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Differentiation of *Epinephelus moara* from *E. bruneus* by improved nest-tetra-primer-specific PCR

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Abstract

Epinephelus moara and *E. bruneus* are closely related species in the genus *Epinephelus* (Perciformes, Serranidae). Their morphological similarity, changing color pattern at different stages and living conditions make them difficult to be differentiated. To identify these two species, an improved nest-tetra-primer-specific PCR assay was developed. Three specific molecular markers, the control region NC1 (394 bp), species-specific internal region ND2-M (268 bp) and ND2-B (122 bp), were identified in the mitochondrial ND2 gene from these two grouper species. Five markers were also discovered in the ITS1 regions of their nuclear ribosomal DNA, which were the control regions NC2 (588 bp) and NC3 (563 bp), and species-specific internal regions rDNA-M (426 bp), ITS1-M (488 bp) and ITS1-B (304 bp). This method provided a highly specific, precise, reliable and rapid molecular marker technique to discriminate between the two grouper species, as well as a new way of DNA identification to differentiate closely related species in fishes.

Keywords: *Epinephelus moara*; *E. bruneus*; ND2 gene; ITS1 region; Nest-tetra-primer-specific PCR

1. Introduction

Epinephelus moara T. & S. and *E. bruneus* B., commonly known as kelp grouper and longtooth grouper, respectively, are of considerable importance in the commercial and artisanal fisheries along the southeast coast of China. They are bottom-associated fishes on rocky reefs and mud bottom and are widely distributed in tropical and subtropical seas, such as the seas in Korea, Japan (north to Hegura-jima Island, 37°50′N) and China (south to Hong Kong and Hainan Island, east to Taiwan) [1,2]. Being quite similar in the external characters and distribution of these two species, there are a lot of disputes about their classification. Some researchers regarded them as one species, *E. moara* being identified as the juveniles of *E. bruneus* [2,3], while most others classified them to be two species based on the morphological characters [4–6]. Our former observations also confirmed them to be different species by comparing and analyzing their backgrounds of morphology [7]. However, because their morphologies are similar and their colors and stripes usually change at different developmental stages or in different living environments, it is really quite difficult to differentiate between them only by their external characters. In order to classify these two species, a precise, rapid and sensitive method needs to be established.

Tetra-primer ARMS PCR (tetra-primer amplification refractory mutation system PCR) is a rapid, simple and economical technique for analyzing a number of allelic-specific SNPs. It adopts certain principles of the tetra-primer method and the amplification refractory mutation system (ARMS) [8]. Recently, it has extensively been used in SNP scoring and genotypic research on disease-related genes [9–12]. In this study, a modified nest-tetra-primer-specific PCR was

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established for the identification of the closely related grouper species, *E. moara* and *E. bruneus*. By amplifying species-specific internal sequence regions of the mitochondrial ND2 gene and the nuclear ribosomal ITS1 region, some specific molecular markers were identified for differentiating between them. It was the first attempt to develop species-specific molecular markers for species discrimination in fishes by exploring sequence polymorphism.

2. Materials and methods

2.1. Sampling and DNA isolation

All samples were collected from the fish market of Xiamen in China from March 2005 to December 2007. Totally, 47 individuals, 23 *E. moara* with a standard length of 18.8–30.7 cm and 24 *E. bruneus* of 14–34.6 cm, were used in this study. Species identification and nomenclature were as follows: "*Grouper of the world*" [2], "*Fish Taxology*" [4] and "*Systematic Synopsis of Chinese Fishes*" [6]. Some epaxial musculature was excised from fresh specimens of each species and immediately preserved in 95% ethanol. Total genomic DNA was extracted from the central portion of the tissues using the phenol/chloroform procedure [13].

2.2. PCR amplification and nucleotide sequencing

The ND2 gene and ITS1 region were amplified by PCR with common forward and reverse primers. The primers used to amplify the ND2 gene were ND2-1L (5′-AAGGGC CACTTTGATAGAGTG-3′, designed with reference to the partial nucleotide sequences from the mtDNA of Perciformes [GenBank Accession No. AB101290]) and ND2-1H (5′-GT(A/G)AGT(A/G)(T/C)GGGGG(C/T)TTTTGC (C/T)CA-3′) [14]. The primers for the ITS1 region were ITS-1F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-1R (5′-CGCTGCGTTCTTCATCG-3′) [15]. PCR was carried out on a Model 9700 thermal cycler with a 25 μl reaction volume containing 12.8 μl double-distilled water, 2.5 μl *Ex*Taq buffer (Mg^{2+} free), 1.8 μl Mg^{2+} (2.5 mM), 1.2 μl dNTP (2.5 mM each), 2.5 μl of each primer (10 μM), 0.2 μl *Ex*Taq DNA polymerase (5 U/μL, TaKaRa) and 2.0 μl total genomic DNA (about 100 ng). The PCR was performed using cycling conditions of an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 45 s, 72 °C for 1 min 30 s (ND2)/94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min (ITS1 region), and a final extension at 72 °C for 10 min.

PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide for band characterization via ultraviolet transillumination. Double-stranded PCR products of ND2 used in this study had been sequenced in our laboratory (GenBank Accession Nos. DQ862077 and DQ862084). The ITS1 region amplicons were excised and purified from the agarose gel using a MinElute Gel Extraction Kit (Qiagen). Subsequently, purified PCR products were cloned into the pMD18-T vector (TaKaRa). These ligation mixtures were used to transform competent *Escherichia coli* strain DH5-α (TOYOBO). Transformed bacterial cells were incubated on Amp LB plates. Positive clones, which had amplicons of sizes similar to those of the corresponding bands, were selected for sequencing on a Model 3730 DNA sequencer (Invitrogen, Shanghai, China). Meanwhile, the same clones were kept for control.

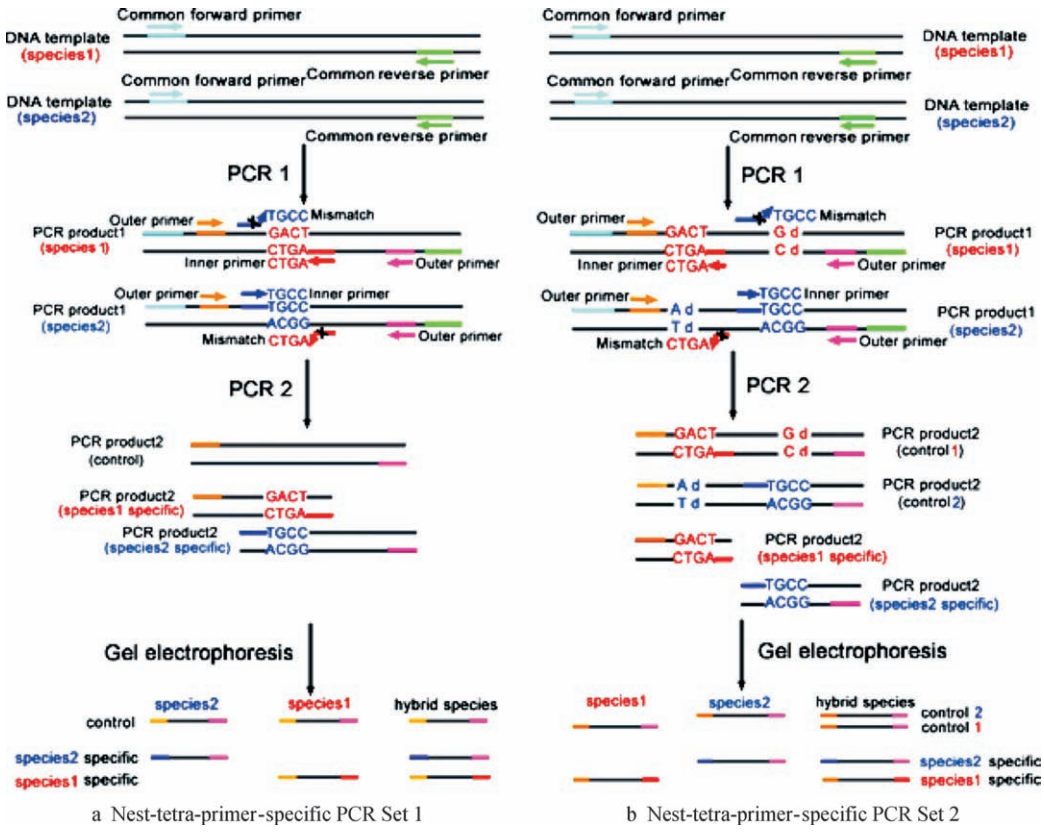
The DNA sequences were edited and analyzed using the softwares DAMBE and Genetyx. After splicing with the ContigExpress software and correcting with peak Chroma, all sequences were compared (using the algorithm BLAST*n*) with those available via the NCBI database (http://www.ncbi.nlm.nih.gov).

2.3. Designing of primers

The inner primers were designed at consecutively multi-base mutation or deletion sites (Fig. 1(a) or (b), it could be the same site or different), which were near the end of primer 3′. Two outer primers were positioned laterally on the conservative regions of the chosen sequence. Moreover, they should be at different distances from the multi-base mutation or deletion sites. It made the amplified species-specific fragments different in length and can be easily detected by gel electrophoresis (Fig. 1). The outer primers were not only the primers for positive control, but also the primers for being paired with the inner primers to selectively amplify species-specific markers. The inner primers were used to detect the occurrence of successive mutations. The more the mutation sites were close to the 3′-end of the inner primers, the higher the amplification specificity was. After being compared with many different sequences, sequences of the ND2 gene and ITS1 region were selected to discriminate these two grouper species. Two sets of primers were designed (Table 1) at the consecutive mutation sites or deletion sites (Fig. 2).

2.4. Amplification and verification of the species-specific molecular markers

First, the ND2 gene and ITS1 region were amplified using universal primers as mentioned in Section 2.2. Then, the PCR products were 1:20 diluted. To discriminate between *E. moara* and *E. bruneus*, a 25 μl nest-tetra-primer-specific PCR reaction was performed as follows: 10.05 μl double-distilled water, 2.5 μl *Ex*Taq buffer (Mg^{2+} free), 1.8 μl Mg^{2+} (2.5 mM), 1.5 μl dNTP (2.5 mM each), 5% DMSO, 0.2 μl *Ex*Taq DNA polymerase (5 U/μl, TaKaRa), 0.4 μl of each outer primer (10 μM), 2.2 μl of each specific internal primer (10 μM) and 2.5 μl diluted PCR product (about 100 ng). The PCR was performed with the following cycling parameters: initial denaturation at 94 °C for 4 min, 22 cycles (first set) of 94 °C for 30 s, Ta1 (Ta1, Ta2 referred to Table 1) for 30 s, 72 °C for 1 min, and 25 cycles of 94 °C for 30 s, Ta2 for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The positive con-



a Nest-tetra-primer-specific PCR Set 1 b Nest-tetra-primer-specific PCR Set 2

Fig. 1. Schematic presentation of nest-tetra-primer-specific PCR. PCR 1 and 2 indicate objective sequences PCR pre-amplification and tetra-primer-specific PCR amplification, respectively. The deletion of bases is represented by "d". The specificity is conferred by a mismatch (represented by ×) between the 3′-terminal base of an inner primer and the template.

Table 1
Tetra-specific PCR primers of the ND2 gene and ITS1 region.

Primers	Sequences (5′–3′)	Annealing temperature
A: ND2-Fo178	TGGACTAGGAACCACCATTACACTCA	
ND2-Ro572	TTTGAAGAAATAGGGCGAAGGGTGCT	Ta1 = 51 °C
ND2-RiM446	AGAGGGCAAGAATCACCATAGTAATT	Ta2 = 54 °C
ND2-FiB450	TGAAGGTTGGCCTTGCCCCACTA	
B: ITS-1Fo	TCCGTAGGTGAACCTGCGG	
ITS-1Ro	CGCTGCGTTCTTCATCG	Ta1 = 53 °C
ITS-FiM100	GCTGCTGCTGCTGCTGCTGTCATT	Ta2 = 61 °C
ITS-RiB300	TTGACATTAGGAGGTTCCGAGGGCC	

F, forward; R, reverse; o, outer (common); i, inner (specific); M, *E. moara*; B, *E. bruneus*.

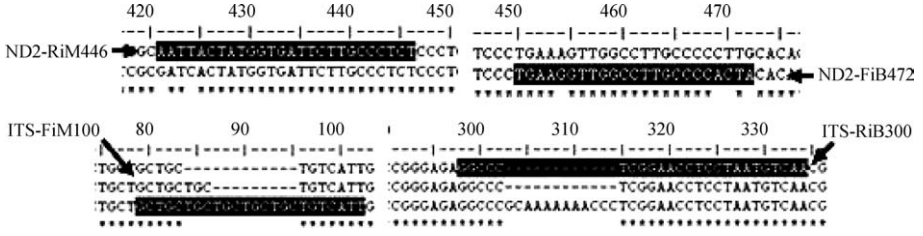


Fig. 2. Design of species-specific inner primers of tetra-primer-specific PCR in the ND2 gene and ITS1 region.

trol was performed with the same assay, using the positive clone as the DNA template.

To validate the accuracy and evaluate the sensitivity of the specific molecular markers, the nest-tetra-primer-specific PCR was performed with all 47 sampled individuals. PCR products were directly checked by 2.0% agarose gel electrophoresis, stained with ethidium bromide, detected and recorded by the Bio-Rad gel imaging system (GAS7001B).

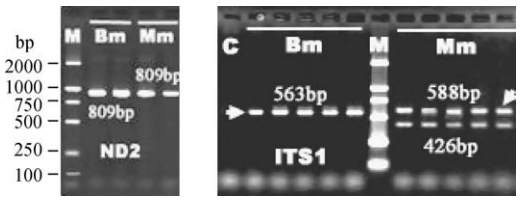


Fig. 3. Representative agarose gel electrophoretograms of the ND2 gene (left) and ITS1 region (right). C and M represent negative control and MarkerDL2000, respectively. Mm and Bm mean the amplification products of a mixture of all individuals genomic DNA from *E. moara* and *E. bruneus*. Arrows show the ITS1 region.

3. Results

3.1. Sequences analysis

PCR products were sequenced and submitted to GenBank directly. Similarities of the ND2 gene and ITS1 region between *E. moara* and *E. bruneus* were 94.932% and 92.749%, respectively. The whole sequence contained 809 bp of the partial ND2 gene (Fig. 3), of which no insertion or deletion of base pairs was found after alignment. There was a non-coded region of 139 bp in the upstream of the ND2 gene, which covered mitochondrial $tRNA^{Glu}$ and $tRNA^{Met}$ genes and a non-coded region between the $tRNA^{Met}$ gene and the ND2 gene. Both ITS1 regions were registered in GenBank under the Accession Nos. FJ176797 and FJ176798. In the ITS1 sequences of *E. moara* and *E. bruneus*, n1–87 and n1–19 in their upstream were parts of the 18S rDNA sequence, and n572–588 and n547–563 in their downstream were parts of the 5.8S rDNA sequence. The results searched through GenBank showed that the sequence homology of the ITS1 region between n88–571 of *E. moara* and n65–596 of *E. salmonoides* (GeneBank Accession No. AB375591) was 80%, and the sequence homologies between n20–284 of *E. bruneus* and n1–302 of *E. salmonoides*, and n311–546 of *E. bruneus* and n311–546 of *E. salmonoides* were 79% and 85%, respectively. In addition, a specific fragment of rDNA-M (426 bp) (Fig. 3), with no homologous sequence being found, was amplified from *E. moara*.

The results of sequencing in a number of grouper individuals revealed that both the ND2 gene and ITS1 region sequences in these two species were relatively stable within species but different between species. They were very suitable for the identification of closely related species by nest-tetra-primer-specific PCR. Consecutive multiple base mutations appeared on n421 and n472 in the ND2 gene and deletions appeared on n84–95 and n303–314 in the ITS1 region of these two grouper species (Fig. 3), which were suitable for the design of specific inner primers.

3.2. Detection of specific molecular markers

The results of the experiments with different concentration ratios (12:1, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1) between inner and outer primers showed that the amplified effect was best at the concentration ratio of 6:1. This ratio was then optimized to 5.5:1. Three specific bands appeared in the ND2 gene (Fig. 4). On the first cycle, the internal sequence NC1 (394 bp) was produced by the amplification of two outer primers, and it was used as the template for the following species-specific amplification. Specific bands ND2-M (268 bp) of *E. moara* and ND2-B (122 bp) of *E. bruneus* were generated in the second cycle. Internal sequence NC2 (588 bp) and specific band ITS1-M (488 bp) were the amplified products of the ITS1 region in *E. moara*, and internal sequence NC3 (563 bp) and specific band ITS1-B (304 bp) were of the ITS1 region *in E. bruneus*. A specific band rDAN-M (426 bp) was also amplified in *E. moara* (Fig. 4).

3.3. Verification of the species-specific molecular markers

The results of verification tests showed that the frequency of occurrence of the species-specific molecular markers in *E. moara* was 100% in all 23 tested *E. moara*, and 0% in all 24 tested *E. bruneus*, and the frequency of occurrence of the molecular markers in *E. bruneus* was 100% in all 24 tested individuals and 0% in all 23 tested *E. moara*. No polymorphism was found within species. The results of control groups with positive clones as the template were also fully consistent. It illuminated that the species-specific bands were the sequencing regions and that they were unique in each grouper species. It can be further deduced from the results that, in the south sea of Fujian,

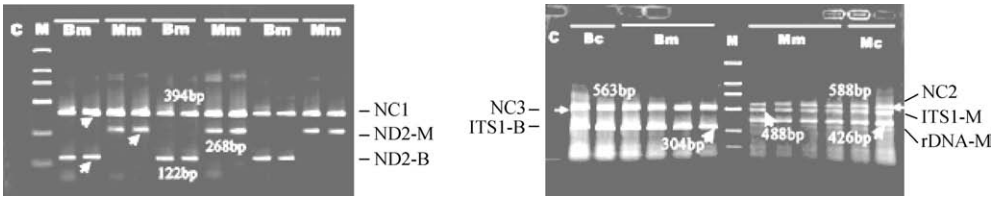


Fig. 4. Agarose gel electroretograms of the ND2 gene (left) and ITS1 region (right) by nest-tetra-primer-specific PCR. Mc and Bc are PCR products of the positive clone in *E. moara* and *E. bruneus*, respectively. Arrows show species-specific molecular markers of the two kinds of groupers. Other notes are the same as in Fig. 3.

China, *E. moara* and *E. bruneus* had different niches and that no hybridization occurred between them.

4. Discussion

Nest-tetra-primer-specific PCR is based on the principle of tetra-primer ARMS PCR [8]. In order to produce species-specific bands and thus rapidly and accurately identify the closely related species, appropriate genes or genes are selected and pre-amplified by nest-PCR, and then amplified by tetra-primer-specific PCR. Pre-amplification by nest-PCR enhances the stability and specificity of tetra-primer PCR, which can rule out false-positive results and simplify the process of optimization. In nest-tetra-primer-specific PCR, consecutively multi-base mutations are generally introduced into the inner primers, and the mutation sites should be near the 3'-end of the primers to enhance the specificity. The annealing temperature is mainly decided by the inner primers. In addition, the concentrations and ratios of the inner primers and outer primers should be adjusted to improve the accuracy and sensitivity of detection [8,9,12].

At present, studies on fish phylogeny and taxonomy are usually based on the proximity relationship of investigated species. Appropriate DNA sequences from the mtDNA or nuclear genome were selected as the molecular genetic markers. Mitochondrial genes suitable for species relationship studies are mainly 12S rRNA and 16S rRNA [16], Cytb [17,18], ND2 [14,19], COI [20], ATPase6 and ATPase8 [21,22], and the control region (D-loop) [23,24]. Nuclear genes are generally used for evolutionary relationship studies between far-related species [25]. Introns or interval sequences between exons, such as the moderately conservative sequence ITS1 region of the ribosomal gene cluster, have higher evolutionary rates and are chosen as the genetic markers for the closely related species [15,26–28]. Grouper classification has been a difficult problem in fish taxonomy. Because grouper has a short inter-species differentiation time and close phylogenetic relationships, choosing faster evolutional gene fragments will be helpful to reveal the relationship between species [18]. In this study, several genes, including mitochondrial 16S rRNA, Cytb and ND2, nuclear 18S rDNA, 5SrDNA and ITS1 region sequences in *E. moara* and *E. bruneus*, were sequenced and compared. Because the results showed that the ND2 gene and ITS1 region were conservative within species and different between species, they were chosen for the nest-tetra-primer-specific PCR.

Groupers are economically important species in tropical and subtropical seas [2]. In recent years, the molecular genetic research on groupers was mainly focused on their population genetics, phylogeny and taxonomy. Maggio et al. analyzed the genetic population structure of *E. marginatus* in the waters of the Mediterranean and Atlantic by the ND2 and Cytb sequences and RFLP [29]. Craig et al. investigated the evolutionary relationships of 42 American grouper species based on mitochondrial 16S rDNA sequences [30]. Ding et al. studied the molecular phylogenetic relationships of more than 30 grouper species from the South China sea and part of the Indian Ocean [18]. Wang et al. examined the genetic relationship between *E. malabaricus* and *E. coioides* and their hybrid offspring based on their morphological criteria and multi-molecular techniques RAPD and AFLP [31]. In this paper, an improved nest-tetra-primer-specific PCR was successfully established and it was first and successfully used for quick and accurate identification of *E. moara* and *E. bruneus*. The method would not only be applicable to identify other grouper species, but also provide a new way to identify the other closely related fish species. This method identifies different fish species only by PCR amplification and gel electrophoresis and no sequencing is needed anymore. The method has the advantages of quickness, stabilization and accuracy. Compared with the traditional morphological method, it could avoid the impacts of the environment, phenotypic characters of fish and individual developmental stages. Moreover, it could be used to confirm the parental sources and to identify the existence of hybrids.

Acknowledgements

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