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Molecular protocol for authentication of snappers (Lutjanidae-Perciformes) based on multiplex PCR

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ABSTRACT

Fraud involving fish products is regularly reported and investigated using genetic methods. However, no such approach has been applied to snappers, despite the commercial relevance of these fish. This study proposes an authentication protocol with multiplex PCR for three species of snappers (*Lutjanus purpureus*, *L. synagris*, and *Ocyurus chrysurus*). The protocol yielded a distinct triple-banding pattern for *L. purpureus*, whereas *L. synagris* and *O. chrysurus* showed a double banding pattern of different sizes, thereby allowing differentiation of the three species. The protocol was validated using fillets labeled as snapper or "Pargo" in Brazil, previously identified using DNA sequencing, amongst which substitution with *Rhomboplites aurorubens* was detected. When subjected to the new protocol, banding characteristic of *L. purpureus* were detected whilst *R. aurorubens* generated only the control band. Our study provides a practical tool for investigating substitutions and might assist in quality control and increase food safety for consumers.

1. Introduction

The scientific literature reports numerous cases of species substitution in fish-derived food products, revealing that this type of fraud is recurrent across the world (Carvalho, Palhares, Drummond, & Frigo, 2015; Cawthorn, Steinman, & Witthuhn, 2012; Filonzi, Chiesa, Vaghi, & Marzano, 2010). Mistakes might result, for example, from misidentification of taxa with similar morphologies or transformations that fish undergo during processing, where species become indistinguishable due to the loss of diagnostic traits. However, a large number of substitutions are intentional, motivated by financial gain, in cases where species with higher commercial value are substituted with those of a lower value (Cutarelli et al., 2014; Di Pinto et al., 2013; Marko et al., 2004).

Species substitutions, whether accidental or intentional, could cause health problems, such as unintended exposure to allergens (Cohen et al., 2009), and could compromise the efficiency of conservation programs when endangered species are involved (Palmeira et al., 2013). In addition, consumers suffer economic losses when they purchase species with a lower value because of inaccurate or falsified labels (Armani, Castigliego, Tinacci, Gianfaldoni, & Guidi, 2012; Galal-Khallaf, Ardura, Mohammed-Geba, Borrel, & Garcia-Vazquez, 2014; Wong & Hanner, 2008).

Forensic genetics has proved useful in identification and investigation of species substitutions in fish products. Sequencing of DNA fragments has undoubtedly been useful in the search for fraud (Carvalho et al., 2015; Di Pinto et al., 2013; Marko et al., 2004), it is recognized as being effective and, as it has gained popularity, it has also become less costly to perform.

However, other methods exist that do not require DNA sequencing, but which are also very efficient as well as being more practical and rapid. These protocols are based on species-specific banding patterns observed directly in concentrated gels (Hellberg & Morrisey, 2011).

These patterns result from either conventional polymerase chain reaction (PCR), which amplifies DNA segments that intrinsically contain a recognizable banding pattern, such as 5S ribosomal DNA (rDNA) (Rodrigues-Filho et al., 2011; Sales, Rodrigues-Filho, Haimovici, Sampaio, & Schneider, 2011), or they can be produced by multiplex assays, a potential tool for authenticating fish products (Armani et al., 2012; Castigliego, Armani, Tinacci, Gianfaldoni, & Guidi, 2015; Catanese, Manchado, Fernández-Trujillo, & Infante, 2010).

Multiplex PCR is a practical alternative for routine use in fishing industries and the retail sector, as well as by surveillance agencies for controlling quality and protecting the consumer (Armani et al.,

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2012; Asensio, 2008; Castigliego et al., 2015). These practices are important considering the growth in demand for fish-based foods and the diverse range of seafood products available (FAO, 2014).

Species of Lutjanidae have high economic importance throughout their distribution areas (Schwartz, 2007; Cervigón, 1993; MPA, 2010, 2012, 2013). There are also reports of species substitution for this group (Hellberg & Morrisey, 2011; Marko et al., 2004; Stiles, Kagan, Lahr, Pullekines, & Walsh, 2013; Wong & Hanner, 2008). A molecular approach using banding patterns of DNA for identification of snappers from the Western Atlantic was proposed by Veneza et al. (2014), However, the authors found a non-specific banding pattern for the ribosomal 5S gene in this group.

Therefore, the present study aimed to develop a rapid authentication protocol, based on multiplex PCR, which provided distinct banding pattern for the three main snappers caught on the Eastern Atlantic coast, especially in Brazil: *Lutjanus purpureus*, *L. synagris*, and *Ocyurus chrysurus*.

2. Materials and methods

2.1. Sampling

A total of 101 samples of snappers were used, of which 26 were sequenced in our previous work, Veneza et al. (2014), which selected a DNA reference sequence of 1130 bp from the cytochrome oxidase subunit I (COI) gene. The set of 26 samples included nine species from the family Lutjanidae: *L. purpureus*, *L. synagris*, *L. jocu*, *L. buccanella*, *L. vivanus*, *L. cyanopterus*, *L. analis*, *O. chrysurus*, and *R. aurorubens*, collected along the coast of Brazil (Table 1) (Veneza et al., 2014).

Using this database, primers were developed for *L. purpureus*, *L. synagris*, and *O. chrysurus* for standardizing a multiplex PCR reaction.

Of the 75 remaining samples, 45 were used to test the specificity of the primers developed here, corresponding to five samples for each of the nine species, and 30 samples, 10 from each of the three species to be authenticated, were used to evaluate the reproducibility of the banding pattern of the target species.

Additionally, 15 samples of fillets labeled as snapper (*L. purpureus*) purchased from a supermarket in Bragança (state of Pará, northern Brazil) were used, totaling 116 samples. These fillet samples were stored in Eppendorf tubes containing 90% alcohol, preserved, and refrigerated at −20 °C. The fillet samples were previously identified via DNA sequencing and then used to validate the protocol proposed in this study.

2.2. DNA Isolation, Amplification, and sequencing

Total genomic DNA was extracted with the Wizard Genomic® Kit (PROMEGA, Madison, USA), according to the manufacturer's instructions, for all samples (exception of the 26 sequences of Veneza et al. (2014)). To assess the quality of the isolated DNA, the samples were stained with GelRed and subjected to 1% agarose gel electrophoresis for 30 min at 60 V and were then visualized with a transilluminator.

Table 2
Primers used in the multiplex reaction and their characteristics. [1]Reverse primer common to the target species of this study (Palumbi & Benzie, 1991); [2]Primers for amplifying the control band (Palumbi et al., 1991).

Primer	Sequence	Volume (μL)
COILpu280	5′-GGACAGTCTACCCGCCCCTAGCAGGC-3′	1
COIOch430	5′-GTATCAAACGCCCCTATTCGTC-3′	1.5
COILsy920	5′-TATCTCCCAATACCAAACACCCCTG-3′	1
COIA[1]	5′-AGTATAAGCGTCTGGGTAGTC-3′	1
16SL1987[2]	5′-GCCTCGCCTGTTTACCAAAAAC-3′	0.5
16SH2609[2]	5′-CCGGTCTGAACTCAGATCACGT-3′	0.5

DNA sequencing of the COI gene was used to identify the 15 samples of fillets. The COIFishF2 and COIFishR2 primers (Ward, Zemlak, Innes, Last, & Hebert, 2005) were used to isolate the target genomic region. The PCR reaction mix consisted of a 15 μL final volume with the following composition: 2.4 μL of mixed DNTPs (1.25 mM); 1.5 μL of buffer (10x); 0.6 μL of $MgCl_2$ (50 mM); 0.6 μL of each primer (50 ng/μL); approximately 100 ng of total DNA; 0.1 μL of Taq Recombinant DNA polymerase (Invitrogen; 5 U/μL); approximately 100 ng of total DNA and purified water to complete the final reaction volume. The amplification conditions for this reaction were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles with denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 1 min, and a final extension of 3 min at 72 °C.

After the PCR, the successfully amplified samples were sequenced using the dideoxyterminal method (Sanger, Nichlen, & Coulson, 1977), with reagents of the Big Dye Kit (ABI Prism™ Dye Terminator Cycle Sequencing Reading Reaction – Life Technologies). The DNA sequences were obtained in the ABI 3500 automated capillary sequencer (Applied Biosystems).

2.3. Computational analyses

The DNA sequences from the fillets were edited, aligned, and corrected in BioEdit (Hall, 1999) and then added to the database used for designing the multiplex PCR primers developed in this study. Thus, a new database was created with sequences of an approximate length of 600 bp, equivalent to the barcode portion of the COI gene and consisting of the set of 26 sequences generated by Veneza et al. (2014), in addition to the sequences obtained here for the fillets analyzed.

To identify the sampled fillets, the new database was analyzed in the MEGA 6 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), in which a neighbor-joining (NJ) phylogenetic tree was generated using the evolutionary Kimura-2-Parameters (K2P) model (Kimura, 1980), usually adopted in studies of DNA

Table 1
Sampling used in the primer design for authenticating *L. purpureus*, *L. synagris*, and *R. aurorubens*.

Species	No. of Samples	Accession Codes
L. purpureus	6	KF633328; KF633327; KF633326; KF633325; KF633324; KF633323.
L. synagris	3	KF633285; KF633284; KF633283
L. vivanus	3	KF633332; KF633331; KF633330
L. buccanella	2	KF633392; KF633391
L. analis	3	KF633344; KF633343; KF633345
L. jocu	2	KF633373; KF633372
L. cyanopterus	1	KF633393
O. chrysurus	3	KF633393; KF633266; KF633265
R. aurorubens	3	KF633264; KF633263; KF633262
Total	26	