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Development of EPIC-PCR Markers for *Lutjanus purpureus* (Lutjanidae-Perciformes) and their Potential Applicability in Population Analyses

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ABSTRACT

In the present study, a novel set of eight EPIC primers were developed for *Lutjanus purpureus* and assayed in five other marine teleosts including three lutjanids, one scianid and one anablepid. Most of the genomic regions used in this study presented genetic diversity indexes equal or greater than the intragenic regions commonly used in population genetics studies. Moreover, six out of eight markers showed cross-amplification with other taxa. Thus, the primers described here may be used to elucidate questions at the intraspecific level for a large number of taxa.

Key words: EPIC-PCR, intron, *Lutjanus,* Southern Red Snapper.

INTRODUCTION

Advances in DNA sequencing technology have provided access to information of a large number of loci in non-model organisms, and have promoted great significant advances in population genetics and related fields. On the other hand, approaches that employ a limited number of independent and variable genomic regions are useful for describing evolutionary processes that act on a given taxonomic group (Bowen et al. 2014).

Microsatellites are the most commonly used molecular markers in intraspecific studies, and for population-level analyses because they have a high level of polymorphism (Zhang and Hewitt 2003) and generally reflect population dynamics that have occurred in recent periods (see Morin et al. 2004, Guichoux et al. 2011).

Another class of markers that has been widely used at the population level is the single-copy nuclear DNA sequences (e.g., introns). Introns are untranslated intragenic regions of the nuclear genome that may include polymorphism capable of revealing information regarding the population structure of taxa. Thus, introns are suitable for use in population genetics and phylogeography studies (Zhang and Hewitt 2003).

Moreover, it has been postulated that DNA sequence-based markers provide more accurate descriptions of historical demographic processes

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compared with those obtained using microsatellites (Brumfield et al. 2003). Thus, the combination of microsatellite markers and introns is of great importance for understanding the evolutionary history of species in different historical periods (Zhang and Hewitt 2003).

In the present study, we used the exon-primed intron-crossing (EPIC-PCR) (Lessa 1992) technique to develop a set of novel nuclear markers for *Lutjanus purpureus* that are amplifiable across the Lutjanidae family and other marine teleosts. EPIC is based on the design of primers anchored in the adjacent exons, in order to amplify and sequence the intron (Li et al. 2010). Given the high rate of substitution for the majority of introns, EPIC-PCR has been used to investigate patterns of intraspecific genetic variation in many taxa, including teleosts (Gaither et al. 2010, 2011, Li et al. 2010, Silva-Oliveira et al. 2012, Chow and Yanagimoto 2016). Lutjanidae and several other groups of marine teleosts are organisms that have wide geographic distributions, which make them good models for phylogeographic and population genetic analyses. In addition, isolation and characterization of microsatellite markers has frequently been performed in Lutjanidae (Pinsky and Palumbi 2014). However, a limited number of population analyses have been conducted using introns (i.e., Gaither et al. 2010). Moreover, snappers are important fishery resources; therefore, studies aimed at their genetic characterization are fundamental for delimitation and management of stocks.

MATERIALS AND METHODS

Primers were developed through the EPIC-PCR technique, based on sequences available in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/). We made a manual search in GenBank for nuclear sequences of Lutjanidae and model species of closely taxa in order to find potential regions for primers design using as main criteria of choice the information about the position of introns and exons (Table I), with intron fragments ranging from 200-1000 bp. The primers were designed using the software FastPCR (Kalendar et al. 2009) using the default parameters.

The primers developed in the present study (Table I) were initially used on specimens of *L. purpureus* collected along the coast of the state of Pará (North of Brazil), to characterize the levels of polymorphism. For North coast of Brazil, there is a single stock, with elevated levels of variation (Sousa-Júnior et al. 2002, Gomes et al. 2008, 2012). With the aim to demonstrate that these markers could be used in different groups and phylogenetically distant taxa, cross-amplification was tested in five other marine teleosts (two indvidual per species) including other lutjanids (*Lutjanus synagris*, *Rhomboplites aurorubens*, *Ocyurus chrysurus*), a Sciaenidae (*Cynoscion jamaicensis*), and Anablepidae (*Anableps anableps*). All individuals used in the present analysis were purchased in municipal markets of the north coast of Brazil, marketed dead.

The DNA was isolated using the phenol-chloroform protocol (Sambrook et al. 1989). The amplicons were amplified by PCR using the primers described in Table I. We also amplified six genomic regions previously used in lutjanids (Table II) for comparisons purpose. The PCR reactions were standardized to a final volume of 15 μL, which contained 200 μM of dNTPs, 1 x of buffer (200 mM Tris-HCl- pH 8.0, 500 mM KCl) , 3 mM of $MgCl_2$, 5 mM of each primer, approximately 50 ng of DNA template, 0.5 U Taq Polymerase and water up to the reaction final volume. The amplification conditions were: 95°C for 3 minutes followed by 35 cycles at 95°C for 35 seconds, 45 seconds (see Table I for Annealing), 80 seconds at 72°C, and a final cycle of 3 minutes at 72°C. Positive PCRs were sequenced using the dideoxy method (Sanger et al. 1977) and the ABI

TABLE I
Description for the markers developed in the present study.

Locus (Abbreviation)	Primers	Sequence 5'-3'	Annealing (◦C)	GenBank Access Code
Delta 6 desaturase - Intron 8 (Delta 8)	Delt6 F8	TTACTACCTTCGCTACCTGTGCT	64	*Sparus aurata* (AF525692)
	Delt6 R8	AGTCACCCACACAAACCAGTKAC		
Delta 6 desaturase- Intron 10 (Delta 10)	Delt6 F10	GTTCAGCGGACACCTCAACT	50	*Sparus aurata* (AF525692)
	Delt6 F10	CACACAGTGCGTGGACAAG		
Myostatin - Intron1 (Myo 1)	Myo 1F	ATGAGCATGCCATCACAGAG	64	*Lates calcarifer* (EF672685)
	Myo 1R	ATGCGATTGGCTTGAAACTT		*Lutjanus russellii* (JQ068866)
Myostatin - Intron2 (Myo 2)	Myo 2F	GCATCGAGATTAACGCCTTC	64	*Lates calcarifer* (EF672685)
	Myo 2R	GGCCCTCTGAGATCTTCACC		*Lutjanus russellii* (JQ068866)
Trypsin - Intron1 (Try 1)	Try 2F	CCTGATCTCCAGCACCTGGGTK	60	*Lutjanus fulvus* (AB738891)
	Try 2R	GATGTCATTGTYCAGGTTGCSGCT		
LWSCO - Intron1 (LWSCO 1)	LWSCO 1F	GCTGATCTGGGAGAGACAGTTT	60	*Lutjanus johnii* (FJ824756)
	LWSCO 1R	CATTTGGCATCAAACTTGACAT		
Insulin-like Growth Factor (IGF 1)	FC-F1	AGCGCTCTTTCCTTTCAGTG	59	*Dicentrarchus labrax* (GQ924783)
	FC-R1	CRCACAGCAGTAGTGAGAGG		
Interferon - Intron2 (Int 2)	Int 2F	GTACAGMCAGGCGTCCAAAGCAT	64	*Dicentrarchus labrax* (AM946400)
	Int 2R	GTTCTCCTCCCATGATGCMGAG		*Sparus aurata* (FM882244)

3500 XL automatic sequencer. All DNA sequences are available in Genbank under the accession codes KT869380 to KT869495 and in Figshare: (http://dx.doi.org/10.6084/m9.figshare.1564736;http://dx.doi.org/10.6084/m9.figshare.1564735).

The sequences were edited with the BioEdit software (Hall 1999), and aligned using the parameter default of ClustalW (Thompson et al. 1994) available in BioEdit. Heterozygous insertion and deletion events were resolved in the Mixed Sequences Reader (Chang et al. 2012). The gametic phase was reconstructed with PHASE (Stephens et al. 2001), implemented in the DNAsp v. 5.10. (Librado and Rozas 2009), based on 1,000 burn-in iterations, 1,000 main iterations, thinning interval of 1 and using a threshold of 0.6. In certain cases, haplotypes returning with a probability lower than 0.6 were resolved by the Clark's method (Clark 1990). In order to assess the level of polymorphism of the markers, the number of polymorphic sites, haplotype diversity (h) and nucleotide diversity (π) were estimated using DNAsp software (Librado and Rozas 2009).

RESULTS AND DISCUSSION

In the present study, a set of eight EPIC-PCR markers (Table I) for *L. purpureus* ranging from 172 to 637 bp were developed, amplified and sequenced for *L. purpureus* (Table II).The success rate of positive PCR amplification remained near 90% for all loci, except for Interferon - Intron 2, and IGF 1 (≈ 70%). Almost all of these markers (except Interferon - Intron 2), presented polymorphism (Table II). For the polymorphic markers, the number of segregating sites ranged between 2 (LWSCO 1) and 12 (Insulin-like Growth Factor – Intron 1). The genetic diversity values ranged from 0.090 (LWSCO 1) to 0.94 (Delta 6 – Intron 10) for h values and from 0.053% (LWSCO 1) to 1.053% (Try 1) for π values.

Six out of eight of the EPIC-PCR markers developed here have been successfully amplified in other species (Table II). Thus, the markers reported here are useful for population genetics and phylogeographic studies at a vast taxonomic level.

All the markers showed genetic variation levels similar to or higher than intragenic regions previously used in other marine teleosts, including lutjanids (e.g., Gaither et al. 2010, 2011, da Silva et al. 2015) (Table II). In certain cases, the genetic variation values were comparable to the variation indicated by mitochondrial regions. For example, Delta 10 showed a genetic diversity value (h) of 0.94, which was similar to the values commonly presented by hypervariable regions of mitochondrial DNA. Even for the other markers (except Int 2, LWSCO 1 h = 0.09), the levels of observed genetic variation (h ≥ 0.75; Table II) were comparable or higher than the polymorphism presented by mitochondrial genome segments commonly used

TABLE II
Statistics for the markers analyzed in the present study (for *L. purpureus*), and the results of cross-amplifications.

Locus	N	bp	Nh	S	h ± sd	π (in %) ± sd	Indel	Results of cross-amplification
[1]Delta 8	20	571	11	11	0.871 ± 0.028	0.557± 0.039	1	Positive: *L. synagris, R. aurorubens, O. chrysurus, C. jamaicensis*
[1]Delta10	20	535	18	13	0.94 ± 0.018	0.7 ± 0.058	3	Positive: *L. synagris, R. aurorubens, A. anableps*
[1]Myo1	22	363	13	10	0.857 ± 0.033	0.876 ± 0.049	3	Positive: *L. synagris, R. aurorubens, O. chrysurus, C. jamaicensis, A. anableps*
[1]Myo2	22	637	10	8	0.782 ± 0.042	0.272 ± 0.028	1	Positive: *L. synagris, R. aurorubens, O. chrysurus,C. jamaicensis, A. anableps*
[1]Try1	22	259	9	7	0.755 ± 0.049	1.053 ± 0.046	2	Positive: *L. synagris, R. aurorubens, O. chrysurus,C. jamaicensis, A. anableps*
[1]LWSCO1	22	172	3	2	0.090 ± 0.059	0.053 ± 0.035	0	Positive: *L. synagris, R. aurorubens, O. chrysurus,C. jamaicensis, A. anableps*
[1]FC1	20	468	14	12	0.849± 0.039	0.465± 0.050	2	Negative
[1]Int2	22	530	1	0	0	0	0	Negative
[2]ANT 1	19	290	3	2	0.104±0.067	0.036±0.024	0	Not aplicable
[2]Gh 5	22	146	3	3	0.369±0.075	0.308±0.085	0	Not aplicable
[2]Cyt B	21	779	10	11	0.862±0.057	0.301±0.038	0	Not aplicable
[2]ND4	21	515	10	13	0.776±0.093	0.322±0.067	0	Not aplicable

N = sample size; bp= base pairs; Nh = number of haplotypes; S = polymorphic sites; h = haplotype diversity; π = nucleotide diversity; and sd = standard deviation.
[1]Markers developed in the present study.
[2]Markers available from literature (i.e., Hassan et al. 2002, Jarman et al. 2002, Sevilla et al. 2007, Arevalo et al. 1994, Bielawski and Gold 2002).

in intraspecific studies, such Cytochrome B and ND4.

In addition, with the development of coalescent methodologies used for estimation of evolutionary parameters of taxa, the use of multiple genomic regions has been growing in popularity (Li et al. 2010). In this way the use of nuclear sequences such as variable introns, has become very common in the study of shallow evolutionary process including species delimitation, population analysis and population dynamics (Li et al. 2010). Thus, the markers described in the present study may provide robust information to elucidate the evolutionary processes that act on marine fish populations and help to identify and accurately delimit fish stocks.

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