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PRIMER NOTE

Noncoding mitochondrial loci for corals

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Abstract

We developed five degenerate primer pairs for the amplification and sequencing of two noncoding regions found in the mitochondrial genome of corals. These primers amplify products ranging from 380 to 950 bp, and work in a wide variety of scleractinian taxa from both the Pacific and Caribbean. Based on our initial analysis of ~300 sequences from 13 scleractinian taxa, both these noncoding regions appear to have equivalent levels of variability to the most variable of previously published coral mitochondrial loci, but work in a wider variety of taxa. We believe these primers will be of use to coral biologists studying questions above the level of species; as with other mithochondrial DNA markers in corals, these loci will likely provide little resolution for within-species studies.

Keywords: degenerate primer, mtDNA, noncoding region, octocoral, scleractinia, variable sequence

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In most animal taxa studied to date, the mitochondrial genome tends to accumulate sequence changes more rapidly than that of the nuclear genome (Brown *et al*. 1979), lending to the widespread use of mitochondrial genes for a variety of phylogenetic studies (reviewed by Avise 2004). Cnidarian mitochondrial DNA (mtDNA), however, evolves much slower than that of most other metazoans and has greatly limited our ability to do studies of phylogeography and phylogenetics in this group (Shearer *et al*. 2002). The recent completion of phylogenetically diverse cnidarian mitochondrial genomes (Medina *et al.* 2006) allowed us to design degenerate primers for amplification of the noncoding regions of the *nad5* intron (Fig. 1). The *nad5* intron is unusual in that it contains a large coding portion of the mitochondrial genome flanked by two noncoding regions of significant length for potential use at lower taxonomic levels (van Oppen *et al*. 2002). We felt that these noncoding regions held the greatest promise to find more variable regions of mitochondrial sequence in corals for future use in phylogeographic studies. Therefore, we developed primers to amplify these coral mtDNA 'introns' in the hope that they may accumulate substitutions at a pace suitable for examining population structure and phylogeography within species of scleractinian corals. Because the flanking regions of these noncoding regions are known to be highly conserved across divergent taxa, we developed degenerate primers aimed at being universal for scleractinian taxa.

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Partial mitochondrial DNA sequences from seven distantly related scleractinian corals (*Mussa angulosa*, *Porites* sp., *Siderastrea radians*, *Acropora* sp., *Pavona clavus*, *Astrangia* sp., and *Agaricia* sp.) were aligned for the region of interest based on both nucleotide and amino acid sequences. PRIMER 3 (Rozen & Skaletsky 2000) was used to develop degenerate primer pairs from the protein alignments in the genes flanking the noncoding regions (Table 1). Primer pairs that most consistently yielded clean single bands are noted in Table 2 along with their approximate expected product size and annealing temperature. Oligonucleotide primers were ordered from Integrated DNA Technologies and tested across a broad range of coral taxa (Table 3).

DNA was extracted from *Acropora acuminata*, *A. yongei*, *Acropora* spp., *Pocillopora damicornis*, *Pocillopora eydouxi*, *Pocillopora danae*, and *Pocillopora* spp. using an extraction protocol adapted from (Rowen & Powers 1991). Briefly, a 5 mm^3 piece of coral tissue is digested for 2–3 h in 200 μL of DNAB (0.4 M NaCl, 50 mM Na_2EDTA pH 8.0) + 1% SDS + 10 μL proteinase K (10 μg/mL) on a shaker at 55 °C. An equal volume of 2X CTAB (cetyltrimethyl ammonium bromide) + 10 μL/mL β-mercaptoethanol is then added, and the

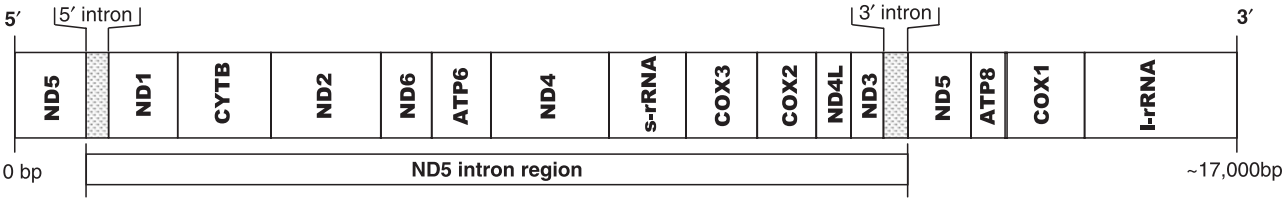

Fig. 1 Location of non-coding regions in a linearized depiction of the scleractinian mitochondrial genome.

Table 1 Degenerate primers for noncoding mitochondrial regions of scleractinian corals. Position refers to the position in the alignment where the sequence begins

Locus	Position in alignment	Primer sequence (5′–3′)	T_m (°C)	Length (bp)
nad5-5′ intron	NAD5_700F	F: YTGCCGGATGCYATGGAG	60.7	18
	NAD5_316F	F: GGGGAYCCTCATRTKCCTCG	60.6	20
	NAD1_157R	R: VCCATCYGCAAAAGGCTG	60.5	18
	NAD1_445R	R: ARCCCAATCGAAACYTCATAACT	60.1	23
nad5-3′ intron	NAD3_118F	F: TCKGCWTATGARTGTGGDTT	60.3	20
	NAD3_225F	F: YTTTYTTTTYCCYTGGTGYG	59.9	20
	NAD3_259F	F: ATTKCHCCYTTYGGKTTTTG	59.0	20
	NAD5_44R	R: ATAAAAAVACACCYGCCG	59.4	18
	NAD5_215R	R: ACARGCMACMACCATATAYC	59.6	20
	NAD5_9R	R: TGRATTAARGCRGAMACC	59.5	18

Table 2 Degenerate primer pairs for noncoding mitochondrial regions of corals that yield the most consistent PCR amplification products

Primer pair	Primer	Approximate product size	T_a (°C)
ND51a	NAD5_700F NAD1_157R	~500 bp	57
ND51b	NAD5_700F NAD1_445R	~750 bp	48
ND51d	NAD5_316F NAD1_445R	~950 bp	48
ND35a	NAD3_118F NAD5_44R	~380 bp	55
ND35b	NAD3_118F NAD5_215R	~550 bp	55

T_a, annealing temperature.

tube is vortexed before being incubated at 65 °C for an additional 30–60 min. Samples are allowed to cool, and an equal volume of chilled chloroform is added prior to vortexing well. The samples are then left on a rotating platform for 2–3 h. Finally, the supernatant is precipitated with 95% EtOH, pelleted by centrifugation, and subsequently washed with 70% EtOH. DNA is resuspended in 50 µL deionized water (dI) before making 1–50 dilutions (approximate final concentration of ~5 ng/µL) in dI for subsequent use as template in all polymerase chain reactions (PCRs).

QIAGEN DNeasy Tissue Kit was used to extract DNA from the following taxa: *Siderastrea radians*, *Porites porites*, *Pavona clavus*, *Agaricia humilis*, *Mussa angulosa*, *Montipora capitata*, *Porites compressa*, *and Pocillopora meandrina*. We followed the manufacturer's protocol except that both buffers AW1 and AW2 were used to wash the DNA twice instead of the recommended single rinse. For the first elution, 50 µL of buffer AE was added to QIAGEN DNeasy spin columns and incubated at room temperature for 1 min before centrifugation. The second elution was then performed by adding 200 µL buffer AE to spin columns and incubating the column at 55 °C for 10 min prior to centrifugation. The first elution was discarded, and all subsequent PCRs were performed using the second elution at full strength as the DNA template (approximate final concentration of ~5 ng/µL). Optimal annealing temperatures for each primer set were determined empirically by temperature gradient PCR on a Bio-Rad MyCycler Thermal Cycler. Each 25 µL PCR contained 1 µL of DNA template, 2.5 µL of 10× ImmoBuffer, 0.1 µL IMMOLASE DNA polymerase (Bioline), 3 mM $MgCl_2$, 10 mM total dNTPs, 13 pmol of each primer, and deionized H_2O to volume. Hot-start PCR amplification was performed as follows: 95 °C for 10 min (1 cycle), 95 °C for 30 s, annealing temperature (see Table 2) for 30 s, and 72 °C for 60 s (35 cycles) followed by a final extension at 72 °C for 10 min (1 cycle). PCR products were visualized using 1.0% agarose gels (1 × TAE) stained with Gelstar. PCR products were treated with 2 U of exonuclease I and 2 U of shrimp alkaline phosphatase

Table 3 Mitochondrial sequences generated from a broad range of scleractinia with select primer pairs. Accession numbers listed in the table are representative sequences submitted to NCBI

Taxon	Location	ND51a	ND51b	ND51d	ND35a	ND35b	ND35c
Acropora acuminata	Waikiki Aquarium	DQ351245	DQ351246			DQ351243	DQ351244
Acropora spp.	Waikiki Aquarium	DQ351249	DQ351250			DQ351247	DQ351248
Acropora yongei	Waikiki Aquarium	DQ351253	DQ351254			DQ351251	DQ351252
Agaricia humilis	Beenwood reef, Florida	DQ351256			DQ351255		
Montipora capitata	Coconut Island	DQ351257					
Mussa angulosa	Caribbean	DQ351258					
Pavona clavus	Eastern Panama	DQ351260			DQ351259		
Pocillopora damicornis	Coconut Island			DQ351261			
Pocillopora danae	Easter Island			DQ351262			
Pocillopora eydouxi	Tahiti			DQ351263			
Pocillopora meandrina	NWHI			DQ351264			
Porites asteroides	Caribbean	DQ351266			DQ351265		
Porites compressa	Coconut Island	DQ351269	DQ351270			DQ351267	DQ351268
Porites porites	Florida	DQ351272			DQ351271		
Siderastrea radians	Beenwood reef, Florida	DQ351274			DQ351273		

Table 4 Uncorrected ('p') distance matrix in scleractinian mitochondrial 5′ intron

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*A. acuminata*	—														
2	*A.* spp.	0.00	—													
3	*A. yongei*	0.00	0.00	—												
4	*A. humilis*	0.166	0.166	0.166	—											
5	*M. capitata*	0.060	0.060	0.060	0.148	—										
6	*M. angulosa*	0.563	0.563	0.563	0.562	0.556	—									
7	*P. clavus*	0.174	0.174	0.174	0.024	0.150	0.571	—								
8	*P. damicornis*	0.409	0.409	0.409	0.436	0.418	0.640	0.462	—							
9	*P. danae*	0.409	0.409	0.409	0.436	0.418	0.640	0.462	0.000	—						
10	*P. eydouxi*	0.409	0.409	0.409	0.436	0.418	0.640	0.462	0.000	0.000	—					
11	*P. meandrina*	0.409	0.409	0.409	0.436	0.418	0.640	0.462	0.000	0.000	0.000	—				
12	*P. asteroides*	0.135	0.135	0.135	0.180	0.133	0.550	0.186	0.392	0.392	0.392	0.392	—			
13	*P. compressa*	0.167	0.167	0.167	0.175	0.166	0.548	0.181	0.390	0.390	0.390	0.390	0.021	—		
14	*P. porites*	0.171	0.171	0.171	0.183	0.171	0.556	0.189	0.394	0.394	0.394	0.394	0.032	0.010	—	
15	*S. radians*	0.132	0.132	0.132	0.165	0.139	0.551	0.170	0.396	0.396	0.396	0.396	0.103	0.091	0.092	—

(Exo:SAP) using the following thermocycler profile: 37 °C for 60 min, 80 °C for 10 min. Cleaned PCR products were then cycle-sequenced using BigDye Terminators (PerkinElmer) run on an ABI-3100 automated sequencer. Resulting sequences were inspected and aligned by eye using SEQUENCHER version 4.5 (Gene Codes).

Primer pairs that yielded the cleanest PCR bands in the trial run were applied to multiple taxa and the products were sequenced in both directions. Annealing temperatures used are given in Table 2. Approximate length of the expected PCR product is shown in Table 2. Table 3 highlights the primer combinations that worked best in our tests.

Uncorrected 'p' distance matrices were generated with PAUP 4.0b10 (Swofford 2003) and are located in Tables 4 and 5 for both the 5′ and the 3′ Introns, respectively. Our results suggest that these noncoding regions have approximately the same mutational rate as the rest of the mitochondrial genome of corals (reviewed by Shearer *et al*. 2002). We had hoped to discover an mtDNA region of sufficient variability to perform phylogeographic studies in corals. Unfortunately, our comparison of ~300 sequences amplified from 13 taxa using these primers suggests that the degree of sequence variation is not dramatically higher than currently available variable mitochondrial primers for corals (e.g. the putative control region, van Oppen *et al*. 2002). However, we have had greater success amplifying target regions in a broad range of coral taxa using these primers than with previously published mtDNA primers for cnidarians. Thus, we expect these primers will be useful to researchers looking at relationships above that of species in a wide range of scleractinian corals, but will likely be of little use for within-species comparisons.

Table 5 Uncorrected ('p') distance matrix in scleractinian mitochondrial 3′ intron

		1	2	3	4	5	6	7	8	9
1	*A. acuminata*	—								
2	*A.* spp.	0.000	—							
3	*A. yongei*	0.000	0.000	—						
4	*A. humilis*	0.261	0.261	0.261	—					
5	*P. clavus*	0.229	0.229	0.229	0.063	—				
6	*P. asteroides*	0.144	0.144	0.144	0.235	0.197	—			
7	*P. compressa*	0.144	0.144	0.144	0.235	0.197	0.000	—		
8	*P. porites*	0.166	0.166	0.166	0.271	0.233	0.040	0.040	—	
9	*S. radians*	0.578	0.578	0.578	0.571	0.549	0.479	0.479	0.513	—

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