggest that these parents with the 500 bp band were heterozygous at this locus.

For the GGAG-2 (450/430/410) locus, however, the band patterns of the hybrid larvae were inconsistent with Mendelian inheritance. When a colony with a single 450 bp was mated to a 450/410 double banded colony, all hybrid larvae had the 450/410 double bands (Fig. 3D, Table 3) even though just the 450 bp band would expected in some of the larvae. Also, all hybrid larvae from a colony with a single 430 bp band mated with a 450/410 double-banded colony had all three alleles (450/430/410). As mentioned above, this locus appears to consist of multiple-copy sequences in tandem or different loci.

Nuclear ITS Sequence Analyses

Internal transcribed spacers DNA sequences were obtained from nine to 12 colonies from each of three taxa in Panama and two to six colonies of the three taxa in the Bahamas. There were a total of 34 variable positions (but no indels) within the 440 bp sequenced. No individual had more than two alleles. A phylogenetic tree using these sequences, together with 14 sequences representing the three taxa in the Florida Keys (seven colonies from *M. annularis*, three for *M. franksi*, and four for *M. faveolata* [obtained from GenBank; U59903-U59906, AF013727-AF013734; Medina et al. 1999]), showed no evidence of reciprocal monophyly (Fig. 4), nor evidence of sorting by geographic region. Bootstrap values were less than 30% for all branches. Nevertheless, statistical analyses revealed significant differences between *M. faveolata* and the other two taxa in Panama, although there were no significances among the three taxa in the Bahamas (Table 2) or in Florida (data not shown).

Mitochondrial Sequence Analyses

Noncoding region

Among the 891 bp of sequence for 68 individuals, there were 21 substitutions and one deletion observed; only eight of the substitutions occurred more than once (Table 4). The most common sequence (24 individuals, type I) was frequent in *M. annularis* and *M. franksi* in both Panama and the Bahamas. It also occurred in two individuals of *M. faveolata* from the Bahamas. There were seven other sequences in *M. franksi* and *M. annularis* that differed from the most common sequence by single bp substitutions (type II, III, IV, V, VI, VIII, XI), but only one of these (type II) occurred more than once (primarily in *M. annularis* in both Panama and the Bahamas). Most individuals of *M. faveolata* had one of four sequences (XIII, XIV, XV, XVI) that also differed from the most common sequence by a single bp substitution or deletion. Two of these were found at both sites, and one (XIII) was common; none of these sequences were found in either *M. annularis* or *M. franksi*.

Many individuals of *M. franksi* (and one *M. annularis*) had sequences that were more divergent. In Panama, nearly half of *M. franksi* had one of two related sequences that differed by three or four bp (types X and X′). Another three individuals from Panama and one from the Bahamas had a sequence that differed by two bp (types IX, XII).

Statistical analyses showed that the three Panamanian taxa were significantly different from one another, but that there were no significant differences among the three taxa in the Bahamas (Table 2), although the numbers of analyzed colonies of *M. franksi* and *M. faveolata* in the Bahamas were relatively small. This regional difference is also reflected in the fact that for both *M. franksi* and *M. faveolata*, there were significant differences between Panama and the Bahamas (Table 2).

COI

COI sequences (1239 bp) from eight colonies of *M. annularis*, six of *M. franksi*, and seven of *M. faveolata* (only Panamanian corals were analyzed) were identical with but two exceptions. The different sequences were from *M. franksi* and *M. faveolata*, and each differed by just a single bp substitution (data not shown).

Morphological Analyses

In Panama, the three taxa were morphologically distinct (Fig. 5). The most divergent was *M. faveolata*, which was always well separated from *M. franksi* and *M. annularis*. *Montastraea franksi* and *M. annularis* were also distinct, although the difference was reduced in the lagoonal habitats of Bocas del Toro.

In the Bahamas, in contrast, no clear separation among the three taxa was seen (Fig. 5). *Montastraea faveolata* from the Bahamas and Panama were similar, but both *M. franksi* and *M. annularis* converged on the micromorphology of *M. faveolata* in the Bahamas.

Discussion

Species Boundaries in Panama

Montastraea faveolata versus the other two taxa

Many independent characters separate *M. faveolata* from *M. annularis* and *M. franksi* in Panama. In this study, we documented significant frequency differences for the three AFLP loci (Fig. 2; Tables 1, 2), nonoverlapping genotypes in the noncoding region of mitochondrial DNA (Table 4), statistically significant differences in ITS allele frequencies (Table 2), and consistent three-dimensional morphological differences at the level of the corallite (Fig. 5). These data are consistent with crossing experiments in Panama between *M. faveolata* and the other two taxa, which showed clear evidence for strong gametic barriers to fertilization (Knowlton et al. 1997; Levitan et al. 2004), as well as with earlier genetic analyses using protein electrophoresis (Knowlton et al. 1992).

Although these data broadly argue against the presence of substantial gene flow, the GGAG-1 (920/880) locus merits additional discussion. No individuals identified in the field as *M. faveolata* or *M. franksi* exhibited the double bands expected of an F1 hybrid between *M. faveolata* and either of the other two taxa, but eight of 54 *M. annularis* exhibited

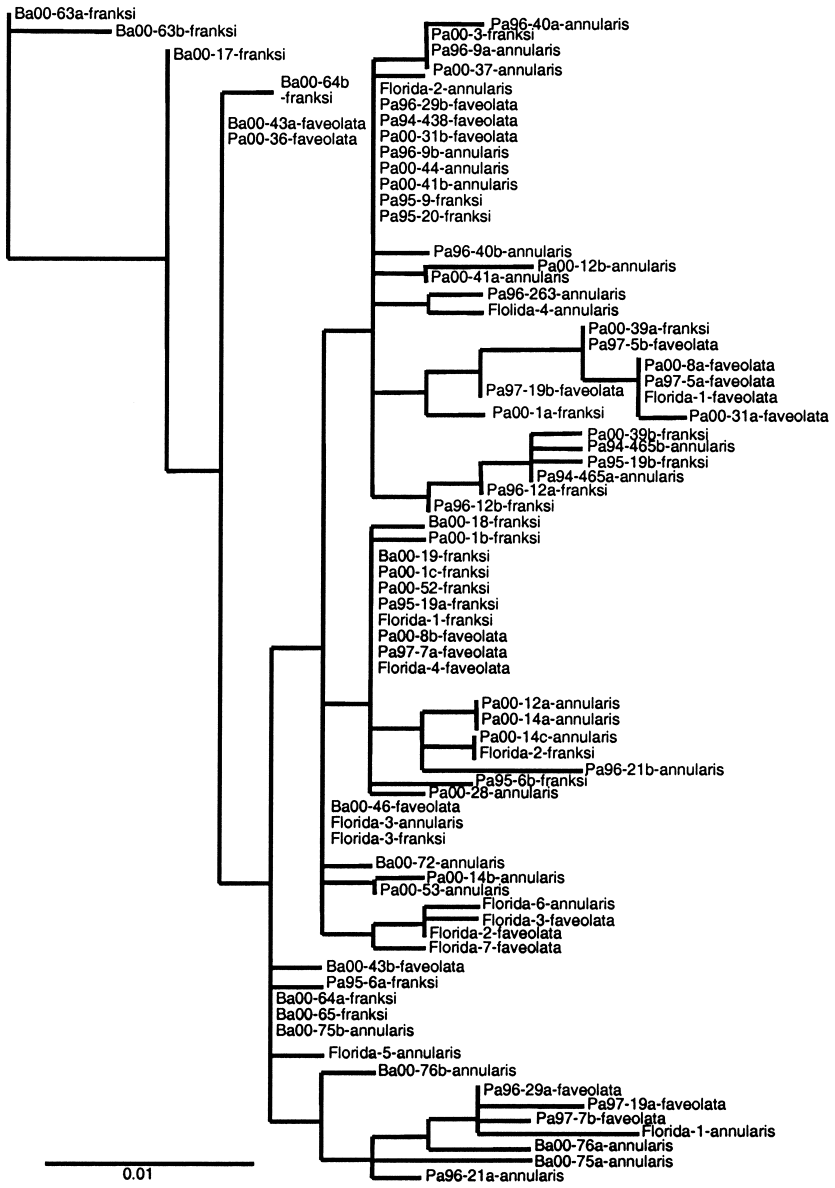

FIG. 4. Internal transcribed spacer tree constructed by maximum likelihood for colonies sequenced in this study and sequences for the three taxa from Florida in GenBank. See methods for details of tree construction. Accession numbers AB65299-AB065364. In sample codes, Ba, Bahamas; Pa, Panama.

the 920/880 genotype and four other individuals were homozygous for the 880 allele typical of *M. faveolata*. Moreover, limited DNA sequences of 880 alleles revealed no differences between those found in *M. faveolata* and those found in *M. annularis* (data not shown). This interspecific allelic identity could be interpreted as reflecting recent hybridization between *M. faveolata* and *M. annularis*. However, little variation was observed among sequences from either the 880 bp or 920 bp bands, so that interspecific identity of the 880 allele is not unexpected. Moreover, the strong frequency differences between these two taxa at the GGAG-2 (450/430/410) locus (Table 1) and other genetic loci (Table 2) suggest that gene introgression does not occur frequently. This, together with data on reproductive barriers (Levitan et al. 2004), makes ancestral polymorphism a more likely explanation for the appearance of the GGAG-1 880 allele in over 20% of the *M. annularis* individuals from Panama.

Montastraea annularis versus M. franksi

Earlier studies based on protein electrophoresis suggested that *M. annularis* and *M. franksi* are more closely related to each other than they are to *M. faveolata* (Knowlton et al. 1992; Weil and Knowlton 1994), and this conclusion was supported by our results. *Montastraea annularis* and *M. franksi* were more similar morphologically (Fig. 5, especially in the lagoonal habitats of Bocas del Toro) and exhibited no significant frequency differences for the ITS and GGAG-2 alleles (Table 2). Moreover, reproductive studies suggest that of all the possible scenarios for interspecific hybridization

TABLE 4. DNA nucleotide substitutions in the mitochondrial noncoding region (891 bp) and three-locus AFLP genotypes, listed left to right as GGAG-1, GGAG-2 and AGAA.

		Bp position 11122233334445688 89922406812682595408 27395645545449304 7762	Type	AFLP markers		
M. annularis						
Panama	M01-2	AACGATACTCACTGACGGGTT	I	A	A	*
	M01-3	.....	I	A	A	A
	M01-5	.....	I	A/B	A	A
	M01-7	.....	I	A	A	A
	M01-9	.....	I	A	A	A
	M01-10	.....	I	A	A	A
	Pa00-53	.....	I	A	A	A
	Pa01-23	.....	I	A	A	A
	M01-4	.....G.....	II	A/B	A	A
	M01-6	.....G.....	II	A	A	A
	M01-8	.....G.....	II	B	A	*
	M01-11	.....G.....	II	B	A	A
Bahamas	Ba00-3	.....	I	A	A	A
	Ba00-42	.....	I	A	A	A
	Ba00-45	.....	I	A	A	A
	Ba00-50	.....	I	A	A	A
	Ba00-72	.....	I	A/B	A	A
	Ba00-85	.....	I	A	A	A
	Ba00-35	.....	I	A	A	A
	Ba00-73	.....G.....	II	A	A	A
	Ba00-75	.....G.....	II	A	A	*
	Ba00-13	.....T.....	III	A	A	*
	Ba00-14	.....C.....	IV	A	A	A
	Ba00-40	.....T.....	V	A	*	*
	Ba00-79	.....T.....	VI	A	A	A
	Ba00-77	G..A.....C..	VII	A	A	*
M. franksi						
Panama	F01-1	.....	I	A	A	*
	M01-1	.....	I	A	A	*
	M01-5	.....	I	A	A	*
	M01-7	.....	I	A	A	*
	Pa00-3	.....	I	A	A	A
	F01-3	.....G.....	II	A	A	*
	M01-4	.....T.....	VIII	A	A	*
	F01-5	.....G.....A....	IX	A	A	*
	F01-7	.....G.....A....	IX	A	A	*
	Pa01-32	.....G.....A....	IX	A	A	*
	F01-4	.....AC....C.	X	A	A	*
	F01-6	.....AC....C.	X	A	A	A
	F01-8	.....AC....C.	X	A	A	A
	F01-9	.....AC....C.	X	A	A	*
	M01-3	.....AC....C.	X	A	A	*
	M01-6	.....AC....C.	X	A	A	A
	Pa01-5	.....AC....C.	X	A	A	*
	Pa01-33	.....AC....C.	X	A	A	*
	M01-2	.....AC....CC	X′	A	A	*
Bahamas	Ba00-68	.....	I	A	A	A
	Ba00-95	.....	I	A	A	*
	Ba00-70	.....C.....	XI	A	A	A
	Ba00-65	..T.....A...	XII	A	A	A
M. faveolata						
Panama	M01-1	.....G.....	XIII	B	A/B	*
	M01-3	.....G.....	XIII	B	A/B	*
	M01-4	.....G.....	XIII	B	A/B	*
	M01-5	.....G.....	XIII	B	A/B	*
	M01-6	.....G.....	XIII	B	A/B	*
	M01-7	.....G.....	XIII	B	A/B	*
	M01-9	.....G.....	XIII	B	A/B	*
	M01-10	.....G.....	XIII	B	A/B	*
	Pa01-28	.....G.....	XIII	B	A/B	A
	Pa00-9	....C.....	XIV	B	B	A
	M01-12	....C.....	XIV	B	A/B	A

TABLE 4. Continued.

		Bp position 11122233334445688 89922406812682595408 27395645545449304776 2	Type	AFLP markers
Bahamas	FF3R	.....	I	B B A
	Ba00-30	.....	I	A A A
	Ba00-1	.....G.....	XIII	B A/B *
	FKA4L	.....G.....	XIII	B B A
	Ba00-2	....C.....	XIV	B A/B A
	Ba00-4	....C.....	XIV	B A/B *
	FF3L	.T.....	XV	B B A
	T7[a]	.....----------	XVI	B A/B A

[a] T7 has a gap region from 266 to 848 bp.

among the three taxa, the most likely is the fertilization of *M. franksi* eggs by *M. annularis* sperm under conditions of low gamete movement (Levitan et al. 2004).

However, there were statistically significant differences between *M. annularis* and *M. franksi* at two AFLP loci (Tables 1, 2; Fig. 2A) and at the mitochondrial noncoding region (Tables 2, 4). The AGAA (500/null) AFLP locus exhibited especially strong differences in the frequency of the most common alleles (in Panama 43 of 52 colonies of *M. franksi* lacked this band, whereas 50 of 54 colonies of *M. annularis* had the 500 bp band). Although interpretation is made more difficult by the fact that individuals with the 500 bp band could be either homozygotes or heterozygotes, if we assume H-W equilibrium and calculate F_{ST} on the basis of estimated allele frequencies, the pattern of significant differences between the two species does not change. At the mitochondrial noncoding region, all *M. annularis* had one of two haplotypes (I and II), whereas most *M. franksi* had four other haplotypes (Table 4). Thus the sharing between *M. franksi* and *M. annularis* of types I and II in the mitochondrial noncoding region seems most likely to be due to shared ancestral polymorphism.

Species Boundaries in the Bahamas

Bahamian members of the *M. annularis* complex exhibit intermediate colony morphologies not seen in many Carib-

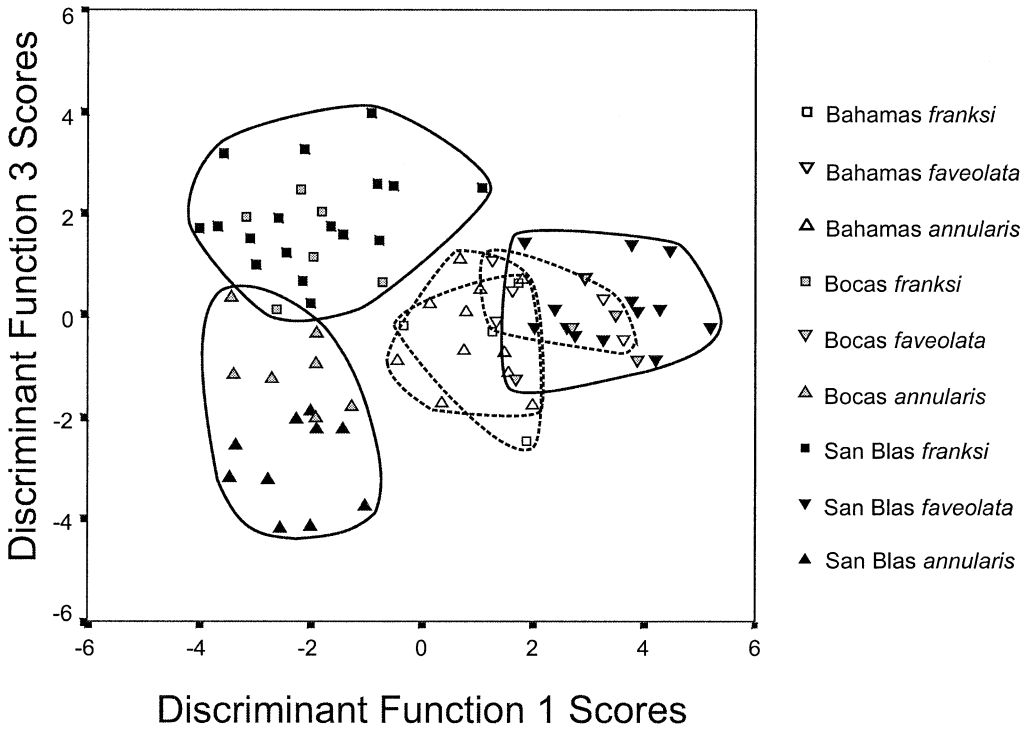

FIG. 5. Canonical discriminant analysis of three dimensional landmark data on samples of the three species from Panama and from the Bahamas. Each point on the plot represents one colony. Solid polygons enclose colonies of the same species from Panama; dotted polygons enclose colonies of the same species from the Bahamas. The first three discriminant functions in the analysis were statistically significant; function one accounts for 49.0% of the variance, function two for 23.0%, and function three for 17.9%. Relative development of major versus minor costae (X16, X1) and septum height (Y23, Y8) are strongly correlated with function one; costa length (X12, X4, X19), minor septum length (X15), and wall thickness (X6, X20, X14) are inversely correlated with function three.

bean localities (see Methods and below), and they also exhibit far less clear-cut hierarchies in aggressive interactions (pers. obs.). These differences are consistent with our observations of overlap in micromorphological characters and genotypes in the Bahamas, although our reproductive data (Levitan et al. 2004) do not show parallel patterns of reduced barriers to gene flow in the Bahamas.

Morphology

At the level of the corallite, the three taxa are much less distinctive in the Bahamas than they are in Panama (Fig. 5). This is primarily because the micromorphologies of *M. annularis* and *M. franksi* converge on that of *M. faveolata*, which does not differ substantially between the two sites.

Colony morphologies are more difficult to quantify but can be assessed qualitatively. Individuals with typical *M. faveolata* genotypes are relatively similar morphologically in the Bahamas and Panama. *Montastraea annularis* colonies differ dramatically in the two locations, however; in Panama this species has a consistent columnar morphology, whereas in the Bahamas, colonies that spawn late but have *annularis/franksi*-like genotypes have morphologies ranging from columns to large heads to plates, sometimes within a single colony. *Montastraea franksi* is also unusual in the Bahamas, where it occurs in shallow, clear water (3 m). In Panama, *M. franksi* is largely absent in the shallow clear-water habitats of the San Blas, and in the murkier waters of Bocas del Toro, shallow-water *M. franksi* have colony morphologies and colorations that do not resemble those of the Bahamas. Nevertheless, shallow-water *M. franksi* from the Bahamas resemble *M. franksi* from Panama in their highly aggressive behavior (pers. obs.) and early spawning time (Levitan et al. 2004).

Genetics

Montastraea faveolata was genetically distinct in the Bahamas, but to a lesser degree than in Panama (Fig. 2; Tables 1, 2, 4). Two of the three AFLP loci exhibited highly significant differences between *M. faveolata* and the other two taxa (Table 2), and there was only a single individual with the GGAG-1 880/920 genotype expected of an F_1 hybrid between *M. faveolata* and either *M. annularis* or *M. franksi*. However, two *M. faveolata* in the Bahamas had the type I mitochondrial noncoding genotype typical of the other taxa, and one of these individuals (Ba00-30, Table 4) also had the AFLP three-locus genotype typical of *M. annularis* and *M. franksi* in the Bahamas. There were also no differences between *M. faveolata* and the other two taxa in ITS genotype frequency distributions (Table 2). The blurring of species boundaries between *M. franksi* and *M. annularis* in the Bahamas was even stronger, as these taxa were indistinguishable genetically (Tables 1, 2, 4; Fig. 2). Thus, ancestral polymorphism alone does not appear to explain the genetic pattern observed.

Reproduction

As described elsewhere, patterns of spawning and gametic compatability were remarkably similar in Panama and the Bahamas (Levitan et al. 2004). There were no significant differences in spawning time and most aspects of gametic compatibility. Indeed, the only difference observed between sites in gametic incompatibility was in the direction of greater gametic incompatibility between *M. franksi* and *M. annularis* in the Bahamas. Thus, the three taxa have comparable reproductive isolating mechanisms at the two sites. This stands in contrast to the data presented here for genetics and morphology, in which the three species were more similar in the Bahamas than they were in Panama. A plausible explanation for this discrepancy lies in the details of landforms and current regimes, which may at times permit more extensive interactions between heterospecific gametes in the Bahamas (Levitan et al. 2004, see also below).

The Montastraea annularis Complex in Time and Space

The most likely ancestor of the *M. annularis* species complex occurred in the Dominican Republic 4.5–5.5 million years ago (MYA), and was morphologically similar to *M. franksi* (Budd and Klaus 2001). Two of the three modern species (*M. franksi*, *M. faveolata*) first appear in the fossil record in Costa Rica in deposits dated at 2.9–3.5 MYA, and are morphologically distinct throughout this period (Budd and Klaus 2001). There are also questionable occurrences in Curaçao at localities dated at 5.6–3 MYA (Budd et al. 1998). Two to three undescribed fossil species were closely related to each of these two modern species, suggesting that the modern species are survivors of previously more diverse clades. The third modern species, *M. annularis*, does not appear unambiguously in the fossil record until about 500 thousand years ago in Barbados, and is more closely related to *M. franksi* than to *M. faveolata* (Pandolfi et al. 2002). Earlier, there are *M. annularis*-like forms in Panama dated at 1.8–2.2 MYA, in Jamaica dated at 1.4–2 MYA, and in Curaçao dated at 2–2.6 MYA (Budd et al. 1998; Budd and Klaus 2001). The relative recency of these divergences, especially that between *M. annularis* and *M. franksi*, undoubtedly contributes to the difficulties associated with resolving their species status.

Analysis of fossil material also supports the distinctiveness of late Pleistocene Bahamian members of the *M. annularis* complex (A. F. Budd and J. M. Pandolfi, unpubl. ms.). Taxa identified based on colony morphology as columnar, massive, and organ-pipe (now extinct) largely overlap in corallite morphology (and most closely resemble modern *M. annularis* sensu stricto), whereas elsewhere (e.g., the Dominican Republic) the three forms are distinctive in the Pleistocene (Klaus and Budd 2003). Indeed, the massive form from late Pleistocene Bahamian reefs may not correspond to *M. faveolata*, whereas Pleistocene massive colonies from elsewhere clearly do (A. F. Budd and J. M. Pandolfi, unpubl. ms.).

Genetic analyses are consistent with some aspects of the fossil record. First, all genetic analyses (Weil and Knowlton 1994; this study) suggest that *M. franksi* and *M. annularis* are sister taxa. The presence of highly divergent alleles within the mitochondrial noncoding region of *M. franksi* also supports its being a basal group, as suggested by the fossil record. The relationship between the fossil record and geographic patterns of genetic diversity is harder to understand, however,

because the fossil record points to origins in the central to south Caribbean, whereas genetic diversity is higher to the north. In the case of the noncoding mitochondrial region, both *M. annularis* and *M. faveolata* are substantially more diverse in the Bahamas than they are in Panama, where no unique alleles for either species were observed (Table 4). Unusual genetic variation was also seen at the GGAG-2 (450/430/410) locus in the Bahamas, where approximately 25% of the *M. annularis* and *M. franksi* individuals had an additional, highly divergent 450 allele (or locus). One possible explanation for the greater genetic diversity exhibited by Bahamian corals could be introgression from now extinct forms.

Resolving these paradoxes will depend on additional studies of corals at other geographic locations. Preliminary genetic data from Puerto Rico and Bermuda for the mitochondrial noncoding region suggest that there are consistent differences between northern parts of the range of these corals and Panama. For example, using the same analyses as in Table 2, Puerto Rico and Bermuda are indistinguishable from the Bahamas, whereas Puerto Rico and Bermuda are both highly distinct with respect to Panama (for Puerto Rico, $P < 0.01$ for *M. franksi* and *M. faveolata*; for Bermuda, $P < 0.01$ for *M. franksi*, the only species collected).

Already, however, these new analyses clarify why previous studies have come to different conclusions with respect to the status of these three species. The primary disagreement has centered on the extent to which they are comparably distinct in the northern (Florida, Bahamas; Szmant et al. 1997; Medina et al. 1999) and southern (Panama; Knowlton et al. 1992; Weil and Knowlton 1994; Lopez et al. 1999) parts of their ranges. (An earlier report [van Veghel 1994] that reproductive barriers are reduced to the south, in Curaçao, was not supported by our observations [Levitan et al. 2004], and in other aspects corals from Curaçao and Panama are very similar [Knowlton et al. 1992; van Veghel and Bak 1993; Weil and Knowlton 1994]). Neither do our reproductive analyses (Levitan et al. 2004) support the idea that the intrinsic potential for hybridization is higher in the north (Szmant et al. 1997). However, our studies do indicate that corals to the north are distinct genetically, and in addition exhibit morphological and genetic patterns consistent with introgression (the former also supported by analyses of Manica and Carter 2000).

Similar geographic patterns have been described in a number of organisms. For example, genetic differences between the Bahamas and the Caribbean have recently been reported for a goby (Taylor and Hellberg 2003), and geographic variation in the extent of introgression is well established in a number of groups (e.g., Arnold 1997; Howard et al. 1997; Shaw 2002; Bierne et al. 2003). Our results are also consistent with Veron's (1995) contention that reticulate evolution will vary regionally.

The Importance of Reticulate Evolution in Corals

Although evidence for the importance of hybridization is increasing in animals (Arnold 1997; Dowling and Secor 1997), most animal groups are not thought to be dominated by the kinds of reticulate processes that clearly play an extremely important role in plants. However, Veron (1995) has forcefully argued that hybridization is the major force influencing the evolution of mass-spawning corals, particularly in the Indo-West Pacific region due to its vast geographic scale. His suggestion that conventional species concepts do not apply to corals because of rampant hybridization has prompted a reevaluation of the nature of species in corals, as well as considerable controversy.

The genus *Acropora*, the most species rich genus of corals, has received the greatest study in this regard. In the Pacific, extensive breeding studies indicate that many morpohologically defined taxa that spawn synchronously are capable of interbreeding in the laboratory (e.g., Willis et al. 1997; Hatta et al. 1999; van Oppen et al. 2002; Marquez et al. 2002a,b). Moreover, studies of nuclear and mitochondrial genes reveal that many species, particularly those capable of interbreeding in the laboratory, are intermingled on phylogenetic trees (Hatta et al. 1999; van Oppen et al. 2001, 2002; Marquez et al. 2002b). On the other hand, differences in spawning time isolate some species, others are known to be reproductively isolated even when they cannot be resolved genetically or are morphologically very similar, and some appear to be isolated based on genetic data even though reproductive studies provide little evidence for clear barriers (van Oppen et al. 2001, 2002; Marquez et al. 2002b; Fukami et al. 2003). Thus, although the data for the Pacific suggest that reticulate evolution has played a role in the evolutionary history of Pacific *Acropora*, there is little support for rampant hybridization on the scale suggested by Veron (1995). In the Caribbean, in contrast, only three putative species of *Acropora* occur—*A. palmata*, *A. cervicornis*, and *A. prolifera*. The latter has long been suspected of being an F_1 hybrid of the other two, and this has recently been confirmed (van Oppen et al. 2000; Vollmer and Palumbi 2002). However, the degree to which backcrossing and introgression occurs appears limited, although its extent remains a source of dispute (Vollmer and Palumbi 2002; Miller and van Oppen 2003). The more limited evidence for introgression in Atlantic *Acropora* may reflect a more ancient divergence between these taxa; although *A. palmata* and *A. cervicornis* date back only a few million years as species, the lineages from which they derive have been distinct for at least 17 million years (Budd et al. 1994b).

Data on other corals are more limited. Diekmann et al. (2001) argued for reticulate evolution in the Caribbean brooding coral *Madracis*, but support for this was based largely on the lack of resolution in ITS genes. Our own analyses of ITS (Fig. 4) suggest that such data must be interpreted with caution, and the fact that morphological data clearly separate at least one of the *Madracis* species that cannot be separated genetically calls into question the adequacy of ITS sequences for resolving these taxa. The only other genus to receive extensive study is the Pacific coral *Platygyra*. In this case, no genetic differences (allozymes) and no reproductive barriers were found between taxa that were readily distinguished morphologically (Miller 1994; Miller and Benzie 1997; Miller and Babcock 1997). Once again, this has been interpreted as evidence for reticulation.

A major problem for elucidating the role of reticulate evolution in corals is their apparently slow rate of molecular (particularly mitochondrial) evolution, exemplified by little intraspecific variation and low levels of interspecific diver-

gence (Romano and Palumbi 1996; Snell et al. 1998; van Oppen et al. 1999; Shearer et al. 2002). In *Montastraea*, for example, members of the *M. annularis* complex and *M. cavernosa* differ in the mitochondrial COI gene by only 2.4% (Medina et al. 1999), despite the fact that these lineages have been distinct for at least 24 million years. Indeed, complete mitochondrial sequences of two individuals of each of the three members of the *M. annularis* complex revealed only 29 variable positions for 16, 134 bp (H. Fukami and N. Knowlton, unpubl. ms.). Thus negative evidence, e.g., the fact these taxa are identical at COI (Medina et al. 1999; this study), says little about species status. Slow rates of molecular evolution also affect phylogenetic interpretations. Although slow rates of evolution do not affect the rate at which genes become reciprocally monophyletic per se, they do affect the number of genes with adequate variation that are available for study. Theory suggests that only about one-third of trees will support reciprocal monophyly after N generations following the interruption of gene flow, where N is population size (Tajima 1983, summarized in Harrison 1998). Given the large population sizes and long generation times of many corals, the absence of reciprocal monophyly when only small numbers of genes are examined is not surprising and not particularly informative. Low rates of molecular evolution also make it difficult to distinguish ancestral polymorphisms from recent hybridization.

In sum, corals are unusually challenging with respect to understanding species boundaries. Even in the absence of gene flow, species may be difficult to distinguish for the reasons summarized above. When boundaries are or have been porous in parts of species' ranges, as the data for the *Montastraea annularis* complex in the Bahamas suggest, the problems multiply. A multifaceted approach, involving detailed analysis of genetics, reproduction, morphology, and the fossil record across the geographic range of the taxa in question, is required in such cases. Such an analysis has not been completed for the *M. annularis* complex and has not been attempted for most other coral species.

Acknowledgments

B. Shiplock, C. Swanson, D. Carlon, D. Kline, K. McGhee, T. McGovern, and E. Weil helped collect the corals. The genetics and field research was supported by grants from the National Science Foundation to NK (OCE98-11124) and to DL (OCE99-11225). A. Lopez Perez and S. C. Wallace helped with digitizing colonies using the Reflex microscope. The morphometrics research was supported by a grant from the National Science Foundation (EAR97-25273) to AFB. Two anonymous reviewers and C. Cunningham made many very helpful suggestions.

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