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Molecular Identification of *Wallago attu* (Bloch and Schneider, 1801) and its Phylogenetic Association with other Silurids Based on Mitochondrial DNA

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Key words
Catfish, Molecular identification, Phylogenetic connection, Siluridae, Mitochondrial DNA, Pakistan

ABSTRACT

The assessment of genetic diversity and population genetic structure is highly important for species that are economically and ecologically valuable, threatened, or of global conservation concern. Mitochondrial DNA (mtDNA) analysis can be successfully used in species identification and population genetics studies due to its own maternally inherited DNA and its genome evolves faster, making it useful for evolutionary study. The Siluridae family comprises species with diverse morphology and is widely distributed in Eurasia and Africa. The aim of this study is the genetic characterization of *W. attu* using the DNA barcoding approach and to ascertain its phylogenetic relationship with other Silurid species based on analysis of mitochondrial protein coding and non-coding genes, as a conservative management tool. A total of 227 mitochondrial COI barcodes, 59 Cytb, 91 16S rRNA, and 40 D-Loop sequences were obtained from Siluridae family individuals. The average length of the sequences was above 600 base pairs and, in all studied genes, mean inter-specific genetic distance was greater than the mean intra-specific distance in this family. Nucleotide composition of the mitochondrial genes was showed average amount of G+C content (~46.00%). The sequencing data revealed rich genetic diversity among fish specimens of family Siluridae. We conclude that Cytb, 16S rRNA and D-Loop region equally contribute to reconstruct phylogenetic relationship. Monophyly of Siluriformes was supported by all applied phylogenetic methods. *W. attu* holds significant economic value and is classified as Vulnerable by the IUCN. Its conservation status is at risk due to its importance and the changing environmental conditions. The study of *W. attu* was reported for the first time from Azad Kashmir, Pakistan, useful for different databases, as a future needed conservation management support in such remote area.

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INTRODUCTION

Biodiversity is a key factor for the functioning and efficiency of an ecosystem. Pakistan is an agricultural country, hosts a great deal of species diversity. In recent years, traditional methods of species identification have been complemented by the use of molecular markers. This supplementation is due to the difficulty in precisely identifying species based solely on morphology, often caused by the absence of certain morphological features and a shortage of taxonomic expertise (Tsoupas *et al*., 2022). As compared to morphological study, a 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 (Kochzius, 2009). Certain mitochondrial genes are well characterized and are the promising markers for exact identification of animal species, stock maintenance, and construction of evolutionary connection (Xiao *et al*., 2001; Perdices *et al*., 2004; Gilles *et al*., 2001; 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 (Hubert *et al*., 2008).

DNA barcoding has become the most popular approach for the species identification and the assignment of specimens throughout their life stages. In animals, an app. 660 base pair (bp) fragment of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene has been chosen

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as standardized barcode marker (Hebert *et al*., 2003a, b). Much prosperous works have been carried out on fish diversity for both freshwater and marine fishes using DNA barcoding (Chakraborty and Ghosh, 2014; Lakra *et al*., 2016; Zhang and Hanner, 2011). Various scientists have been preferred the DNA barcode as a primary source for standardizing the diversity and range of fish species, due to huge data of COI in the barcode of life data (BOLD) systems database (Hebert *et al*., 2003a; April *et al*., 2011; Puckridge *et al*., 2013). It is an important genetic marker which is used for a global bio-identification system and would be sufficient to differentiate all, or at least the vast majority of animal species (Elmeer *et al*., 2012). It provides authentic and accurate identification of fish species and is useful for fish conservation, its sustainability and management (Ortega-Ortiz *et al*., 2014; Gilles *et al*., 2001; Durand *et al*., 2002). However, there is still a limited number of studies on DNA barcoding of *Wallago attu* (Sajjad *et al*., 2023).

W. attu is a fast-growing catfish belongs to family Siluridae under the order siluriformes. It is commonly known as helicopter catfish or *wallago* catfish. It is among the largest, voracious and predatory of the local catfish, which thrives well in rivers and tanks. It migrates from the rivers to small streams, canals, and flood points during the flood season. It is rather sluggish and stays at the bottom of water in search of food (Ng, 2010). It has good market demand as a food fish having high nutritional value and high protein content in its flesh (Sharma and Simlot, 1971; Devadasan *et al*., 1978; Gupta, 2015). It is also popular as a good sport fish (Talwar and Jhingran, 1991).

W. attu lives through large parts of South and Southeast Asia. On the Indian subcontinent, its range includes all the major rivers of India, Pakistan, Bhutan, Nepal, Myanmar and Bangladesh as well as the island of Sri Lanka. To the Northwest, its range extends beyond Pakistan into Iran and Afghanistan (Babare *et al*., 2013; Kumbar and Lad, 2014; Coad, 2015; Siraj *et al*., 2016). Even though it is a hardy fish in nature, its populations are now rapidly declining due to over harvesting, environmental degradation, pollution and lack of proper management.

Mangla Dam (33.12 N, 73.39 E) is situated in the Mirpur District of Azad Jammu and Kashmir, and downstream from the Jhelum District in Punjab, Pakistan, at a maximum elevation of 630 meters. It spans an area of 26,500 hectares (Ali *et al*., 2011). The Mangla Reservoir yields an estimated annual fish production ranging from 247 to 853 metric tons (MFD, 2002). The reservoir's fish population consists of both indigenous and exotic species, totaling 57 species of which 54.4 percent are Cyrpinids, 19.3 percent are Silurids, and 26.3 percent belong to other classes. These fish species are classified into nine orders, 17 families, and 44 genera (Mirza *et al*., 2013). Of note, *W. attu* holds significant economic value among these species. It is commonly known as "Malee" and is classified as vulnerable by the IUCN 3.1 (Ng *et al*., 2019). Its conservation status is at risk due to its importance and the changing environmental conditions (Hussain *et al*., 2016).

While substantial progress has been made in documenting fish species diversity in AJK based on morphological studies, the diversity of fish species has not been fully explored, especially in the Mangla Dam. The primary objective of this study was genetic characterization of *W. attu* using the DNA barcoding approach and to ascertain its phylogenetic relationship with other species in the Silurids. For this purpose, the study utilizes two protein-coding genes (*COI, Cytb*) and two non-coding regions, namely 16s rRNA (highly conserved) and the D-Loop (highly variable), all located in the mitochondria. Additionally, this study aims to establish an NCBI reference database for *W. attu* in Azad Kashmir, Pakistan.

MATERIALS AND METHODS

Sampling

During the field sampling process, we obtained permission from the relevant authorities responsible for managing the reservoir to collect fish specimens. Since the reservoir is subject to the protective measures of the Department of Wildlife and Fisheries of the Government of Azad Jammu and Kashmir, we were granted permission to collect a limited set of 20 samples in 2021. These samples were gathered from six different locations within the Mangla Reservoir, situated along the Jhelum River in the Mirpur District of Azad Kashmir, Pakistan. On average, three individuals were collected from each locality (Fig. 1). To ensure unbiased genetic analysis, we selected only six samples out of the 20, all of which had successfully undergone sequencing for all four genes. The collected fish were anesthetized by immersing them in a 1% benzocaine solution in water and subsequently euthanized with an overdose of benzocaine. The specimens were preserved in ethanol (95%) and were initially identified on the basis of morphology by using the available taxonomic keys (Mirza *et al*., 1989; Jayaram, 2010). After analysis, the voucher specimens were deposited in the Zoological Museum Hall at the University of AJK, Pakistan.

DNA extraction and PCR amplification

After the identification based on morphology, the identified samples were further processed for molecular identification. Approximately 0.1 g of muscle tissue was removed from each fish sample, immediately preserved

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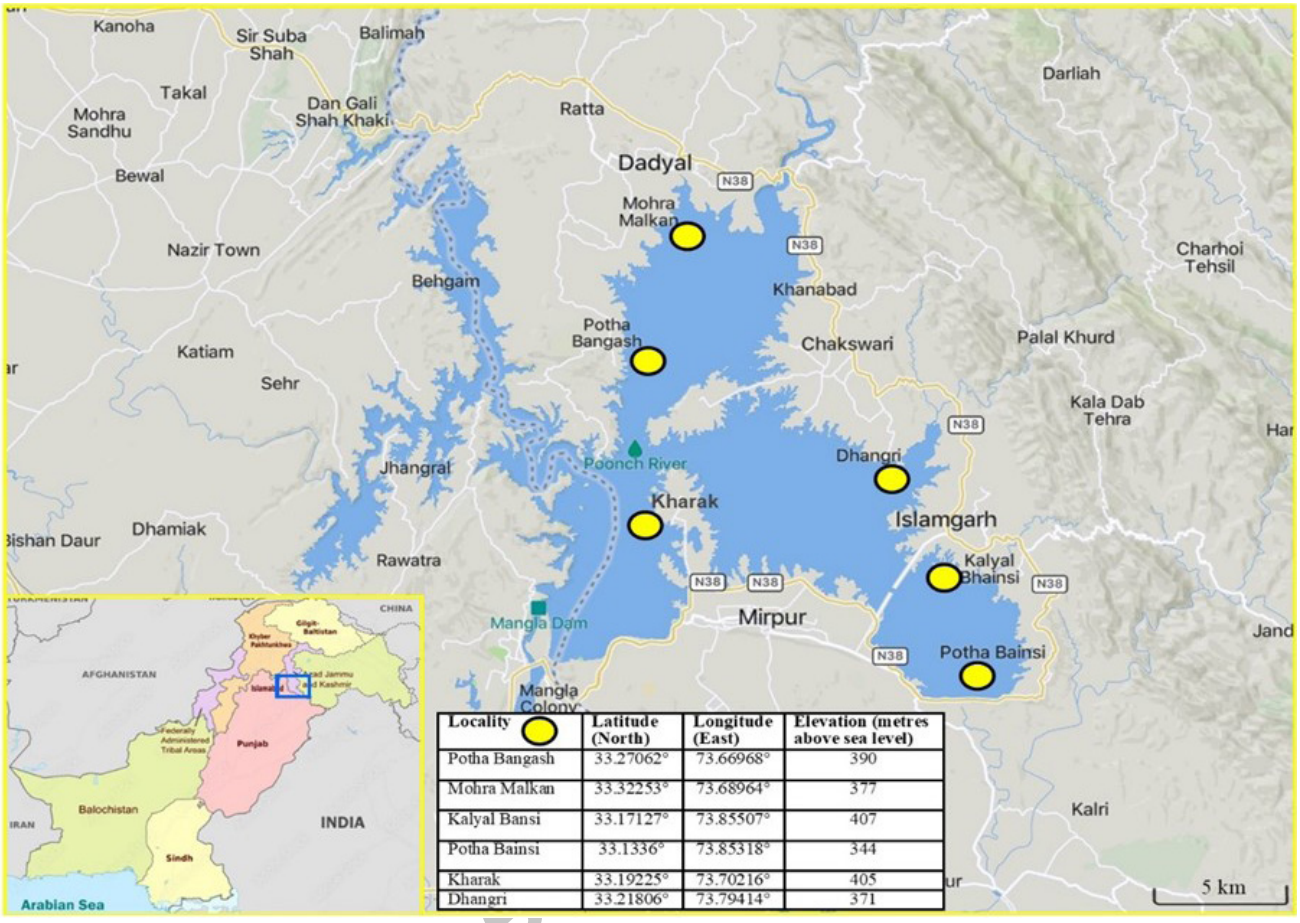

Locality	Latitude (North)	Longitude (East)	Elevation (metres above sea level)
Potha Bangash	33.27062°	73.66968°	390
Mohra Malkan	33.32253°	73.68964°	377
Kalyal Bansi	33.17127°	73.85507°	407
Potha Bainsi	33.1336°	73.85318°	344
Kharak	33.19225°	73.70216°	405
Dhangri	33.21806°	73.79414°	371

Fig. 1. Sampling sites of Mangla reservoir for the collection of *W. attu* specimens (Google map).

in 95% ethanol, and stored at -20°C until genetic analysis was performed. Genomic DNA extraction was performed by using standard phenol-chloroform extraction protocol of Sambrook *et al*. (1989). The extracted DNA was run on 1% agarose gels and visualized with a UV transilluminator. The results were recorded with a gel documentation system and quantified with a spectrophotometer at the 260/280nm wavelengths. Primer-3 online tool (Koressaar *et al*., 2018) was used to design the species-specific primers of all four genes and synthesized from MACROGEN Inc., Seoul, Korea. Forward and reverse primer used for CO1, Cytb, 16S rRNA and D-Loop region are

COI-F=5`-TTG GTA AAG AAT TGG GTC TCC-3`,
R=5`-CAG TCT ACT AAT TCG AGC AGA-3`;
Cytb-F=5`-AGG CGC CAC AGT AAT TAC AAA-3`,
R=5`-TAT CAT TCT GGC TTG ATG TGG-3`;
16S rRNA-F=5`-ACC GTG CAA AGG TAG CGCA-3`,
R=5`-TAA TCG TTG AAC AAA CGA ACC-3` and
D-Loop-F=5`-CCT ACA TGC ATA ATT TAC TAT G-3`,
R = 5`-TCA ACA CAC ATC GCT ATT CG-3`,

respectively.

The thermocycler (SimpliAmp) was used for Polymerase chain reaction comprised of 25 µL reaction mixture comprised of 2.5 µL of MgCl2, 2.5 µL of taq buffer, 0.5 µL of dNTPs (10 mM), 3 µL DNA, 1 µL of each of reverse and forward primers, 0.5 µL of Taq DNA polymerase, and 14 µL of pre-sterilized H_2O were incubated following the thermal cycle: initially template DNA was denatured at 95°C for 3 min. comprising of 35 cycles each of DNA denaturation for 5 min at 95°C, 30 sec of annealing at different temperature (51-58°C) for primers binding to complementary DNA template, and 1 min. at 72°C for DNA synthesis. Final extension was done for 5 min at 72°C.

PCR product purification and standard sequencing

The PCR product was purified using the Exo-SapIT Affymetrix purification kit. Bidirectional nucleotide sequencing was conducted on an ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems; Foster City, CA,

USA) utilizing gene-specific forward and reverse primers. Sequence editing was performed with the BioEdit program (http://www.mbio.ncsu.edu/BioEdit) to determine nucleotide and amino acid variants. Alignment of the sequences was carried out using the ClustalW (Thompson *et al*., 1994) algorithm in the MegAlign program within the LaserGene software package (DNAStar, Inc., Madison, WI). The voucher specimens were identified by using the barcode of life data system (BOLD) search engine and by Basic Local Alignment Search Tool (BLAST) of Genbank/ NCBI to confirm the morphological identification.

Analysis of genetic relationship

The refined sequences of target genes were then analyzed to determine the genetic relationship of the species using the Kimura 2-parameter method, with 1000 replicates of the bootstrap method. MEGA X (Kumar *et al*., 2018) was utilized to examine the nucleotide and amino acid composition, nucleotide substitutions, codon usage pattern, and relative synonymous codon usage (RSCU) of the genes under study. The same software was used to compute the intraspecific and interspecific pairwise genetic distances, conserved sites, variable sites, as well as parsimony-informative and singleton sites. DnaSP v5.10.01 (Rozas *et al*., 2003) was used to calculate the non-synonymous mutation rate (Ka) and synonymous mutation rate (Ks), haplotype diversity (Hd), nucleotide diversity (Π), and polymorphic sites (S).

Phylogenetic analysis

Phylogenetic trees were constructed using two approaches. The Neighbor-Joining (NJ) method was employed in MEGA X, and the BEAST analysis was conducted in BEAST 2.6.0 (Bouckaert *et al*., 2014). The NJ tree was generated using the Kimura 2-Parameter (K2P) model, with each node supported by 1000 bootstraps. BEAST analysis used a lognormal relaxed molecular clock algorithm under the calibrated Yule model and a gamma distribution under the HKY model. Posterior distributions of parameters were estimated using 1,000,000 Markov chain-Monte Carlo (MCMC) generations, sampled every 1000 generations, with a 20% burn-in in the TreeAnnotator 2.6.0 program. The consensus tree was visualized using the FigTree program (https://github.com/rambaut/fgtree/releases). Furthermore, a Median Joining Network (MJN) was constructed using Network 10.2 software (Bandelt *et al*., 1999) to depict the haplotypes.

RESULTS

Total genomic DNA of *W. attu* was extracted and then PCR and sequencing were carried out. All 24 sequences, including COI, Cytb, D-Loop, and 16S rRNA sequences (six sequences for each gene), were obtained in this study, and submitted to the Genbank database (Table I).

All the studied *COI* gene sequences of *W. attu* were identified through nucleotide BLAST, shown the maximum similarity (E-value is equal to 0) to reference sequences and verifying the lack of nuclear copies of mitochondrial origin (numts). The pattern of nucleotide distribution of A, T, C and G was T > C > A > G: percentage of A+T (54.30%) was greater than G+C (45.80%). Percentage of G+C nucleotide bases at the first, second and third codon positions for all the *W. attu* sequences (studied + retrieved from NCBI) were 57.60%, 42.80% and 36.90%, respectively. At 1st codon position, the highest number of GC content were observed among three codon positions. The usage frequency of all the bases are significantly different except C which was almost similar among all codon positions.

The usage of T (17.70%) was lowest as compared to other bases A (24.8%), C (26.10%), and G (31.50%) at 1st codon position. While, at 2nd codon position, usage of T (42.00%) was highest, and G (13.60%) was lowest. Similarly, at 3rd codon, nucleotide bases C (27.10%), T (28.00%), and A (35.10%) were higher and G (9.80%) base was lowest, presenting the anti-G bias pattern. K2P model was applied to calculate the mean pair-wise genetic distance (0.018±0.002) and average number of nucleotide differences (6.843) of all the *W. attu* specimens. The sequences larger than 550 bps were included in analysis. Multiple alignment of nucleotide sequence analysis of the entire dataset that

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Table I. Gene title, species code, voucher ID and accession numbers of *W. attu* subjected to genetic analysis.

Species	Code	Vocher ID	*COI*	*Cyt b*	D-loop	*16S rRNA*
W. attu	WA-1	FLZ-01-UAJK-2021	MN495551	MN495539	MN495545	MN495557
	WA-2	FLZ-02-UAJK-2021	MN495552	MN495540	MN495546	MN495558
	WA-3	FLZ-03-UAJK-2021	MN495553	MN495541	MN495547	MN495559
	WA-4	FLZ-04-UAJK-2021	MN495554	MN495542	MN495548	MN495560
	WA-5	FLZ-05-UAJK-2021	MN495555	MN495543	MN495549	MN495561
	WA-6	FLZ-06-UAJK-2021	MN495556	MN495544	MN495550	MN495562

total no. of sites (removing the gaps and missing data) were 470 of 696, sites with alignment gaps or missing data were 226 that were excluded from analysis. 437 of 470 sites were conserved, 33 of 470 sites were variable, 6 of 470 sites were parsimony informative, and 27 singleton sites were saw. The transition/transversion rate ratios are k1= 1.93 (purines) and k2= 3.69 (pyrimidines). The overall transition/transversion ratio is R = 1.56, where R = [A*G*k1 + T*C*k2]/[(A+G)*(T+C)]. The 0-fold degenerate sites are 346, and 4-fold sites are 31 of 470.

The average number of identical pairs (ii) was 601, of which 202, 202 and 198 were found at the first, second and third codon positions. Transitional pairs (si= 03) were found to be more common than transversional pairs (sv= 01). Both transitional and transversional pairs were most common at the third codon position (si= 003 and sv= 01).

Intraspecific genetic variations ranged from 0 to 7.9%, with mean value of 1.0%; the maximum genetic distance of 7.9% was found in *W. attu* (OK256189.1), and the genetic distance of all other species was less than 2.4%. The distances between species was 4.20-20.40%, with mean value of 14.10%, the more genetic distance was observed between *W. leerii* and *W. attu* (17.80%) as compared to genetic distance between *W. micropogon* and *W. attu* (4.20%). The minimum interspecific genetic distance of most *Wallago* sequences was greater than the maximum intraspecific genetic distance. Between genera, the genetic distance was 13.30-19.30%, with the mean value of 17.00%. The best fit model for *COI* gene was GTR+G with 17026.630 BICs values and 12659.508 AICc values.

In *W. attu*, commonly used codon is CUA (12) and its usage patterns is illustrated in Table II. Through genetic analysis it was depicted that COI gene coded 20 amino acids, and most frequently used amino acid is leucine (33) while, the least commonly used is Lysine (1). The polar amino acids (Tyr, Cys, Ser, Asp, and Glu) were greater as compared to non-polar (Ala, Ile, Leu, Phe, and Val). Relative synonymous codon usage calculated the total 209 codons and the most and least overused codons are ACA (3.04%), GUC (0.3%), respectively.

Siluridae family

In family Siluridae, 227 COI barcodes (larger than 600 bps) were retrieved from 07 genera and 25 species and their accession numbers are listed in Supplementary Table SI. Average length of *COI* sequences were 417, among these, the 54 were conserved sites. There were 363 variable sites of which 291 (80.16%) were parsimony informative sites, and 72 (19.84%) were singleton variable. The ratio of average base frequencies for COI gene of family Siluridae was A= 25.6 %, T=28.3%, C=29.0%, and G=17.0%.

Table II. The relative synonymous codon usage of *COI* gene.

Amino acid	Codon	Number	Frequency (%)	Amino acid	Codon	Number	Frequency (%)
Phe	UUU	8	1.10	Tyr	UAU	2	0.98
Phe	UUC	6	0.90	Tyr	UAC	2	1.02
Leu	UUA	3	0.56	*	UAA	0	0.00
Leu	UUG	2	0.36	*	UAG	0	0.00
Leu	CUU	9	1.66	His	CAU	1	0.51
Leu	CUC	4	0.67	His	CAC	3	1.49
Leu	CUA	12	2.19	Gln	CAA	5	2.00
Leu	CUG	3	0.56	Gln	CAG	0	0.00
Ile	AUU	11	1.52	Asn	AAU	5	1.03
Ile	AUC	5	0.64	Asn	AAC	4	0.97
Ile	AUA	6	0.84	Lys	AAA	1	2.00
Met	AUG	4	1.00	Lys	AAG	0	0.00
Val	GUU	5	1.33	Asp	GAU	1	0.45
Val	GUC	1	0.30	Asp	GAC	5	1.55
Val	GUA	5	1.51	Glu	GAA	1	1.05
Val	GUG	3	0.86	Glu	GAG	1	0.95
Ser	UCU	3	1.69	Cys	UGU	0	0.00
Ser	UCC	3	1.63	Cys	UGC	0	0.00
Ser	UCA	3	1.65	*	UGA	5	3.00
Ser	UCG	0	0.00	Trp	UGG	0	1.00
Pro	CCU	6	1.52	Arg	CGU	0	0.00
Pro	CCC	5	1.43	Arg	CGC	0	0.00
Pro	CCA	4	1.05	Arg	CGA	3	6.00
Pro	CCG	0	0.00	Arg	CGG	0	0.00
Thr	ACU	1	0.33	Ser	AGU	1	0.47
Thr	ACC	2	0.64	Ser	AGC	1	0.56
Thr	ACA	9	3.04	Arg	AGA	0	0.00
Thr	ACG	0	0.00	Arg	AGG	0	0.00
Ala	GCU	3	0.56	Gly	GGU	2	0.42
Ala	GCC	10	1.65	Gly	GGC	4	0.94
Ala	GCA	10	1.63	Gly	GGA	6	1.24
Ala	GCG	1	0.15	Gly	GGG	6	1.39

The percentage of A+T content (53.9%) was higher as compared to G+C content (46.0%). The nucleotide diversity (π) was 0.156±0.007 (mean±SD) and the average value of k (pairwise nucleotide differences) was 65.08 and the rate of transitions are greater than transversions. A total of 54 transitions and 28 transversions pairs were found within the dataset. Seventy one haplotypes were obtained in

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all sequences and their Hd was 0.964±0.006. The general topology of the median-joining network constructed for *COI* gene sequences of family Siluridae was presented in Figure 2.

A total of 71 haplotypes were derived from the family Siluridae with 32 (45.07%) of them being unique haplotypes and 39 (54.92%) were shared haplotypes. Hap3 recorded the highest distribution frequency with 33 individuals of *W. attu*. Studied sequences having Hap9 don't share any haplotype with other individuals. Shared haplotypes present in center, while the unique ones diverge from central area.

Catfish phylogeny

In order to determine the phylogenetic relationships of *W. attu* and other genera within the family Siluridae, we utilized newly generated CO1 sequences, as well as publicly available CO1 DNA sequences from the NCBI GenBank database. Phylogenetic trees were constructed using two different approaches: the Neighbour Joining (NJ) method implemented in MEGAX and BEAST 2.6.0. Initially, a tree was constructed using the NJ method based on K2P model. This analysis encompassed a total of 227 nucleotide sequences, which included both experimental and reference sequences retrieved from NCBI, each with

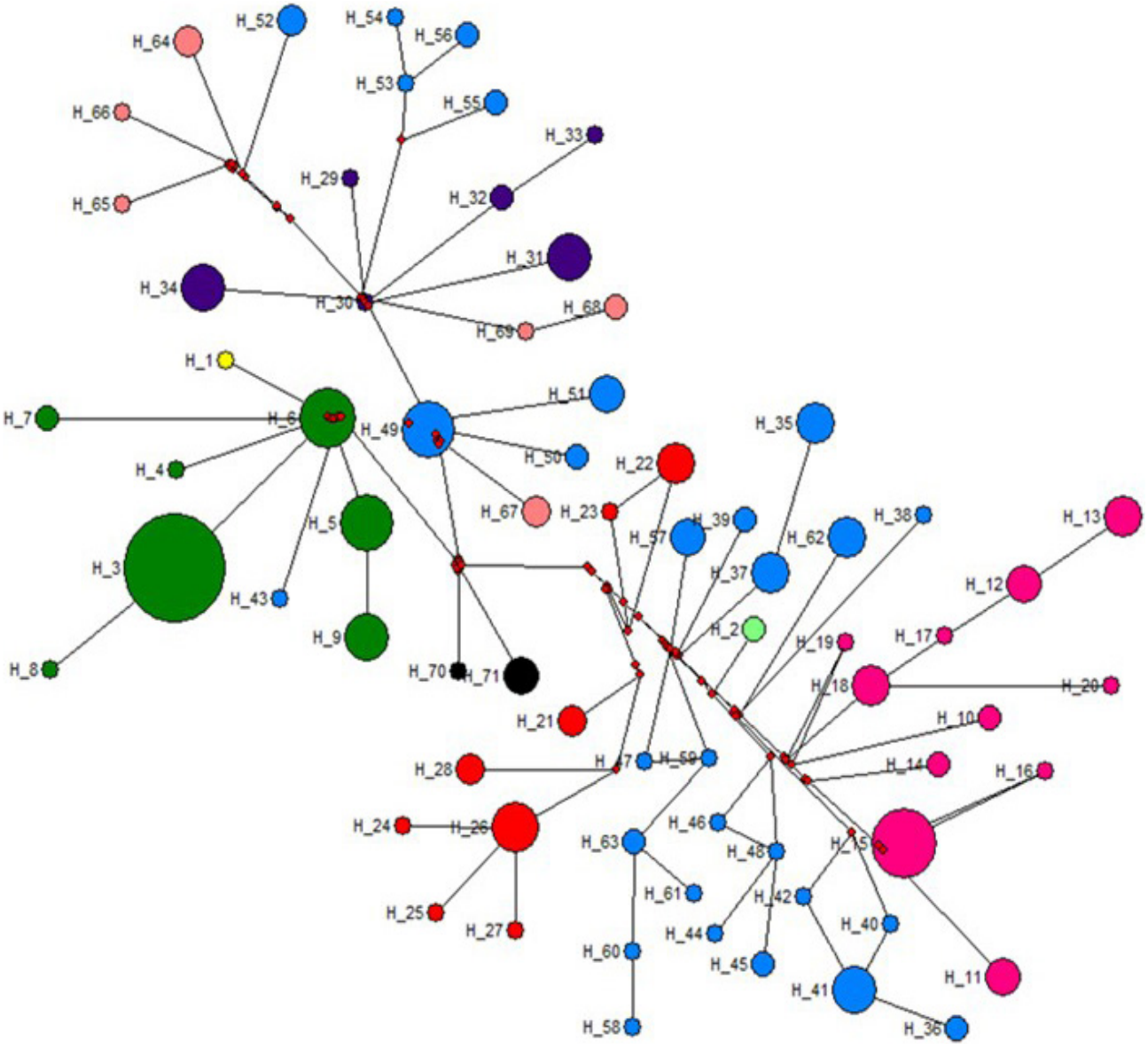

Fig. 2. Haplotype network of family Siluridae through MJN and their circle size representing the haplotype frequency.

accession numbers. Within the family Siluridae, all genera formed distinct clades, each with a substantial bootstrap support value of over 70%. All the analyzed sequences of *W. attu*, both from our study and international sources, were grouped together with strong bootstrap support. Notably, we observed a close genetic relationship between *Wallago* and the *Ompok pabu*, resulting in the formation of a monophyletic clade. Furthermore, all other genera (*Kryptopterus*, *Phalacronotus*, *Pterocryptis*, and *Silurus*) formed their separate clades. *Ompok hypophthalmus* was positioned at the base of the tree with a robust bootstrap support of over 95% and a posterior probability of 98% (Fig. 3A).

A more detailed representation of this phylogenetic tree can be found in BEAST v2.6.2 and was visualized using FigTree v1.4.4. Notably, the visualization was based on haplotypes exclusively. We derived a total of 71 haplotypes from the initial set of 227 nucleotide sequences, and these haplotypes are depicted in Fig. 3B. The family Siluridae was divided into two major clades. Clade I consists of four genera: *Wallago*, *Silurus*, *Ompok*, and *Phalacronotus*. All of our studied sequences form a single haplotype, which is Hap_9, and they cluster together with other haplotypes (Hap_8, 11, 13, and 34) of *Wallago attu* from India, Bangladesh, and Pakistan. Clade II was further divided into two subclades, Sub-clade I and Subclade II. Subclade I contained the following genera: *Silurus*, *Pterocryptis*, *Ompok*, *Kryptopterus*, *Wallago*, and *Belodontichthys*. However, most species of the genera *Wallago*, *Silurus*, *Phalacronotus*, *Ompok*, *Kryptopterus*, *Belodontichthys*, and *Pterocryptis* were clustered into Subclade II. Most individuals of the same species were grouped together, confirming the previous taxonomy based on morphology. However, there were a few exceptions. For example, sequences from different species formed the same cluster, while a few other samples of the same species formed a separate cluster.

Cytochrome b gene

Multiple alignments of 58 sequences of *Cytb* gene resulted in a consensus length of 406 out of 841 base pairs. Of the 406 sites (excluding sites with gaps/ missing data), 240 were constant, 152 variable characters were parsimony informative, and 14 variable characters were singleton. The model contained the nucleotide substitution estimated nucleotide frequencies A = 27.4, C= 30.5, G= 14.5, and T=27.7; nucleotide substitution rate matrix A–C=0.031, A–G=0.075, A–T=0.026, C–G=0.013, and C–T=0.284. The best fit model for Cytb was TN93+G+I. The proportion of invariable sites was estimated to be zero and the shape of the gamma parameter was estimated to be 0.138. The estimated Ts/Tv bias (R) was = 2.62. The average evolutionary divergence of all the sequence pairs is 0.162±0.01. Average number of pairwise differences was 59.14. The ML, and NJ methods of analysis generated almost identical relationships. Total no. of 26 haplotypes were observed with 0.952±0.01 haplotype diversity.

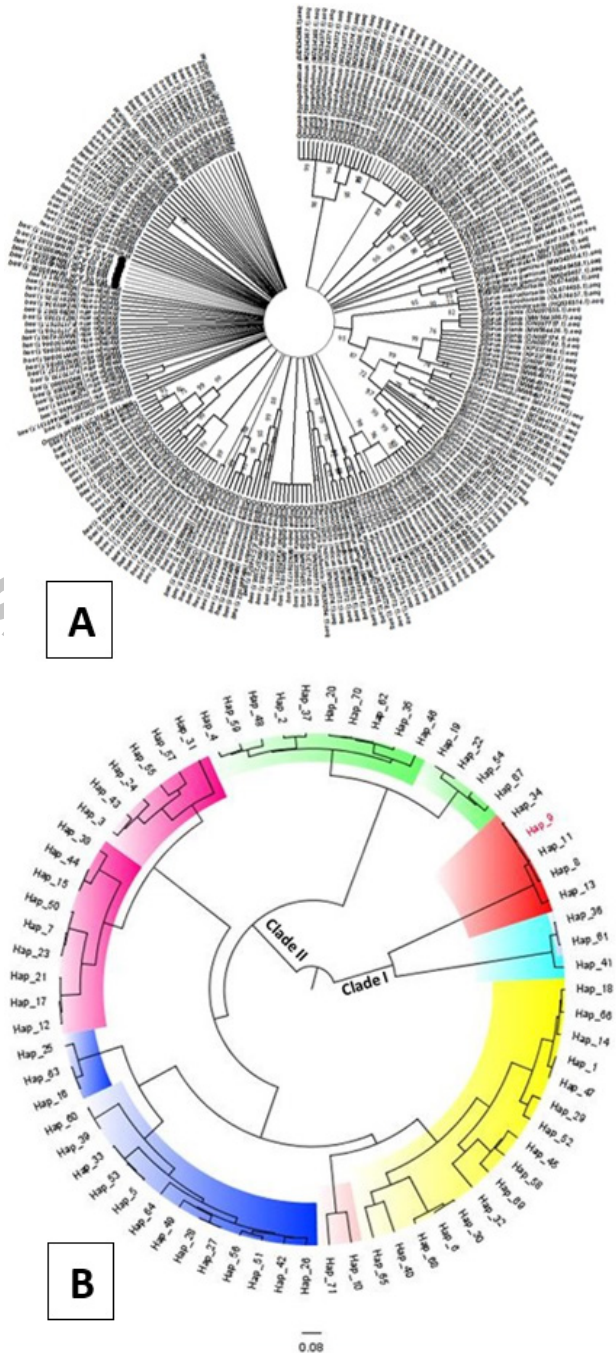

Fig. 3. A: Phylogenetic tree was reconstructed based on the Neighbor-Joining (NJ) method using *CO1* sequences. B: A Bayesian tree was reconstructed with 71 CO1 haplotypes of Family *Siluridae* by using the BEAST program.

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16S rRNA *gene*

For the phylogeny of family Siluridae, 575 bps were aligned from 91 specimens of all the species. Based on the 16s rRNA analysis, we detected 29 haplotypes with 0.956±0.007 haplotype diversity. Total number of sites (excluding sites with gaps/ missing data) were 352 of 575. 281 of 352 (79.82%) sites were monomorphic, 71 of 352 (20.17%) sites were polymorphic, 63 of 352 (17.89%) sites were parsimony informative, and 8 (2.27%) singleton sites were present. The percentage of nucleotide bases were T (22.30%), C (23.80%), A (31.50%) and G (22.40%). The base composition analysis for the 16s rRNA sequence showed that the average T, C, and G content were almost same and the average A content was the highest; the AT content (53.80%) was higher than the GC content (46.20%). The intraspecific genetic distance within 13 species ranged from 0.000 to 0.128 with an average of 0.065. The proportion of invariable sites was estimated to be zero and the shape of the gamma parameter was estimated to be 0.05. The estimated transition/transversion bias (R) was 3.34.

Displacement region

All the studied and available sequences of family Siluridae retrieved from NCBI database (above 500 bps) were collected and their size ranged from 544 to 910 bps. After re-moving the indels (Insertions and deletions) from left and right hypervariable domain, the 103 sites were found invariable and 380 were observed as variable. Out of 380 sites, 374 were parsimony-informative while 06 were singletons. Average percentage of nucleotide bases consist of 31.3–35.7% (A), 29.3–31.6% (T), 13.2–16.5% (G) and 19.5–22.5% (C), emphasizing A+T rich vertebrate mtDNA. The inter-specific K2P genetic distances was 0.321±0.03 and the average value of k was 155.04. 28 total no. of haplotypes were obtained with 0.959±0.02 haplotype diversity. A total of 66 transitions, and 163 transversions sites were found within the dataset. The best-fit nucleotide substitution model for D-Loop was T92+G+I with lowest AICc values.

DISCUSSION

In current study, two protein coding (COI, Cytb), one highly conserved (16S rRNA) and one highly variable (D-Loop) gene of mitochondrial DNA are included. These genes were identified by using nucleotide BLAST on NCBI database as per Lalitha and Chandavar (2018). All the sequences showed the maximum similarity (E-value is equal to 0) with reference sequences, validating the absence of numts. NUMTs are shuffling of mtDNA into nuclear genome and it was noticed that presence of numts can cause the biodiversity misestimating (Sorenson and Quinn, 1998). They also recommended to apply newly designed primers using reference sequences, accordingly, we designed and apply the new primers for all genes of *W. attu* instead of using universal primers.

Mitochondrial DNA fragments can be used for the authentic identification and discrimination of unknown or closely related species (Dawnay *et al*., 2007). *COI* gene, having high levels of conservation within species, used as DNA barcoding fragment with high efficiency in species identification already reported in Japanese marine fishes (Zhang and Hanner, 2011). Indian freshwater fishes (Chakraborty and Ghosh, 2014), Snow trout (Akhtar *et al*., 2017) and cyprinids of Pakistan (Ayesha *et al*., 2019). Multiple alignment of *COI* gene resulted in 33 variable sites, 6 were parsimony informative, and 27 were singleton. The observed nucleotide pair frequencies were alike to the fishes of Pakistan (Akhtar *et al*., 2017; Ayesha *et al*., 2019). Both tran-sitional and transversional pairs were highest at the third codon position. The overall base composition in *COI* gene was 25.1% % A, 27.5% C, 29.2% T, and 18.3% G.

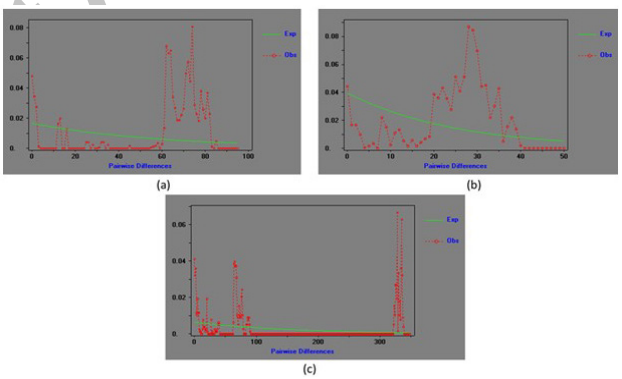

Fig. 4. Mismatch distribution analysis was conducted based on (a) *Cytb* gene, (b) *16s rRNA*, and (c) D-loop region. The expected frequency under the hypothesis of population expansion model was shown by continuous green line while, the observed frequencies were represented by red dotted line.

The usage of T (17.70%) was lowest as compared to other bases A (24.8%), C (26.10%), and G (31.50%) at 1st codon position. While, at 2nd codon position, usage of T (42.00%) was highest, and G (13.60%) was lowest. Similarly, at 3rd codon, nucleotide bases C (27.10%), T (28.00%), and A (35.10%) were higher and G (9.80%) base was lowest, presenting the anti-G bias pattern and similar patterns have also been observed in Ophichthyidae and Soleidae (Wang *et al*., 2014; Bingpeng and Zlrx, 2017; Bingpeng *et al*., 2018). Many other fishes also have such type of nucleotides pattern with little differences (Akhtar

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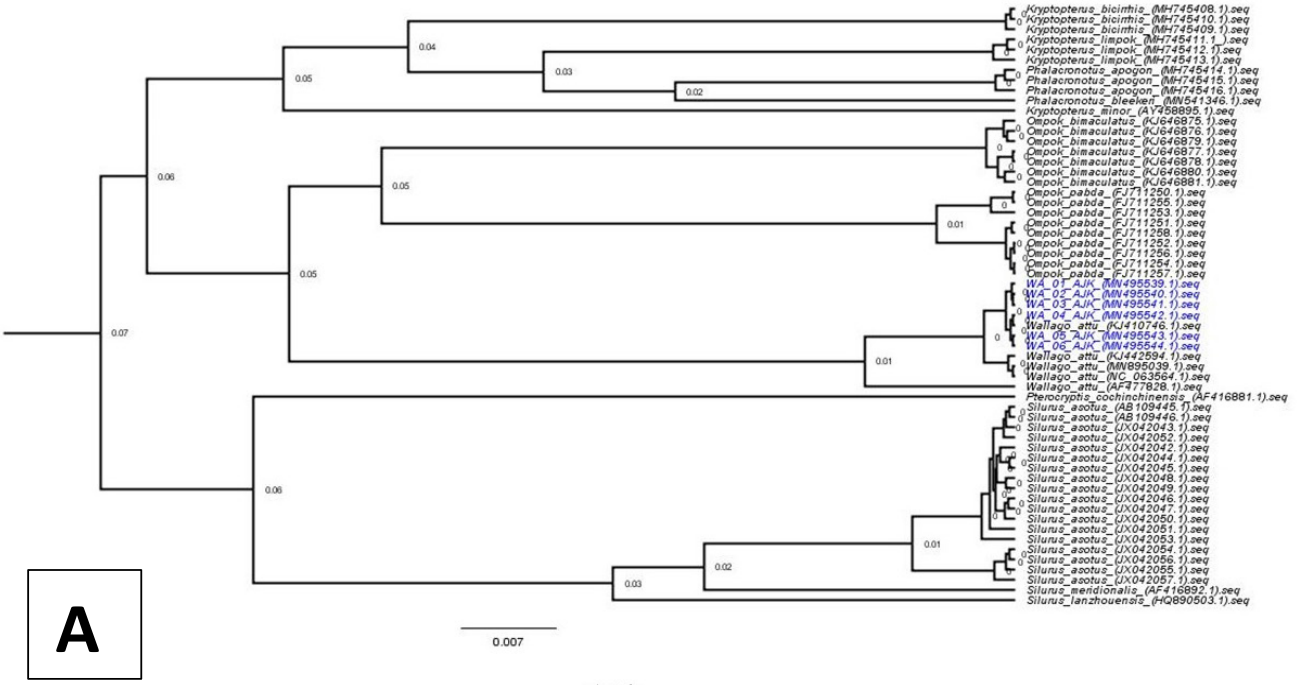
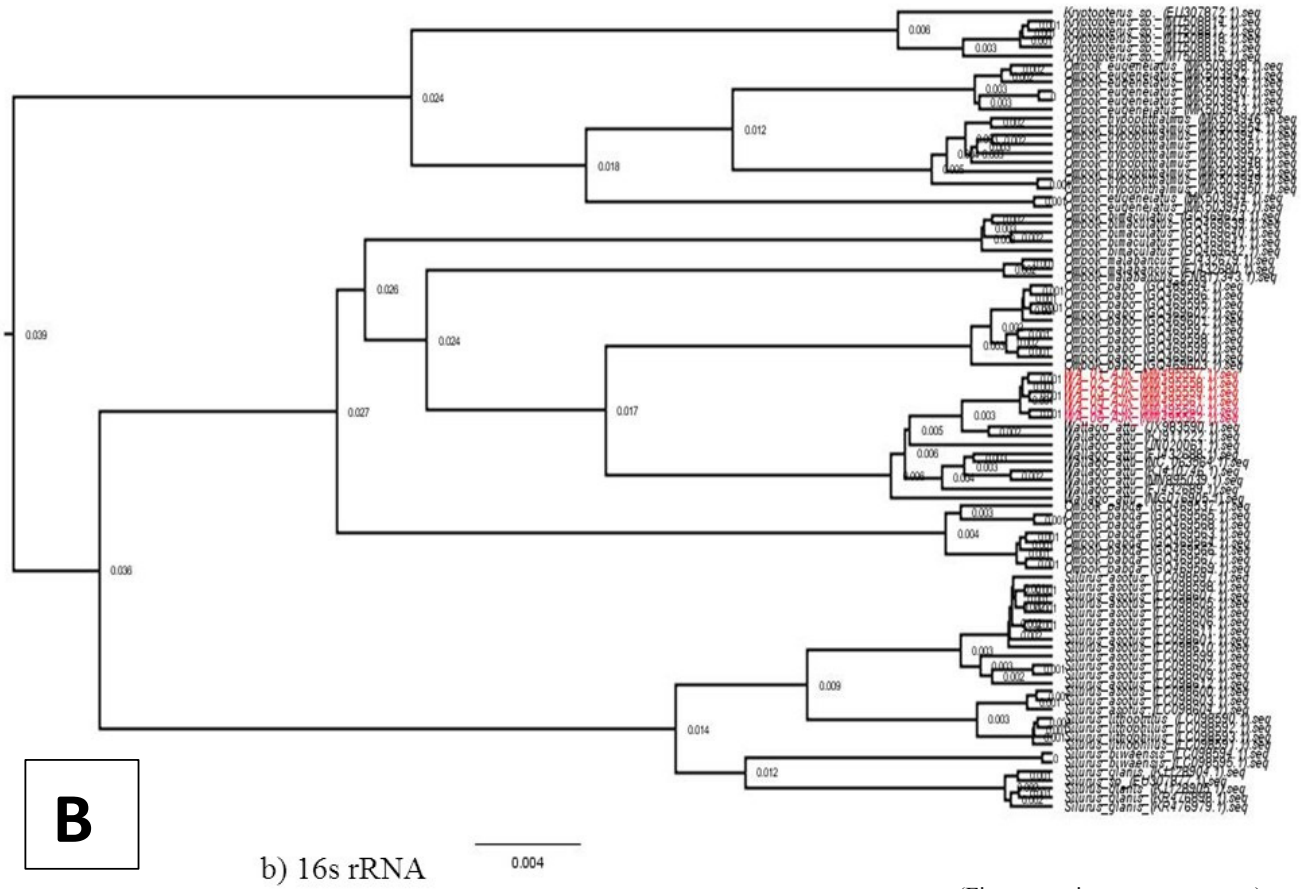


a) Ctb



b) 16s rRNA

(Figure continues on next page)

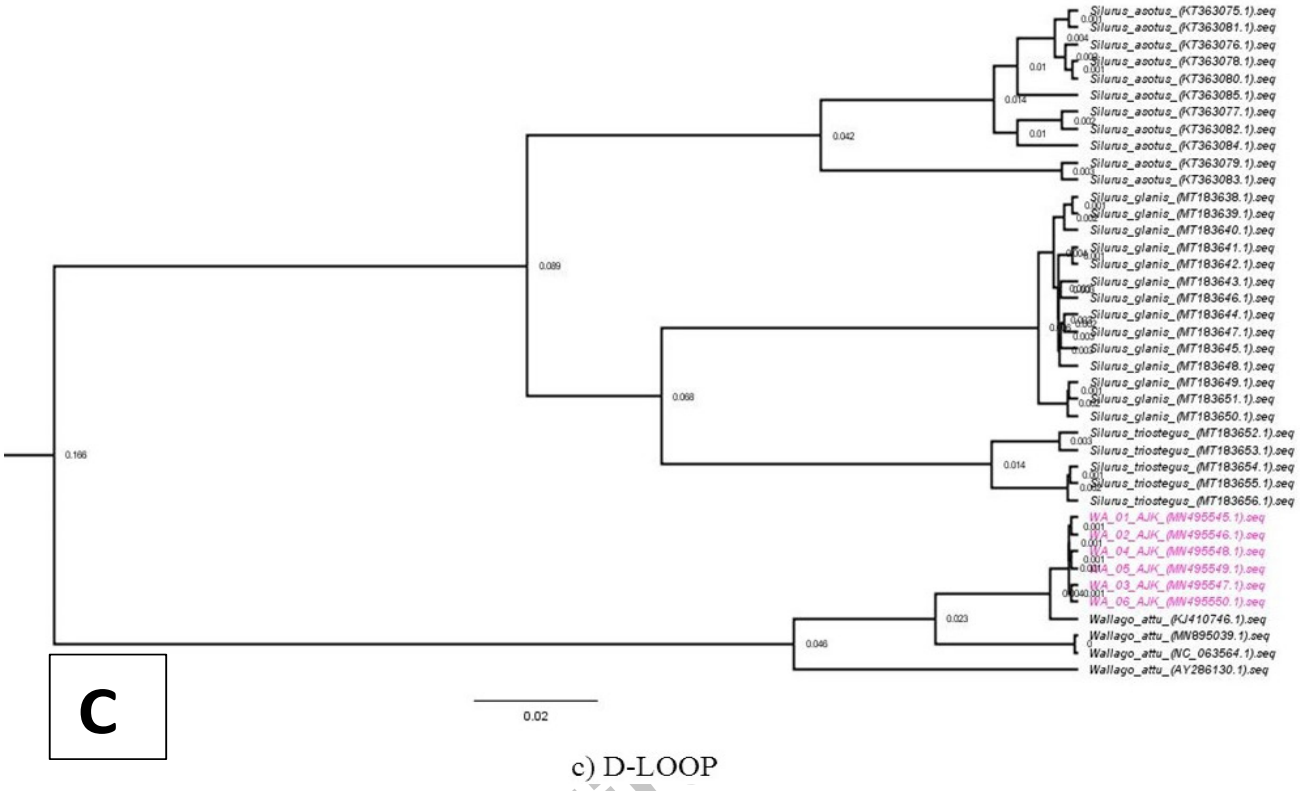


Fig. 5. Phylogenetic analysis of the representative Siluridae family, generated for (A) *Cytb* gene, (B) *16S rRNA*, and (C) D-Loop region. The phylogenetic tree was constructed using BEAST software. The studied sequences are highlighted in blue, red, and purple, respectively.

and Ali, 2016) and presented an observable anti-G bias observed in teleost fishes (Gilles *et al*., 2001; Zhu *et al*., 2013). In all sequences, an overall excess of thymine nucleotides compared to other nucleotide bases was observed, which has also been reported in other fish species (Sajjad *et al*., 2023; Meyer and Paulay, 2005; Karim *et al*., 2018). The authenticity of DNA barcoding depends on both intra and interspecific divergence. DNA barcoding attempts to identify the boundaries to define species, and also estimate the divergence between the closely related species (Hebert *et al*., 2003b; Chakraborty and Ghosh, 2014). In current study, the mean intraspecific distance was 1.0%, compared with 14.10% for species within genera; this result corresponds to the DNA barcoding principle that interspecific divergence sufficiently outscores intraspecific divergence (Bingpeng *et al*., 2018).

The base composition analysis of the COI, Cytb, 16S rRNA, and D-Loop region revealed that A+T contents were higher than G+C content, which is a result similar to the results found in Australian (Ward *et al*., 2005), Canadian (Hubert *et al*., 2008), Cuban (Lara *et al*., 2010), Taiwan (Bingpeng *et al*., 2018) and Pakistani fish species (Akhtar *et al*., 2020). Similarly, transitional substitutions rate was higher that follow the previous reports in teleost fish (Akhtar *et al*., 2020; Singh *et al*., 2012; Khan *et al*., 2016; Barik *et al*., 2018). The proportion of sites estimated to be invariable is 0.001%, specifying that mitochondrial genes are more virtually variable. The observed average number of pairwise differences (k) in Cytb, 16s rRNA and D-Loop was 59.14, 24.24, 155.04 while the observed variance of k was 641.32, 98.26 and 19883.21, respectively. Similarly, the Ramos-Onsins and Rozas (R2) was 0.175 in Cytb, 0.169 in 16S and 0.202 in D-Loop region. It was observed that the rate of mutation and substitutions was significantly high-er in observed values as compared to expected one (Fig. 4).

In the present study, we investigated the intraspecific genetic diversity of *W. attu* based on *mtDNA* gene sequences. The results revealed low nucleotide and haplotype diversity, which might reflect the impact of human activities, including habitat destruction, pollution, and overfishing (Whitfeld and Elliott, 2002). Genetic diversity can directly indicate the potential and ability of species to adapt to environmental changes (Frankham et

Table III. D-Loop region of family Siluridae with four termination associated sequence (TAS) and its reverse compliments (RC), Box, and conserved sequence blocks-D (CSB-D) were highlighted.

Species name	TAS-1	TAS-2	TAS-3	TAS-4	Box	CSB-D	RC-1	RC-2
S. asotus (KT363075.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363076.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363077.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363078.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363079.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363080.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363081.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363082.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363083.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363084.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. asotus (KT363085.1)	TACAT	TACAT	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183638.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183639.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183640.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183641.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183642.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183643.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183644.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183645.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183646.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183647.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183648.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. glanis (MT183649.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCT	ATGTA	AGGTA
S. glanis (MT183650.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCT	ATGTA	AGGTA
S. glanis (MT183651.1)	TACAT	TACAT	AACAT	TACAT	GTGGGGG	TGCTGGCATCTGGTTCT	ATGTA	AGGTA
S. triostegus (MT183652.1)	TACAT	GGCAC	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. triostegus (MT183653.1)	TACAT	GGCAC	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGTA	AGGTA
S. triostegus (MT183654.1)	TACAT	GGCAC	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGATCC	ATGTA	ATGTA
S. triostegus (MT183655.1)	TACAT	GGCAC	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGATCC	ATGTA	ATGTA
S. triostegus (MT183656.1)	TACAT	GGCAC	TACAT	TACAT	GTGGGGG	TACTGGCATCTGGATCC	ATGTA	ATGTA
WA 01 AJK (MN495545.1)	TACAT	AACAC	TACAT	CCCAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGCA	ATGTA
WA 02 AJK (MN495546.1)	TACAT	AACAC	TACAT	CCCAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGCA	ATGTA
WA 03 AJK (MN495547.1)	TACAT	AACAC	TACAT	CCCAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGCA	ATGTA
WA 04 AJK (MN495548.1)	TACAT	AACAC	TACAT	CCCAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGCA	ATGTA
WA 05 AJK (MN495549.1)	TACAT	AACAC	TACAT	CCCAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGCA	ATGTA
WA 06 AJK (MN495550.1)	TACAT	AACAC	TACAT	CCCAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGCA	ATGTA
W. attu (AY286130.1)	TACAT	AACAT	TACAT	CCCAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGCA	ATGTA
W. attu (KJ410746.1)	TACAT	TACAT	TACAT	CCCAT	GTGGGG-	TACTGGCATCTGGTTCC	ATGCA	ATGTA
W. attu (MN895039.1)	TACAT	TACAT	TACAT	CCCAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGCA	ATGTA
W. attu (NC_063564.1)	TACAT	TACAT	TACAT	CCCAT	GTGGGGG	TACTGGCATCTGGTTCC	ATGCA	ATGTA

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al., 2002; Spielman *et al*., 2004). Since *W. attu* has been classified as vulnerable by the IUCN, it is essential to pay more attention to its conservation. Our study focused on a single species, and our results showed relatively low genetic differences. These findings are consistent with previous studies by Mudumala *et al*. (2011) and Sajjad *et al*. (2023). In the mtDNA genome, D-Loop is non-coding and highly variable region with fast evolutionary rate (Dowling *et al*., 2008). Different domains in family *Siluridae* i.e., the termination associated sequence (TAS) domain, the central conserved sequence block (CSB-D) domain and its reverse complement (RC), and GTGGGGG BOX were also detected. In these sequences, we identified four TAS motif – TACAT – in 5′ region and its 'ATGTA' was also found near the 5`end. Though, D-Loop region is rapidly evolving and highly variable part of mtDNA, structurally, it consist of these domains as found in freshwater turtles of order- Geoemydidae (Jiang *et al*., 2011) and Trionychidae (Xiong *et al*., 2010). TAS domain and its RC near 5` end were recorded in turtles (Xiong *et al*., 2010) and also reported in current study but these motifs are not highly conserved in this family. TAS motifs were also reported in coregonid species and it was suggested that these motifs are involved in termination of replication process (Brzuzan and Ciesiels, 2002).

Phylogenetic tree analysis showed identical categorization regarding morphology and taxonomy, alongside non-significant differentiation at taxonomic levels. Siluriformes is among the most ecologically and economically important orders (Nelson, 2006; Ha *et al*., 2018). From an evolutionary perspective, it has been used as the model system to investigate historical biogeography (Kappas *et al*., 2016). It was reported that the phylogenetic positions of its families, such as Siluridae, Schilbeidae, Malapteruridae, Bagridae, Mochokidae, and Plotosidae, remained undefined (Hardman, 2005; Sullivan *et al*., 2006). In the current study, we have focused only on the *Siluridae* family. Phylogenetic trees were constructed based on mitochondrial Cytb, 16S rRNA, and D-Loop regions to understand relationships among closely related genera and species of the Siluridae family. The combined phylogenetic tree, with 58 Cytb (Fig. 5A), 91 16S rRNA (Fig. 5B), and 40 D-Loop (Fig. 5C) regions of the Siluridae family, revealed that *W. attu* formed a single group with other *W. attu* specimens retrieved from NCBI. At the genus level, a close link of *Wallago* with the genus *Ompok* was well resolved as a monophyletic clade and showed a sister relationship, while all the remaining genera (*Kryptopterus*, *Phalacronotus*, *Pterocryptis*, and *Silurus*) formed a separate clade. The most obvious difference observed was the arrangement between inter-generic relationships among the current Silurid species. In all the genes (Cytb, 16S rRNA, and D-Loop), the values of node ages ranged from 0 to 0.07, indicating that the divergence events are recent in the Siluridae family compared to older evolutionary divergences. The smaller the value, the more recent the divergence event. It is also suggested that these genera have diverged from their most recent common ancestors relatively recently in evolutionary history. The branch length between species is deeper than within species, similar to findings reported by Meyer and Paulay (2005).

A GTGGG-box (common to euteleosts), was also identified (Table III), and reported by Syed *et al*. (2016) in Indian Schizothoracinae. CSB-D was positioned downstream to other domains, and its sequence was TACTGGCATCTGGTTCC with the few intraspecific variation- transitions (C to T at 17th residues in *S. glanis*). Nilsson (2009) and Wang *et al*. (2011) also reported these region with the same relative position in other vertebrates. The consensus sequence of conserved sequence blocks in Siluridae was highly conserved and consistent with other reported data (Liu, 2002; Jin-Liang *et al*., 2006).

CONCLUSIONS

The continuous increase in available DNA barcodes facilitates the ongoing enhancement of DNA barcoding efficiency and the resolution of previously conflicting or unresolved cases. This is the first comprehensive attempt to develop a DNA based reference library for *W. attu* from Azad Kashmir, Pakistan. It is clearly indicated that the use of DNA barcodes for the identification of fish species is promising. The use of partial sequences of Cytb, 16S rRNA and D-Loop genes also clearly identify and separate *W. attu* samples from other genera of Siluridae. These genes also strengthen the results of DNA barcoding. This study has proved the authenticity of the molecular approach using mitochondrial DNA in the identification and phylogenetic analysis of family Siluridae. Therefore, present study considerably added the data of mtDNA for Pakistani catfish on Genbank database which was not available before. The Jhelum River hotspot of biodiversity, including fish, is under a high human impact pressure in the Mangla Dam area. While, *W. attu* is naturally a hardy fish, its populations are currently declining rapidly due to overharvesting, environmental degradation, pollution, and the lack of proper management. The observation of low genetic and haplotype diversity in *W. attu* also suggests a significant need for fisheries management and conservation efforts. New modern assessment and management tools based approaches, methods and techniques are highly needed for this area conservation. It is essential to implement monitoring and mitigation measures to address

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potential threats to fish populations and their habitats. Protecting and preserving this unique ecosystem holds paramount significance in maintaining ecological balance, supporting local livelihoods, and safeguarding the rich aquatic biodiversity for the benefit of future generations.

DECLARATIONS

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IRB approval

All experimental procedures were approved by the Board of Advanced Studies and Research (BASR), University of Azad Jammu and Kashmir, and were conducted in accordance with international guidelines for the care and use of laboratory animals.

Ethical approval

All animal experimental procedures were conducted in accordance with local and international regulations. The international regulation is the Wet op de dierproeven (Arti-cle 9) of Dutch Law (International). The Board of Advanced Studies and Research at the University of Azad Jammu and Kashmir, Muzaffarabad, granted permission to conduct this study. The Board of Advanced Studies and Research at the University of Azad Jammu and Kashmir in Muzaffarabad, Pakistan provided the permit to conduct this study. No specific permission was required for the collection sites.

Data availability statement

The data that support the findings of this study are openly available in repository under Accession: MN495539-MN495562 https://www.ncbi.nlm.nih.gov/nuccore MN495539-MN495562. All other sequences used in the study were retrieved from NCBI for comparative analysis.

Consent to participate

No human subjects or other vulnerable groups have been used for the study.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI or AI-assisted technologies were used in the preparation of this manuscript.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20240920112623

Statement of conflicts of interest

The authors have declared no conflict of interest.

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Supplementary Material

Molecular Identification of *Wallago attu* (Bloch and Schneider, 1801) and its Phylogenetic Association with other Silurids Based on Mitochondrial DNA

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Supplementary Table SI. The accession numbers of specimens subjected to genetic analysis.

COI	*CYTB*	*16S rRNA*	*D-Loop*
Belodontichthys dinema (JF781186.1)	*Kryptopterus bicirrhis (MH745408.1)*	*Kryptopterus sp. (EU307872.1)*	*Silurus asotus (KT363075.1)*
Belodontichthys dinema (JF781187.1)	*Kryptopterus bicirrhis (MH745409.1)*	*Kryptopterus sp. (MT508814.1)*	*Silurus asotus (KT363076.1)*
Belodontichthys dinema (MH633771.1)	*Kryptopterus bicirrhis (MH745410.1)*	*Kryptopterus sp. (MT508815.1)*	*Silurus asotus (KT363077.1)*
Belodontichthys dinema (MH757447.1)	*Kryptopterus limpok (MH745411.1)*	*Kryptopterus sp. (MT508816.1)*	*Silurus asotus (KT363078.1)*
Belodontichthys truncatus (MK448174.1)	*Kryptopterus limpok (MH745412.1)*	*Kryptopterus sp. (MT508817.1)*	*Silurus asotus (KT363079.1)*
Kryptopterus bicirrhis (KU568889.1)	*Kryptopterus limpok (MH745413.1)*	*Kryptopterus sp. (MT508818.1)*	*Silurus asotus (KT363080.1)*
Kryptopterus bicirrhis (KU568890.1)	*Kryptopterus minor (AY458895.1)*	*Ompok bimaculatus (GQ469623.1)*	*Silurus asotus (KT363081.1)*
Kryptopterus bicirrhis (MG981087.1)	*Ompok bimaculatus (KJ646875.1)*	*Ompok bimaculatus (GQ469639.1)*	*Silurus asotus (KT363082.1)*
Kryptopterus bicirrhis (MH732875.1)	*Ompok bimaculatus (KJ646876.1)*	*Ompok bimaculatus (GQ469640.1)*	*Silurus asotus (KT363083.1)*
Kryptopterus bicirrhis (MH732876.1)	*Ompok bimaculatus (KJ646877.1)*	*Ompok bimaculatus (GQ469641.1)*	*Silurus asotus (KT363084.1)*
Kryptopterus bicirrhis (MH732877.1)	*Ompok bimaculatus (KJ646878.1)*	*Ompok bimaculatus (GQ469642.1)*	*Silurus asotus (KT363085.1)*

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COI	***CYTB***	***16S rRNA***	***D-Loop***
Kryptopterus cheveyi (MK049459.1)	*Ompok bimaculatus (KJ646879.1)*	*Ompok eugeneiatus (MK503938.1)*	*Silurus glanis (MT183638.1)*
Kryptopterus cheveyi (MK448117.1)	*Ompok bimaculatus (KJ646880.1)*	*Ompok eugeneiatus (MK503939.1)*	*Silurus glanis (MT183639.1)*
Kryptopterus geminus (MK049457.1)	*Ompok bimaculatus (KJ646881.1)*	*Ompok eugeneiatus (MK503940.1)*	*Silurus glanis (MT183640.1)*
Kryptopterus geminus (MK049458.1)	*Ompok pabda (FJ711250.1)*	*Ompok eugeneiatus (MK503941.1)*	*Silurus glanis (MT183641.1)*
Kryptopterus geminus (MW343554.1)	*Ompok pabda (FJ711251.1)*	*Ompok eugeneiatus (MK503942.1)*	*Silurus glanis (MT183642.1)*
Ompok bimaculatus (MH749364.1)	*Ompok pabda (FJ711252.1)*	*Ompok eugeneiatus (MK503943.1)*	*Silurus glanis (MT183643.1)*
Ompok bimaculatus (MK359880.1)	*Ompok pabda (FJ711253.1)*	*Ompok eugeneiatus (MK503944.1)*	*Silurus glanis (MT183644.1)*
Ompok bimaculatus (MK359953.1)	*Ompok pabda (FJ711254.1)*	*Ompok eugeneiatus (MK503945.1)*	*Silurus glanis (MT183645.1)*
Ompok bimaculatus (MK448086.1)	*Ompok pabda (FJ711255.1)*	*Ompok hypophthalmus (MK503946.1)*	*Silurus glanis (MT183646.1)*
Ompok bimaculatus (MK448128.1)	*Ompok pabda (FJ711256.1)*	*Ompok hypophthalmus (MK503947.1)*	*Silurus glanis (MT183647.1)*
Ompok bimaculatus (MK448129.1)	*Ompok pabda (FJ711257.1)*	*Ompok hypophthalmus (MK503948.1)*	*Silurus glanis (MT183648.1)*
Ompok bimaculatus (MK448132.1)	*Ompok pabda (FJ711258.1)*	*Ompok hypophthalmus (MK503949.1)*	*Silurus glanis (MT183649.1)*
Ompok bimaculatus (MK448182.1)	*Phalacronotus apogon (MH745414.1)*	*Ompok hypophthalmus (MK503950.1)*	*Silurus glanis (MT183650.1)*
Ompok bimaculatus (MK572383.1)	*Phalacronotus apogon (MH745415.1)*	*Ompok hypophthalmus (MK503951.1)*	*Silurus glanis (MT183651.1)*
Ompok bimaculatus (MK572384.1)	*Phalacronotus apogon (MH745416.1)*	*Ompok hypophthalmus (MK503952.1)*	*Silurus triostegus (MT183652.1)*
Ompok bimaculatus (MK681757.1)	*Phalacronotus bleekeri (MN541346.1)*	*Ompok hypophthalmus (MK503953.1)*	*Silurus triostegus (MT183653.1)*
Ompok bimaculatus (MK814495.1)	*Pterocryptis cochinchinensis (AF416881.1)*	*Ompok hypophthalmus (MK503954.1)*	*Silurus triostegus (MT183654.1)*
Ompok bimaculatus (MK814498.1)	*Silurus asotus (AB109445.1)*	*Ompok malabaricus (FJ432679.1)*	*Silurus triostegus (MT183655.1)*
Ompok bimaculatus (MN083156.1)	*Silurus asotus (AB109446.1)*	*Ompok malabaricus (FJ432680.1)*	*Silurus triostegus (MT183656.1)*
Ompok bimaculatus (MN255382.1)	*Silurus asotus (JX042042.1)*	*Ompok malabaricus (FN811343.1)*	*WA 01 AJK (MN495545.1)*
Ompok eugeneiatus (MH732878.1)	*Silurus asotus (JX042043.1)*	*Ompok pabda (GQ469537.1)*	*WA 02 AJK (MN495546.1)*
Ompok eugeneiatus (MH732879.1)	*Silurus asotus (JX042044.1)*	*Ompok pabda (GQ469563.1)*	*WA 03 AJK (MN495547.1)*
Ompok eugeneiatus (MH732880.1)	*Silurus asotus (JX042045.1)*	*Ompok pabda (GQ469564.1)*	*WA 04 AJK (MN495548.1)*
Ompok eugeneiatus (MH732881.1)	*Silurus asotus (JX042046.1)*	*Ompok pabda (GQ469565.1)*	*WA 05 AJK (MN495549.1)*
Ompok eugeneiatus (MH732882.1)	*Silurus asotus (JX042047.1)*	*Ompok pabda (GQ469566.1)*	*WA 06 AJK (MN495550.1)*
Ompok eugeneiatus (MH732883.1)	*Silurus asotus (JX042048.1)*	*Ompok pabda (GQ469567.1)*	*Wallago attu (AY286130.1)*

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COI	***CYTB***	***16S rRNA***	***D-Loop***
Ompok eugeneiatus (MH732884.1)	*Silurus asotus (JX042049.1)*	*Ompok pabda (GQ469568.1)*	*Wallago attu (KJ410746.1)*
Ompok eugeneiatus (MH732885.1)	*Silurus asotus (JX042050.1)*	*Ompok pabda (GQ469569.1)*	*Wallago attu (MN895039.1)*
Ompok hypophthalmus (MH732886.1)	*Silurus asotus (JX042051.1)*	*Ompok pabo (GQ469594.1)*	*Wallago attu (NC_063564.1)*
Ompok hypophthalmus (MH732887.1)	*Silurus asotus (JX042052.1)*	*Ompok pabo (GQ469595.1)*	
Ompok hypophthalmus (MH732888.1)	*Silurus asotus (JX042053.1)*	*Ompok pabo (GQ469596.1)*	
Ompok hypophthalmus (MH732889.1)	*Silurus asotus (JX042054.1)*	*Ompok pabo (GQ469597.1)*	
Ompok hypophthalmus (MH732890.1)	*Silurus asotus (JX042055.1)*	*Ompok pabo (GQ469598.1)*	
Ompok hypophthalmus (MH732891.1)	*Silurus asotus (JX042056.1)*	*Ompok pabo (GQ469599.1)*	
Ompok hypophthalmus (MK473379.1)	*Silurus asotus (JX042057.1)*	*Ompok pabo (GQ469600.1)*	
Ompok hypophthalmus (MZ634366.1)	*Silurus lanzhouensis (HQ890503.1)*	*Ompok pabo (GQ469601.1)*	
Ompok hypophthalmus (MZ634367.1)	*WA 01 AJK (MN495539.1)*	*Ompok pabo (GQ469602.1)*	
Ompok hypophthalmus (MZ634368.1)	*WA 02 AJK (MN495540.1)*	*Ompok pabo (GQ469603.1)*	
Ompok hypophthalmus (MZ634369.1)	*WA 03 AJK (MN495541.1)*	*Silurus asotus (LC098597.1)*	
Ompok hypophthalmus (MZ634370.1)	*WA 04 AJK (MN495542.1)*	*Silurus asotus (LC098598.1)*	
Ompok hypophthalmus (MZ634371.1)	*WA 05 AJK (MN495543.1)*	*Silurus asotus (LC098599.1)*	
Ompok hypophthalmus (MZ634372.1)	*WA 06 AJK (MN495544.1)*	*Silurus asotus (LC098600.1)*	
Ompok hypophthalmus (MZ634373.1)	*Wallago attu (AF477828.1)*	*Silurus asotus (LC098601.1)*	
Ompok hypophthalmus (MZ634374.1)	*Wallago attu (KJ410746.1)*	*Silurus asotus (LC098602.1)*	
Ompok malabaricus (HQ009494.1)	*Wallago attu (KJ442594.1)*	*Silurus asotus (LC098603.1)*	
Ompok malabaricus (HQ009495.1)	*Wallago attu (MN895039.1)*	*Silurus asotus (LC098604.1)*	
Ompok malabaricus (MK238497.1)	*Wallago attu (NC_063564.1)*	*Silurus asotus (LC098605.1)*	
Ompok malabaricus (MW193708.1)		*Silurus asotus (LC098606.1)*	
Ompok malabaricus (ON076066.1)		*Silurus asotus (LC098607.1)*	
Ompok malabaricus (ON799404.1)		*Silurus asotus (LC098608.1)*	
Ompok pabda (LC314575.1)		*Silurus asotus (LC098609.1)*	
Ompok pabda (LC741140.1)		*Silurus asotus (LC098610.1)*	
Ompok pabda (MK359903.1)		*Wallago_attu_(NC_063564.1)*	
Ompok pabda (MK572385.1)		*Wallago attu (MN895039.1)*	
Ompok pabda (MK572386.1)		*Wallago attu (MG076905.1)*	

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COI	*CYTB*	*16S rRNA*	*D-Loop*
Ompok pabda (MN200457.1)		*Wallago attu (KJ911222.1)*	
Ompok pabda (MN259183.1)		*Wallago attu (KJ410746.1)*	
Ompok pabda (MN520013.1)		*Wallago attu (JX983590.1)*	
Ompok pabda (ON166101.1)		*Wallago attu (JN020061.1)*	
Ompok pabo (KX455911.1)		*Wallago attu (FJ432689.1)*	
Ompok pabo (KX946739.1)		*Wallago attu (FJ432688.1)*	
Ompok pabo (KX946740.1)		*WA 06 AJK (MN495562.1)*	
Ompok pabo (KY290090.1)		*WA 05 AJK (MN495561.1)*	
Ompok pabo (KY290091.1)		*WA 04 AJK (MN495560.1)*	
Ompok pabo (KY290092.1)		*WA 03 AJK (MN495559.1)*	
Ompok pabo (LC314570.1)		*WA 02 AJK (MN495558.1)*	
Ompok pabo (MK572387.1)		*WA 01 AJK (MN495557.1)*	
Ompok pabo (MK572388.1)		*Silurus_sp_(EU307877.1)*	
Ompok pabo (MN259173.1)		*Silurus_lithophilus_ (LC098593.1)*	
Ompok pabo (MT812128.1)		*Silurus_lithophilus_ (LC098592.1)*	
Ompok pabo (MT812129.1)		*Silurus lithophilus (LC098593.1)*	
Ompok pabo (MT812130.1)		*Silurus biwaensis (LC098594.1)*	
Ompok pabo (MT812131.1)		*Silurus biwaensis (LC098595.1)*	
Ompok pabo (MT812132.1)		*Silurus glanis (KJ128904.1)*	
Phalacronotus apogon (MH732892.1)		*Silurus glanis (KJ128905.1)*	
Phalacronotus apogon (MH732893.1)		*Silurus glanis (KR476898.1)*	
Phalacronotus apogon (MH732894.1)		*Silurus glanis (KR476979.1)*	
Phalacronotus apogon (MH732895.1)		*Silurus asotus (LC098610.1)*	
Phalacronotus apogon (MH732896.1)		*Silurus asotus (LC098611.1)*	
Phalacronotus apogon (MH732897.1)		*Silurus asotus (LC098612.1)*	
Phalacronotus apogon (MH745414.1)			
Phalacronotus apogon (MH745415.1)			
Phalacronotus apogon (MH745416.1)			
Phalacronotus bleekeri (OM345063.1)			

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COI	*CYTB*	*16S rRNA*	*D-Loop*
Phalacronotus bleekeri (OM345064.1)			
Phalacronotus bleekeri (OM345065.1)			
Phalacronotus bleekeri (OM345066.1)			
Phalacronotus bleekeri (OM345067.1)			
Phalacronotus bleekeri (OM345068.1)			
Phalacronotus bleekeri (OM345069.1)			
Phalacronotus bleekeri (OM345070.1)			
Pterocryptis anomala (MT424989.1)			
Pterocryptis anomala (MT424990.1)			
Pterocryptis anomala (MT424991.1)			
Pterocryptis anomala (MT424992.1)			
Pterocryptis anomala (MT424993.1)			
Pterocryptis anomala (MT424994.1)			
Pterocryptis anomala (MT424995.1)			
Pterocryptis anomala (MT424996.1)			
Pterocryptis anomala (MT424997.1)			
Pterocryptis anomala (MT424998.1)			
Pterocryptis anomala (MT424999.1)			
Pterocryptis anomala (MT425000.1)			
Pterocryptis anomala (MT425001.1)			
Pterocryptis barakensis (MN830284.1)			
Pterocryptis indicus (MN991970.1)			
Pterocryptis indicus (MN991971.1)			
Pterocryptis indicus (MN991972.1)			
Pterocryptis indicus (MN991973.1)			

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COI	*CYTB*	*16S rRNA*	*D-Loop*
Pterocryptis indicus (MN991974.1)			
Pterocryptis wynaadensis (KX946772.1)			
Pterocryptis wynaadensis (KX946773.1)			
Pterocryptis wynaadensis (KX946774.1)			
Silurus asotus (MT571988.1)			
Silurus asotus (MT571990.1)			
Silurus asotus (MT571991.1)			
Silurus asotus (MT884747.1)			
Silurus asotus (MT884748.1)			
Silurus asotus (MZ871108.1)			
Silurus asotus (MZ871109.1)			
Silurus asotus (MZ871110.1)			
Silurus asotus (MZ871111.1)			
Silurus asotus (MZ871112.1)			
Silurus glanis (KP237864.1)			
Silurus glanis (KP237865.1)			
Silurus glanis (MW564386.1)			
Silurus glanis (MW564426.1)			
Silurus glanis (MW649711.1)			
Silurus glanis (MW649712.1)			
Silurus glanis (MW649713.1)			
Silurus glanis (MW649714.1)			
Silurus glanis (MW649715.1)			
Silurus glanis (ON097387.1)			
Silurus glanis (ON097574.1)			
Silurus glanis (ON097655.1)			
Silurus glanis (ON097757.1)			
Silurus glanis (ON097782.1)			
Silurus meridionalis (MF123166.1)			
Silurus meridionalis (MK477621.1)			
Silurus meridionalis (MT572018.1)			
Silurus meridionalis (MT572019.1)			
Silurus meridionalis (MT572020.1)			
Silurus meridionalis (MT884749.1)			
Silurus meridionalis (MT884750.1)			
Silurus meridionalis (MZ871113.1)			
Silurus meridionalis (MZ871114.1)			
Silurus meridionalis (OL494309.1)			
Silurus microdorsalis (HQ536514.1)			

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COI	***CYTB***	***16S rRNA***	***D-Loop***
Silurus microdorsalis (OL674455.1)			
Silurus microdorsalis (OL674456.1)			
Silurus microdorsalis (OL674457.1)			
Silurus soldatovi (MT413345.1)			
WA 01 AJK (MN495551.1)			
WA 02 AJK (MN495552.1)			
WA 03 AJK (MN495553.1)			
WA 04 AJK (MN495554.1)			
WA 05 AJK (MN495555.1)			
WA 06 AJK (MN495556.1)			
Wallago attu (FJ170767.1)			
Wallago attu (FJ170768.1)			
Wallago attu (FJ170769.1)			
Wallago attu (FJ170770.1)			
Wallago attu (FJ170771.1)			
Wallago attu (HQ009498.1)			
Wallago attu (HQ009499.1)			
Wallago attu (JX983591.1)			
Wallago attu (KJ911223.1)			
Wallago attu (MG981086.1)			
Wallago attu (MH197257.1)			
Wallago attu (MK359980.1)			
Wallago attu (MK577971.1)			
Wallago attu (MK714085.1)			
Wallago attu (MK814491.1)			
Wallago attu (MK814509.1)			
Wallago attu (MK814520.1)			
Wallago attu (MN259185.1)			
Wallago attu (MT670295.1)			
Wallago attu (MT812143.1)			
Wallago attu (MT812144.1)			
Wallago attu (MT812145.1)			
Wallago attu (MT812146.1)			
Wallago attu (MT812147.1)			
Wallago attu (MW074290.1)			
Wallago attu (MW150844.1)			
Wallago attu (MW150845.1)			
Wallago attu (MW150846.1)			
Wallago attu (MW326662.1)			
Wallago attu (MZ312383.1)			
Wallago attu (MZ461935.1)			

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COI	*CYTB*	*16S rRNA*	*D-Loop*
Wallago attu (MZ895367.1)			
Wallago attu (MZ895368.1)			
Wallago attu (MZ895369.1)			
Wallago attu (MZ913725.1)			
Wallago attu (MZ913726.1)			
Wallago attu (OK256189.1)			
Wallago attu (ON109396.1)			
Wallago attu (OP048833.1)			
Wallago attu (OP048834.1)			
Wallago attu (OP048835.1)			
Wallago attu (OP048836.1)			
Wallago attu (OP048837.1)			
Wallago attu (OP048838.1)			
Wallago attu (OP048839.1)			
Wallago attu (OP048840.1)			
Wallago attu (OP048841.1)			
Wallago attu (OP048842.1)			
Wallago attu (OP048843.1)			
Wallago attu (OP048844.1)			
Wallago attu (OP048845.1)			
Wallago attu (OP048846.1)			
Wallago attu (OP048847.1)			
Wallago attu (OP048848.1)			
Wallago attu (OP048849.1)			
Wallago leerii (MN992975.1)			
Wallago leerii (MN992976.1)			
Wallago micropogon (MK448131.1)			

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