Pakistan J. Zool., vol. 53(3), pp 1099-1109, 2021 DOI: https://dx.doi.org/10.17582/journal.pjz/20190906180937

Genetic Diversity of *Schizothorax*, *Tor*, and *Mystus* spp. in Khyber Pakhtunkhwa, Pakistan: Species of Economic Importance

Muhammad Qayash Khan[1,2], Muhammad Zubair Anjum[2], Muhammad Adnan[3], Abbas Khan[1], Hafsa Zahid[1], Javed Nawab[4], Sher Zaman Safi[5], Muhammad Ishaq Ali Shah[6], Atif Kamil[7] and Abid Ali[1]*

[1]*Department of Zoology, Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa, Pakistan*
[2]*Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Punjab, Pakistan*
[3]*Department of Zoology, University of Peshawar, Peshawar Khyber Pakhtunkhwa*
[4]*Department of Environmental Science, Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa, Pakistan*
[5]*Interdisciplinary Research Centre in Biomedical Materials (IRCBM), COMSATS Lahore, Pakistan*
[6]*Department of Chemistry, Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa, Pakistan*
[7]*Department of Biotechnology Abdul Wali Khan University Mardan.*

Article Information
Received 06 Septemebr 2019
Revised 12 December 2019
Accepted 04 March 2020
Available online 30 March 2021

Authors' Contribution
MQK, MZA and AA designed the study and experiments. MQK, MA, MZA, AK, HZ, JN, MIAS, SZS and AA, performed the experiments. MQK, HZ and AA performed the *in silico* analysis. All the authors performed critical revision and approved the final manuscript.

Key words
***Schizothorax, Tor, Mystus*, Diversity, Khyber Pakhtunkhwa, Pakistan**

ABSTRACT

Among closely related fish species the taxonomic ambiguities arose as a result of morphological similarities which not only conceal the genetic diversity but also pose problems in fisheries management and conservation practices. The purpose of the present study was a molecular characterization of fish species of economic importance belonging to the genus *Schizothorax, Tor* and *Mystus* collected from various rivers of Khyber Pakhtunkhwa, Pakistan. The samples were morphologically identified, its mitochondrial DNA was extracted and subjected to molecular characterization by amplifying and sequencing the mitochondrial cytochrome c oxidase 1 (*CO1*) gene. The sequencing data revealed rich genetic diversity among fish specimens belonging to the genus *Schizothorax.* For the genus *Tor,* as was commonly believed of having two species (*Tor putitora* and *Tor macrolepus*), the sequencing data was not in agreement with the morphological identification and hence negated the existence of *T. macrolepus*. The genus *Mystus* was also found to be comprised of a single species: *M. armatus.* The *COI* sequence of *M. armatus* reported for the first time during this study will be a useful addition to the database. The present study strongly recommends the effectiveness of *COI* barcoding for species delineation over the traditional taxonomic methods.

INTRODUCTION

Pakistan is an agricultural country where majority of the population relies on agriculture products. Agriculture plays a key role in the economy of the country which contributes about 21% to the total GDP (Azam and Shafique, 2017). The freshwater natural resources of Pakistan in the form of rivers, streams, natural lakes, and dams are valuable and have a great potential for fisheries and aquaculture practices. Generally, freshwater fish species are considered as one of the important and valuable food source across the globe. Freshwater fish fauna of Pakistan is widely distributed and is comprised of 193 fish species of which 43 fish species are endemic (Rafique and Khan, 2012; Khan *et al*., 2018). The river Indus, its tributaries and many other glacial fed streams constitute an excellent freshwater natural habitat for the survival of about 180 freshwater fish species (Mirza and Mirza, 2014).

The environmental contaminants, habitat degradation, extensive use of insecticides, pesticides and herbicides are among the serious issues for the aquatic biodiversity and its conservation in Pakistan. For instance, atrazine herbicides have significantly affected the hematological and biochemical indices of fishes (Ali *et al*., 2018).

* Corresponding author: uop_ali@yahoo.com
0030-9923/2021/0003-1099 $ 9.00/0

The importance of indigenous fish fauna cannot be negated and threats to their habitat and spawning grounds due to anthropogenic and environmental contamination is a well-documented fact. With this in mind, the documentation and accurate identification of fish species are not only required to detect market fraud but also to assist their management and conservation practices. Traditional methods of morphological identification can be misleading and erroneous (Wong and Hanner, 2008). Fish species belonging to the same genus possess remarkable morphological similarities which pose problems in their accurate identification at least for a non-experienced worker (Tahir and Akhtar, 2016).

The fishes included in sub-family Schizothoracine are commonly known as snow trout or Himalayan trout, comprising of 15 genera and over 100 species across the world (Mir *et al*., 2013). The taxonomy of snow trout has been studied extensively (McClelland, 1839; Hora, 1936; Jhingran, 1991; Kullander *et al*., 1999). The taxonomic ambiguity among species of the genus *Schizothorax* due to their remarkable morphological similarities has been well documented (Chandra *et al*., 2012; Mir *et al*., 2013) For example, *Shizothorax plagiostomus* is very similar to *Shizothorax labiatus*, both species are syntopic and are therefore usually misidentified. On the other hand, *Tor putitora* (Golden Mahaseer) is an expensive and national fish of Pakistan, which is distributed throughout South and South East Asia. A total of twenty species have been identified and recognized within this genus across Asia, with a wide distribution from Himalayan to Malaysia and Indonesia (Chen and Yang, 2004). The morphological similarities in the species belonging to the genus *Tor* have been reported and *Tor yingjiangensis* has long been misidentified as an allopatric species, *T. putitora* Hamilton, 1822 (ZiMing and JunXing, 2004). In Khyber Pakhtunkhwa (KP) Pakistan, before this study, the morphological identification of the species of the genus *Tor* led us to believe that it was comprised of two species: *T. putitora* and *Tor macrolepus*.

Species belong to the genus *Mystus* are small to medium size and are distributed throughout Asia. The distribution range of *Mystus armatus* extended to Myanmar and Bangladesh (Hora, 1931; Jayaram and Sanyal, 2003). Previous studies have confirmed the existence of 44 species belonging to the genus *Mystus* (Jayaram and Sanyal, 2003; Ferraris, 2007). Prior to this study, *M. armatus* in Pakistan was identified on the basis of morphological characters and it was one of the least studied species. Molecular characterization was therefore essential to confirm if there is any genetic diversity. In such scenarios, where morphological identification fails or is misleading, molecular approaches have been proved useful and an effective tool for exact and flawless identification at species level (Arnot *et al*., 1993; Floyd *et al*., 2002).

Molecular based approach is a powerful tool for species identification as it can be applied to organisms at any stage of their life from egg to adult or even to their remains without taking into consideration their morphological characters (Kochzius, 2009). Certain mitochondrial genes are well characterized and are the promising markers for exact identification of fish species in contrast to the nuclear genes (Hubert and Hanner, 2015). The reason being that mitochondrial DNA is only 15-20 Kb in size, present in high copy number and is in a more stable circular form. The properties of mitochondrial DNA including only 15-20 Kb in size, presenting in high copy number and in a more stable circular form not only help in efficient extraction but also eases the downstream analysis (Hubert *et al*., 2008). The application of *COI* barcoding for the identification of freshwater and marine fishes has been demonstrated and proved effectively (Wang *et al*., 2012).

The economically important fish species of the genus *Schizothorax* are abundantly found in cold and semi-cold waters while those of *Tor* and *Mystus* genera are confined to semi-cold and warm waters of Pakistan. Very little is known about their genetic diversity, therefore, the study was aimed to investigate the genetic diversity at species level in the genus *Schizothorax* captured from District Kohistan while *Tor* and *Mystus* from District Mardan, KP, Pakistan.

MATERIALS AND METHODS

A total of 540 *S. plagiostomus* were collected from different localities along river Indus in District Kohistan (35°14’60.00” N, 73°29’59.99” E) during 2016. On the other hand, 352 *T. putitora* and 180 *M. armatus* were collected from different localities along Balar Stream in District Mardan (34°34’82.50” N, 72°27’28.20” E) during 2017. Fish specimens across all localities were collected by using 5-panels of gill net 6×25 feet (mesh size 1 cm, 1.5 cm, 2 cm, 2.5 cm and, 3 cm) during the first week of each month. The latitude and longitude of the collection stations were extracted from Google Earth Pro and the site map was developed by using the ArcGIS version 10.3.1 (Fig. 1). Collected fish species were carefully identified on the basis of morphology by using the available taxonomic keys (Jayaram, 1981; Kullander *et al*., 1999). The dorsal finrays (D), pectoral finrays (P), ventral finrays (V), anal finrays (A) and lateral line scales were counted.

DNA was separately extracted from the muscle tissues of fish samples collected from each station using a genomic DNA extraction kit following the manufacturer’s instructions (Thermo Scientific GeneJET). Shortly, muscle

tissue (20 mg) were grinded gently by a sterilized mortar and pestle, placed in an Eppendorf tube, suspended in 180 µl of digestion solution followed by the addition of 20 µl of proteinase K, vortexed and incubated for three h at 56 °C. After incubation, RNase-A solution (20 µl) was added, vortexed gently and left for ten min at room temperature. Lysis solution (200 µl) was added and vortexed for a short interval till a homogenous mixture was obtained. In the following step, 400 µl of 50 % ethanol was added to the lysate, thoroughly mixed, transferred to a column containing a collection tube and centrifuged for a min at 6000 × g. The flow-through was discarded, the column was transferred to a new collection tube (2 ml) and washed with washing buffers. Finally, the genomic DNA was eluted by addition of 200 µL of elution buffer to the column, incubated for two min at room temperature and centrifuged for 1 min at 8000× g to collect the DNA. The eluted DNA was stored at -20 ºC for downstream use.

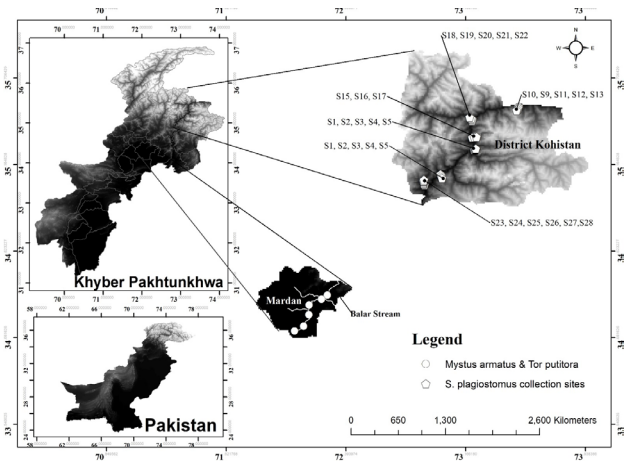

Fig. 1. Map showing different sampling sites of collected fish spp.

Nucleotide sequences for *COI* gene of *Shizothorax* spp. were downloaded from NCBI, aligned in Bio-Edit v 7.0.5 and on the basis of highly conserved regions a pair of primer (forward FP1: CTAACCACAAAGACATTGGTACCC, reverse RP1; CCTGAGTAGTGGC TACAACGTGAG) was designed for the amplification of *CO1* gene. For the amplification of *CO1* gene of *Tor* and *Mystus* species, a pair of universal primer (F1: TCAACCAACCACA AAGACATTGGCAC and R1: TAGACTTCTGGGTGGCCAAAGAATCA) were used as reported previously (Ward *et al*., 2005). Dream *Taq* Green PCR Master Mix (2X) (Thermo-Scientific) was used for amplification of the *CO1* gene. Each PCR reaction (25 µl) contained 12.5 µl Master mix (2X), forward and revese primers (1 µl each), 2 µl template DNA and 8.5 µl PCR water. The reaction mixture was thoroughly mixed and quickly transferred to a thermocycler (HT, ILF, UK). The parameters for amplification of *CO1* gene for *Schizothorax* spp. were set to initial denaturation at 95 °C for 2 min followed by 35 cycles of amplification (denaturation at 95 °C for 30 sec, annealing at 58 °C for 45 sec and extension at 72 °C for 45 sec) followed by final extension at 72 °C for 4 min. Similar parameters were followed for *Tor* and *Mystus* spp. except the annealing temperature was set to 54 °C and the final extension lasted for 10 min. The amplified target DNA was loaded parallel to 1 Kb ladder marker (Thermo-scientific) onto an agarose gel (1.5 % W/V) containing ethidium bromide, electrophoresed and visualized by a transilluminator (UVP Bio-Doc, California, USA) for confirmation of the expected product. The confirmed PCR products (640 bp, 431 bp, and 648 bp for *Schizothorax* spp., *T. putitora* and *M. armatus*) were cleaned by using a purification kit (GeneClean II from Qbiogene) following the manufacturer's protocol. The final purified products were sent to Macrogen (Korea) in order to determine its nucleotide sequences.

After sequencing, the obtained crude sequences were trimmed by using Bio-Edit v. 7 (Hall, 1999) to remove any contamination from primers. The obtained trimmed sequences were scanned in NCBI GeneBank through BLAST against non-redundant nucleotide database for homologous sequences deposited for the same gene (Altschul *et al*., 1990). The obtained sequences and homologous sequences were aligned and edited using GeneDoc v. 5.1 (Nicholas *et al*., 1997) and Bio-Edit version 7 (Hall, 1999). Aligned sequences were used to make a phylogenetic tree by using neighbor joining (NJ) method in MEGA v. X (Molecular Evolutionary Genetics Analysis) (Kumar *et al*., 2018). The bootstrap confidence intervals for each branching pattern were calculated from 1000 replicates by resampling (Tamura *et al*., 2013). The values of average sequence divergence and nucleotide composition were calculated by using MEGA v. X.

RESULTS

During this study, two areas of KP were targeted for the collection of fish species of economic importance posing taxonomic ambiguities during identification and characterization. Preliminary identification of all the collected specimens were done by using dichotomous keys and fin formula which have confirmed *Schizothorax* spp. as *S. plagiostomus* from river Indus in District Kohistan, *Tor* spp. as *T. putitora* and *T. macrolepus* and *Mystus* spp. as *M. armatus* from Balar stream in District Mardan.

Fish samples collected from river Indus were observed with elongated, silvery golden colored, heavy spots, minute

scales, rounded abdomen, mouth with inferior suctoral disc, two pairs of rostral and maxillary short barbells and blunt snout. Lateral line was complete having dorsal fin toward anterior wherase its last unbranched fin ray was serrated internally. Specific features of the fins were thoroughly recorded according to the extracted fin formula (D III, 7; P 16-17; V 10; A II, 5; C19- 20; L1 105) (Jayaram, 1981; Kullander *et al*., 1999), and the specimens were identified as *S. plagiostomus*. Specimens of *Tor* spp. collected from Balar stream were morphologically identified by applying the meristic method to count different structures in the selected organs of the fish body and to extract the fin formula (Jayaram, 1981; Kullander *et al*., 1999). On the basis of fin formula, collected specimens were identified as *T. putitora* (D IV, 8; A III, 5; P 17-18; V I 8; Ll 25-28) and *T. macrolepis* (D IV, (7-9); P15-18; V I, (7-8); A II, (5-6); Ll (24-28)) (Jayaram, 1981; Talwar and Jhingran, 1991; Mirza, 2004). Similarly, the morphological description of *M. armatus* was also made through meristic method by applying the fin formula (D II, 7; P I, 9; VI, 5; A III, 8; C 19) (Jayaram, 2010).

The amplified DNA from each specimen collected in each location was sequenced and deposited to NCBI genebank (accession numbers: *Schizothorax* spp., MK587530, MK430415, MK627707, MK559419, MK421950, MK430414, MK640501, MK645807, MK421950, MK430414, MK795688, MK883813, MT020509, MT020510, MT020511, MT020512, MT020513, MT020514, MT020515, MT020516, MT020517, MT020518, MT020519, MT020520; *T. putitora,* MN515234; *M. armatus,* MN715235). After trimming, each of the *CO1* sequences yielded an average of 640 bp sequence for *S. plagiostomus* (Table I), 431 bp sequence for *T. putitora* (Table II) and 648 bp sequence for *M. armatus* (Table III). The *COI* sequence analysis revealed the average nucleotide frequencies for *S. plagiostomus* as 28.6% T, 28.2% C, 25.7% A and 17.5% G (Table I). In case of *T. putitora* 28.91% T, 26.72% C, 27.45% A and 16.79% G (Table II), while *M. armatus* has shown a percent ratio of nucleotides as 29.78% T, 27.47% C, 26.54% A and 16.2% G (Table III). The obtained sequences and their homologous sequences, collected after BLAST, were subjected to multiple sequence alignment that showed highly conserved regions among these sequences and with those collected from NCBI GenBank deposited for same species from neighbor countries like China and India.

In the case of *Schizothorax* spp. the average sequence divergence was estimated 0.029 to 0.07 showing high sequence diversity among specimens morphologically identified as *S. plagiostomus* (Table IV). In case of *Tor* spp. all obtained sequences were found identical and were considered as a single sequence. The average sequence divergence was estimated 0.002 to 0.007 among *Tor* spp. showing highest sequence similarity with other *T. putitora* sequences (Table V). These findings negated the existence and distribution of *T. macrolepus* in KP. In case of *Mystus* spp. all obtained sequences were also identical and therefore considered as a single sequence. For *M. armatus*, the NCBI database was searched with no sequence similarities thus the present work was the first attempt using *COI* partial gene for species delineation and sequence addition to the database. The average sequence divergence of *M. armatus* with other *Mystus* spp. retrieved from NCBI was estimated as 0.003 to 0.142 (Table VI).

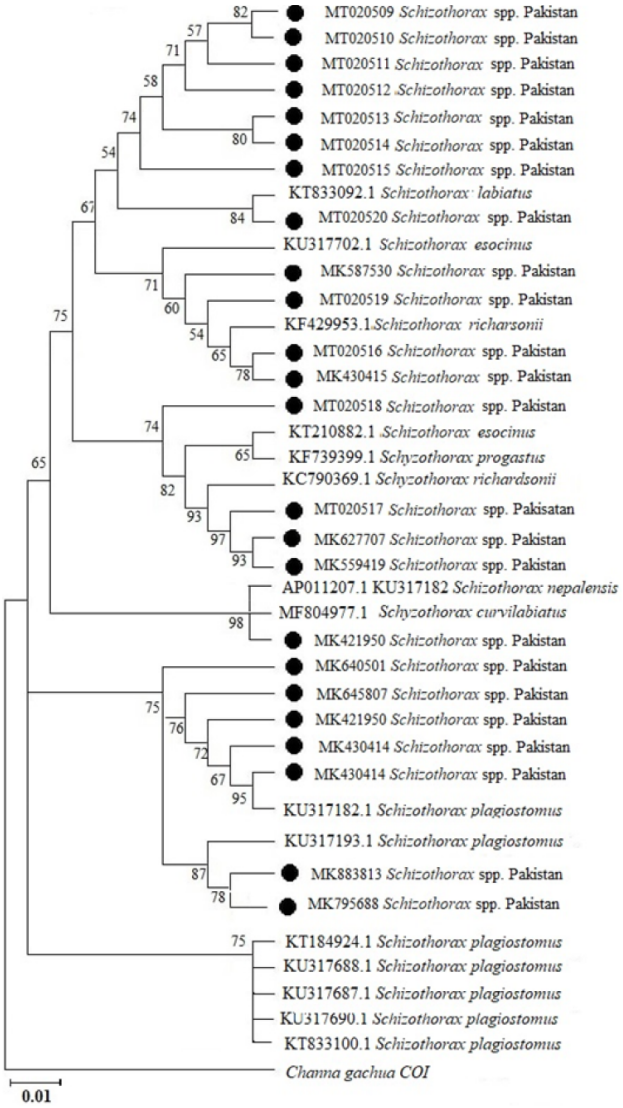

Fig. 2. Phylogenetic tree constructed using NJ method based on *COI* sequences shows the evolutionary relationship of obtained sequences from *Schizothorax* spp.

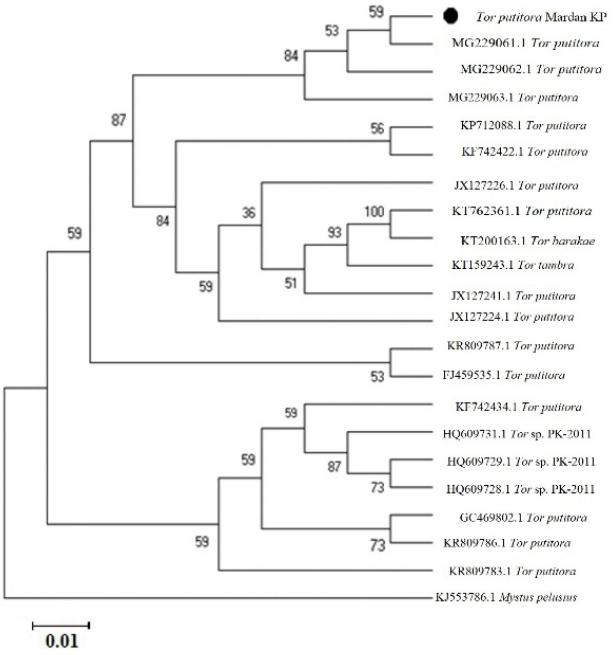

Fig. 3. Phylogenetic tree based on *COI* sequences showing the evolutionary relationship of *Tor* spp. constructed by using NJ method.

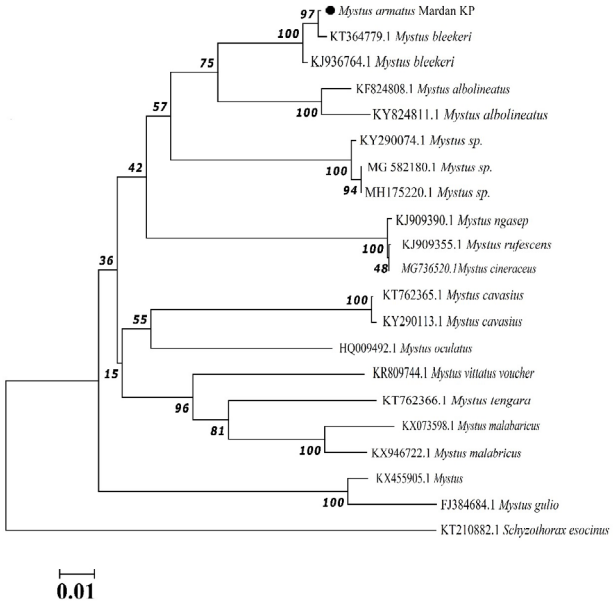

Fig. 4. Phylogenetic tree based on *COI* sequences showing the evolutionary relationship of *Mystus* spp. constructed by using NJ method.

The phylogenetic analysis was separately performed for each genus using obtained sequences in MEGA v. X. In case of *S. plagiostomus*, the *COI* sequences clustered into various clades and sub-clades belonging to different species in the genus *Schizothorax* (Fig. 2). In case of *T. putitora, COI* sequence clustered closely with *T. putitora* reported from Azad Jammu and Kashmir forming a distinct clade which distantly clustered with other *T. putitora* sequences reported from India and China (Fig. 3). In the present study, the *COI* sequence of *M. armatus* were clustered with *M. bleekari* from India. Furthermore, BLAST search has confirmed an addition of a novel *M. armatus* sequence to the NCBI database in the present study (Fig. 4).

DISCUSSION

Numerous fish species have been misidentified due to their taxonomic similarities with several other species belonging to the same genus (Chu and Chen, 1989; Shan *et al*., 2000; Hebert *et al*., 2003; ZiMing and JunXing, 2004; Hebert and Gregory, 2005). Therefore, molecular studies are essential to accurately identify these fishes at species level. Species belong to *Schizothorax* genus are mainly inhabiting Himalayan and sub-Himalayan region, *T. putitora* naturally distributed throughout the rivers (and associated reservoirs) of the South Himalayan drainage from Pakistan (Jha *et al*., 2018). The *Mystus* spp. have been reported from the south and southeast Asian countries including Bangladesh, India, Myanmar, Pakistan, Sri Lanka, Indonesia, Malaysia, Singapore, Thailand and Vietnam (Ng, 2010). The *Tor* spp. and *Mystus* spp. have been proved to be very useful for its taxonomy as well as for the investigation of its evolutionary pattern (Ahmad *et al*., 2014; Bashir *et al*., 2016). The present study is the first attempt to explore the species identification and genetic diversity of *Schizothorax* spp. inhabiting river Indus and its tributaries in District Kohistan and *T. putitora* and *M. armatus* in Balar Stream District Mardan KP, Pakistan. Collective approach using traditional keys and molecular method were adopted to explore species delineation and phylogenetic relationships.

The DNA barcoding based on *COI* divergence has been aimed to build an association between molecular and morphological taxonomists (Hebert and Gregory, 2005). The molecular characterization of *S. plagiostomus*, *M. armatus* and *T. putitora* applied during this study proved authentic and reliable for species level identification. It is noteworthy to mention that all the collected species of *Schizothorax*, *Tor*, and *Mystus* were preliminary characterized on the basis of their morphological identification as *S. plagiostomus*, *T. putitora*, *T. macrolepus* and *M. armatus*. However, the molecular characterization revealed high species diversity within the genus *Schizothorax* including *S. labiatus*, *Schizothorax nepalensis*, *Schizothorax progastus, Schizothorax curvilabiatus,*

Table I. Average composition of AT/GC of *Schizothorax* spp. sequences collected from different localities.

Collection site	COI sequences	Latitude	Longitude	T(U)	C	A	G	Total
Jalkot Nallah	S 6	35.25602	73.219306	28.7	27.8	25.9	17.7	640
Jalkot Nallah	S 21	35.25686	73.222654	28.8	27.9	25.8	17.5	640
Jalkot Nallah	S 9	35.2583	73.225873	28.8	28	25.7	17.5	640
Jalkot Nallah	S 15	35.26096	73.227611	27.9	28.7	24.1	19.4	640
Jalkot Nallah	S 2	35.26299	73.230723	28.7	28	25.7	17.6	640
Gul Bagh Nallah	S 28	35.10315	73.002975	28.8	28	25.7	17.5	640
Gul Bagh Nallah	S 22	35.09397	73.00761	28.9	28	25.6	17.5	640
Gul Bagh Nallah	S 16	35.08392	73.009177	28.8	27.9	25.7	17.6	640
Gul Bagh Nallah	S 23	35.07798	73.012803	28.6	28	25.7	17.6	640
Sazin Gah Nallah	S 20	35.5274	73.507279	28.9	27.8	25.7	17.6	640
Sazin Gah Nallah	S 1	35.5219	73.50567	27.8	30.8	25	16.3	640
Sazin Gah Nallah	S 27	35.51519	73.504618	28.9	27.9	25.9	17.3	640
Sazin Gah Nallah	S 17	35.5099	73.498653	25.4	31.5	24.1	19	640
Sazin Gah Nallah	S 14	35.50281	73.493482	28.7	27.8	25.8	17.6	640
Barseen Nallah	S 13	35.33321	73.207202	28.8	28.1	25.8	17.4	640
Barseen Nallah	S 12	35.3337	73.22145	28.9	27.8	26	17.2	640
Barseen Nallah	S 18	35.33685	73.235098	28.1	28.9	26.4	16.6	640
Kandia Nallah	S 10	35.43698	73.206497	28.7	27.9	25.8	17.7	640
Kandia Nallah	S 5	35.44264	73.208558	28.8	28.1	25.8	17.4	640
Kandia Nallah	S 26	35.44841	73.206456	28.6	28	25.9	17.5	640
Kandia Nallah	S 11	35.45229	73.200662	28.7	28.3	25.7	17.3	640
Kandia Nallah	S 8	35.45998	73.19019	28.8	28	25.9	17.3	640
Dubair Nallah	S 19	35.03751	72.898914	28.8	27.9	25.6	17.7	640
Dubair Nallah	S 3	35.04324	72.894365	29	27.8	25.8	17.4	640
Dubair Nallah	S 24	35.04877	72.894086	28.9	28	25.6	17.5	640
Dubair Nallah	S 26	35.05601	72.892563	28.7	28.3	25.7	17.3	640
Dubair Nallah	S 25	35.06114	72.893936	28.7	28	25.8	17.5	640
Dubair Nallah	S4	35.06758	72.896403	28.8	27.9	25.9	17.5	640
Average				28.6	28.2	25.7	17.5	640

Table II. Average composition of AT/GC of *T. putitora* sequences collected from different localities.

Collection Site	COI Sequence	Latitude	Longitude	T	A	C	G	Total
Rustam	*T. putitora*	34.34825	72.27282	28.91	27.45	26.72	16.79	431
	T. putitora			28.91	27.45	26.72	16.79	431
	T. putitora			28.91	27.45	26.72	16.79	431
	T. putitora			28.91	27.45	26.72	16.79	431
Chargulli	*T. putitora*	34.323102	72.235972	28.91	27.45	26.72	16.79	431
	T. putitora			28.91	27.45	26.72	16.79	431
	T. putitora			28.91	27.45	26.72	16.79	431
Bakhshali	*T. putitora*	34.28614	72.155027	28.91	27.45	26.72	16.79	431
	T. putitora			28.91	27.45	26.72	16.79	431
Bala Garhi	*T. putitora*	34.226454	72.154324	28.91	27.45	26.72	16.79	431
	T. putitora			28.91	27.45	26.72	16.79	431
Toru	*T. putitora*	34.154009	72.120519	28.91	27.45	26.72	16.79	431
	T. putitora			28.91	27.45	26.72	16.79	431
Magam Nullah	*T. putitora*	34.124292	72.062368	28.91	27.45	26.72	16.79	431
	T. putitora			28.91	27.45	26.72	16.79	431
Average	-	-	-	28.91	27.45	26.72	16.79	431

Table III. Average composition of AT/GC of *Mystus* spp. sequences collected from different localities.

Collection Site	COI Sequence	Latitude	Longitude	T	A	C	G	Total
Rustam	*M. armatus*	34.34825	72.27282	29.78	26.54	27.47	16.2	648
	M. armatus			29.78	26.54	27.47	16.2	648
Chargulli	*M. armatus*	34.323102	72.235972	29.78	26.54	27.47	16.2	648
	M. armatus			29.78	26.54	27.47	16.2	648
	M. armatus			29.78	26.54	27.47	16.2	648
	M. armatus			29.78	26.54	27.47	16.2	648
Bakhshali	*M. armatus*	34.28614	72.155027	29.78	26.54	27.47	16.2	648
	M. armatus			29.78	26.54	27.47	16.2	648
	M. armatus			29.78	26.54	27.47	16.2	648
Bala Garhi	*M. armatus*	34.226454	72.154324	29.78	26.54	27.47	16.2	648
	M. armatus			29.78	26.54	27.47	16.2	648
Toru	*M. armatus*	34.154009	72.120519	29.78	26.54	27.47	16.2	648
	M. armatus			29.78	26.54	27.47	16.2	648
Magam Nullah	*M. armatus*	34.124292	72.062368	29.78	26.54	27.47	16.2	648
	M. armatus			29.78	26.54	27.47	16.2	648
Average				29.78	26.54	27.47	16.2	648

Table IV. Average evolutionary divergence between obtained sequences and homologous sequences of *Schizothorax* spp. from NCBI.

Species	NCBI-GenBank accession No	Regions	Specimen sequences from River Indus	Average evolutionary divergence
S. plagiostomus	KU317693.1	AJK Pakistan	S 1 FWP- S28 FWP	0.03
S. plagiostomus	KT833100.1	China	S 1 FWP- S28 FWP	0.031
S. plagiostomus	KU317682.1	AJK Pakistan	S 1 FWP- S28 FWP	0.031
S. plagiostomus	KT184924.1	Dir Pakistan	S 1 FWP- S28 FWP	0.031
S. plagiostomus	KU317690.1	AJK Pakistan	S 1 FWP- S28 FWP	0.033
S. plagiostomus	KU317688.1	AJK Pakistan	S 1 FWP- S28 FWP	0.033
S. plagiostomus	KU317687.1	AJK Pakistan	S 1 FWP- S28 FWP	0.033
S. esocinus	KT210882.1	Dir Pakistan	S 1 FWP- S28 FWP	0.032
S. progastus	KF739399.1	India	S 1 FWP- S28 FWP	0.032
S. labiatus	KT833092.1	China	S 1 FWP- S28 FWP	0.032
S. esocinus	KU317702.1	AJK Pakistan	S 1 FWP- S28 FWP	0.036
S. nepalensis	AP011207.1	Nepal	S 1 FWP- S28 FWP	0.039
S. richardsonii	KU695220.1	India	S 1 FWP- S28 FWP	0.03
S. richardsonii	KF429953.1	India	S 1 FWP- S28 FWP	0.029
S. curvilabiatus	MF804977.1	China	S 1 FWP- S28 FWP	0.07
S. richardsonii	KC790369.1	India	S 1 FWP- S28 FWP	0.033

Table V. Average evolutionary divergence between obtained sequences and homologous sequences of *T. putitora* from NCBI.

Species	NCBI-GenBank accession No	Regions	Specimen Sequences from Balar Stream	Average Evolutionary Divergence
T. putitora	MG229063.1	Muzaffarabad, PK	*T. putitora*	0.002
T. putitora	MG229062.1	Muzaffarabad, PK	*T. putitora*	0.002
T. putitora	MG229061.1	Muzaffarabad, PK	*T. putitora*	0.002
T. putitora	KP712088.1	China	*T. putitora*	0.007
T. putitora	KF742434.1	India	*T. putitora*	0.002
T. putitora	KR809783.1	India	*T. putitora*	0.002
T. putitora	GQ469803.1	India	*T. putitora*	0.002
T. putitora	GQ469802.1	India	*T. putitora*	0.002
T. putitora	KR809786.1	India	*T. putitora*	0.002

Table VI. Average evolutionary divergence between obtained sequences and homologous sequences of *M. aramatus* from NCBI.

Species	NCBI-GenBank accession No	Regions	Specimen sequences from Balar Stream	Average evolutionary divergence
M. bleekeri	KJ936764.1	India	*M. armatus*	0.008
M. bleekeri	KT364779.1	Bangladesh	*M. armatus*	0.003
M. albolineatus	KF824808.1	India	*M. armatus*	0.060
M. albolineatus	KF824811.1	India	*M. armatus*	0.092
M. ngasep	KJ909390.1	India	*M. armatus*	0.130
M. cavasius	KT762365.1	Bangladesh	*M. armatus*	0.136
M. tengara	KT762366.1	Bangladesh	*M. armatus*	0.142

Schizothorax richardsonii and *Schizothorax esocinus*. In the present study, the estimated average sequence divergence confirmed the presence of *M. armatus* showing sequence similarity with *M. bleekari* reported from Bangladesh and India. This similarity with *M. bleekari* might be due to the unavailability of *M. armatus* sequences in the NCBI database. In the case of *Tor* spp. the specimen's preliminary identified as *T. putitora* and *T. macrolepus* through morphological features, however, molecular approach has confirmed their species signature as *T. putitora* only. Similar studies have been reported showing *T. macrolepis* misidentified as *T. putitora* from river Indus (Chu and Chen, 1989; Shan *et al*., 2000).

All the obtained sequences were A+T rich which is in agreement with previous reports for *S. plagiostomus* (Johns and Avise, 1998). For *S. plagiostomus* average A+T contents of obtained sequences in the present study were found as 54.2 % and 45.8 % as G+C contents (Ward *et al*., 2005; Lakra *et al*., 2011; Vineesh *et al*., 2013; Bashir *et al*., 2016). For *T. putitora* A+T contents were calculated as 56.35% and 43.5% as G+C which is accordance with previous reports (Esa *et al*., 2008). In case of *M. armatus* the A+T contents were 56.33 % and 43.67% for G+C and is in accordance (45.4 %) with the individuals of the same order (*i.e.* Siluriformes). Also, a strong correlation between the GC contents for *COI* gene and entire mitochondrial genome have been already reported (Min and Hickey, 2007).

According to the phylogenetic analysis, our obtained sequences clustered with various *Schizothorax* spp. (Fig. 2). The genetic diversity showed that the collected *Schizothorax* spp. have evolutionary relationship with *Schizothorax* spp. from India, China and Nepal (Chandra *et al*., 2012). The genetic diversity of *T. putitora* species collected from the Balar Stream showed closest genetic similarity with *T. putitora* previously reported from Azad Jammu Kashmir, Pakistan and India (Laskar *et al*., 2013; Yang *et al*., 2015) (Fig. 3). *M. armatus* collected from the Balar Stream of Mardan District showed close sequence resemblance with the species *M. blekeeri* from India and

Bangladesh (Fig. 3).

The phylogenetic analysis of the *COI* gene during the present study has confirmed various *Schizothorax* species in the study area mostly misidentified as *S. plagiostomus*. In case of *Tor* and *Mystus* spp., the collected specimens were identified as *T. putitora* and *M. armatus* respectively, by the application of DNA barcoding through the use of *COI* gene. This is in agreement to the finding of Hebert *et al*. (2003) who reported that phylogenetic analysis through *COI* barcoding is an effective tool for the identification of an organism at species level.

CONCLUSIONS

This is the first ever molecular approach for the identification of *Schizothorax* species in river Indus and *T. putitora* and *M. armatus* species in Balar stream Mardan, KP, Pakistan. To the best of our knowledge, the *M. armatus COI* gene sequence is reported for the first time during this study. This study has proved the authenticity of the molecular approach using *COI* barcoding in the identification of *Schizothorax, Tor* and *Mystus* spp. Further molecular studies are highly recommended to investigate fish species of economic importance commonly inhabiting various streams and rivers in Pakistan.

ACKNOWLEDGMENTS

The authors appreciate the financial support provided by Higher Education Commission (HEC) and Pakistan Science Foundation (PSF) for ongoing research facilities in the laboratory.

Statement of conflict of interest

The authors have declared no conflict of interests.

REFERENCES

Ahmad, S.M., Bhat, F.A., Balkhi, M.U.H. and Bhat, B.A., 2014. Mitochondrial DNA variability to explore the relationship complexity of *Schizothoracine* (Teleostei: Cyprinidae). *Genetica*, **14**: 507-516. https://doi.org/10.1007/s10709-014-9797-y

Ali, F., Khan, M.Q., Anjum, M.Z. and Khattak, I., 2018. Toxic effect of atrazine herbicide on the hematological indices of snow carp (*Schizothorax plagiostomus*): An indigenous fish species of economic importance. *Fresenius environ. Bull.*, **27**: 3075-3080.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J., 1990. Basic local alignment search tool. *J. mol. Biol*., **215**: 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Arnot, D.E., Roper, C. and Bayoumi, R.A., 1993. Digital codes from hypervariable tandemly repeated DNA sequences in the *Plasmodium falciparum* circumsporozoite gene can genetically barcode isolates. *Mol. Biochem. Parasitol*., **61**: 15-24. https://doi.org/10.1016/0166-6851(93)90154-P

Azam,. A. and Shafique, M., 2017. Agriculture in Pakistan and its impact on economy: A review. *Int. J. Advan. Sci. Technol.,* **103**: 47-60. https://doi.org/10.14257/ijast.2017.103.05

Bashir, A., Bisht, B.S., Mir, J.I., Patiyal, R.S. and Kumar, R., 2016. Morphometric variation and molecular characterization of snow trout species from Kashmir valley, India. *Mitochond. DNA Part A*., **27**: 4492-4497. https://doi.org/10.3109/19401736.2015.1101537

Chandra, S., Barat, A., Singh, M., Singh, B.K. and Matura, R., 2012. DNA bar-coding of Indian cold water fishes of genus *Schizothorax* (family: Cyprinidae) from Western Himalaya. *World J. Fish. Mar. Sci*., **4**: 430-435.

Chen, Z. and Yang. J., 2004. A new species of genus *Tor* from Yunan China (Teleostei: Cyprinidae). *Environ. Biol. Fishes*, **70**: 185-191. https://doi.org/10.1023/B:EBFI.0000029360.57048.92

Chu, X.L. and Chen, Y.R., 1989. *Yunnan Yu Lei Zhi (The fishes of Yunnan, China) Part I*. Cyprinidae., Science Press, Beijing.

Esa, Y.B., Siraj, S.S., Daud, S.K., Rahim, K.A.A., Japning, J.R.R. and Tan, S.G., 2008. Mitochondrial DNA diversity of *Tor tambroides* Valenciennes (Cyprinidae) from five natural populations in Malaysia. *Zool. Stud*., **47**: 360-367.

Ferraris, C.J., 2007. Checklist of catfishes, recent and fossil (Osteichthyes: Siluriformes), and catalogue of siluriform primary types. *Zootaxa*, **1418**: 1-628. https://doi.org/10.11646/zootaxa.1418.1.1

Floyd, R., Abebe, E., Papert, A. and Blaxter, M., 2002. Molecular barcodes for soil nematode identification. *Mol. Ecol*., **11**: 839-850. https://doi.org/10.1046/j.1365-294X.2002.01485.x

Hall, T.A., 1999. *Bioedit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt.* Paper presented at the nucleic acids symposium series. **41**: 95-98.

Hebert, P.D., Cywinska, A. and Ball, S.L., 2003. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B. Biol. Sci.,* **270**: 313-321. https://doi.org/10.1098/rspb.2002.2218

Hebert, P.D. and Gregory, T.R., 2005. The promise of DNA barcoding for taxonomy. *Syst. Biol*., **54**: 852-

859. https://doi.org/10.1080/10635150500354886
Hora, S.L., 1931. On two small collection of fishes from Burma. *Rec. Indian Mus*., **33**: 1-2.
Hora, S.L., 1936. On a further collection of fish from Naga Hills. *Rec. Indian Mus*., **38**: 317-331.
Hubert, N. and Hanner, R., 2015. DNA barcoding, species delineation and taxonomy: A historical perspective. *DNA Barcodes*, **3**: 44-58. https://doi.org/10.1515/dna-2015-0006
Hubert, N., Hanner, R., Holm, E., Mandrak, N.E., Taylor, E., Burridge, M., Watkinson, D., Dumont, P., Curry, A., Bentzen, P. and Zhang, J., 2008. Identifying Canadian freshwater fishes through DNA barcodes. *PLoS One*, **3**: 1-8. https://doi.org/10.1371/journal.pone.0002490
Jayaram, K.C., 2010. *The freshwater fishes of the Indian region*. Narendra Publication Company., New Delhi, pp. 616.
Jayaram, K.C., 1981. *Freshwater fishes of India, Pakistan, Bangladesh, Burma and Sri Lanka*. Zoological Survey of India.
Jayaram, K.C. and Sanyal, A., 2003. A taxonomic revision of the fishes of the genus *Mystus scopoli* (family: Bagridae): Records of the zoological survey of India. *Zool. Surv. India*, **207**: 1-136.
Jha, B.R., Rayamajhi, A., Dahanukar, N., Harrison, A. and Pinder, A.C., 2018. *Tor putitora*. The IUCN Red List of Threatened Species, 2018.
Jhingran, V.G., 1991. *Fish and Fisheries of India*. Hindustan Publ. Co., New Delhi. pp. 727.
Johns, G.C. and Avise, J.C., 1998. Tests for ancient species flocks based on molecular phylogenetic appraisals of sebastes rockfishes and other marine fishes. *Evolution*, **52**: 1135-1146. https://doi.org/10.1111/j.1558-5646.1998.tb01840.x
Khan, M.Q., Anjum, M.Z., Akhter, S., Khattak, I. and Ali, A., 2018. Diet composition and seasonal fluctuations in the feeding habit of snow barbel (*Schizothorax plagiostomus*) in River Indus, Pakistan. *Acta Sci. Vet.*, **46**: 1-8. https://doi.org/10.22456/1679-9216.82439
Kochzius, M., 2009. *The future of fisheries science in North America: Trends in fishery genetics. Springer*, **31**: 453-493. https://doi.org/10.1007/978-1-4020-9210-7_24
Kullander, S.O., Fang, F., Delling, B. and Ahlander, E., 1999. *The fishes of the Kashmir Valley, River Jhelum, Kashmir Valley: Impacts on the aquatic environment.* Swedmar Göteborg, pp. 99-167.
Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol*., **35**: 1547-1549. https://doi.org/10.1093/molbev/msy096
Lakra, W.S., Verma, M.S., Goswami, M., Lal, K.K., Mohindra, V., Punia, P., Gopalakrishnan, A., Singh, K.V., Ward, R.D. and Hebert, P., 2011. DNA barcoding Indian marine fishes. *Mol. Ecol. Resour.*, **11**: 60-71. https://doi.org/10.1111/j.1755-0998.2010.02894.x
Laskar, B.A., Bhattacharjee, M.J., Dhar, B., Mahadani, P., Kundu, S. and Ghosh, S.K., 2013. The species dilemma of northeast Indian mahseer (Actinopterygii: Cyprinidae): DNA barcoding in clarifying the riddle. *PLoS One*, **8**: 1-11. https://doi.org/10.1371/journal.pone.0053704
Mcclelland, J., 1839. Indian cyprinidae. *Asiat Res*., **19**: 217-471.
McCusker, M.R., Denti, D., Van Guelpen, L., Kenchington, E. and Bentzen, P., 2013. Barcoding Atlantic Canada's commonly encountered marine fishes. *Mol. Ecol. Resour.,* **13**: 177-188. https://doi.org/10.1111/1755-0998.12043
Min, X.J. and Hickey, D.A., 2007. DNA barcodes provide a quick preview of mitochondrial genome composition. *PLoS One,* **2**: 325. https://doi.org/10.1371/journal.pone.0000325
Mir, F.A., Mir, J.I. and Chandra, S., 2013. Phenotypic variation in the snow trout *Schizothorax richardsonii* (Gray, 1832) (Actinopterygii: Cypriniformes: Cyprinidae) from the Indian Himalayas. *Contrib. Zool*., **82**: 115-122. https://doi.org/10.1163/18759866-08203001
Mirza, M.R. and Mirza, Z.S., 2014. Longitudinal zonation in the fish fauna of the Indus River in Pakistan. *Biologia*, **60**: 149-152.
Mirza, M.R., 2004. *Freshwater fishes in Pakistan, 2nd edition*. Urdu Science Board, Lahore, Pakistan.
Tahir, M. and Akhtar, S., 2016. Services of DNA barcoding in different fields. *Mitochond. DNA*, **27**: 4463-4474. https://doi.org/10.3109/19401736.2015.1089572
Ng, H.H., 2010. *Wallago attu*. The IUCN Red List of Threatened Species, version 2014. pp. 3.
Nicholas, K.B., Nicholas, H.B. and Deerfield, D.W., 1997. GeneDoc: Analysis and visualization of genetic variation. *EMBnet News*, **4**: 14.
Rafique, M. and Khan, N.U.H., 2012. Distribution and status of significant freshwater fishes of Pakistan. *Rec. Zool. Surv. Pakistan*, **21**: 90-95.
Shan, X.H., Lin, R.D., Yue, P.Q. and Chu, X.L., 2000. Barbinae. In: *Fauna Sinica, Osteichthyes, Cypriniformes III* (ed. P.Q. Yue). Science Press, Beijing, China. pp. 3-170.

Talwar, P.K. and Jhingran, A.G., 1991. *Inland fisheries of India and adjacent countries*. Vol. I & II, pp. 1-1158.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. MEGA 6 molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol*., **30**: 2725-2729. https://doi.org/10.1093/molbev/mst197

Vineesh, N., Mohitha, C., Bineesh, K.K., Kumar, R.G., Gopalakrishnan, A. and Basheer, V.S., 2013. DNA barcoding and taxonomic notes on the genus *Macolor* (Perciformes: Lutjanidae) from Indian waters. *J. Ocean Sci. Found,*., **13**: 16-26.

Wang, Z.D., Guo, Y.S., Liu, X.M., Fan, Y.B. and Liu, C.W., 2012. DNA barcoding South China Sea fishes. *Mitochondrial DNA*., **23**: 405-410. https://doi.org/10.3109/19401736.2012.710204

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R. and Hebert, P.D., 2005. DNA barcoding Australia's fish species. *Philos. T. R. Soc. B.,* **360**: 1847-1857. https://doi.org/10.1098/rstb.2005.1716

Wong, E.H.K. and Hanner, R.H., 2008. DNA barcoding detects market substitution in North American seafood. *Fd. Res. Int*., **41**: 828-837. https://doi.org/10.1016/j.foodres.2008.07.005

Yang, L., Sado, T., Hirt, M.V., Pasco-Viel, E., Arunachalam, M., Li, J., Wang, X., Freyhof, J., Saitoh, K., Simons, A.M. and Miya, M., 2015. Phylogeny and polyploidy: Resolving the classification of cyprinine fishes (Teleostei: Cypriniformes). *Mol. Phylog. Evol.,* **85**: 97-116. https://doi.org/10.1016/j.ympev.2015.01.014

Ziming, C. and Junxing, Y., 2004. A new species of the genus *Tor* from Yunnan, China (Teleostei: Cyprinidae). *Environ. Biol. Fish*, **70**: 185-191. https://doi.org/10.1023/B:EBFI.0000029360.57048.92