Identification of *Epinephelus malabaricus* and *Epinephelus coioides* using DNA markers

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**Abstract**

Using multi-molecular marker technologies and based on morphological criteria, the genetic relationship between *Epinephelus malabaricus* and *E. coioides* was examined in the hope of resolving the long-standing issue of identifying these two species. Results showed that ¹ 1. *E. coioides* and *E. malabaricus* should be identified as two species; the consistency of mitochondrial DNA cytochrome b gene sequence between *E. malabaricus* and *E. coioides* is 94.4% ² and the genetic similarity by AFLP was 0.753 ³. Hybridization exists between *E. malabaricus* and *E. coioides*; the specific RAPD and AFLP fragments are found to be useful in the identification of these two species and the genetic properties both with exterior and inheritance of hybrid is significantly biased to the male parents and ³. ³ AFLP was a potentially powerful tool in constructing the genetic linkage map for these two groupers.

**Key words** *Epinephelus malabaricus*, *Epinephelus coioides*, hybridize, molecular identification

1 Introduction

Both *Epinephelus malabaricus* and *E. coioides* belong to the genus *Epinephelus* Perciformes Serranidae Epinephelinae. They are warm water reef fishes that have similar distribution ranges as well as similar morphological characteristics which have resulted in many confusions and misidentifications of these two species. Chen and Zhang 2001 Ding et al. 2003 Heemstra and Randall 1993. The mixing of the two species in aquaculture practice especially the mixing of brood stocks in the breeding process increased the possibility for genetic introgression of these two species which by itself caused even more confusions in their indentification.

In this study we estimated the phylogenetic relationships between these two species of groupers using several molecular marker technologies ¹ to identify the molecular markers that could better distinguish these two groupers and ² to explore if there was hybridization between these two groupers at the DNA level. The results of this study will provide important information for the conservation of grouper genetic resources and the continuation of grouper culturing.

2 Materials and methods

2.1 Fish sampling

Sixteen groupers were collected from Xiamen and Hainan fish markets. Muscle tissues were preserved in 100% ethanol and stored at −20°C until analysis.
2.2 Morphology identification

According to the morphological taxonomic criterion of FAO Guideline Groupers of the World the diagnostic features of the two species are as follows: *E. malabaricus* is covered with dark brown or black also with irregular white spots and blotches on the head and body and has black dense spots on the lower jaw and gular area and has the number of pyloric caeca above eighty while *E. coioids* is covered with orange or reddish brown no white spots or blotches no spots on the lower jaw and gular area and the number pyloric caeca about 50 ~ 60. Hereby among the 16 groupers sampled five have the features of *E. malabaricus* and six have the features of *E. coioids*. However the remaining five groupers seemed to have diagnostic features from both sides two of them have the reddish brown spots of *E. malabaricus* and the pyloric caeca number 80 ~ 90 of *E. coioids* three of them have dark brown spots of *E. coioids* and the pyloric caeca number about 50 ~ 60 of *E. malabaricus*. On the basis of the above observations we devided the 16 samples into four groups for DNA molecular level identification. These four groups were Dd in Xx group there is *E. malabaricus* in Dx group there is *E. malabaricus*’ pigment pattern and *E. coioids*’ number of pyloric caeca and in Xd group there is *E. coioids*’ pigment pattern and *E. malabaricus*’ number of pyloric caeca.

2.3 Methods

2.3.1 Total DNA extraction

Total DNAs were extracted following the phenol/chloroform protocol of Sambrook et al. with some modifications. For each fish sample 15 ~ 20 mg of muscier tissue was transferred to a 1.5 mL tube and 1 mL of TE buffer 10 mmol/dm$^3$ Tris-HCl pH 8.0 with 1 mmol/dm$^3$ EDTA pH 8.0 was added mixed and then the mixture was centrifuged and the upper phase was discarded to get rid of the ethanol in the sample. Then 600 µL DNA extraction buffer 570 µL TE buffer pH 8.0 + 30 µL 30% SDS and 3 µL of proteinase K 20 mg/cm$^3$ were added to the tube and the mixture was incubated at 55°C until digestion is complete. After incubation two phenol-chloroform-isopentanol 25 : 24 : 1 extractions were performed followed by one chloroform extraction. DNA was precipitated by adding two volumes of cold ethanol. DNA was collected by a brief centrifugation and washed once with 70% ethanol. Then it was air-dried redissolved in water and kept at 4°C overnight. The DNA concentration was measured with the ZF-401 spectrophotometer for absorption at 260 nm and estimated using the following formulation sample DNA concentration equaling 50 multiplied by OD$^260$ multiplied by times of original volumen after dilution. Adjust the DNA sample concentration to about 10 µg/mm$^3$ and store at −20°C.

2.3.2 Mitochondrial DNA amplification and sequencing

The mitochondrially encoded cyt b gene was obtained with the following primers 28-For 5’-CGAACGTGATAGAAAAACATGTTG-3’ Meyer et al. 1990 and 34-Rev 5’-AACTGAGCCCTCAGAATGATATTTGTCCTCA-3’ Cantatore et al. 1994. PCR amplification was carried out in a 50 µL reaction volume. PCR cycles were as follows 5 min at 94°C 30 cycles of 30 s at 94°C 30 s at 52°C and 45 s at 72°C and final elongatin for 5 min at 72°C stored at 4°C until use. To verify the existence of the aim fragment PCR products were separated electrophoretically on a 1.2% agarose gel in TBE buffer stained with ethidium bromide. Then both strands of the purified DNA were sent to the Shanghai Invitrogen Biotechnology Company in China where they were sequenced using an ABI Prism 377...
2.3.3 RAPD amplification and detection

Amplification was performed in a 25 μL reaction volume. The following cycle program was adopted: an initial 5 min at 94°C followed by 30 s at 94°C, 45 s at 36°C, 90 s at 72°C for 45 cycles, and 5 min at 72°C for a final extension. The amplified products were separated and detected on a 1.5% agarose gel stained with ethidium bromide. The gels were photographed under UV light.

2.3.4 AFLP reaction and detection

Amplified fragment length polymorphisms (AFLP) procedures were essentially based on Vos et al. (1995). An aliquot of the total DNA (8 μL) was digested at 37°C overnight using 1 μL MseI and 1 μL EcoRI in a 25 μL reaction. Ligation was then started by adding a 10 μL mix of MseI and EcoRI adapters and 1 x ligation buffer and it continued at 37°C for about 10 h. Preamplification PCR was performed in 25 μL volumes with 2 μL diluted restricted/ligated DNA x PCR buffer (1.5 mmol/dm³ MgCl₂, 0.2 mmol of each dNTP, 30 ng pre-E 5'-GACTGCGTACCAATTCA primer and 30 ng pre-M 5'-GATGAGTCCTGAGTAAC). PCR was carried out with 2 min initial denaturation at 94°C followed by 25 cycles of denaturing 94°C, 30 s, annealing 56°C, 30 s, and extension 72°C, 1 min. Selective amplification PCR was performed in 25 μL volumes with 1 μL preamplification product as template 1 x PCR buffer with 1.5 mmol/dm³ MgCl₂, 0.2 mmol of each dNTP, 30 ng MseI primer and 10 ng EcoRI primer. PCR consisted of 2 min denaturation 94°C, 13 cycles of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C, with the annealing temperature decreasing from 65°C by 0.7°C increments in cycles 2 ~ 13. This was followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. Nine reproducible primer combinations were used: E1M4 [AAC + CGA], E3M1 [ACG + CAC], E3M2 [ACG + CTG], E3M3 [ACG + CCA], E3M4 [ACG + CGA], E4M1 [ATG + CAC], E4M2 [ATG + CTG], E4M3 [ATG + CCA], and E4M4 [ATG + CGA]. The PCR products were electrophoresed on a 6% polyacrylamide 19:1 BIS:7 mol/dm³ urea gel at 80 W for 120 min, and then silver stained.

2.4 Data analysis

Sequences homology and divergence among the obtained partial sequences of cyt b were analyzed using a GENETYX program package. AFLP fingerprints were scored for presence 1 or absence 0 and transformed into the 0/1 binary character matrix. Then genetic distances and indexes of genetic similarity were calculated by importing the data matrix into the popgene 1.32 version software. Finally, clustering analysis was performed using the unweighted pair group method clustering algorithm UPGMA.

3 Results

3.1 mtDNA sequence analysis

A 500 bp fragment of the mitochondrial cyt b gene was obtained from the PCR amplification of the genome DNA of four groups of 16 individual groupers using the special primers. Sequencing results revealed that two types of sequences named A and B respectively were present in the four groups of groupers — Sequence A in Groups Dd and Xd, Sequence B in Groups Xx and Dx. The pairwise nucleotide divergence between Sequences A and B was 5.6%. Comparison of the sequence and morphological characteristics of the four sample groups was shown in Table 1.
Table 1. Comparison of the sequence and morphological characteristics of the four sample groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dd</th>
<th>Dx</th>
<th>Xd</th>
<th>Xx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphological feature</td>
<td>body color</td>
<td>spots</td>
<td>with irregular white blotches</td>
<td>jaw</td>
</tr>
<tr>
<td></td>
<td>brown</td>
<td>brown</td>
<td>dark brown</td>
<td>dark brown</td>
</tr>
<tr>
<td></td>
<td>small</td>
<td>dark brown</td>
<td>small</td>
<td>dark brown</td>
</tr>
<tr>
<td></td>
<td>with yes</td>
<td>no</td>
<td>big</td>
<td>reddish brown</td>
</tr>
<tr>
<td></td>
<td>dark spots</td>
<td>dark spots</td>
<td>no dark spots</td>
<td>no dark spots</td>
</tr>
<tr>
<td></td>
<td>reddish brown with irregular white blotches</td>
<td>no dark spots</td>
<td>no dark spots</td>
<td>no dark spots</td>
</tr>
<tr>
<td>Sequence of mtDNA cyt b</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Number of samples</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

3.2 RAPD analysis

For the RAPD analysis, a total of ten fish were examined: three from Dd group, two from Dx, two from Xd, and three from Xx. A total of 95 loci were scored from 14 primers. The number of resolved amplified fragments varied from 3 to 11 with the size range varying from 100 to 1,000 bp. Out of the 14 primers used, five primers Table 2 detected group-specific bands while the other nine did not. Two of these banding patterns produced by Primers 1120 and 1127 respectively were illustrated in Fig. 1 and in each banding pattern several bands were found to be exclusive to certain fish groups in the Primer 1120 graph 700 bp band to Xd and Xx groups 1,500 bp band to Dx and Xx groups and 1,200 bp band to Dx, Xd and Xx groups in the Primer 1127 graph 850 bp band to Dx and Xx groups 1,400 bp band to Dx group and 1,800 bp band to Dd, Dx and Xd groups Fig. 1. Using these group-specific bands we can distinguish the four groups of groupers.

Table 2. Arbitrary primers showing different amplified products in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ – 3’</th>
<th>Number of special band</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1046</td>
<td>GTCGGAGTGG</td>
<td>2</td>
</tr>
<tr>
<td>S1120</td>
<td>ACCAACCCAGG</td>
<td>3</td>
</tr>
<tr>
<td>S1127</td>
<td>TCGCTGCGGA</td>
<td>3</td>
</tr>
<tr>
<td>S1132</td>
<td>AACGGCCGTC</td>
<td>1</td>
</tr>
<tr>
<td>S1135</td>
<td>TGATGCACGCT</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 1. The electrophoresis patterns of RAPDs for four group samples. M is DL 2000 DNA marker from top to down fragments size were 100, 250, 500, 750, 1,000, 2,000 bp.
3.3 AFLP analysis

AFLP analysis of ten individuals, three fish of Dd group, two fish of Dx, two fish of Xd, and three fish of Xx, using nine primer combinations produced a total of 641 countable bands ranging in size from 100 to 750 bp corresponding to an average of 71.2 bands per combination. Of these bands, 35.02% (215 in total) was polymorphic. Of the 641 bands, 426 bands were found in all four groups; 215 bands were found to be exclusive to specific groups: 115 to Dd and Dx groups; 70 to Xd and Xx groups; 18 to Dx and Xd groups; and only 12 to Dd and Xx groups. Each of the nine primer combinations produced group-specific bands which could be used for distinguishing the four groups of groupers. For example, six diagnostic loci were produced using the E4M4 primer combination Fig. 2. In Fig. 2 we can see that Bands A, B, and C pointed by arrows only occurred in Dd and Dx groups; Bands E and F only in Xd and Xx groups; Band D only in Dx and Xx and Xd groups. Using these group-specific bands, we can distinguish the four groups of groupers.

3.4 Genetic relationship analyses

The pairwise genetic similarity and genetic distances among these four groups were estimated by analyzing the data matrix of AFLP Table 3. Genetic similarities between each two groups ranged from 0.924 to 0.753. Groups Dd and Dx had the smallest pairwise genetic similarity and the largest pairwise genetic distance.

![AFLP band patterns generated by E4M4 primers for four groups samples.](image)

Table 3. Pairwise genetic similarity and genetic distances among these four groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Dd</th>
<th>Dx</th>
<th>Xd</th>
<th>Xx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dd</td>
<td>0.867</td>
<td>0.873</td>
<td>0.753</td>
<td></td>
</tr>
<tr>
<td>Dx</td>
<td>0.082</td>
<td>0.811</td>
<td>0.889</td>
<td></td>
</tr>
<tr>
<td>Xd</td>
<td>0.141</td>
<td>0.207</td>
<td>0.889</td>
<td></td>
</tr>
<tr>
<td>Xx</td>
<td>0.275</td>
<td>0.124</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: Genetic similarity above diagonal and genetic distances below diagonal.

On the basis of the pairwise genetic distances analysis to describe the relationships among the four groups in Fig. 3. The UPGMA dendrogram re-
revealed two clades: Groups Dd, Dx in one and Groups Xd, Xx in the other. The genetic similarity between Dd and Dx was larger than Xd and Xx. We also noticed that groups within the same clade had similar colour patterns.

![Fig. 3. UPGMA analysis among these four groupers.](image)

## 4 Discussion

Colour pattern and the number of pyloric caeca are the main morphological characteristics in the grouper identification (Heemstra and Randall, 1993). However, as identification features, both colour and pyloric caeca exhibit certain weaknesses. Many groupers alter their colour patterns within a few seconds when "frightened" or "stressed". And post-mortem changes in colour pattern can obscure the normal pattern of the live fish. As for the number of pyloric caeca in spite of its reliability, the death of the sample is unavoidable. Accurate species identification is vital for effective germplasm resource conservation and management. Several obstacles exist in the discrimination of *E. malabaricus* and *E. coioides*. In addition to the similarity of their morphological characteristics, their reproduction behaviors also resemble each other. The mixing of the two species in aquaculture practice, especially the mixing of brood stocks in the breeding process, increased the possibility for genetic introgression of these two species which by itself caused even more confusions and difficulties in their identification, seed-protection and breeding. While RAPD technology has been widely applied in species identification and population genetic analysis due to its convenience, Chen et al. (2002), Zheng and Liu (2004) AFLP marker is gaining more and more attention for its higher reliability and sensitivity. Capable of providing large information, AFLP is suitable for species identification at both inter- and intra-species levels. It has also been widely used in many other fields like molecular phylogeny and interspecific relationship assessment (Zhang et al., 2005). This study obtained species-specific markers for *E. coioides* and *E. malabaricus* and their interspecific hybrid employing RAPD and AFLP technologies.

Analysis of mtDNA cyt b sequence showed that the pairwise nucleotide divergence value between *E. coioides* and *E. malabaricus* was 5.6%, which is much smaller than the average value in genus *Epinephelus* 12%, yet larger than the value between populations in *E. coioides* intraspecies 0.8%. It is also larger than the divergence value between other closely related *Epinephelus* species such as *E. areolatus* and *E. akaara* 3.3%, *E. radiatus* and *E. epistictus* 4.5%. Therefore, we concluded that *E. coioides* and *E. malabaricus* belong to two species though they might diverge only a short time ago. In addition to that, AFLP analysis also suggested that the same result see Table 3.

For many years, strict maternal inheritance and mitochondrial DNA (mtDNA) which occur in only one type in certain individuals are the basic characteristics of animal mtDNA inheritance (Xiao and Zhang, 2000; Zhang et al., 1992). On the basis of this principle, mtDNA has been widely applied in species identification and exploration of the origin of species. In this study, we observed that the fish in the Dx group not only have the morphological characteristics of *E. malabaricus*—covered with dark brown or black, black dense spots on the lower jaw—but also of *E. coioides*—irregular white spots or blotches, the number pyloric caeca about 50 ~ 60. However, since their mtDNA cyt b sequence is consistent with that of *E. coioides*, and mtDNA has a strict maternal inheritance, we concluded that the fish in the Dx group might be hybrid descendants of *E. coioides*. 
E. malabaricus fish were also amplified in their hybrid offspring, indicating that Mendel’s law applies as well. In addition, AFLP markers contain rich polymorphic loci. Hence, AFLP will have great potentials in the construction of genetic linkage maps of these two groupers. As to the few abnormal AFLP markers appearing in DxD or Dd/Xd, we think it is mainly because of our sampling method. All the fish we sampled were about the same age and the putative hybrid descendants DxD and Xd groups were not the direct offspring of the fishes in Xx and Dd groups. Thus, it will not be a surprise to see some unexpected AFLP markers in the DxD/Xd group. Besides, even in direct hybrid offspring, there are still possibilities that abnormal AFLP markers might occur owing to the possible genetic material mutations—duplication, deletion, inversion, and translocation—at either the genetic or chromosome level or both. Zhang et al. 2005; Yang et al. 2005. These non-Mendelian markers must be considered when constructing the genetic linkage map using AFLP markers.

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