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Research Article

Molecular Identification and Genetic Diversity of *Notopterus notopterus* (Pallas, 1769) Using Mitochondrial COI Gene and RAPD Markers in Riverine Water of Pakistan

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WA collected samples, conducted experiments, data analysis and typing. MN revised the manuscript. WA designed experiment and wrote the article.

Keywords
DNA barcoding, Molecular identification, Genetic divergence, Mitochondrial COI, RAPD markers, *Notopterus notopterus*

Abstract | Identification and genetic diversity is important to explore biodiversity and conservation of species. Here we identified a commercially important fish *Notopterus notopterus* and analysed its genetic diversity. For the purpose of identification 15 specimens of *N. notopterus* were collected from Satluj, Ravi, Indus, Jehlum and Chenab rivers of Pakistan and barcoded with mitochondrial COI gene. BLAST analysis confirmed 100% identity of *Notopterus notopterus* with reference sequences belonging to NCBI GenBank database. Genetic diversity was analysed by using PCR amplification of 75 specimens of *N. notopterus* with five RAPD markers. RAPD markers data indicated high genetic diversity 0.5832 in Satluj while lowest genetic diversity 0.2173 in Chenab. High genetic polymorphism 60.52% observed in Satluj while lowest genetic polymorphism 23.07% observed in Chenab. Shannon's Information Index (I) found 0.6281 in Satluj while 0.3162 in Chenab. Nei's genetic diversity (h) among five riverine population was observed high in Satluj 0.5832 while lowest in Chenab 0.2173 which reflecting high genetic diversity in Satluj while lowest genetic diversity in Chenab. UPGMA dendrogram revealed that Indus and Jehlum riverine populations, and Satluj and Ravi populations of *N. notopterus* has close genetic similarity. Maximum likelihood tree revealed that *N. notopterus* identified from Pakistan has close genetic link with *N. notopterus* reported from Indo-Myanmar and India. Present study successfully reported molecular identification and genetic diversity of *N. notopterus*. Moreover, low genetic diversity reported in Chenab is matter of concern for fish resource managers and need to take constructive steps for its conservation.

Novelty Statement | DNA barcode base molecular identification and genetic diversity of *N. notopterus* evaluated from Satluj, Chenab, Jhelum, Ravi and Indus River of Pakistan through mitochondrial COI gene and RAPD markers reported first time from Pakistan. Low genetic diversity reported in Chenab River matter of concern for fish resource managers and need to take constructive steps for its conservation.

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Introduction

Fish identification is critically important for conservation and management of fisheries. DNA barcode base molecular identification is an efficient approach of species identification (Suresh *et al.*, 2022; Sajjad *et al.*, 2023) as compare to traditional identification method. Traditional identification methods are less effective and produce ambiguities in identification of morphologically similar species (Naz *et al.*, 2023). Mitochondrial COI gene particularly used for identification of species due to its high mutation rate and nucleotide variability. Mitochondrial

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COI gene is maternally inherited and quick enough to distinguish closely related morphologically similar fish species (Habib *et al*., 2021).

Genetic diversity data of natural populations of fish is essential to observe the genotypic variation of species. Genetic drift, mutation and migration are main causes of genetic variations in fishes (Zhao *et al*., 2011). Different methods of biotechnology used to analyse genetic diversity in freshwater fish populations (Yousefian *et al*., 2011). DNA markers universally used for evaluation of genetic diversity of freshwater fishes. Randomly Amplified Polymorphic DNA (RAPD) markers significantly used for analysis of genetic diversity (Popoola *et al*., 2014). The proper management is necessary to maintain genetic stock of fish in freshwater reservoirs which achieved by analysing genetic diversity (Zhao *et al*., 2011; Carlson *et al*., 2015).

Notopterus notopterus (Pallas, 1769) belongs to family Notopteridae found in Pakistan, Bangladesh, India and Indo-Myanmar. *N. notopterus* is a commercially important fish used in both fresh and dried forms for human consumption in Asia (Abbas *et al*., 2013). In Pakistan, the population of *N. notopterus* is declining quickly due to uncontrolled fishing, habitat destruction and water pollution (Abbas *et al*., 2013). However, genetic study on *N. notopterus* is crucial for its conservation.

According to best of our information, DNA barcode base molecular identification and genetic diversity of *N. notopterus* was not studied yet from Pakistan. Therefore, to fill this gap, we identify *Notopterus notopterus* fish and evaluated its genetic diversity from five main rivers of Pakistan. Moreover, morphological and meristic characteristics of *N. notopterus* were also studied in present study.

Materials and Methods

Collection of specimens

A total of 90 specimen of *Notopterus notopterus* fish were collected during the year 2020-2021 from Chenab, Indus, Jhelum, Ravi and Satluj rivers of Punjab, Pakistan (Figure 1). Among 90 collected specimens, *N. notopterus* 15 specimens used for molecular identification while other 75 *N. notopterus* specimens used for genetic diversity analysis. Sampling location and river wise number of specimen collection provided in Table 1.

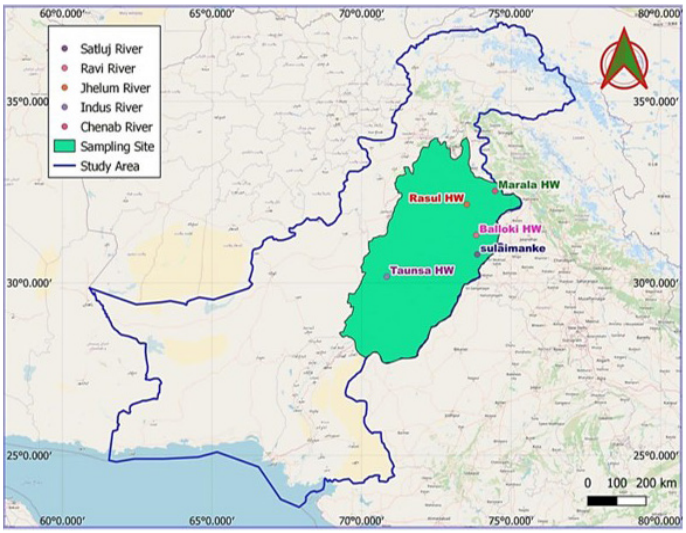

Figure 1: Sampling site and study area of *Notopterus notopterus* fish of Pakistan.

The collected fish euthanized (kill) by immersing in concentrated Tricaine methane sulfonate (MS-222) 250 mg/L solution for 30 min (Popovic *et al*., 2012). After exposure, fish lose consciousness and stop breathing. Then fish specimens stored at -4°C for further studies.

Then specimens were analysed in the Laboratory of Fisheries, Institute of Zoology, Bahauddin Zakariya University Multan, Pakistan.

Morphometric study

Morphometric and meristic characters of collected specimens accomplished with the standard keys used for *N. notopterus* by Talwar and Jhingran (1991). Total body length, stranded length, head length, fins length and meristic features measured and counted followed Rahman (1989) and Galib *et al*. (2009).

Table 1: Detail of *N. notopterus* specimen collection.

***N. notopterus* specimens collection detail**				
Population	**Specimen collection point**	**Longitude/ Latitude**	**Specimens used for identification using mitochondrial COI**	**Specimens used for RAPD diversity**
River Chenab	Marala Headworks	74° 27' 51.5"/32° 40' 22.4"	3	15
River Indus	Taunsa Headworks	70°50' 57.1"/ 30° 30' 46.1"	3	15
River Jhelum	Rasul Headworks	73°31' 15.1"/ 32° 40' 49.0"	3	15
River Ravi	Balloki Headworks	73° 51' 44.7"/31° 13' 13.5"	3	15
River Satluj	Sulaimanke Headworks	73° 51' 58.6"/30° 22' 40.3"	3	15
Total			15	75

Table 2: Mitochondrial cytochrome oxidase I (COI) and RAPD primers used for PCR amplification.

Detail of mitochondrial cytochrome oxidase I (COI) and RAPD primers			
Primer	**Primer sequence**	**GC percentage**	**Number of fragments**
Mitochondrial cytochrome oxidase I (COI) gene			
FishF1-COI	5′-TCAACCAACCACAAAGACATTGGCAC-3′	46%	530 base pairs
FishR1-COI	5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′	48%	530 base pairs
Randomly amplified polymorphic DNA (RAPD) markers			
OPY-02	CATCGCCGCA	70%	04-11
OPY-05	GGCTGCGACA	70%	03-10
OPY-07	AGAGCCGTCA	60%	04-11
OPY-11	AGACGATGGG	60%	05-08
OPY-16	GGGCCAATGT	60%	05-10

DNA extraction

50 mg fish fin tissue sample removed from each specimen. Then DNA of 90 specimens extracted with Phenol chloroform DNA extraction technique followed Garg et al. (2014) and Chowdhury *et al.* (2016). The isolated DNA was stored at -20°C until further analyses.

PCR amplification of mitochondrial gene and RAPD markers

PCR amplification of mitochondrial gene successfully completed by using the primers FishF1 COI and FishR1 COI followed Shen et al. (2016) and Naeem and Hassan (2019). The FishF1 COI and FishR1 COI primer sequences and GC percentage detail provided in Table 2. Total PCR reaction solution volume was 25µl for each PCR reaction, containing 1.5µl DNA template, 12.5µl PCR Master Mix (TaqNova-Red BLIRT S.A.), 0.1µl F1 and 0.1µl R1 CO1 primer concentrations with 10.8µl nuclease free water used. The PCR thermal cycler conditions were set as the initial denaturation for 2 min at 95°C followed by 35 complete cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 54°C and extension for 1 min at 72°C. Final extension was set for 10 min at 72°C (Ghouri *et al.*, 2020). The success of PCR amplification checked by running the PCR products on 2% (w/v) agarose gel.

The amplicons of randomly amplified polymorphic DNA were amplified using five primers OPA-02, OPA-05, OPA-07, OPA-11 and OPA-16 followed by Laxmi *et al.* (2013) for screening *N. notopterus* samples (Table 2). Total 25µl PCR reaction volume made with DNA template 1.5µl, Master Mix 12.5µl (TaqNova-Red BLIRT S.A.), 0.3µl primers (each) and 10.8µl sterile water. The PCR amplification conditions set as initial denaturation at 94°C for 5 min, 40 complete cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min. A final extension step at 72°C for 7 min (Laxmi *et al.*, 2013). The PCR amplicon products were checked on 1.5% agarose gel buffered with 1X TAE buffer and visualized under UV light after staining in 1µl/ml ethidium bromide. The gel containing PCR products stained with ethidium bromide to detect the polymorphisms. The amplicon bands of each gel photographed with Gel Documentation System PHOTONYX DARKROOM (NTGB/1003). DNA marker (Thermo fisher) 100bp ranging 100bp-2000bp in size as standard to determine the molecular weight of each amplification band.

Mitochondrial COI gene sequence and genetic connectivity analysis

PCR purified products of 15 fish specimens sent to First BASE Laboratories Sdn Bhd, Malaysia and barcoded successfully. The barcoded sequences aligned and trimmed to same length by using MEGA-X software (Tamura *et al.*, 2011). *N. notopterus* barcoded COI sequences processed through BLAST (Basic Local Alignment Search Tool) analysis of nucleotide database of NCBI (National Centre for Biotechnology Information) and examined the accurate identity match. All barcoded sequences submitted in GenBank Database as reference.

Maximum likelihood (ML) tree used to infer the genetic link and genetic similarity among COI barcode sequences of *N. notopterus* populations using MEGA-X software (Kumar *et al.*, 2018).

Statistical analysis of RAPD genetic diversity and phylogeographic relationship

POPGENE Software version 3.1 was used to analyses RAPD diversity (Souza-Shibatta *et al.*, 2022). The genetic diversity data was analysed by calculating Nei's gene diversity (h) (Zhu *et al.*, 2022), and Shannon's information index (I) followed Mukhopadhyay and Bhattacharjee (2014). UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram constructed to show the genetic link among five rivers using Past4.03 software.

Results and Discussion

Study of morphometric characteristics

Notopterus notopterus has highly compressed body. The body outline is equally convex from both ventral and dorsal sides. Min scales are present at surface of body.

N. notopterus have short dorsal fin and long anal fin. The colour of body observed silvery white (Figure 2). Total body length was 19.9 cm (mean), stranded length 18.7 (mean), head length was 3.5 cm (mean) and eye size 19.9% (mean) of head length. Summary of morphological and meristic characteristics (mean) provided in Table 3.

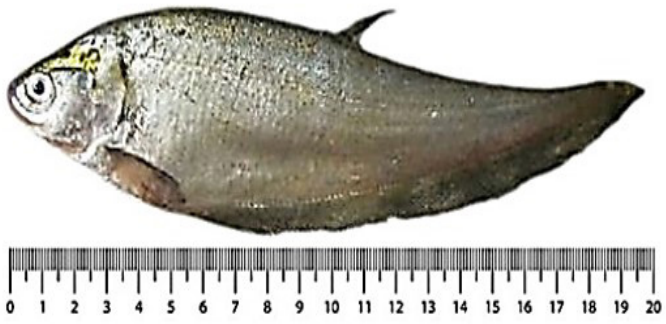

Figure 2: *Notopterus notopterus* fish identified in present study (Lateral view).

Gene barcoding, nucleotide sequence analysis and molecular identification success

In the present study, fifteen *Notopterus notopterus* fish specimens were barcoded successfully with mitochondrial COI gene. All barcoded sequences submitted to the GenBank database. GenBank Accession of submitted sequences provided in Table 4. The barcoded sequences aligned with final sequence fragment of 530 base pairs. All specimen barcode data and associated sequence information recorded in the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). BLAST result confirmed 100% identity of *Notopterus notopterus* with relevant sequences of GenBank databases (Table 4).

Genetic connectivity of Notopterus notopterus fish identified from Pakistan

A tree of genetic connectivity was constructed using 15 DNA barcode sequences of *N. notopterus* of current study and 5 sequences of *N. notopterus* were taken from GenBank database of previously reported neighbouring countries; India, Bangladesh and Indo-Myanmar. Maximum likelihood (ML) genetic tree produced two distinct clusters. Cluster-I was an assemblage of *N. notopterus* species identified from Pakistan with *N. notopterus* species reported from Indo-Myanmar which revealed that both species have high genetic connectivity and similarity. Cluster-II showed assemblage of *N. notopterus* species identified from Pakistan with *N. notopterus* species reported from India. This assemblage under same cluster indicated a high genetic similarity and genetic connectivity between both species. This genetic connectivity indicated that *N. notopterus* fish identified from Pakistan has close genetic linkwith *N. notopterus* fish of Indo-Myanmar and India (Figure 3).

Table 3: Morphometric and meristic characteristic of *N. Notopterus*.

Morphometric parameters	*Notopterus notoptrus* analysed	Morphological and meristic identity evidences	Identified species
Morphological characters			
Body formation	Highly compressed	Highly compressed (Galib *et al.*, 2009)	*Notopterus notoptrus*
Body outline ventrally	Equally convex	Equally convex (Galib *et al.*, 2009)	
Body outline dorsally	Equally convex	Equally convex (Galib *et al.*, 2009)	
Scales	Min	Min (Rahman, 1989)	
Dorsal fin	Short	Short (Rahman, 1989)	
Anal fin	Lengthy	Lengthy (Rahman, 1989)	
Body coloration	Silvery white	Silvery white (Galib *et al.*, 2009)	
Lateral line	Complete	Complete (Galib *et al.*, 2009)	
Morphometric characters			
Total length	19.9 cm	20.0 cm (Talwar and Jhingran, 1991)	*Notopterus notoptrus*
Standard length	18.7 cm	18.7 cm (Talwar and Jhingran, 1991)	
Scales	230-235	230-240 (Galib *et al.*, 2009)	
Head length	3.5 cm	3.5 cm (Galib *et al.*, 2009)	
Body height	35.2% of Standard length and 32.1% of total length	35.5% of Standard length and 32.4% of total length (Galib *et al.*, 2009)	
Eye	19.9 % of head length	20% of head length (Galib *et al.*, 2009)	
Meristic characters			
Anal fins	100-103	100-104 (Rahman, 1989)	*Notopterus notoptrus*
Pectoral fins	16-17	16-17 (Rahman, 1989)	
Anal + caudal fins	101-107	101-107 (Talwar and Jhingran, 1991)	
Dorsal fins	8-9	7-9 (Talwar and Jhingran, 1991)	

Table 4: BLAST analysis of mitochondrial COI gene barcode sequences of *Notopterus notopterus*.

River name	Specimen collected	GenBank accession of submitted sequences	NCBI		
			Identity (%)	E- value	Identified species name
Chenab	1	MZ328226	100	0.0	*Notopterus notopterus*
	2	MT434341	100	0.0	*Notopterus notopterus*
	3	MZ328225	100	0.0	*Notopterus notopterus*
Indus	1	MZ343242	100	0.0	*Notopterus notopterus*
	2	MZ343243	100	0.0	*Notopterus notopterus*
	3	MZ343298	100	0.0	*Notopterus notopterus*
Jehlum	1	MZ343251	100	0.0	*Notopterus notopterus*
	2	MZ343252	100	0.0	*Notopterus notopterus*
	3	MZ343254	100	0.0	*Notopterus notopterus*
Ravi	1	MZ329064	100	0.0	*Notopterus notopterus*
	2	MZ329667	100	0.0	*Notopterus notopterus*
	3	MZ329040	100	0.0	*Notopterus notopterus*
Satluj	1	MZ343295	100	0.0	*Notopterus notopterus*
	2	MZ342920	100	0.0	*Notopterus notopterus*
	3	MZ343294	100	0.0	*Notopterus notopterus*

Table 5: RAPD diversity analysis of five population of *N. notopterus*.

Marker	Polymorphic and monomorphic genetic amplification among five populations of *N. notopterus*																			
	Ravi population				Satluj population				Jehlum population				Indus population				Chenab population			
	Polymorphic locus	Monomorphic locus	RAPD total loci	Polymorphic locus %	Polymorphic locus	Monomorphic locus	RAPD total loci	Polymorphic locus %	Polymorphic locus	Monomorphic locus	RAPD total loci	Polymorphic locus %	Polymorphic locus	Monomorphic locus	RAPD total loci	Polymorphic locus %	Polymorphic locus	Monomorphic locus	RAPD total loci	Polymorphic locus %
OPY-02	04	03	07	57.14	04	02	06	66.66	03	04	07	42.85	02	04	06	33.33	01	03	04	25.00
OPY-05	04	02	06	66.66	06	03	09	66.66	01	04	05	20.00	01	04	05	20.00	01	04	05	20.00
OPY-07	03	04	07	42.85	04	03	07	57.14	01	03	04	25.00	02	03	05	40.00	01	04	05	20.00
OPY-11	03	04	07	42.85	03	03	06	50.00	03	05	08	37.50	03	05	08	37.50	02	05	07	28.57
OPY-16	03	05	08	37.50	06	04	10	60.00	02	03	05	40.00	02	05	07	28.57	01	04	05	20.00
Total	17	18	**35**	48.57	23	15	**38**	60.52	10	19	**29**	34.48	10	21	**31**	32.25	06	20	**26**	23.07

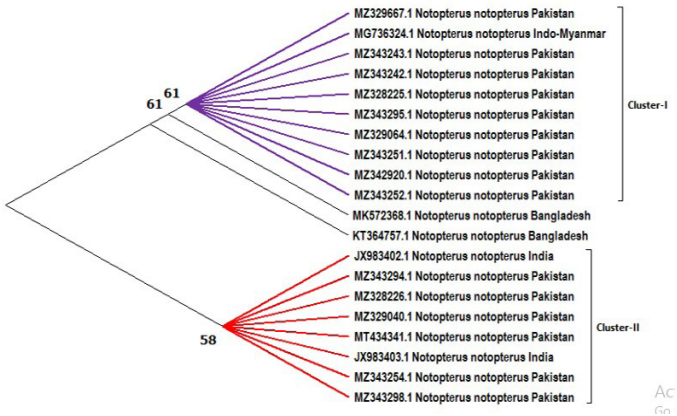

Figure 3: Maximum likelihood tree analyses of *Notopterus notopterus* identified from Pakistan with *Notopterus notopterus* reported from neighbour countries (India, Bangladesh and Indo-mayanmar). The node values represent the confidence level of 2000 bootstrap replicates using MEGA-X.

Results of RAPD polymorphic and monomorphic DNA amplification

RAPD five markers produced a total of 66 polymorphic bands and 93 monomorphic bands among five populations. *N. notopterus* 15 specimens from each population amplified with five RAPD markers. A total number of 17, 23, 10, 10 and 06 polymorphic bands detected in populations of Ravi, Satluj, Jehlum, Indus and Chenab, respectively. Monomorphic bands were detected 18, 15, 19, 21 and 20 in populations of Ravi, Satluj, Jehlum, Indus and Chenab, respectively (Table 5). DNA band range varied from 3-11 and the DNA fingerprints of PCR amplified product showed the band size of DNA ranged from 300-1500 bp (Figure 4A-E).

RAPD five primers were produced polymorphic 66 (41.50%) bands among five population (75 specimens) of *N. notopterus*. In Ravi River population 48.57%

polymorphic bands detected, in Satluj River population 60.52%, in Jehlum River population 34.48%, in Indus River 32.25% population and in Chenab River population 23.07% polymorphic bands were detected (Table 5).

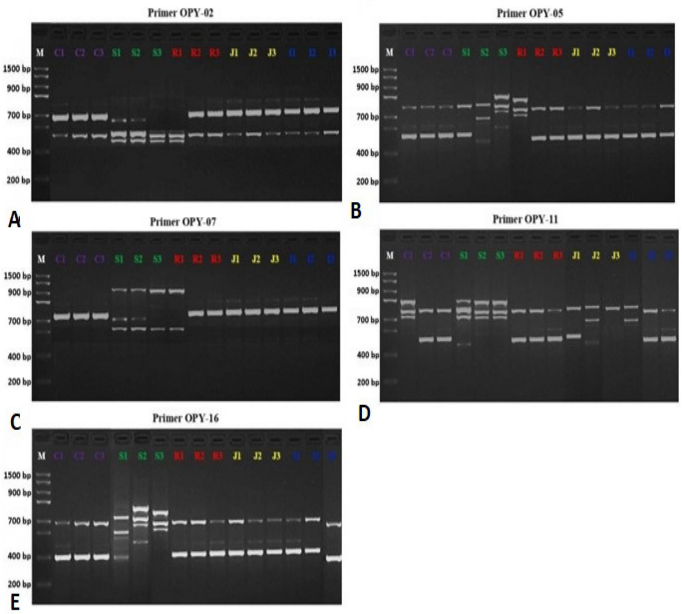

Figure 4: DNA fingerprints in *N. notopterus* of Chenab, Satluj, Ravi, Jehlum and Indus using 5 RAPD markers A OPA-02. B OPA-05. C OPA-07. D OPA-11. E OPA-16. Samples C1-C3 for Chenab, S1-S3 for Satluj, R1-R3 for Ravi, J1-J3 for Jehlum, I1-I3 for Indus River for each primers and M is molecular marker (Ladder).

RAPD diversity and genetic differentiation

RAPD five primers produced 159 total scorable bands consisting 66 (41.50%) polymorphic and 93 (58.50%) monomorphic bands. The pattern of genetic variation among five populations observed as Satluj > Ravi > Jehlum > Indus > Chenab from high to low (Table 5). Genetic differentiation (GST) among five populations was observed GST= 0.6160, total heterozygosity (HT) 0.6223±0.0012 and intra-population heterozygosity was observed (HS) 0.4132±0.1023.

The highest Nei's genetic diversity (h) was observed 0.5832 in population of Satluj followed by Ravi 0.5234, Jehlum 0.4624, Indus 0.2572 and Chenab 0.2173 (Table 6). Results of Shannon's information index (I) revealed the same pattern of genetic diversity as 0.6281, 0.5833, 0.4523, 0.3971and 0.3162 for populations of Satluj, Ravi, Jehlum, Indus and Chenab respectively (Table 6).

Analysis of genetic distances using RAPD markers

Based on RAPD data the maximum genetic distance 0.4142 found between Jhelum and Satluj while the lowest genetic distance found 0.1360 between Satluj and Ravi (Table 6).

UPGMA dendrogram construction for analysis of genetic connectivity among rivers

UPGMA dendrogram produced two distinct clusters. Cluster-I includes Satluj and Ravi River which indicated that populations of Satluj and Ravi has close genetic link. Cluster-II consist of two subclusters; Subcluster-I includes Indus and Jehlum, which indicated that populations of Indus and Jehlum has close genetic link while Sub-cluster-II observed independent consist of Chenab population alone (Figure 5).

Genetic relationship between populations

Nei's co-efficient of genetic identity (I) was used to calculate the genetic relatedness among related fish species. Results of Nei's co-efficient of genetic identity (I) of RAPD data analysis showed a distant high relatedness among Satluj and Ravi populations D= 0.2123 of *N. notopterus* whereas genetic distance between Jehlum, Indus and Chenab populations was observed 0.1711.

Fish Identification and evaluation of genetic diversity is vital for fisheries conservation and management (Suresh *et al*., 2022). Fish identification is also important for sustainability of aquatic ecosystem. Traditional fish identification techniques are ordinary because these techniques cannot identify morphologically similar species accurately (Hou *et al*., 2022). Therefore, DNA barcode of mitochondrial COI gene now used efficiently for identification of species (Habib *et al*., 2021).

Morphometric and meristic characters of *N. notopterus* reported in Table 3 of present study found similar with the standard characters reported by Talwar and Jhingran (1991). Total body length, stranded length, head length, fins length and meristic features also found similar to reference characters reported by Rahman (1989) and Galib *et al*. (2009).

In present study, fifteen *N. notopterus* specimens were barcoded with 530 base pairs, which confirmed the identification of fish species as *Notopterus notopterus* Table 4 as Naeem and Hassan (2019) used 650 base pairs for identification of species. *N. notopterus* fifteen barcode sequences produced in this study submitted in GenBank Database, which may use as reference for future studies. Record keeping of DNA barcode sequences is the basic objective of GenBank Database (Naeem and Hassan, 2019; Zhang and Hanner, 2012).

Figure 3 of ML tree in present study indicated that *N. notopterus* fish identified from Pakistan has close genetic link with *N. notopterus* fish reported from India and Indo-mayanmar as ML tree used by Naeem and Hassan (2019) and Mazhar and Saif (2022) to report phylogenetic relationship previously.

Table 6: Parameters of genetic diversity variations of *N. notopterus*.

Parameters of genetic diversity variations for *N. notopterus* among rivers				Nei's (1978) Unbaised genetic diversity and genetic distance					
Riverine population	**Number of specimens**	**Nei's gene diversity (h)±SD**	**Shannon's information index (I) ± SD**	**River**	**Ravi**	**Satluj**	**Jehlum**	**Indus**	**Chenab**
Ravi	15	0.5234±0.0112	0.5833±0.1523	Ravi	****	0.1360	0.3319	0.2192	0.3782
Satluj	15	0.5832±0.0143	0.6281±0.1842	Satluj	0.1360	****	0.4142	0.1652	0.2314
Jehlum	15	0.4624±0.0213	0.4523±0.1163	Jehlum	0.3319	0.4142	****	0.1860	0.2319
Indus	15	0.2572±0.0243	0.3971±0.1289	Indus	0.2192	0.1652	0.1860	****	0.3278
Chenab	15	0.2173±0.0135	0.3162±0.1327	Chenab	0.3782	0.2314	0.2319	0.3278	****

In present study, five RAPD primers were used (Table 2) and observed a total number of 159 bands as compared to Lal *et al.* (2006), who reported 1344 bands with five RAPD primers while Neekhra *et al.* (2014) used 6 RAPD primers and reported 746 amplified bands in *Cyprinidae*.

Figure 4A-E of current study presented DNA fingerprints of PCR amplified product band range from 300-1500 bp as Garg *et al.* (2014) reported genetic diversity in *N. notopterus* using DNA fingerprints.

RAPD analysis data of Table 5 showed the genetic diversity in *N. notopterus*. The number of bands produced per primer varied from 3 to 11 (Table 2). In present study, amplified DNA band size in *N. Notopterus* observed 100bp-1500 bp as Lal *et al.* (2006) reported band range of 250-2900 in *N. notopterus* and *C. chitala*. Table 5 of present study also revealed that RAPD five primers were produced 66 (41.50%) polymorphic bands of *N. notopterus*. In Ravi River population, 48.57% polymorphic bands were detected, in Satluj River population 60.52%, in Jehlum River population 34.48%, in Indus River 32.25% population and in Chenab River population 23.07% polymorphic bands were detected as Laxmi *et al.* (2013) reported the percentage of polymorphic bands to analysis of genetic polymorphism in *N. notopterus*.

The average heterozygosity (H) of alleles is the evaluation of genetic variation in population (Sahoo *et al.*, 2023). Zhu *et al.* (2022) used parameter of Nei's genetic diversity (h) to estimate a wide variety of organism's diversity with RAPD markers. Table 6 of present study showed the highest Nei's genetic diversity (h) 0.5832 in population of Satluj River followed by Ravi 0.5234, Jehlum 0.4624, Indus 0.2572 and Chenab 0.2173 as Laxmi *et al.* (2013) reported average heterozygosity in *N. notopterus*.

In Table 6, Shannon's information index (I) showed the pattern of genetic diversity as 0.6281, 0.5833, 0.4523, 0.3971 and 0.3162 for populations of Satluj, Ravi, Jehlum, Indus and Chenab as Laxmi *et al.* (2013) reported using Shannon's information index (I) in *N. notopterus*.

Fish species genetic relatedness can be determined by estimating the genetic distance and the genetic identity by Nei's co-efficient of genetic identity (I) (Laxmi *et al.*, 2013). Nei's co-efficient of genetic identity (I) of RAPD data analysis showed a high relatedness among Satluj and Ravi populations D=0.2123 of *N. notopterus* in present study whereas genetic relatedness between Jehlum, Indus and Chenab populations was observed 0.1711 as Laxmi *et al.* (2013) used Nei's co- efficient of genetic identity (I) to report the genetic relatedness in *N. notopterus*.

Fish genetic diversity could be due to mutations (Liu *et al.*, 2022). Moreover, rate of species extinction is rapid in small size population (Wang *et al.*, 2019). Therefore, low genetic diversity is consequences of limited founder stocks and inbreeding in a population (Wu and Yang, 2012). Continued and uncontrolled fishing from riverine water could intensely decrease size of effective population and a cause of losing genetic diversity (Garcia-Cisneros *et al.*, 2017). There is no specific data about larval dispersal rate, sex ratio and sex biased fishing pressure on *N. notopterus* reported from Pakistan.

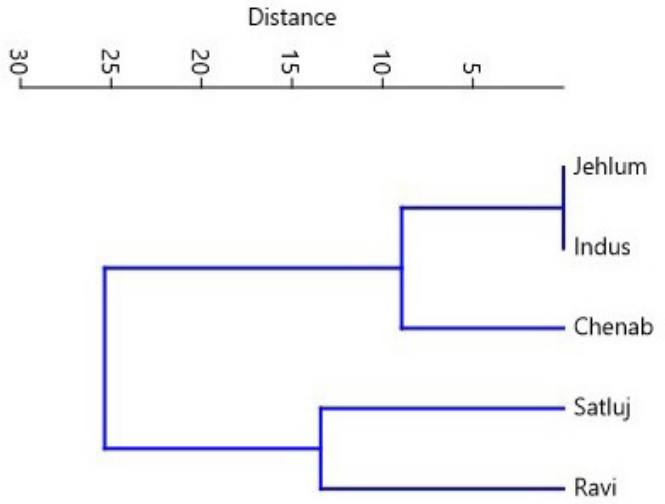

Figure 5: UPGMA dendrogram based on RAPD polymorphic data showed genetic connectivity among riverine populations of Ravi, Satluj, Jehlum, Indus and Chenab.

Figure 5 of current study resented UPGMA dendrogram that showed the genetic connectivity of

N. notopterus populations among five rivers. UPGMA dendrogram revealed that Indus and Jehlum riverine populations, and Satluj and Ravi populations has close genetic similarity as Laxmi *et al.* (2013) used UPGMA dendrogram to report genetic connectivity among riverine populations.

Conclusions and Recommendations

Present study successfully reported the identification and genetic diversity of *Notopterus notopterus* fish from five main rivers (Chenab, Indus, Jhelum, Ravi and Satluj) of Pakistan through DNA barcode of mitochondrial COI gene and RAPD markers. RAPD markers data indicated that Satluj and Ravi produce high degree of genetic diversity while River Chenab had lowest genetic diversity. Therefore, population of *N. notopterus* in Chenab rivers need to be protect and conserved. Moreover, *N. notopterus* fish identified from Pakistan has close genetic similarity with *N. notopterus* fish reported from India and Indo-Myanmar. According to best of our knowledge, present study is first attempt to identify *N. notopterus* fish on molecular basis from Pakistan. The barcode sequences of mitochondrial COI gene of *N. notopterus* have submitted directly into GenBank database, which used as reference for future studies. Moreover, genetic diversity data generated from this study provide a valuable information for fisheries resource manager to take immediate steps in conservation of *N. notopterus* population in Chenab River to achieve sustainability.

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Ethics statement

All the methods carried out in line with international norms for animals. The study approved in meeting of Board of Studies (BOS) Zoology dated 06-03-2020. The Advance Study and Research Board (ASRB) issued letter in approval of study with No. Acad/Scholar's File/582. dated 23-01-2021. Bahauddin Zakariya University Multan, Pakistan.

Conflict of interest

The authors have declared no conflict of interest.

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