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DISCRIMINATION OF SPECIES IN THE *MONTASTRAEA ANNULARIS* COMPLEX USING MULTIPLE GENETIC LOCI

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ABSTRACT

Montastraea annularis, M. franksi and *M. faveolata* are a complex of recently distinguished coral species whose status remains controversial due to the lack of fixed differences. Here we report on two types of genetic analyses for these taxa: 1) DNA sequences of two nuclear genes [internal transcribed spacers of rDNA (ITS-1, ITS-2) and a ß-tubulin intron] and 2) a preliminary screening of the entire nuclear genome using amplified fragment length polymorphisms (AFLP). There was very little variation within or among the three species in DNA sequences. Only 3 of 300 nucleotide positions in ITS-1, 3 of 350 positions in ITS-2, and 3 of 350 positions in the ß-tubulin intron had large amounts of polymorphism. At some of these sites there were allele frequency differences among the species, but no diagnostic substitutions. Sequences from polymorphic sites in the ß-tubulin intron also showed evidence of heterozygosity. Pilot experiments with AFLP yielded stronger evidence of genetic boundaries among these species. Two AFLP primers gave patterns of band presence or absence that were potentially diagnostic for *M. faveolata* (n=7) versus *M. franksi* (n=7); *M. annularis* (n=4) may also have diagnostic patterns, but the differences between it and *M. franksi* were quantitative (band intensity) rather than qualitative.

INTRODUCTION

Species are the fundamental unit of biodiversity because they represent cohesive and independent lineages, often with distinct habitats, life histories and physiologies. In the sea, however, many of the most conspicuous and best known "species" appear to be heterogeneous assemblages of ecologically and genetically distinct forms (Knowlton 1993).

The species problem is particularly acute for scleractinian corals, as there is no consensus on the number of species in many of the important genera. This is true even in sympatry, where the criteria for species designations based on different species concepts (biological and phylogenetic) are in rough accord (Cracraft 1989; Knowlton and Weigt, in press). Veron (1995) argues that these debates reflect the fact that species of corals are "arbitrary" (pg 33).

There are two reasons why corals might pose problems for species discrimination: First, species may be reproductively well isolated, but exhibit much overlap in the characters used to tell them apart. Phenotypic plasticity (Willis 1985), slow rates of change in molecular characters (Romano and Palumbi 1996), relatively recent origins (Budd et al 1994) and long generation times may all contribute to difficulty in recognizing coral species. Second, species boundaries in corals may be muddled because of extensive and complex patterns of hybridization (Veron 1995). Many corals participate in mass-spawning events that potentially provide numerous opportunities for interspecific fertilizations (Willis et al. in press). Progeny of such crosses often survive (Willis et al. in press), perhaps because the relatively simple morphology of corals imposes few developmental constraints.

Montastraea annularis sensu lato provides an important example for exploring these issues. It is the dominant reef builder of the Caribbean and has been so for the past 2 million years (Budd et al. 1994). Because it has been widely used as a model system, more is known about its biology than any other Caribbean coral. Nevertheless, its taxonomic status remains controversial.

For decades, *M. annularis* was considered the archetypal generalist. Extensive variability in colony morphology exhibited over a wide depth range was believed to be an adaptive response to differing light levels, despite the apparent absence of intermediates (Graus 1977). More recently, a number of features were found to covary with the different types of colony morphologies in Panama and Curaçao, including allozymes, aggressive behavior, ecology, life history, growth rate, corallite morphometrics, and stable isotopes (Knowlton et al. 1992; Van Veghel and Bak 1993, 1994; Van Veghel and Kahmann 1994).

In response to these discoveries, Weil and Knowlton (1994) resurrected two previously synonymized species, *M. faveolata* and *M. franksi*, making, together with *M. annularis sensu stricto*, a total of three species in the complex. This perspective has been strengthened by the recent documentation of potential temporal and developmental barriers to cross-fertilization among the three species in Central America (Knowlton et al. in press). Nevertheless, these studies do not rule out the possibility of hybridization, and the need for diagnostic characters remains. Therefore, we have screened a number of molecular techniques to see if fixed genetic differences among the species exist (Avise 1994; Hillis et al. 1996). Here we summarize our preliminary findings.

MATERIALS AND METHODS

General approach

The fossil record of *M. annularis sensu lato* (Budd et al. 1994) suggests that the members of this complex diverged over the last two to four million years. Thus our choice of molecular approaches (Table 1) was dictated by the need to find genetic markers or sequences with relatively high rates of divergence.

Table 1: Molecular genetic approaches for sibling species identification. Detailed descriptions for each method are listed in a-Avise (1994); b-Hillis and Dixon (1991), Hillis et al. (1996); c-White et al. (1990); d-Palumbi and Baker (1994), Palumbi (1996); e- Sültmann et al. (1995); f-Vos et al. (1995).

I.	Mitochondrial DNA[a,b]
II.	rDNA genes and spacers[c]
III.	Intron characterization[d]
IV.	Anonymous nuclear loci[a,e]
V.	Amplified Fragment Length Polymorphism (AFLP)[f]
VI.	Random Amplified Polymorphic DNA (RAPD)[a]

We used gametes instead of somatic tissue because *Montastraea* gametes lack algal symbionts (Szmant 1991) and are thus a good source of "clean" DNA. We focused initially on *M. franksi* and *M. faveolata*, since allozyme data (Knowlton et al. 1992) and fertilization studies (Knowlton et al. in press) suggested that *M. annularis* and *M. franksi* are more closely related and thus potentially more difficult to distinguish. Hence, our sample sizes for *M. annularis sensu stricto* are smaller.

Mitochondrial DNA sequences (Method I) are obvious candidates for analysis (Moore 1995), but to date we have only amplified a conserved 16S rDNA segment using the Polymerase Chain Reaction (PCR) (Innis et al. 1990). PCR amplification of more rapidly evolving mtDNA regions has been hampered by a lack of suitable primers. Moreover, recent studies allude to a slower evolutionary rate for coral mtDNA than that found in other animals (Pont-Kingdon et al. 1995; Romano and Palumbi 1996).

The search for variation in specific nuclear genes (Methods II and III) has been more successful, although once again the low level of variation and the difficulty in designing primers that work with coral genes have proved to be stumbling blocks. For the rDNA region, we found informative polymorphisms in the ITS regions (Pleyte et al. 1992; and in more detail below). No diagnostic differences were found in roughly 800 bp of large subunit rDNA sequences, however, and the intergenic spacer (IGS) between individual rDNA gene clusters could not be amplified. Degenerate PCR primers for regions of conserved amino acid composition in actin, enolase, elongation factor-1, pyruvate kinase, creatine kinase-1 and tubulin genes were designed or obtained from the literature (Palumbi 1996). However, only tubulin primers were successful in PCR (see below).

Five anonymous nuclear loci were isolated, screened, and sequenced (Method IV), but the presence of multiple copies and large amounts of intraspecific variation limited their usefulness for this project. Two remain as potentially informative, but they will not be discussed further here.

AFLP (Method V) shows considerable potential for distinguishing closely related species (see below). A modification of RAPD-PCR (Method VI) was applied using a simple sequence primer (Gupta et al. 1994) with limited success.

Sample preparation

Coral DNA was obtained from *Montastraea* gametes (sperm and oocytes) collected during mass spawning events in 1994 and 1995 in the San Blas Islands, Panama. Corals of the three species were collected from Cayos Limones and brought to the Smithsonian Tropical Research Institute field station [see Fig. 1 in Weil and Knowlton (1994)]. The corals were identified to species in the field based on colony morphology (Knowlton et al. 1992; Weil and Knowlton 1994).

On nights of possible spawning, individual corals were placed in separate buckets, kept in the dark, and monitored every twenty minutes for spawning. Eggs probably remain unfertilized using this procedure, because mixtures of eggs and sperm from a single colony rarely produce planulae (see Knowlton et al. in press for details). However, our molecular analyses of nuclear genes should reveal heterozygosity in parental colonies where it occurs, because samples from individual colonies consist of many eggs and many sperm.

Gamete bundles were aspirated from the water surface into 100-200 ml plastic jars. The aspirate was then washed with filtered (0.2 µm) seawater over plankton netting (102 µ) to separate sperm and eggs. Eggs were washed from the screen into test tubes. Excess sea water was removed by pipette from below the buoyant eggs, which were then frozen (-196°C) following addition of an equal volume of "L" buffer (0.2M EDTA pH 8.0, 0.01M Tris-Cl pH 7.6, 0.02M NaCl) (Sambrook et al. 1989). Wash water containing the sperm was poured into centrifuge tubes and spun for 5 min in a clinical centrifuge with a fixed angle rotor (4475 g). Sperm from a single individual were then combined by serially resuspending the pellets in < 5 ml of filtered sea water or L buffer, and frozen as above. Following return to the laboratory, gamete samples were stored at -80°C. We used a standard DNA extraction protocol of cell lysis with 1% SDS followed by proteinase K digestion at 50°C, successive phenol/chloroform extractions, and ethanol precipitation (Sambrook et al. 1989).

Primers and PCR

Nuclear introns were isolated by designing degenerate oligonucleotide primers according to the protocol of Palumbi and Baker (1994). Both ITS-1 and ITS-2 regions were amplified as a single 704 bp PCR product, using a 54°C annealing temperature for 45 sec, and the universal primers ITS-5 [5' GGAAGTAAAAGTCGTAACAAGG 3'] and ITS-4 [5' TCCTCCGCTTATTGATATGC 3'] (White et al. 1990). The ß-tubulin coding and intron gene sequences were amplified with the degenerate primers tub1050 [5' GGNWCNGGNATGGGAACNCT 3'] and tub1525rc [5' ATCATGTTCTTNGCRTCNAACAT 3']. This region corresponds to amino acid positions 119-244 in the protozoan *Eimer tenella* (GenBank accession no. U19609).

Each PCR (30-50 µl) contained 10-100 ng purified DNA template, 100 µM of each dNTP, 2.5 mM $MgCl_2$, 10 mM Tris-HCL pH 8.9, 50 mM KCl, 10 µM primer, and 1.25 U Taq polymerase (Stratagene or ProMega). PCR profiles varied according to the method (Table 1) or oligonucleotide primers (Ransom Hill Biosciences) used, but generally would consist of a cycle of 94°C for 30 sec, 50-60°C for 30 sec and 72°C for 1-1.5 min repeated 28-33 times. Negative controls lacking coral DNA were included in all pilot PCR assays to monitor possible contamination (Innis et al. 1990; Palumbi 1996). PCR products were purified from agarose by GeneClean (Bio 101, La Jolla) or Gel-ase (EpiCentre Technologies) digestion.

Copy number determination

To assess the copy number of nuclear loci, we used candidate clones and PCR products as probes in Southern blot hybridizations. Probes were labeled with $^{[32]}$P-dCTP according to standard methods (Sambrook et al. 1989). Total coral genomic DNA was digested with 6 bp-cutting restriction enzymes, which have recognition sites at approximately 4.0 kb intervals. Thus, a typical probe (ca. 2.0 kb) to single copy loci is expected to detect only a few (1-2) bands and should require several days of autoradiographic exposure for detection on X-ray film (Sambrook et al. 1989). In contrast, intense smears or many bands after only 12-24 hours exposure indicated multiple copies of a locus and elimination of the candidate DNA segment from further consideration (except rDNA ITS sequences).

DNA sequencing, cloning, and sequence analysis

DNA sequences were obtained by cycle-sequencing reactions run on a 373A automatic DNA sequencer (Applied Biosystems Inc). Cloned inserts generally gave more reliable DNA sequences. However, direct sequencing of PCR products also allowed the opportunity to pinpoint locations of indels (insertion/deletion mutations), because heterozygous individuals for an indel caused ensuing downstream sequences to appear frameshifted, and consequently unreadable from the unprocessed automated sequence output (chromatographs). When direct sequencing of PCR products produced ambiguous results, fragments were subcloned into pGem-T vectors (Promega) and then resequenced.

Representative *Montastraea franksi* ITS-1 and ITS-2 and *M. faveolata* ß-tubulin sequences have been deposited in GenBank under accession nos. U60605 and U60604, respectively. All nucleotide positions in this paper refer to these two sequences, unless otherwise stated. Specific DNA polymorphisms will be referred to in the text by their gene, nucleotide position, and residue type (A, C, G, or T) (e.g. ß-tubulin/484/A).

To detect homology to previously characterized DNA sequences, *Montastraea* sequences were compared with data in sequence databases such as EMBL and GenBank (release 95) with BLAST (Altschul et al. 1990). Alignments, protein coding regions, and percent similarities were determined using MacVector (Scientific Imaging Systems),

SeqEd (ABI), Sequencer 3.1 (Kessing, unpubl.), or PILEUP in UWGCG (Genetics Computer Group 1994).

Amplified Fragment Length Polymorphism (AFLP)
The AFLP method was employed following standard procedures (Zabeau and Vos 1995; Vos et al. 1995; Mueller et al. 1996). Briefly, coral DNA was digested with restriction endonuclease Pst I and simultaneously ligated with a short 15-17 bp double-stranded oligonucleotide adapter, possessing a compatible 3' sticky end which did not restore the Pst I recognition site (underlined below):

5' CTCGTAGACTGCGTACATGCA 3'
3' GCATCTGACGCATGT 5'

This combined reaction produces a genomic population of restriction fragments containing the known adapter sequence at both termini. Complete digestion/ligation was monitored by running aliquots on agarose gels. A single oligonucleotide primer complementary to the 15 bp adapter was then used in subsequent PCRs. However, to amplify a smaller subset of the total template pool, arbitrary bases were added to the 3' end of the PCR primer with the sticky end. Both the length and nucleotide composition of this random base "extension" determine which genomic fragments become amplified. Thus, two different individuals with identical restriction sites for Pst I will produce distinct AFLP patterns if they differ in the 3' extension of the PCR primer. To date, we have tested six "adapter-complementary" primers with 3 or 4 bp extensions: -ATT 3', -ATG 3', -ACGC 3', -GAC 3', -GAG 3' and -ATG 3'. The latter two gave the best balance of total band yield and polymorphism in *Montastraea*. PCR products were resolved on 1.2-1.4% agarose TBE gels.

RESULTS

rDNA ITS-1 and ITS-2 DNA sequences
PCR with the ITS-4 and ITS-5 primers typically yielded large amounts of a single 704 bp fragment (Fig. 1A). Southern blot hybridizations with these ITS PCR products confirmed a high copy number for rDNA genes in *Montastraea*. We have assumed that rDNA and accompanying ITS sequences evolve by "concerted evolution", a process which homogenizes different ITS unit repeats within the tandemly repeated rDNA locus in many eukaryotic taxa (Dover 1982; Hillis and Dixon 1991).

DNA sequencing of this product revealed that the 5.8S rRNA gene (ca. 87% similarity with previously characterized genes of fungi) resided between ITS-1 & ITS-2 (Fig. 1A). Precise ITS boundaries with 5.8S rRNA were inferred from previously studied rDNA loci (Hibbet et al. 1995). Database searches failed to detect significant conservation between *Montastraea* ITS sequences and other entries in GenBank. Significant variation among ITS copies of single individuals (such as indel mutations) was also absent based on the sequence clarity of PCR products.

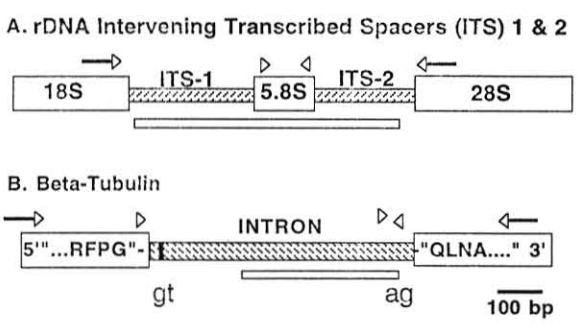

Fig. 1: Structure of rDNA ITS-1 and ITS-2 (A) and ß-tubulin (B) gene regions. Large boxes denote coding sequences. Arrows and arrowheads indicate primers used for PCR and DNA sequencing, respectively. White bars indicate regions analyzed in detail. The black bar in (B) marks a 3 bp deletion found in some individuals. Upper case letters in (B) denote the last four amino acids adjacent to the exon/intron junction, and gt and ag the consensus nucleotides for the beginning and end of the intron.

Mean intraspecific DNA sequence divergences in the most variable ITS-1 and ITS-2 regions (ca. 300 and 350 bp) were relatively low: 0.9 and 0.7% in *M. franksi*, 1.1 and 0.8% in *M. faveolata*, and 1.7 and 0.9% in *M. annularis*; ITS-2 had the smaller of the two mean values in all three species. Mean interspecific differences for ITS-1 and ITS-2, respectively, were 1.2 and 0.9% (*M. franksi-M. annularis*), 1.2 and 0.9% (*M. franksi-M. faveolata*), and 1.5 and 1.0% (*M. annularis-M. faveolata*). The highest individual pairwise divergence was 3.4% (between two *M. annularis*). A transition/transversion bias of 1.5-3.0 was evident in most pairwise comparisons.

Most of the polymorphisms at individual nucleotide sites were rare, being observed in three or fewer of the 23 sequences obtained. A summary of the most variable ITS sites is shown in Table 2. The variation observed at polymorphic sites was generally limited to only two of the four possible nucleotides, which is consistent with the observed low DNA substitution rate.

Variation across different positions within ITS-1 and ITS-2 appeared to be non-independent. Across three positions only two patterns were observed: ITS-1/190/T + ITS-1/227/T + ITS-2/454/A or ITS-1/190/C + ITS-1/227/A + ITS-2/454/G (T+T+A or C+A+G). A similar dichotomy was observed between two other positions within ITS-2: ITS-2/336/G + ITS-2/456/G or ITS-2/336/C(or T) + ITS-2/456/C (G+G or C+C). However, these two sets of patterns sort independently in that T+T+A and C+A+G are each found with G+G and C+C. The most conspicuous difference among the species in these patterns was the relative rarity of C+A+G in *M. franksi* (1 of 10 individuals) and perhaps *M. annularis* (0 of 4 individuals) compared to *M. faveolata* (5 of 9 individuals).

ß-tubulin DNA sequences
Two bands of approximately 1.5 and 1.1 kb in size were visualized after initial amplification with the ß-tubulin-1050 and -1525 primers. Preliminary sequence analysis and BLAST queries revealed strong similarity of both PCR products to ß-tubulin. Multiple tubulin genes are expected since they comprise a multi-gene family in many eukaryotes (Cleveland and Sullivan 1986; Hillis et al. 1996). Only the 1.1 kb fragment was extensively analyzed.

DNA sequencing revealed the presence of a 594 bp intron and about 306 and 163 bp of flanking 5' and 3' coding sequence (Fig. 1B). Both coding sequences exhibited more than 90% amino acid and 80% DNA identity to sea urchin and human ß-tubulin sequences in GenBank, which abruptly ended at exon/intron boundaries at nt pos 307 and 896 (Fig. 1B) in all three species. The intron was also identified by the presence of stop codons in all six possible reading frames beyond the junctions, precluding any coding sequences larger than 30 amino acids in length. Comparison of ß-tubulin exon/intron junctions in other invertebrates suggests that those of *Montastraea* are novel. For example, the ß-tubulin gene of the nematode *Haemonchus contortus* (GenBank accession no. X80046) possesses two smaller introns in the same overlapping region of the *Montastraea* ß-tubulin gene.

Unlike either ITS-1 or ITS-2, the *Montastraea* ß-tubulin intron possessed frequent homopolymer stretches of dA or dT, underscoring the strong base compositional bias of up to 64% dA/dT found in some individuals. This finding was replicated in the DNA sequences of anonymous nuclear loci (data not shown), and thus appears to be a general property of some coral nuclear DNA loci.

Table 2: Patterns and positions of variable nuclear DNA polymorphisms in different nuclear loci among *Montastraea* species. Column numbers refer to base positions within the reference sequences deposited in GenBank (see text). Entries with >1 base indicate detection of heterozygotes (see text). Nucleotides in lower case indicate an equivocal base call (low signal in sequence chromatograph). Italics denote sequences derived from clones. Bold letters denote the most common base, with its frequency of occurrence in a single species in parentheses. N's denote unavailable data. Alignments using default parameters in PILEUP were used (Genetics Computer Group 1994), since very few gaps were encountered at these loci. All ITS sequences have been read from both strands, while tubulin samples marked with * had indels and usually could be read in only one direction. AFLP data are grouped by the extension primer used (GAG or ATG; see text) and scored by presence or absence; parentheses indicate weak AFLP bands.

ID	*Montastraea*	ITS-1			ITS-2			ß-Tubulin				AFLP Bands			
												GAG		ATG	
No.	species	190	227	265	336	454	456	484	709	713		880	920	630	750
438	*faveolata*	T	T	G	G	A	G	*G*	*T*	*G*		N	N	+	+
410	*faveolata*	T	T	A	G	A	G	*G*	*T*	*G*		+	-	+	+
428	*faveolata*	C	A	G	C	G	C	A/g	T	A/G	*	+	-	+	+
406	*faveolata*	T	T	A	T	A	C	**A**	*C*	*A*		N	N	N	N
425	*faveolata*	C	A	G	C	G	C	G	T	G	*	+	-	+	+
409	*faveolata*	C	A	G	N	G	C	*A*	*T*	*A*		+	-	+	+
37	*faveolata*	C/T	A	G	N	N	N	A/g	T	A/G	*	+	-	+	+
23	*faveolata*	T	T	A	G	A	G	A	C	A	*	N	N	N	N
490	*faveolata*	C	A	G	G	G	G	N	T	A/G	*	+	-	+	+
Most	**common base**	**C**/T(.5)	**A**(.55)	**G**(.67)	**G**(.75)	**G/A**(.5)	**G/C**(.5)	**A**(.55)	**T**(.78)	**G/A**(.5)					
459	*franksi*	C	A	G	G	G	G	A	C	A	*	-	+	-	(+)
467	*franksi*	T	T	A	G	A	G	*A*	*C*	*A*		(+)	+	-	(+)
426	*franksi*	T	T	A	G	A	G	*A*	*C*	*A*		-	+	-	(+)
408	*franksi*	T	T	A	C	A	C	A	c/t	A/G	*	-	+	-	(+)
312	*franksi*	T	T	A/g	C	A	C	a/g	c/t	A/G		-	+	-	-
417	*franksi*	T	T	A	G	A	G	N	N	N		-	+	-	+
19	*franksi*	T	T	A	N	A	G	A	C	A	*	(+)	+	N	N
20	*franksi*	T	T	A	N	A	G	a/g	C/T	A/G	*	N	N	N	N
3	*franksi*	T	T	G	G	A	G	A	C	A	*	N	N	N	N
15	*franksi*	T	T	G	G	A	G	N	N	N		N	N	N	N
13	*franksi*	N	N	N	N	N	N	A	C	A	*	N	N	N	N
Most	**common base**	**T**(.9)	**T**(.9)	**A**(.65)	**G**(.75)	**A**(.9)	**G**(.8)	**A**(.88)	**C**(.83)	**A**(.83)					
464	*annularis*	T	T	G	G	A	G	G	C	A/G	*	N	N	N	N
465	*annularis*	T	T	A	G	A	G	g	c	g	*	+	+	-	+
7	*annularis*	T	T	A	G	A	G	g/a	c/t	A/g	*	N	N	N	N
457	*annularis*	T	T	G	G	A	G	G	T	G	*	N	N	-	+
Most	**common base**	**T**(1.0)	**T**(1.0)	**G/A**(.5)	**G**(1.0)	**A**(1.0)	**G**(1.0)	**G**(.88)	**C**(.62)	**G**(.75)					

Within the most divergent 350 bp at the 3' end of the ß-tubulin intron, mean intraspecific nucleotide divergence was 0.3% in *M. franksi* (n=9), 0.8% in *M. faveolata* (n=9), and 0.6% in *M. annularis* (n=3). Intraspecific divergence decreased to about 0.6% for *M. faveolata* when one individual (#23) with many unique substitutions was excluded. Interspecific divergences ranged from 0.8-0.9% between pairs of species. A 3 bp deletion (CCG) was found 30 bp downstream of the 5'intron/exon junction in a number of individuals (asterisked in Table 2); this deletion made it difficult to sequence the gene in both directions using the primers shown in Fig 1B. Other isolated insertions were also evident (e.g. pos. 712 in *M. faveolata* #23). None of these sorted differentially among the three species.

As with ITS, most of the polymorphisms at individual nucleotide positions were rare. Only three positions (484, 709, and 713) were highly polymorphic (Table 2). Again as with ITS, sites appeared to covary in such a way as to generate two common patterns: 484/G + 713/G (G+G) or 484/A + 713/A (A+A). Both G+G and A+A individuals could have either C or T at position 709. There was no obvious correspondence in patterns between the ITS and B-tubulin loci, which suggests non-linkage and independent transmission of these loci.

Many individuals appeared to be heterozygous. Heterozygosity was inferred from the presence of two superimposed peaks of equivalent height in the sequence chromatograph data (Table 2). As expected, putative heterozygotes in ß-tubulin sequences were concentrated at rare polymorphic sites, whose variability was independently identified with cloned DNA. This pattern was not seen in ITS sequences, suggesting that some superimposed double peaks, especially in ITS, are a result of sequencing noise. Rarity of genuine heterozygotes in ITS is expected if gene conversion regularly homogenizes sequences within individuals.

Genomic fingerprinting with AFLP

In AFLP analyses (Fig. 2, also summarized in Table 2), species-specific bands ranging from about 600 bp to 1.0 kb were observed. The absence of the 920 bp band in Fig. 2A and the presence of the 630 bp band in Fig. 2B appeared to be diagnostic for separating *M. faveolata* and *M. franksi*. *Montastraea annularis* and *M. franksi* seem to be more similar, as expected. There was a marked quantitative difference in the intensity of the 750 bp band in Fig. 2B which was confirmed in two additional individuals of *M. annularis* (data not shown). The 880 bp band of *M. annularis* in Fig. 2A may also prove to exhibit quantitative differences with *M. franksi* upon further sampling. Because the allelic status and coding identity of these AFLP bands are currently unknown, however, the interpretation of quantitative differences is more problematic.

Each sample was carefully monitored for complete digestion of total genomic DNA. Artifacts arising from partial digestion seem unlikely in these gels since several "conserved" AFLP bands were concomitantly observed with the variable bands in most individuals and among *Montastraea* species. Also, at least one of these conserved bands in each gel was of lower molecular weight than the species-specific diagnostic bands (Fig. 2).

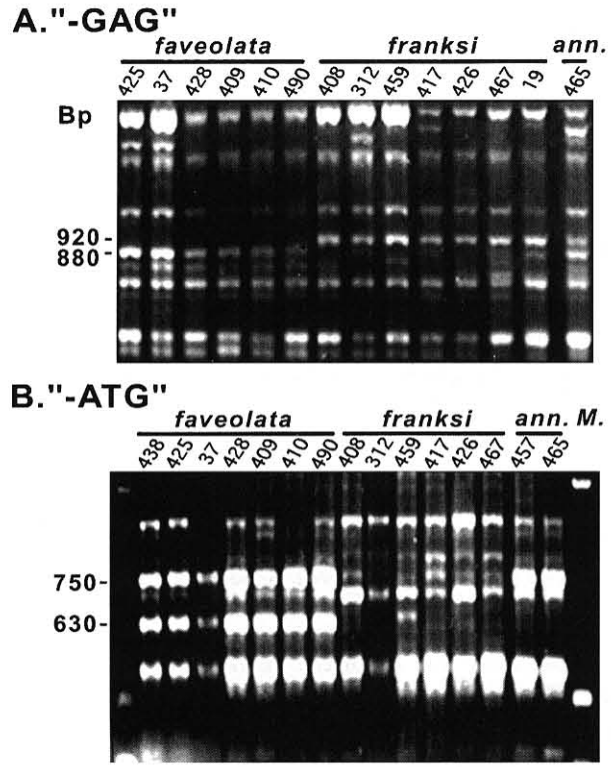

Fig. 2: AFLP banding patterns for the three *Montastraea* species. "GAG" and "ATG" refer to the identities of the extension primers used in the AFLP-PCR. Molecular sizes (in bp) indicate positions of species-specific diagnostic bands. Only the 0.4-1.0 kb portion of the 1 kb size standard (M) is shown.

DISCUSSION

Combined DNA sequence data (1764 bp) from two separate nuclear loci revealed low variability with little evidence for differentiation of the proposed *Montastraea* species. This may reflect large population sizes and low substitution rates for species like these corals (Ohta 1992; Palumbi 1992). Ancestral polymorphisms that have not become reciprocally monophyletic among taxa could explain the patterns of variation observed in the ITS and ß-tubulin loci (Avise 1994). It is also unknown which factors (e.g. selective constraints, differential mutation pressure) may have caused the extremes in base composition and patterns of non-independent substitutions within genes that we observed.

Although still preliminary, the apparently greater success of the AFLP technique in discovering diagnostic genetic loci probably stems from its ability to "cast a wider net" across the genome (Vos et al. 1995; Mueller et al. 1996). It should also be emphasized that our AFLP results were produced after screening only six extension primers. Note that only a fraction of potential AFLP variation was surveyed in this study. To illustrate, the total number of all possible 3 and 4 bp extension primers is 64 and 256, respectively. Furthermore, two different 3 or 4 bp primers can be combined in a single PCR to yield 16,384 different types of reactions.

AFLP avoids several potential artifacts associated with RAPD-PCR, another genome-wide assay (Williams et al. 1990; Hadrys et al. 1992), because of its higher specificity. Nevertheless, screening of only high quality coral DNA, such as that extracted from gametes, is recommended until the reliability of AFLP methods with coral somatic tissues has been established.

AFLP data can be used to estimate the degree of concordance (Avise and Ball 1990) among a number of independent genetic loci. The greater the number of bands showing alternate patterns of fixation or strong frequency differences between taxa, the less likely is extensive gene flow between them. Because this technique is relatively inexpensive and rapid, it is ideally suited for initial surveys of coral taxa whose status is questionable. Once evidence for species-specific patterns has been obtained, however, screening a variety of defined loci for differences in DNA sequences may be justified, particularly for determining the natural frequency of hybridization and for comparing DNA from lower quality sources. However, the small amount of variation we observed suggests that finding informative DNA sequences in closely related coral species remains a difficult task.

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