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Identification of *Plectropomus leopardus*, *Promicrops lanceolatus* and *Cromileptes altivelis* using species-specific TaqMan real-time PCR

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ABSTRACT

Plectropomus leopardus, *Promicrops lanceolatus* and *Cromileptes altivelis* are high-value groupers in chinese aquatic product market and are substituted by cheaper ones sometimes due to economic profit. In this study, real-time PCR methods for identification of these groupers were developed. Species-specific primers and probes were designed on the basis of cytochrome oxidase I (COI) mitochondrial DNA sequences of groupers and other fishes. The specificity of the real time PCR assays were tested with a total of 51 specimens of fish, meat and poultry. Amplifications were observed in specific species only. The methods developed were accurate and rapid and offered the potential to detect fraudulent or unintentional mislabeling of these species in routine seafood authentication analysis.

1. Introduction

Seafood mislabeling has been widely reported throughout the world (Bénardcapelle et al., 2015; Cawthorn, Duncan, Kastern, Francis, & Hoffman, 2015; Filonzi, Chiesa, Vaghi, & Marzano, 2010; Galal-Khallaf, Ardura, Mohammed-Geba, Borrell, & Garcia-Vazquez, 2014; Khaksar et al., 2015; Muñozcolmenero, Blanco, Arias, Martinez, & Garciavazquez, 2016; Nagalakshmi, Annam, Venkateshwarlu, Pathakota, & Lakra, 2015) and the accurate description or labeling of food is increasingly important to consumers. Highly valuable fish species and products are particularly susceptible to be substituted due to the economic profits arising from selling cheaper species as high-value ones. The processed products are more vulnerable to fraudulent labeling since the morphological characters are lacking, by gutting, heading, slicing, filleting and chopping. Therefore, it is difficult to identify clearly the species of processed products based on their morphology. For this reason, accurate and rapid method is required that could identify fresh or processed products to species level, and would be useful and reliable for official regulatory use.

Grouper is a highly appreciated and expensive group of fishes and a high incidence of species substitution and mislabeling was detected in grouper products. Di Pinto et al. (2012) investigated 56 samples of fillet fish products labeled as grouper (*Epinephelus marginatus*), and found 48/56 (85.7%) fillets of grouper (*E. marginatus*) were mislabeled. Asensio, González, Pavón, García, and MartínAn (2008), Asensio, Samaniego, Gonzalez, Garcia, and Martin (2008) revealed that only 14 of 52 commercial fish fillets samples were confirmed to be grouper. Jacquet and Pauly (2008) discovered 70% of fish samples labeled 'grouper' were actually another species. *Plectropomus leopardus* (miniatus grouper, leopard coral grouper), *Promicrops lanceolatus* (giant grouper) and *Cromileptes altivelis* (mouse grouper, humpback grouper) are most high value groupers in southeast Asia. The substitution incidence of them, by similar but cheaper ones, such as cheaper groupers, *Siniperca chuatsi*, *Channa argus*, *Lateolabrax japonicus* and *Oreochromis niloticus*, has increased in recent years and the situation becomes more and more serious.

DNA-based techniques have been used to investigate the identification of the grouper species recently. For instance, Trotta et al. (2005) developed two PCR-based methods to discriminate *Epinephelus* and *Mycteroperca* species from substitute species, Nile perch and wrech fish. However, the grouper species Tratta used were not the same as those in China. *Plectropomus, Promicrop* and *Cromileptes* species had no amplification and could not be detected using this method (data not shown). Asensio, González, et al. (2008), Asensio, Samaniego, et al. (2008) applied an indirect ELISA and a multiplex PCR procedure to detect *E. marginatus* mislabeling in the fish market. The same research group also established a PCR method based on the 12S ribosomal RNA gene to authenticate commercial frauds in *E. marginatus* fillets (Asensio,

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González, Rojas, García, & MartínAn, 2009). Using microsatellite markers, Rodrigues and his colleagues were able to identify *E. lanceolatus*, *C. altivelis* and *E. fuscoguttatus* (Rodrigues, Shigeharu, & Ch'ng, 2011). Chen et al. (2012) used real-time PCR method to differentiate 10 groupers from other fishes, while the grouper species could not be discriminated. Ulrich et al. (2013, 2015) reported real-time nucleic acid sequence-based amplification (RT-NASBA) targeting mitochondrial 16S rRNA gene to detect grouper species, and developed a handheld genetic sensor which could be performed outside of the lab. But, species *C. altivelis* and *P. leopardus* were not included in this study. Chen et al. (2014) provided PCR and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for discrimination of 10 groupers based on mitochondrial cytb gene sequences. However this method is not practical for processed products, as it requires a relatively large PCR product; and also has difficulty in identification of samples containing multiple species.

So far, a number of DNA-based methods have been used in detecting species substitution on the commercial market. Among them, PCR-RFLP and species-specific PCR are the most commonly used techniques in fish species identification due to their high reproducibility, high specificity, sensitivity, and easy to operate. PCR sequencing and PCR-RFLP protocols are relatively complicated and require substantial time for sequencing or restriction processing. Compared these two methods, species-specific real-time PCR has the advantages to be faster and more easily. It eliminates the need of post-PCR processing steps and reduced chance of cross-contamination. Along with the development of PCR chips and real-time capabilities, the species-specific real-time PCR method has the potential to be applied to high-throughput operations. The aim of this study was to develop simple, reliable, and quick real-time PCR methods for identification of *P. leopardus*, *P. lanceolatus* and *C. altivelis* in species substitution detection and perform market surveys in China.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total of 51 specimens of 51 species, including 41 fish species (*Epinephelus akaara*, *Epinephelus awoara, Epinephelus coioides, E. fuscoguttatus, Epinephelus moara, Epinephelus quoyanus, P. lanceolatus, P. leopardus, Plectropomus maculates, C. altivelis, L. japonicus, S. chuatsi, Hapalogenys mucronatus, Acanthopagrus schlegelii, Acanthopagrus latus, Pagrus major, Parapristipoma trilineatum, Oplegnathus fasciatus, Priacanthus tayenus, Lutjanus malabaricus, Larimichthys crocea, Sciaenops ocellatus, Parastromateus niger, Psenopsis anomala, Pampus minor, Nemipterus marginatus, Siganus fuscescens, O. niloticus, C. argus, Micropterus salmoides, Thunnus albacares, Salmo salar, Gadus morhua, Rhynchobatus djiddensis, Dasyatis zugei, Eleutheronema tetradactylum, Takifugu oblongus, Cheilinus undulates, Mustelus manazo, Prionace glauca* and *Hypophthalmichthys nobilis*) and 10 muscle samples of meat and poultry (pork, beef, donkey, horse, venison, mutton, rabbit, duck, goose and chicken) were used to test the primer pairs and probes. There are 10 individuals of *P. leopardus*, *P. lanceolatus* and *C. altivelis*. All samples were obtained from Xiamen, China. Fish samples were purchased from aquatic products wholesale market, supermarket and restaurant, and taxonomically identified by an ichthyologist at Third Institute of Oceanography, State Oceanic Administration. Meat and poultry samples were purchased from supermarkets. A small portion of muscle was excised from specimens and stored at −20 °C.

Total DNA was extracted from approximately 100 mg of tissue sample using DNeasy Blood & Tissue Kit (Qiagen) in accordance with the manufacturer's Spin-Column protocol. DNA concentrations were measured using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific). Extracts were stored at −20 °C.

2.2. Primer and probe design

Partial COI sequences of 30 fish species (Table 1) available from Genbank were aligned using Clustal X 1.83 (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) to visualize sequences unique to *P. leopardus*, *P. lanceolatus* and *C. altivelis*, respectively. Species-specific primer pairs and probes for each species were designed manually and screened for hairpins, homo- and cross-dimers using OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer). The primer and probe sequences, as well as the corresponding amplicon sizes, are listed in Table 2. Primers and probes were synthesized by TAKARA Ltd. The probes were synthesized with a 6-FAM (6- carboxyfluorescein) reporter dye at the 5′ end and a TAMRA (6- carboxytetramethylrhodamine) quencher molecule at the 3′ end.

2.3. Species-specific TaqMan PCR

TaqMan real-time PCR reactions with species-specific primers and probes, targeting a region within COI gene, were used to detect *P. leopardus*, *P. lanceolatus* and *C. altivelis*, respectively. The PCR amplification reactions were carried out in a Mx3005P real-time PCR instrument (Agilent) in a total volume of 25 μL. Each reaction mixture contained 12.5 μL of *Taqman* 2× Fast Start Universal Probe Master (Rox) (Roche), 5 μL of template DNA (2.5–25.0 ng/μL), 1 μL of each primer pair (10 μmol/L), 1 μL of probe (10 μmol/L), and 4.5 μL of distilled water. The PCR thermoprofiles were as stated in Table 3. Fluorescence data were acquired and analyzed with the MxPro-Mx3005P software (version 4.1.0). Each PCR run included positive DNA controls, a non-template control (only the master mix and PCR grade water), and a negative DNA control. All samples were run in duplicate.

3. Result

3.1. Species-specific primer and probe sets design and DNA extraction

By aligning the COI sequences of 30 species, we were able to design species-specific primers and probes (Table 1) to differentiate three high value groupers from other fish species.

All DNA extracted from the samples had absorbance ratios 260/280 nm of 1.8–2.0, indicative of high purity. The amplification of all DNA extracts was confirmed by the standard mitochondrial 16SrRNA gene primers 16Sar and 16Sbr (Palumbi, 1996), as well as the PCR thermocycle profile (Chen et al., 2012).

3.2. Specificity of primers and probes

To test the specificity of the methodology, and inspect the cross-reactivity with many common species to avoid false positives, DNAs extracted from the targets and 50 non-target species were used as templates. The real-time PCR reactions showed successful amplification only in *P. leopardus* (Fig. 1), *C. altivelis* (Fig. 2) and *P. lanceolatus* (Fig. 3), respectively. None of the non-target species tested showed amplification, demonstrated that the PCR systems did not cross-react with DNA samples from other non-target species. To verify positive amplification in all individuals and avoid false negative results, every species-specific primer and probe set was investigated using 10 specimens of *P. leopardus*, *C. altivelis* or *P. lanceolatus*. Good positive real-time PCR results obtained in all