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Molecular Determination of Species Boundaries in Corals: Genetic Analysis of the *Montastraea annularis* Complex Using Amplified Fragment Length Polymorphisms and a Microsatellite Marker

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Abstract. Analyses of DNA have not been widely used to distinguish coral sibling species. The three members of the *Montastraea annularis* complex represent an important test case: they are widely studied and dominate Caribbean reefs, yet their taxonomic status remains unclear. Analysis of amplified fragment length polymorphisms (AFLPs) and a microsatellite locus, using DNA from sperm, showed that *Montastraea faveolata* is genetically distinct. One AFLP primer yielded a diagnostic product (880 bp in *M. faveolata,* 920 bp in *M. franksi* and *M. annularis*) whose homology was established by DNA sequencing. A second primer revealed a 630 bp band that was fixed in *M. faveolata,* and rare in *M. franksi* and *M. annularis;* in this case homologies were confirmed by Southern hybridizations. A tetranucleotide microsatellite locus with several alleles exhibited strong frequency differences between *M. faveolata* and the other two taxa. We did not detect comparable differences between *M. annularis* and *M. franksi* with either AFLPs (12 primers screened) or the microsatellite locus. Comparisons of AFLP patterns obtained from DNA from sperm, somatic tissues, and zooxanthellae suggest that the technique routinely amplifies coral (animal) DNA. Thus analyses based on somatic tissues may be feasible, particularly after diagnostic differences have been established using sperm DNA.

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Abbreviations: AFLP - amplified fragment length polymorphism, a registered trademark of Keygene.

Introduction

The recognition of species boundaries in sympatry is straightforward in principle, because the absence of interbreeding implies the existence of at least some fixed genetic differences between taxa (Avise and Ball, 1990). However, the number of such differences may be very small if the isolation of taxa is recent or the rate of evolution is slow. If in addition sporadic hybridization occurs, the problem of defining species becomes particularly difficult (*e.g.,* Howard *et al.,* 1997).

Closely related coral species appear to be especially challenging in this regard (Veron, 1995; Knowlton and Weigt, 1997). Species boundaries are in flux for a number of well-studied groups (*e.g.,* Miller and Babcock, 1997; Miller and Benzie, 1997; Odorico and Miller, 1997; Willis *et al.,* 1997; Knowlton and Budd, unpubl.), and it is unclear whether these controversies are due to the technical challenge of finding diagnostic characters between generally similar but reproductively isolated taxa, or alternatively, to the blurring of species boundaries by hybridization (Veron, 1995; Knowlton and Weigt, 1997; Willis *et al.,* 1997). Molecular methods have great potential to resolve the nature of species boundaries because of the large number of unambiguous characters they provide (Avise, 1994).

A clear example of these issues is presented by the proposed members of the *Montastraea annularis* species complex: *M. annularis* (formerly morphotype I or columnar morph), *M. faveolata* (formerly morphotype II or massive morph), and *M. franksi* (formerly morphotype III or bumpy morph) (Knowlton *et al.,* 1992; Van Veghel and Bak, 1993;

Weil and Knowlton, 1994). In sympatry, these taxa differ in colony morphology, growth rate, stable isotope chemistry, aggressive behavior, allozymes, corallite structure, and life history (Tomascik, 1990; Van Veghel and Bak, 1993, 1994; Van Veghel, 1994; Van Veghel and Kahmann, 1994; Weil and Knowlton, 1994; Van Veghel and Bosscher, 1995; Van Veghel *et al.,* 1996; Szmant *et al.,* 1997; Knowlton and Budd, unpubl.). Such concordance of suites of independent characters in sympatric taxa strongly suggests reproductive isolation (Avise and Ball, 1990), and differences in the timing of spawning and apparent barriers to interspecific fertilization (Knowlton *et al.,* 1997) also support this interpretation (but see Szmant *et al.,* 1997). Overall, these data support separate species status regardless of the species concept used (Templeton, 1989; Cracraft, 1989; Mallet, 1995; Knowlton and Weigt, 1997).

Nevertheless, a preliminary molecular survey revealed no fixed DNA sequence differences among these taxa in two regions that might, *a priori,* be expected to have them: the ITS regions of rDNA and an intron in a β-tubulin gene (Lopez and Knowlton, 1997). Sequence-based methods can only be used to examine a limited stretch of DNA, however, and methods that screen a larger proportion of the genome appear to offer greater promise (Lopez and Knowlton, 1997). One such approach is analysis of amplified fragment length polymorphisms (AFLPs), which screens for polymorphisms at, or adjacent to, restriction endonuclease sites (Zabeau and Vos, 1995). In a preliminary survey, we found evidence for potentially diagnostic differences between *M. faveolata* and *M. franksi* using two AFLP primers (Lopez and Knowlton, 1997).

In the present study we wished to (1) determine whether these apparently diagnostic AFLP differences hold up when sample sizes are increased, (2) screen additional AFLP primers to see if any show promise for distinguishing *M. annularis* and *M. franksi,* (3) determine, using Southern hybridization and DNA sequencing, whether apparently similar AFLP bands are indeed homologous, and (4) assess whether diagnostic polymorphisms detected using high-quality DNA derived from sperm could also be seen in more readily collected, but potentially less pure, somatic tissue samples.

During earlier work on tubulin introns (Lopez and Knowlton, 1997), we also uncovered a tetranucleotide microsatellite locus (here called Mfra-gttt1) in a genomic clone derived from *M. franksi.* Microsatellite or simple repeat loci have become increasingly important tools in evolutionary and population studies because of their high levels of polymorphism and codominant inheritance (Jarne and Lagoda, 1996). Here we report on evidence for allelic frequency differences at this locus among the *Montastraea* taxa.

Materials and Methods

Sample acquisition and DNA preparation

All corals were collected from the San Blas Islands, Panama. Colonies were identified to species in the field, based on colony morphology, and brought to waters near the laboratory shortly before the anticipated date of spawning (Knowlton *et al.,* 1997). At dusk, each colony was placed in a separate container; spawning generally occurred 2–4 h after sunset, and the gamete bundles were collected immediately after release. The gamete bundles from each container were washed separately over plankton netting. The eggs were retained on the netting, while the sperm passed through with the wash water, which was collected and centrifuged. The pelleted sperm were quick frozen (details in Lopez and Knowlton, 1997). Abundant DNA (hundreds of micrograms) was extracted from 1–2 ml of highly concentrated sperm solution using standard techniques (Sambrooks *et al.,* 1989), as previously described (Lopez and Knowlton, 1997).

Sperm provide an ideal source of coral DNA (McMillan *et al.,* 1988), but they cannot be collected routinely. However, high molecular weight DNA is difficult to extract from somatic tissues (McMillan *et al.,* 1988) and may be contaminated by DNA from symbiotic dinoflagellates (zooxanthellae), which the gametes of *Montastraea* lack (Szmant, 1991). To determine whether DNA extracted from somatic tissues is of sufficient quality for AFLP analyses, we compared the analyses of DNA from sperm with those of DNA from somatic tissues from the same colonies. DNA from somatic samples was extracted according to the protocol of Rowan and Powers (1991), except that tissue was removed from 25–50 cm^2 of coral with an airbrush at 75–100 psi and suspended in 5–20 ml of L buffer (100 m*M* EDTA, 10 m*M* Tris-Cl, pH 7.6). To enrich for coral (animal) DNA within somatic tissues, frozen samples were ground in a glass homogenizer 5–10 times and centrifuged in an RT6000B Sorvall centrifuge at 50–100 $\times$ *g* for 10 min at room temperature. This spin was repeated one or two times for samples especially rich in zooxanthellae. The animal-enriched DNA was then incubated for 3 h in 20–50 μg/ml proteinase K with 1% SDS (final concentration), followed by successive phenol:chloroform extractions (Sambrook *et al.,* 1989). DNA that remained resistant to restriction digestions was further purified by the GeneClean (Bio 101) protocol. To clarify further the potentially confounding contribution of zooxanthellae in coral somatic samples, we also analyzed zooxanthella DNA provided by Rob Rowan. This DNA came from other colonies of *Montastraea* (primarily *M. faveolata*) from the same region, and was not necessarily entirely free of coral (animal) DNA.

AFLP-PCR

The AFLP method and preparation of templates using the Pst I adapter system have been described in detail (Mueller *et al.,* 1996). Genomic DNA was cut at specific 6-base recognition sequences by the Pst I restriction enzyme, and then a synthetic, 21 bp adapter was ligated to the ends of the fragments. The polymerase chain reaction (PCR) was then used to amplify these restriction fragments, using primers matching the adapter sequence. To limit the number of different fragments that are amplified (and hence improve the clarity of the resulting products), several additional, arbitrarily chosen bases were added to the PCR primers at their 3′ ends. These additional bases (by which primers are identified, *e.g.,* ATG or GGAG) overlap with genomic DNA beyond the restriction site, and amplify the subset of fragments that contain the additional nucleotides.

We used the same methods and extension primers (ATG, GGAG) as previously reported (Lopez and Knowlton, 1997; note, however, that in our earlier report, the GGAG primer was incorrectly listed as GAG). We also used primers with the following 3′ extensions: ATT, GAC, GTG, ATC, TGT, ACT, TTG, AGC, TAG, and ACGC. The PCR profile for all AFLP extension reactions was 94°C/45 s, 60°C/60 s, and 72°C/90 s for 30 cycles, using an MJ Research PTC-100 or PTC-200 thermocycler. Typically, a "preamplification" PCR was performed with an extension primer possessing one additional base (A, C, G or T) (Vos *et al.,* 1995). This reaction enriches for the subset of amplifiable templates possessing the extra nucleotide, improves the targeted band signal, and reduces background. The preamplification PCR was run with the same AFLP profile as above, but with fewer (20) cycles. However, this preamplification protocol did not improve the clarity of the patterns for the GGAG primer. The best electrophoretic resolution of PCR products was obtained with 1.2%–1.4% agarose/TBE gels (containing at least 50% Metaphor agarose, FMC) run at 5.4 V/cm. The agarose-based technique used here and in our previous study does not require radioactive nucleotides, is less toxic, and is relatively easy to perform (Mueller *et al.,* 1996; Lopez and Knowlton, 1997), although it yields fewer discrete bands per lane (6–12) than the original polyacrylamide gel electrophoresis (PAGE) method (Vos *et al.,* 1995) due to poorer resolution of fragments of less than 400 bp. All AFLP analyses shown here were performed more than once to ensure reproducibility, and AFLP-PCRs with no DNA added served as controls for contamination.

AFLP banding patterns and DNA sequences (see below) were analyzed with RFLPscan (Scanalytics), BLAST (Altschul *et al.,* 1990), and MAPD (Yuhki and O'Brien, 1990; Stephens *et al.,* 1992). Only bands in the 0.3–1.6 kb size range were considered, since variation in higher molecular weight bands is more difficult to interpret due to potentially inconsistent amplification of large DNA fragments and the possibility of incomplete restriction enzyme digestion. RFLPscan (Scanalytics) converted band patterns into binary presence/absence characters for each sample and computed distance estimates for pairwise comparisons based on band sharing (Nei and Li, 1979).

Cloning, Southern hybridization, and sequencing

Both gel-purified and cloned AFLP fragments were used as hybridization probes and as sequencing templates. Specific AFLP bands were dissected from low melting temperature agarose (NuSieve/Metaphor, FMC) gels, added to 200 μl of distilled H_2O, and melted at 65°C. When this DNA was used as a template in a "re-amplification" PCR with the original primer to obtain more material, PCR products were visualized on an agarose minigel to verify that a single band of the correct size had been amplified. Alternatively, AFLP fragments were cloned directly into pGEM-T vectors (Promega). Probes for Southern hybridizations were labeled with $\alpha[^{32}P]$-dCTP via nick-translation to a specific activity of 10^6–10^7 cpm/μg (Sambrook *et al.,* 1989).

After separation on agarose gels, AFLP products were blotted onto nylon membranes (Duralon, Stratagene) according to standard procedures (Southern, 1975; Sambrook *et al.,* 1989). Higher molecular weight fragments (> 1 kb) appeared to be transferred to membranes less efficiently than smaller fragments, probably because of the relatively high gel concentrations of agarose (1.2%–1.4%) that were used. After hybridization and stringent washing (in 0.1 X SSC and 0.5% SDS at 50°C) (Sambrook *et al.,* 1989), filters were exposed to Kodak XAR-2 X-ray film, generally for 2–3 days.

Informative fragments that were generated using the GGAG primer were either directly sequenced after cleaning the PCR product with QIAquick PCR purification kits (Quiagen), or sequenced from plasmid-cloned fragments purified with Wizard miniprep kits (Promega). Sequencing reactions were run on automated DNA sequencers (ABI 373A or 377, Perkin Elmer), initially using primers complementary to T7 or SP6 promoter regions, following the standard cycle sequencing protocols (ABI, Perkin Elmer) used previously (Lopez and Knowlton, 1997). The following primers were then designed and used to obtain complete sequences for the GGAG 880 and 920 bp fragments: 5′ CCCTGATCAGTATTTTGGG 3′ (880i), 5′ TTGGAATATTTGCCTTACCG 3′ (880f), and 5′ GGAGGGCTCTGTTATTCTATC 3′ (880r). The 880f primer (slightly internal to 880i) matches available *Montastraea* sequences, and when used with primer 880r yielded products of 837 or 804 bp.

Microsatellite analyses

The microsatellite locus Mfra-gttt1 was initially detected in a clone (tub29A) derived from *M. franksi* (no. 426) that was recovered while we were screening for taxonomically

informative β-tubulin introns (Lopez and Knowlton, 1997). Its occurrence and polymorphism in other *Montastraea* species were determined by designing the following 2 oligonucleotide primers, which are complementary to the genomic sequences flanking Mfra-gttt1: Sput1f–5′ AAACA TACGG CCAGT GCTGG 3′ and Sput2rc – 5′ GAAAA GAGCA ATCTT TTGTA TGGTG 3′. The PCR profile used for Mfra-gttt1 amplification from genomic DNA was 94°C/40 s, 60°C/45 s, and 72°C/60 s for 30 cycles. All PCRs shown here were reproducible and included negative controls. The resolution of PCR products was better when 4.0% agarose (Metaphor, FMC) TBE gel electrophoresis was used, and banding patterns were confirmed by polyacrylamide gel (10%) electrophoresis using the entire PCR product (approximately 1 μg DNA).

Results

AFLP band patterns

Band patterns produced using the GGAG primer showed a clear diagnostic difference between *M. faveolata* and *M. franksi* (Fig. 1A), which confirms our previous results obtained with smaller sample sizes (Lopez and Knowlton, 1997). The 920 bp GGAG band was absent from, and the 880 bp band was present in, all 16 *M. faveolata* tested (including 6 previously analyzed), while the reciprocal pattern occurred in 15 *M. franksi* (including 7 previously analyzed). A third band, migrating at around 850 bp, may also occur at significantly different frequencies in the two taxa, but our sample sizes are too limited to test for this.

The ATG primer also provided evidence of genetic difference between *M. faveolata* and *M. franksi:* as previously reported (Lopez and Knowlton, 1997), the 630 band was characteristically present in the former and absent from the latter (Fig. 1B). In this case, however, increasing the sample size indicated that this difference between the species is not fixed: the 630 bp band was present in all 16 individuals of *M. faveolata* tested (including 7 from the previous study), but it also appeared in one individual of *M. franksi* (no. 19, lane 9). The remaining 14 *M. franksi* (including 6 previously studied) lacked this band.

In contrast to the clear differences separating *M. faveolata* from *M. franksi,* no diagnostic bands separated *M. franksi* and *M. annularis.* This was true, not only for the ATG (Fig. 1B) and GGAG primers (five *M. annularis* analyzed, data not shown), but also for 10 additional primers that were screened (data not shown). The ATT primer yielded band patterns with the strongest quantitative differences (Fig. 2), but the differences are not statistically significant by a chi-square test, once a Bonferroni correction (Rice, 1989) for the total number of bands examined is applied (see legend Fig. 2). Moreover, mean average percent difference (MAPD; Yuhki and O'Brien, 1990) in ATT band-sharing values among samples of *M. annularis* (28%) and among samples of *M. franksi* (22%) were very similar to the value calculated for interspecific comparisons between these taxa (27%).

Homology of bands

Southern hybridization with DNA probes derived from the 630 bp ATG bands provided further insights into the nature of genetic differences between *M. faveolata* and *M. franksi.* In general, results were better when the hybridization probes were derived from DNA clones. Multiple bands were labeled when probes were derived from gel-isolated ATG bands, suggesting that the 630 bp bands were contaminated with fragments of different molecular weight.

Southern hybridizations showed that the 630 bp ATG bands found in all *M. faveolata* and one *M. franksi* (no. 19) (Fig. 3A) are homologous (Fig. 3B). Moreover, another *M. franksi* (no. 20) possessed a higher molecular weight fragment that also hybridized to the 630 bp ATG probe (Fig. 3B). This larger fragment may be the same one visible in Figure 3A, and probably arose by one or more DNA insertions at the 630 bp locus. Unfortunately, similar Southern hybridizations using the diagnostic 880 GGAG fragment as a probe were unsuccessful due to our inability to use the preamplification protocol.

We therefore used DNA sequences to evaluate the homologies of the 880 and 920 bp GGAG bands. Partial DNA sequences were initially obtained from both 5′ and 3′ termini of the GGAG 880 (*M. faveolata*) and GGAG 920 (*M. franksi*) fragments. These preliminary sequences permitted the design of PCR primers by which the corresponding locus was amplified from genomic DNA. The GGAG 880 locus was amplified consistently from samples of *M. faveolata* (Fig. 4A), but a larger band appeared for *M. franksi* (Fig. 4A) and *M. annularis* (data not shown) when the same primers were used. DNA sequencing confirmed the homology of PCR products for 9 *M. franksi,* 6 *M. annularis,* and 7 *M. faveolata* (sequences deposited in Genbank AF110114-AF110129, AF112346-AF112351). Three insertions or deletions (22, 8, and 3 bp) constituted the primary differences between *M. faveolata* and the other two taxa (Fig. 4B), as would be expected from the estimated size differences in the 880 and 920 bp bands. There were also 5 nucleotide substitutions [4 transitions, 1 transversion in 837 bp within the GGAG 920 fragment (see methods); data not shown] that distinguished *M. faveolata* from *M. annularis* and *M. franksi.* The sequences exhibited d(AT) contents of 58%–64%, and did not resemble sequences in current databases (GenBank and EMBL; April 1998). The lack of significant open reading frames in the sequences suggests that they do not represent protein-encoding regions.

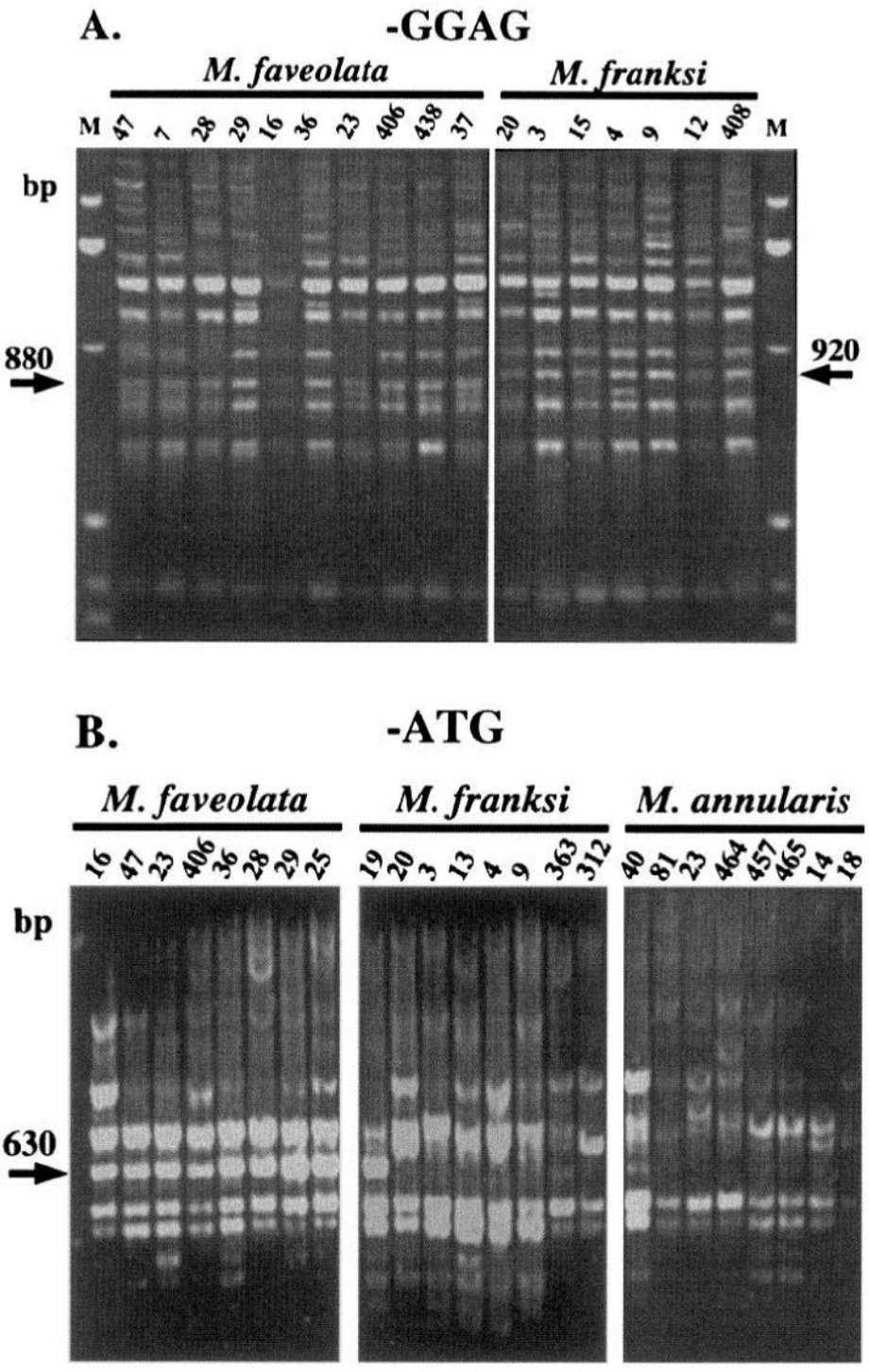


Figure 1. AFLP band patterns. Samples are grouped by species, and individual sample numbers are indicated above each lane. A 1.0 kb ladder (Gibco/BRL, Bethesda) was used as the molecular weight standard. (A) AFLP patterns derived with the GGAG primer. Species-specific bands at 880 and 920 bp are indicated by arrows. (B) AFLP patterns derived with the ATG primer. The 630 bp band is indicated by an arrow.

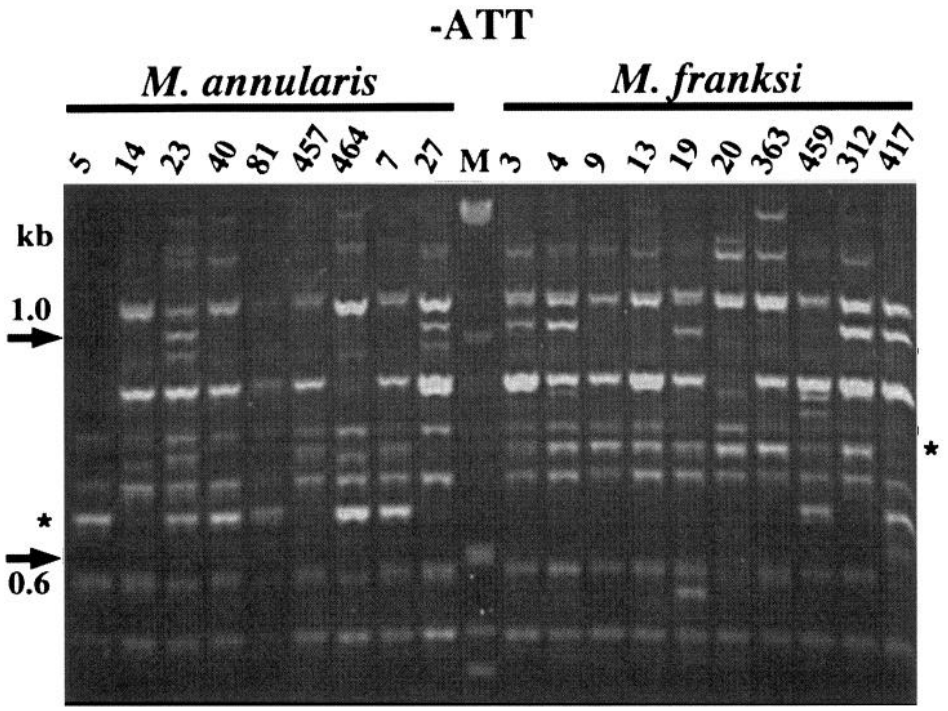


Figure 2. Comparison of *Montastraea franksi* and *M. annularis* AFLP patterns obtained with the ATT primer. Polymorphic bands showing the greatest frequency differences between the species are marked by asterisks. The most extreme frequency difference (band indicated by upper asterisk, present in 5 of 13 versus 12 of 13 individuals of *M. annularis* and *M. franksi,* respectively; not all samples shown) was individually significant (chi-square = 6.1, $P < 0.02$). However, this difference is not significant when a Bonferroni correction (Rice, 1989) for the total number of bands (18) is applied (P must be less than 0.003).

Comparisons of band patterns from gametes, somatic tissues, and zooxanthellae

The ATG patterns for DNA derived from sperm and from somatic tissue were generally consistent, and the taxonomically informative 630 bp band was conspicuous in analyses of somatic tissues from *M. faveolata* (Fig. 5A). Reproducibility for the GGAG primer was poorer due to our inability to use the preamplification protocol, but diagnostic GGAG bands at 920 and 880 bp were visible in analyzed samples of DNA from somatic tissues from *M. franksi* and *M. faveolata,* respectively (Fig. 5B). This suggests that AFLP analyses can be informative with somatic tissues, especially once diagnostic patterns have been established with sperm samples. The general lack of higher molecular weight AFLP bands from analyses of somatic tissue compared to those of gamete samples may be due to degradation during DNA purification of somatic samples (*e.g.,* McMillan *et al.,* 1998) or to the presence of contaminants that interfered with the reactions, but these bands were typically not scored. Some differences between somatic and gamete samples (*e.g.,* for *M. franksi* no. 467 in Fig. 5A) cannot currently be explained.

To determine how zooxanthella-derived bands in particular might confuse the interpretation of analyses of somatic tissues, we obtained AFLP bands from DNA purified from zooxanthella types A, B, and C from *Montastraea* (see Rowan and Knowlton, 1995) (Fig. 6). There may be some potential for confusion between the diagnostic 630 bp band in *M. faveolata* and similarly-sized bands from zooxanthella types A and B, although these zooxanthella bands may in fact be due to coral (animal) contamination. In general, the similarity of the gamete and somatic tissue samples (Fig. 5) and the difference between zooxanthella-enriched and zooxanthella-absent (sperm) samples (Fig. 6), suggest that for these corals, the AFLP technique primarily amplifies coral (animal) DNA from somatic tissue samples.

Microsatellite locus

Analysis of a clone from *M. franksi* derived from a PCR amplification product using primers for β-tubulin revealed a microsatellite locus (Mfra-gttt1) whose core repeat sequence (GTTT) was perfectly repeated 9 times (EMBL accession number AJ223626). It is similar (but not identical) to simple repeats in other scleractinian corals (McMillan *et al.,* 1991). Analysis of additional samples using the same primers revealed that a smaller allele (approximately 160 bp, its size presumably due to

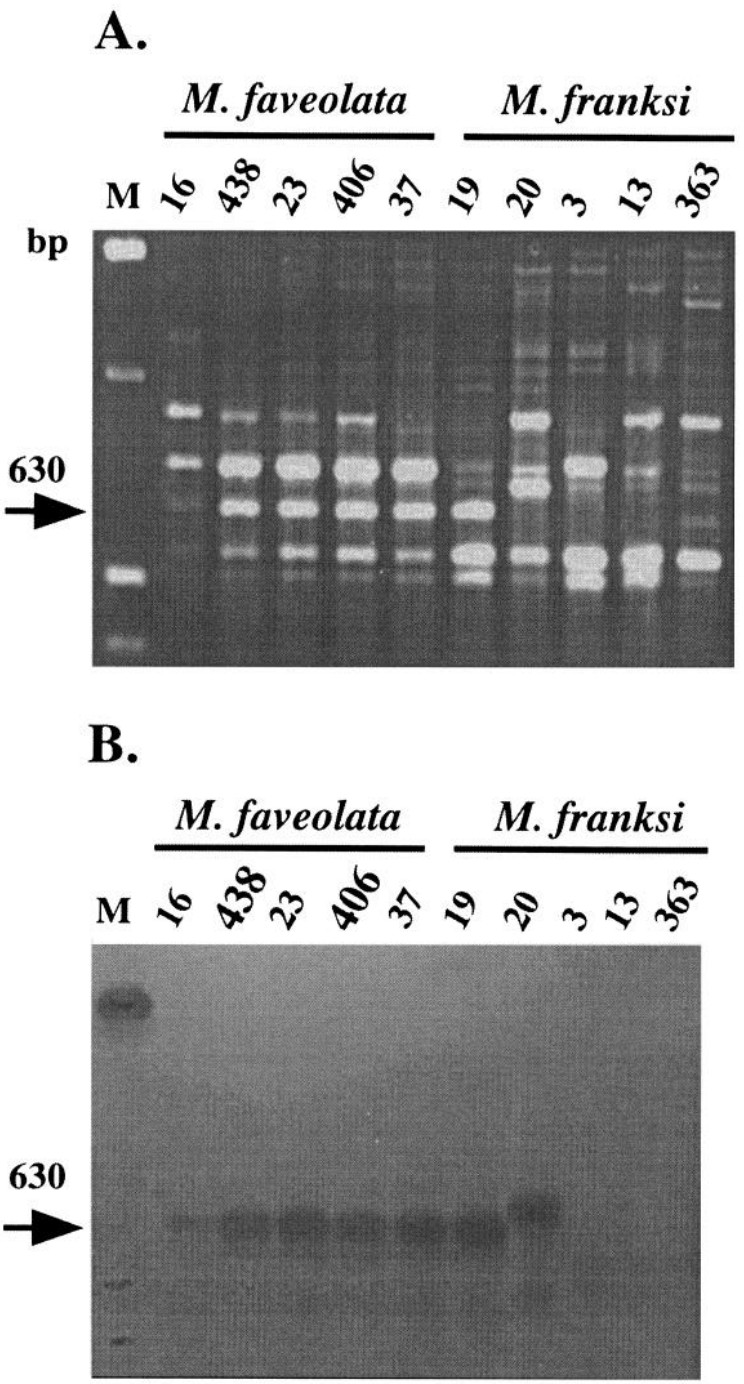
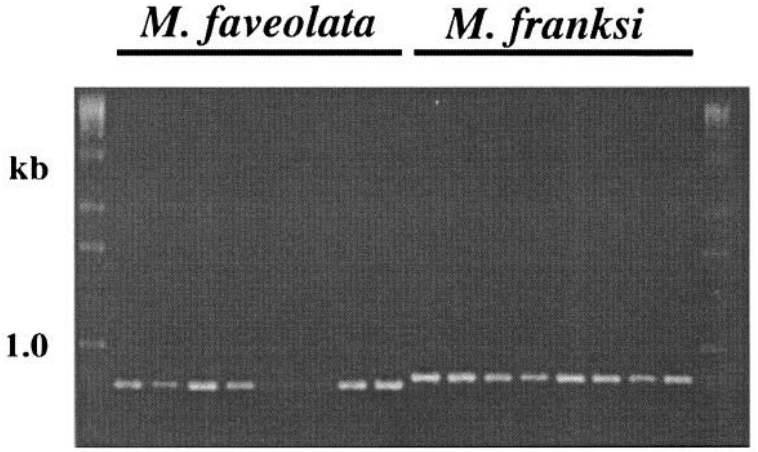


Figure 3. Southern hybridization experiments with the ATG 630 bp fragment to determine band homologies. (A) Ethidium bromide-stained agarose gel used for Southern blotting, showing typical AFLP patterns obtained using the ATG primer. (B) Autoradiograph produced with probe for the ATG 630 bp fragment. A cloned 630 bp ATG fragment was radiolabeled and used for probing the filter of the gel in (A). This fragment also hybridized to the 1.6 kb fragment in the marker lane (M).

a loss of 2–3 repeat units) is the most common allele in both *M. franksi* and *M. annularis* (Fig. 7; Table I). Two individuals appeared to be heterozygous for the 160 bp and 169 bp alleles (*i.e.*, two bands amplified; data not shown). One sample (from *M. annularis* no. 27, Fig. 7) yielded three bands (160 bp, 169 bp, and an intermediate band migrating between them); this pattern suggests the presence of an additional locus, although it could be a

A.



B.

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                          131          141
                           |            |
M. franksi      ACCTATTTCCCTAAA ATTTCTCGC
M. franksi      ............... .........
M. franksi      ............... .........
M. annularis    ............... .........
M. annularis    ............... .........
M. faveolata    .--------------- --------.
M. faveolata    .--------------- --------.
M. faveolata    .--------------- --------.
M. faveolata    .--------------- --------.
M. faveolata    .--------------- --------.

                361          371     501          511
                 |            |       |            |
M. franksi      GAGTTTAACAACT       AACCTTCTGCGTT
M. franksi      .....A.......       .............
M. franksi      .....A.......       .............
M. annularis    .....A.......       .............
M. annularis    .....A.......       .............
M. faveolata    .--------....       ....---......
M. faveolata    .--------....       ....---......
M. faveolata    .--------....       ....---......
M. faveolata    .--------....       ....---......
M. faveolata    .--------....       ....---......
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Figure 4. Agarose gel and sequence alignments showing the differences between the 880 and 920 bp GGAG fragments. (A) Fragments amplified from genomic DNA of *Montastraea faveolata* and *M. franksi* using the *Montastraea*-biased primers. (B) Sequence alignment showing the regions that generate the difference in size of the 880 and 920 bp fragments.

PCR artifact. In contrast, most samples of *M. faveolata* yielded a higher molecular weight smear above 220 bp, rather than discrete 160 or 169 bp bands, when using the same primers and PCR conditions ("null" alleles, Fig. 7). Overall, this microsatellite locus suggests that genetic differences exist between *M. faveolata* and the other two taxa, but determining the precise nature of these differences would require further analyses.

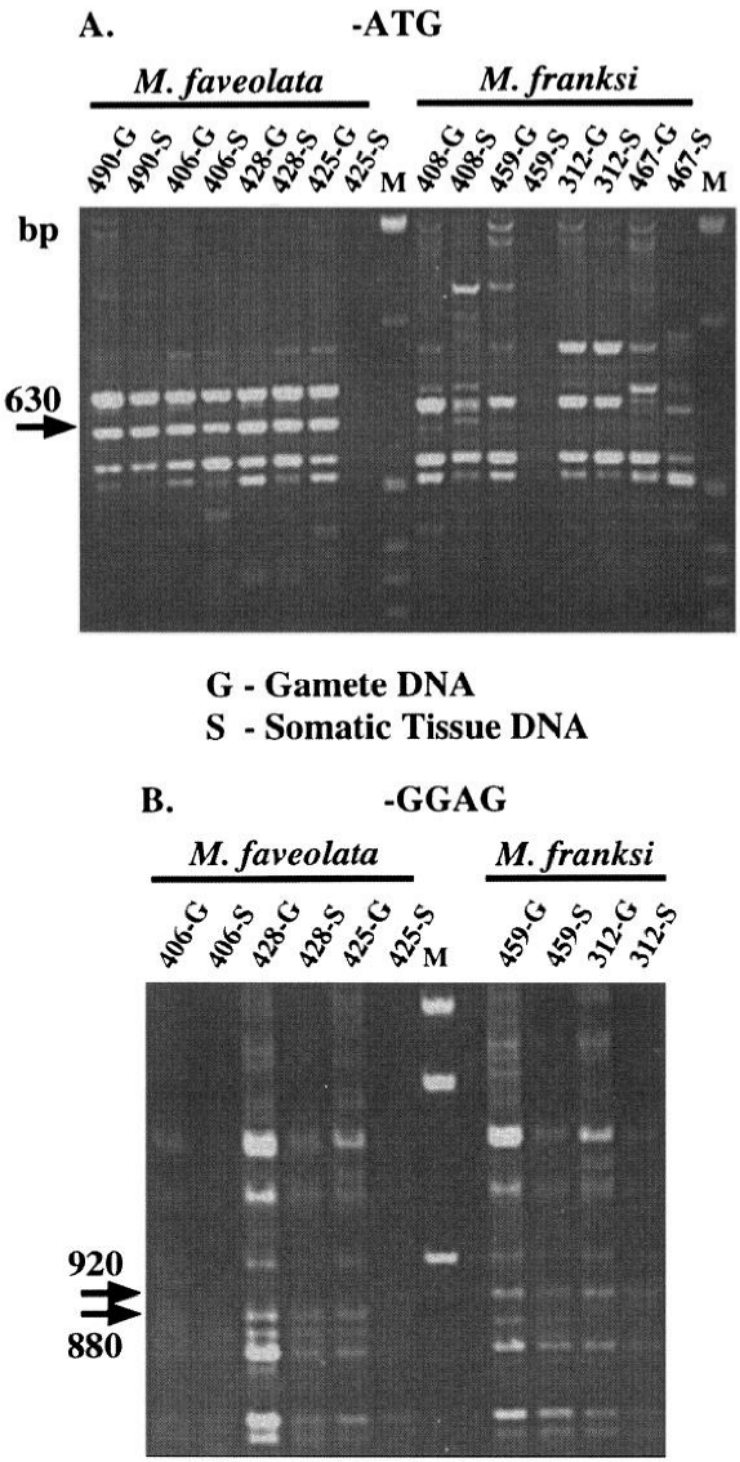

Figure 5. Comparison of AFLP patterns from gametic (G) and somatic (S) tissue samples. DNA derived from sperm and from somatic tissue of the same *Montastraea* colony were analyzed in parallel AFLP-PCRs, using identical conditions. (A) Results from ATG primer. (B) Results from GGAG primer. Diagnostic bands are identified by arrows.

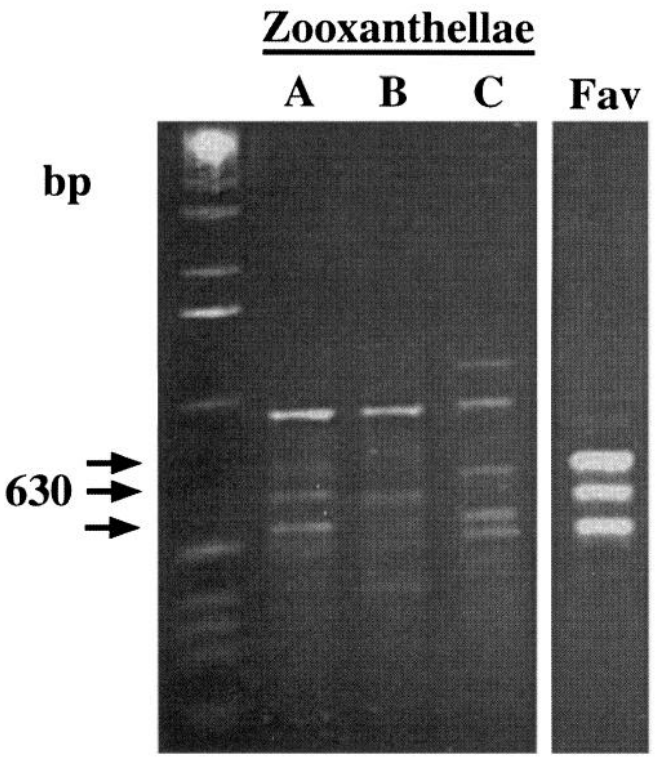

Figure 6. AFLP assay of zooxanthella samples. The ATG primer was used after PCR preamplification of zooxanthella templates (see methods). Identical conditions for AFLP analyses were used on both zooxanthellae and coral (*Montastraea faveolata*) DNA samples. Three faint bands (indicated by arrows) obtained from Type A zooxanthellae appear similar to the three dominant AFLP bands obtained from *M. faveolata* (550, 630, 750 bp) shown in Fig. 1B; these may be due to coral (animal) contamination of the zooxanthella DNA.

Table I

Mfra-gttt1 allele distributions in members of the Montastraea annularis *complex*

	Allele size*/pattern				
Species	160	169	160/169	Null	Total
M. franksi	8	1	1	1	11
M. annularis	7	2	1	1	11
M. faveolata	0	1	0	11	12

* Sizes indicated are approximate (see legend Fig. 7).

Discussion

Status of the three members of the Montastraea annularis *complex*

When the specific status of taxa in sympatry is questionable, multiple, independent, fixed differences provide compelling evidence for the lack of effective interbreeding (Avise and Ball, 1990). The reciprocal presence/absence pattern for the GGAG 880 and 920 bp bands appears to represent one such fixed difference between *M. faveolata* and the other two taxa. In addition, strong frequency differences at the ATG 630 locus and failure to amplify the 160 or 169 bp alleles at the microsatellite locus in most *M. faveolata* also point to the distinctiveness of this species. The significance of these genetic differences is further supported by other biological differences that distinguish the taxa (Tomascik, 1990; Hayes, 1990; Knowlton *et al.,* 1992; Van Veghel and Bak, 1993, 1994; Van Veghel, 1994; Van Veghel and Kahmann, 1994; Van Veghel and Bosscher, 1995; Van Veghel *et al.,* 1996; Weil and Knowlton, 1994; Szmant *et al.,* 1997; Knowlton *et al.,* 1997; Knowlton and Budd, unpubl.).

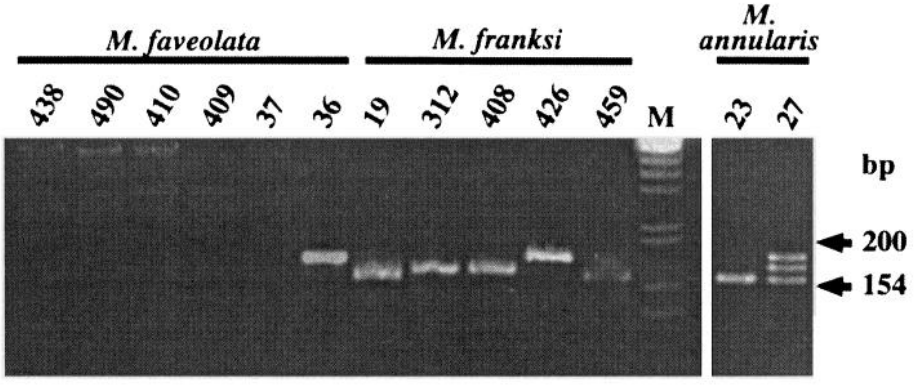

Figure 7. A subset of the *Montastraea* samples assayed for the Mfra-gttt1 microsatellite locus. Sample number and species identity for each coral colony are shown above gel. Sizes refer to two bands in the molecular weight markers (M). Representative "null" Mfra-gttt1 patterns are shown in the first five lanes. Samples from *M. annularis* were run on a separate gel. Samples from two individuals of *M. franksi* (nos. 312, 408) yielded bands that appear to be slightly larger than 160 bp; confirmation of their distinctiveness would require additional analysis.

The nature of a species boundary between *M. annularis* and *M. franksi* remains much more problematic. No technique used to date has revealed fixed genetic differences between them, despite marked differences in both aggressive behavior (Weil and Knowlton, 1994; Van Veghel and Bak, 1993) and the timing of spawning (Knowlton *et al.,* 1997; Szmant *et al.,* 1997). More than rare hybridization would presumably erode the predictable association between colony morphology and these other biological characteristics, but genetic evidence supporting this otherwise reasonable argument is lacking. Nevertheless, negative results for any single gene is weak evidence to support synonymizing species, particularly when, as is the case here, other types of data point to the existence of reproductive barriers. A sobering example of the limitations of negative genetic evidence is provided by Howard *et al.* (1997), who found only six species-specific markers distinguishing two species of oaks, despite having screened 700 10-bp primers.

Molecular characters also provide an ideal means for statistically analyzing the probability of encountering particular combinations of characters, including those that would be expected in an F1 hybrid (Lessios and Pearse, 1996; Boeklen and Howard, 1997; Suchanek *et al.,* 1997; Foltz, 1997). The only individual with an atypical allele for its species (*M. franksi* no. 19, with the ATG 630 bp band characteristic of *M. faveolata;* Fig. 1B) had the typical *M. franksi* band size for GGAG (fig. 2A in Lopez and Knowlton, 1997). This suggests that this individual is not an FI hybrid, although this pattern could reflect introgression. Using these and additional loci to screen for hybrids in natural populations will allow us to determine whether Veron's (1995) proposal of frequent hybridization applies to this species complex. If F1 hybrids are not detected in large surveys, then the rare occurrence of atypical alleles at some loci probably reflects the fact that ancestral polymorphisms have not yet been completely sorted with respect to current species boundaries (Pamilo and Nei, 1988; Moore, 1995).

The finding of genetic differences between *M. faveolata* and the other two taxa in Panama should allow us to determine whether the same patterns occur at other locations within the range of these species. Of particular interest will be sites in the northern Caribbean. Fertilization studies in the Florida Keys do not reveal clear barriers between *M. faveolata* and the other taxa (Szmant *et al.,* 1997), in contrast to results from similar studies in Panama (Knowlton *et al.,* 1997; Levitan and Knowlton, pers. obs.). Occasional colonies that exhibit mosaic growth forms between *M. faveolata* and *M. annularis* have also been observed in both the Bahamas (Knowlton, pers. obs.) and the Dry Tortugas (E. Weil, pers. comm.). The same primers that amplified the Mfra-gttt1 microsatellite locus in Panama amplified a similar 169 bp band in two *Montastraea* colonies of uncertain taxonomic status from the Florida Keys, suggesting that at least some of the markers we have developed for corals from Panama will have broad geographic utility (Cook *et al.,* 1991).

Molecular genetic analyses of scleractinian corals

Until recently, protein electrophoresis was the primary tool for genetic studies of corals, primarily at the level of species (Ohlhorst, 1984; Ayre *et al.,* 1991; Weil, 1993; Potts *et al.,* 1993; Stobart and Benzie, 1994; Weil and Knowlton, 1994; Garthwaite *et al.,* 1994; Miller and Benzie, 1997) and population (Stoddart, 1984a, 1984b; Heyward and Stoddart, 1985; Willis and Ayre, 1985; Ayre and Willis, 1988; Hunter, 1993; Hellberg, 1994). More recently, DNA-based techniques have been used to determine higher level phylogenies (McMillan and Miller, 1990; McMillan *et al.,* 1991; Chen *et al.,* 1995; Veron *et al.,* 1996; Romano and Palumbi, 1996, 1997), and to analyze or recognize species and populations (McMillan and Miller, 1989; Beauchamp and Powers, 1996; Odorico and Miller, 1997; Lopez and Knowlton, 1997; Hunter *et al.,* 1997; Takabayashi *et al.,* 1998). This is, in principle, straightforward given the wide applicability of the methods; but in practice, the scleractinian coral genome has provided several surprises that remain poorly understood. For example, Romano and Palumbi (1996) used mitochondrial 16S rDNA sequences to define two distantly related clades, whose 29% sequence divergence implied a split predating the origin of coral skeletons 240 million years ago. Nevertheless, three individuals contained sequences from both of these highly divergent clades. Odorico and Miller (1997) also found highly divergent ITS and 5.8S nuclear rDNA sequences within single individuals of several *Acropora* species. These patterns could be interpreted as evidence for evolutionary reticulation. However, extensive inter-individual variation without intra-colony variation has also been reported (*e.g.,* 31% sequence variation among 12 individuals of *Stylophora pistillata;* Takabayashi *et al.,* 1998). Individual genes can also show quite different evolutionary patterns in different coral taxa: ITS sequences exhibit modest to considerable variability between congeneric species in *Acropora* (Odorico and Miller, 1997), *Porites* (Hunter *et al.,* 1997), and *Balanophyllia* (Beauchamp and Powers, 1996), but very little between members of the *Montastraea annularis* complex (Lopez and Knowlton, 1997; Szmant *et al.,* 1997). Identical 16S rDNA sequences for corals in different genera (Romano and Palumbi, 1996) are also surprising.

When genetic variation is low, sequencing individual genes may be less efficient than the use of approaches that screen broadly across the genome. Of these, analysis of AFLPs has considerable promise because it is straightforward, relatively inexpensive, and accessible. It is also probably more reproducible than RAPDs, and therefore more suitable for analyses of field samples (*e.g.,* Janssen *et al.,* 1996; Huys *et al.,* 1996; Majer *et al.,* 1996; Folkertsma *et*

al., 1996; this study). Many AFLP loci have already been shown to be inherited in a Mendelian fashion (Vos *et al.,* 1995), although like RAPDs they are dominant markers. Although allozymes remain a valuable tool because of their codominant inheritance and accessibility, the relatively small number of potential loci that can be reliably scored in scleractinians limits their usefulness for discriminating very similar species.

AFLP loci can also be further explored using the standard techniques of molecular biology. These more time-consuming and expensive steps are recommended whenever potential inherent biases in PCR-based methods have not been explored (Vos *et al.,* 1995). More detailed analysis is also essential for understanding the genetic basis of different band patterns and confirming which bands are homologous. The results of our studies of *Montastraea* support the importance of such additional analyses.

For example, the GGAG band pattern differences between *M. faveolata* and the other taxa could in principle have been due to a difference at one locus (resulting in change in fragment size), or differences at two loci (each with a visible band and a null allele). The ability of primers based on the 880 bp band to amplify what appears to be the 920 bp band and the homology of sequences from these amplifications support the former interpretation. When there are many differences between taxa, and distinguishing taxa is the only goal, then knowing the exact number of independent loci is perhaps not a serious issue. However, when there are relatively few loci that distinguish taxa (as is the case here), or when one wishes to recognize hybrids, understanding the basis of observed differences is particularly important.

Interpreting similarity between bands can be likewise complex due to the possibility of comigration of non-homologous fragments (Rieseburg, 1996; Grosberg *et al.,* 1996). Thus we cannot be sure that the AFLP bands shared between *M. annularis* and *M. franksi* are homologous, although this seems likely based on the overall genetic similarity of these two taxa (Van Veghel and Bak, 1993; Weil and Knowlton, 1994; this study). Assessing homology is particularly important in the interpretation of unusual banding patterns—for example, the ATG 630 bp band in a single individual of *M. franksi,* which was found to be homologous to the ATG 630 bp band characteristic of *M. faveolata.*

DNA-based methods for analysis of intraspecific gene flow will be especially difficult when species themselves are poorly defined. For *Montastraea,* there may be a narrow technical window between methods that can detect differences among species, and methods that can detect differences among populations or clones within species (*e.g.,* Coffroth, 1997; Sites and Crandall, 1997). This should be a high priority for future work, as effective conservation biology depends on determining whether regions are genetically interconnected to the extent predicted by current patterns (Roberts, 1997).

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