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Polymorphic Microsatellite Loci for Endemic *Mussismilia* Corals (Anthozoa: Scleractinia) of the Southwest Atlantic Ocean

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In the Southwest Atlantic, coral reefs are unique due to their growth form, low species richness, and a high level of endemic coral species, which include the most important reef builders. Although these reefs are the only true biogenic reefs in the South Atlantic Ocean, population genetic studies are still lacking. The purpose of this study was to develop a suite of microsatellite loci to help gain insights into the population diversity and connectivity of the endemic scleractinian coral with the largest distributional range along the Southwest Atlantic coast, *Mussismilia hispida*. Fourteen microsatellite loci were characterized, and their degree of polymorphism was analyzed in 33 individuals. The number of alleles varied between 4 and 17 per loci, and H_o varied between 0.156 and 0.928, with 2 loci showing significant heterozygote deficiency. Cross-amplification tests on the other 2 species of the genus (*Mussismilia braziliensis* and *Mussismilia harttii*) demonstrated that these markers are suitable for studies of population diversity and structure of all 3 species of *Mussismilia*. Because they are the most important reef builders in the Southwest Atlantic, the developed microsatellite loci may be important tools for connectivity and conservation studies of these endemic corals.

Subject areas: *Conservation genetics and biodiversity; Population structure and phylogeography*

Key words: *conservation genetics, endemic species, hypervariable markers, Mussidae*

Coral reefs along the Southwest Atlantic coast are unique due to their low scleractinian species richness combined with a high degree of endemic species (36%), which include the 3 most important reef building species (*Mussismilia hispida*, *Mussismilia braziliensis*, and *Mussismilia harttii*; Laborel 1969). As with most coral reefs around the world, these coral reefs are suffering from a variety of anthropogenic impacts, such as overfishing, eutrophication, among others. Although at slow pace compared with many reef ecosystems, conservation efforts are being undertaken, for instance, with the establishment of marine protected areas (Castro and Pires 2001) and coral reefs environmental education programs (Projeto Coral Vivo; http://coralvivo.org.br/).

One of the key objectives in marine conservation has been to understand population connectivity (Cowen and Sponaugle 2009) because it maintains high levels of genetic diversity and prevents the reduction of the effective population size, which are, both, crucial in terms of resilience to natural and anthropogenic disturbances (Frankham 2005). The knowledge of genetic diversity and gene flow among populations of corals can help reef conservation management by determining, for example, if a population is suffering from bottleneck or inbreeding depression and which populations are source and sink of recruits (Baums 2008). Additionally, the knowledge of genetic diversity and connectivity can help determine the ideal size and location of marine protected areas (Baums 2008, Underwood et al. 2009).

The degree of reef connectivity can be assessed through estimates of gene flow using molecular tools (Roberts et al. 2006). In the case of scleractinian corals, the most efficient molecular tool for studying population connectivity has been the use of hypervariable markers, such as

microsatellites (Van Oppen and Gates 2006, Baums 2008), because mitochondrial markers are extremely conserved in these animals (Shearer et al. 2002). So far, there are no studies that have assessed genetic diversity or gene flow among coral populations in the Southwest Atlantic coast, even though these are the only true biogenic coral reefs in the South Atlantic Ocean.

The coral *M. hispida* is endemic to the Atlantic coast of South America and is one of the most important reef builders with the greatest distribution along the coast (>2400 km; Castro and Pires 2001). This species occurs over a wide latitudinal and ecological range, occurring from the state of Maranhão (00°53'00"S; 044°16'00"W) southward to São Paulo state (24°19'18"S; 46°10'57"W), Brazil. *Mussismilia hispida* is a hermaphroditic broadcast spawner that liberates gamete bundles yearly at different times of the year depending on the locality (Pires et al. 1999; Neves and Pires 2002). This asynchrony in gamete production among localities corroborates the necessity of population genetics studies to better understand the larval dispersal capacity of this species. Compared with *M. hispida*, the other 2 species have more restricted distributions, with *M. harttii* occurring from the state of Ceará southward to Bahia state and *M. braziliensis* being endemic to Bahia, Brazil (Castro and Pires 2001). The present study developed a suite of microsatellite loci for *M. hispida*, which were also tested and proved to be polymorphic in the other 2 *Mussismilia* species (*M. braziliensis* and *M. hartii*). These loci will be important tools for the assessment of genetic diversity and population structure of these species. Such studies could greatly help the evaluation of the conservation status of the species and future management strategies along the Brazilian coast.

Materials and Methods

Sampling and DNA Extraction

Because *M. hispida* harbors a symbiotic dinoflagellate (zooxanthellae = *Symbiodinium* spp.), zooxanthellae-free animal genomic DNA was obtained from sperm, which was collected during spawning on 6th August 2010. Colonies of *M. hispida* were collected from Recife de Fora reef in Porto Seguro, Bahia state, Brazil (16°24'S; 038°59'W) and placed in a semiclosed tank system at "Projeto Coral Vivo" base in Arraial d'Ajuda, Bahia, Brazil. During the release spawning, 5–10 gamete bundles, free of zooxanthellae, were collected from one of the colonies and placed in 15-ml centrifuge tubes, which were filtered through a 50-µm plankton mesh in order to separate the sperm from the high lipid oocytes. The sperm with seawater were centrifuged to concentrate the sperm and placed in a lysis solution of CHAOS (4 M Guanidine Thiocyanate, 0.5% n-Lauroylsarcosine Sodium, 25 mM Tris–HCl pH 8.0, 0.1 M B-mercaptoethanol; Fukami et al. 2004). The developed microsatellite loci were tested on 33 individuals of *M. hispida* collected also at Recife de Fora, Bahia, Brazil. Additionally, loci were tested for polymorphism in 4 individuals each of *M. braziliensis* and *M. harttii*, collected at the same locality as *M. hispida*. DNA extractions of all specimens were performed with the Phenol:Chloroform method described in the study by Fukami et al. (2004).

Microsatellite Development

Microsatellites were isolated from an enriched partial genomic library following the protocol of Bloor et al. (2001). The high molecular weight genomic DNA (10 µg) was digested with the restriction enzyme Sau IIIA (Jena Bioscience), which was then ligated to phosphorylated double-stranded linkers and size selected (between 500 and 1000 bp) through excision from a 2% agarose gel. The DNA was purified using a GFX PCR DNA and Gel Band Purification Kit (GE) following the manufacturer's instructions. Fragments were hybridized with biotinylated $(CA)_{12}$ and $(CAA)_8$ probes and isolated using streptavidin-coated magnetic beads (Invitrogen). A PCR primed with the forward linker oligo was used for enrichment of DNA containing microsatellites. Enriched fragments were cloned using pGEM-T vectors (Promega) and One Shot TOP10 competent cells (Invitrogen). Recombinant clones were identified by black/white screening on S-Gal/LB/Agar (Sigma-Aldrich) plates with ampicillin (100 µg/mL). The presence of a microsatellite insert was confirmed by 2 or more PCR products after amplification using the forward linker oligo and (nonbiotinylated) microsatellite oligos as primers. Forty-three positive clones were sequenced in both directions in an ABI3500 (Applied Biosystems) automated sequencer. Sequences were edited using SeqMan (DNAStar). Sequences from all 43 clones have been deposited at GenBank (accession numbers KJ577491–KJ577533) in compliance with the Journal's data archiving policy.

Twenty-eight pairs of primers flanking microsatellite regions were designed using the Primer3 Input program (http://frodo.wi.mit.edu/) together with the OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/applications/oligoanalyzer/). Because we used the tailed primer method (Schuelke 2000), all forward primers were synthesized with a M13 tail at their 5' end (TGT AAA ACG ACG GCC AGT), so that primers labeled with different dyes (6-FAM, VIC, NED, or PET) could anneal to the newly replicated strand during PCR reactions. Hence, all PCR reactions contained 3 primers: A tailed forward primer (with the M13 tail), a labeled primer (M13 with VIC, NED, PET, or 6-FAM fluorescent dyes), and a reverse primer. Each PCR reaction consisted of 1U GoTaq (Promega), 1× PCR Buffer (Promega), 0.20 mM dNTPs (Invitrogen), between 1.5 and 2.5 mM $MgCl_2$ (Table 1), 10 µg bovine serum albumin (Invitrogen), 0.2 µM of tailed primer, 0.4 µM of labeled primer, and 0.8 µM of reverse primer in 10 µL reactions with approximately 5–10 ng of DNA template. Cycling conditions were 95 °C, 3 min, 5 cycles at 95 °C, 30 s; 52 °C–62 °C (Table 1), 30 s; 72 °C, 45 s, 30 cycles at 92 °C, 30 s; 52 °C–62 °C, 30 s; 72 °C, 55 s, and a final extension at 72 °C for 30 min. PCR products were pooled with GS600-LIZ size standard (Applied Biosystems) and sized using the automated sequencer ABI3500 (Applied Biosystems). The same labeled

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Table 1 Description of the 14 loci developed for *Mussismilia hispida* (N = 33), with their respective GenBank Accession Numbers, showing which loci amplified individuals of *Mussismilia braziliensis* (Mbr, N = 4) and *Mussismilia harttii* (Mha, N = 4) and how many alleles were found for each species

Locus/GenBank Accession Number	T_a (°C)	$MgCl_2$ (mM)	Size range (bp)[a]	N	A	H_e	H_o	F_{IS}	P value*	Mbr	Mha
Mhi1/KF609532	60	2.5	170–204	33	9	0.638	0.697	−0.094	0.221	4	3
Mhi2/KF609533	56	1.5	176–404	33	17	0.894	0.697	**0.223**	**0.007**	4	7
Mhi4/KF609534	62	2.5	86–239	33	7	0.811	0.667	0.180	0.032	2	4
Mhi5/KF609535	56	1.5	163–393	28	16	0.928	0.643	**0.311**	**0.004**	5	4
Mhi14/KF609536	56	2.0	219–295	33	9	0.761	0.788	−0.036	0.457	3	4
Mhi16/KF609537	58	2.0	174–197	31	5	0.156	0.129	0.172	0.207	2	1
Mhi17/KF609538	54	2.5	168–184	33	4	0.443	0.455	−0.026	0.525	2	4
Mhi18/KF609539	52	2.5	164–306	29	12	0.644	0.552	0.146	0.064	2	2
Mhi20/KF609540	58	2.5	172–231	32	16	0.854	0.742	0.133	0.046	3	4
Mhi21/KF609541	52	2.5	192–221	31	10	0.840	0.581	**0.312**	**0.004**	4	4
Mhi23/KF609542	58	2.0	217–257	33	14	0.902	0.727	**0.196**	**0.007**	3	5
Mhi24/KF609543	54	2.0	113–160	27	11	0.850	0.926	−0.092	0.189	1	3
Mhi26/KF609544	54	2.0	164–220	33	11	0.856	0.849	0.009	0.543	2	4
Mhi27/KF609545	56	2.0	151–181	33	9	0.819	0.849	−0.036	0.457	x	x
Across loci					**10.7**	**0.664**	**0.731**	**0.107**	**0.004**		

An x denotes unsuccessful amplification in Mbr and Mha; T_a = primer's annealing temperature; N = number of individuals genotyped; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; F_{IS} = inbreeding coefficient—significant departures from HWE, as verified by False Discovery Rate procedures, are in bold.

[a]Allele size discounting the tailed extension of the primers.

*Unadjusted P values.

primer was used for each microsatellite locus across all amplifications to avoid problems of dye shift (Sutton et al. 2011). Genotypes were determined using the program GeneMapper v. 4.1 (Applied Biosystems).

Data Analyses

Observed and expected heterozygosities were calculated using the program Genetix 4.04 (Belkhir et al. 2002), whereas deviations from Hardy–Weinberg (HW) and linkage equilibrium conditions were tested using FSTAT (Goudet 1995). HWE test was based on permutations of alleles among individuals using the inbreeding coefficient F_{IS} (Weir and Cockerham 1984; Goudet 1995). The occurrence of null alleles was investigated using the program Micro-Checker (Van Oosterhout et al. 2004). In all multiple tests, the false discovery rate (Pike 2011) was used to verify significance levels (Table 1).

Results and Discussion

The Brazilian coast not only has the only true biogenic coral reef system in the South Atlantic Ocean, but it also has a unique reef system characterized by distinct growth forms (i.e., *Chapeirões*) occurring in areas with exceptionally high levels of sedimentation (Leão et al. 1988). Even though the most important reef builders along the coast are endemics, population genetics studies are inexistent for Southwestern Atlantic corals, possibly due to the lack of adequate markers (Ridgway and Gates 2006).

In the present study, 14 loci were developed and used to analyze 33 individuals of the endemic scleractinian coral *M. hispida* (see Supplementary Material online). Of the 14 pairs of primers developed, 3 were dinucleotide (Mhi1, Mhi2, and Mhi18) and 11 trinucleotide, a similar proportion found by Casado-Amezúa et al. (2011) using di- and trinucleotide probes for the coral *Cladocora caespitosa* in the Mediterranean.

An average of 11 alleles were found among all 14 loci with a minimum of 4 (Mhi17) and a maximum of 17 (Mhi2). H_o values varied between 0.156 and 0.928, whereas H_e values varied between 0.129 and 0.926 (Table 1). Four loci (Mhi5, Mhi21, Mhi23, and Mhi2) had significant heterozygote deficiency in the HWE deviation analyses. Heterozygote deficiencies in microsatellite analyses seem to be common among scleractinian corals (Underwood et al. 2006; Van Oppen et al. 2007, among others) and could be due to restricted gamete dispersal, inbreeding, or the Wahlund effect (Maier et al. 2005; Underwood et al. 2007). Two of those loci (Mhi5 and Mhi21) had high significant homozygote excesses in the Micro-Checker analyses, suggesting the possible presence of null alleles (Table 1). Results of the Micro-Checker showed no evidence of scoring errors or large allele dropout for all 14 analyzed loci.

In cross-amplification analyses with individuals of *M. braziliensis* and *M. harttii*, 13 of the 14 loci amplified in both species (Table 1). Most of the amplified loci also proved polymorphic in these species, with the exception of Mhi16 in *M. harttii* and Mhi24 in *M. braziliensis*, although this could be due to the low number of individuals sampled. The successful transferability extends the use of these markers to the 2 other extant species of the genus *Mussismilia*, making them useful for studies of gene diversity, connectivity, and conservation of the 3 most important coral reef builders of the Southwest Atlantic.

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Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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