RAPD FINGERPRINTING OF THREE SPECIES OF GROUPER (*Epinephelus* spp.) FROM MAKASSAR STRAIT, SOUTH SULAWESI, INDONESIA

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ABSTRACT

Grouper belonging to the epinepheline serranid family has promising aquaculture potential for food and ornamental fish production. Random amplified polymorphic DNA (RAPD) technique was employed in this study to determine the genetic variability and species differentiation of groupers from genus *Epinephelus* (*E. areolatus*, *E. merra*, and *E. suillus*). Groupers (N=52) were collected from Makassar Strait, South Sulawesi, Indonesia. Of 34-screened RAPD primers, ten primers (OPA-02, OPA-06, OPA-08, OPA-10, OPA-15, OPA-16, OPA-17, OPA-18, OPA-19, and CA-05) were selected to generate the RAPD fingerprinting of groupers. Results indicated that the ten primers revealed different RAPD profile among three species. Similarity index obtained by pairwise comparison was 0.62 ± 0.07 for *E. areolatus*, 0.58 ± 0.11 for *E. merra* and 0.80 ± 0.11 for *E. suillus*. Polymorphism and genotype number detected were 51.3% ± 8.7% and 3.8 ± 0.6 in *E. areolatus*; 56.8% ± 14.6% and 4.1 ± 1.1 in *E. merra*; and 60.2% ± 11.8% and 3.8 ± 0.9 in *E. suillus*, respectively. Genetic distance level of individuals ranges from 0.19—0.42, 0.14—0.50, and 0.02—0.30 for *E. areolatus*, *E. merra*, and *E. suillus*, respectively. The genetic distance between *E. merra* and *E. areolatus* (D=0.52) was closer compared with *E. merra* and *E. suillus* (D=0.67). A total of 33 RAPD markers differentiating the three species of groupers were detected. The study presents the RAPD technique as the useful genetic marker for assessment of genetic diversity and species differentiation in groupers.

KEYWORDS: RAPD fingerprinting, genetic variability, grouper, species differentiation

INTRODUCTION

Groupers (*Epinephelus* spp.) are commercially important marine fishes in Southeast Asian countries. Among these groupers, *E. areolatus*, *E. merra*, and *E. suillus* are commonly found in the local and export market in Indonesia. For *E. suillus*, it may be reported as *E. tauvina* for previous studies. Most of these species for both aquarium and consumption are caught from the wild. In some areas the species are rare as a result of over exploitation. Taxonomic classification based on the morphological characteristics of the groupers is still troublesome since some groupers have similar in their morphological appearance. Confusion is particularly seen in identification of *E. malabaricus*, *E. suillus*, and *E. salmoides* (Doi *et al.*, 1991). Thus, the genetic variability and species differentiation of groupers are necessary to be investigated in order to design and to implement adequate management, breeding program and genetic improvement strategies.

Recent advances in molecular techniques have provided new approaches in determining species identification of fishes. To date, genetic analyses have become an increasingly important component of taxonomic studies in fisheries biology. This approach has advantage of allowing access the characters which are usually unaffected by environmental conditions and that can permit a direct indication of the reproductive status of morphological divergent forms.

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A new molecular technique called Random Amplified Polymorphic DNA (RAPD) developed by Williams *et al.* (1990) is based on the amplification of DNA with arbitrary primer in polymerase chain reaction (PCR). The development of the PCR technique has promised a highly sensitive method, since a few DNA are enough to obtain accurate patterns and radioactivity is no longer necessary. The RAPD method has been shown to generate informative fingerprinting in relatively short time and no prior knowledge of DNA sequences is required (Welsh & McCelland, 1990). Significant genetic differentiation based on RAPD marker in various fish species has been noted in many studies (Dinesh *et al.*, 1993a; Foo *et al.*, 1995; Bielawski & Pumo, 1997; Coccone *et al.*, 1997; Koh *et al.*, 1999; Liu *et al.*, 1999), but no published data on genetic variation of the three species of groupers is available so far. Since the RAPD technique can detect DNA variability between strains or species, it has been applied for genetic differentiation of three species of the genus *Oreochromis* and four other sub-species of *O. niloticus* (Bardakci & Skibinski, 1994; 1998); three species of *Anguilla* spp. (Takagi & Taniguchi, 1995); three species of tilapia (*O. japonicus, O. australis*, and *O. mossambicus*) (Dinesh *et al.*, 1993b); *Penaeus monodon* and *Metapeneus ensis* (Meruane *et al.*, 1997).

This study aimed to employ the RAPD in association with PCR to examine the usefulness of RAPD analysis in detecting the genetic variability and species differentiation of grouper species (*E. areolatus, E. merra*, and *E. suillus*). Data on genetic variability among three species may identify the new basis of taxonomic identification of grouper.

MATERIALS AND METHODS

Fish Samples

E. areolatus (N=17), *E. merra* (N=19), and *E. suillus* (N=16) collected from Makassar Strait, South Sulawesi, Indonesia were used in this study. The range of their total length and body weight varied from 14.0 to 24.3 cm and 50 to 186 g for *E. areolatus*, 11.5 to 28.9 cm and 16 to 310 g for *E. merra* and 18.7 to 32.5 cm and 107 to 324 g for *E. suillus*. The samples were morphologically identified according to Randall *et al.* (1987) and Kohno *et al.* (1990). Approximately 50 mg of muscle tissue was taken out from each sample and preserved in 250 mL of Tris-NaCl-EDTA-SDS-Urea (TNES-Urea) buffer. This preservative consists of 6 M urea; 10mM Tris-HCl; 125 mM NaCl; 10 mM EDTA; and 1% (w/v) sodium dodecyl sulfate; at pH 7.5 (Asahida *et al.*, 1996). The samples were transported at ambient temperature from the field and kept under room temperature in the laboratory prior to DNA extraction.

DNA Extraction

Genomic DNA was extracted from preserved muscle tissue by Phenol-Chloroform method developed by Parenrengi *et al.* (2000). The genomic DNA was electrophoresed at a 0.8% (w/v) horizontal agarose gel at 55 volts for 1-2 hours in 1XTBE (Tris-Borate-EDTA) buffer. The gel staining was done in 0.5 µg/mL of ethidium bromide for 20—30 minutes and then washed with distilled water for 5—10 minutes. Purity of genomic DNA was estimated by measuring in a spectrophotometer at the wavelength of 260 nm and 280 nm. The purity of DNA was quantitatively estimated from the ratio between the reading of absorbency at 260 nm and 280 nm (OD_{260}/OD_{280}) and also qualitatively observed through the appearance of the single band formed on the agarose gel.

Primer Screening

A total of 34 arbitrary primers were screened for a randomly single individual fish in order to find the suitable primer for DNA amplification of grouper. The sequence, nucleotide length, and GC content of these primers are listed in Table 1. Among them, ten primers (OPA-02, OPA-06, OPA-08, OPA-10, OPA-15, OPA-16, OPA-17, OPA-18, OPA-19, and CA-05), based on the clarity and sharpness bands formed on agarose gel, were selected to use for further analysis.

DNA Amplification

The total reaction volume of 25 mL was used with the final concentration containing 1X PCR buffer; 3.5 mM $MgCl_2$; 0.4 mM Biotools dNTPs mix; 0.4 mM primer; 2.0 units Biotools taq DNA polymerase; 50 ng template DNA. The DNA amplification was performed using a GeneAmp PCR system 2400 (Perkin Elmer) programmed for 45 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing temperature at 36°C, 1 min of primers extension at 72°C, and a final extension of 2 min at 72°C. Amplification of PCR products were separated in a horizontal electrophoretic gel system.

Mixture of 10 ml PCR product and 2.5 mL loading dye was run on a 2.0% agarose gel electrophoresis at 55 volts in 1XTBE for 2—3 hours and then stained with 0.5 mg/mL of ethidium bromide for 20—30 minutes. Gel electrophoresis was washed with distilled water for 5—10 minutes prior to photographing with Image Master VDS (Pharmacia-Technology).

Data Analysis

The molecular weight of fragments was estimated based on the standard of DNA banding pattern from GeneRuler DNA ladder marker (Fermetas). The fragment that was present for all individuals in three species of *Epinephelus* genus was considered as a genus-specific marker. The fragments were valued as polymorphic when they were absent in some samples but changes in banding intensity were not valued as polymorphic. Different banding pattern generated by each primer in the same species of grouper was considered as a different genotype. Presence of fragment was scored as 1 while absence of band was scored as 0 at a particular position or distance migrated on the gel. A data matrix of 1's and 0's was entered into the data analysis package. Data analysis was performed using the program RAPDistance Package Software Version 1.04 (Amstrong *et al.*, 1994) and Numerical Taxonomy and Multivariate Analysis System (NTSYS) Version 1.80 (Rohlf, 1994). The genetic similarity index was calculated across all possible pairwise comparisons of individuals using the formula: $S_{xy} = 2n_{xy}/n_x + n_y$ (Nei & Li, 1979). Where n_{xy} is number of band shared by individual x and y; n_x and n_y are the number of band scored for each individual. The index of similarity was used to calculate the genetic distance value and to construct the dendrogram. The dendrogram was constructed using Unweighted Pair-Group Method of Aritmethic (UPGMA) employing Sequential, Agglomerative, Hierarchical and Nested Clustering (SAHN) from NTSYS.

Data of polymorphism and genotype number among three species of groupers were analyzed by one-way ANOVA from MSUSTAT-pc program. When ANOVA identified differences, multiple comparisons among means were made with least significant difference (LSD). Statistical significance was determined by setting the aggregate type at 5% ($P<0.05$) for each set of comparisons.

RESULTS DAN DISCUSSIONS

Results

DNA Extraction

The genomic DNA was successfully extracted from preserved grouper muscle tissue by Phenol-Chloroform method. A clear single band formed on a 0.8% agarose gel stained with 0.5 mg/mL of ethidium bromide indicates the high purity of genomic DNA obtained. The purity of genomic DNA obtained were 2.00 ± 0.14 for *E. areolatus*, 2.07 ± 0.13 for *E. merra* and 2.14 ± 0.22 for *E. suillus*.

Primer Screening

Twenty-eight out of 34 screened primers (82.4%) were observed to yield visible amplification products, while the other primers gave negative results (Appendix 1). Based on the clear bands, ten primers (OPA-02, OPA-06, OPA-08, OPA-10, OPA-15, OPA-16, OPA-17, OPA-18, OPA-19, and CA-05) were selected for further analysis on different species of grouper. A typical RAPD amplification yielded from one to seven visible fragments and their size of DNA amplification product is within the range of 150—1,700 bp. This may indicate the limitation in the resolving power of the agarose gel at the lower molecular weight as well as the inefficiency of the PCR extension reaction at higher molecular weights. The result showed that the primer screening plays an important step prior to primer selection for further study.

RAPD Profile

Ten primers generated different number and size of fragment for genus *Epinephelus*. Two to eight fragments were observed in *E. merra* and *E. areolatus*, while two to nine fragments were detected in *E. suillus*. The size of fragments ranging from 350 to 3,000 bp for *E. areolatus*, 200 to 3,000 bp for *E. merra* and 300 to 1,700 bp for *E. suillus* were detected in this present study. The fragment number and size generated by ten primers are presented in Table 1.

Polymorphism and number of genotype among three species of grouper were not significantly different ($P>0.05$). Polymorphic fragments was 60.2% ± 11.8% in *E. suillus*, 56.8% ± 14.6% in *E. merra* and 51.3% ± 8.7% in *E. areolatus*. Number of genotype observed in this study also showed slight variation among species of *Epinephelus* spp. ($P>0.05$). Percent

Table 1. Fragment number and size of DNA amplification in *E. areolatus*, *E. merra*, and *E. suillus* generated by ten primers

Primer	Number of fragment			Size range of fragment (bp)		
	E. areolatus	*E. merra*	*E. suillus*	*E. areolatus*	*E. merra*	*E. suillus*
OPA-02	3--4	4--6	5--7	450–2000	600--1500	550–1700
OPA-06	5--8	3--6	2--5	400–2500	400--1500	250–1500
OPA-08	4--6	4--6	3--9	300–1100	300--1500	300–1400
OPA-10	2--8	5--7	4--7	550–3000	200--3000	300–1500
OPA-15	5--7	2--4	3--6	350–1600	350--1500	350–1500
OPA-16	3--7	5--6	3--7	350–1200	300--950	300–1500
OPA-17	3--6	3--5	3--5	550–1600	500--1300	400–1700
OPA-18	6--7	4--6	6--7	350–1100	350--1000	300–1200
OPA-19	4--7	5--8	4--6	350–1600	400--1250	320–1300
CA-05	7--8	5--7	3--6	300–1200	300--1000	300–1500

polymorphic fragment and number of genotypes of *E. areolatus*, *E. merra*, and *E. suillus* for each primer are presented in Table 2. The ten primers produced different RAPD banding patterns on different species of grouper. Figure 1 shows the RAPD fingerprinting of grouper generated by the ten primers from two representative samples of *E. areolatus*, *E. merra*, and *E. suillus*, respectively.

Similarity Index

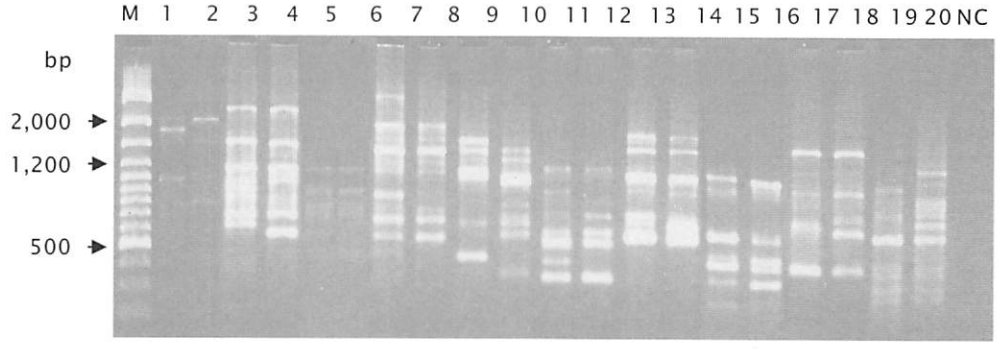
Similarity index of grouper was 0.62 ± 0.07 for *E. areolatus*, 0.58 ± 0.11 for *E. merra* and 0.80 ± 0.11 for *E. suillus*. Among three species of grouper, *E. suillus* showed the highest similarity index, followed by *E. areolatus* and *E. merra*.

Table 2. Polymorphism and number of genotype detected in groupers (*Epinephelus* spp.) by ten primers

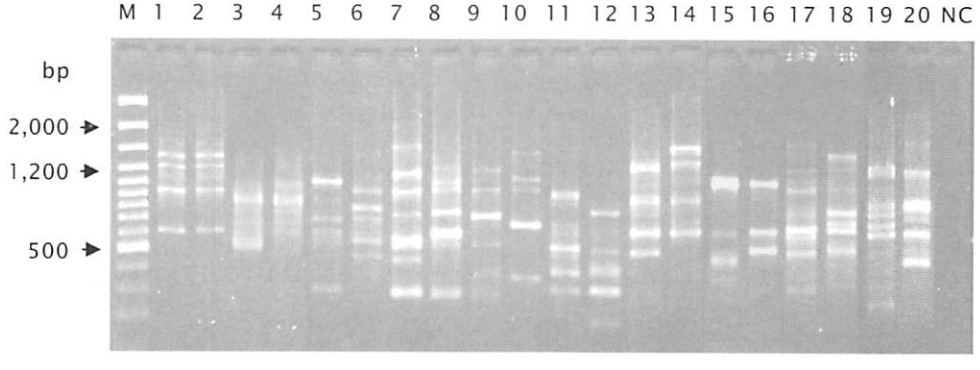
Primer	Polymorphism (%)[2]			Number of genotype[2]		
	E. areolatus	*E. merra*	*E. suillus*	*E. areolatus*	*E. merra*	*E. suillus*
OPA-02	66.7	75	42.9	4	4	3
OPA-06	44.4	57.1	60	4	5	4
OPA-08	50	50	66.7	4	4	5
OPA-10	62.5	50	62.5	5	3	4
OPA-15	50	85.6	75	4	6	5
OPA-16	57.1	57.1	66.7	3	3	5
OPA-17	50	60	71.4	4	5	3
OPA-18	37.5	33.3	37.5	3	3	3
OPA-19	50	50	62.5	4	5	3
CA-05	44.4	50	57.1	3	3	3
Average[1]	51.3 ± 8.7[a]	56.8 ± 14.6[a]	60.2 ± 11.8[a]	3.8 ± 0.6[a]	4.1 ± 1.1[a]	3.8 ± 0.9[a]

[1] Values are mean ± SD

[2] Values in the same row with different superscripts are significantly different (P<0.05)



(A)



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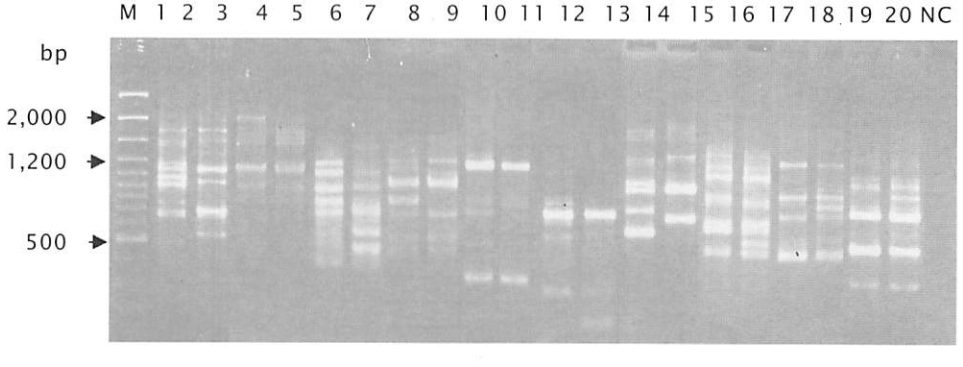


(C)

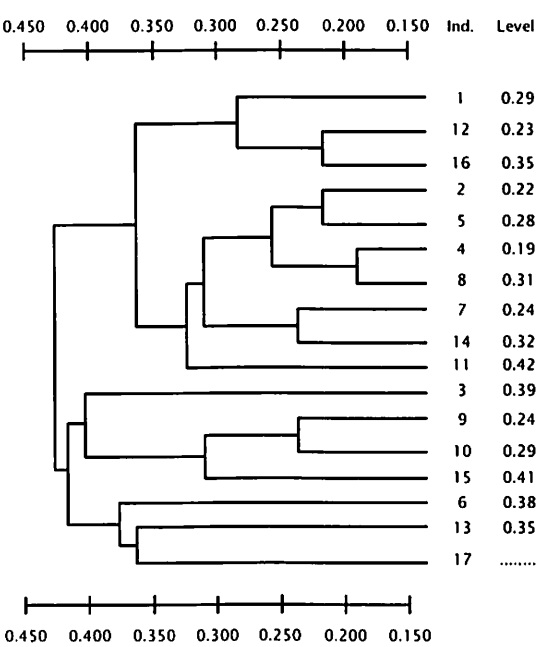
Figure 1. RAPD banding patterns of two representative samples of *E. areolatus* (A); *E. merra* (B); and *E. suillus* (C) generated by various primers. GeneRuler 100bp DNA ladder plus from Fermentas (lane M), samples generated by primer OPA-02 (lanes 1-2), OPA-6 (lanes 3-4), OPA-08 (lanes 5-6), OPA-10 (lanes 7-8), OPA-15 (lanes 9-10), OPA-16 (lanes 11-12), OPA-17 (lanes 13-14), OPA-18 (lanes 15-16), OPA-19 (lanes 17-18), CA-05 (lanes 19-20) and negative control (lane NC)

Genetic Distance

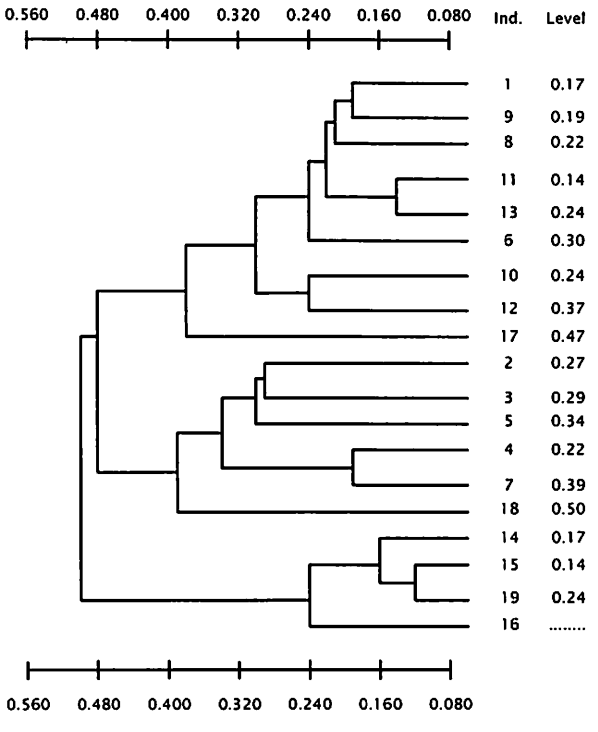
Genetic distance level (D) of groupers was 0.31 ± 0.07 for *E. areolatus*, 0.27 ± 0.10 for *E. merra* and 0.09 ± 0.08 for *E. suillus*. This low genetic distance within individual of this species indicates that all individuals are genetically closely related. Figure 2 shows the dendrogram among individuals of *E. areolatus*, *E. merra*, and *E. suillus* using UPGMA analysis based on the Nei and Li's indices. The dendrogram of *E. merra* and *E. suillus* showed the similar structure in individual clustering. There were two individual clusters obtained from these species where one cluster shows the dominant cluster in population (11 individuals in *E. suillus*; 15 individuals in *E. merra*). While, *E. areolatus* dendrogram is evenly distributed from 17 individuals of two main clusters. Genetic distance among three species within the genus *Epinephelus* was 0.52—0.67. The UPGMA cluster analysis summarizing the genetic relationship among three species of *Epinephelus* spp. based on the genetic distance levels is shown in the dendrogram of Figure 3. The cluster analysis shows that *E. areolatus* species are closer to *E. merra* (D=0.52) than to *E. suillus* (D=0.67).

Diagnostic Marker

The presence of genus-specific marker was observed in three species of the genus *Epinephelus* for ten primers. This marker was used as a diagnostic genetic marker to differentiate the three species of grouper. Five fragments (950bp in OPA-02; 550bp and 700bp in OPA16; 600pb in OPA-08; 650bp in OPA-17) were considered as genus specific markers of this present study, since they were present in all three species of groupers (*Epinephelus* spp.). Most of the diagnostic markers of *E. merra* (700bp, 950bp in OPA-06; 550bp in OPA-10; 450bp, 500bp, 600bp, 1000bp in OPA-18; 900bp in OPA-19; 650bp, 800bp, 1000bp in CA-05) were also obtained in *E. areolatus* but these diagnostic markers were absent in *E. suillus* (Appendix 2). The presence of certain fragment was also observed in each species of groupers. Seven fragments (2000bp in OPA-02; 1000bp, 600pb in OPA-06; 850bp in OPA-10; 1400bp in OPA-15; 1400bp; 300bp in OPA-19) in *E. areolatus*, five fragments (1100bp in OPA-06; 900bp in OPA-08; 400bp in OPA-16; 900bp in OPA-17; 500bp in OPA-19) in *E. merra* and nine fragments (900bp in OPA-06; 650bp in OPA-16; 850bp in OPA-17; 1200bp, 850bp,



2A



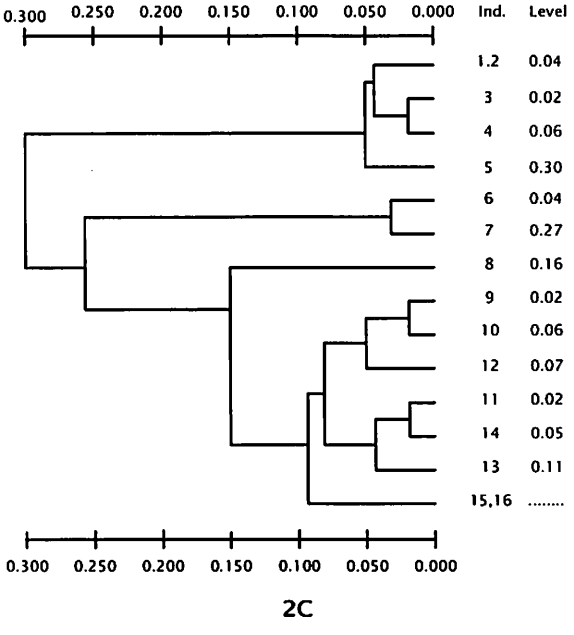


Figure 2. UPGMA cluster analysis of *E. areolatus* (A), *E. merra* (B), and *E. suillus* (C), based on the genetic distance generated from Nei and Li's indices

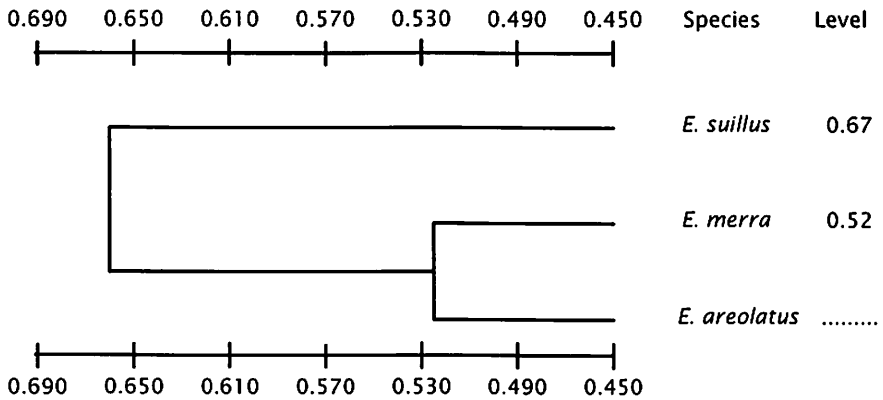


Figure 3. UPGMA cluster analysis based on the genetic distance generated from Nei and Li's indices of three species within the genus *Epinephelus*

550bp in OPA-18; 1700bp, 750bp in OPA-19; 850bp in CA-05) in *E. suillus* were detected in this study (see Appendix 2).

DISCUSSION

DNA Extraction

Linacero *et al.* (1998) noted that quantification of DNA is an important step in many procedures where it is necessary to know the amount of DNA that is present when performing technique such as RAPD. It is suggested to use the genomic DNA ranging from 1.8—2.0 for PCR requirement in amplification of DNA. However, in this present study, genomic DNA in slight lower or more than the range (1.6—2.2) was not found to strongly influence to the banding pattern on agarose gel. Apun et *al.* (2000) also reported that the ratio OD_{260}:OD_{280} ranging from 1.53—1.96 was sufficiently pure for PCR reaction. The genomic DNA of more than 2.0 was suspected to be contaminated with phenol and other organic matter residue derived from the preservative and DNA extraction, while lower than 1.8 was perhaps contaminated with protein. Since the genomic DNA obtained was not degraded and no smearing band was detected on the gel, the DNA amplification of genome is suspected to produce a good RAPD banding pattern. The genomic DNA of fish obtained from Phenol-Chloroform extraction was compatible with mtDNA, restriction enzyme and RAPD analysis (Smith *et al.*, 1997; Heist & Gold, 1999).

For application of the Phenol-Chloroform technique in preserved muscle tissue by TNES-Urea, the sample homogenization is not required and incubation for cell lysis only needs a short time (compared to the fresh tissue lysis) due to the activity of the cell lysis during the preservation periods. The clear band formed on agarose gel indicates that the buffer preserved DNA well. While preserving the DNA samples, this buffer perhaps worked also to lyse the muscle tissue, since the buffer contains the chemicals such as Tris-base, NaCl, EDTA, and SDS which are commonly used in lysis buffer for DNA extraction. Urea in this buffer also played the important role in preserving DNA for extraction purposes as well as in improving the DNA amplification. The addition of 7 M urea on the 8% (w/v) resolving gels in SDS-PAGE improved the amplified fragment separation considerably in tiger barb, guppy, tilapia and salmon (Dinesh I, 1993a; 1993b). The TNES-Urea is suitable for DNA extraction of fish muscle tissue that are rich in cellular endonucleases, since urea is an inhibitor of these enzymes and also as an activator of Proteinase K (Asahida *et al.*, 1996). The TNES-Urea is also the most reliable buffer for tissue preservation and DNA extraction of Japanese flounder, *Paralichthys olivaceus* and Atlantic hearing, *Clupea harengus* (Asahida *et al.*, 1996). They found that this preservative offers advantages for preserving the fish muscle tissue with the DNA yield is 0.5—2.6

mg of total DNA/mg tissue after the tissue was preserved for 3 years.

Primer Screening

The primer screening plays an important step prior to primer selection for RAPD studies. This is a useful preliminary study in order to find the most suitable primer for amplifying the genomic DNA. The failure in producing the amplification fragment is due to the poor discrimination of the primer between the alternative-priming site of different nucleotide sequence. This is due to the limitation in the resolving power of the agarose gel at the lower molecular weight as well as the inefficiency of the PCR extension reaction at higher molecular weights as explained by Bowditch *et al.* (1993). Smith *et al.* (1997) reported that a certain percentage of arbitrary primer gave poor results and sometimes did not give any DNA amplification at all. Williams *et al.* (1990) reported that the some polymorphisms were clear and easy to score, but other bands appeared to produce ambiguous fragments. Bowditch *et al.* (1993) also reported that for any given particular genome, a certain percentage of RAPD primers produced unsatisfactory amplification results. However, the same primer may work perfectly well on the next genome tested, so the failure of the particular primer/template combination is more likely because of the lack of readily amplifiable segments in the template DNA rather than a systematic problem with the primer.

From the result of the study, it was found that there was no relationship between the length of the primers and the number of amplified products. The number of fragments generated is dependent on the primer sequence rather than to the nucleotide length. Similar result was obtained from the study on the guppy, *P. reticulata* (Dinesh *et al.*, 1994). This present study showed that the good fragments of amplification the grouper DNA was obtained from the primers containing 60% or slightly more than 60% of GC bases in their sequences in comparison with the lower GC content. Williams *et al.* (1990) demonstrated that the importance of the GC primer content in order to get the clarity of the banding patterns. They also reported the minimum primer length to detect amplification in ethidium bromide-stained agarose gel was nine bases as well as a minimum of 40% GC content of primer is generally used. Fritsch *et al.* (1993) also reported that primers with a high GC content (more than 60%) produced better results for generating RAPD profile than the other primers.

RAPD Profile

In this study, different RAPD profile and high polymorphism (51.3%—60.2%) were revealed within species of *Epinephelus* spp. by various primers. The high polymorphism detected in each species could be indicative a low level of inbreeding. This is a general agreement that the samples were collected from the wild population. In addition, the high variability based on the polymorphism for each species indicated that the high probability of success in genetic improvement of these species through the breeding selection method. Asma (1999) reported the different number and size of fragment revealed by different primers between varieties of tiger barb, *P. tetrazona*. The number and size of amplification products ranged from one to nine and 210 to 255bp, respectively. A range of the molecular weight fragments amplified by the four different primers (OPA-14, OPA-17, OPA-18, and OPA-19) on genomic DNA of Malaysian River catfish, *M. numerus* was 200—2000bp (Lim, 1998). According to Liu *et al.* (1999), the RAPD marker was highly reproducible for both channel catfish and blue catfish in size range from 200-1500bp.

Different species of tilapine fishes showed the variation in polymorphic bands (Mwanja *et al.*, 1996). The results of their investigation on five species of tilapia showed that the number of polymorphic bands was 49 to 81% for *Oreochromis niloticus*, 33% to 78% for *O. esculentus*, 64%—78% for *O. leocostictus*, 70% for *Saratherodon galilaeus* and 55%—75% for *Tilapia zilli*. Four strains of channel catfish and three strains of blue catfish also exhibited a low level of polymorphism (5%—10%), even though distinct phenotypes were observed and inherited for each of these strains (Liu *et al.*, 1999). A total of 14 RAPD markers were obtained from crosses of guppy, *P. reticulata* (Foo *et al.*, 1995). Of these markers, eight (60.0%) of them were polymorphic. Interspecific hybridization between brown trout and Atlantic salmon (Elo *et al.*, 1997) involving three primers (OPA-11, OPA-19, and OPB-08) generated polymorphism as well as producing different banding patterns between species.

To produce reliable and useful RAPD profile, the following areas need to be considered. It

is recommended to use more primer to minimize the risk of misinterpretation in a genetic analysis since different RAPD fragments have similar size. The mixed intensity of RAPD fragment produced each primer can be overcome by optimization of protocol along with an experimental strategy for obtaining reproducible RAPD banding pattern. To avoid this problem, PCR reaction for DNA amplification such as genomic DNA, $MgCl_2$, taq polymerase, dNTP, primer, annealing temperature and cycle number for DNA amplification have been standardized on the previous study on PCR optimization for RAPD marker of grouper.

Similarity Index

The genetic similarity estimations among individuals of the three species of grouper were used to assess their phylogenetic relationship. The high similarity index obtained in the three species of grouper in this present study suggests that there is a close relationship among individuals for each species. *E. suillus* showed the closest genetic relationship among individuals, followed by *E. areolatus* and *E. merra*. The relatively different genetic similarity of the three species of tilapia was also revealed by the RAPD analysis (Dinesh *et al.*, 1996). They found that Aureus tilapia showed the most divergent RAPD profile while the Mozambique and Nile tilapia were closely related to each other than to the Aureus tilapia. High genetic similarity was reported on the guppy, *P. reticulata* (Foo *et al.*, 1995). The mean similarity value between individuals of the Green Snakeskin and Three-quarters Black variety was 0.66 ± 0.066. Different similarity index was also revealed from different variety of tiger barb, *P. terrazona* (Asma, 1999). The similarity coefficient was found to be high between normal and green variety of *P. tetrazona* (0.7313—0.8322) while low value was detected between green and yellow variety (0.3333—0.6278) for both Perak and Johor populations. The mean pairwise similarity between brown trout, *Salmo trutta* and nonanadromous salmon, *Salmo salar* was 0.338 generated by three random primers (Elo *et al.*, 1997).

Genetic Distance

Genetic distance estimates obtained in the present study suggest that *E. areolatus* is genetically closer to *E. merra* than to *E. suillus*. This is also supported by the high degree of morphological similarity between both species of *E. merra* and *E. areolatus*. Dinesh *et al.* (1996) showed the phylogenetic relationship between three species of tilapia. They found that the relatively higher genetic similarity (0.59 ± 0.07) between Mozambique and Nile tilapia than between the Aureus and Mozambique pair (0.48 ± 0.07) was obtained. Also, the genetic similarity between Aureus and Nile (0.46 ± 0.09) was significantly higher compared with that of Aureus and Mozambique tilapia (0.38 ± 0.07). Furthermore, the study by Bardakci & Skibinski (1994) on tilapia using RAPD analysis linked Mosambique tilapia with the sub species of Nile tilapia, with Aureus tilapia as the outgroup.

The result obtained from analysis of genetic distance indicates that the species understudied are distinguished well-defined gene pools. The genetic distance obtained from three species of genus *Epinephelus* varied between 0.52—0.67. Smith and Chesser (1981) noted that mean genetic distance between species is usually greater than 0.20 and is higher than the genetic distance within individuals. The significant differences between species are also shown in denrogram of UPGMA cluster analysis (Figure 3). The genetic distance between species was found to be higher that the genetic distance within individuals of each species (0.31 ± 0.07 in *E. areolatus*, 0.27 ± 0.10 in *E. merra* and 0.09 ± 0.08 in *E. suillus*). The result of this present study is in agreement with the finding of Smith & Chesser (1981).

The different genetic distance on various fish species was reported on tilapia (Bardakci & Skibinski, 1994), discus (Koh *et al.*, 1999), and hilsa shad (Dahle *et al.*, 1997) where the range in genetic distance was 0.04—0.34, 0.07—0.18, and 0.08—0.16 respectively. Asma (1999) found the low genetic distance level on tiger barb, *P. tetrazona* among individuals from different varieties. The genetic distance values ranged from 0.048—0.294 for normal variety from Perak population; 0.032—0.833 for green variety from Perak population; 0.030—0.286 for yellow variety from Perak population; 0.043—0.529; for normal variety from Johor population; 0.033—0.304 for green variety from Johor population and 0.034—0.429 for yellow variety from Johor population.

Some authors have reported the utility of RAPD markers as a source of phylogenic information between species. Takagi & Taniguci (1995) reported the occurrence of polymorphism for three species of eels

(*Anguilla japonicus, A. australis,* and *A. bicolor*) employing three random primers (OPA-11; OPA-12; and OPA-16). RAPD fingerprinting was also used to assess the genetic diversity among four wild forms and five cultivated varieties of discus, *Symphysodon* spp. (Koh *et al.*, 1999). They found that the cultivated varieties was genetically closer to the three *S. aequiefasciata* wild forms (blue, green, and brown form) with a mean genetic distance of 0.033 than to *S. discus* (Heckel) with a mean genetic distance of 0.105. This shows that *S. aequiefasciata* wild form was the genetic origin of the cultivated varieties.

Diagnostic Marker

All of ten primers produced the diagnostic markers for three species of genus *Epinephelus*. Eleven fragments were present in both *E. merra* and *E. areolatus* but they were absent in *E. suillus*. This result was also acceptable with the genetic differences between *E. merra* and *E. areolatus* was closer than to *E. suillus*. In addition, five fragments (OPA02-950bp; OPA08-950bp; OPA16-700bp; OPA16-550bp; OPA17-860bp) obviously indicate as genus-specific marker of *Epinephelus* since they are present in all the three species from the same genus of grouper studied. This result demonstrates the useful marker in genetic differentiating between the species of grouper. Williams *et al.* (1998) also reported that a total of 15 diagnostic markers were used to identify the subspecies of largemouth bass, *Micropterus salmoides*. On the other hand, different result was shown in study on red mullet, *Mullus barbatus* where the four selected primers (OPA-02, OPA-09, OPE-11, and OPE-12) failed to produce the specific marker for discriminating of different populations (Mamuris *et al.*, 1998) and none of the unique fragment was found in each population of hilsa shad (Dahle *et al.*, 1997).

CONCLUSIONS

A total of 75 fragments for *E. suillus* and *E. areolatus;* while 72 fragments for *E. merra* were revealed by ten RAPD primers. Number of genotype detected was slightly different between species of grouper. Primer OPA-15 in *E. suillus* and in *E. merra*, and OPA-10 in *E. areolatus* produced the highest polymorphism. *E. suillus* was observed to show high polymorphism (60.0%), followed by *E. merra* (56.9%) and *E. areolatus* (52.0%). Similarity index of the three species of grouper was 0.80 ± 0.11 for *E. suillus*, 0.62 ± 0.07 for *E. areolatus* and 0.58 ± 0.11 for *E. merra*. Level of genetic distance among individuals of *E. suillus* was 0.02—0.30, while *E. merra* and *E. areolatus* were 0.14—0.50 and 0.199—0.42, respectively. Dendrogram shows that species of *E. merra* was genetically closer to *E. areolatus* (0.52) than to *E. suillus* (0.67). A total of 11 diagnostic markers (700bp, 950bp in OPA-06; 550bp in OPA-10; 450bp, 500bp, 600bp, 1000bp in OPA-18; 900bp in OPA-19; 650bp, 800bp, 1000bp in CA-05) were present in *E. merra* and *E. areolatus*, but they were absent in *E. suillus*. Five fragments (950bp in OPA-02, 600bp in OPA-08, 550bp and 700bp in OPA16, and 650bp in OPA-17) were distinguished as genus-specific markers of grouper.

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Appendix 1. Primer code, sequence, nucleotide length and GC content used in Random Amplified Polymorphic DNA analysis for primer screening

No[1]	Primer[2]	Sequence 5' to 3'[3]	Nucleotide length	GC Content (%)
1	OPA-01+	CAGGCCCTTC	10-mer	70.0
2	OPA-02+*	TGCCGAGCTG	10-mer	70.0
3	OPA-03+	AGTCAGCCAC	10-mer	60.0
4	OPA-04+	AATCGGGCTG	10-mer	60.0
5	OPA-05+	AGGGGTCTTG	10-mer	60.0
6	OPA-06+*	GGTCCCTGAC	10-mer	70.0
7	OPA-07+	GAAACGGGTG	10-mer	60.0
8	OPA-08+*	GTGACGTAGG	10-mer	60.0
9	OPA-09+	GGGTAACGCC	10-mer	70.0
10	OPA-10+	GTGATCGCAG	10-mer	60.0
11	OPA-11+	CAATCGCCGT	10-mer	60.0
12	OPA-12+	TCGGCGATAG	10-mer	60.0
13	OPA-13+	CAGCACCCAC	10-mer	70.0
14	OPA-14+	TCTGTGCTGG	10-mer	60.0
15	OPA-15+*	TTCCGAACCC	10-mer	60.0
16	OPA-16+*	AGCCAGCGAA	10-mer	60.0
17	OPA-17+*	GACCGCTTGT	10-mer	60.0
18	OPA-18+*	AGGTGACCGT	10-mer	60.0
19	OPA-19+*	CAAACGTCGG	10-mer	60.0
20	OPA-20+	GTTGCGATCC	10-mer	60.0
21	NUSZG-01+	TTGCGTCCA	9-mer	55.6
22	NUSZG-02	GCACTGTCT	9-mer	55.6
23	NUSZG-03	GGTAACGCCC	10-mer	70.0
24	NUSZG-04+	GGAGCTGGC	9-mer	77.8
25	NUSZG-09	TGCCTGTGGGGAATCC	16-mer	62.5
26	NUSZG-10	TATGTAAAACGACGGCCAGT	20-mer	45.0
27	CA-01+	TTTTTTAGCCTTTTTTGAGC	20-mer	30.0
28	CA-02+	AAATGTTGAGGAAAAAAAGT	20-mer	25.0
29	CA-05+*	TTCCCACGGATCATGATCAATCAA	24-mer	410
30	CA-08	TCCACAAAAGTGGATCCAGATCTAGATCCA	30-mer	43.0
31	Primer-40+	GTTTTCCCAGTCACGAGGTTGTA	23-mer	47.8
32	Primer-50+	TTGTGAGCGGATAACAATTTC	21-mer	38.1
33	DALRP+	CACACAGGAAACAGCTATGAC	21-mer	417
34	DALP-22+	GTTTTCCCAGTCACGACGC	19-mer	57.9

[1]No. 1-20 =Primers purchased from Operon Technologies Inc., 21-30= Commercially synthesized primers from Gibco-BRL, 31-34=Commercially synthesized primers from Genosys.

[2]+ =Primers yielded visible amplification products, *=Selected primers used for further analysis

[3]Abbreviations: A=Adenine; C=Cytosine; G=Guanine; and T=Thymine

Appendix 2. Diagnostic markers of species within the genus *Epinephelus*

Primers	Diagnostic marker (bp)		
	E. areolatus	*E. merra*	*E. suillus*
OPA-02	2,000	-	-
	950	950	950
OPA-06	-	1,100	-
	1,000	-	-
	950	950	-
	-	-	900
	700	700	-
	600	-	-
OPA-08	-	900	-
	600	600	600
OPA-10	850	-	-
	550	550	-
OPA-15	1,400	-	-
OPA-16	700	700	700
	-	-	650
	550	550	550
	-	400	-
OPA-17	-	900	-
	-	-	850
	650	650	650
OPA-18	-	-	1,200
	1,000	1,000	-
	900	-	900
	-	-	850
	600	600	-
	-	-	550
	500	500	-
	450	450	-
OPA-19	-	-	1,700
	1,400	-	-
	900	900	-
	-	-	750
	-	500	-
	300	-	-
CA-05	1,000	1,000	-
	-	-	850
	800	800	-
	650	650	-