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CRYPTIC SPECIES OR INTRAGENOMIC VARIATION: IMPLICATIONS FOR THE MILLEPORES

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ABSTRACT

Fire corals of the genus *Millepora* are ubiquitous in tropical western Atlantic reefs. Two distinct morphologies of *Millepora*, currently classified as separate species, exist off the coast of the Bahamas. *M. complanata* have broad, smooth blades and prefer shallow waters whereas *M. alcicornis* have finer, knobby branches and prefer deeper waters. However, due to extensive phenotypic plasticity in the group, a wide range of intermediate growth forms exist that do not fit into either species group.

In order to untangle the evolutionary relationship of the millepores, we initiated a phylogenetic analysis. A maximum likelihood tree constructed from 36 rDNA sequences showed that the millepores separated into two distinct clades suggesting that millepores may be reproductively isolated cryptic species.

Although, phylogenetic analysis based on DNA has helped untangle evolutionary relationships, one of the major concerns with using rDNA is the existence of variability among the repeated rDNA units, which may cause extensive differentiation even within single individuals. The problem concerns the assumption that these repeated sequences have undergone concerted evolution, a process involving homogenization of individual repeats of a multigene family. We have analyzed the variability within rDNA repeats in order to ascertain the level of intragenomic variability within the millepores.

We analyzed twenty rDNA clones isolated from a single *Millepora complanata* colony and found low intragenomic sequence divergence ranging from 0-0.087%. When these 20 clones were compared to *M. exaesa*, the rDNA sequence divergence ranged from 0.649-0.655%. Construction of an unrooted phylogenetic tree also indicated low levels of intragenomic rDNA variation within the millepores. Although intragenomic sequence variation was low, DGGE analysis showed the existence of rare rDNA variants.

These results indicate that using rDNA is a useful tool for phylogenetic constructions in the millepores as long as the level of intragenomic variability is determined.

INTRODUCTION

For centuries, biologists have attempted to group organisms based on shared characteristics in order to understand the evolutionary relationships of the tremendous diversity of life. This construction of evolutionary relationships (phylogenies) is called systematics and allows biologists to ascertain patterns of relationships among species.

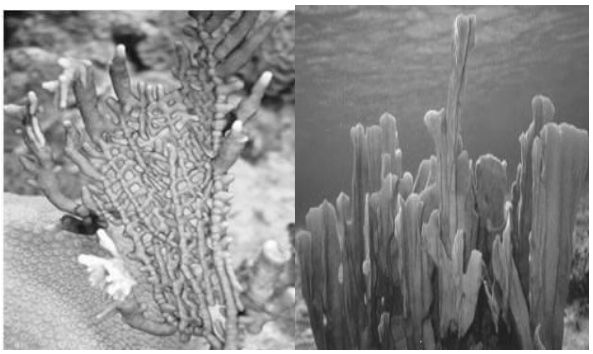
Traditionally, phylogenies are constructed based on similarities. The more similar organisms are (more shared characters), the more closely related they are presumed to be. The opposite is also true. There are numerous problems associated with constructing phylogenies based on similarities. Phylogenetic relationships are estimated using morphological, behavioral, and other phenotypic characters. However, these characters may not accurately represent evolutionary relationships because evolution is not

always divergent (Hendry, 2009; Schluter, 2009). Two species can independently evolve the same features due to similar habitats and favored adaptations. Therefore, two species that are not closely related may end up more phenotypically similar to each other than to their closest relatives.

The problem of understanding evolutionary relationships between organisms is particularly acute in reef-building coral assemblages that serve as the foundation of complex reef ecosystems. The evolutionary history and current speciation in this diverse class of animals remain paradoxical. For example, most corals reproduce in annual, synchronous mass spawning events that provide numerous opportunities for interspecific hybridization. Vollmer and Palumbi (2002) examined *Acropora cervicornis* (elkhorn coral), *A. palmata* (staghorn coral), and *A. prolifera* (fused staghorn coral). They demonstrated that *A. prolifera* was a first-generation hybrid descendent of *A. cervicornis* and *A. palmata* and did not deserve a separate species designation. Hence, coral diversity can occur not only by conventional species formation, but also through inter-species hybridization.

Further compounding the problem is that morphological traits used to construct phylogenies are not useful for corals. Coral taxonomic classification (as well as our current understanding of coral evolution) is based upon morphological characters of the calcareous skeleton (Wallace and Willis, 1994). Unfortunately, the calcareous skeleton of many marine organisms shows considerable phenotypic plasticity. The architecture of the coral skeleton is affected by environmental factors such as underwater irradiance, water motion, water temperature and sedimentation (Barnes and Chalker, 1990). Additionally, calcification rates are affected by lunar, diurnal and seasonal fluctuations (Barnes and Lough, 1989).

The coral phylogenetic issue is particularly problematic in the calcareous hydrozoan coral, *Millepora*, which is one of the most common skeleton-forming animals on reefs. This group of corals, known as "fire-coral" because of its painful sting, is represented by multiple species and is nearly ubiquitous on reefs in the Atlantic, Indian and Pacific Oceans. Millepores are important reef framework builders, second only to the scleractinia (stony or hard) corals (Lewis, 2006). The morphology of the millepores is highly variable and shows phenotypic plasticity (Stearn and Riding, 1973; Lewis, 2006, Tepper et al. 2012). The various growth forms of *Millepora* (Figure 1) in the Caribbean range from thinly encrusting sheets and delicate dendroid branches for *M. alcicornis*, to thicker, rigid bladed forms for *M. complanata* (Stearn and Riding, 1973). It is this variation in morphology that has led to constant controversy about millepore classification. Currently, species designation within the genus is mainly based on growth form, geographical distribution and morphological differences such as surface texture, nematocyst structure, and the size and shape of pores (Lewis, 2006).



A **B**

Figure 1. Photographs depicting the typical growth forms of the species of Millepora found in the Bahamas. ***A*** *Millepora alcicornis* ***B*** *Millepora complanata.*

Tepper et al. (2012) examined the evolutionary relationship of the two commonly found millepores (*M. complanata* and *M. alcicornis*) in the northern Caribbean off the coast of San Salvador, Bahamas. In addition to these two recognizable species, they reported the existence of numerous intermediate forms, of which the specific species status is unknown. Because of the wide range of growth forms (Figure 2), the question arises whether the blade (*M.*

complanata) and branching (*M. alcicornis*) forms are separate species or represent phenotypic variations of one highly variable species.

Abundance surveys were conducted on various patch reefs off the coast of San Salvador in order to address this question (Tepper et al., 2012). Millepore abundance surveys at some reefs suggested that the two standard morphologies were utilizing different habitats since the blade form (*M. complanata*) was found predominately in shallow waters and the branch form (*M. alcicornis*) was found in deeper waters. At other reef locations, they reported the occurrence of all growth forms in mutual proximity. Because both morphs resided in the same location on the reef, they concluded that morphological variation in the millepores is not primarily a response of a single species to environmental differences and that genetic differences exist between the growth forms.

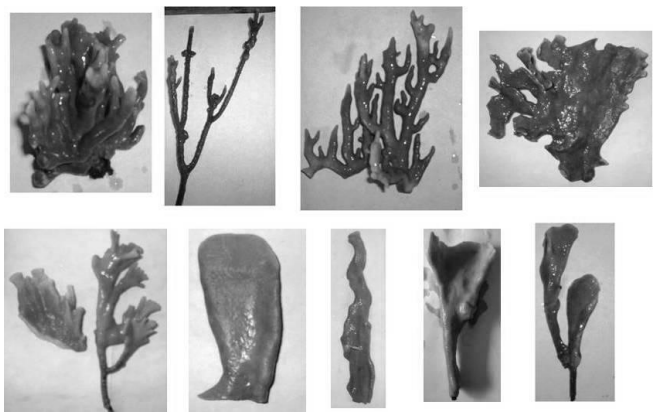


Figure 2. Photographs depicting the wide range of millepore growth forms found on reefs around San Salvador, Bahamas.

The recent use of molecular genetics has established a more accurate assessment of evolutionary relatedness. The inherent uncertainty in using morphological characters as a way to establish phylogenetic relationships can be aided by using genetic markers to distinguish between closely related growth forms. Among the most widely used genetic markers are the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). Ribosomal RNA genes are organized in clusters of hundreds to thousands of tandem repeats per chromosome, each of which consists of three coding regions (18S, 5.8S and 28S), two internal transcribed spacer regions (ITS -1 and ITS-2), one external transcribed spacer (ETS), and one non-transcribed spacer (NTS) region (Prokopowich et al., 2003). The coding regions are evolutionarily conserved and have been used for phylogenetic inference for phylum-level distinctions.

The two ITS regions have been used for detecting differences between conspecifics and are useful in studying closely related individuals due to their rapid evolutionary rates (Hillis and Dixon, 1991). Takabayashi et al. (1998) reported that the ITS region varies from 2 to 31% in different coral species, making this region ideal for comparative analyses between populations. Meroz-Fine et al. (2003) utilized DNA sequence information from the ITS region of the Red Sea fire-coral, *Millepora dichotoma*, to show that the currently recognized single species with two growth forms (blade and branching) was two distinct species. Forsman et al. (2009) reported that they were unable to use morphological characters to identify species diversity in the coral genus *Porites*. Using rDNA ITS regions and mitochondria gene markers, they revealed numerous cryptic patterns of species diversity in *Porites*.

Although phylogenetic analysis based on rDNA has helped untangle evolutionary relationships, the use of rDNA sequences has proven to be problematic because of the existence of variability among the repeated units, which may cause extensive differentiation even within single individuals (Vollmer and Palumbi, 2004). The problem concerns the assumption that these repeated sequences have homogenized via concerted evolution, a process involving homogenization of individual repeats of a multigene family (Zimmer et al., 1980; Elder and Turner, 1995; Sang et al., 1995; Harris and Crandall, 2000; Harpke and Peterson, 2006). Concerted evolution is assumed to produce a uniform sequence in all repeats in a given species. Two mechanisms, unequal crossover and gene conversion, have been proposed to contribute to the process

of concerted evolution (Elder and Turner, 1995). However, evidence exists that concerted evolution may not occur in all species. Vollmer and Palumbi (2004) showed that concerted evolution did not completely homogenize rDNA arrays, thereby accounting for the intragenomic variation they observed in rDNA repeats for the scleractinian coral *Acropora* leading to the construction of false phylogenies. LaJeunesse and Pinzón (2007), using a single colony of *Acropora valida*, reported the existence of 23 unique ITS-2 sequences out of 29 that were sequenced, some differing by up to 28%. Dorado and Sánchez (2009), using the gorgonian coral *Pseudopterogorgia bipinnata*, reported an ITS-2 sequence variation of 14.4% among 37 samples. With the observed high levels of intragenomic variation, using rDNA to construct phylogenies can be problematic. However, phylogenetic analyses of the ITS regions for corals in the genera *Pavona*, *Platygyra*, *Porites* and *Siderastrea* (Lam and Morton, 2003; Moothien et al., 2005; Forsman et al., 2005) demonstrated clear phylogenetic relationships due to the lack of sequence variability in the ITS regions. These results indicate that using the ITS region of rDNA is still applicable as long as the level of intragenomic variability is determined.

Here we report that millepore intraspecific rDNA sequence divergence is consistently lower than interspecific sequence divergence. This implies that concerted evolution is operating in the millepores, and rDNA regions have the potential to be used as a species-specific molecular marker capable of distinguishing millepore species. These results substantiate our conclusions that the millepores in the northern Caribbean are represented by two cryptic species.

METHODS

Study Site and Sample Collection

Millepores were collected from reefs surrounding the island of San Salvador, Bahamas, which is located on the eastern edge of the Bahamas Island chain. Patch reefs included for collection were Lindsay Reef (24°00’32”N, 74°31’59”W), Rocky Point Reef (24°06’25”N, 74°31’17”W), and French Bay (23°56’59”N, 74°32’50”W). Lindsay Reef and Rocky Point Reef are shallow reefs with maximum depths of approximately 5m. French Bay has both shallow (1-5m) and deep (5-10m) patch reefs.

Coral samples were randomly collected from each of the aforementioned reefs by removing a small piece, approximately 4 sq. cm in size. Samples of the various growth forms were held in a flow-through seawater tank for no more than two days before coral DNA was isolated.

DNA Extraction, PCR Amplification and DNA Sequencing

Genomic DNA was isolated from colonies of *Millepora* using a procedure modified from Rowan and Powers (1991) and Lopez et al. (1999). Coral tissue was removed by repeatedly blasting the skeleton with a 50cc syringe containing L buffer (100mM EDTA, 10mM tris, pH 7.6). Coral tissue was centrifuged at 3500 rpm for 10 minutes; the resulting pellet was washed in 10mL of L buffer and re-centrifuged. The tissue pellet was resuspended in 900µL of L buffer and macerated manually with a tissue homogenizer. The homogenate was centrifuged twice at 500 rpm for 10 minutes in order to separate the coral tissue from the liberated zooxanthellae symbiont. Following the addition of 1% (w/v) SDS to the supernatant, the lysate was incubated at 65°C for 30-60 minutes. Pro K (0.5 mg/mL) was added and the lysate was incubated at 37°C for at least 6 hours. NaCl (0.8M) and CTAB (1% w/v) were added and samples were incubated at 65°C for 30 minutes. Nucleic acids were precipitated twice in 70% (v/v) ethanol and 3M sodium acetate (pH 5.2) and centrifuged. Following resuspension of the pellet in dH_2O, the DNA was briefly centrifuged and the supernatant was retained.

ITS rDNA PCR amplification was performed using 100-300ng of template, 60pmol of the coral specific primers A18S (5’GATCGAAC-GGTTTAGTGAGG3’) and

the universal primer ITS 4 (5'TCCTCCGCTTATTGATATGC3') (Takabayashi et al., 1998), 10X Tfl PCR buffer (Promega, Madison, WI), 2.0mM $MgSO_4$, 0.1mM dNTP and 1U of Tfl polymerase. The primers amplify approximately 824 nucleotides (Tepper et al. 2012), which includes 148 nucleotides of the 3' end of the 18S rDNA gene, the entire ITS-1 (242 nucleotides), 5.8S rDNA gene (158 nucleotides) and ITS-2 (216 nucleotides) sequences and the 5' end of the 28S rDNA gene (60 nuleotides). The PCR profile was: 1 cycle of 94°C for 2 minutes; 30 cycles of 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes; and 1 cycle of 72°C for 5 minutes (Meroz-Fine at al., 2003). Amplified PCR products were run on 1.2% (w/v) low melting agarose gels. A discreet, prominent band at 824 bp was excised and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Purified PCR products were ligated into pGEM-T vectors following the manufacturer's protocol (Promega) and transformed into competent JM109 *E. coli* host cells (Z-Competent Transformation Kit, Zymo Research, Orange, CA). Following blue-white selection, positive colonies were harvested and plasmid DNA was isolated using the Zyppy Plasmid Miniprep Kit (Zymo Research). Plasmids containing ITS rDNA inserts were sequenced in both directions with 3.0 pmol of M13 forward and reverse primers. Reagents and reaction conditions for sequencing were as specified by the USB Thermo Sequenase Cycling Sequencing Kit (USB, Cleveland, OH). PCR products were run in 5.5% KB^{Plus} Gel Matrix acrylamide using a LI-COR 4300 DNA Analyzer (LI-COR, Lincoln, NE). Sequence reaction products were analyzed using e-Seq V3.0 (LI-COR). A BLAST query of the National Center of Biological Information's (NCBI) sequence database confirmed that the sequences were of the ITS regions and most similar to other *Millepora* samples.

Phylogenetic Analysis

Maximum likelihood trees were produced using MEGA 4.0.1. The sequence of *Millepora exaesa* (Odorico and Miller, 1997) was used for the outgroup (GenBank, accession no. U65484) and 1000 bootstrap replicates were performed. Nucleotide percent substitution was also calculated from sequence data and compared within and between morphologies using MEGA 4 (Kumar et al., 2008, The Biodesign Institute, Tempe, AZ).

Intragenomic Sequence Variation

A single blade of *Millepora complanata* was broken into four pieces (Figure 3) and DNA was extracted, PCR amplified, cloned and sequenced as described above. From each coral fragment (A-D), five clones were isolated and sequenced.

Sequences were manually aligned to correspond with published alignments. Percent divergence calculations and unrooted phylogenetic reconstructions (based on Maximum Parsimony) were implemented in ClustalW and ClustalX version 2.0 (Larkin et al. 2007). Independent phylogenetic analyses were performed for the combined rDNA fragments (18S, 5.8S, and 28S), and each ITS region using corrected pair-wise percent divergence. Gaps in the data because of insertions/deletions (indels) and microsatellite regions (primarily in the ITS regions) were treated as missing. Percent divergence calculations were based on pair-wise treatments of sequences to better account for length variation because of indels and microsatellites.

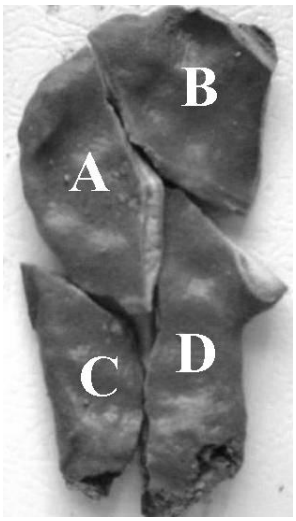


Figure 3. A single M. complanata colony was broken into four pieces. Five rDNA ITS clones were isolated from each piece and within individual DNA sequences were compared.

Denaturing Gradient Gel Electrophoresis (DGGE)

Amplification and primers for PCR-DGGE analysis were as described above except ITS 4 was modified with a 39 bp GC clamp (Sheffield et al. 1989) (5'CGCCCGCCGCGCC-CCGCGCCCGTCCCGCCGCCCCCGCCCTC CTCCGCTTATTGTATG3'). PCR products were run on 6% polyacrylamide denaturing gels containing a 40-80% gradient (2.8M urea/16% formamide to 5.6M urea/32% formamide). Gels were pre-run at 90 volts and 55°C for 30 minutes. Once PCR products were loaded, the gels were pre-heated at 60°C for 10 minutes without voltage followed by 150 volts at 60°C for 20-24 hours. The gels were stained for 1-2 hours in TAE (40 mM tris, 10mM sodium acetate, 1mM EDTA, pH 8) containing ethidium bromide (5 μg/mL) and destained in fresh TAE buffer for 30 minutes. Following staining, the gels were photographed under UV light.

RESULTS

Phylogenetic Analysis

Maximum likelihood (ML) analysis of rDNA sequences produced consensus topologies with two major clades (Figure 4). The two clades each contained members of all three morphologies (bladed-*M. complanata,* branched-*M. alcicornis,* and intermediate growth forms). Bootstrap values for the two main clades of the tree are represented as a percentage and were 75% for both clades.

Ribosomal DNA Variation within Individuals and between Species

Twenty different clones were isolated and sequenced from a single colony of *M. complanata* (Figure 3). Of the 20 rDNA clones, 11 (55%) were identical in sequence. The amplified rDNA fragment (781 bp, not including primers) contained partial fragments of 18S (127 bp) and 28S (39 bp) rDNA, all of the 5.8S (158 bp), and the ITS rDNA regions, ITS-1 (242 bp) and ITS-2 (215 bp). No length variation was observed in any of the rDNA sequences. The average sequence divergence among clones within a single *M. complanata* colony was 0.025% with a range of 0-0.087% (Table 1). The expected Taq polymerase cloning error rate is approximately 0.01% (Eckert and Kunkel, 1990). Within individual rDNA variation was observed across four of five rDNA gene regions. The lone exception was the partial 18S rDNA sequence, which displayed no variation in the twenty clones. The levels of within-individual variation were highest for the ITS regions, reaching 0.172% in ITS-2 and 0.017% in ITS-1. Among the three rDNA genes, the variation ranged from 0 (for all three rDNA genes) to 0.026% for the partial 28S rDNA fragment.

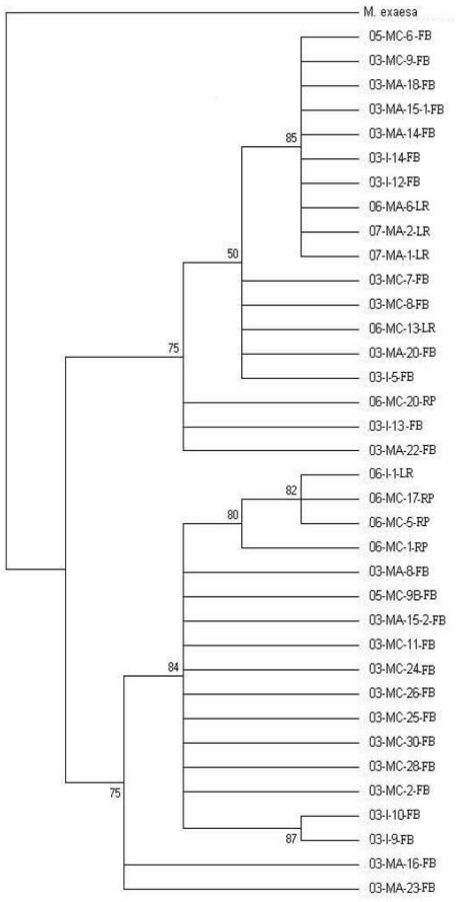


Figure 4. Maximum likelihood tree showing bootstrap values (branchpoints) for 36 samples of Millepora from the full range of phenotypes. 03, 05 or 06 denote the year the sample was collected (2003, 2005 or 2006). MA = Millepora alcicornis, MC = Millepora complanata, I = intermediate growth form. Numbers after the species designations represent the sample number. FB = French Bay, LR = Lindsay Reef and RP = Rocky Point. M. exaesa was used as the outgroup.

Phylogenetic reconstructions based on maximum parsimony were used to analyze the level and pattern of diversity among sequence variants generated from the 20 *M. complanata* clones. Unrooted phylogenies of the cloned rDNA sequences are presented in Figure 5. All 20 sequences from *M. complanata* were tightly clustered together and showed little divergence. The published sequence of *M. exaesa* (Odorico and Miller, 1997) occupied a distant phylogenetic position in the reconstruction, indicating its divergence from *M. complanata*.

	Millepora complanata	***Millepora exaesa***
Overall rDNA Avg (range)	0.025 (0-0.087)	0.653 (0.649-0.655)
18S rDNA Avg (range)	0 --	0.126 --
ITS-1 rDNA Avg (range)	0.012 (0-0.017)	0.696 (0.694-0.703)
5.8S rDNA Avg (range)	0.003 (0-0.013)	0.356 (0.354-0.361)
ITS-2 rDNA Avg (range)	0.047 (0-0.172)	0.691 (0.689-0.703)
28S rDNA Avg (range)	0.005 (0-0.026)	0.054 (0.051-0.077)

Table 1. Ribosomal DNA variation (percent divergence). Average pairwise sequence divergence (%) among 20 sequenced M. complanata clones showing levels of rDNA variation within an individual coral colony and between 20 sequenced clones of M. complanata and a single M. exaesa sequence from Odorico and Miller (1997).

Maximum sequence divergence between *M. complanata* and *M. exaesa* was 0.655% overall and 0.703% in ITS-1 and ITS-2 (Table 1). As observed for within colony variation, interspecific rDNA variation was highest in the ITS regions and lower in the rDNA genes.

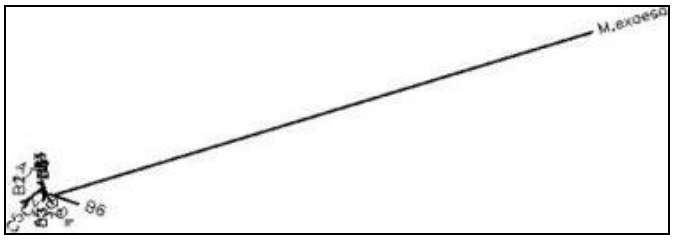


Figure 5. Unrooted phylogenetic reconstruction using Maximum Parsimony based on 20 rDNA clones isolated from a single colony of M. complanata and compared to a single M. exaesa sequence from Odorico and Miller (1997).

Assessment of Intragenomic Variation using DGGE

Although the average rDNA sequence divergence was low, denaturing gradient gel electrophoresis (DGGE) analysis demonstrated the existence of intragenomic variants for the rDNA sequences. The amplified rDNA sequences of a single colony of both bladed and branched forms showed both dominant and rare variants of rDNA (Figure 6). Although only two individuals were analyzed, DGGE is a sensitive method useful in detecting intragenomic variants with up to one nucleotide difference. The DGGE gel results show that although there are variant rDNA forms, the low levels of genetic divergence observed are a result of a prevalent rDNA form and a minor variant form. The results for the bladed sample (MC-3 in Figure 6) indicate that the low levels of sequence divergence and the tight clustering of the 20 cloned rDNA fragments observed in the unrooted tree are the result of low representation of the rare variant form. Additionally, the sequence difference(s) in the rare variant and dominant forms are presumably minor. Although intragenomic rDNA variation is high in some genera of corals (Vollmer and Palumbi, 2004 for *Acropora* and Dorado and Sánchez, 2009 for *Pseudopterogorgia*), the low levels of sequence divergence in rDNA observed here make PCR amplification followed by rDNA cloning and sequencing a reliable method for understanding the phylogenetic relationship of the millepores.

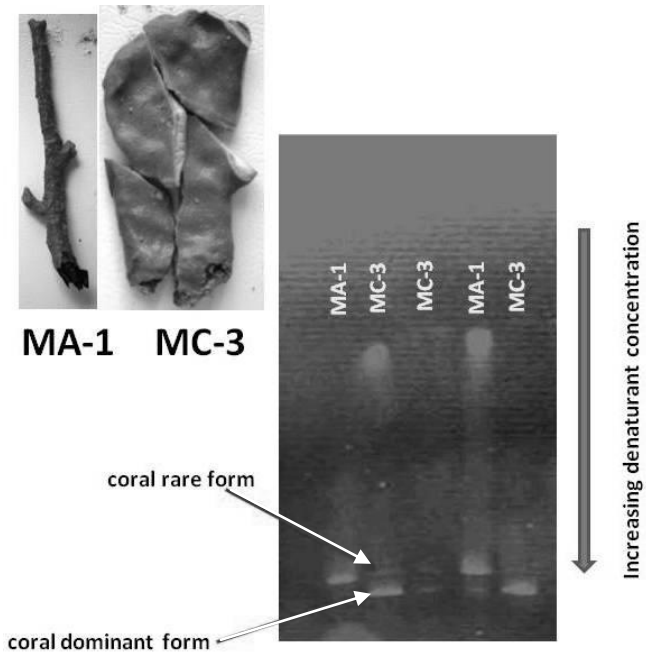


Figure 6. PCR-DGGE fingerprint analysis of the amplified rDNA region from both branched (MA-1) and bladed (MC-3) Millepora forms. Arrows indicate the appearance of prominent and rare rDNA sequences.

DISCUSSION

The taxonomy of the millepores is currently based on morphological characters and does not take into account genetic differences that may be present (de Weerdt, 1981). Results from the rDNA sequence comparison of the two purported species of *Millepora* and the intermediate growth forms show that the three morphologies are very closely related. However, the rDNA ITS region exhibits considerable divergence when compared to the reproductively isolated *M. exaesa* found in the Red Sea.

The phylogenetic tree (Figure 4) generated from these sequences demonstrated the presence of two clades, each containing members of all three morphologies, which further suggests the current taxonomy of *Millepora* may not be accurate. The analysis suggests the existence of two cryptic clades that are reproductively isolated. The intermediate

growth forms do not appear to be a result of hybridization.

Eukaryotic genomes typically contain hundreds to thousands of tandemly repeated copies of the 18S, 5.8S and 28S genes, and the two internal transcribed spacers, ITS-1 and ITS-2 (Prokopowich et al., 2003). As part of a multigene family, the numerous copies of rDNA are expected to become homogenized through mechanisms of concerted evolution (Elder and Turner, 1994). Homogenization leads to greater similarity among members of a repeated sequence family within a species, than between species and is the reason why rDNA sequences have been extensively used for species-level phylogenetic analysis (Hillis and Dixon, 1991). However, basing species-level phylogenetic reconstructions on ITS regions of rDNA can be problematic because of incomplete homogenization due to the existence of polymorphisms among repeated units, which may cause extensive differentiation even within single individuals (Suh et al., 1993).

Phylogenetic studies based on rDNA have been used extensively to provide numerous insights into scleractinian coral evolution (Lopez and Knowlton, 1997, Odorico and Miller, 1997, Lam and Morton, 2003, Chen et al., 2004, Forsman et al., 2005, and Moothien et al., 2005). However, Vollmer and Palumbi (2004) indicated that individual coral colonies contain high levels of intragenomic variation, and rDNA sequences may not be suitable for species-level phylogenetic analysis. Although most of the intragenomic variation was uncovered in the genus *Acropora*, the results implied that the rate of concerted evolution, and hence homogenization, of repeated sequences is quite variable in corals. This high rate of intragenomic variation may be due to interspecific hybridization as well as incomplete concerted evolution in some coral species (Vollmer and Palumbi, 2004).

The level of intragenomic variation present in *Millepora* is low as seen in the pairwise percent divergence data (Table 1). In addition to the low levels of average sequence divergence in the three conserved rDNA genes (0% for the partial 18S, 0.003% for the complete 5.8S and 0.005% for the partial 28S), the sequence divergence for both ITS regions was also extremely low (0.012% for ITS-1 and 0.047% for ITS-2), indicating that concerted evolution mechanisms may be in operation in the millepores. This conclusion is also supported by the tight clustering of intragenomic sequences in the unrooted phylogenetic tree (Figure 5). The low level of intragenomic variation makes sense because the secondary structures of both ITS-1 and ITS-2 play a major role in the maturation of rRNA and little tolerance of changes are expected as a result of the ITS splicing machinery (Cote and Peculis, 2001). Hence, only variants carrying minor changes in DNA sequence are tolerated. This assumption is supported by the closely related prominent and rare variants observed for both bladed and branched forms of *Millepora* on the DGGE gel (Figure 6). Since DGGE analysis is based on the melting characteristics of the double-stranded PCR products, variant bands that are located close together are expected to have small changes in DNA sequence.

On the other hand, the pairwise sequence percent divergence data for the bladed form of *Millepora* compared to *M. exaesa* ranged from a low of 0.051% for the partial 28S to a high of 0.703% for both ITS-1 and ITS-2 (Table 1).

Our study validates the use of rDNA as a phylogenetic tool to determine species-level distinctions in the millepores and confirms the work of Chen et al. (2004) who showed that high intragenomic rDNA sequence diversity seems to be unique to *Acropora*. The percent sequence divergence data clearly showed that there are low levels of intragenomic rDNA variation in *Millepora*, suggesting that the phylogenetic signals are informative for species-level comparisons. Similar patterns of low intragenomic variation have been reported for other corals (*Platygyra,* Lam and Morton, 2003; *Siderastrea*, Forsman et al., 2005; and *Pavona*, Moothien Pillay et al., 2005).

Although the millepores may be an exception, other lines of evidence indicate that traditional cloning methods overestimate the intragenomic diversity (Lajeunesse and Pinzón, 2007). In order to avoid using phylogenetic

markers that can lead to false phylogenies, excising bands from DGGE gels for sequence analysis allows for the detection of prevalent and rare intragenomic variants that may be scored as distinct phylogenetic signals by traditional cloning methods (Dorado and Sánchez, 2009).

Although most phylogenetic studies use primary DNA sequence information, Chen et al., (2004) have shown that rRNA secondary structures are useful in phylogenetic reconstructions because they contain characters not found in the primary structure. The transcript folding structure of the ITS-2 region provides the proper orientation for the ribosomal coding regions when they are processed into small and large rRNA. Functional ITS sequences fold themselves to form secondary structures that are conserved and can be used as diagnostic indicators of taxonomic difference. ITS predicted secondary structure has been useful in providing reliable phylogenetic information in many corals including scleractinians (with the exception of *Acropora*) (Chen et al., 2004), the hexacoral *Zoanthus*, (Aguilar and Reimer, 2010), and octocorals (Grajales et al., 2007 and Dorado and Sanchez, 2009). Grajales et al. (2007) reported that predicted ITS-2 secondary structure led to the construction of new phylogenetic relationships for the octocoral species, *Eunicea*. We are beginning to examine whether predicted rRNA ITS-2 secondary structure can aid in untangling the phylogenetic relationships of the millepores.

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