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Molecular discrimination of five Mahseer species from Indian peninsula using RAPD analysis

Vindhya MOHINDRA*, Praveen KHARE, Kuldeep K.LAL, Peyush PUNIA, Rajeev K.SINGH, Anindya Sundar BARMAN, W.S.LAKRA

National Bureau of Fish Genetic Resources (ICAR), Canal Ring Road, P.O.Dilkhusha, Lucknow-226002 (UP), India

Abstract The genetic relatedness between five species of Mahseer group (*Tor putitora*, *Tor tor*, *Tor khudree*, *Tor mosal mahanadicus* and *Neolissochilus hexagonolepis*) was examined by Random Amplified Polymorphic DNA (RAPD) analysis for the first time. Out of the sixty-nine random primers tested, eleven primers generated scoreable patterns in all the five species. The comparative RAPD profiles revealed that the combination of RAPD markers could discriminate the species, except between *T. mosal mahanadicus* and *T. putitora*, which had a similar RAPD profile. UPGMA analysis depicted three distinct clusters; one formed by *T. putitora*, *T. mosal mahanadicus* and *T. tor*, the second by *T. khudree* and the third of *Neolissochilus hexagonolepis*. The taxonomic status of *T. mosal mahanadicus* is the subject of disagreement between authors; it has been considered as a subspecies of *T. khudree* and *T. tor*. The results demonstrated that the *T. mosal mahanadicus* of the river Mahanadi is closer to *T. putitora* than to the other *Tor* species studied and reassessment of its systematic position is required [*Acta Zoologica Sinica* 53 (4): 725 – 732, 2007].

Key words Mahseer, India, RAPD markers, Molecular discrimination, Clusters

使用 RAPD 分析辨别印度半岛的五种结鱼

Vindhya MOHINDRA* Praveen KHARE Kuldeep K.LAL Peyush PUNIA Rajeev K.SINGH Anindya Sundar BARMAN W.S.LAKRA

National Bureau of Fish Genetic Resources (ICAR), Canal Ring Road, P. O. Dilkhusha, Lucknow-226002 (UP), India

摘 要 用 RAPD 分析研究了结鱼种组(黄鳍结鱼 *Tor putitora*,结鱼 *Tor tor*,库德里结鱼 *Tor khudree*, *Tor mosal mahanadicus* 和墨脱四□□ *Neolissochilus hexagonolepis*)5 个物种的遗传关系。在所测试的 69 个随机引物中,11 个引物能够在所有 5 个物种中扩增出稳定的条带。RAPD 带型显示,综合使用这些 RAPD 标记能够区分这 5 个物种,但 *Tor mosal mahanadicus* 和黄鳍结鱼享有相似的带型。UPGMA 分析揭示出 3 个独特的分支,第一支由黄鳍结鱼、*Tor mosal mahanadicus* 和结鱼组成,第二支是库德里结鱼,第三支是墨脱四□□。*Tor mosal mahanadicus* 的分类地位在不同学者间存在分歧,被认为是库德里结鱼或结鱼的亚种,但我们的结果表明,相对而言,Mahanadi 河中的 *Tor mosal mahanadicus* 与黄鳍结鱼的进化关系更近,因此有必要对其系统分类地位进行重新评估[动物学报 53 (4): 725 – 732, 2007]。

关键词 结鱼 印度 RAPD 标记物 分子辨别 聚类

The group, Mahseer, comprises medium to large sized freshwater fishes distributed in South and Southeast Asia including Indonesia, Java, Malaysia, Laos, Myanmar, Indian peninsula, Pakistan and South China (Menon, 1992; Roberts, 1999; Chen and Yang, 2004). Mahseer inhabits fast moving streams in uplands and foothill regions. Mahseers (subfamily: Cyprininae) are commercially important game as well as highly esteemed table fish; mahseers fetch a high market price and are potential candidate species for aquaculture (Ogale, 2002). Inclusion of Mahseer in polyculture and cage

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* Corresponding author. E-mail: nbfgr@sancharnet.in, kulvin100@yahoo.co.in

culture has been suggested by Tripathi (1995)*. Successful breeding of different mahseer species in captivity (Ogale, 2002; Ingram et al., 2005) is significant for aquaculture development and conservation of natural populations. In developing strategies for aquaculture and propagation assisted rehabilitation of mahseer species, there is a need to resolve taxonomic ambiguities(Nguyen et al., 2006).

The common genera of the group are *Tor*, *Neolissochilus* and *Naziritor* (*Tor cheilynoides* McClelland). The genus *Neolissochilus* was proposed to accommodate *N. hexagonolepis* (Rainboth, 1985) and genus, *Naziritor*, for *T. zhobensis* found in the Zhob River in West Pakistan (Mirza and Javed, 1985). In India, their natural distribution is across the Himalayan region from Kashmir to Northeastern states, as well as in central Indian rivers like Narmada and River Mahanadi (Sen and Jayaram, 1982; Talwar and Jhingran, 1992) and rivers of peninsular India (Jayaram, 2005). There are seven valid species reported and the diagnostic characters of the genus have been described by Desai (2003). These are *Tor putitora* (Hamilton), *Tor tor* (Hamilton), *Tor khudree* (Sykes), *Tor progeneius* (McClelland), *Tor mosal* (Sykes), *Tor mussallah* (Sykes), *Tor kulkarnii* (Menon) and two subspecies *Tor khudree malabaricus* (Jerdon) and *Tor mosal mahanadicus* (David) (Desai, 2003). The taxonomy, biology, distribution, culture potential and other prospects of mahseer of Indian subcontinent have been reviewed by Shrestha (1997) and Desai (2003).

Osteological and morphological characters, especially the proportion of head length to body depth, are the common criteria used to classify the species (Talwar and Jhingran, 1992). The plasticity in morphological characters among mahseer species has been the source of taxonomic ambiguities leading to disagreement between researchers with respect to the validity of certain species as well as subspecies. *Tor khudree malabaricus*, considered to be a subspecies of *Tor khudree* (Desai, 2003), has been found to exhibit RAPD profiles different from *T. khudree* (Silas et al., 2005). Therefore the authors considered *T. khudree malabaricus* as a separate species, *T. malabaricus* in place of subspecies. *Tor mosal mahanadicus*, reported only from River Mahanadi in the Deccan plateau (David, 1953) was also referred as *Tor khudree mahanadicus* (Menon, 1992) and *Tor tor mahanadicus* (Sugunan, 1995). In the Fish Base (Froese and Pauly, 2007) *T. mosal*, is referred as a synonym of *T. putitora*, while others consider *T. mosal* as separate species. The exploration of *Tor* genus has also yielded descriptions of new species; *T. yingjiangensis* from the River Yunnan, China (Chen and Yang, 2004); *T. tambra*, *T. sinensis* and *T. ater* from Mekong Basin, Laos (Roberts, 1999); *T. remadeviae* (River Parnbar) and *T. moyarensis* (River Moyar) from the Western Ghats, India (NATP, 2004**). Nguyen et al. (2006) have highlighted the need to address the taxonomic status of *T. douronensis* in Malaysia.

Molecular markers can be of vital importance to complement other taxonomic tools in validation of species in the genus *Tor* and to resolve ambiguities. The RAPD-PCR technique has been used for species identification and determination of phylogenetic relationships in a wide range of organisms including fishes (Callejas and Ochando, 2001; Das et al., 2005). The RAPD-PCR technique amplifies random segments of genomic DNA using a single short primer of arbitrary sequence and is suited for differentiating the nonspecific populations, particularly where the morphological characters do not permit an unambiguous identification of species (Dahle et al., 1997).

In the present study, RAPD profiles of five Indian Mahseer species were analyzed, to determine the species discriminating markers and the genetic relatedness between the species. RAPD genotype data is also evaluated to determine the status of one subspecies *Tor mosal mahanadicus* with respect to other *Tor* species.

1 Material and methods

1.1 Samples

Blood samples of five species of Mahseer i.e. *T. putitora*, *T. tor*, *T. khudree*, *T. mosal mahanadicus* and *N. hexagonolepis* were collected (Fig. 1, Table 1) and analyzed. For sample collection, distribution and diagnostic keys (Desai, 2003) were followed for localization and identification of different species.

Table 1 Details of collection sites and Sample size of different mahseer species studied

Species	Number of Sample	Collection sites	Latitude/longitude
Tor putitora (TP)	10	R. Ganga, Ajetpur.	30° 16′N/78°17′E
Tor tor (TT)	10	R. Narmada, Tawa.	22°38′N/77°28′E
Tor khudree (TK)	10	Valvan reservoir Lonawala.	18°45′N/73°24′E
Tor mosal mahanadicus (TmM)	20	R. Mahanadi, Sonapur.	20°50′N/83°56′E
Neolissochilus hexagonolepis (NeH)	10	R. Jiabharli, Balukpong.	27°28′N/94°15″E

* Tripathi SD, 1995. Summary of Proceedings of 4th Workshop on Conservation of Mahseer.

** NATP, 2004. Germplasm inventory, evaluation and gene banking of freshwater fishes. National Agricultural Technology, World Bank funded Project MM, No.27/28/98/NATP/MM-Ⅲ: 18 – 32.

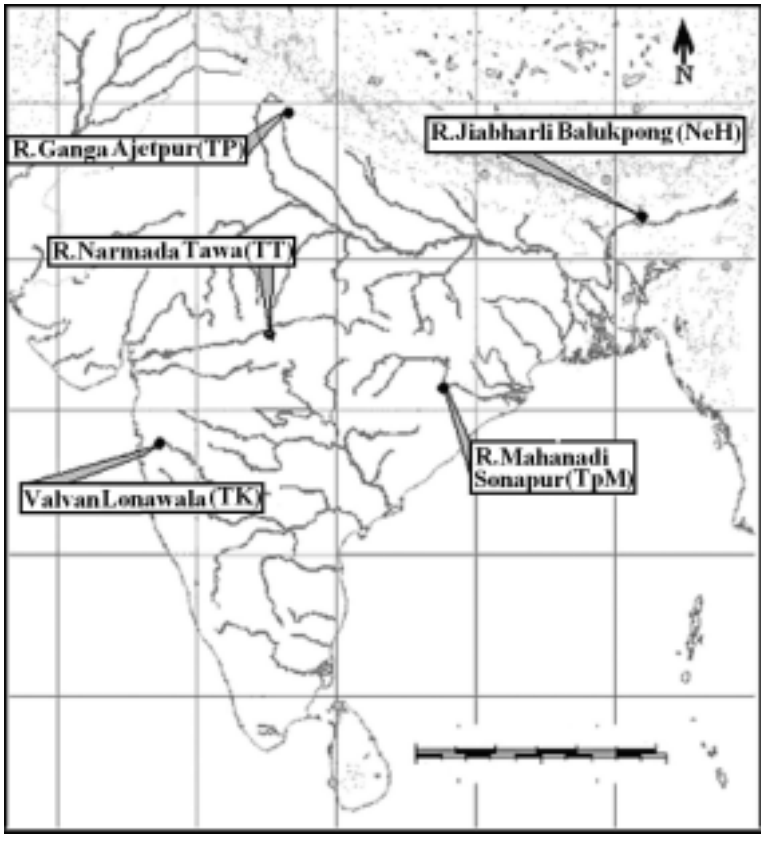

Fig.1 Map showing different collection sites for Mahseer from India

TP: *Tor putitora*. TT: *Tor tor*. TK: *Tor khudree*. TmM: *Tor mosal mahanadicus*. NeH: *Neolissochilus hexagonolepis*.

1.2 RAPD-PCR analysis

Total genomic DNA was extracted from ethanol fixed blood samples, following the procedure of Ruzzante et al. (1996), with minor modifications. A total of 69 arbitrary primers with random sequence (Operon Ltd, USA) were used to screen suitable primers for each species. PCR amplifications were performed in a thermal cycler (MJ Research, PTC-200), in a final volume of 25 μl, containing 50 ng genomic DNA, 1X PCR buffer (10 mmol/L Tris-HCl, pH 9.0; 50 mmol/L KCl; 0.01% gelatin), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, 5 pmol primer and 1.5 units *Taq* DNA polymerase. PCR reactions were carried out following a strict standardized protocol. Amplification conditions were 94℃ for 5 min followed by 40 cycles at 94℃ for 1 min, 40℃ for 1 min, and 72℃ for 2 min, with a final extension of 72℃ for 2 min. After amplification, the band patterns generated by each primer were visualized through gel electrophoresis in 1.5% agarose gels (7 × 10 cm), containing ethidium bromide and TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA), for 2 hours 30 minutes at a constant 70 V. To ensure that the amplified DNA bands originated from genomic DNA, and not primer artifacts, negative control was carried out for each set of reaction. Each reaction was repeated at least twice to confirm the reproducibility of bands and the consistent bands were taken for further analysis. Non-reproducible and generally weaker bands were not included in the analysis. Molecular weight of each band was estimated using a standard molecular marker (*EcoR* Ⅰ/digested λ DNA) with Image Master 1D Elite V3.01 (Amersham Biosciences, Hong Kong). All fragments were designated by the primer name followed by its size (bp). Each individual was scored for the presence or absence of particular amplified band. The RAPD profiles for the five species were classified according to Callejas and Ochando (2001). The monomorphic loci present in individuals of all species were termed as 'group diagnostic markers' and polymorphic as 'group exclusive' markers. The species diagnostic loci were present in all the specimens of one species and 'shared diagnostic loci' in all the specimens of two or more species but absent in the remaining species. Individual RAPD genotype data was analyzed using the software TFPGA (Ver. 1.3, Miller 1997) and MEGA (Ver. 3.1; Kumar et al., 2004). Partition of variance among population tested using AMOVA (Excoffier et al., 1992) using ARLEQUIN (ver. 2.0, Schneider et al., 2000). The shared loci, conserved among all the individuals of two or more species and absent in the remaining species, could be employed as diagnostic characters, at higher taxonomic levels (Callejas and Ochando, 2001). A UPGMA dendrogram

was constructed on basis of Nei's (1972) genetic distance, and the significance of nodes was calculated through the 1000 bootstraps repetitions.

2 Results

Out of a total of 69 primers tested, 30 primers amplified scorable band patterns and eleven primers (OPA-11, -18; OPB-5, -10, -12, -13; OPH-07, -08, -18; OPAC-15 and OPAH-06) amplified bands in all the five mahseer species used in the study (Fig.2). These primers amplified a total of 270 bands ranging from 200 to 2 000 bp, which were assigned to 80 RAPD loci (Table 2). The number of RAPD loci generated per primer per species varied between 5 and 11, with a mean of 7.2 loci per primer.

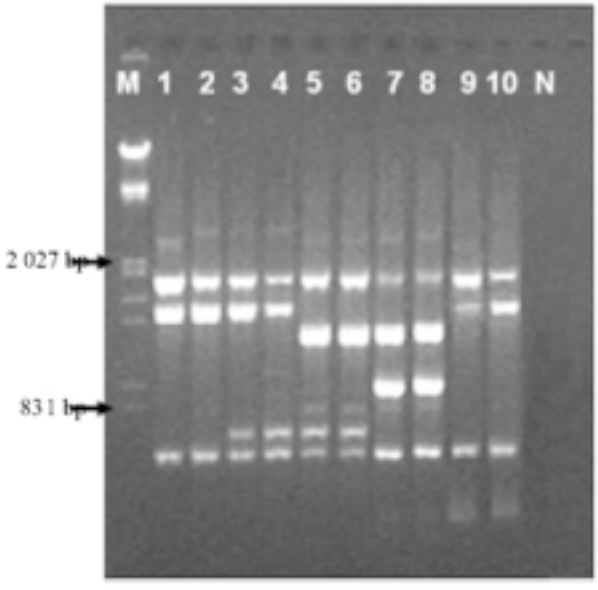

Fig.2 RAPD bands amplified by primers OPH-07 in Mahseer

M: Standard molecular weight marker, λ DNA *EcoR* Ⅰ/*Hind* Ⅲ. 1, 2: *Tor putitora*. 3, 4: *Tor tor*. 5, 6: *Tor khudree*. 7, 8: *N. hexagonolepis*. 9, 10: *Tor mahanadicus*. N: Negative

Out of a total of 80 loci, 30 were present in all five species investigated. Of these, 13 monomorphic loci were considered as 'group diagnostic markers' and 17 polymorphic as 'group exclusive' markers (Table 3). Eight genus exclusive loci for *Tor* genus were observed, which were absent in *N. hexagonolepis*, and 19 markers were present in one or more species of both the genus.

In the genus *Tor*, species diagnostic loci (present in all the individuals of only one species) were observed in *T. tor* (OPH18-646), *T. khudree* (OPA11-2503, OPAC15-2904, OPAH06-1647) and *N. hexagonolepis* (OPH07-925, OPA18-952, OPAH06-1842, OPB05-570). 'Species exclusive markers', present only in a particular species in medium or low frequencies, were detected in *T. putitora* (OPB13-1340, OPAC15-271),

Table 2 RAPD profile of the Mahseer species for 80 loci

Loci	Mahseer Species				
	TP	TT	TK	NeH	TmM
OPH 07-1851	–	–	–	+ (40)	–
OPH 07-1774	+ (70)	+	+	+ (90)	+ (79)
OPH 07-1295	+ (80)	+	+ (10)	+ (20)	+
OPH 07-1205	–	+ (20)	+	+	–
OPH 07-925	–	–	–	+	–
OPH 07-821	–	–	+ (70)	–	–
OPH 07-701	–	+	+ (80)	–	–
OPH 07-621	+	+	+	+	+
OPH 18-1269	–	–	–	+ (90)	–
OPH 18-1100	+ (80)	–	+	+ (10)	+ (92)
OPH 18-921	+ (90)	+	+	+ (70)	+ (88)
OPH 18-828	+ (90)	+	+	+ (90)	+
OPH 18-786	–	–	+ (80)	+ (90)	–
OPH 18-704	+	+	+ (90)	+	+
OPH 18-692	+ (50)	–	+ (10)	+ (60)	+ (92)
OPH 18-646	–	+	–	–	–
OPH 18-559	–	+	–	–	+ (79)
OPA 18-1836	+	+	+ (40)	–	+ (53)
OPA 18-1573	+	+	+	+	+ (53)
OPA 18-1560	–	+	+	+	–
OPA 18-1272	+	+	+	+	+
OPA 18-952	–	–	–	+	–
OPA 18-886	+	+	+ (90)	+	+
OPA 11- 3239	–	+	–	+	+ (13)
OPA 11-2503	–	–	+	–	–
OPA 11-1827	–	–	–	+ (50)	–
OPA 11-1476	+	+	+	–	+ (71)
OPA 11-1242	+	+	+	–	+ (71)
OPA 11-977	+	+	–	–	+ (67)
OPA 11-863	+	+	+ (80)	–	+ (63)
OPA 11- 638	+ (80)	–	–	–	+ (71)
OPA 11-542	+ (70)	+	+ (50)	+ (70)	+ (70)
OPA 11-512	–	–	+ (80)	+ (40)	–
OPH 08-1960	+	+ (90)	+ (20)	–	+
OPH 08-1559	+	+ (90)	+	+ (80)	+
OPH 08-1450	–	+ (10)	–	+ (70)	+ (63)
OPH 08-1061	+ (60)	+ (90)	+	+ (30)	+
OPH 08-962	+ (80)	+ (40)	–	+ (30)	+ (33)
OPH 08-741	+	+	+ (30)	+ (40)	+ (46)
OPH 08-642	+	–	+	+	+ (04)

续表（**Continued**）

Loci	Mahseer Species				
	TP	TT	TK	NeH	TmM
OPH 08-575	+ (80)	+	+ (90)	+ (30)	+
OPH 08-489	+ (20)	+ (40)	+	+ (80)	+
OPH 08-367	+	+	+	+	+
OPH 08-283	+	+	+	+	+
OPB 13-1340	+ (80)	–	–	–	–
OPB 13-1158	+ (90)	+	+ (70)	+ (80)	+
OPB 13-1051	–	–	–	+ (80)	–
OPB 13-895	+	+	+	+	+
OPB 13-675	–	–	–	+ (80)	–
OPB 13-568	+	+	+ (70)	+ (40)	+
OPAC15-2904	–	–	+	–	–
OPAC15-2407	–	–	+ (70)	+	–
OPAC15-1421	+	+	–	+ (60)	+
OPAC15-905	–	–	–	+ (70)	–
OPAC15-695	+	+	–	–	+
OPAC15-443	+	+	–	+ (10)	+
OPAC15-271	+ (30)	–	–	–	–
OPAH06-1842	–	–	–	+	–
OPAH06-1647	–	–	+	–	–
OPAH06-1316	+	+	+	–	+
OPAH06-659	–	+	+	–	+ (25)
OPAH06-600	+	–	–	+	+ (74)
OPAH06-249	+	+	+	–	+
OPAH06-185	+	+	–	–	+
OPB12-1338	+	+	+	+	+
OPB12-796	–	+ (40)	+ (50)	–	+ (75)
OPB12-532	+	+	+	+ (20)	+
OPB12-335	+ (50)	+	+	+ (50)	+ (70)
OPB12-259	+ (50)	+	+ (60)	–	+ (75)
OPB10-1152	+	+	+	+	+
OPB10-519	+	+	+	+	+
OPB10-423	–	–	–	+ (50)	–
OPB10-362	+	+	+	+	+
OPB10-295	+	+	+	+	+
OPB05-1142	+	+	+	+	+
OPB05-765	+	+	+	+	+
OPB05-570	–	–	–	+	–
OPB05-442	+	+ (60)	–	–	+ (85)
OPB05-366	–	+ (70)	–	–	+ (80)
OPB05-290	+	+	+	+	+

TP: *Tor putitora*. TT: *Tor tor*. TK: *Tor khudree*. TmM: *Tor mosal mahanadicus*. NeH: *Neolissochilus hexagonolepis*.

Presence (+) and absence (-) of loci is indicated. Values in paranthesis depict the proportion (%) of individuals that possess the genotype. Cells which do not show any value in paranthesis indicate that all the samples exhibited that genotype.

T. khudree (OPH07-821, OPH18-786) and *N. hexagonolepis* (OPH07-1851, OPH18-1269, OPA11-1827, OPB13-1051, -675, OPB10-423, OPAC15-905). The analysis of molecular variance (Table 4) of two specific groups (i.e. genus *Tor* and *Neolissochilus*) indicated that the variance among populations within groups and within populations was statistically significant ($P < 0.001$). Nei's (1972) genetic distance between pairs of species varied from 0.0944 to 0.5696 1 (Table 5) and UPGMA analysis revealed the formation of a cluster of the four species of the genus *Tor* distinct from *N. hexagonolepis* (Fig.3).

3 Discussion

This study on five species of Mahseer revealed a RAPD profile in *T. mosal mahanadicus* similar to that observed in *T. putitora* in all the eleven primers studied. Within the genus *Tor*, *T. putitora* and *T. mosal mahanadicus* were most closely related (node 1) and joined with *T. tor* (node 2). *T. khudree* formed another cluster (node 3). The bootstrap values supporting the nodes 1, 2, 3 and 4 were significant (93% to 100%). The distinct robust cluster formed by *T. mosal mahanadicus* with *T. putitora* indicated that the former is genetically closer to *Tor putitora* than the other *Tor* species studied. David (1953), quoted by Shreshta (1997), described *T. mosal mahanadicus* with characteristic head length larger than body depth as a variety of *T. mosal* which has head length equal to body depth. However, Sen and Jayram (1982) have described head length bigger than body depth as a diagnostic characteristic of *Tor putitora*. The proportion of head length to body depth is key parameter in the classification of the genus *Tor*. The meristic character described in a key (Sen and Jayaram, 1982; Desai, 2003) to the genus *Tor* also revealed a high degree of overlap between *T. putitora* [D, 12 (3/9); P.19; V.9; A.8 (3/5); C.19; L. l.25 – 28; L. tr. 31/2/31/2] and *T. mosal mahanadicus* [D, 13 (4/9); P. 17; V.9; A8. (3/5); C.19; L. 1. 25 – 27; L. tr. 31/2/31/2.]. *T. mosal* as a valid species has also been questioned and synonymies with *T. tor* (Menon, 1999) and in Fishbase it is synonymies with *T. putitora* (Froese and Pauly, 2007). The RAPD analysis and meristic similarity, clearly establish the genetic affinity of *T. mosal mahanadicus* with *T. putitora* and argue against *T. mosal mahanadicus* being a subspecies of *T. khudree* (Menon, 1992) and *T. tor* (Sugunan, 1995). However, this further raises the question, of whether the Mahanadi *Tor* is a subspecies or a differentiated genetic stock of *T. putitora*. The genetic affinity and pattern of genetic differentiation between *T. mosal mahanadicus* and *T. putitora* provide adequate evidence to recommend the re-examination of systematic position of the Mahanadi *Tor*.

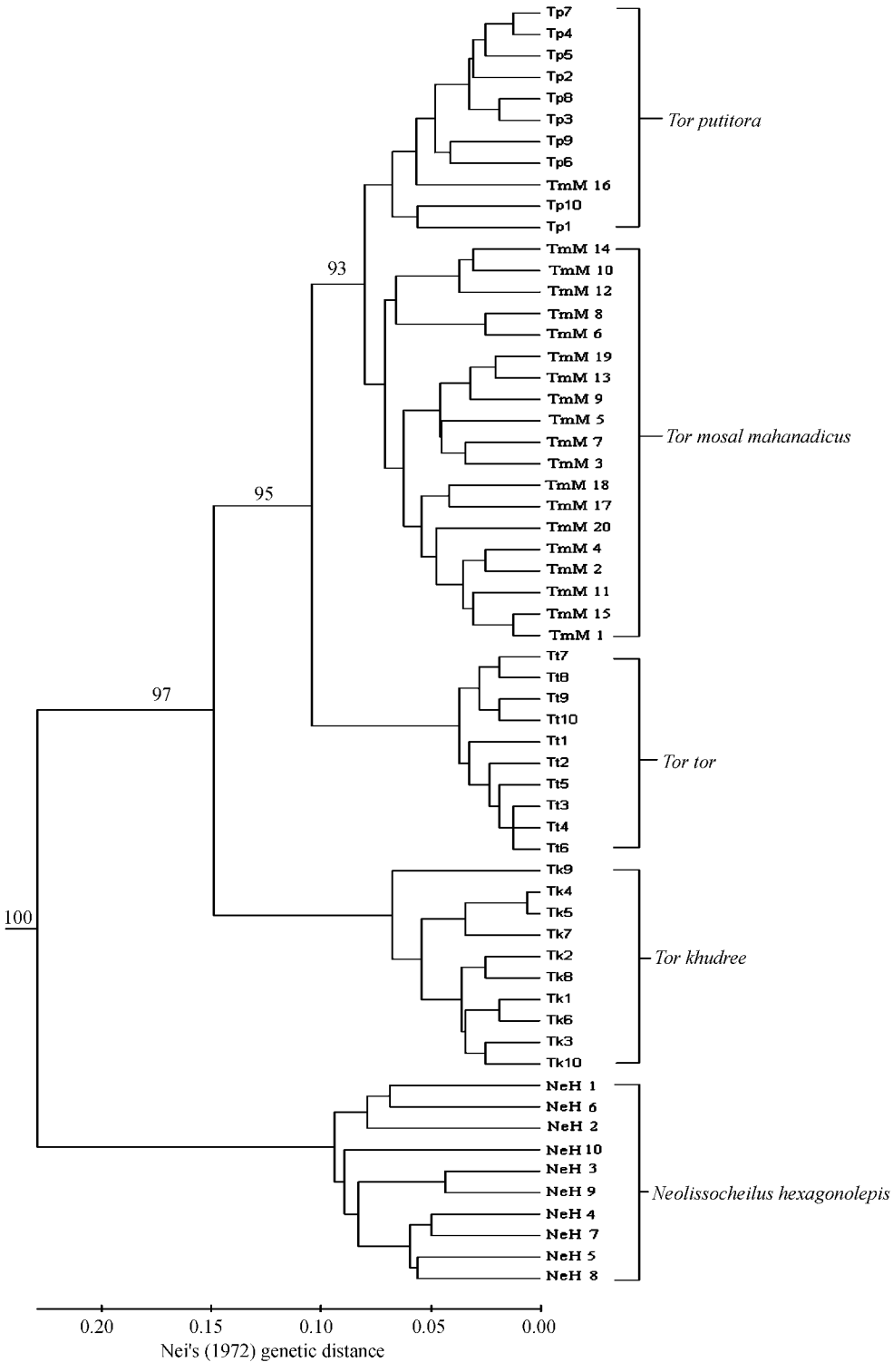

Fig.3 UPGMA dendogram (individual level) on the basis of Nei's (1972) genetic distance

Values at nodes represent proportion of similar replicates in Mahseer species (four species from genus *Tor* and one species from genus *Neolissocheilus*).

The study highlighted that the species of Mahseer studied can be identified through the combination of RAPD markers. However, inclusion of more species as well as other molecular markers will help to ascertain the time of divergence and the phylogenetic relatedness of different *Tor* species.

Table 3 Details of the different diagnostic and exclusive RAPD markers, in five mahseer species studied

Shared diagnostic	Group diagnostic	Group exclusive
OPH 07-1205 (TT, TK, NeH)	OPH07-621	OPH07-1774, -1295
OPH 07-701 (TT, TK)	OPA18-1272	OPH18-921, -828, -704
OPH 18-1100, 692 (TP, TK, NeH, TmM)	OPH08-367, -283	OPA18-1573, -886
OPH 08-642 (TP, TK, NeH, TmM)	OPB13-895	OPA11-542
OPH 18-786 (TK, NeH)	OPB12-1338	OPH08-1559, -1061, -741
OPH 18-559 (TT, TmM)	OPB10-1152, -519, -362, -295	OPH08-575, -489
OPA 18-1836 (TP, TT, TK, TmM)	OPB05-1142, -765, -290	OPB13-1158, -568
OPA 18-1560 (TT, TK, NeH)		OPB12-532, -335
OPA 18-952, OPB 13-675 (TM, NeH)		
OPA 11-3239 (TT, NeH, TmM)		
OPA 11-977 (TP, TT, TmM)		
OPA 11-512 (TK, NeH)		
OPH 08-1450 (TT, NeH, TmM)		
OPH 08-962 (TP, TT, NeH, TmM)		
OPA 11-632 (TP, TmM)		

TP: *Tor putitora*. TT: *Tor tor*. TK: *Tor khudree*. TmM: *Tor mosal mahanadicus*. NeH: *Neolissochilus hexagonolepis*.

Table 4 Partition analysis of molecular of variance (AMOVA) for five Mahseer species studied

Source of variation	Variance components	Percentage of variation	Fixation Indices
Among groups	6.21673 Va	39.86	FCT : 0.39856
Among populations within groups	5.23797* Vb	33.58	FSC : 0.55834
Within populations	4.14333* Vc	26.56	FST : 0.73437
Total	15.59803		

* $P < 0.0001$

Table 5 Nei's (1972) Genetic distance in five species of Mahseer studied

	TP	TT	TmM	TK
TT	0.1580			
TmM	0.0944	0.1593		
TK	0.2802	0.2924	0.2220	
NeH	0.4465	0.5696	0.4144	0.4210

TP: *Tor putitora*. TT: *Tor tor*. TK: *Tor khudree*. TmM: *Tor mosal mahanadicus*. NeH: *Neolissochilus hexagonolepis*.

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