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TECHNICAL NOTE

New nuclear primers for molecular studies of Epinephelidae fishes

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Abstract The EPIC strategy was used in the present study in order to develop molecular markers for *Epinephelus itajara*. Twelve primers were designed for six genomic regions. The results of the study provide a number of appropriate new nuclear DNA markers for future genetic studies in several endangered/overexploited grouper species. The description of these markers will also expand the number of molecular tools available for the reconstruction of the life history of the different grouper species, in particular those in urgent need of effective protection measures.

Keywords Goliath grouper · Intron · Nuclear markers

The development of effective strategies for the protection and management of population units demands a holistic approach that integrates data on the life-history strategy of the species with the ecological characteristics of the populations (Begg and Waldman 1999). In this context, the genome can offer important insights for the development of effective strategies in the conservation of endangered species by providing estimates of genetic variability (Lee et al. 2010; Guerier et al. 2012).

The family Epinephelidae includes a number of commercially-important fish species that are found in subtropical and tropical regions throughout the World (Sadovy and Eklund 1999). Unregulated fishery exploitation, together with the biological characteristics of these species, contributes to the difficulties of conserving stocks (Sadovy and Eklund 1999; Frias-Torres 2006). Of the 20 epinephelids occurring in Brazil, two species, *Epinephelus itajara* (Goliath grouper) and *Mycteroperca marginata* (Dusky grouper), have the most preoccupying conservation status, being classified by IUCN, as critically endangered and endangered, respectively.

The EPIC (exon-primer intron-crossing) strategy has been widely used to sequence introns, primarily because it uses conserved regions of exons to anchor the primers for the sequencing of the adjacent introns (Lee et al. 2010). In the present study, nuclear primers are described for the Atlantic Goliath grouper (*E. itajara*) based on specimens collected in Caeté estuary (North Brazilian region, Pará state). Cross amplification for a number of different species from Epinephelidae was evaluated.

Based on the EPIC-PCR procedure, twelve new primers were designed, using the FastPCR program (Kalendar et al. 2009), based on conserved flanking regions between the exons of four Epinephelidae species available in the NCBI (Table 1).

Total DNA was isolated using a DNeasy kit (Qiagen). PCR protocols were done according to those reported in

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Table 1 Description of primers used in this study

Gene	Intron	Primer	Annealing temperature (°C)	Primer sequences (5′–3′)	Genebank sequence source (access number)
Myostatin	1	MYOS-F	58–60	GCTTCTCGACCAGTACGACGTG	*Dicentrarchus labrax, Epinephelus coioides* (AY839106, AY856860)
		MYOS-R		GCATCAGGCGGGAGATTTGCAGG	
Growth hormone	2	GH-F -int2	49–50	GAGTTCAACATCTCCACCTG	*Epinephelus akaara, Epinephelus coioides, Epinephelus lanceolatus* (AY326406, AY513647, EU280321)
	3	GH-R-int2		TCAGAGTTACAGAAGTCCTG	
		GH-F-int3		GCAGGACTTCTGTAACTCTG	
		GH-R-int3		TTTCTGGGAGCAGAACCTCCGG	
Interferon regulatory factor 2β	1	INTERFERON-F	65	GCGTCATGGCTGGGACCTGGAG	*Epinephelus coioides* (FJ828966)
		INTERFERON-R		GTCTTTGGATCTGGACGG	
Cytocrhome P 450 α aromatase	1	P450AROint1	53.5	GCCTGGAGCCACACAGACA	*Epinephelus akaara, Epinephelus coioides*, (AY547354, AY510711)
		P450AROint1		GAACCTCACATATGACAGAAGTG	
Cytocrhome P450 β aromatase	2	GBARO-F-int2	50	CGGGTCTGGATTAATGGCGAGG	*Epinephelus* coioides, *Monopterus albus* (AY510712, EU840259)
		GBARO-R-int2	54	TGCTCCCAAATCTGGCTGTG	
	4	GBARO-F-int4		TTCCTCAGGGTGCCGCTCAA	
		GBARO-R-int4		GCCATCCAATCTTGAAG	
Transcription factor SOX-9	1	SOX9-F-int1	*	AACGCGGAACTCAGCAAA	*Epinephelus akaara, Epinephelus coioides* (AY676309, GQ232762)
		SOX9-R-int1		CGAAGTAGAGAAGCGCCC	
Thyroid hormone receptor B protein mRNA	1	THRBP-F-int1	60	GAGGATGAAGTCTTGGCCTTG	*E. coioides* (EF502004)
		THRBP-R-int1		GGCCTCATCTTTCCAGCGGG	
	3	THRBP-F-int3		AGTTACCTGGACAAGGATGAG	
		THRBP-R-int3		TGCACTTCTTGAAGCGACA	
	4	THRBP-F-int4		GAATGTCGCTTCAAGAAGTG	
		THRBP-R-int4		GTGGGCTCCAGTCGGTC	
	6	THRBP-F-int6		GCCATAACCCGAGTGGTGGAC	
		THRBP-R-int6		CTCGCAGTGACATGATCTC	

* No positive amplifications reported

Pinho et al. (2010). The amplification conditions were: 3 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at the hybridization temperature recommended for the species, and 2 min at 72 °C, with final extension of 7 min at 72 °C (Table 1). The samples were sequenced using the dideoxyterminal method with a DYEnamicTM ET dye Terminator kit (Amersham Pharmacia Biotech, Inc., UK) in an ABI 3500 sequencer (Applied Biosystems).

Positive results were obtained for five gene fragments using twelve tested primers (Table 2). The fragments obtained for *E. itajara* varied in size between 153 and 560 base pairs (bp). The fragment that was amplified positively for the largest number of taxa (four of the five species analyzed) was intron 1 of the Myostatin gene and intron 2 of the Growth Hormone. The least successful locus amplification was intron 1 of Cytochrome P450, which was amplified in only two species (*E. itajara* and *M. marginata*). Two markers (intron 2 and intron 4) were amplified from the Cytochrome P450 β Aromatase gene, with both providing positive results. Specifically, intron 2 was amplified in *C. fulva*, *E. adscensionis* and *E. itajara*, while intron 4 was amplified in *C. fulva*, *E. adscensionis*, *E. itajara*, and *E. morio*. The others markers returned unsatisfactory results.

In order to test the genetic diversity of the newly-developed markers, 20 specimens of *E. itajara* from Caeté estuary were sequenced for all the primers (Table 2). The sequences obtained from the samples were aligned using the Bioedit program (Hall 1999), while haplotypes were reconstructed using Phase v2.1 (Stephens et al. 2001), run in Dnasp v5.00.7 (Librado and Rozas 2009). Runs consisted of 1,000 iterations of burn-in and 1,000 main iterations, with a thinning interval

Table 2 Summary statistics obtained for the markers analyzed in this study

Marker	N	*h*	*S*	π %	Hd	bp	Rm	Indels (size)	Positive PCR cross-amplification
Myos- int1	40	4	3	0.060	0.317	560	0	0	*E. adscencionis*, *C. fulva* e *E. morio*
GH-int2	40	2	1	0.059	0.097	166	0	0	*E. adscencionis*, *E. morio* e *M. marginata*
Gbaro-int2	40	1	0	0	0	153	NC	0	*E. adscencionis* e *C. fulva*
Gbaro-int4	40	2	1	0	0	237	0	3 (2/1/1)*	*E. adscencionis*, *C. fulva* e *E. morio*
P450Aro-int1	40	6	4	0.49	0.600	186	0	5 (1/1/1/1/1)*	*M. marginata*

* Number between parenthesis represent the number bases of each single indel

π = nucleotide diversity

N Number of sequences; *h* number of haplotypes; *S* segregating sites; *Hd* haplotype diversity; *bp* base pairs; *Rm* number of recombination events; *Indels* insertion or deletion; *NC* not calculated

of 1. The same program was used to calculate the polymorphism for each marker generated by *E. itajara* samples (Table 2), as well as the standard statistical summary and the minimum number of recombination events (Rm). Genetic distance was calculated by Mega v.5 (Tamura et al. 2011). All the sequences were deposited in the GenBank (JX535501–JX535514).

While all the markers sequenced were relatively short, it was possible to identify polymorphisms in the Goliath grouper. The results indicate that the intron 1 of Myostatin and intron 1 of Cytochrome P450 α Aromatase are potentially useful markers for the development of population and phylogeographic studies. This conclusion was reinforced by the fact that the genetic diversity observed was greater than the values reported for other nuclear genetic marker in studies of *E. itajara* populations, such as Intron S7 (Craig et al. 2009).

The Myostatin marker presented better amplification pattern in all the species analyzed. The genetic distances recorded between species using this intron varied between 1.8 and 3.7 %, indicating that Myostatin has considerable potential for phylogenetic studies.

In the specific case of the epinephelids, the majority of the previous genetic studies of phylogenetic, phylogeographic or population parameters have been based exclusively on mitochondrial markers (Silva-Oliveira et al. 2008). Craig et al. (2009) is the single exception by combining mitochondrial and nuclear DNA. However the marker used (intron S7) was not polymorphic.

Thus present study offers new nuclear markers in which they might be used genetic studies of *E. itajara*, *E. adscensionis*, *E. morio*, *C. fulva*, and *M. marginata*, and probably may be applicable to other epinephelid species. These markers may also provide a more informative database for population biology evidences as identification of fishery stocks that require urgent and effective protection.

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