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Development of PCR-based DNA markers for identification of hybrids of *Tor putitora* and *Tor khudree*

Rutuparnna Priyadarshini[1], Pavan-Kumar A.[1], Vivek Viswas Rao[2], Dhalongsaih Reang[1], [1]Gireesh-Babu P.[1] and Chaudhari A.[1]

[1] *Division of Fish Genetics and Biotechnology, ICAR-Central Institute of Fisheries Education, Mumbai*
[2] *Mahseer Fish Hatchery, Lonavala, Maharashtra*

Abstract

The genetic identification and characterization of *Tor putitota, Tor khudree* and their hybrids hold profound significance in both fundamental biological exploration and practical domains like conservation biology and fisheries management. This study endeavors to establish a panel of PCR-based DNA markers capable of accurately discerning between *T. putitora, T. khudree*, and their hybrid counterparts. Controlled conditions were employed to generate hybrids of *T. putitora* and *T. khudree*, serving to validate the developed markers. For hybrid identification, primers were devised targeting the nuclear DNA region of ITS1-5.8S-ITS2-28S rRNA. Post-optimization, the primers MHITSF and MH28SR yielded 700 bp and 500 bp amplicons for pure *Tor khudree* and *Tor putitora*, respectively. Remarkably, *Tor putitora* x *Tor khudree* hybrids displayed both 700 bp and 500 bp amplicons. These markers present a valuable tool for screening mahseer hybrids, thereby enhancing their management and conservation prospects.

Keywords:
DNA markers, *Tor putitora*, *Tor khudree*

***Corresponding author:**
pavankumar@cife.edu.in

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Introduction

India is one among the major biodiverse countries and harbours the greatest number of endemic freshwater fish in continental Asia. The country is blessed with a wide variety of freshwater fish, among which the family Cyprinidae consists of three genera, namely Tor, Neolissochilus, and Naziritor. These are often referred to as mahseer, which are endemic to South-East Asia. Due to their large size, mahseer are classified among the 20 mega fishes of the world and are often called the "tiger of the water" and the "King of Indian Aquatic systems."

In India, *Tor* species were originally described from Himalayan rivers and the Brahmaputra (Sehgal, 1971). Genetic identification and characterization of species and their hybrids play a pivotal role in both fundamental biological research and applied fields such as conservation biology and fisheries management. In this context, the genus Tor holds particular significance due to its ecological importance and economic relevance in many regions. The ability to accurately distinguish between these species and their hybrids is of utmost importance for biodiversity conservation, understanding evolutionary dynamics, and sustainable fisheries management.

Hybridization, which involves the interbreeding of genetically distinct species, can lead to novel genotypic combinations and phenotypic traits that might impact the ecological roles and adaptive potentials of the involved species. High incidence of natural hybridization has been

reported in families of Cyprinidae and Centrarchidae (Scribner *et al.*, 2000). However, hybridization can also result in the introgression of undesirable genetic traits, posing challenges for species integrity and local adaptation. The hybrids between *T. putitora* and *T. khudree* are of particular interest due to their intermediate morphological traits, which can complicate their identification using traditional methods.

To address these challenges, molecular tools such as the polymerase chain reaction (PCR) have emerged as powerful techniques for accurate species and hybrid identification in cyprinids (Pallipuram, 2021; Rehbein, 2013). By targeting specific genetic markers, PCR-based approaches can provide precise and reproducible insights into the genetic makeup of individuals, enabling the differentiation of pure species and hybrids. In this study, our aim is to develop a set of PCR-based DNA markers that can reliably distinguish between *T. putitora, T. khudree,* and their hybrids. This will contribute to a deeper understanding of their hybridization dynamics and facilitate effective conservation strategies.

Materials and methods

Five samples of adult *Tor putitora* and *Tor khudree* were collected from the Ganga River [Ramnagar (29.39N, 79.12 E), Uttarakhand], and the Periyar River [Chalakudy (10.05 N, 76.15E) Kerala], respectively. The samples were identified up to species level using the morphological keys provided by Talwar and Jhingran (1991). These samples were used as reference samples to ascertain the pure species.

Male brooders of *Tor putitora* and female brooders of *Tor khudree,* each weighing approximately 1.5-2.0 kg, were reared in separate ponds within the premises of the Mahseer fish farm located in Lonavala. These brooders were stimulated to release milt and eggs through the application of OvaprimTM, following the methodology outlined by Pandey *et al.* (1998). To collect mature eggs from the female *Tor khudree,* gentle pressure was applied to their abdomens. Subsequently, these eggs were fertilized with the milt from the male *Tor putitora,* combining the two using feathers. The resulting fertilized eggs underwent a hatching process for five days at a temperature of 20-24°C. This identical procedure was replicated to create hybrids of female *Tor putitora* and male *Tor khudree.* Following hatching, the fry were preserved in absolute alcohol, while the remaining fry were sacrificed and discorded to prevent their introduction into natural water bodies.

The total genomic DNA was isolated from the muscle tissue of the pure (muscle tissue) and hybrid (hatchling) species using Phenol-chloroform method (Sambrook and Russell, 2006). Quality and quantity of the isolated DNA was assessed using agarose gel electrophoresis on 1% agarose gel. Partial genes of mitochondrial cytochrome *c* oxidase subunit I (COI) and nuclear genes Recombination activating gene (RAG) and 18S ribosomal RNA genes were amplified using the reported primers (Ward *et al.,* 2005; Lopez *et al.,* 2004; Singh *et al.,* 2009). The PCR was performed in a 25 µl reaction volume containing 100 ng template DNA, 10 pmol of each specific primer, 200 mM of each dNTPs, 1.0 units of Taq DNA polymerase and 1xTaq buffer containing 1.5 mM $MgCl_2$.The amplification reaction was carried out in 0.2ml PCR tubes in a heated lid thermocycler. The amplicons were purified and sequenced in both the directions using PCR primers (Agrigenom, Kochi).

The COI partial gene sequences were manually assembled using Gene Runner V 3.0 software. Open reading frame of COI gene was predicted using NCBI ORF Finder tool to confirm the absence of stop codons in the sequence. Later, the sequences were subjected to BLAST (Basic Local Alignment Search Tool) analysis with NCBI database and BOLD (Barcode of Life Database) to confirm the species. Sequences were assigned to taxon based on the sequence similarity and genetic distance values. Accordingly, a threshold value of 99-100% for sequence similarity (0-1% divergence) was used to assign sequence to species level. The Neighbour-joining (NJ) trees were constructed to assess the reliability of identification including samples and reference sequences with 1000 bootstrap replications using MEGA6 (Saitou and Nei 1987, Tamura *et al.,* 2013).

For identification of hybrids, novel primers were designed for the nuclear DNA region of ITS1-5.8S-ITS2-28S rRNA. Comparative analysis of the reported sequences of Tor putitora (GU568350-54) and Tor khudree (GU568366-70) showed ~ 100 bp deletion (70 bp deletion at a stretch and sequential deletion of 15 bp each) specific to Tor putitora only (Fig. 14-15). Accordingly, novel PCR primers, MHITSF:5'CATCGACACTTTGAACG GACTTTGC-3'MH28SR: 5'-CTTACTTATATGCTTAAATTCAGCG-3 were designed to amplify this region. The PCR amplification was carried out at 94°C for 3min for initial denaturation, denaturation at 94°C for 45sec, annealing at 52°C for 30sec and extension at 72°C for 1min.

Results and discussion

The COI sequences (~650 bp) of pure species were scrutinized for the presence of insertions, deletions, and stop codons. The absence of stop codons and indels remained consistent across all amplified COI sequences, indicating that Nuclear DNA sequences originating from mitochondrial DNA sequences (NUMTs) were not sequenced. Similarly, the quality of the nuclear 18S rDNA (~1500 bp) sequence was verified by assessing the Phred score of each nucleotide. Only bases with a Q value exceeding 30 were retained in the sequence, while low-Q value end sequences were discarded. The BLAST analysis of both the gene sequences of *T. putitora* and *T. khudree* displayed a 99-100% sequence similarity with *T.*

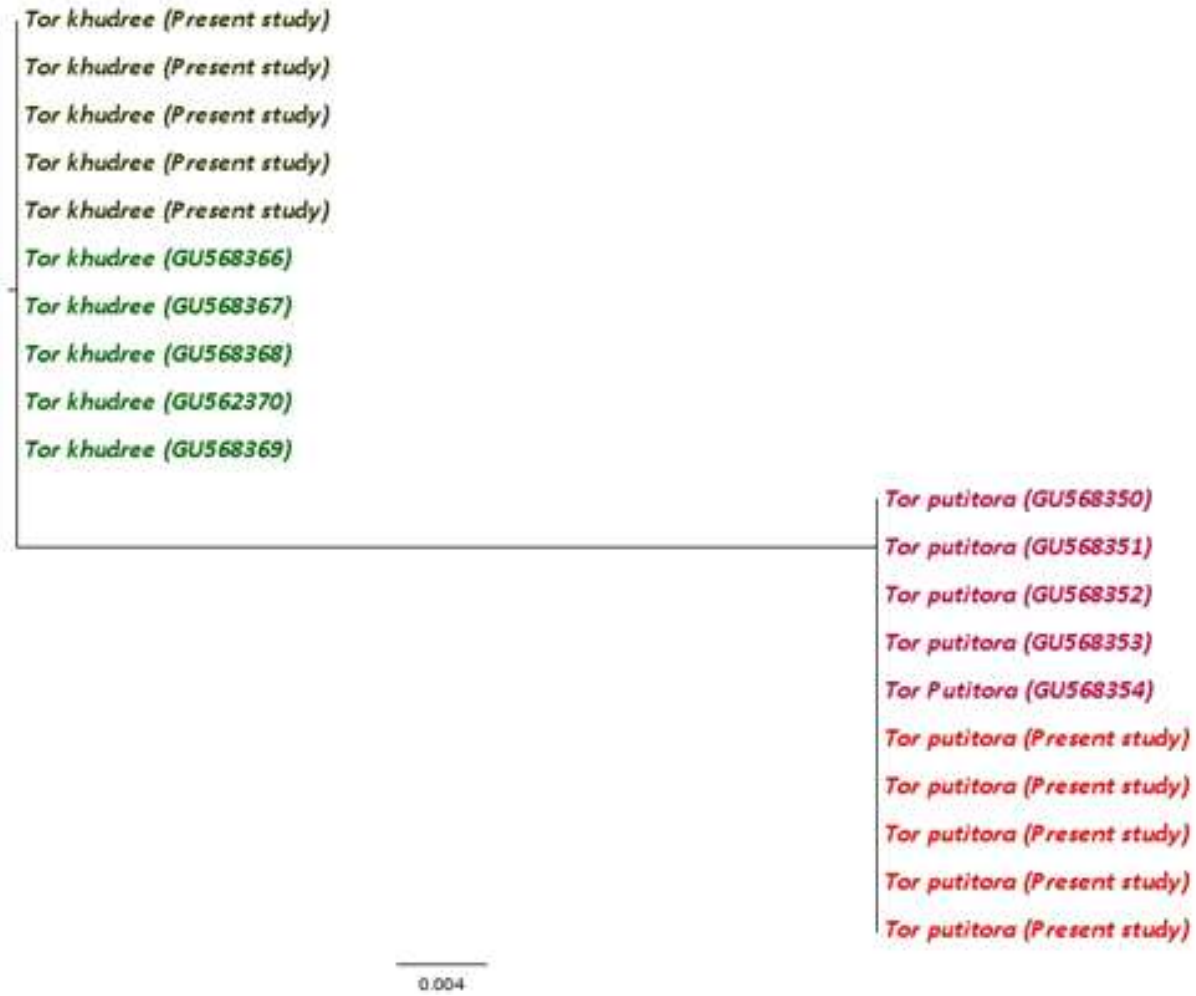

Fig. 1. Neighbour joining analysis of Nuclear ^{18}S rDNA of *Tor putitora* and *Tor khudree*

putitora and *T. khudree,* respectively. The neighbor-joining analysis using 18S rDNA exhibited distinct clustering of the *Tor putitora* and *Tor khudree* specimens from this study with their respective reported sequences (Fig. 1).

For the nuclear ITS1-5.8S-ITS2-28S region, the anticipated PCR product size for *Tor khudree* and *Tor putitora* was 600 and 500 bp, respectively. Following optimization, the primers MHITSF and MH28SR produced amplicons of 700 bp and 500 bp for pure *Tor khudree* and *Tor putitora,* respectively. Meanwhile, hybrids of T*or putitora* X T*or khudree* displayed amplicons of both 700 bp and 500 bp (Fig. 2).

Molecular markers with moderate to high selection pressure (moderate to low polymorphism), such as ribosomal RNA genes (18S rRNA) and protein-coding genes (recombination activating gene, Elongation factor), have been utilized to detect hybrids of different catfish species (Porto-Foresti et al., 2013; Hashimoto *et al.*, 2012; Vaini *et al.,* 2014). In the present study, the 18S rRNA and ITS1-5.8S-ITS2-28S ribosomal region were employed to develop molecular markers for hybrid detection. The Internal Transcribed Spacer sequence regions are situated within the rDNA gene complex of all eukaryotic cells, specifically between 18S-5.8S rDNA genes as ITS1 and 5.8S-28S rDNA genes as ITS2. Due to its rapid evolutionary rate, the non-coding ITS2 region has been harnessed as a molecular marker for species delineation (Prasad *et al*., 2009; Li *et al.,* 2010). Notably, based on conserved regions in ITS2 across eukaryotes, it has been implicated in pre-rDNA maturation (Coleman and Mai, 1997; Joseph *et al.,* 1999). However, ITS sequences exhibit less conservation, and the absence of variable repeat units in ITS2 has been identified as a contributing factor to size variation among species (Nolan and Cribb, 2005).

In the current study, a ~100 bp deletion observed in the internal transcribed spacer 2 (ITS2) sequences of Tor putitora was utilized to create markers for hybrid identification. Liang *et al*. (2016) previously reported a 70 bp deletion in the ITS2 region of Leiocassis longirostris (catfish: Bagridae) and employed it to differentiate hybrids of yellow catfish (*Tachysurus fulvidraco)* and Chinese long snout catfish (*Leiocassis longirostris*).

However, for pure *Tor khudree* species, the observed amplicon size exceeded the expected size (700 bp instead of 600 bp). The cause of this size variation needs to be validated by sequencing the two amplicons of 700 bp and 500 bp. Furthermore, additional markers, such as microsatellites and species-specific primers through RAPD, should be developed to enhance the resolution of hybrid identification.

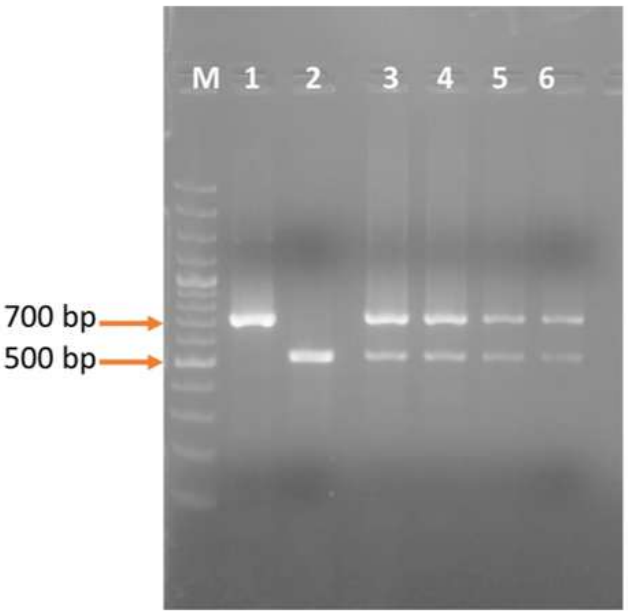

Fig.2. Identification of hybrids of *Tor putitora* X *Tor khudree* using PCR amplification of ITS1-5.8S-ITS2-28S rRNA gene. Lane M: 100bp DNA ladder; Lane 1: *Tor khudree*; Lane 2: *Tor putitora*; Lane 3-6: Hybrids of *T. putitora X T. khudree.*

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