GENETIC PROFILING OF GROUPERS OF THE GENUS *EPINEPHELUS* (FAMILY: SERRANIDAE) USING DNA-LEVEL MARKERS

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सारांश

दुनिया भर पाई जानेवाली ग्रूपर मछलियाँ महंगी हैं. इपेनेफेलस वंश की ये मछलियाँ अपने बाह्य आकृति और अभिलक्षणों से जाति निर्धारण में अडचन पैदा करती है. आनुवंशिकी तरीका रान्डम आंप्लिफैड पोलिमोरफिक डी एन ए (RAPD) से इसकी सात जातियाँ याने कि *ई. डायाकान्थस* , *ई. एयरोलोटस, ई. क्लोरोस्टिग्मा, ई. ब्लीकेरी, ई. कोइओडस, ई.टविना, ई. मलबारिकस* के जेनेटिक प्राफाइल का विकास किया गया. यह प्रयास इस वंश के वर्गीकरण केलिए अत्यंत उपयोगी साबित हुआ है. 4 प्राइमर (ओ पी ए 01 , ओ पी ए 07, ओ पी एफ 08 और ओ पी एफ 10) से विकसित किए 4 फिंगर प्रिंट स्थायी, पुनरुत्पादनीय होने के साथ साथ सभी जातियों के जातिनिर्धारण केलिए उचित अंकक साबित हुए. इन चार प्राइमरों से कुल 59 RAPD लोसि विकसित किए जो 70 से 4500 बेस पेअर आकार के थे. चयन की गई 7 मछली जातियों के आनुवंशिक संबंध दिखाने केलिए आनुवंशिक दूरी मूल्य याने कि जेनेटिक डिस्टन्स वाल्यू के आधार पर UPGMA डन्ड्रोग्राम विकसित किया गया. जातियों के बीच में से और जातियों के अंदर आनुवंशिकी दूरी कम थी. संबंधों में सब से अधिक आनुवंशिक दूरी *ई. मलबारिकस* और *इ. डयाकान्थस* ने दिखाई. *ई. कोइओइड्स* और *ई. टाविना* के बीच, *ई. मलबारिका* और *ई. क्लोरोस्टिग्मा* के बीच और *ई. क्लोरोस्टिग्मा* और *ई. ब्लीकेरी* के बीच निकटतम आपसी आनुवंशिक संबन्ध दिखाए पडे. इपेनेफेलस मछलियों की 5 जातियों में आकार-रूप अध्ययन मल्टिवेरियेट सांख्यिकी विश्लेषण के ज़रिए किया गया . इसके परिणाम भी RAPD विश्लेषण के सदृश्य थे.

ABSTRACT

Groupers are economically important marine fishes having world wide distribution. Phenotypic identification of species of the genus *Epinephelus* is often confusing due to overlapping of morphological characters. The Random Amplified Polymorphic DNA (RAPD) fingerprinting was used to develop genetic profiles in seven species of *Epinephelus*, such as *E. diacanthus, E. areolatus, E. chlorostigma, E. bleekeri, E. coioides, E. tauvina*, and *E. malabaricus* with a view to ratifying their taxonomic status. The RAPD fingerprints generated with four primers (OPA 01, OPA 07, OPF 08 and OPF 10) were consistent, reproducible and yielded species-specific diagnostic markers in all the species. A total of 59 RAPD loci in the size range of 70-4500 bp were produced from all the four arbitrary primers. UPGMA dendrogram was constructed based on genetic distance values to show the genetic relationships among seven species. Intraspecies genetic distance values were significantly lower than interspecies values. *E. malabaricus* was observed to be most distantly related to *E. diacanthus* and *E. bleekeri*. A very close genetic relationship was seen among *E. coioides, E. tauvina* and *E. malabaricus* and also between *E. chlorostigma* and *E. bleekeri*. Within species genetic polymorphism was highest in *E. chlorostigma* and lowest in *E. tauvina*. Multivariate statistical analysis of truss network landmark distance measures was also done to differentiate five species of *Epinephelus* based on their body size and shape variation. Results of Principal Component Analysis and Discriminant analysis were in conformity with those of RAPD analysis.

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INTRODUCTION

1. INTRODUTION

Groupers of the genus, *Epinephelus*, Family Serranidae, are economically important and highly valued marine food fishes. They are distributed through out the tropical and temperate seas of the world. The Family Serranidae comprises of three subfamilies; Serraninae, Anthiinae and Epinephelinae. Groupers are classified in 14 genera of the Epinephelinae, which includes at least half of the approximately 449 species in the Serranidae.

Groupers are not only important for food but also for ornamental purposes. Both small and large species are kept in aquariums. Groupers form a major component of artisanal fisheries resources. The species under the genus *Epinephelus* are very important as far as mariculture of finfishes is concerned. Fast growth, disease resistance and high market value attracted the farmers as well as the researchers to develop a culture technology. The whole aquaculture system of groupers including feeding, spawning, larval rearing and grow-out production has been standardized. Considerable progress in the culture of few species has been achieved in many countries like Peoples' Republic of China, Hong Kong, Malaysia, Singapore, Taiwan, Thailand, Republic Korea, etc. The major species being cultured world wide are *E. coioides, E. malabaricus, E. tauvina, E. staiatus, E.akaara, E. fuscoguttatus*. In India, Groupers are widely distributed in the coastal waters. More than 28 species have been reported, under the genus *Epinephelus,* along East and West coasts of India and in the Islands of Lakshadweep in the Arabian Sea and in the waters of Andaman and Nicobar Islands in the Bay of Bengal (James *et al.*, 1996).

Groupers are bottom associated fishes and most species occur on coral reefs, but some live in estuaries or on rocky reefs. Juveniles of some species are found in seagrass beds and adults of few species prefer sandy or silty areas. Majority of the species inhabit depths less than 100 m and juveniles are often found in tide-pools. Groupers are highly carnivorous fishes and usually feed on a variety of fishes, larger crustaceans, and cephalopods and few are plankton feeders. They exhibit

protogynous hermaproditism. After spawning as a female for one or more years, the fish changes sex and thereafter functions as a male.

One major problem with grouper is the identification of species because of the closely similar and often overlapping morphological features. Generally groupers are identified by their colour pattern and/or a suite of morphological characters like body shape, configuration and size of the fins, the shape and relative size of the head and various parts of the head and body, the number of fin rays, scales and gill rackers.

Except in large adults of some species, the colour pattern of most groupers is usually distinguishing enough to identify the particular species but intra-specific variation in colour pattern is most common and this makes identification difficult. Juveniles of some species look completely different from adults of the same species. In species with dark spots, the spots become smaller and more numerous with growth. These groupers have an ability to alter their colour pattern in few seconds, depending on the mood of the fish. Many groupers have a "fright" or "stress" pattern of white blotches or bars. Post-mortem changes in colour pattern can obscure the normal pattern of the live fish. Hence, morphologically based classification is often confusing. Their wide distribution and colour variation make it difficult not only to identify grouper species visually, but also to record the catch statistics for each species. Selection of precise pair for breeding purpose is another difficulty with grouper identification.

Thus, there is a need for supporting techniques to identify species along with morphological characteristics and it is also essential to determine genetic profiling of grouper populations, which is most important for the design of adequate management programs. Truss morphometrics is one of many tools available for identification of fish stocks (Ihssen *et. al.,* 1981). However, the technique has also found application in differentiating fish species (Cavalcanti *et al.*, 1990; Golubtsov *et al.,* 1999). The truss is a system of vertical, horizontal and oblique distances measured between preselected anatomical landmarks, which are points identified on the basis of local morphological features and chosen to divide the body into functional units (Bookstein *et. al.*, 1985). This method has advantages over conventional morphometric character sets that usually comprise length, depth and

width measurements. Conventional morphometric measurements are redundant (most are along the longitudinal axis) and have no geometric properties, whereas the truss network provides measurements that cover the entire body and represents the shape of the animal (Strauss and Bookstein, 1982). The principal component *analysis (PCA) and dicriminant analysis of truss landmarks distance measures can* reveal morphometrics relatedness among species/stocks. The idea of using truss network analysis in the present study was to examine whether this technique is useful in identifying the species of groupers based on the shape variation by comparing with the results of DNA markers.

One alternative for gathering information on genetics of grouper species is the use of molecular DNA-level markers. Recently, a number of easily assayable and highly valuable genetic markers such as Isozymes, Random Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), *Variable Number of Tandem* Repeats (VNTRs: minisatellite and microsatellite) and Single Stranded Conformational Polymorphisms (SSCPs) have been developed (Hallerman and Beckman, 1988; Wright, 1993; Fergusson, 1994). These markers, in conjunction with the discovery of a polymerase chain reaction (PCR) capable of rapid DNA multiplication, have a wide range of potential applications in fisheries and aquaculture.

For the present study Random Amplified Polymorphic DNA (RAPD) technique, a method of DNA fingerprinting analysis, was used which requires no previous knowledge about the target sequence. Instead, *RAPD relies on the* presence of low stringency priming sites for single arbitrary primer on both strands of DNA molecule close enough to permit DNA amplification. This technique involves detection of polymorphisms by randomly amplifying multiple regions of the genome using single primer and then determining their nucleotide sequence. The number and size of amplification products depends on the complementarities of the sequence of the particular primer and template DNA. RAPD products are compared after separation of the DNA by *gel-electrophoresis, with bands of identical sexes being* used as an identifying characteristic of species or strains. This method is rapid which requires only a small amount of DNA, relatively easy to perform, involves no

radioactivity and the cost is low compared to other genetic markers. The main objective of this study is to reveal the taxonomic status of seven grouper species available along Indian waters and also to develop species-specific diagnostic markers using RAPD fingerprinting.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Taxonomic Status of Groupers

The composition and phylogenetic relationship of the family serranidae were discussed by Johnson (1983, 1988), Kendall (1984), and Leis (1986). Johnson proposed that the serranidae comprises three subfamilies: Serraninae, Anthiinae, and Epinephelinae. He further divided the Epinephelinae into five tribes such as Niphoninae, Epinephelini, Diploprioni, Liopropomini, and Grammistini. Johnson (1988) hypothesized that the Diploprioni, Liopropomini, and Grammistini constitute a monophyletic group that is most closely related to the Epinephelini and that this group of four tribes is the sister taxon on the Niphoni. Heemstra and Randall (1993) have divided the family Serranidae into five subfamilies: Serraninae, Anthiinae, Niphoninae, Epinephelinae, and Grammistinae.

About 100 species are reported worldwide under the genus *Epinephelus* and artificial propagation of about 13 species of *Epinephelus* has been achieved (Wang, 1997).Twenty eight species of groupers are recorded from around the seas of India (James *et al.*, 1996). Wide distribution, identical morphological characteristics and intraspecific colour variation made identification of grouper species difficult (Heemstra and Randall, 1993) as briefly mentioned below.

2.1.1. *Epinephelus areolatus* (Forsskal, 1775)

It has often been confused with *E. chlorostigma* (Heemstra and Randall, 1993), which is also covered with brown spots and has modally one more dorsal and pectoral fin rays, 2 more gill rackers, and smaller, more numerous, dark brown spots, with the largest dark spots on body about half of the size of the pupil, also the dark spots are closer together, with the pale interspaces forming a pale network on the head, body and median spines.

2.1.2. *E. bleekeri* (Vaillant, 1878)

This species was twice misidentified and then 3 names were given to this species (Heemstra and Randall, 1993).

2.1.3. *E. chlorostigma* (Valenciennes, 1828)

This species was misidentified with two other species such as *E. polylepis* and *E. gabriellae*. This species complex is characterized by their truncate or emarginated caudal fin, colour pattern of small closely set dark brown spots covering all but ventral parts of head and body and all the fins, preopercle subangular with numerous small platelets, operculum with a straight upper edge and 2 rows of teeth on sides of lower jaw. The other two species of this complex, *E. gabriellae* and *E. polylepis* were described as new species (Randall and Heemstra, 1991; Morgans, 1982).

2.1.4. *E. coioides* (Hamilton, 1822)

It is often misidentified for *E. malabaricus* and *E. tauvina*. The colour pattern of all three species are similar (Chan, 1968; Tan and Tan, 1974; Randall *et al.*, 1978; Morgans, 1982; Sainsbury *et al., 1985*) but the dark spots of *E. malabaricus* are smaller, blackish brown (not reddish brown or brownish orange, as on *E. coioides*), and remain distinct in preservative. *E. malabaricus* also has irregular white spots on the head and body (no white spots on *E. coioides*). *E. tauvina* often has a black blotch (larger than eye) on body at base of last 4 dorsal fin spines and extending onto lower part of fin. Juveniles have the dark spots on the median fins. *E. tauvina* also has a longer jaw (upper jaw length 21-24%of standard length, versus 17-20% in *E.coioides*), usually more gill rackers and no bony platelets on lateral side of first gill arch (Heemsra and Randall, 1993).

2.1.5. *E. diacanthus* (Valenciennes, 1828)

It is an important component of the grouper fishery along Kerala coast and off Bombay coast. This species is similar to the allopatric species *E. sexfasciatus* and *E. stictus* (Randall and Heemstra, 1991). Records of *E. diacanthus* from the western pacific are based on misidentifications of *E. stictus*

(Chan, 1968, Katayama, 1988), or *E. sexfasciatomaculos* (Burgess *et al.,* 1988; Shen, 1984*)*. *E. sexfasciatus*, the sister species of *E. diacanthus* differs in having black spots on the median fins, fewer scales (lateral line 46-51, lateral scale series 82-96), a smaller head and deeper caudal peduncle. *E. stictus* has numerous black spots on the head and front part of the body, fewer scales and auxillary scales (Heemstra and Randall, 1993).

2.1.6. *E. longispinis* (Kner, 1864)

It is similar to *E. maculates* in counts of fin rays, scales and gill rackers, and also in morphometric features, elevated anterior dorsal-fin spines and a distinct step like indentation on ventral edge of maxilla. Juveniles are somewhat similar in colour pattern, brown with small-scattered dark spots on body, larger dark spots on fins and irregular white spots and blotches on head and body (Randall and Heemstra, 1991).

2.1.7. *E. malabaricus* (Bloch and Schneider, 1801)

E. malabaricus is known from the Red sea and Indo-Pacific area. It was often misidentified with *E. coioides* (Heemstra, 1991) and *E. tauvina* (Kyushin *et al.*, 1977; Morgans, 1966, 1982; Tan *et al.*, 1982). Morgans (1966) distinguished *E. malabaricus* from *E. coioides* and *E. tauvina* but he used the wrong names for these species; his *E. tauvina* is *E. malabaricus* and he described *E. tauvina* as a new species, *E. chewa*. He identified *E. coioides* as *E. malabaricus* but also appears to have mistaken large (>150cm total length) specimens of *E. lanceolatus* for *E. malabaricus* ((Heemstra and Randall, 1993).

2.1.8. *E. tauvina* (Forsskal, 1775)

E. tauvina is most important species for mariculture and is a major component of artisanal fisheries but separate catch statistics are not available for this because of the confusion with *E. malabaricus* and *E. coioides.* Most of the literature concerning *E. tauvina* that was published before 1984 was based on misidentifications of *E. coioides, E. malabaricus* or *E. lanceolatus* (Heemstra and Randall, 1993). Randall and Bentauvia (1983) incorrectly listed *E. salonotus* as a synonym of *E. tauvina*, this error was corrected by Heemstra and Randall (1984)

who recognized *E. salonotus* as a synonym of *E. spilotoceps*. Randall and Heemstra (1991) have discussed the many misidentifications of *E. tauvina*. Detailed identification characters of these species are discussed by Heemstra and Randall (1993).

2.2. Truss Morphometry

Truss network analysis has been used to differentiate cryptic/sibling species in addition to their wider application for delineation of stock / strain structure of many fishes. It was Strauss and Bookstein (1982), who proposed this method of sampling linear distances by creating a box truss network between landmarks as a more comprehensive representation of form. Several researchers have compared performance of traditionally measured finfish dimensions to box-truss distances and found that trussed data resulted in more accurate classification of individuals (Strauss and Bookstein, 1982; Winans, 1987; Schweigert, 1990; Roby *et al.,* 1991). Principal Component Analysis (PCA) of size-corrected truss distances can yield a picture on the relatedness of different species of fish (Rohlf and Bookstein, 1987). Calvacanti *et al.* (1990) studied comparative morphometrics of three species of sciaenids namely: *Ctenosciana gracilicirrhus, Paralonchurus braciliensis and Micropogonias furnieri* by multivariate analysis of truss networks. Principal component analysis of interlandmark distances defined by the truss system showed that the *Ctenosciana gracilicirrhus* are different form those of the two species in relation to shape, being more similar to the individuals of *Micropogonias furnieri* in relation to size. Creech (1992) investigated species status of *Atherina boyeri* and *A. presbyter* using multivariate morphometrics.

Canonical Variate Analysis (CVA) is one of the most important and widely used multivariate statistical techniques in biological research. The procedure was developed by R.A. Fisher in 1936 and further expanded by M.S. Bartlet, P.C. Mahalanobis and C.R. Rao to examine several significant problems relevant to systematic biology. These include separation of groups of morphologically similar organisms; ascertaining of patterns of character covariation, such as size and shape patterns between groups; assessment of intergroup affinities; and allocation of individuals to pre-existing groups. CVA is discussed widely in modern text books on multivariate analysis (Kshirsagar, 1972). However, most treatments stress algebraic, computational and inferential aspects, rather than geometrical understanding

(Dempster, 1969). Campbell and Atchley (1981) described the geometry of canonical variate analysis, Mahalanobis D^2 and Principal Component Analysis (PCA).

Calvacanti and Lopes (1993) studied morphological differentiation among five species of serranid fishes namely *Diplectrum formosum, D. radiale, Dules auriga, Epinephelus guaza* and *Mycteroperca rubra.* They used multivariate statistical techniques such as principal components analysis and Canonical variate analysis for analyses of measurements obtained from truss networks based on anatomical landmarks and found that traditional measurement systems used in fish morphometrics are inadequate, not covering areas and axes of variation important for discriminating among groups. Canonical variate analysis was useful in discriminating these species. Calvacanti *et al.* (1999) used landmark based morphometric analysis to examine the relation between body form and feeding habit in six species of marine fishes belonging to two subfamilies of the family Serranidae *viz.*, Serraninae: *Dules auriga, Diplectrum formosum, D. radiale* and Epinephelinae: *Epinephelus marginatus, Mycteroperca acutirostris* and *M. bonaci.* They observed that there is a significant difference among species with respect to the uniform components, but failed to separate taxonomic groups related to these components, and species were instead separated on the basis of body height and caudal peduncle length. Similar study was conducted in lethrinid fishes (Carpenter, 1996) and three spine sticklebacks (Walker, 1996, 1997) using geometric morphometrics for the analysis of landmark data.

2.3. Genetic Markers

Technological advances in molecular biology and biochemistry have led to the development of a variety of genetic markers that can be used to address questions of relevance to the management and conservation of fish species. Genetic markers have been applied to three fisheries areas in particular stock structure analysis, aquaculture and taxonomy/systematics with varying degrees of success (Carvalho and Hasser 1994, Ward and Grew, 1994).

Genetic markers can be categorised based on their transmission and evolutionary dynamics (Park and Moran, 1994). Nuclear markers such as allozymes, Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs) and Variable Number of Tandem Repeats (VNTRs) are

biparentally inherited. Mitochondrial DNA (mtDNA) markers are maternally inherited and non-recombining such that they have one quarter the genetic effective population size (*Ne*) of nuclear markers. This reduced *Ne* makes mtDNA particularly sensitive to detecting reductions in genetic variation as the result of founder events and population bottlenecks. Furthermore, mtDNA retains a history of past isolations for a longer period relative to nuclear DNA (Billington and Hebert, 1991). However, the maternal inheritance of mtDNA limits its ability to provide information on male component of populations.

The need to detect genetic variation has fuelled the development of novel markers systems in fisheries biology. The detection for genetic variation among individuals (i.e. homozygotes and heterozygotes at nuclear loci, different mtDNA haplotypes) is a requirement in all applications of genetic markers. Some application will also require the partitioning of variation among groups of individuals (i. e. groups having different allele for haplotype frequencies). The choice of a genetic marker system should be based on the characteristics of a particular species (interacting with the attributes of the markers type) rather than how recently they have been developed (Ferguson and Danzmann, 1998). In fact, a combination mitochondrial and nuclear markers is the most powerful approach (Ward and Grewe, 1994).

Application of genetic markers for species identification and evaluation of genetic heterogeneity in groupers is limited. *Epinephelus chlorostigma* exhibited significant tissue-specific expression of allozymes (Manxian and Lintao, 1996). In another study using allozymes, low genetic variation was detected in *E. merra* (Planes *et al.*, 1997). Levels of mtDNA variability in red grouper, *E. morio* were among the lowest reported from marine fishes (Gold *et al.*, 1998). Bakar and Azizah (2000) studied RAPD profiles in *E. bleekeri* and *E. coioides* from Malayasia. Using microsatellite DNA analysis, Stevenson *et al.* (1998) found no stock separation among localized populations of Nassau grouper (*E. striatus*) within western tropical Atlantic. Nugroho *et al.* (1998) have developed GT Repeats microsatellite to study genetic polymorphism in *E. merra* and found them useful as markers for studying genetic polymorphism in other species including *E. bonthoides, E. fuscoguttatus, E. ongus* and *E. coramandelicus.* Enzymatic polymorphism in the population of *E. marginatus* was reported (Goarant, 1998).

2.3.1. Molecular taxonomy

Molecular techniques have become a major tool for systematic ichthyologists at the species level and above, but these approaches may also be useful to fishery biologists for taxonomic problems ranging between species and population levels (Chow *et al.*, 1993). These workers expressed difficulty in the identification of larvae among lutjanid species owing to their close similarities. Analysis of distribution of eggs and larvae for life history and recruitment studies of the fishery has been hampered by the inability to identify these stages of most snapper species. Molecular genetic markers significantly increased the number of eggs and larvae that could be unambiguously identified.

The U.S. Endangered Species Act of 1973 affords protection to three categories of endangered taxa-species, subspecies and populations – but existing notions about this taxonomic distinctions based on morphological analysis have often been revised following molecular analysis (O'Brien and Mayr, 1991). In protecting endangered fishes, molecular analysis offers the potential to provide (i) taxonomic recognition of groups showing little evolutionary differentiation and (ii) lack of taxonomic recognition of phylogenetically distinct forms (Avise, 1989). Molecular taxonomy is considered appropriate and necessary in the identification of (i) cryptic members of species complexes that can usually only be discriminated by expert morphological analysis and (ii) members of closely related species that can only be identified at a particular life stage (Black, 1996).

2.3.2. Random Amplified Polymorphic DNA (RAPD)

Williams *et al.* (1990) and Welsh and McClelland (1990) described a novel PCR based method termed RAPD fingerprinting. This technique allows detection of DNA polymorphisms by randomly amplifying multiple regions of the genome by PCR using single arbitrary primers designed independent of target DNA sequence (Williams *et al.*, 1990, 1993; Welsh and McClelland, 1990; Hadrys, 1992). RAPD fingerprinting technique is robust, simple, fast, sensitive and particularly suited to problems where the genome is anonymous or the quantity of genomic DNA

available is limited. It can also be used for analysis of museum specimens and rare fishes using DNA isolated from scales and clipped fins without destroying the whole organisms.

Since the RAPD technique involves enzymatic amplification of target DNA by PCR using arbitrary primers it is also called Arbitrary Primed PCR (AP-PCR) or DNA Amplification Fingerprinting (DAF). This method overcomes some technical limitations of the earlier fingerprinting methods and has wide applications including genetic fingerprinting of bacteria, plants, few animal species and humans (Welsh and McClelland, 1990; Williams *et al.*, 1990; Caetano-Anolles, *et al.*, 1991; Carlson *et al.*, 1991; Chalmers *et al.*, 1991; Echt *et al.*, 1992; Schneider *et al.*, 1997). Estimations of genetic similarity at the intra-specific level (Caetano-Anolles *et al.*, 1991; Hadrys *et al.*, 1992; Johnson *et al.*, 1994), creating linkage maps (Rafalski *et al.*, 1991), locating disease resistant genes (Martin *et al.*, 1991; Michelmore *et al.*, 1991) and identification of sex-specific marker in fishes (Iturra *et al.*, 1998; Griffiths *et al.*, 2000; Kovacs *et al.*, 2001).

RAPD analysis has been used to determine genetic diversity and introgression in plant (Dawson *et al.*, 1993; Mc Coy and Echt, 1993) insect (Hadrys *et al.*, 1992), and mammalian (Woodward *et al.*, 1992) populations. The RAPD analysis has also been used to evaluate genetic diversity in tilapia (Naish *et al.*, 1995; Bardakci and Skibinski 1994; Dinesh *et al.*, 1993, 1996; Degani *et al.*, 2000). It has also been used to study the genetic variation in pacific cod (Saitoh, 1998) and *Penaeus monodon* (Garcia and Benzie, 1995). Genetic variation among different strains of ornamental fishes has been revealed by RAPD–PCR. The RAPD markers used in studies of ornamental fishes such as angel fish (Degani *et al.*, 1997), Guppy (Foo *et al.*, 1995), Zebra fish (Johnson *et al.*, 1994) and Koi (Jackson *et al.*, 2000).

RAPD methodology has been shown to be useful in preliminary pedigree analysis (Dinesh *et al.*, 1993a) and in the detection of phenotypic-specific DNA polymorphic markers in different colour mutants of two freshwater aquarium fishes (Dinesh *et al.*, 1993a; 1993b). AP-PCR has been used for the detection of DNA polymorphism of few fish species including colour mutants of tiger barb, *Barbus tetrazona* and guppy, *Poecilia reticulata* (Dinesh *et al.*, 1993b). Kubota *et al.*, (1992)

used this method for the detection of radiation induced DNA-damages in Japanese medaka fish *Oryzias latipes*.

2.3.3. Population genetic RAPD markers

Identification and characterisation of population units are imperative for fisheries management because an efficient resource utilisation can best be achieved when managing at the population level. The possible neglect of genetic diversity in fisheries management decisions might have been due to lack of tools for the determination of genetic variation and to placing emphasis on environmentally induced characters such as conventional morphological characters used for evaluating variation among populations.

RAPD variations have been widely used to investigate population structure of different types of fish such as tilapia (Bardakci and Skibinski, 1994), Discus (Koh *et al*., 1999), stripped bass (Bielawski and Pumo, 1997) Hilsa shad (Dahle *et al.,* 1997), goat fish (Mamuris *et al*., 1998), Pacific cod (Saitoh, 1998), Largemouth bass (Williams *et al.,* 1998), catfish (Yoon and Kim , 2001), edible oyster (Hirschfeld *et al*., 1999), fresh water shrimp (D'Amato and Corach, 1996) and Prawns (Tassanakajon *et al.,* 1997). The results of their investigations suggest that RAPD analysis might be more sensitive to reveal variation within the fish populations. The technique also offers the possibility of carrying out compatibility analysis with unlimited number of primers, each detecting variation at several regions of the genome. The dominant quality of RAPD polymorphism together with high sensitivity to amplification, and the possible difficulty of comparing results between laboratories, has however, impeded the wide use of RAPDs in population analysis (Dahle *et al.,* 1997). Potential applications of RAPD in genome analysis of scombroid fishes were mentioned (Jayasankar and Dharmalingam, 1997a) and results of a preliminary study on stock structure analysis of Indian mackerel (*Rastrelliger kanagurta*) from east and west coasts of India were reported (Jayasankar and Dharmalingam, 1997b).

2.3.4. RAPD and species identification

The RAPD technique was first employed by Williams *et al.* (1990) to examine human DNA samples, while they found that the information returned for an

individual RAPD analyses was quite low, studies using multiple markers to define a genome yielded the best results. This technique was also used by Mulcahy *et al.* (1993) in an examination of apple cultivars. Using the RAPD technique, they were able to distinguish eight distinct apple cultivars. The RAPD results were fairly consistent within each group, which indicates that RAPD markers are likely to provide reliable identifications. Meruane *et al.* (1997) reported potential application of RAPD markers for identification of species and evaluation of polymorphisms in penaeid prawns.

Bardakci and Skibinski (1994) applied RAPD technique and allozyme analysis to identify species and subspecies of tilapia and the results showed that RAPD markers are more successful in distinguishing subspecies of *Oreochromis niloticus* than allozyme analysis (Seyoum, 1990; Seyoum and Kornfield, 1992). Dinesh *et al.* (1996) also found that RAPD markers are useful for species identification in tilapia. Partis and Wells (1996) used RAPD to investigate it as a potential fish species identification method for eight species of fish such as barramundi, Nile perch, John dory, Mirror carp, Silver dory, Pinkey Oreo, Warty Oreo and Smooth Oreo. RAPD profiles generated were consistent within barramundi. Species-specific profiles were also generated for the other seven species analysed by RAPD. Williams *et al.*, (1998) used RAPD to identify largemouth bass subspecies and their intergrades and they found that RAPD analysis was more sensitive than traditional histochemical agarose gel electrophoresis for identification of large mouth bass subspecies because of the increased number of markers visualized with this technique. Taxonomic relationship between four species of the family mullidae was studied using three genetic markers such as allozymes, RAPD and mtDNA. RAPDs have proved more reliable in determining the taxonomic status of the four species compared to other two methods (Mamuris *et al.*, 1999).

RAPD technique showed high power of resolution for phylogenetic analyses within the genus *Anguilla* and this technique has differentiated four species of eels (Lehmann *et al.*, 2000). Species specific markers were identified for three species of *Anguilla* using RAPD analyses and found that this technique can also be used for identification of eels larvae leptocephali in which morphological differentiation is not easy (Takagi and Taniguchi, 1995). Six species of sturgeons

were identified by this method and the results obtained were in good agreement with their geographical distribution and inhabitancy system (Comincini *et al.*, 1998).

Efficient differentiation and classification of closely related species and varieties will be very useful in ornamental fishes such as Discuss which is most expensive and popular aquarium fish. The current classification of two species and four subspecies of Discuss are debatable and lacking in evidence. A study on RAPD fingerprinting of Discuss undertaken by Phang *et al.* (1996) to investigate its classification suggested a single species and possibly only two subspecies. Borowsky *et al.* (1995) studied genetic variation among three species of the genus *Xiphophorus* by applying RAPD analysis. Dinesh *et al.* (1996) used RAPD to differentiate three species of tilapia such as *Oreochromis aureus, O. mossambicus* and *O. niloticus*. DNA profiles generated in each species of tilapia were unique and this study presented RAPD markers as a new class of useful genetic markers for assessment of genetic diversity and species differentiation in tilapia. Similarly RAPD was useful in identifying four species of puffer fish in *Fugu*. The result showed that each species has its own unique amplified genome pattern which might be used to identify different species of *Fugu* (Chao *et al.*, 2001).

For the routine sympatric species identification of fishes during the youngster stages or of egg distribution, without prior information on allozyme or mtDNA structures, the RAPDs could be more promising than any other molecular methods employed, as shown in the case of four species of Mullidae namely *Mullus barbatus* L., *Mullus surmuletus* L., *Upeneus moluccensis* and *Pseudopeneus prayensis* (Mamuris *et al.*, 1999). RAPD markers have clearly differentiated *Epinephelus bleekeri* and *E. coioides* from Malaysia (Bakar and Azizah, 2000) and these workers have commented on the potential of RAPD technique for systematic investigation at the species level for the genus *Epinephelus*.

2.3.5. Inheritance of RAPD markers

The Mendelian inheritance of RAPD markers remains poorly understood. Several studies have demonstrated that RAPD markers can be inherited as Mendelian loci (Williams *et al.*, 1990; Welsh *et al.*, 1990; Echt *et al.*, 1992; Hunt and Page, 1992; Roy *et al.*, 1992; Kazan *et al.*, 1993, Levitan and Gosberg, 1993;

Rothuirizen and Van Wolferan, 1994; Foo *et al.*, 1995; Liu *et al.*, 1998; Chong *et al.*, 2000). In contrast, other studies have shown that RAPD fragments are not always inherited in Mendelian fashion and that non-parental bands can occur in offspring (Carlson *et al.*, 1991; Hunt and Page, 1992; Reiter *et al.*, 1992).

Foo *et al.* (1995) used RAPD fingerprinting for the analysis of genomic polymorphisms of two varieties of guppies, *Poecilia reticulata* and results demonstrated to show full penetrance and to follow dominant Mendelian inheritance; Numerous RAPD markers can be generated for their ready use in pedigree studies (Hallerman and Beckmann, 1988; Echt *et al.*, 1991; Welsh *et al.*, 1991 a) and rapid construction of a genetic linkage map (Postlethwait *et al.*, 1994). The linkage map can also provide DNA markers for sex determination (Foo *et al.*, 1995).

2.3.6. Reproducibility of RAPD markers

One of the most important factors determining the applicability of RAPD for gene mapping analysis is its reproducibility (Hadrys *et al.*, 1992; Reidy *et al.*, 1992; Scott *et al.*, 1992; Powell *et al.*, 1995). A prerequisite for carrying out RAPD-PCR investigations is to establish an optimized standard technique (Bechmann, 1994). Each reaction component should always have an identical origin (Schierwater and Ender, 1993) and standard PCR conditions (Bardakci and Skibinski, 1994; Shweder *et al.*, 1995) and equipment (He *et al.*, 1994). Very important are also the precise adjustment of template DNA concentration (Williams *et al.*, 1993), avoidance of contamination and the inclusion of purity tests (Pammi *et al.*, 1994). The reproducibility of RAPD banding patterns is not otherwise guaranteed (Schierwater and Ender, 1993).

Dinesh *et al.* (1995) reviewed reproducibility of RAPD markers. The first difficulty with RAPD products is the generation of unreliable products in identical PCR and the second difficulty is the use of DNA preparations that are not of identical quality and concentration. They tested the first variable factor in generating RAPD fingerprints by repeating the amplification reaction thrice under identical conditions for several selected primer-template combination from four fish species (tiger barb, common carp, Mozambique tilapia, and *Atlantic salmon*). The RAPD markers generated by three separate amplification reactions under identical conditions were

100% reproducible. They also observed the reproducibility of the RAPD fragment detection by electrophoresing the same amplification products in three separate urea dPAGE gels using identical conditions and found that the RAPD markers over the entire range of 600-3000 bp were 100% reproducible. However, for about five RAPD markers in the remaining size range of 200-600 bp, they were detectable in two out of three PAGE gels. These low molecular weight RAPD fragments were quantitatively very minor RAPD fragments. The inconsistency in the detection of the minor RAPD fragments is probably due to the staining procedure. To test the second variable factor of DNA quality and concentration on RAPD fingerprints, they amplified different concentrations of template DNA (0.1, 0.4 and 1.0 μg) in three tiger barb and guppy individuals. Highly reproducible RAPD profiles are obtained over a wide range of template DNA concentrations (Dinesh *et al.,* 1995). Jayasankar and Dharmalingam (1997a) observed that a wide range of 1-200ng template DNA did not make any difference in RAPD fingerprints of scombroid fishes.

Saitoh (1998) observed reproducible band pattern in pacific cod, *Gadus macrocephalus* even when different template DNA comes from the same individual with a different batch of reagents used. Bielawski *et al.* (1995) believe that using multiple concentrations of template DNA during optimization of RAPD program parameters resulted in substantially improved reproducibility of RAPD data.

Penner *et al.* (1993) reported that six different laboratories, using five identical primers, amplified different size ranges of RAPD markers in oat cultivars. They found that the variation in the size ranges of RAPD markers was predominantly contributed by small (< 450 bp) and large (>1600 bp) RAPD fragments that were not always reproduced. They also identified that variation in RAPD profiles was due mainly to the fact that different thermal cyclers can have different temperature cycling profiles. Thus, a change of as little as 1^0C of annealing temperature can lead to quantitatively different results in RAPD analysis (Penner *et al.,* 1993). Another two studies comparing the RAPD fingerprints generated by employing different DNA polymerases in the scombroid fishes (Jayasankar and Dharmalingam, 1997a), in the sugarcane, *Saccharum spontaneum* (Sobral and Honeycutt, 1993) and in the cladoceron, *Daphnia galeata*, (Schierwater and Ender, 1993), clearly demonstrated

that the reproducibility of RAPD fingerprint patterns also depends on the type of DNA polymerase used in the amplification reaction.

Under identical amplification conditions, RAPD profiles for any particular primer-template DNA combination is highly reproducible over a wide range of template DNA (Dinesh *et al.*, 1995). They also anticipate that the information available on the reproducibility of RAPD fingerprinting would increase the comparability of diagnostic RAPD markers generated in different laboratories. The compatibility of RAPD markers will be of prime importance for providing a regional or central database service on RAPD markers for purposes such as species diagnostics, detection of molecular markers linked to economic traits, measurement of genetic variation and establishment of genetic similarity at different taxonomic levels (Schierwater and Ender, 1993; Kresovich *et al.*, 1992).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Truss Morphometrics

3.1.1. Data collection

A total of 190 individuals of five grouper species, namely *E. areolatus* (n=30; TL range: 305-360 mm; Plate 1), *E. bleekeri* (n=30; TL range: 281-320 mm; Plate 2), *E. chlorostigma* (n=50; TL range :300-375 mm; Plate 3), *E. diacanthus* (n=40; TL range: 275-345 mm; Plate 4), and *E. longispinis* (n=40; TL range: 293-348 mm; Plate 5) were collected from commercial fish landing centre at Thoppumpady Fishing Harbour, Kochi, during December 2001 to March 2002 for truss morphometric study. The landings of these fishes were from the coasts of Mumbai, Goa, Karnataka, Kerala and Lakshadweep islands along Arabian'sea. They were mainly caught with Hook-and-line, trawl and sometimes in traps. Preliminary identification of the species was done using the taxonomic key of Heemstra and Randall (1993).

Measurements were based on a truss network protocol (Strauss and Bookstein, 1982; Winans, 1984; Bookstein *et. al.*, 1985) anchored at ten homologous anatomical landmarks (Fig. 1). Landmarks refers to: (1) Anterior tip of the snout on the upper jaw; (2) the most posterior part of the neurocranium (beginning of scaled nape); (3) origin of pelvic fin; (4) origin of spinous dorsal fin; (5) origin of anal fin; (6) origin of soft dorsal fin; (7) insertion of anal fin; (8) insertion of 2nd dorsal fin; (9) insertion of 1st ventral caudal fin ray; and (10) insertion of 1st dorsal caudal fin ray. The fish was placed on a water resistant paper and the body postures and the fins were teased into a natural position. Around the outline of the fish form ten anatomical landmarks as described above were identified. Each landmark was identified by making a hole with a dissection needle in the water resistant paper along its respective location. These points were transferred to a graph sheet. The distance (D) was calculated from the X and Y co-ordinates using the relationship,

$$D = \sqrt{(x_1-x_2)^2 + (y_1-y_2)^2}$$

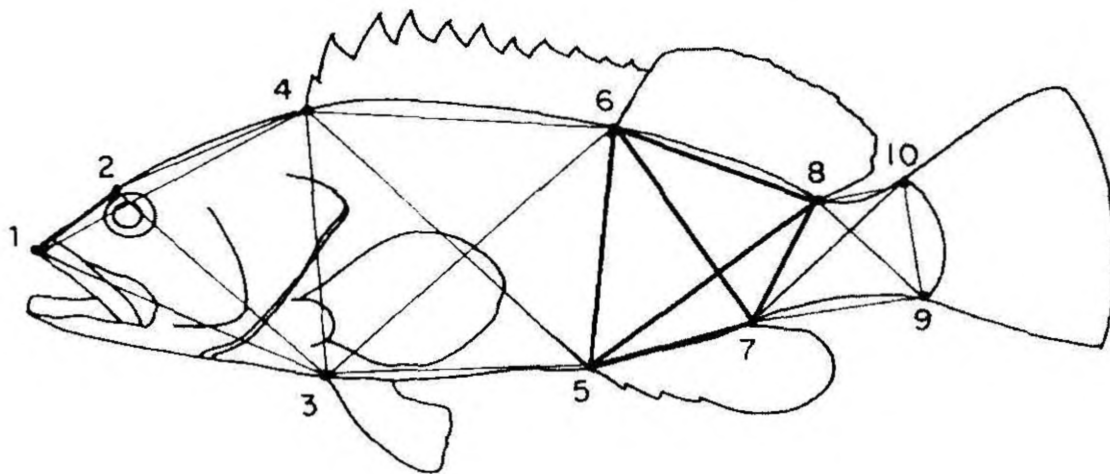


Fig. 1. Outline drawing of *Epinephelus* sp showing the locations of the 10 anatomical landmarks (numbered points) and morphometric distance measures recorded on each individual.

3.1.2. Data analysis

Principal component Analysis of 21 truss network distances was carried out using SYSTAT 7.0 (Morrison, 1990). Principal Component I (PC I) and Principal Component II (PC II) scores were plotted as XY scatters with PC I on the X-axis and PC II on the Y-axis. The clusters were further analysed by a "Size correction" method of Rohlf and Bookstein (1987). Sheared PC I and PC II scores were plotted as XY scatter diagram with PC I on the X-axis and PC II on the Y-axis. Discriminant analysis of truss landmark distances was done using SYSTAT 7.0 package.

3.2. RAPD

3.2.1. Collection of tissue samples

A total of 70 grouper individuals were collected from the commercial fish landing centres at Thopumpady Fisheries Harbour (Kochi, Kerala) and Mandapam (Tamilnadu), as well as from brood stock maintained at Fisheries Harobour Laboratory of CMFRI, Kochi. Ten individuals each of *E. areolatus* (Plate 1), *E. bleekeri* (Plate 2), *E. chlorostigma* (Plate 3), *E. diacanthus* (Plate 4), *E. coioides* (Plate 6), *E. malabaricus* (Plate 7), and *E. tauvina* (Plate 8) were selected for RAPD analysis. A minimally invasive technique was used to collect tissue samples for DNA extraction. Live specimens need not be sacrificed for tissue sampling by adopting this sampling technique. About 150 to 200 mg of caudal fin clippings were taken from each individual and preserved in 95% ethanol. They were stored at -85^{0}C till they could be used for the extraction of DNA.

3.2.2. DNA extraction from caudal fin clippings

Total genomic DNA was extracted from caudal fin clippings following the method described by Jayasankar and Dharmalingam (1997a) with some modifications. Approximately 50mg of frozen tissue were minced, homogenised in 1600μl digestion buffer (10mM Tris HCl, 25mM EDTA, 100mM Nacl, 0.5% SDS and pH 8.0) and were transferred to 5.0ml volume centrifuge tubes. Four hundred micro litres of 10% SDS (1.0g/ml) and 20μl proteinase-K (10 mg/ml) solution were added to the homogenised sample, gently mixed and incubated in a water bath at 55^{0}C for 2 to

Plate 1. *Epinephelus areolatus*

Plate 2. *Epinephelus bleekeri*

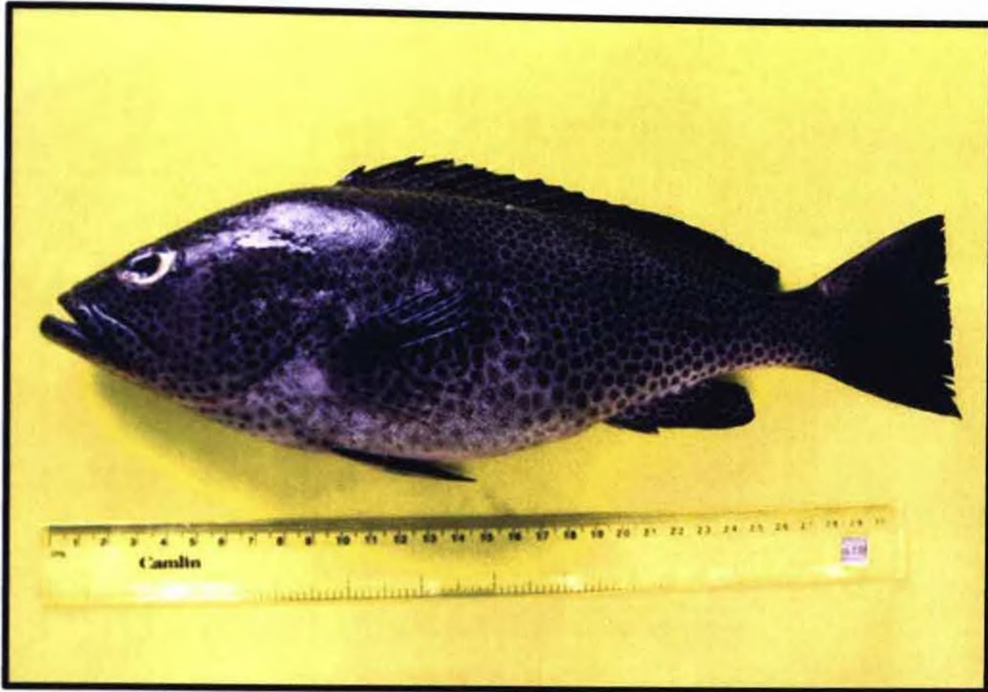


Plate 3. *Epinephelus chlorostigma*

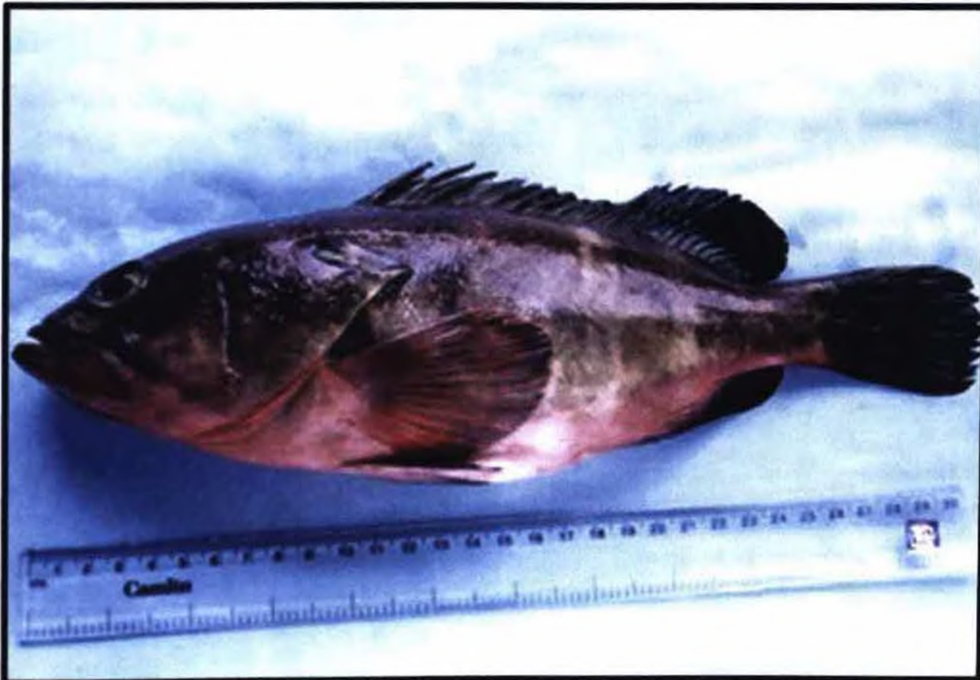


Plate 4. *Epinephelus diacanthus*

Plate 5. *Epinephelus longispinis*

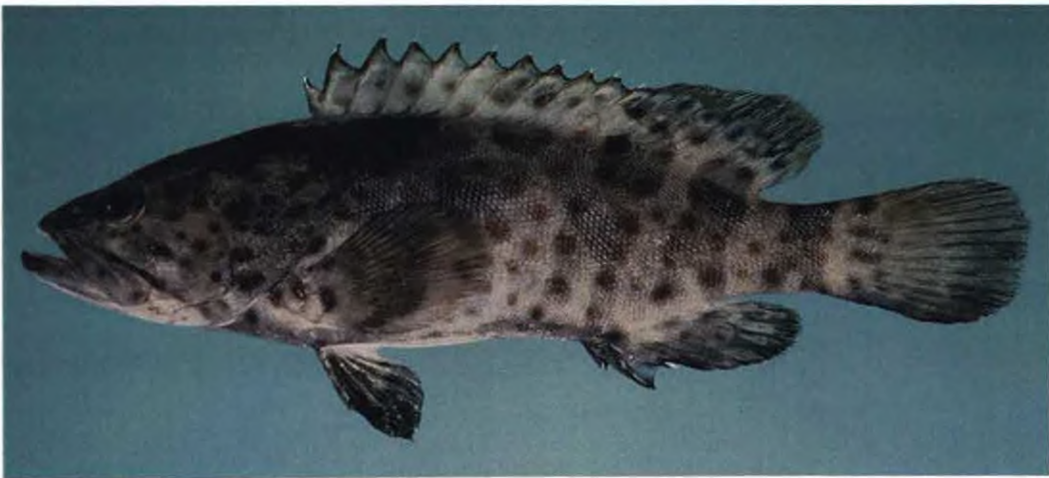


Plate 8. *Epinephelus coioides*

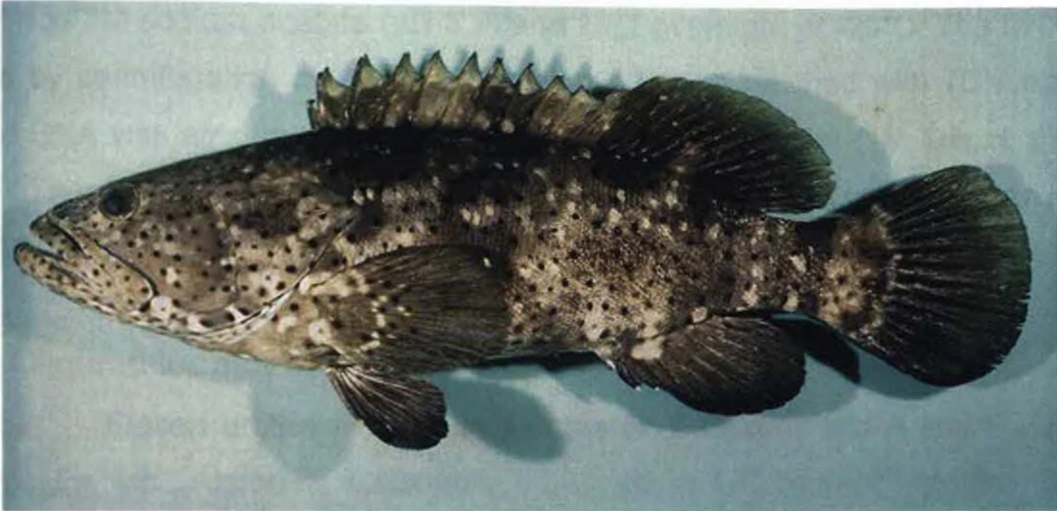


Plate 7. *Epinephelus malabaricus*

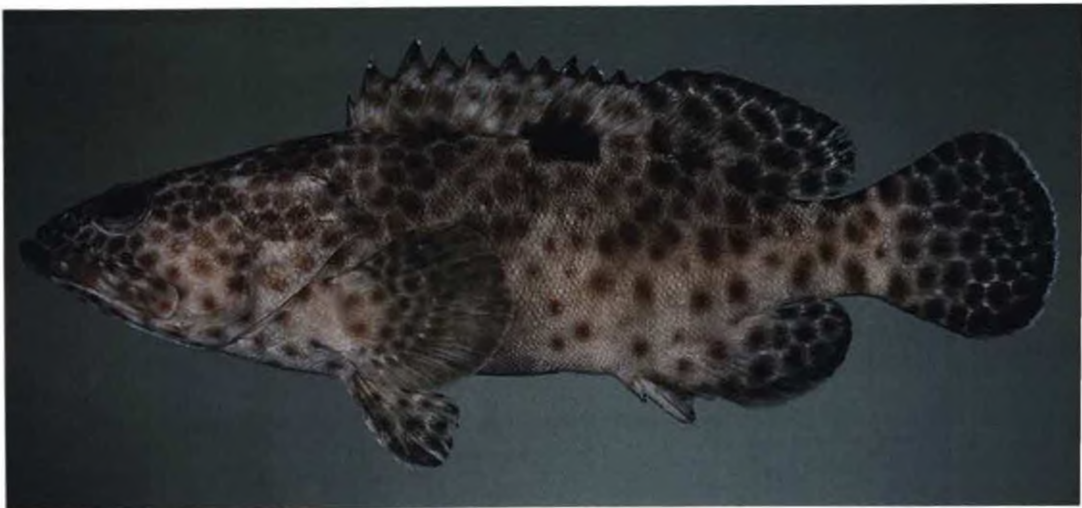


Plate 8. *Epinephelus tauvina*

2 ½ hrs with periodic agitation. After incubation, DNA was purified by successive extraction with buffered phenol, phenol: chloroform: iso-amyl alcohol (25:24:1) and chloroform: Iso-amyl alcohol (24: 1) respectively. DNA was precipitated with ice-cold ethanol and 3M sodium acetate (pH 5.2) and kept overnight at -20^{0}C. The DNA was pelleted by centrifugation at 9K for 10 min at 4^{0}C and washed with 70% ethanol. Pelleted DNA was air dried and resuspended in 50µl TE buffer (1M Tris cl, pH 8.0; 0.5M EDTA, pH 8.0). Quality and quantity of DNA were checked by 0.8% agarose gel electrophoresis and spectrophotometer. Extracted DNA was stored at -20^{0}C.

3.2.3. Oligonucleotide arbitrary primers

Eleven arbitrary decamer primers from Operon kits A and F (Operon Technologies Inc., Alameda, California, USA) with a GC-content between 60 and 70% were used for PCR amplification. The details of the primers are given in table 1.

3.2.4. PCR amplification of DNA

PCR was carried out in a total reaction volume of 25µl containing 10 to 15ng DNA, 1X PCR buffer, 0.2mM dNTPs, 10pM primer, and 1U *Taq* DNA polymerase (Bangalore Genei, India).

PCR was performed using thermal cycler, Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer, USA) programmed for an initial denaturation of 30s at 94°C followed by 45 cycles each consisting of 30s at 94°C (Denaturation), 30s at 36°C (Annealing) and 120s at 72 °C (Extension). A final extension was carried out at the same temperature for 7min and followed by pausing file at 4 °C until they could be used or stored. PCR products were stored at 4 °C in refrigerator.

A total of eleven primers were screened initially to check amplification, repeatability and robustness of bands and four were selected for further analysis of all samples based on these qualities.

Table 1. Decamer primers used for the study (Operon Technologies Inc., Alameda, California, USA)

Primer Codes	Sequence 5' to 3'	Molecular weight	GC-content [%]
OPA 01	CAGGCCCTTC	2955	70
OPA 03	AGTCAGCCAC	2988	60
OPA 05	AGGGGTCTTG	3090	60
OPA 07	GAAACGGGTG	3108	60
OPA 09	GGGTAACGCC	3044	70
OPA 11	CAATCGCCGT	2979	60
OPF 02	GAGGATCCCT	3019	60
OPF 04	GGTGATCAGG	3099	60
OPF 06	GGGAATTCGG	3099	60
OPF 08	GGGATATCGG	3099	60
OPF 10	GGAAGCTTGG	3099	60

3.2.5. Agarose gel electrophoresis

Approximately 10µl of PCR amplification products were mixed with 2µl loading dye (0.05 %(w/v) 0.1M EDTA, pH 8.0; 0.5%(w/v) sodium lauryl sulphate) and loaded in 1.5% agarose gels containing 1x TBE buffer (0.09M TrisHcl, 0.09M Boric acid, 0.0025M EDTA, pH 8.3) and were electrophoresed at 100V for 2 ½ to 3h in a horizontal slab electrophoretic unit. Gels were stained in Ethidium bromide solution (1 µg/ml) for 30 min. Bioprofil, a charge coupled device (CCD) video camera imaging system (Vilber Lourmet, France), was used to document the gels.

3.2.6. Analysis of RAPD data

Size of RAPD bands was determined by comparison with a λ DNA digested with *Eco* RI / *Hin*d III molecular weight marker. For all primers, Presence (1) or absence (0), of a fragment was scored manually on the photograph by two persons separately, and RAPD patterns of individuals were compared within and between species. Bioprofil (Bio-1D) was used to calculate the fragment sizes of the RAPD bands with reference to molecular size markers.

The 'species-specific diagnostic' markers are defined in the present study as those RAPD bands which are exclusive to a species for a given primer.

The similarity index between all possible pair-wise comparisons of individuals was calculated using the formula:

$$S_{xy}= 2n_{xy}/(n_x + n_y),$$

Where, n_x and n_y are the number of RAPD fragments in individuals x and y, and n_{xy} is the number of fragments shared between those individuals (Nei, 1978). Genetic distances between paired individuals or species were also calculated using Nei (1978).

Phylogenetic relationships between individuals or populations of seven grouper species were constructed using the unweighed pair-group method of analysis (UPGMA) (Sneath and Sokal, 1973) contained in the NEIGHBOR program of PHYLIP ver 3.57c, based on Nei's (1978) genetic distance values calculated for all primers.

3.2.7. Statistical analysis

Test of differences in intraspecies and interspecies genetic distance coefficients among the seven species of groupers were made by one-way analysis of variance (ANOVA). Comparisons of intraspecies genetic distance values (within species) with interspecies genetic distance values (between species) were made by paired *t*-test. The statistical analyses were carried out using the statistical software package SYSTAT version 7.0.

RESULTS

4. RESULTS

4.1. Sheared Principal Component Analysis (SPCA)

Sheared Principal Component Analysis (SPCA) was done for 4 groups of *Epinephelus* each containing 3 species per group. This is to know the morphological relatedness among species, which would help to ratify their taxonomic status. In all the groups, *E. diacanthus* formed a separate cluster, while there was relatively more closeness of clusters of other species (Figs 2, 4, 6 and 8).

SPCA was done for all five species together and scatter plot is shown in Figure 10. Component loadings of the five sheared principal components are given in Table 2. Analysis of covariance matrix of size adjusted truss measurements for all species indicated that the first five PCs explained about 86.6% of variance of the morphometric characters. The PC I explained 49.0% of the variation and exhibited component loadings that differed in magnitude with respect to characters. The PC I represents size and PC II represents size-corrected shape. Strong positive loadings were associated with caudal peduncle depth (9-10), depth between end of anal and end of 2nd dorsal fins (7-8) and distance between pelvic and anal fins (3-5).

The second principal component (PC II) explained 20% of additional variation and had strong negative loadings for the distance between end of 2nd dorsal fin and dorsal caudal peduncle (8-10), end of 2nd dorsal fin and ventral insertion of caudal fin (8-9) and end of anal fin to ventral insertion caudal fin (7-9). Strong positive loadings were associated with second dorsal fin length (6-8), anal fin length (5-7) and also body depth at the end of 1st dorsal fin and insertion of anal fin distance (5-6), between end of 1st dorsal fin and end of anal fin (6-7) and end of anal fin and end of 2nd dorsal fin (7-8). For PC II, contrasts were limited to measures in the 2nd dorsal fin and anal fin area only.

The remaining three PCs explained 17.6% of additional variation. A plot of PC I and PC II scores (Fig. 10) showed among grouper species, *E. diacanthus* was clearly separated from rest of the species along PC II. Though the clusters of other four species are separated, it was less marked.

4.2. Analysis of Discriminant Functions

Canonical variate analysis of truss morphometric data was also carried out for the same four groups separately as SPCA was done. Standardized canonical discriminate functions were calculated and drawn the scatter plots for each group with Canonical Variate 1 (CV 1) against Canonical Variate 2 (CV 2) (Figs. 3, 5, 7, and 9).

Similarly, standardized discriminant functions were calculated for all five species together and the results are shown in Table 3. Scatter plots were drawn based on all the three canonical variates to show the relationships among each species (Fig. 11). CV 1 and CV 2 show maximum loadings for anal fin length (5-7) and distance between end of anal fin and end of 2nd dorsal fin (7-8). Strong negative values were associated with the distance between end of first dorsal fin and end of anal fin (6-7). This indicates that maximum variation is associated with the area encompassing the vertical, horizontal and diagonal distance measures between the 2nd dorsal fin and anal fin (the bold truss network box shown in Fig. 1).

Canonical plots of CV 1 and CV 2 and CV 1 and CV 3 shows clear separation of *E. diacanthus* from rest of the species (Fig. 11). In the plot between CV 1 and CV 2, *E bleekeri* has also exhibited perceptible separation from the rest.

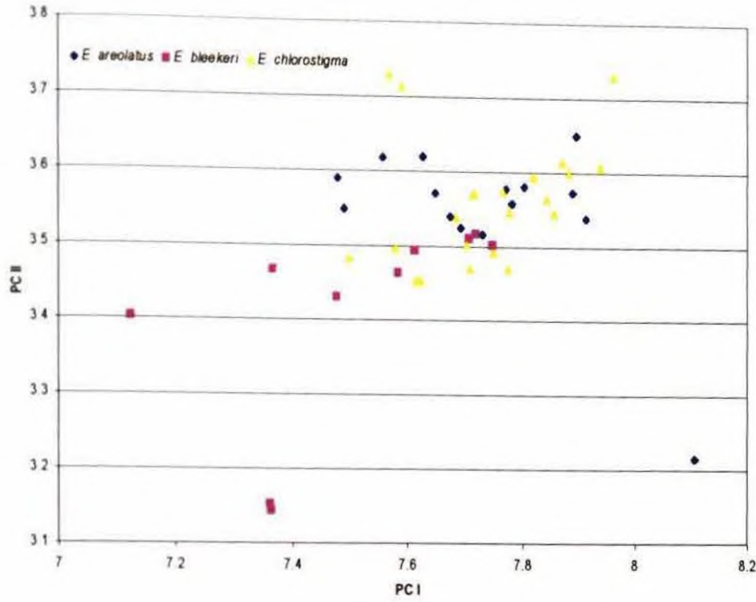


Fig. 2. Sheared PCA of truss network landmarks of *Epinephelus areolatus, E. bleekeri* and *E. chlorostigma*.

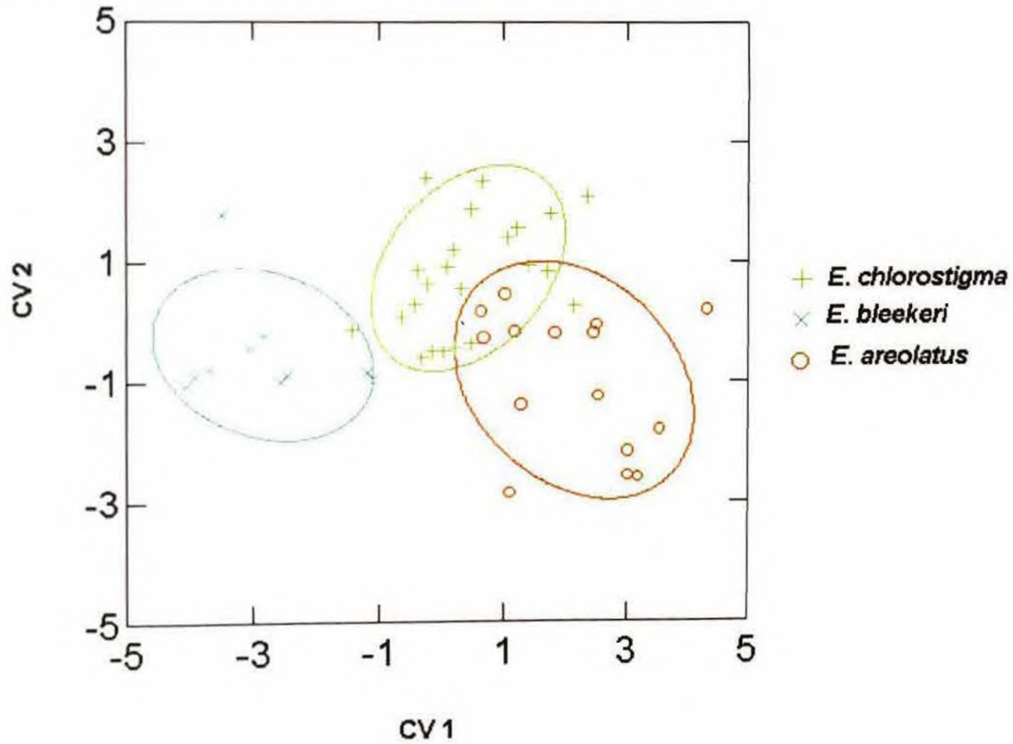


Fig. 3. Canonical scatter plot of truss landmarks of *Epinephelus areolatus, E. bleekeri* and *E. chlorostigma*.

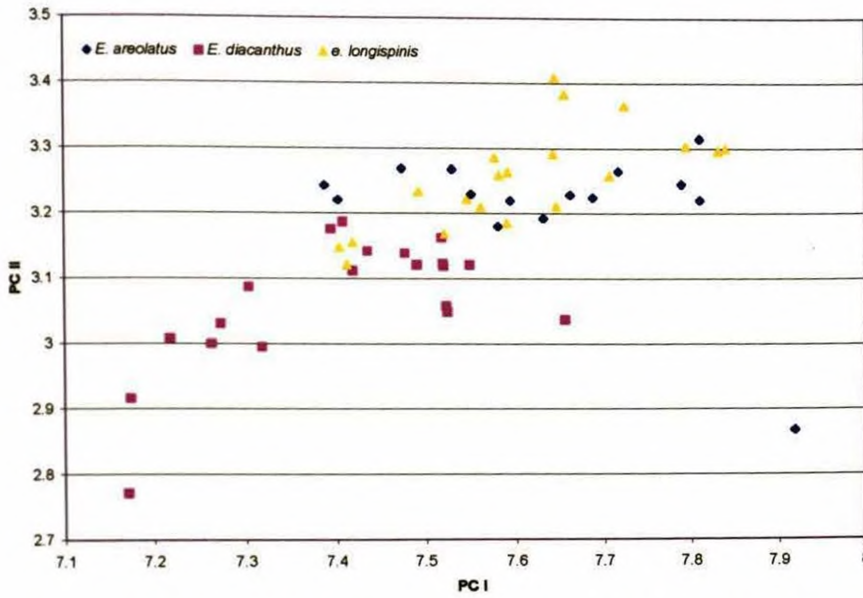


Fig. 4. Sheared PCA of truss network landmarks of *Epinephelus areolatus, E. diacanthus* and *E. longispinis.*

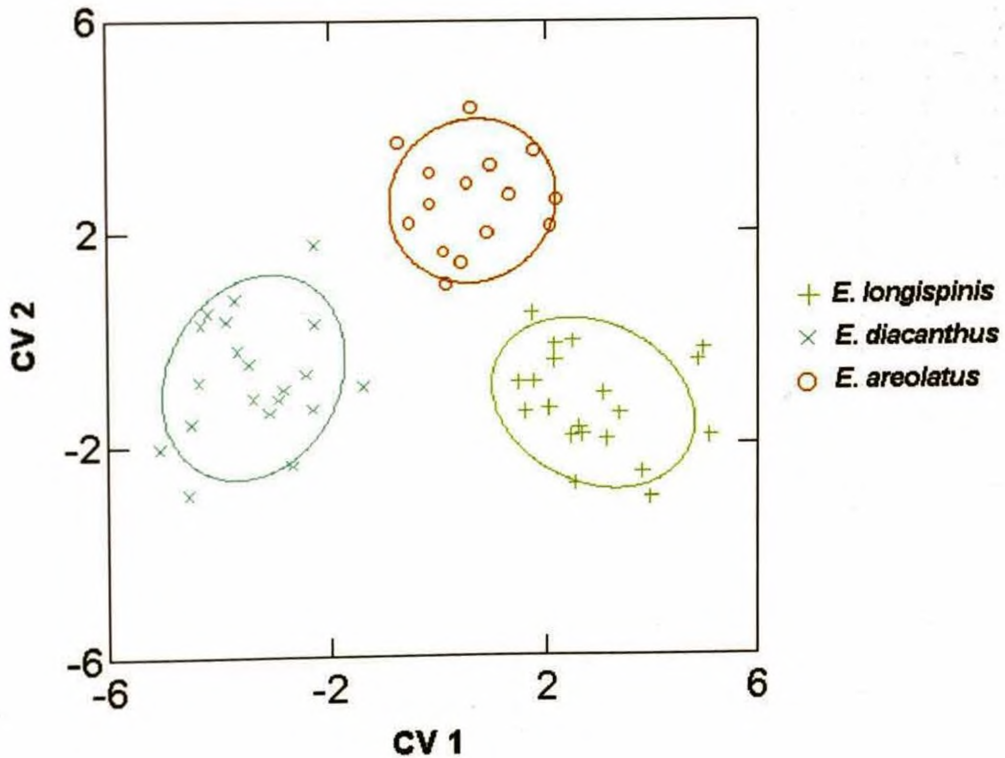


Fig. 5. Canonical scatter plot of truss landmarks of *Epinephelus areolatus, E. diacanthus* and *E. longispinis.*

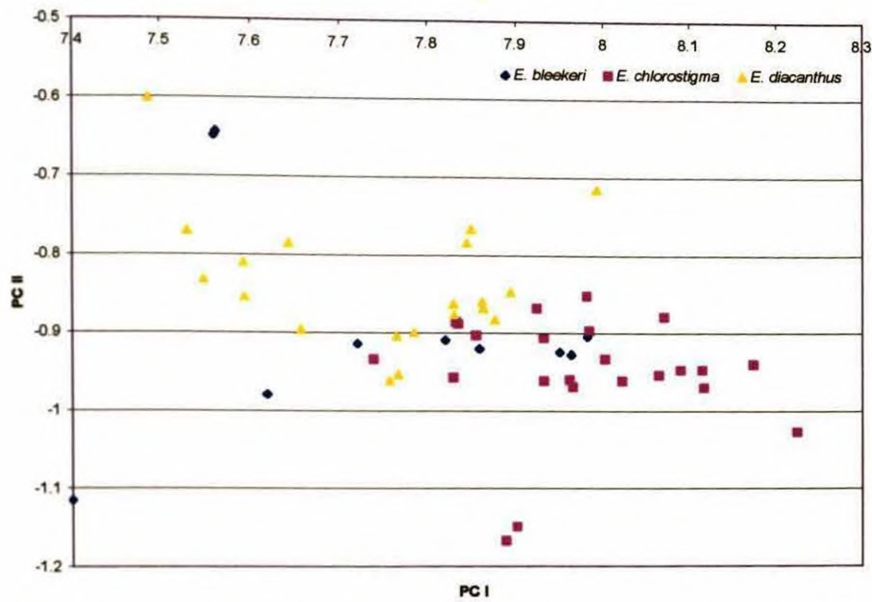


Fig. **6**. Sheared PCA of truss network landmarks of *Epinephelus bleekeri, E. chlorostigma* and *E. diacanthus.*

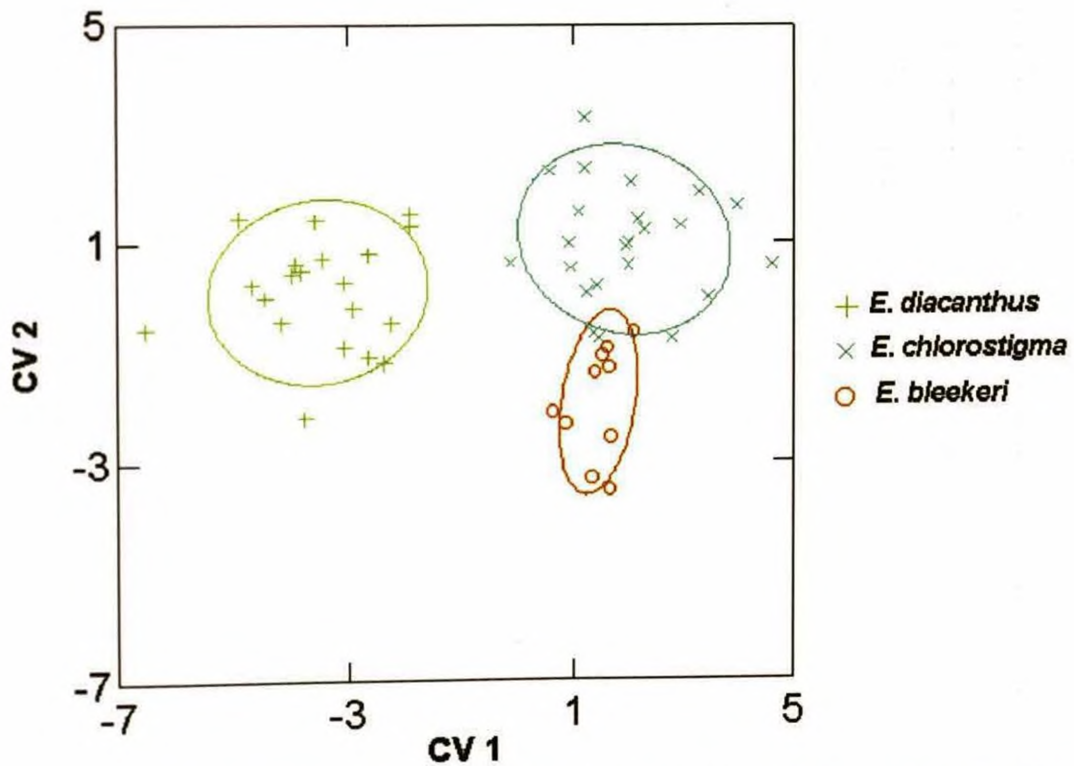


Fig. **7**. Canonical scatter plot of truss landmarks of *Epinephelus bleekeri, E. chlorostigma* and *E. diacanthus.*

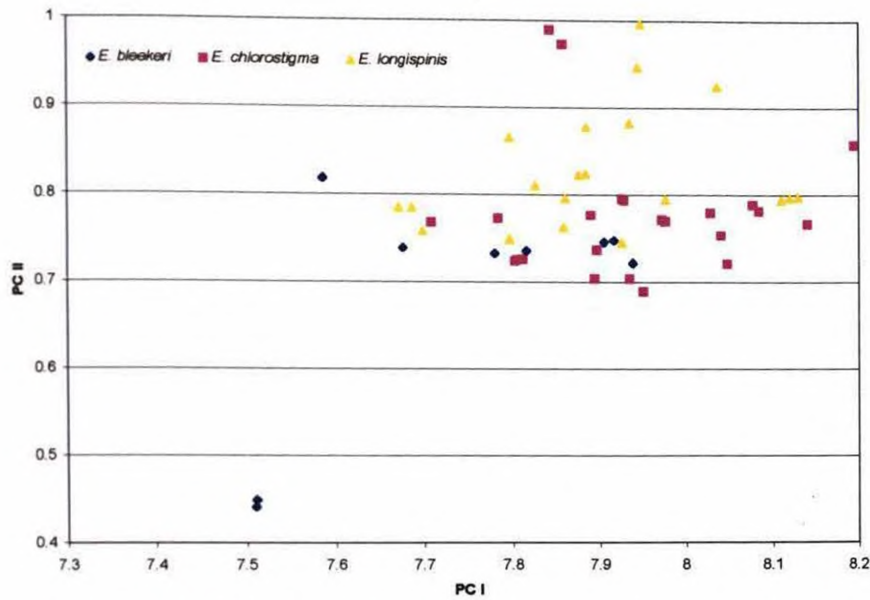


Fig. 8. Sheared PCA of truss network landmarks of *Epinephelus bleekeri*, *E. chlorostigma* and *E. longispinis*.

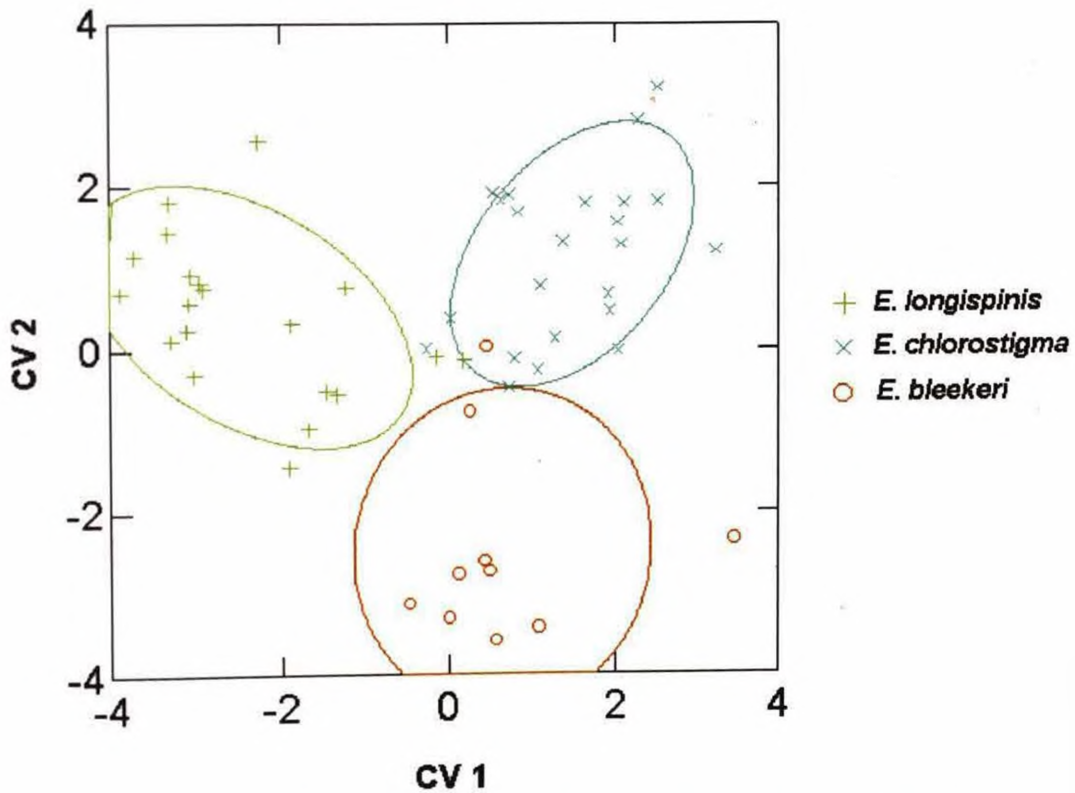


Fig. 9. Canonical scatter plot of truss landmarks of *Epinephelus bleekeri*, *E. chlorostigma* and *E. longispinis*

Table 2. Component loadings of the first five sheared principal components for truss morphometric characters of *Epinephelus areolatus, E. bleekeri, E. chlorostigma, E. diacanthus* and *E. longispinis*

Character	Principal Component				
	I	II	III	IV	V
1-2	0.210307	0.014831	0.084911	0.136118	0.600126
1-3	0.168257	0.011945	-0.08828	0.032282	0.143948
1-4	0.173254	0.013961	-0.21474	0.008147	0.184655
2-3	0.184682	0.015754	-0.09196	0.026468	0.162675
2-4	0.161344	0.015164	-0.34011	-0.05122	0.033764
3-4	0.190729	-0.00686	-0.18869	0.035612	0.2115
3-5	0.247336	-0.02609	-0.46691	0.096247	-0.18894
3-6	0.21073	-0.00082	-0.26581	0.074532	-0.11243
4-5	0.214042	0.033349	-0.15272	0.068161	-0.06476
4-6	0.201193	-0.02583	-0.21572	0.183879	-0.2591
5-6	0.227327	0.105413	0.017182	0.007904	-0.04615
5-7	0.156663	0.223888	0.188154	-0.42457	0.147549
5-8	0.222266	0.234226	0.130761	-0.21497	0.020381
6-7	0.206802	0.155814	0.09355	-0.0429	0.09208
6-8	0.142809	0.133242	0.180231	0.225262	0.341699
7-8	0.314982	0.277698	0.061639	-0.05551	-0.18019
7-9	0.18835	-0.13466	0.102867	0.204221	0.121622
7-10	0.24052	-0.05647	0.196324	0.289619	-0.11634
8-9	0.308939	-0.23373	0.043349	-0.66972	-0.11765
8-10	0.175318	-0.8278	0.119907	-0.03319	0.103175
9-10	0.318668	-0.00141	0.507361	0.255124	-0.39774
Percent of variance explained	49.15	19.9	8.25	5.63	3.66

Table 3. Standardized canonical discriminant functions of the truss morphometric characters of *Epinephelus areolatus, E. bleekeri, E. chlorostigma, E. diacanthus* and *E. longispinis*

Character	Standardized Discriminant Functions (Canonical variates)		
	1	2	3
P1P2	0.284	1.334	2.499
P1P3	-0.077	0.444	2.34
P1P4	0.197	-3.619	-4.856
P2P3	-0.935	-0.629	-2.907
P2P4	-0.989	3.337	3.627
P3P4	-0.253	-0.175	0.553
P3P5	-0.773	0.524	-0.919
P3P6	0.395	0.13	0.682
P4P5	0.835	-1.165	2.614
P4P6	-0.828	0.71	-1.318
P5P6	3.116	2.56	-0.351
P5P7	6.518	4.764	-0.612
P5P8	-10.978	-7.37	0.234
P6P7	-3.948	-3.272	0.555
P6P8	2.972	1.699	-0.52
P7P8	6.649	4.003	-0.56
P7P9	-0.332	-1.525	-0.472
P7P10	1.589	1.606	0.016
P8P9	2.476	2.869	-0.337
P8P10	-2.48	-2.301	0.317
P9P10	-0.664	-0.845	0.024

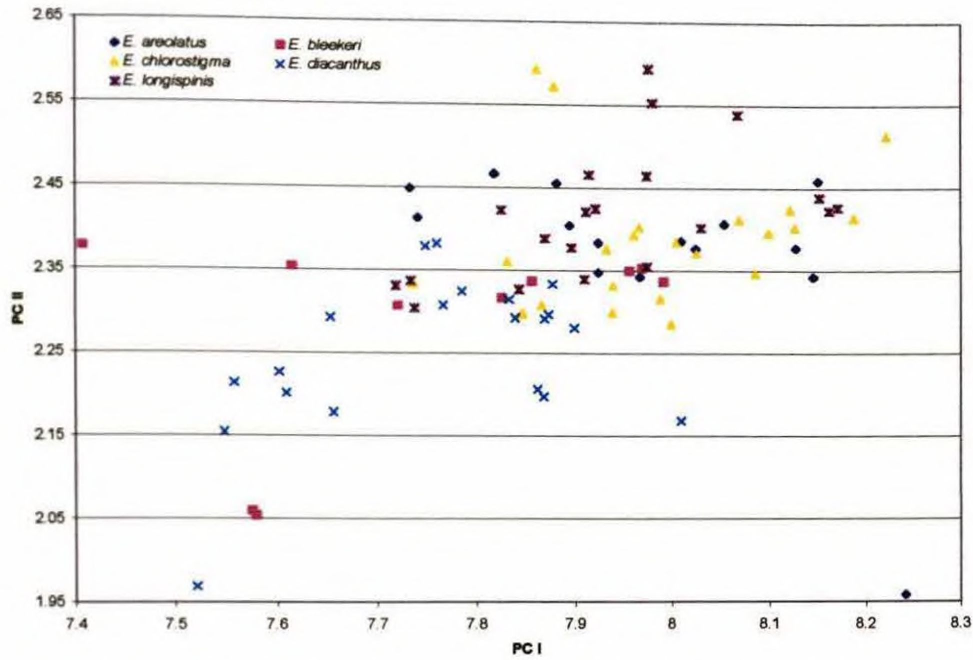


Fig. **10.** Sheared PCA of truss network landmarks of *Epinephelus areolatus, E. bleekeri, E. chlorostigma, E. diacanthus* and *E. longispinis.*

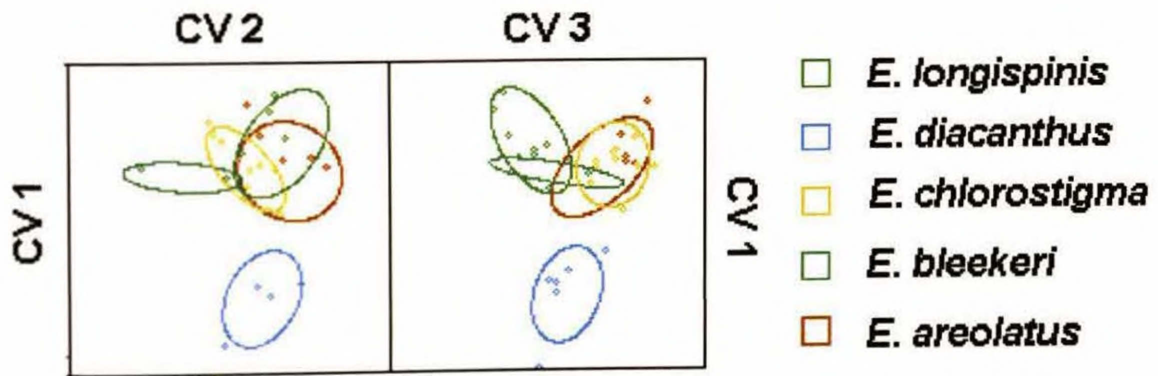


Fig. **11.** Canonical scatter plot of truss landmarks of *Epinephelus areolatus, E. bleekeri, E. chlorostigma, E. diacanthus* and *E. longispinis.*

4.3. RAPD

A total 11 arbitrary primers were screened with 7 grouper species (Table 1). Except OPA 03, all other primers produced bands. However, only four primers OPA 01, OPA 07, OPF 08 and OPF 10 have been selected, for final analysis of all individuals of seven species considering reproducibility, robustness and sharpness of banding patterns. These four primers could generate between 2 and 12 stable and clear loci. There were 59 fragments altogether and the fragment sizes were within 70-4500 bp. Only those fragments having molecular weight ranging from 100 to 2000 bp have been selected for analysis since they were more reproducible and robust. On an average, every primer generated 14.8 fragments. Figures 12, 13, 14 and 15 show the RAPD fingerprints (profiles) for different individuals of *Epinephelus* spp.

From primers OPA 01, OPA 07, OPF 08 and OPF10, 208, 213,138 and 162 RAPD genotypes were generated respectively. The average number of genotypes per primer was 30.3 in *E. diacanthus*, 26.0 in *E. areolatus,* 21.5 in *E. chlorostigma*, 17.8 in *E. bleekeri,* 19.5 in *E. coioides*, 26.8 in *E. tauvina* and 30.8 in *E. malabaricus*.

4.4. Genetic Identity and Genetic Distance

Pair-wise Genetic Identity (GI) and Genetic Distance (GD) values were calculated for each primer based on Nei (1978) (Tables 4, 5, 6 and 7).

Primer OPA 01 showed maximum GI value of 0.872 between *E. tauvina* and *E. malabaricus*, 0.841 between *E. chlorostigma* and *E. bleekeri* and 0.791 between *E. coioides* and *E. tauvina*. Lowest GI value observed was 0.458 between *E. bleekeri* and *E. tauvina*. High GD values were seen between *E. bleekeri* and *E. tauvina*, *E. bleekeri* and *E. malabaricus* and also between *E. tauvina* and *E. diacanthus* (Table 4). Based on these GD values UPGMA dendrogram was constructed to show the genetic relationship between species (Fig. 16A).

Primer OPA 07 showed high GI value between *E. tauvina* and *E. coioides* (0.938), between *E. bleekeri* and *E. tauvina* (0.898) and between *E. bleekeri* and *E. chlorostigma* (0.893) Low GI values were between *E. diacanthus* and *E. malabaricus* (0.482) and between *E. bleekeri* and *E. malabaricus* (0.566). GD values calculated for OPA 07 primer showed highest value between *E. malabaricus* and *E. diacanthus*, while the lowest between *E. bleekeri* and *E. malabaricus*. UPGMA dendrogram was constructed to show the genetic relationships between species (Fig. 16 B).

The primer OPF 08 showed high GI between *E. bleekeri* and *E. coioides* (0. 935) and between *E. chlorostigma* and *E. coioides* (0.895). *E. areolatus* and *E. tauvina* showed the lowest GI between them (0.454). The GD was high between *E. tauvina* and *E. areolatus* and between *E. malabaricus* and *E. diacanthus*, where as low between *E. coioides* and *E. bleekeri* and between *E. coioides* and *E. malabaricus* (Table 6). UPGMA dendrogram was constructed based on GD values to show the relationship between species (Fig. 17 A).

GI values calculated for OPF 10 primer showed maximum similarity between *E. tauvina* and *E. coioides* and between *E. chlorostigma* and *E. tauvina*. Low GI was observed between *E. bleekeri* and *E. malabaricus*. The GD values were high between *E. malabaricus* and *E. bleekeri* (0.567) and between *E. bleekeri* and *E. diacanthus* (0.536). Lowest GD was between *E. tauvina* and *E. coioides* (0.083) and between *E. malabaricus* and *E. tauvina* (0.135) (Table 7). UPGMA dendrogram was constructed based on GD values to show the relationship between species (Fig. 17 B).

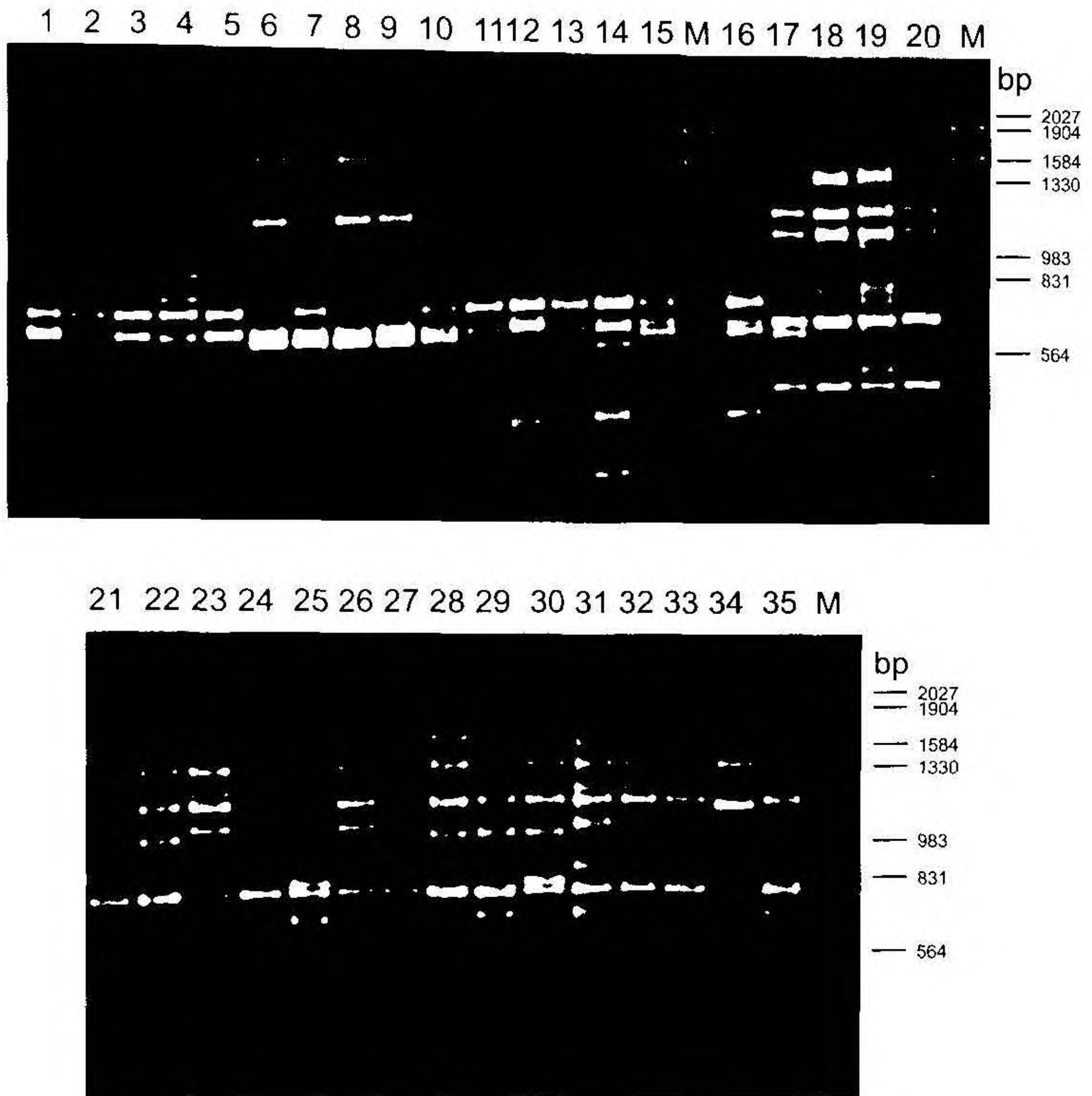


Fig. 12. RAPD fingerprints generated by OPA 01 primer in different individuals of *Epinephelus* spp. Lanes 1-5: *E. diacanthus*; lanes 6-10: *E. areolatus*; lanes 11-15: *E. chlorostigma*; lanes 16-20: *E. bleekeri*; lanes 21-25: *E. coioides*; lanes 26-30: *E. tauvina*; lanes 31-35: *E. malabaricus* and lane M:λ DNA marker double digested with *Eco* RI / *Hind* III.

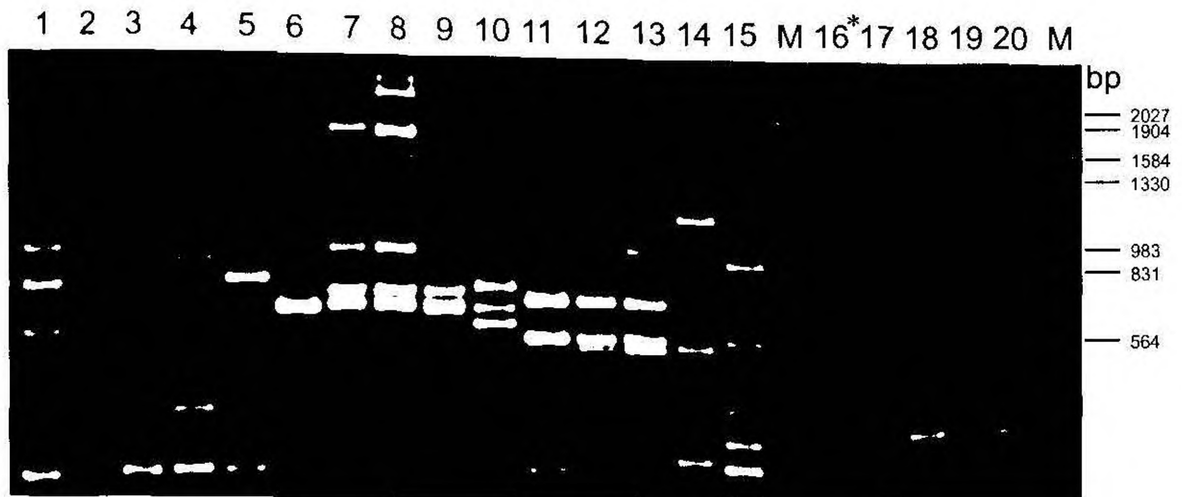
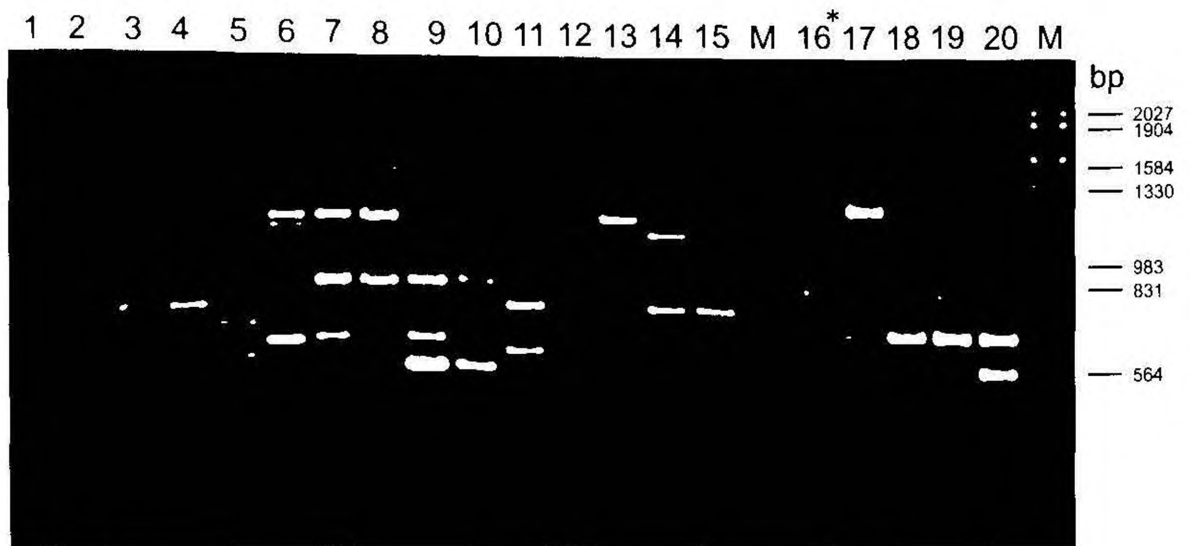


Fig. 13. RAPD fingerprints generated by OPA 07 primer in different individuals of *Epinephelus* spp. Lanes 1-5: *E. diacanthus*; lanes 6-10: *E. areolatus*; lanes 11-15: *E. chlorostigma*; lanes 16-20: *E. bleekeri*; lanes 21-25: *E. coioides*; lanes 26-30: *E. tauvina*; lanes 31-35: *E. malabaricus* and lane M: λ DNA marker double digested with *Eco* RI / *Hind* III.

* not taken for analysis.



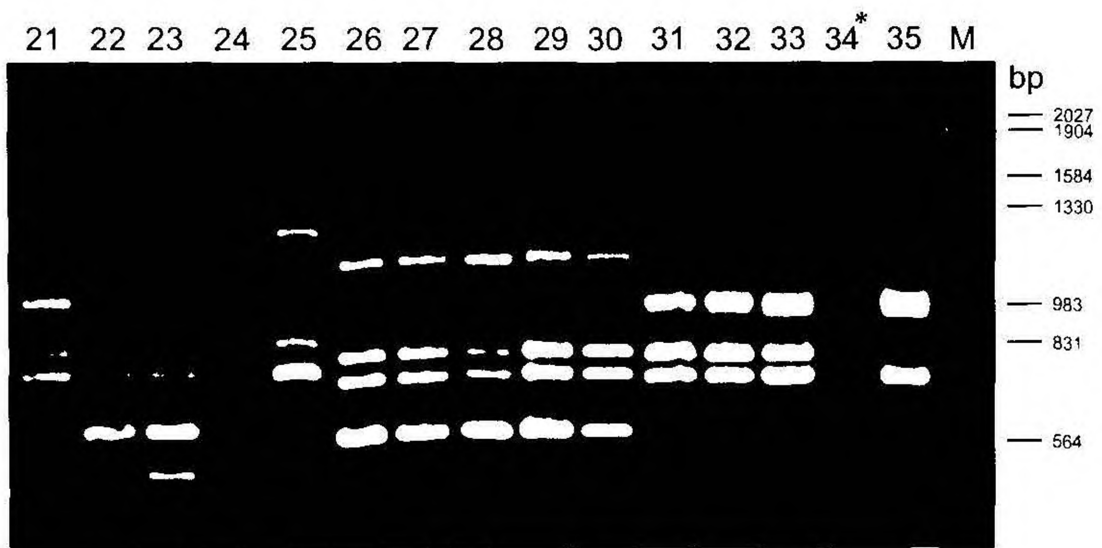
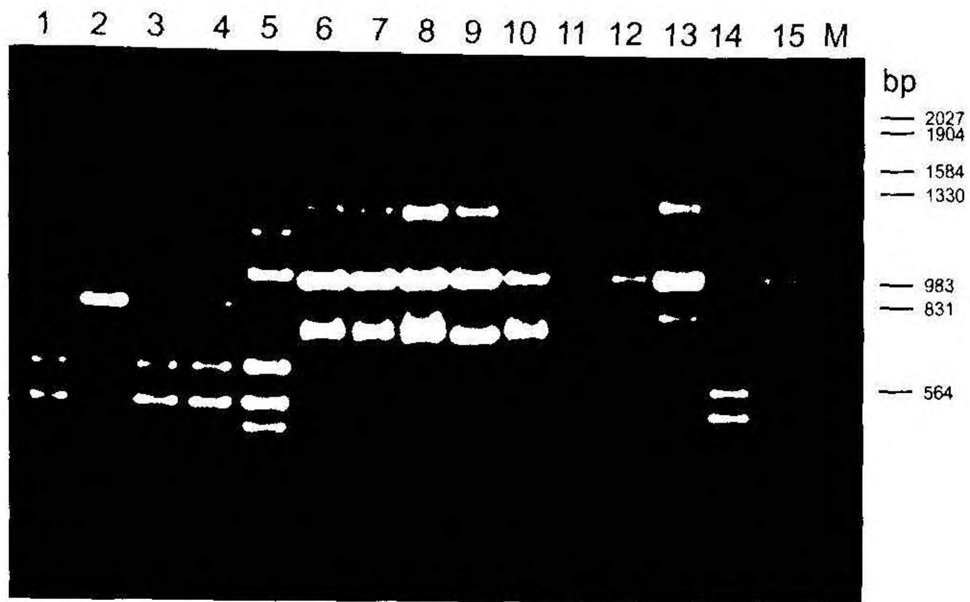


Fig. 14. RAPD fingerprints generated by OPF 08 primer in different individuals of *Epinephelus* spp. Lanes 1-5: *E. diacanthus*; lanes 6-10: *E. areolatus*; lanes 11-15: *E. chlorostigma*; lanes 16-20: *E. bleekeri*; lanes 21-25: *E. coioides*; lanes 26-30: *E. tauvina*; lanes 31-35: *E. malabaricus* and lane M:λ DNA marker double digested with *Eco* RI / *Hind* III.
* not taken for analysis.



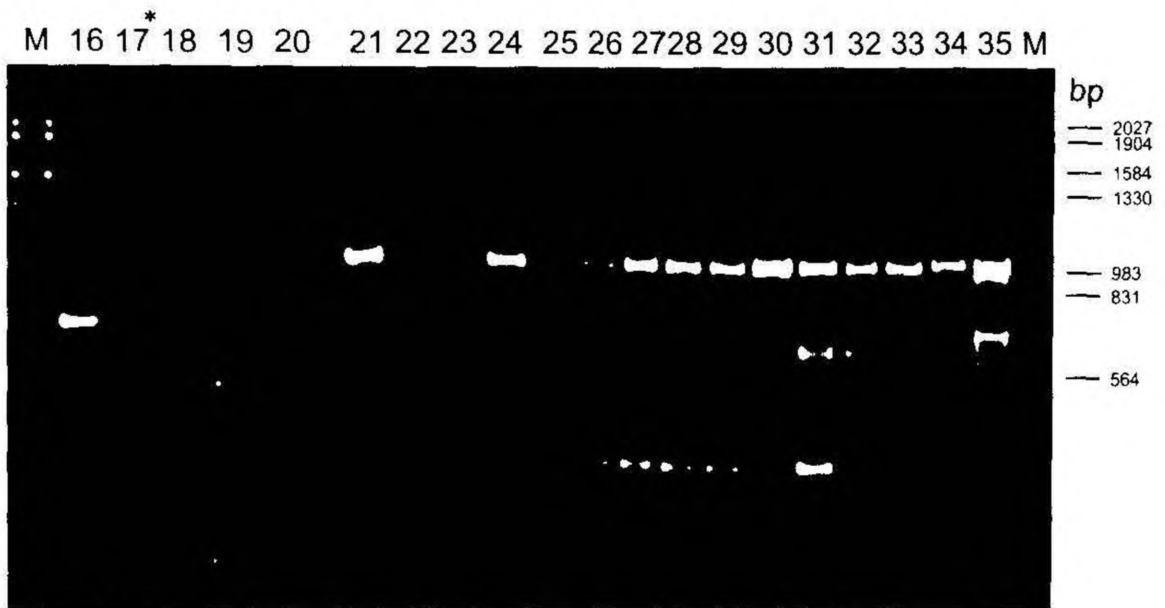


Fig. 15. RAPD fingerprints generated by OPF 10 primer in different individuals of *Epinephelus* spp. Lanes 1-5: *E. diacanthus*; lanes 6-10: *E. areolatus*; lanes 11-15: *E. chlorostigma*; lanes 16-20: *E. bleekeri*; lanes 21-25: *E. coioides*; lanes 26-30: *E. tauvina*; lanes 31-35: *E. malabaricus* and lane M: λ DNA marker double digested with Eco RI / Hind III.

* not taken for analysis.

Table 4. Data showing pair-wise comparison of Genetic Identity (above diagonal) and Genetic Distance (below diagonal) of *Epinephelus* spp based on Nei (1978) calculated for OPA O1 primer

Species ID	*E.dia*	*E.are*	*E.chl*	*E.ble*	*E.coi*	*E.tau*	*E.mal*
E.dia	****	0.6449	0.7515	0.5598	0.7559	0.4905	0.6362
E.are	0.4387	****	0.7686	0.6310	0.7606	0.6447	0.6739
E.chl	0.2857	0.2632	****	0.8406	0.7345	0.5114	0.5667
E.ble	0.5802	0.4604	0.1736	****	0.6156	0.4580	0.4654
E.coi	0.2799	0.2736	0.3086	0.4852	****	0.7914	0.7935
E.tau	0.7124	0.4389	0.6706	0.7809	0.2339	****	0.8720
E.mal	0.4523	0.3947	0.5680	0.7649	0.2313	0.1369	****

E.dia = E. diacanthus; E. are = E. areolatus; E. chl = E. chlorostigma; E.ble = E. bleekeri; E.coi = E. coioides; E. tau = E. tauvina; E. mal = E. malabaricus

Table 5. Data showing pair-wise comparison of Genetic Identity (above diagonal) and Genetic Distance (below diagonal) of *Epinephelus* spp based on Nei (1978) calculated for OPA O7 primer

Species ID*	*E.dia*	*E.are*	*E.chl*	*E.ble*	*E.coi*	*E.tau*	*E.mal*
E.dia	****	0.6430	0.8866	0.7802	0.7402	0.6329	0.4824
E.are	0.4416	****	0.8319	0.6988	0.7540	0.6311	0.5979
E.chl	0.1203	0.1841	****	0.8932	0.8478	0.7178	0.5742
E.ble	0.2482	0.3584	0.1130	****	0.7892	0.6768	0.5660
E.coi	0.3009	0.2824	0.1651	0.2368	****	0.9377	0.8213
E.tau	0.4574	0.4603	0.3315	0.3904	0.0643	****	0.8982
E.mal	0.7290	0.5143	0.5548	0.5691	0.1969	0.1074	****

*Species ID is referred in Table 4.

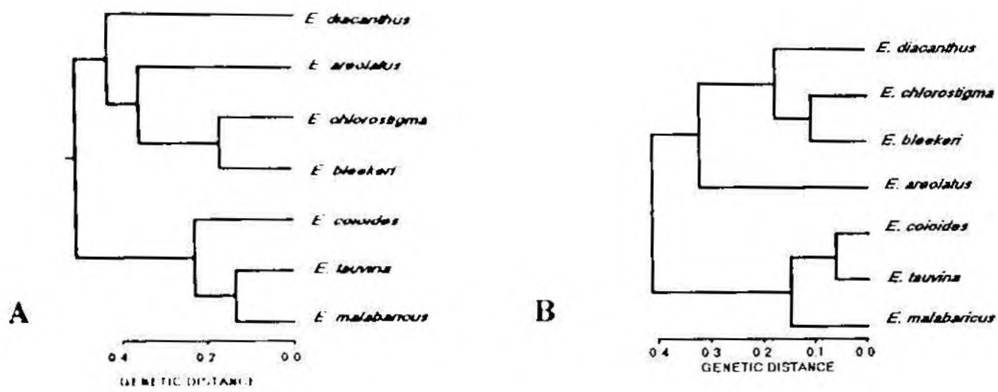


Fig. 16. UPGMA dendrogram of *Epinephelus* spp based on genetic distance values (Nei, 1978) calculated from data for primers OPA 01 (A) and OPA 07 (B).

Table 6. Data showing pair-wise comparison of Genetic Identity (above diagonal) and Genetic Distance (below diagonal) of *Epinephelus* spp based on Nei (1978) calculated for OPF O8 primer

Species ID*	*E.dia*	*E.are*	*E.chl*	*E.ble*	*E.coi*	*E.tau*	*E.mal*
E.dia	****	0.6253	0.7086	0.7699	0.7584	0.6756	0.5146
E.are	0.4695	****	0.7033	0.8462	0.7514	0.4537	0.7229
E.chl	0.3444	0.3519	****	0.8661	0.8994	0.6598	0.6986
E.ble	0.2615	0.1670	0.1437	****	0.9354	0.6146	0.6944
E.coi	0.2766	0.2858	0.1060	0.0668	****	0.7613	0.8066
E.tau	0.3922	0.7904	0.4158	0.4867	0.2727	****	0.5895
E.mal	0.6645	0.3244	0.3587	0.3647	0.2150	0.5284	****

*Species ID is referred in Table 4.

Table 7. Data showing pair-wise comparison of Genetic Identity (above diagonal) and Genetic Distance (below diagonal) of *Epinephelus* spp based on Nei (1978) calculated for OPF 10 primer

Species ID*	*E.dia*	*E.are*	*E.chl*	*E.ble*	*E.coi*	*E.tau*	*E.mal*
E.dia	****	0.6394	0.6977	0.5851	0.6462	0.7743	0.7614
E.are	0.4473	****	0.8496	0.6998	0.7665	0.8137	0.8326
E.chl	0.3599	0.1630	****	0.7710	0.7855	0.8505	0.7347
E.ble	0.5359	0.3569	0.2600	****	0.8095	0.7704	0.5670
E.coi	0.4366	0.2659	0.2415	0.2114	****	0.9205	0.7823
E.tau	0.2557	0.2062	0.1619	0.2609	0.0829	****	0.8739
E.mal	0.2725	0.1833	0.3083	0.5674	0.2456	0.1348	****

*Species ID is referred in Table 4.

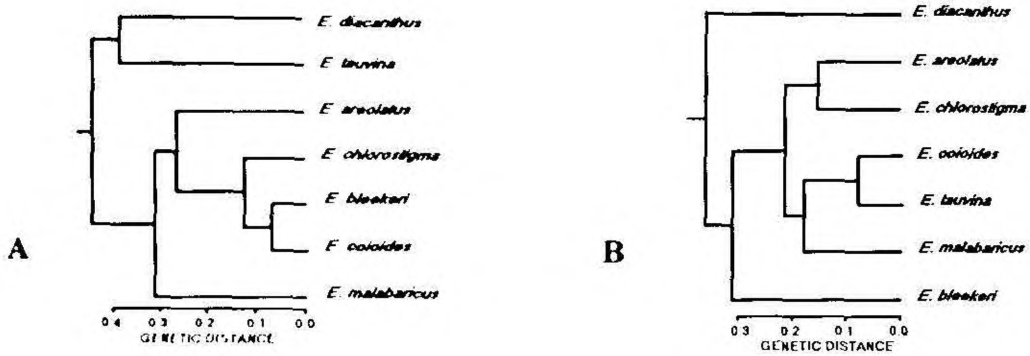


Fig. 17. UPGMA dendrogram of *Epinephelus* spp based on genetic distance values (Nei, 1978) calculated from data for primers OPF 08 (A) and OPF 10 (B).

Average pair-wise GI and GD values were calculated for all the four primers together. The GI was high between *E. coioides* and *E. tauvina* (0.857) and between *E. chlorostigma* and *E. bleekeri* (0.845), low similarity values were between *E. bleekeri* and *E. malabaricus* (0.568) and between *E. diacanthus* and *E. malabaricus* (0.591). *E. malabaricus* was highly diverged from *E. bleekeri* with maximum GD of 0.565 and *E. malabaricus* was distant from *E. diacanthus* (0.526). Low GD was observed between *E. coioides* and *E. tauvina* (0.154) followed by *E. chlorostigma* and *E. bleekeri* (0.168), showing that they are closely related (Table 8). Dendrogram was constructed from the combined data for all the four primers (Fig. 18).

GI and GD within species of *Epinephelus* were calculated and results are shown in Table 9. Based on these values, dendrogram was constructed to show the genetic relationships among different individuals of *Epinephelus* (Fig. 19). Different individuals of the each species were by and large grouped with their respective species clusters. However, few specimens of *E. chlorostigma, E. bleekeri* and *E. coioides* did not belong to their own species clusters (Fig. 19).

4.5. Intraspecies and Interspecies Genetic Distance

The intraspecies GD values obtained by pair-wise comparisons of different individuals in each species are given in table 9. The intraspecies GD values for the seven grouper species were tested by one-way ANOVA and found to be significantly different ($p<0.01$) (Table 10).

The interspecies GD values were obtained by pair-wise comparisons of different individuals of each of the seven grouper species (Table 9). The Interspecies GD values estimated for the seven grouper species were tested by one-way ANOVA and found to be highly significantly different ($p<0.01$) (Table 11).

Statistical analysis was also carried out to test for differences in intraspecies and interspecies GD values. Mean intraspecies GD was lower (0.305) than interspecies (0.365). In theory, the intraspecies GD values are expected to be lower than the interspecies GD values. Hence, a two-sample *t*-test was performed to test whether the intraspecies GD values differed from interspecies GD values. Results showed that the intraspecies GD (number of pair-wise comparisons=70,

mean=0.305) was significantly lower (t=2.871, df= 69, P<0.005) than the interspecies GD values (number of pair-wise comparisons= 504, mean=0.365) thus confirming the theoretical expectation.

Based on Nei (1973), genetic diversity within the species was calculated and data are shown in table 12. High genetic diversity was observed in *E. coioides* (0.1658) and also in *E. chlorostigma* (0.1527). *E. tauvina* and *E. malabaricus* exhibited lower genetic diversity values of 0.0936 and 0.1025, respectively. *E. chlorostigma* showed highest level of polymorphism (49.15%) while the lowest level of polymorphism was observed in *E. tauvina* (25.42%). Data for other species are given in Table 12.

4.6. Species-Specific Diagnostic Markers

Several RAPD fragments show fixed frequencies in each of the seven species of grouper (Figs. 12, 13, 14 and 15). These could be used as species-specific markers to distinguish grouper species from Indian waters (Table 13). All the species are having at least one diagnostic marker from each primer, with some of the primers yielding two. All the species-specific markers were below 1700 bp. Maximum number of diagnostic markers were observed in *E. bleekeri* and minimum in *E. tauvina* and *E. malabaricus*.

Table 8. Data showing pair-wise comparison of Genetic Identity (above diagonal) and Genetic Distance (below diagonal) of *Epinephelus* spp based on Nei (1978) calculated for all primers

Species ID*	*E.dia*	*E.are*	*E.chl*	*E.ble*	*E.coi*	*E.tau*	*E.mal*
E.dia	****	0.6387	0.7707	0.6748	0.7238	0.6377	0.5910
E.are	0.4483	****	0.7921	0.7099	0.7580	0.6422	0.6966
E.chl	0.2604	0.2331	****	0.8451	0.8098	0.6788	0.6323
E.ble	0.3934	0.3426	0.1683	****	0.7779	0.6313	0.5685
E.coi	0.3233	0.2771	0.2109	0.2512	****	0.8576	0.7993
E.tau	0.4500	0.4428	0.3875	0.4599	0.1537	****	0.8181
E.mal	0.5259	0.3615	0.4584	0.5648	0.2240	0.2008	****

*Species ID is referred in Table 4.

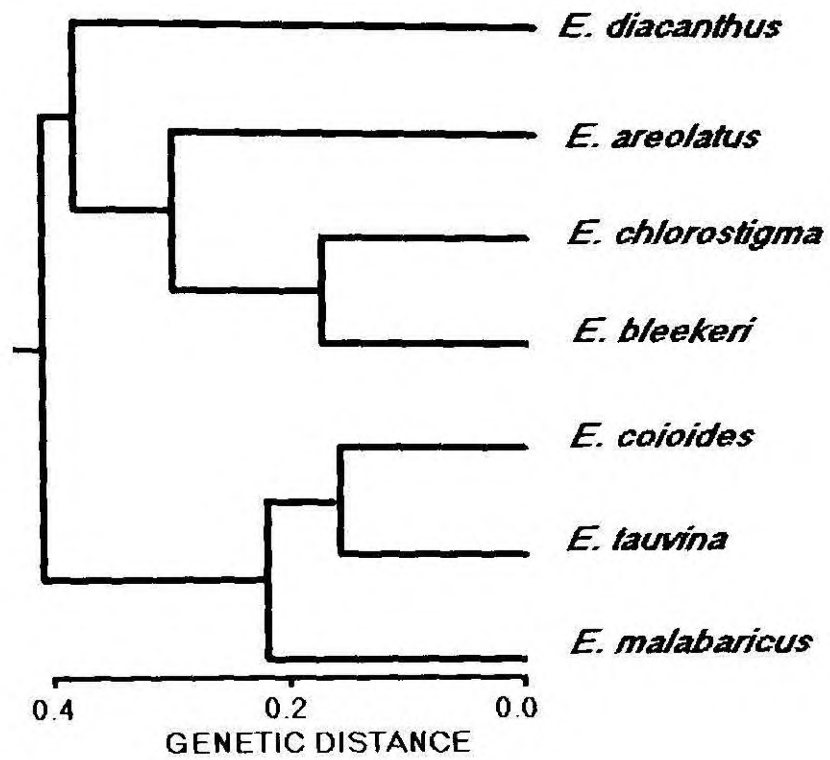


Fig. 18. UPGMA dendrogram of *Epinephelus* spp based on values of genetic distance (Nei, 1978) calculated from data for all primers.

Table 9. Genetic Identity (above diagonal) and Genetic Distance (below diagonal) matrices of different individuals of *Epinephelus* spp based on Nei (1978) calculated from RAPD markers for all primers

	E.dia1	*E.dia2*	*E.dia3*	*E.dia4*	*E.dia5*	*E.are1*	*E.are2*	*E.are3*	*E.are4*	*E.are5*	*E.chl1*	*E.chl2*	*E.chl3*	*E.chl4*	*E.chl5*	*E.ble1*	*E.ble2*	*E.ble3*	*E.ble4*	*E.ble5*
E.dia1	****	0.847	0.813	0.813	0.898	0.508	0.457	0.474	0.525	0.593	0.491	0.576	0.644	0.695	0.542	0.390	0.525	0.542	0.525	0.491
E.dia2	0.166	****	0.830	0.830	0.780	0.525	0.474	0.457	0.508	0.644	0.542	0.661	0.661	0.712	0.627	0.474	0.542	0.559	0.610	0.542
E.dia3	0.206	0.186	****	0.966	0.813	0.491	0.407	0.390	0.441	0.610	0.576	0.559	0.593	0.712	0.695	0.441	0.542	0.559	0.542	0.542
E.dia4	0.206	0.186	0.034	****	0.780	0.525	0.440	0.424	0.474	0.644	0.542	0.593	0.627	0.744	0.729	0.407	0.576	0.593	0.576	0.542
E.dia5	0.107	0.249	0.206	0.250	****	0.542	0.491	0.474	0.525	0.661	0.491	0.542	0.576	0.662	0.542	0.424	0.525	0.474	0.491	0.491
E.are1	0.676	0.643	0.710	0.643	0.612	****	0.915	0.864	0.881	0.779	0.474	0.695	0.763	0.576	0.627	0.441	0.542	0.559	0.576	0.542
E.are2	0.782	0.745	0.899	0.819	0.710	0.088	****	0.949	0.898	0.729	0.525	0.644	0.713	0.491	0.542	0.525	0.491	0.508	0.559	0.525
E.are3	0.745	0.782	0.942	0.859	0.745	0.146	0.052	****	0.949	0.712	0.474	0.593	0.661	0.508	0.525	0.508	0.542	0.491	0.508	0.508
E.are4	0.643	0.676	0.819	0.745	0.643	0.126	0.107	0.052	****	0.763	0.457	0.644	0.678	0.559	0.576	0.457	0.559	0.542	0.559	0.559
E.are5	0.522	0.440	0.494	0.440	0.414	0.249	0.316	0.340	0.271	****	0.559	0.712	0.678	0.627	0.644	0.457	0.593	0.542	0.593	0.525
E.chl1	0.710	0.612	0.551	0.612	0.710	0.745	0.643	0.745	0.782	0.581	****	0.644	0.610	0.559	0.678	0.695	0.423	0.508	0.559	0.525
E.chl2	0.551	0.414	0.581	0.522	0.612	0.364	0.440	0.522	0.440	0.340	0.440	****	0.864	0.678	0.796	0.407	0.712	0.695	0.711	0.678
E.chl3	0.440	0.414	0.522	0.466	0.551	0.271	0.340	0.414	0.389	0.389	0.494	0.146	****	0.712	0.729	0.508	0.644	0.695	0.712	0.678
E.chl4	0.364	0.340	0.340	0.293	0.414	0.551	0.710	0.676	0.581	0.466	0.581	0.389	0.340	****	0.678	0.491	0.661	0.576	0.593	0.593
E.chl5	0.611	0.466	0.364	0.316	0.612	0.466	0.612	0.643	0.551	0.440	0.389	0.227	0.316	0.389	****	0.441	0.678	0.661	0.678	0.644
E.ble1	0.942	0.745	0.819	0.899	0.859	0.819	0.643	0.676	0.782	0.782	0.364	0.899	0.676	0.710	0.819	****	0.356	0.441	0.491	0.491
E.ble2	0.643	0.612	0.612	0.551	0.643	0.612	0.710	0.612	0.581	0.522	0.859	0.334	0.440	0.414	0.389	1.033	****	0.678	0.627	0.661
E.ble3	0.612	0.582	0.581	0.522	0.745	0.581	0.676	0.710	0.612	0.612	0.676	0.364	0.364	0.551	0.414	0.819	0.389	****	0.915	0.915
E.ble4	0.643	0.494	0.612	0.551	0.710	0.551	0.581	0.676	0.581	0.522	0.581	0.340	0.340	0.522	0.389	0.710	0.466	0.088	****	0.932
E.ble5	0.710	0.612	0.612	0.612	0.710	0.612	0.643	0.676	0.581	0.643	0.643	0.389	0.388	0.522	0.440	0.710	0.414	0.088	0.070	****
E.coi1	0.643	0.612	0.494	0.494	0.581	0.440	0.466	0.494	0.522	0.522	0.522	0.551	0.440	0.522	0.494	0.643	0.782	0.551	0.522	0.467
E.coi2	0.643	0.612	0.612	0.612	0.643	0.612	0.643	0.612	0.643	0.643	0.522	0.612	0.612	0.710	0.551	0.782	0.859	0.551	0.581	0.522
E.coi3	0.522	0.612	0.612	0.612	0.522	0.612	0.710	0.745	0.710	0.581	0.643	0.745	0.551	0.782	0.745	0.782	1.033	0.551	0.581	0.581
E.coi4	0.643	0.676	0.551	0.551	0.643	0.494	0.522	0.494	0.522	0.581	0.522	0.551	0.494	0.522	0.494	0.710	0.782	0.551	0.581	0.522
E.coi5	0.440	0.364	0.522	0.466	0.612	0.466	0.551	0.522	0.494	0.440	0.676	0.364	0.364	0.612	0.522	0.819	0.612	0.414	0.494	0.494
E.tau1	0.522	0.676	0.676	0.612	0.581	0.551	0.643	0.612	0.581	0.522	0.581	0.612	0.551	0.643	0.551	0.782	0.859	0.612	0.643	0.644
E.tau2	0.551	0.643	0.643	0.643	0.551	0.643	0.676	0.643	0.676	0.676	0.611	0.710	0.643	0.745	0.643	0.745	0.986	0.643	0.676	0.612
E.tau3	0.643	0.745	0.676	0.676	0.643	0.612	0.581	0.551	0.581	0.710	0.581	0.745	0.676	0.782	0.676	0.782	1.033	0.745	0.710	0.644
E.tau4	0.494	0.581	0.581	0.581	0.551	0.522	0.612	0.581	0.494	0.612	0.611	0.581	0.522	0.676	0.581	0.819	0.899	0.581	0.612	0.551
E.tau5	0.551	0.643	0.643	0.581	0.676	0.581	0.745	0.643	0.551	0.551	0.819	0.710	0.643	0.676	0.581	1.082	0.819	0.710	0.745	0.745
E.mal1	0.581	0.819	0.676	0.612	0.643	0.551	0.581	0.551	0.522	0.522	0.710	0.745	0.745	0.710	0.676	1.033	0.859	0.745	0.710	0.782
E.mal2	0.581	0.819	0.676	0.612	0.643	0.612	0.643	0.612	0.581	0.581	0.782	0.819	0.819	0.710	0.745	1.133	0.859	0.745	0.782	0.782
E.mal3	0.551	0.782	0.643	0.581	0.612	0.581	0.612	0.581	0.551	0.551	0.745	0.782	0.782	0.676	0.710	1.082	0.819	0.710	0.745	0.745
E.mal4	0.710	0.745	0.819	0.745	0.710	0.494	0.414	0.440	0.522	0.522	0.581	0.676	0.676	0.710	0.676	0.643	0.710	0.819	0.781	0.859
E.mal5	0.710	0.745	0.745	0.676	0.643	0.551	0.522	0.612	0.643	0.643	0.859	0.986	0.745	0.859	0.899	0.643	0.942	0.899	0.782	0.859

Continued/-

Continued from **Table 9**

	E.coi1	E.coi2	E.coi3	E.coi4	E.coi5	E.tau1	E.tau2	E.tau3	E.tau4	E.tau5	E.mal1	E.mal2	E.mal3	E.mal4	E.mal5
E.dia1	0.525	0.525	0.593	0.525	0.644	0.593	0.576	0.525	0.610	0.576	0.559	0.559	0.576	0.491	0.491
E.dia2	0.542	0.542	0.542	0.508	0.695	0.508	0.525	0.474	0.559	0.525	0.441	0.440	0.457	0.474	0.474
E.dia3	0.610	0.542	0.542	0.576	0.593	0.508	0.525	0.508	0.559	0.525	0.508	0.508	0.525	0.440	0.474
E.dia4	0.610	0.542	0.542	0.576	0.627	0.542	0.525	0.508	0.559	0.559	0.542	0.542	0.559	0.474	0.508
E.dia5	0.559	0.525	0.593	0.525	0.542	0.559	0.576	0.525	0.576	0.508	0.525	0.525	0.542	0.491	0.525
E.are1	0.644	0.542	0.542	0.610	0.627	0.576	0.525	0.542	0.593	0.559	0.576	0.542	0.559	0.610	0.576
E.are2	0.627	0.525	0.491	0.593	0.576	0.525	0.508	0.559	0.542	0.474	0.559	0.525	0.542	0.661	0.593
E.are3	0.610	0.542	0.474	0.610	0.593	0.542	0.525	0.576	0.559	0.525	0.576	0.542	0.559	0.644	0.542
E.are4	0.593	0.525	0.491	0.593	0.610	0.559	0.508	0.559	0.610	0.576	0.593	0.559	0.576	0.593	0.525
E.are5	0.593	0.525	0.559	0.559	0.644	0.593	0.508	0.491	0.542	0.576	0.593	0.559	0.576	0.593	0.525
E.chl1	0.593	0.593	0.525	0.593	0.508	0.559	0.542	0.559	0.542	0.441	0.491	0.457	0.474	0.559	0.423
E.chl2	0.576	0.542	0.474	0.576	0.695	0.542	0.491	0.474	0.559	0.491	0.474	0.441	0.457	0.508	0.372
E.chl3	0.644	0.542	0.576	0.610	0.695	0.576	0.525	0.508	0.593	0.525	0.474	0.441	0.457	0.508	0.474
E.chl4	0.593	0.491	0.457	0.593	0.542	0.525	0.474	0.457	0.508	0.508	0.491	0.491	0.508	0.491	0.423
E.chl5	0.610	0.576	0.474	0.610	0.593	0.576	0.525	0.508	0.559	0.559	0.508	0.474	0.491	0.508	0.406
E.ble1	0.525	0.457	0.457	0.491	0.441	0.457	0.474	0.457	0.440	0.339	0.356	0.322	0.339	0.525	0.525
E.ble2	0.457	0.423	0.356	0.457	0.542	0.424	0.373	0.356	0.406	0.441	0.423	0.424	0.441	0.491	0.389
E.ble3	0.576	0.576	0.576	0.576	0.661	0.542	0.525	0.474	0.559	0.491	0.474	0.474	0.491	0.441	0.406
E.ble4	0.593	0.559	0.559	0.559	0.610	0.525	0.508	0.491	0.542	0.474	0.491	0.457	0.474	0.457	0.457
E.ble5	0.627	0.593	0.559	0.593	0.610	0.525	0.542	0.525	0.576	0.474	0.457	0.457	0.474	0.424	0.423
E.coi1	****	0.864	0.796	0.966	0.644	0.729	0.778	0.762	0.746	0.678	0.695	0.695	0.712	0.695	0.662
E.coi2	0.146	****	0.864	0.898	0.712	0.796	0.881	0.864	0.813	0.780	0.729	0.729	0.712	0.695	0.662
E.coi3	0.227	0.146	****	0.796	0.644	0.830	0.847	0.796	0.780	0.780	0.763	0.763	0.743	0.661	0.695
E.coi4	0.034	0.107	0.227	****	0.644	0.763	0.813	0.796	0.780	0.712	0.729	0.729	0.746	0.729	0.627
E.coi5	0.440	0.340	0.440	0.440	****	0.610	0.627	0.576	0.661	0.627	0.508	0.508	0.525	0.508	0.542
E.tau1	0.316	0.227	0.186	0.271	0.494	****	0.915	0.864	0.881	0.847	0.796	0.763	0.780	0.763	0.695
E.tau2	0.249	0.126	0.165	0.206	0.466	0.088	****	0.915	0.898	0.796	0.746	0.712	0.729	0.712	0.678
E.tau3	0.271	0.146	0.227	0.227	0.551	0.146	0.088	****	0.915	0.847	0.763	0.729	0.712	0.729	0.695
E.tau4	0.293	0.206	0.249	0.249	0.414	0.126	0.107	0.088	****	0.864	0.712	0.678	0.695	0.678	0.644
E.tau5	0.389	0.249	0.249	0.340	0.466	0.165	0.227	0.165	0.146	****	0.780	0.780	0.763	0.678	0.678
E.mal1	0.364	0.316	0.271	0.316	0.676	0.227	0.293	0.271	0.340	0.249	****	0.932	0.915	0.796	0.729
E.mal2	0.364	0.316	0.271	0.316	0.676	0.271	0.340	0.316	0.389	0.249	0.070	****	0.983	0.796	0.763
E.mal3	0.340	0.340	0.293	0.293	0.643	0.249	0.316	0.340	0.364	0.271	0.088	0.017	****	0.813	0.746
E.mal4	0.364	0.364	0.414	0.316	0.676	0.271	0.340	0.316	0.389	0.389	0.227	0.227	0.206	****	0.729
E.mal5	0.414	0.414	0.364	0.466	0.612	0.364	0.389	0.364	0.440	0.389	0.316	0.271	0.293	0.316	****

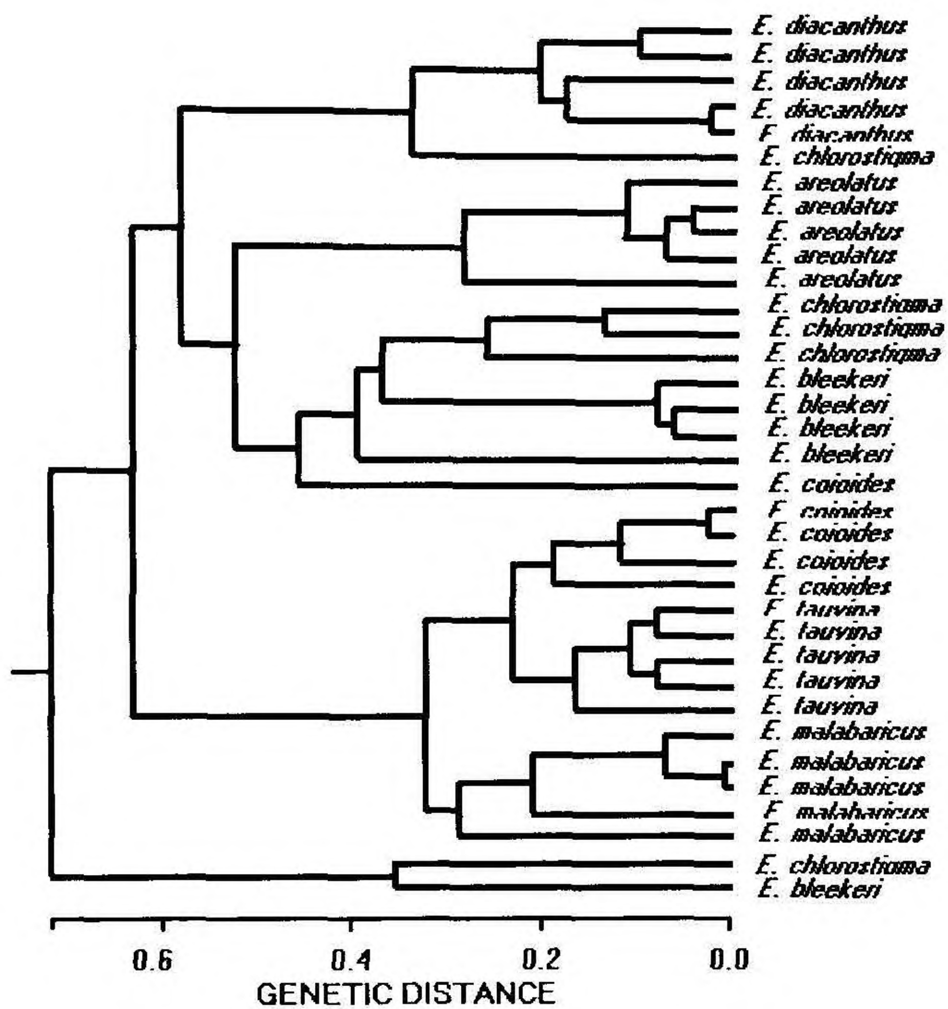


Fig. 19. UPGMA dendrogram constructed based on Nei's (1978) genetic distance calculated from data for all primers, showing the genetic relationships among different individuals of seven species of groupers.

Table 10. Summary of the results of one-way ANOVA to test for differences in intraspecies genetic distance values calculated based on RAPD markers among seven species of *Epinephelus*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Within species	1.2612	6	0.2102	11.4287	0.01	2.2464
Error	1.1587	63	0.0184			
Total	2.4199	69				

Table 11. Summary of the results of one-way ANOVA to test for differences in interspecies genetic distance values calculated based on RAPD markers by pair-wise comparisons of individuals among the seven species of *Epinephelus*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between species	7.1438	20	0.3571	55.2881	0.01	1.5914
Error	3.2561	504	0.0064			
Total	10.399	524				

Table 12. Nei's (1973) Genetic Diversity, No. of polymorphic loci and % polymorphism within each species of *Epinephelus* analyzed by OPA 01, OPA 07, OPF 08 and OPF 10 primers

Species ID*	Genetic Diversity	No. of polymorphic Loci	% polymorphism
E.dia	0.1178	19	32.20
E.are	0.1306	19	32.20
E.chl	0.1527	29	49.15
E.ble	0.1190	19	32.20
E.coi	0.1658	28	47.46
E.tau	0.0936	15	25.42
E.mal	0.1025	17	28.81

*Species ID is referred in Table 4.

Table 13. Species diagnostic RAPD markers in *Epinephelus* spp.

Primer / Species	OPA 01	OPA 07	OPF 08	OPF 10
E. diacanthus	830 & 260	245	660	1160 & 650
E. areolatus	610	745	1250 & 925	910 & 720
E. chlorostigma	775 & 670	650	820	300
E. bleekeri	660 & 430	1320 & 170	600	640 & 120
E. coioides	875	860	815	170
E. tauvina	1610 & 980	905	1130 & 585	245
E. malabaricus	855	365	940	90

DISCUSSION

5. DISCUSSION

Groupers are identified conventionally based on morphological and meristic characters and relies mainly on the meristic counts and pigmentation pattern of the skin (Heemstra and Randall, 1993). The morphological approach is beset with problems including 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*. There is thus the need for supportive techniques to ratify taxonomic status of groupers as they are very important from both fisheries and aquaculture points of view.

Multivariate analysis of morphological measures in fishes is a useful phenotypic approach to support inference of patterns of interspecific diversification (Bronte *et al.*, 1999; Golubtsov *et al.*, 1999). Principal Component Analysis and Discriminant analysis of truss morphometric landmarks are generally used for detecting variations within a species. However, these methods are also useful to evaluate phenetic intermediacy of one taxonomic group among two or more related groups on the basis of size-free shape derived from distance measures (Humphries *et al.*, 1981). The latter workers have done multivariate analysis of size and shape discrimination of *Cyprinodon beltrani* and *C. maya* and commented about its utility with reference to juveniles and occasional hybrids, which are otherwise difficult to precisely identify.

Though the focus of the present study was genetic profiling of grouper species, one of the most advanced phenotypic approaches, namely multivariate analysis of truss morphometric landmarks was carried out to provide supportive evidence for species identification. The present results of SPCA and Canonical Variate Analysis infer that the five species of groupers are distinguished based on the variations in the area encompassing the vertical, horizontal and diagonal distance measures between the second dorsal fin and anal fin.

RAPD markers have been used to investigate the taxonomic status of different groups of fishes such as tilapia (Bardakci and Skibinski, 1994; Dinesh *et al.*, 1996), striped bass (Bielawski and Pumo, 1997), sturgeons (Comincini *et al.*, 1998), large mouth bass (Williams *et al.*, 1998), goatfishes (Mamuris *et al.*, 1999) fugu (Chao *et al.*, 2001), eels (Lehmann *et al.*, 2000) and groupers (Baker and Azizah, 2000) and crustaceans including prawns (Meruane *et al.*, 1997) and mud crabs (Klibunga *et al.*, 2000).

The present study provides the first report on the application of RAPD markers for species identification of groupers from Indian waters. All the four arbitrary primers used gave unique banding patterns for each species. The present results of RAPD analysis demonstrate a separation of gene pools of all the seven species of *Epinephelus*, in which by and large all individuals of each species formed closed monophyletic species clusters. The very low GD value between *E. tauvina* and *E. coioides* (0.1537) and that between *E. bleekeri* and *E. chlorostigma* (0.1683) points to the close proximity of these species. The observation that few specimens of *E. bleekeri*, *E. chlorostigma* and *E. coioides* were not grouped with their respective species clusters indicates that due to similar morphological features, these specimens might have been wrongly identified.

The number of species-specific diagnostic markers observed in *E. bleekeri* and *E. coioides* in the present investigation compares well with those reported for the same species by Bakar and Azizah (2000). All the seven species of grouper had significantly ($P<0.01$) higher interspecies GD values than the intraspecies values. This observation is in conformity with that made in tilapia (Dinesh *et al.*, 1996).

Among the seven species of *Epinephelus*, *E. diacanthus* is clearly differentiated from the rest based on truss morphometry (Tables 2 and 3; Figs 10 and 11) and RAPD fingerprinting (Figs 12,13,14,15 and 18; Table 8). Based on the present results and observations of Heemstra and Randall (1993), it is concluded that there is no confusion in the identity of *E. diacanthus* with reference to the available species of *Epinephelus* in Indian waters.

RAPD fingerprinting has proven itself as a robust, dependable and easy tool to use for identification of grouper species. The RAPD markers developed here can be used by fisheries personel to identify and evaluate genotypes of wild and brood stock *Epinephelus* spp. In the present investigation, reproducibility was tested at various stages of the process, leading to consistent banding patterns with all primer amplifications. Under identical conditions, the RAPD technique, using relatively rapidly evolving DNA regions is informative in taxonomic studies (Schmidt and Westheide, 1998), systematics (Stothard and Rollinson, 1996; Yu and Lin, 1997) and population genetics (Tassanakajon *et al.*, 1997).

Due to overlapping of morphological characters, *E. areolatus, E. chlorostigma* and *E. bleekeri* have often been confused among them, as well as among *E. coioides, E. tauvina* and *E. malabaricus* (Randall and Heemstra, 1991). The fact that between *E. coioides* and *E. tauvina* and between *E. bleekeri* and *E. chlorostigma,* the GD values were as low as 0.154 and 0.168, respectively, raises the question about erection of these species based on current morphological identification key. It is therefore suggested to examine more specimens of these species from different locations to unambiguously establishing their separate species status based on genetic identity.

Further work on the molecular genetic profiling of grouper species commonly used in aquaculture as well as assessing the genetic variation in populations is entailed. This is of great importance in the monitoring and management of any breeding programme. The simplicity, rapidity as well as cost effectiveness of the RAPD technique over the conventional molecular phylogenetic analysis using RFLP and DNA sequencing should encourage its widespread use.

SUMMARY

SUMMARY

- Sheared Principal Component Analysis (SPCA) and Discriminant Analysis of truss landmark distance measures were performed in *Epinephelus areolatus, E. bleekeri, E. chlorostigma, E. diacanthus* and *E. longispinis.*

- *E. diacanthus* is clearly differentiated from other species based on size and shape variations.

- Using truss morphometrics, the five species of the genus *Epinephelus* can be distinguished mainly based on variations in the area encompassing the vertical, horizontal and diagonal distance measures between the second dorsal fin and anal fin.

- A minimally invasive tissue sampling technique has been standardized in grouper which ensures continued survival of these specimens after sampling.

- Arbitrary primers OPA 01, OPA 07, OPF 08 and OPF 10 generated 59 RAPD loci in the size range of 70-4500 bp in the individuals of *E. diacanthus, E. areolatus, E. chlorostigma, E. bleekeri, E. coioides, E. tauvina* and *E. malabaricus.*

- All the major RAPD loci amplified by primers OPA 01, OPA 07, OPF 08 and OPF 10 were found to be reproducible.

- Average number of genotypes per primer was 30.3 in *E. diacanthus*, 26.0 in *E. areolatus*, 21.5 in *E. chlorostigma*, 17.8 in *E. bleekeri*, 19.9 in E. coioides, 26.8 in *E. tauvina* and 30.8 in *E. malabaricus.*

- Based on genetic distance values, *E. malabaricus* was observed to be most distantly related to *E. diacanthus* and *E. bleekeri*, where as very close genetic relation was seen between *E. coioides* and *E. tauvina* and also between

E. chlorostigma and *E. bleekeri*. By and large individuals of each species formed separate clusters indicating their distinct genetic identity.

- Both intraspecies and interspecies genetic distance values were found to be highly significantly different among the seven species of groupers. Interspecies genetic distance values were significantly higher than the intraspecies values.

- Highest and lowest within species genetic diversity was found in *E. coioides* and *E. tauvina*, respectively. *E. chlorostigma* and *E. tauvina* exhibited maximum and minimum levels of polymorphism, respectively.

- All the seven species of groupers have shown at least one species-specific diagnostic marker, with maximum in *E. bleekeri* and minimum in *E. tauvina* and *E. malabaricus*.

- Truss morphometrics has proved to be a useful supportive tool to differentiate among grouper species.

- The present study has clearly demonstrated the utility of RAPD technique for ratification of taxonomic status of *Epinephelus* spp. Misidentification (based on morphological characters) made in three closely similar species was detected in the UPGMA dendrogram generated from RAPD marker data.

- It is suggested to examine more specimens of *Epinephelus tauvina*, *E. coioides*, *E. bleekeri* and *E. chlorostigma* using DNA-level markers for unambiguously establishing their species identity in Indian waters.

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