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Genetic identity of *Tor malabaricus* (Jerdon) (Teleostei : Cyprinidae) as revealed by RAPD markers

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ABSTRACT

Tor malabaricus (Jerdon) is a mahseer species endemic to the Western Ghats. Since its original description, taxonomic position of the species has been extremely confusing. In the present study, Random Amplified Polymorphic DNA (RAPD) markers were used to determine the taxonomic status of *T. malabaricus* collected from Balamore River, Tamil Nadu, India, by comparing its RAPD profile with that of *Tor khudree*. 15 random oligodecamers were used to amplify DNA from *Tor malabaricus* and *Tor khudree* (n=30 each) collected from two geographically isolated localities and a total of 119 amplicons were detected. The RAPD fingerprints generated were consistent, reproducible and yielded 22 species-specific markers (6 for *Tor malabaricus* and 16 for *Tor khudree*). The genetic distance of 0.3429 and the UPGMA dendrogram between the two species indicate that both are not the part of the same gene pool and have to be treated as two distinct species. The application of the result to the taxonomic status and conservation of *Tor malabaricus* is also discussed.

Introduction

Conservation and sustained development of natural living resources and environmental protection have been the focus of extensive scholarly attention in recent times. Approaches for setting conservation priorities are becoming a matter of concern, as the accelerating and potentially catastrophic loss of biodiversity unlike other environmental threats is irreversible. The Western Ghats of India is considered as one of the 34 global biodiversity 'hotspots' owing to its concentration of endemism (Myers *et al*., 2000; Ponniah and Gopalakrishnan, 2000). Nearly 67% of the fishes found in the Western Ghats are endemic (Shaji *et al*., 2000). The biodiversity of the Western Ghats is under threat owing to various anthropogenic activities including illegal fishing practices and deforestation, which is leading to pollution affecting the water quality. There is also the threat from continuous transportation of exotic species such as common carp, silver carp, tilapia,

Pangasius hypophthalmus and African catfish (*Clarias gariepinus*) and fishes from the Indo-Gangetic river systems such as the Indian major carps. The National Bureau of Fish Genetic Resources (NBFGR), Lucknow assessed the conservation status of 98 fish species as per latest IUCN categorisation in a Conservation Assessment Management Plan (CAMP) workshop in 1997 and reported 24% of the evaluated fish fauna is critically endangered; 39% endangered and 13% vulnerable (Ponniah and Gopalakrishnan, 2000). There has been a severe decline of mahseers, the largest freshwater cyprinids and a prime game fish of the genus *Tor* Gray throughout much of their range including the Western Ghats.

The genus *Tor* is one of the most diversified of the family Cyprinidae distributed across Asia. The taxonomic identity and phylogenetic relationships of *Tor* have been a subject of debate over decades because of the types of the morphological variations they exhibit. Hora (1939; 1942; 1943a, b) reviewed and evaluated the status of Indian species of *Tor* and has concluded that taxonomic status of some species were still ambiguous. Later, Menon (1992) attempted a taxonomic study of the genus *Tor* that has again led to many unanswered questions. At the present time, five endemic species of *Tor* have been reported from the Western Ghats: *Tor khudree* (Sykes), *Tor mussullah* (Sykes), *Tor neilli* (Day), *Tor kulkarnii* Menon and *Tor malabaricus* (Jerdon), and the nomenclatural status has been defined for the first four species (Jayaram, 1997; 1999) except for *Tor malabaricus*.

Commonly known as 'Malabar mahseer', *Barbus malabaricus* has an extremely confusing taxonomy. The species was first described by Jerdon (1848) as a new species from the mountain streams of Malabar, which Day (1878) later reported from Courtallam, Tamil Nadu. The generic name of the species was revised to *Tor* Gray (1830) by Smith (1945) and the species was considered as either a synonym of the Deccan mahseer (*Tor khudree*) by Hora (1943) and Menon (1992) or as a sub species – *Barbus* (*Tor*) *khudree malabaricus* by Mac Donald (1944), Silas (1949), Kulkarni (1978) and Sen & Jayaram (1982). The confusion confounded as the CAMP workshop held at NBFGR treated *Tor malabaricus* as a distinct species and assigned the conservation status as "critically endangered" according to latest IUCN categorization (CAMP, 1998).

The authors during a survey along the southern most part of the Western Ghats collected specimens having features approximate to *Tor malabaricus* but different from *Tor khudree*. In spite of their variation in morpho-meristics and colour pattern that could be observed in the fresh specimens of different size groups from *T. khudree*, we felt the need for carrying out a genetic evaluation to define the status of *T. malabaricus*. As in other species, taxonomic definition must be established before effective conservation measures can be applied to *T. malabaricus*.

The development of random amplified polymorphic DNA technique (RAPD) (Williams *et al,* 1990; Welsh and McClelland, 1990) has provided a useful tool for research into genetic variability. It consists of PCR amplification of small, inverted repeats scattered throughout the genome, using a single, short primer of arbitrary sequence. Thus, the genome can be scanned more randomly than with conventional techniques. The ability to examine genomic variation without previous sequence information (Williams *et al,* 1990), the relatively low cost of the technique, and the requirement of only

nanograms of template DNA, are all advantages of RAPD in genetic studies. There is now increasing evidence that the RAPD technique, which has been used in different fields, can detect nuclear variation in fish (Dinesh *et al.*, 1996; Lehmann *et al*., 2000; Callejas and Ochando, 2001; Khoo *et al*., 2002; Govindaraju and Jaysankar, 2004). These studies have shown that RAPD is an extremely sensitive method for detecting DNA variation and for establishing genetic relationships in closely related organisms. In the present work RAPD markers were used to infer the taxonomic status of *Tor malabaricus*.

The morphological definition of both *T. khudree* and *T. malabaricus* are as follows:

***Tor malabaricus* (Jerdon) (Fig. 1 a&b)**

Vernacular name: Malabar mahseer

Fin formula: D iii 9; A ii 5; P i 15; V i 8

Salient features: Body elongate, streamlined with upper profile smooth convex, lower profile slightly arched; body depth at the dorsal origin equal to length of head. Head sharp, eyes rather small, visible from underside of head. Mouth terminal, moderate; lips fleshy, smooth edged, continues in the angle of the mouth with uninterrupted lobe or groove along lower jaw; the lower lip produced into a median lobe of varying length, snout not so prolonged as in the case of Deccan mahseer. Barbels two pairs, maxillaries longer than the rostrals. Dorsal fin inserted slightly near to the caudal fin base than the tip of the snout, with its upper margin slightly concave; its last undivided ray non-osseous feeble, weak and articulated at the tip. Pectoral fins short, much shorter than head; pelvic fins shorter than pectoral not extending to the base of anal. Anal longer than pelvics, not reaching the base of caudal. Caudal fin forked. Scales large, cycloid; lateral line complete with 21 – 24 (usually 22) scales; lateral transverse scale-rows 31/2 – 21/2. Lateral sides of snout covered with a patch of small indistinct tubercles.

Colouration: Dark brown on dorsal side, becoming white on the abdomen. Fins usually brownish yellow or tinged with red, the front edge of dorsal and anal, and upper and lower borders of the caudal dark.

Maximum size: 40cm (present study 25cm).

Conservation status (IUCN): Critically endangered (CAMP,1998).

Distribution: Balamore River, Western Ghats in Kanyakumari Dist., T.N. In Kerala, Kallada River. (other rivers?).

***Tor khudree* (Sykes) (Fig. 2)**

Vernacular name: Deccan mahseer, Blue-finned mahseer, Kuyil (Malayalam)

Fig. 1a. *Tor malabaricus*

Fig. 1b. *Tor malabaricus* (Formalin preserved specimen)

Fig. 2. *Tor khudree*

Fin formula: D iv 9; A ii 5; P i 14; V i 8

Salient features: Body elongate, stream-lined with upper profile convex before dorsal fin, but slightly concave behind it, lower profile slightly arched; body depth equal to length of head. Head sharp, eyes rather small, visible from underside of head. Mouth terminal, moderate; lips fleshy, smooth edged, continues in the angle of the mouth with uninterrupted lobe or groove along lower jaw; the lower lip produced into a median lobe of varying length, it sometimes hypertrophied in specimens living in highly torrential habitats. Barbels two equal pairs; dorsal fin almost in the middle of the body with its upper margin concave; its last undivided ray osseous and modified into a strong, smooth spine. Pectoral fins short, much shorter than head; its outer ray is pointed and straight in males but bend inwards in females. Inner margin of pectoral fin almost straight in males but concave in females; pelvic fins shorter than pectoral not extending to the base of anal. Anal longer than pelvics, rounded near the tip in females, not reaching the base of caudal. Caudal fin forked. Scales large; lateral line complete with 25 – 27 scales; lateral transverse scale-rows 41/2 – 21/2. Lateral sides of snout covered with a patch of small indistinct tubercles.

Colouration: Silvery background with the back and sides above the lateral-line dark bluish, flanks below the lateral-line pale golden-yellow; the belly bluish grey; head dark olive above and creamy yellowish-white below; bases of scales grey with their margins reddish-grey; eyes red. Fins bluish-grey, often tipped with yellowish-pink. Black mahseers are also reported from Mysore and Aruvikkara at Trivandrum.

Maximum size: 100cm; 30kg

Conservation status (IUCN): Vulnerable (CAMP, 1998).

Distribution: Deccan (Mutta-Mulla River, Pune), Godavari River, Western Ghats (Kerala, Karnataka, Maharashtra). In Kerala: Kabani, Chalakkudy, Payaswini, Bavali,

TABLE 1: *Morphometric measurements exhibiting variations in* Tor khudree *and* Tor malabaricus.

Measurements	*Tor khudree* (Mean ± S.D) [From Chalakkudy River; After Shaji & Easa, 2001; n = 30]	*Tor malabaricus* (Mean ± S.D) [From Balamore River; Present study; n = 30]
% of head length in standard length	30.09 ± 1.11[a]	26.51 ± 1.36[a]
% of head length in body depth	105.45 ± 3.32[b]	92.98 ± 5.32[b]
% of snout length in head length	32.54 ± 0.47[c]	37.10 ± 2.12[c]
Length of pectoral fin in% standard length	30.58 ± 2.53[d]	21.59 ± 1.39[d]
Length of anal fin in% standard length	18.98 ± 1.05	21.19 ± 2.05

(a,b,c,d: significant at $P < 0.05$)

Karuvannur, Periyar, Pamba and Neyyar Rivers.

Materials and methods

Specimens were collected from two geographically isolated west flowing river systems of the Western Ghats. The mahseer specimens (15.0 – 28.5cm total length; n = 30) captured from Balamore River, Kanyakumari District, Tamil Nadu ($08^0$12′N; $77^0$22′E) had all the features of Jerdon's Malabar mahseer as mentioned in introduction of this paper and hence they were treated as *Tor malabaricus*. However, they could not be compared with the type specimen of Jerdon due to non-availability of the latter. *Tor khudree* samples (n = 30) were collected at Poringalkuthu, Chalakkudy River, Kerala (10^0 18'N; 76^0 36'E). The occurrence of *T. khudree* in Chalakkudy River has been reported by Menon (1992) and Jayaram (1997, 1999); and the characters of specimens of this species collected from this river in the present study matched with that of *T. khudree*, given by Sykes (1838, 1841) and later by Hora (1943a), Menon (1992) and Jayaram (1999). The blood samples of both the species, collected through caudal puncture, were fixed in 95% ethanol (1:5) and stored at 4°C.

Total DNA was extracted from the blood samples following the procedure of Ruzzante *et al*. (1996) after minor modifications. Samples were treated with SDS, Proteinase K and genomic DNA isolated using phenol: chloroform: isoamyl alcohol extraction and precipitation in ice-cold absolute ethanol. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 15 minutes. After a wash with 70% ethanol, the DNA was air-dried and re-suspended in 100µl TE buffer (10mM Tris, 1mM EDTA, pH 8.0). Concentration and purity of extracted DNA was determined spectrophotometrically at 260nm and 280nm. Samples showing the 1 OD equivalent to 50µg/ml and purity (determined by the ratio of 260 nm and 280 nm) 1.8 alone were taken for further analysis. Extracted DNA was stored at -20^0C until further analysis.

Fifty decamer primers, kits OPA 1-10, OPAA 1-5, OPAC 1-15, OPAH 1-10 and OPB 1-10 (60% G+C content; Operon Technologies Inc., Alameda, USA) were tested on five individuals each from both *Tor khudree* and *Tor malabaricus*. Thirty-seven primers out of 50 produced amplicons; however only 15 primers (Table 2) that produced repeatable, sharp and clearly stained fragments were finally selected to analyse 30 specimens each of two species.

PCR reactions were carried in a PTC 200 gradient thermal cycler (MJ Research, USA) employing the RAPD primers described in Table 2. Amplifications were performed in 25µl reactions containing 1x reaction buffer (100mMTris, 500mM KCl, 0.1% gelatin, pH 9.0), 1.5mM $MgCl_2$,6-8pmoles of primer, 200mM dNTPs, 2U *Taq* DNA polymerase and 25ng of template DNA. To check for DNA contamination, a negative control was set up omitting the DNA from the reaction mixture. The reaction mixture was pre-heated at 95^0C for 3 minutes followed by 40 cycles (94^0C for 3 minutes, 40^0C for 1.30 minutes and 72^0C for 2 minutes). The reaction was then subjected to a final extension at 72^0C for 10 minutes. The resulting products were electrophoretically analyzed through 1.5% agarose gels containing ethidium bromide (5µg/ml) in 1x TBE buffer (pH 8.0) and digitally captured using Image Master VDS. The alleles were designated according to the PCR product size in relation to the molecular marker (λDNA with *Eco*RI /

TABLE 2. *Performance of the Operon Random* Primers in Tor khudree *and* Tor malabaricus

Primer Code	Primer Sequence (5' → 3')	Molecular Weight (Da)	Total no. of bands in Tk & Tm	No. of bands in Tk (p/m)	No. of bands in Tm (p/m)	No. of species-specific bands for Tk (mw in bp)	No. of species-specific bands for Tm (mw in bp)
OPA-02	TGCCGAGCTG	3035	9	9(1/8)	8(1/7)	1(610)	0
OPA-03	AGTCAGCCAC	2988	10	9(5/4)	8(2/6)	1(1150)	1(831)
OPA-07	GAAACGGGTG	3108	7	6(0/6)	4(0/4)	3(1375,1010,590)	1(705)
OPAA-01	AGACGGCTCC	3004	9	9(5/4)	8(5/3)	1(950)	0
OPAC-05	GTTAGTGCGG	3090	8	8(3/5)	4(0/4)	0	0
OPAC-09	AGAGCGTACC	3028	5	5(0/5)	4(0/4)	1(690)	0
OPAC-11	CCTGGGTCAG	3035	8	8(3/5)	6(1/5)	1(410)	0
OPAC-12	GGCGAGTGTG	3115	10	10(1/9)	9(2/7)	3(1480,1250,550)	0
OPAC-15	TGCCGTGAGA	3059	7	5(3/2)	6(3/3)	0	1(1150)
OPAH-05	TTGCAGGCAG	3059	10	9(0/9)	10(0/10)	0	1(990)
OPAH-06	GTAAGCCCCT	2979	7	7(2/5)	5(1/4)	2(1900,1700)	0
OPAH-10	GGGATGACCA	3068	8	6(0/6)	6(1/5)	2(2015,1250)	2(860,564)
OPB-05	TGCGCCCTTC	2946	6	6(1/5)	4(0/4)	0	0
OPB-08	GTCCACACGG	3004	7	6(2/4)	7(5/2)	1(950)	0
OPB-10	CTGCTGGGAC	3035	8	7(3/4)	7(3/4)	0	0
Total			119	110(29/81)	96(24/72)	16	6

'Tk' for *Tor khudree*; 'Tm' for *Tor malabaricus*; 'p' denotes the number of polymorphic bands; 'm' denotes the number of monomorphic bands; 'mw in bp' = molecular weight in base-pairs

*Hin*d III double digest).

The RAPD-PCR technique can produce non-reproducible amplification product (Callejas and Ochando, 2001). Reactions were therefore performed following a strict protocol with standardized conditions. Also, all amplification reactions were carried out at-least thrice in order to make sure consistency and repeatability of fingerprints generated using selected RAPD primers.

For the analysis, RAPD fragments were treated as independent and unweighted characters and a binary matrix was produced whereby the presence or absence of each DNA fragment for each sample was recorded 1 or 0, respectively. Faint or poorly amplified fragments were excluded from the analysis as were very high (above 2000bp) or low (below 300bp) molecular weight. Mathematical formulae used were based on a few assumptions. First, all RAPD fragments scored were 2-allele system, *i.e.*, presence (dominant +/+ and +/-) and absence (recessive -/-) of bands. Second, fragments that migrated at the same position, had the same molecular weight, and stained to the same intensity were homologous bands from the same allele, and the alleles from different loci did not co-migrate. A third assumption is that both the populations conformed to the Hardy-Weinberg equilibrium, $p^2 + 2pq + q^2 = 1$, with frequencies p (dominant, band present) and q (recessive, band absent) (Clark and Lanigan, 1993; Lynch and Milligan, 1994). From the binary matrix, the total number of RAPD fragments, species-specific diagnostic markers and polymorphic bands were calculated for each primer and for all primers. The 'species-specific diagnostic markers' are defined as those RAPD bands that are exclusive to either *Tor khudree* or *Tor malabaricus* for a given primer.

Genetic variability within *Tor khudree* and *Tor malabaricus* and between the two species were estimated from the percentage of polymorphic RAPD loci (%P) and average gene diversity using POPGENE Version 1.31 (Yeh *et al.*, 1999). Pairs of RAPD loci were compared within each species to test for linkage disequilibrium. The %P values were calculated using the criterion for polymorphism of which, the frequency of the most common allele was <0.95. Average gene diversity index (ϕ statistics) also known as average heterozygosity (H) (Nei, 1987; Khoo *et al.*, 2002), is a measurement of genetic variation for randomly mating populations and is analogous to Wright's (1951) Fst statistics (fixation index). H is defined as the mean of heterozygosities (h) for all loci. It is given as $h = 1 - \sum_{i=1}^{m} x_i^2$, where x_i, is the population frequency of the i th allele at a particular locus and m is the number of alleles.

The genetic uniqueness of both *Tor khudree* and *Tor malabaricus* was determined with the help of four parameters such as G_{ST} (coefficient of genetic differentiation), average number of migrants per generation between the two species (Nm), pair-wise genetic similarity index (SI) and Nei's genetic distance (GD) using POPGENE Version 1.31 (Yeh *et al.*, 1999). G_{ST} represented the genetic differentiation between populations/species and Nm was calculated based on G_{ST}. (Nei and Li's 1979). Pair-wise genetic similarity or identity index (SI) among *T. khudree* and *T. malabaricus* were computed and converted by POPGENE into genetic distance (GD) according to Hillis and Moritz's (1990) formula, GD = 1- SI. Genetic similarity index is given as SI =

$2N_{AB}/(N_A+N_B)$ where N_{AB} is the number of bands shared in common by individuals of *Tor khudree* and *Tor malabaricus* and N_A and N_B, the total number of bands for *Tor khudree* and *Tor malabaricus* respectively (Nei and Li, 1979). Intraspecies mean genetic distance measures among the individuals were compared between *T. khudree* and *T. malabaricus* using analysis of variance (ANOVA). The statistical significance of the interspecies genetic distance measure between the two species was evaluated using students' t-test. Cluster analysis was performed and dendrogram plotted based on pair wise genetic distance estimated using the unweighted pair-group method with arithmetic mean (UPGMA) based on Nei (1978), modified from NEIGHBOR procedure of PHYLIP version 3.5c, using POPGENE Version 1.31 (Yeh *et al.*, 1999). To test the confidence level of each branch of UPGMA based dendrogram, the binary data matrix was bootstrapped 1000 times, using WinBoot (Yap and Nelson, 1996). Bootstrap values between 75 and 95 were considered significant and above 95 highly significant (Lehmann *et al.*, 2000).

Results and discussion

In this study, RAPD fingerprinting was used to assess the level of genetic diversity in *T. khudree* and *T. malabaricus* (Table 2; Fig.3-10). A total of 119 reliable fragments were detected using 15 Operon primers, which ranged in size from approximately 2000 to 300bp, using the standard RAPD-PCR amplification protocol followed in the laboratory at NBFGR Cochin Unit. The number of amplicons ranged from 4 (OPA – 07; OPAC – 05, 09; OPB – 05) to 10 (OPAH – 05) in *T. malabaricus* and 5 (OPAC – 09; OPAC – 15) to 10 (OPAC – 12) in *T. khudree*. On average, every primer generated 8 fragments. The amplification results were routinely repeatable even after the DNA was stored at -20^0C for more than 6 months. Several RAPD fragments showed fixed frequencies either in *T. khudree* or *T. malabaricus*. These were used as species-specific markers to distinguish both *Tor* species and could be used in other *Tor* species also. Twenty-two species-specific markers (16 diagnostic bands for *T. khudree* and 6 for *T. malabaricus*) were identified with 12 Operon primers (Table 2; Fig.3-10). These fixed differences at RAPD loci indicate that *T. khudree* and *T. malabaricus* may not be interbreeding. Likewise, shared (common) RAPD fragments found in both *Tor khudree* and *Tor malabaricus* with fixed frequencies (monomorphic) were also observed in all investigated primers, implying their genetically close relationships. In addition, 11 RAPD primers used in the study yielded polymorphic pattern in *T. khudree* and 10 in *T. malabaricus*; as a result, they have potential for use in stock identification and population differentiation analysis within two species of mahseers (Table 2). The percentage of polymorphic bands for each primer ranged from 10.0 to 71.43%. Likewise, the mean percentage of polymorphic bands was lower ranging from 6.25 to 55.56%. No RAPD loci showed significant linkage disequilibrium ($P>0.05$) in both the species. It was therefore assumed that allelic variation at RAPD loci could be considered independent.

Estimates of genetic diversity between two species (G_{ST}) and geneflow (Nm) are given in Table 4. Higher values of overall G_{ST} (0.5729) and very low values of Nm (0.3727) obtained in the present study indicate that *T. khudree*

and *T. malabaricus* have some unique genetic attributes and strong genetic differentiation. Beaumont and Hoare (2003) opined where Ne is small, allele frequencies will differ strongly between populations and F_{ST} (=G_{ST}) will be large. In the present study, high G_{ST} and very low Nm values are supported by the geographic distance (~370 km) between Balamore and Chalakkudy Rivers. The estimates of G_{ST} and Nm in the present study was also similar to those reported for highly differentiated populations/ different species of fish (Beaumont and Hoare, 2003). Average pair-wise similarity index (SI) (=genetic identity) and genetic distance (GD) values were calculated for all 15 primers together. The SI was 0.7097 between *T. khudree* and *T. malabaricus* and GD value 0.3429 (Table 5). A dendrogram was constructed from the combined data for all the primers (Fig.11). The bootstrap values suggested, the species have robust cluster. The genetic distance of 0.3429 between *T. khudree* and *T. malabaricus* is typical that of between-species and not of conspecific populations of teleosts (Avise and Aquadro, 1982; Nevo, 1978; Nevo and

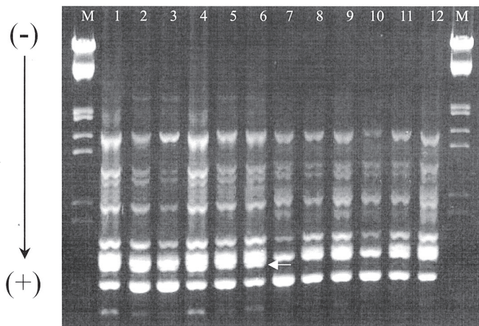

Fig.3. RAPD pattern with primer OPA– 02. Lane no. 1 to 6 – different individuals of *Tor khudree* and lane no. 7 to 12 – different individuals of *Tor malabaricus*. M – marker (λDNA with *Eco* RI / *Hind* III double digest). Arrow indicates species-specific band.

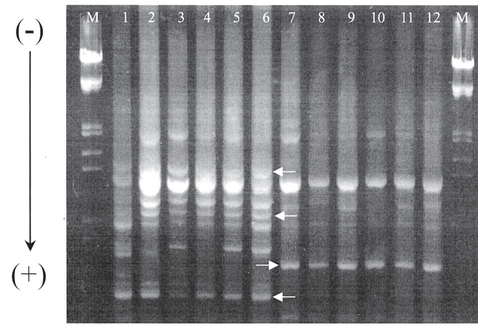

Fig. 5. RAPD pattern with primer OPA – 07. Lane no. 1 to 6 – different individuals of *Tor khudree* and lane no. 7 to 12 – different individuals of *Tor malabaricus*. M – marker (λDNA with *Eco* RI / *Hind* III double digest). Arrows indicate species-specific bands.

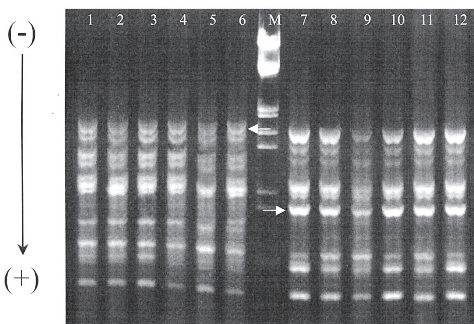

Fig.4. RAPD pattern with primer OPA –03. Lane no. 1 to 6 – different individuals of *Tor khudree* and lane no. 7 to 12 – different individuals of *Tor malabaricus*. M – marker (λDNA with *Eco* RI / *Hind* III double digest). Arrows indicate species-specific bands.

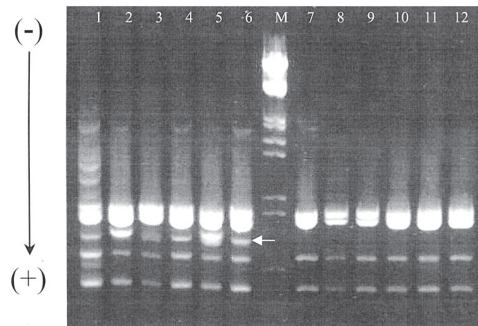

Fig. 6. RAPD pattern with primer OPAC – 09. Lane no. 1 to 6 – different individuals of *Tor khudree* and lane no. 7 to 12 – different individuals of *Tor malabaricus*. M – marker (λDNA with *Eco* RI / *Hind* III double digest). Arrow indicates species-specific band.

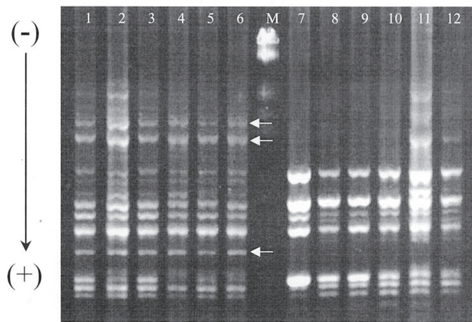

Fig.7. RAPD pattern with primer OPAC – 12. Lane no. 1 to 6 – different individuals of *Tor khudree* and lane no. 7 to 12 – different individuals of *Tor malabaricus*. M – marker (λDNA with *Eco* RI / *Hin*d III double digest). Arrows indicate species-specific bands.

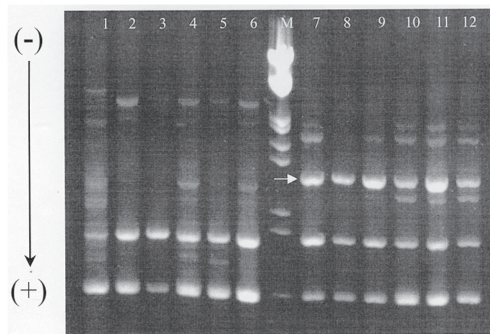

Fig.8. RAPD pattern with primer OPAC – 15. Lane no. 1 to 6 – different individuals of *Tor khudree* and lane no. 7 to 12 – different individuals of *Tor malabaricus*. M – marker (λDNA with *Eco* RI / *Hin*d III double digest). Arrow indicates species-specific band.

Cleve, 1978) and the value is in accordance with the same in other species (Menezies *et al.,* 1993; Smith *et al.*, 1996; Klinbunga *et al.*, 2000a,b; Callejas and Ochando, 2000, 2002; Govindaraju and Jayasankar, 2004).

The results demonstrate that the RAPD technique is a useful tool with great resolving power to discriminate fish species. Smith *et al.* (1996) reported 35.0%, 68.5% and 80.0% differences between king tarakihi and tarakihi; king and porae and tarakihi and porae respectively (Teleostei: Cheilodactylidae) from New Zealand waters using RAPD

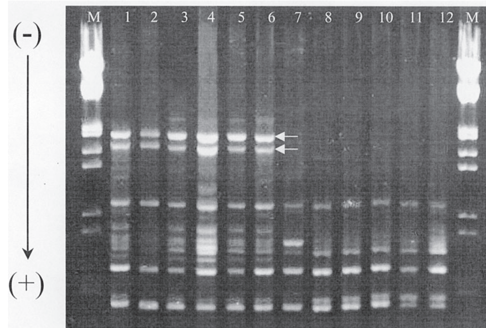

Fig.9. RAPD pattern with primer OPAH – 06. Lane no. 1 to 6 – different individuals of *Tor khudree* and lane no. 7 to 12 – different individuals of *Tor malabaricus*. M – marker (λDNA with *Eco* RI / *Hin*d III double digest). Arrows indicate species-specific

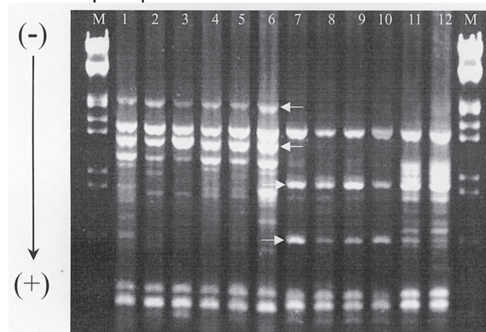

Fig.10. RAPD pattern with primer OPAH –1 0. Lane no. 1 to 6 – different individuals of *Tor khudree* and lane no. 7 to 12 – different individuals of *Tor malabaricus*. M – marker (λDNA with *Eco* RI / *Hin*d III double digest). Arrows indicate species-specific bands.

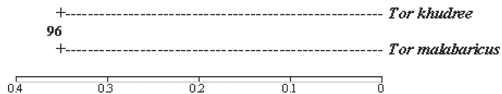

Fig. 11. UPGMA dendrogram of *Tor khudree* and *Tor malabaricus*. Dendrograms are based on genetic distance values calculated by Nei (1978) from data for all primers. Bootstrap estimate (as percentage) is indicated at left side of the branch.

TABLE 3: *Estimates of RAPD variations in* Tor khudree *and* Tor malabaricus

RAPD Primer	*Tor khudree* (Tk)		*Tor malabaricus* (Tm)		Mean Tk & Tm		Across Tk & Tm	
	%P	H	%P	H	%P	H	%P	H
OPA-02	11.11	0.0517	11.11	0.0517	11.11	0.0517	22.22	0.1072
OPA-03	50.00	0.2075	20.00	0.0952	35.00	0.1514	70.00	0.3056
OPA-07	0.00	0.0000	0.00	0.0000	0.00	0.0000	57.14	0.2857
OPAA-01	55.56	0.2691	55.56	0.2110	55.56	0.2401	88.89	0.3723
OPAC-05	37.50	0.1746	0.00	0.0000	18.75	0.0873	50.00	0.2042
OPAC-09	0.00	0.0000	0.00	0.0000	0.00	0.0000	20.00	0.1000
OPAC-11	37.50	0.1816	12.50	0.0618	25.00	0.1217	62.50	0.2366
OPAC-12	10.00	0.0334	20.00	0.0489	15.00	0.0412	40.00	0.1956
OPAC-15	42.86	0.1965	42.86	0.1994	42.86	0.1980	71.43	0.3115
OPAH-05	0.00	0.0000	0.00	0.0000	0.00	0.0000	10.0	0.0500
OPAH-06	28.57	0.1404	14.29	0.0706	21.43	0.1055	57.14	0.2638
OPAH-10	0.00	0.0000	12.50	0.0375	06.25	0.0188	62.50	0.3104
OPB-05	16.67	0.0826	0.00	0.0000	08.34	0.0413	33.33	0.1417
OPB-08	28.57	0.1298	71.43	0.2956	50.00	0.2127	71.43	0.3255
OPB-10	37.50	0.1761	37.50	0.1838	37.50	0.1800	62.50	0.2282
Mean Primers	23.72	0.1095	19.85	0.0837	21.79	0.0966	51.94	0.2292
Across Primers	24.37	0.1118	20.17	0.0843	22.27	0.0995	52.17	0.2296

The percentages of polymorphic loci (%P) and average gene diversity or heterozygosity (H) (Nei, 1987) are listed for each primer. Mean Tk & Tm = average of each primer for Tk & Tm. Across Tk & Tm = actual value for each primer across Tk & Tm. Mean primers = average for both Tk & Tm for 15 primers. Across primers = actual value for Tk & Tm across 15 primers. Sample size, n = 30 individuals each Tk & Tm.

TABLE 4: *Coefficient of genetic differentiation (G_{ST}) and rate of gene flow/migration (Nm) between* Tor khudree *and* Tor malabaricus

Primer Code	Gst	Nm
OPA-02	0.5182	0.4649
OPA-03	0.5047	0.4907
OPA-07	1.0000	0.0000
OPAA-01	0.3553	0.9072
OPAC-05	0.5724	0.3735
OPAC-09	1.0000	0.0000
OPAC-11	0.4857	0.5294
OPAC-12	0.7896	0.1333
OPAC-15	0.3644	0.8721
OPAH-05	1.0000	0.0000
OPAH-06	0.6002	0.3331
OPAH-10	0.9397	0.0321
OPB-05	0.7086	0.2057
OPB-08	0.3466	0.9424
OPB-10	0.2115	1.8645
Over all	0.5729	0.3727

TABLE 5: *Pair wise comparison of Nei's Genetic Identity / Similarity Index (above diagonal) and Genetic Distance (below diagonal) of* Tor khudree *and* Tor malabaricus *based on Nei (1978)*

Species	*Tor khudree*	*Tor malabaricus*
Tor khudree	****	0.7097
Tor malabaricus	0.3429	****

markers. Similarly, genetic distances ranging 0.425 to 0.751 were reported in three mud crab species (*Scylla serrata*, *S. oceanica* and *S. tranquebarica*) from eastern Thailand by RAPD analysis. Govindaraju and Jayasankar (2004) reported genetic distance values ranging 0.154 to 0.460 between seven grouper species from Indian waters using 4 Operon primers. Crossland *et al*. (1993) noted that, some primers indicated greater 'between-taxon' separation than other primers in a study on intertidal *Littorina* spp., but this is not surprising given the varying nature of the genome. Similar results are obtained in the present study also. Out of 15 Operon decamers, four (OPA-03, -07; OPAH-06, -10) exhibited greater differences between *T. khudree* and *T. malabaricus* compared to other primers. Klinbunga *et al*. (2000a) sequenced species-specific RAPD fragments, designed primers and developed "SCAR" (sequence–characterized amplified region) markers, specific to three species of tropical oysters. Similarly, specific "SCAR" markers can be developed for *T. khudree* and *T. malabaricus* from the specific RAPD fragments (Table 2) between the size ranges of 500 – 1000bp.

The analysis of variance (Table 6) revealed that mean genetic distances among individuals within a species did not differ significantly in both the species of mahseers (mean GD *T. khudree* 0.1116; *T. malabaricus* 0.0949; $P>0.05$). The Student's t-test (Table 7) showed that the interspecies genetic distance measures between *T. khudree* and *T. malabaricus* was highly significant (mean interspecies genetic distance = 0.3429; $P<0.001$). The interspecies GD values were significantly higher than the intra-species GD values. In theory, the intraspecies GD values are expected to be lower than interspecies GD values (Dinesh *et al*, 1996; Govindaraju & Jayasankar, 2004). The present results are in concordance with the theoretical expectation. Relatively high mean gene diversity (H) was observed in *T. khudree* (0.1095) compared to *T. malabaricus* (0.0837) with an average of 0.0966 for both the species. Similarly, the level of mean percentage polymorphism (P) was also high in *T. khudree* (23.72%)

TABLE 6: *Summary of results of one-way ANOVA to test intraspecies mean genetic distances among individuals of* Tor khudree *and* Tor malabaricus.

Source of variation	df	SS	MS	F	F value	P
Between species	1	0.0021	0.0021	2.7029	4.1960	0.1114[#]
Error	28	0.0216	0.0008			
Total	29	0.0236				

[#] $P>0.05$.

TABLE 7: Summary of results of Students' t-test for significance in interspecies genetic distance (GD) values based on RAPD markers in *Tor khudree* and *Tor malabaricus*

Mean GD between species	Standard deviation	t	t value	P
0.3429	0.0498	50.6275	2.7238	<0.001*

* Significant at P<0.001.

compared to that of *T. malabaricus* (19.85%) with an average of 21.79% among different primers. The low levels of genetic polymorphism (designated by % P & H estimates) in *T. malabaricus* could be due to small population size and high level of inbreeding in Balamore River.

The present study provides the first report on the application of RAPD markers for species identification of *Tor* species from the Western Ghats. The results of RAPD-PCR analysis demonstrated a distinct separation of gene pools of both the mahseer species, in which the two distinct clusters exhibited highly significant bootstrap values in dendrogram.

The results thus clearly support the validity of *Tor malabaricus* as a distinct management unit (MU)/ species as described by Jerdon in 1848 and it is not to be relegated as a synonym of *T. khudree*. The genetic distance between both species is comparable with those among other congeneric vertebrate species although slightly lower than some other teleosts like *Tilapia* spp. (Dinesh *et al*, 1996) and *Anguilla* spp. (Lehmann *et al.*, 2000). Reproducibility of the RAPD pattern was also tested in the present investigation at various stages of process, leading to consistent banding pattern with all amplified primers. Phylogenetic relationship of *T. malabaricus* with other mahseers can be studied using other molecular markers such as sequence information of mitochondrial DNA genes (16S / 12SrRNA) and nuclear markers such as 18S rRNA genes/ EFIα / internal transcribed spacers (ITS). This will be of great importance in devising conservation and management strategies for this endangered mahseer species. The present study has also opened up following areas for further research:

- Distribution and abundance of *Tor malabaricus* in rivers other than Balamore along the Western Ghat region.
- Biology and life history traits of the species.
- Whether both *Tor khudree* and *Tor malabaricus* co-exist in the same river system.
- Possibilities for captive breeding, river ranching and both *ex-situ* and *in- situ* conservation programmes for *Tor malabaricus*.

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