Indian J. Fish., 61(2) : 28-34, 2014

Resolution of taxonomic ambiguity in groupers (Pisces: Serranidae) by the random amplified polymorphic DNA (RAPD) technique

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ABSTRACT

In this study, a species specific molecular marker RAPD (Random Amplified Polymorphic DNA) was used for resolving taxonomic ambiguity in groupers thereby helping in species identification. Fishes were collected from the coast of Mandapam and Keelakkarai, Tamil Nadu. RAPD patterns were developed for 8 species of groupers and each one had a unique RAPD profile. The phylogenetic relationships of these species were also analysed.

Keywords: Grouper, Phylogeny, RAPD, Taxonomic ambiguity

Introduction

Grouper species are usually identified by their colour pattern as well as meristic characters including pyloric caecae counts (Heemstra and Randall, 1993). Molecular techniques are used as a powerful method to confirm species and also to bring out the evolutionary history. RAPD (Random Amplified Polymorphic DNA) analysis is a technique based on polymerase chain reaction (PCR) amplification of discrete regions of the genome with short oligonucleotide primers of arbitrary sequence (Welsh and McClelland, 1990; Williams *et al*., 1990). This method is simple and quick to perform and most importantly, no prior knowledge of the genetic make-up of the organism in question is required (Hadrys *et al*., 1992). It requires only minute amounts of template DNA with which the complete genome is screened. Genetic polymorphisms can be visualised within 24h from the extraction of genomic DNA. The storage of this genetic material is also easy. RAPD technique has some limitations like limited reproducibility across laboratories, dominance of markers and limited applicability above generic level for systematic studies. Among DNA fingerprinting techniques, it requires the least in technology, labour and expenditure (Caetano-Anolles *et al*., 1991; Hadrys *et al*., 1992; Black, 1993).

RAPD technique has been proven to be a valuable technique for species identification and confirmation in fishes (Dinesh *et al*., 1993; 1995; Bardakci and Skibinski, 1994; Naish *et al*., 1995; Bielawski and Pumo, 1997; Elo *et al*., 1997; Callejas and Ochando, 1998; Liu *et al*., 1998; Williams *et al*., 1998). It can also be used to confirm species identity in groupers (Parenrengi, 2001; Ansenio *et al*., 2002; Upadhyay *et al*., 2006; Saad *et al*., 2012) especially in captive breeding where the stocking of broodstocks based on their morphological appearance has to be supported with other identification techniques to ensure precise pairing (Bakar and Azizah, 2000).

Materials and methods

Collection of specimens

Freshly caught specimens of eight species of groupers were obtained from fishing boats off the coast of Mandapam and Keelakkarai ($9^0$13' N - $9^0$18' N and 78^0 50' E - 79^0 10' E) along the south-eastern coast of Tamil Nadu. The fishes were photographed either in the fishing boats or as soon as they were brought to the landing centres. The species were initially sorted into different groups as per their colour pattern and then identified to the species level based on the morphological and meristic characters especially the pyloric caecae counts (Roy, 2004).

Collection of blood samples

Blood samples (0.25 ml) for DNA extraction were collected from the live fish (20 specimens of each species) immediately after capture, from the caudal vein using sterile syringe rinsed with heparin as anticoagulant. The blood samples were immediately transferred to sterile eppendorf tubes containing 1.25 ml of 95% ethanol, mixed well, sealed with parafilm and stored in a refrigerator until further analysis.

Genomic DNA extraction and quantification

Total DNA was extracted from the blood samples following the procedure of Cenis *et al*. (1993) with minor modifications. In 50 ml centrifuge tubes, 500 µl of blood (stored in 95% ethanol) collected from each specimen was taken and ethanol was decanted by centrifugation at 10,000 rpm for 10 min. The blood cells were washed with high TE buffer (0.1 M Tris, 0.04 M EDTA, pH 8.0) and the TE buffer was decanted by centrifugation at 10,000 rpm for 10 min. In order to lyse the cells, incubation buffer (10 mM Tris, 1 mM EDTA, 0.4 M NaCl, 10% SDS and Proteinase K) was added and incubated at 56 ^{0}C for 2 h. After incubation, the DNA was purified by successive extraction with phenol: chloroform: isoamyl alcohol (25: 24: 1) and chloroform: isoamyl alcohol (25:1) solutions respectively, then centrifuged at 12000 rpm for 15 min. The aqueous supernatant was transferred to a fresh tube and 1/10th volume of 3 M sodium acetate was added. The DNA was precipitated with ice-cold absolute ethanol. The precipitated DNA was pelleted by centrifugation at 1000 rpm for 15 min. After a wash with 70% ethanol, the DNA was vacuum dried and resuspended in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Concentration and purity of extracted DNA was determined spectrophotometrically at 260 nm and 280 nm. Samples showing 1 OD equivalent to 50 µg and purity 1.8 alone were taken for further analysis.

Screening of RAPD primers

Thirty decamer primers *i.e*., 10 each from OPA, OPAH and OPAC series (Operon Technologies, Alameda, USA) were screened in *Epinephelus* species samples.

PCR amplification

Out of the thirty decamer primers used in the study, 8 primers were selected which gave sharp, highly intense and reproducible bands. RAPD–PCR reactions were carried in a PTC 200 gradient thermal cycler (M. J. Research Inc., Water town, Massachusetts, USA) employing the RAPD primers described earlier. Amplification was performed in 25 µl reactions containing 1x extraction buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 6 - 8 pmoles of primer, 200 mM dNTPs, 2 U Taq DNA polymerase (Genei, Bangalore, India) and 25 mg of template DNA. To check for DNA contamination, a negative control was set up omitting the DNA from the reaction mixture. The reaction mixture was preheated at 95 ^{0}C for 3 min. followed by 40 cycles (94 ^{0}C for 3 min., 40 ^{0}C for 1.30 min and 72 ^{0}C for 2 min). The reaction was then subjected to a final extension at 72 ^{0}C for 10 min.

Agarose gel electrophoresis

PCR products were electrophoretically analysed in 1.5% agarose gels, stained with ethidium bromide (5 µg ml^{-1}) in 1x TBE buffer (pH 8.0), visualised and photographed using Image Master VDS (Pharmacia Biotech). The alleles were designated according to the PCR product size in relation to the molecular marker (λ DNA with Eco RI / Hind III double digest).

Analysis of RAPD data

All reproducible and resolvable RAPD fragments were scored directly from the gels. Bands were converted into a binary data matrix in which the presence of a band was scored as '1' and absence as '0'. Mathematical formulae used were based on a few assumptions. First, all RAPD fragments scored were 2 allele systems, *i.e*., presence (dominant) and absence (recessive) of bands. Second, fragments that migrated to the same position had the same molecular weight and which stained to the same intensity were homologous bands from the same alleles. A third assumption was that the grouper populations fit the Hardy-Weinberg equilibrium, $p^2 + 2\,pq + q^2 = 1$, with frequencies p (dominant, band present) and q (recessive, band absent) (Clark and Lanigan, 1993; Lynch and Milligan, 1994). Faint and poorly amplified bands (amplifications were repeated thrice for all samples), were excluded from further analyses. From the binary matrix, the total number of RAPD fragments and polymorphic ones were calculated for each primer and also for all primers.

Genetic distance and cluster analysis

The 'genetic distance (D)' and 'genetic similarity index (S)' between the grouper species were estimated using POPGENE Version 1.32 (Yeh *et al*., 1997). Nei and Li's (1979) pair-wise genetic similarity index (S) among the 8 species of groupers was computed and converted into genetic distance (D) according to Hillis and Moritz's (1990) formula, D = 1 - S. Genetic similarity index is given as: $S = 2\,N_{AB} / (N_A + N_B)$, where, N_{AB} is the number of bands shared in common by individuals A and B,

and N_A and N_B are the total number of bands for A and B respectively (Nei and Li, 1979). S values range from 0 when no bands are shared between the RAPD profiles of two individuals, to 1 when no differences are observed *i.e.*, they are identical. The opposite holds true for D values.

Cluster analysis was performed on pairwise genetic distance estimates using the unweighted pair-group method with arithmetic mean *i.e.*, UPGMA (Sneath and Sokal, 1973) algorithm of PHYLIP Version 3.573c (Felsenstein, 1999). UPGMA dendrograms of the 8 grouper species were plotted using TREEVIEW 1.6.1 (Page, 1996). The binary data matrix was bootstrapped 100 times to test the confidence level of each branch and a consensus tree was then constructed.

Results and discussion

Screening of primers

From the thirty decamer primers (OPA 03 to 12, OPAH 01 to 10 and OPAC 01 to 10) that were screened, eight primers from the OPA and OPAH series were selected. The primers selected were OPA 07, 08, 09, 10, 11, 12 and OPAH 01 and 02 for screening the eight species of groupers *viz.*, *Epinephelus malabaricus, E. coioides, E. polyphekadion, E. diacanthus, E. faveatus, E. merra, E. coeruleopunctatus* and *Cephalopholis formosa.*

RAPD profiles produced by selected primers

All the eight primers selected *i.e.*, OPA 07-12 and OPAH 01-02 produced sharp, highly intense and reproducible bands (Fig. 1a-h). They were also not polymorphic with in a species. A total of 154 bands were produced. The number of fragments (bands) generated per primer varied from 13 - 24. From these eight primers, OPA-07, OPA-08, OPA-12 and OPAH-02 produced easily distinguishable RAPD patterns for each of the eight species and were therefore recommended for differentiation.

Analysis of RAPD data

Genetic distance (D) (Table 1, Fig. 9)

The genetic distance was least between *E. polyphekadion* and *E. diacanthus* (D = 0.2366), followed by *E. faveatus* and *E. merra* (D = 0.2714) and then between *E. malabaricus* and *E. coioides* (D = 0.2863).

The genetic distance was maximum between *E. coioides* and *C. formosa* (D = 0.6039), followed by *E. malabaricus* and *E. merra* (D = 0.5430) and then between *E. coioides* and *E. merra* (D = 0.5129).

Genetic similarity index, 'S' (Table 1, Fig. 9)

The genetic similarity was highest between *E. polyphekadion* and *E. diacanthus* (S = 0.7893) followed by *E. faveatus* and *E. merra* (S = 0.7810) and then between *E. malabaricus* and *E. coioides* (S = 0.7510).

The genetic similarity was least between *E. coioides* and *C. formosa* (S = 0.5467) followed by *E. malabaricus* and *E. merra* (S = 0.5810) and next between *E. coioides* and *E. merra* (S = 0.5987).

Summary of RAPD data (Table 2)

The intra-species genetic distance values in eight species of groupers were determined. Oneway ANOVA was carried out to test for differences in intra-species genetic distance values based on RAPD markers among eight species of groupers. One way ANOVA was also carried out to test for differences in inter-species genetic distance values based on RAPD markers by pairwise comparison of individuals. The inter-species distance (7.220048) was found to be significantly higher ($p < 0.01$) than the intra-species distance (0.167198).

Groupers are generally identified based on the morphological and meristic characters, relying mainly on the meristic counts and pigmentation pattern of the skin (Heemstra and Randall, 1993). The morphological approach is not a very confirmatory one due to the presence of several colour morphs within the species and wide variation in the colour pattern between juveniles and adults of the same species. No single consistent external morphological character has yet been found to differentiate commercially important groupers such as *Epinephelus coioides, E. tauvina* and *E. malabaricus* (Heemstra and Randall, 1993). Supportive techniques are needed to confirm the taxonomic status of groupers, which are very important both from fisheries and aquaculture points of view. The use of pyloric caeca is an invasive method and non-invasive genetic techniques are preferred as they prevented sacrifice of the organism.

The present report is the second one on the application of RAPD markers for species identification of groupers from Indian waters and the first report is of Govindaraju and Jayasankar (2004) in which RAPD fingerprints of 7 species of groupers from the south-west and south-east coasts of India were generated using 4 primers that were consistent, reproducible and yielded species-specific diagnostic markers in all the species. Parenrengi (2001) had earlier differentiated 3 species of groupers from Indo-Malaysian waters using ten selected RAPD primers. Likewise in the present study also,

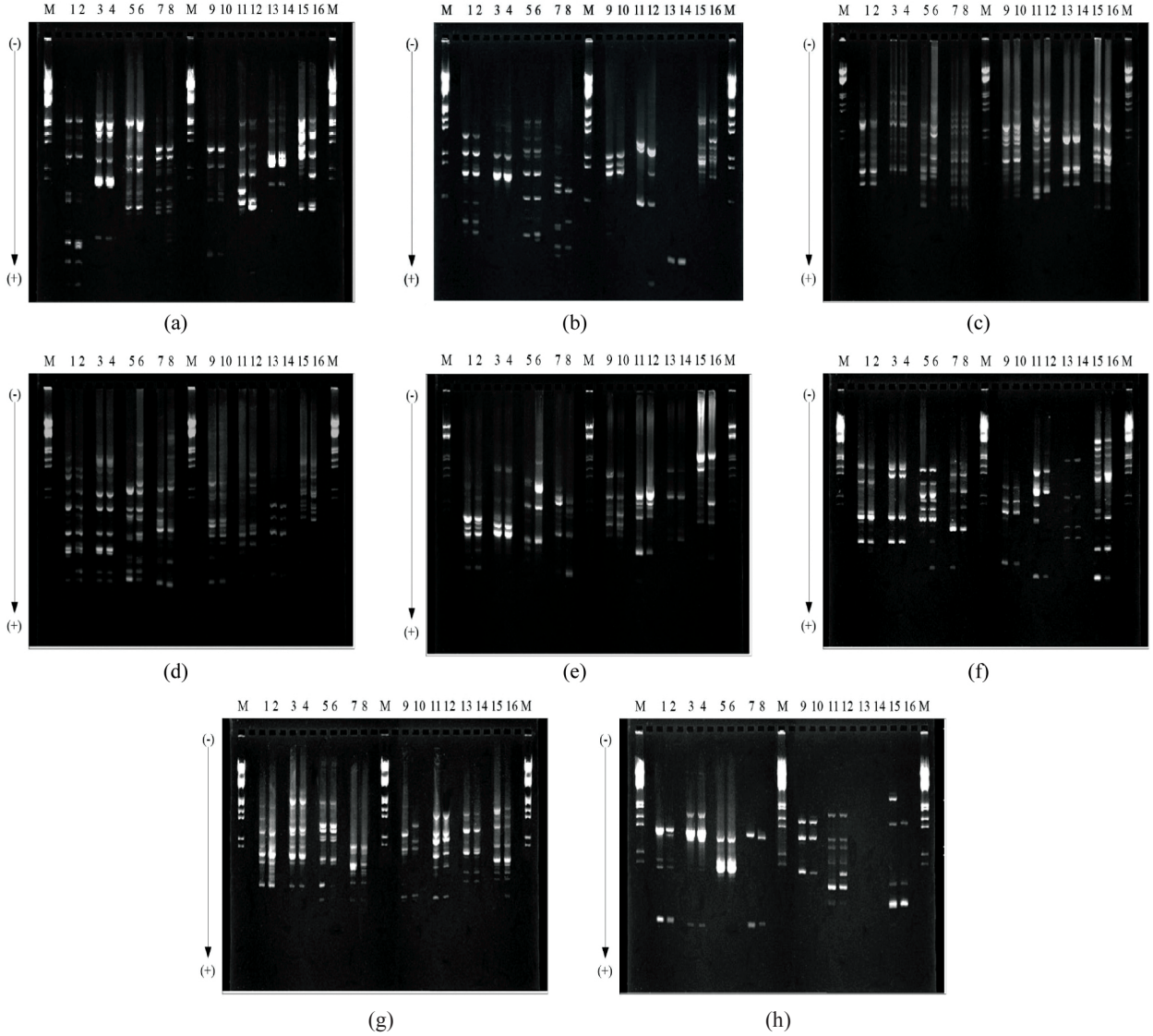

Fig. 8. RAPD pattern of different grouper species with primer OPAH – 02

M - Marker, Lane no. 1 & 2 - *Epinephelus malabaricus,* 3 & 4 - *E. coioides,* 5 & 6 - *E.polyphekadion,* 7 & 8 - *E. diacanthus,* 9 & 10 *E. faveatus,* 11 & 12- *E. merra,* 13 & 14 - *E. coeruleopunctatus*, 15 & 16 - *Cephalopholis formosa*

Table 1. Inter-species similarity index (above diagonal) and genetic distance (below diagonal) of eight species of groupers

Species ID	*E. mal.*	*E. coi.*	*E. pol.*	*E. dia.*	*E. fav.*	*E. mer.*	*E. coe.*	*C. for.*
E. mal.	-	0.7510	0.6877	0.7083	0.6282	0.5810	0.6611	0.6094
E. coi.	0.2863	-	0.6895	0.7139	0.6278	0.5987	0.6473	0.5467
E. pol.	0.3745	0.3718	-	0.7893	0.7223	0.7070	0.7383	0.6457
E. dia.	0.3448	0.3370	0.2366	-	0.7810	0.7683	0.7612	0.7000
E. fav.	0.4649	0.4655	0.3254	0.2471	-	0.7623	0.7171	0.6366
E. mer.	0.5430	0.5129	0.3467	0.2636	0.2714	-	0.6644	0.6226
E. cae.	0.4139	0.4349	0.3035	0.2729	0.3325	0.4088	-	0.6528
C. for.	0.4952	0.6039	0.4375	0.3566	0.4515	0.4739	0.4265	-

E. mal. – Epinephelus malabaricus, E. coi. – E. coioides, E. pol. – E. polyphekadion, E. dia. – E. diacanthus, E. fav. – E. faveatus, E. mer. – E. merra, E. coe – E. coeruleopunctatus C. for. – Cephalopholis formosa

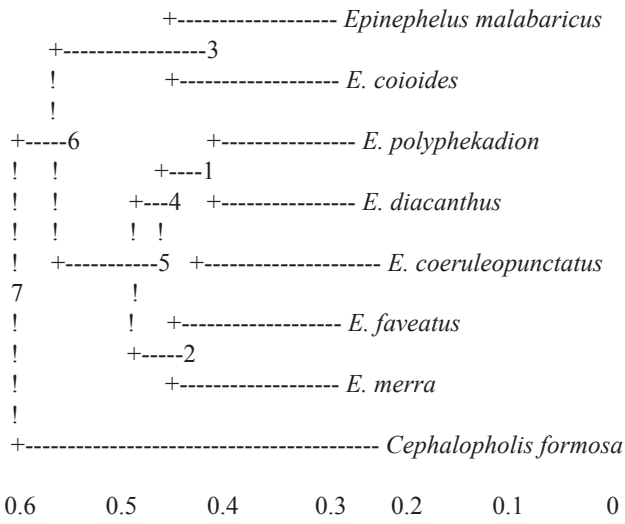

Fig. 9. Dendrogram based Nei's (1978) genetic distance: method = UPGMA modified from NEIGHBOR procedure of PHYLIP Version 3.5

Table 2. Intra-species genetic distance values in eight species of groupers

Species name	Intra-species genetic distance
Epinephelus malabaricus	0.0279
E. coioides	0.0956
E. polyphekadion	0.0487
E. diacanthus	0.0529
E. faveatus	0.0079
E. merra	0.0529
E. coeruleopunctatus	0.0079
Cephalopholis formosa	0.0746

all eight arbitrary primers used, gave unique banding patterns for each species. The results of RAPD analysis demonstrate separation of gene pools of all 8 species of groupers, in which all individuals of each species formed close monophyletic species clusters. The very low GD value between *E. polyphekadion* and *E. diacanthus* (0.2366), between *E. faveatus* and *E. merra* (0.2714) and between *E. malabaricus* and *E. coioides* (0.2863) indicates the proximity of these species. The results of more advanced studies on the phylogenetic relationships of several grouper species of the family Serranidae based on cytochrome b (Ding *et al.,* 2006a) and 16S rDNA mitochondrial sequences (Ding *et al.,* 2006b) as well as DNA barcoding based on cytochrome c oxidase (COI) mitochondrial sequences (Sachithanandam *et al*., 2012) substantiate the present findings.

Because of their overlapping morphological characters, *E. malabaricus, E. coioides* and *E. polyphekadion* have often been confused among themselves, as well as among *E. faveatus* and *E. merra* (Heemstra and Randall, 1993). The present study has segregated these species through the GD values between *E. coioides* and *E. malabaricus,* between *E. coioides* and *E. polyphekadion,* between *E. malabaricus* and *E. polyphekadion* and between *E. faveatus* and *E. merra* which were 0.2863, 0.3718, 0.3745 and 0.2714 respectively. In the present study, reproducibility of RAPD was tested at various stages, leading to consistent banding patterns with all primer amplifications. However, reference RAPD fingerprints in groupers must be prepared only after confirming the identity of the species using its pyloric caeca count and pattern (Roy and Gopalakrishnan, 2011). Following this, the RAPD fingerprint can serve as a reference molecular marker.

Acknowledgements

This study was supported by the National Agricultural Technology Project on groupers (F.No.28(1)/2000-NATP/CGP-II/223, dt.25.05.2001) awarded to late Prof. M. Peter Marian in which the first author worked as a Senior Research Fellow. The authors are also grateful to Mr. T. Saravanan, Mandapam for assistance in specimen collection

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Date of Receipt : 09-08-2011

Date of Acceptance : 08-11-2013