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Research Article

Genetic Diversity and Cryptic Species Identification of Genus *Triplophysa* from River Swat in Malakand Division, Khyber Pakhtunkhwa, Pakistan

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Fish are cold blooded vertebrates' identification on the bases of morphology is not more precise and required high taxonomic expertise therefore molecular identification is used as an alternative and more accurate technique for the identification of fishes. In the current investigation 39 fish specimens were collected from May 2021 to February 2022. With this aim, the recent study was conducted in the freshwaters of River Swat in Malakand Division, Khyber Pakhtunkhwa, Pakistan to explore the genetic diversity of genus *Triplophysa*. DNA was extracted and amplified using gene specific primers. The PCR products carefully were sequenced and Phylogenic analysis was performed using neighbor-joining, maximum likelihood through MEGA software. Nucleotide composition and genetic pairwise distance recommend that the calculated species of the subfamily Nemacheilinae are organically dissimilar. The River Swat is the study area and it is inhabited by three *Triplophysa* species, *Triplophysa naziri, Triplophysa microps,* and *Triplophysa choprai*. The evolutionary tree shows that these species are clearly separated. The mean of total length calculated in the three species of the genus *Triplophysa* such as 8.175 ± 0.198 cm for *Triplophysa naziri*, 10.14 ± 0.35 cm for *Triplophysa microps*, 11.052 ± 0.23 cm for *Triplophysa choprai*. This study provides a model for the improvement of identification in cryptic diversity and field of ichthyofauna.

1. Introduction

Fish are cold blooded lower vertebrate with fins and gills which primarily depend on water for their survival. They have various species which are different from one another because of habitats, size, and morphology. Some species constantly live in a particular environment while others have high capability of migration [1]. Based on Salazar [2]; over half of all living vertebrate species (about 32,000 species) are fish. India there are 2,500 species of fishes of which 930 live in freshwater and 1,570 are marine India one of the mega biodiversity countries in the world and occupies the ninth position in terms of freshwater mega biodiversity [3]. Primarily genetic diversity is used to distinguish between and within different species and populations, and health depends mainly on the expression of immune genes and other physiological indicators. For population survival and evolution, genetic diversity is the core of biological studies. Therefore, fragmentation and degradation of genetic diversity occurring naturally only in a long term without anthropogenic actions and environmental stressors [4]. In Pakistan, DNA based approach has been used for different fish species for diversity and cryptic species identification, including *Schizothorax plagiostomus* using *cytochrome b* gene and *D loop* region of mitochondrial DNA [5]. Among the cryptic species, the Genus *Triplophysa* phenotype is

certainly by biotic features and external environment. Thus, it becomes difficult to differentiate them on morphological bases. *Triplophysa* has a long confusing classification history therefore, the cryptic diversity of the genus *Triplophysa* will be examined in the study. It belongs to the subfamily Nemacheilinae [6]. The diversity of fish is so difficult on morphologically based therefore mitochondrial and nuclear gene sequences have been routinely used because the mitochondrial genomic part is mostly carrying heredity information [7] The current investigation provides information on the genotype of a few Nemachilidae species from Iranian inland waters, including *Paraschistura bampurensis, Oxynoemacheilus kiabii, Turcinemacheilus saadii, Leuciscine cyprinid, Alburnoides bipunctatus and Alburnus alburnus* use mitochondrial gene *COI* [8]. Genus *Triplophysa* fit into Phylum chordata, Superclass Actnopterygii, Class Teleostei, order cypriniformes, family Balitoridae, subfamily Nemacheilinae. The genus *Triplophys*a considered the largest groups of the family Nemacheilinae, with about 130 nominal species worldwide [9–12]. The genus *Triplophysa* distribution in Pakistan has been 4 *Triplophysa* species documented from River Kunhar and River Barandu in Northern Pakistan [13]. There are some doubtful species reported from Pakistan and their presence in the country needs to be confirmed. Some of the species recorded as new from Pakistan have been reported from a single locality and such reports have not been supported by follow-up studies and no information is available regarding their distribution ranges. The taxonomic status of many fish species of the subfamilies Nemacheilinae and Schizothoracinae have remained highly controversial. There are also some nomenclatural and taxonomic problems in the other groups of fishes [14]. Two genera of fishes, including *Schistura* and *Triplophysa* of the subfamily Nemacheilinae have been documented in the fish fauna of Pakistan. But there is still uncertainty and doubt amongst the taxonomists for the identified species [15, 16]. The excellent plans for a morphometric and molecular investigation of the genus Triplophysa to verify doubtfulness among taxonomists. The DNA analysis technology is beneficial for the molecular identification of fish species, such as the molecular characterization *cytochrome c oxidase subunit 1* amplifying and DNA analysis to construct phylogenetic trees and find its divergence and lineage. The current investigation was designed to explore the genetic diversity and cryptic species of the genus *Triplophysa* identified from River Swat in Malakand Division, Khyber Pakhtunkhwa, Pakistan, through morphometric and molecular methods.

2. Materials and Methods

2.1. Study Area. Malakand Division is enclosed by green jungle, high mountains, glaciers, and a large amount of water resources. Swat River flows in District Swat and it provides a good habitat for a diverse group of ichthyofauna. The geographical location of River Swat (R3) is (34°06'60.00" N, 71°42'59.99" E) in Malakand Division, Khyber Pakhtunkhwa, Pakistan as shown in Figure 1.

2.2. Sample Collection. A Total 39 fish specimens were collected from May 2021 to February 2022 with the help of hand nets mesh size 0.5 cm to 1.0 cm from a geographic site in Malakand Division.

2.3. Preservation. The freshly captured specimens were brought to the Laboratory of Zoology department, Centre of Animal Sciences and Fisheries, University of Swat, and preserved in 95% ethanol for molecular study and 10% formalin for identification and confirmation. Each specimen was marked according to locality, time, and date of collection [17].

2.4. Taxonomic, Morphometric and Meristic Parameters Analysis. A dichotomous taxonomic key was prepared for the collected fish samples. This taxonomic key is used for identification of specimens (as shown in Table 1) Each specimen was weighed by a digital scale. The morphometric parameters were measured including Total Length (TL), Standard Length (SL), Forked Length (FL), Head Length (HL), Body Depth (BD), Eye Diameter (ED) and Snout Length (SL), with the help of scale and digital caliper which take in centimeter (as shown in Tables 2 and 3). The meristic counts of each fish specimen were done with the help of a magnifying lens (Table 4). The data was calculated as Mean, Standard deviation was subjected to one-way ANOVA analysis using Microsoft Excel [18].

2.5. Species Identification. The fish specimens were identified by the help of accessible literature and keys [18–22].

2.6. Extraction of DNA, Amplification and Sequencing. The genomic DNA was extracted from the fin tissues of fish by the help of Phenol: chloroform: Isoamyl alcohol method [23]. The excellence quantity and quality of genomic DNA was assessed using 0.8% agarose gel electrophoresis and genomic DNA was preserved at −20°C for future use. For amplification, Polymerase chain reaction (PCR), primers were designed which were based on the original mitochondrial genome and DNA sequences of Nemacheilinae fish. PCR was done to amplified the gene Sequences of *COI*. The *COI* primer pairs with sequences Fishf1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and FishR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) were designed based on mitochondrial genome of fish and amplify 648-563 bp *COI* fragment [24]. The PCR was performed in a 25 μl volume reaction in a thermal Cycler with a mixture containing 1 μl genomic DNA, 0.5 μL forward primer, and 0.5 μl Reversed primer = 1 μl primer, master mix 12.5 μl and the final volume was adjusted with sterile distilled water 10.5 μl. The thermal Cycler parameters were followed as initial denaturation at 94°C for 04 minutes, followed by 35 cycles of 94°C for 30 sec, 56°C for 35 sec, and 72°C for 45 sec and final extension at 72°C for 10 minutes and stored on 4°C. PCR products were run on 1% agarose gel electrophoresis and kept in Gel-Doc system visualized under ultraviolet

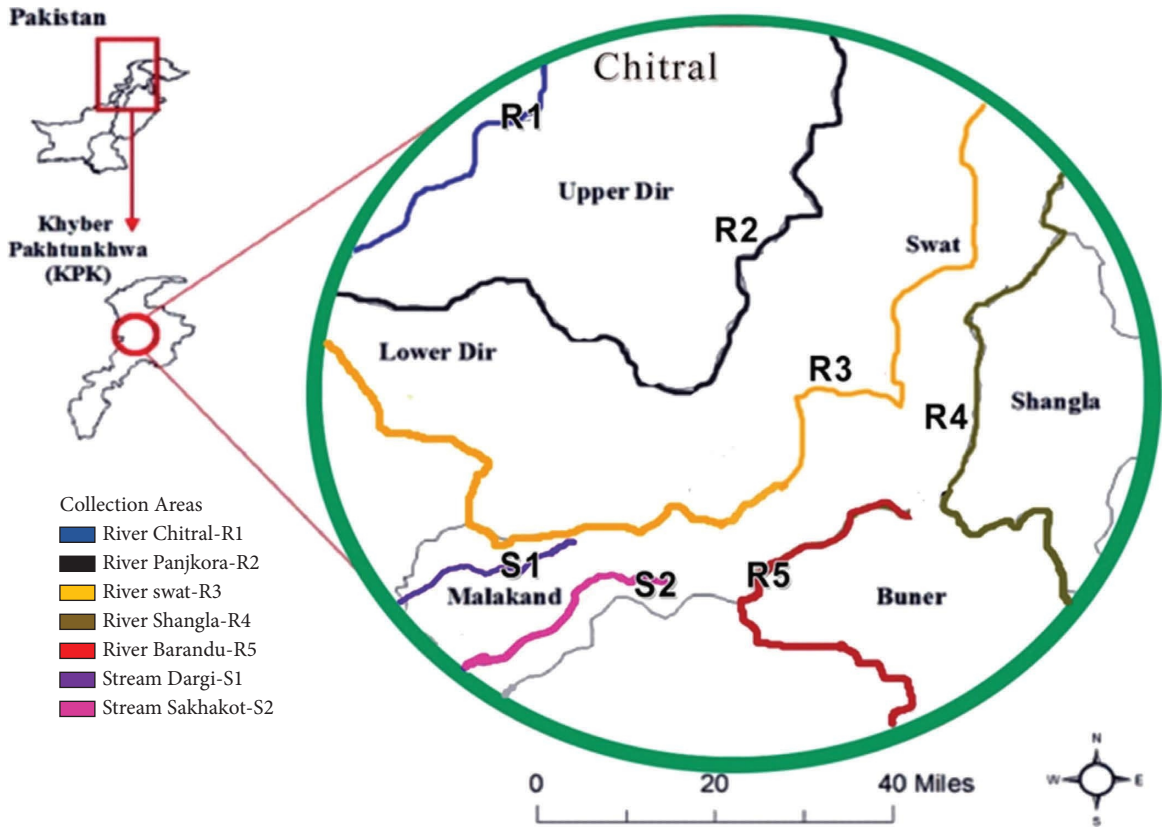

FIGURE 1: The freshwater bodies of Malakand division, Pakistan. These water bodies comprised different rivers and their tributaries including; river Chitral (R1), river Panjkora (R2), river Swat (R3), river Shangla (R4), river Barandu (R5). Dargi stream (S1) and Sakhakot stream (S2).

TABLE 1: The dichotomous key for genus *Triplophysa* fishes of Malakand division, Khyber Pakhtunkhwa, Pakistan.

	Characteristics
1a	Notochord, hollow nerve cord that lies dorsal to the notochord and pharyngeal pouches ----------phylum: chordata
1a (i)	Having living endoskeleton, advanced nervous system, presence of the spinal cords and vertebrae ----------sub-phylum: vertebrata
1a (ii)	The swim bladder is divided into two chamber, minute, unicellular, horny projections known as unculi are commonly present and an alarm substance that is part of a fright reaction ----------super-order: Ostariophysi
1a (iii)	Generally having flexible leptoid scales, the branchiostegal rays evolved from the bones at the base of the branchial cavity and branchiostegal rays class: Actinopterygii
1a (iii) b	No scales on head. No teeth on jaws. Mostly a single dorsal fin. No spine in pectoral fin ----------ordercypriniform
Key to families	
1a	Barbels six and scales on the body if present are non-prominent rather indistinct----------FAMILY NEMACHEILIDAE
Key to genera	
1a	Body bears irregular patterens or spots, caudal peduncle long and cylindrical---------- genus *Triplophysa* Rendahl
Key to species: genus *Triplophysa* [19]	
1a	Caudal fin is truncate or rounded ----------2
B	Forked type caudal fin----------*T. choprai* (Hora)
2a	L. L. incomplete----------*T. microps* (Steindachner)
B	L. L. complete----------*T. naziri* (Ahmad and Mirza)

Table 2: Morphological measurements of collected fish in river Swat, Malakand division, Khyber Pakhtunkhwa, Pakistan.

	Triplophysa naziri	*Triplophysa microps*	*Triplophysa choprai*	Mean	S D
Total length (cm)	8	10.2	11	9.66	1.55
Forked length (cm)	6.5	9.0	9.5	8.33	1.60
Standard length (cm)	5.5	8.3	8.7	7.5	1.74
Head length (cm)	1.6	1.7	1.9	1.73	0.40
Body depth (cm)	1.5	1.5	1.6	1.53	0.25
Eye diameter (cm)	0.29	0.3	0.3	0.175	0.42
Snout length (cm)	2.9	2.57	2.6	2.69	0.18
Weight (gram)	3.21	4	4.9	4.036	0.398

S D = standard deviation.

Table 3: Proportional measurement of genus *Triplophysa* species of *naziri-microps-choprai* complex.

	Triplophysa naziri $n = 10$ Mean ± S D	*Triplophysa microps* $n = 10$ Mean ± S D	*Triplophysa choprai* $n = 10$ Mean ± S D
Total length (cm)	8.175 ± 0.198	10.14 ± 0.35	11.052 ± 0.23
Forked length (cm)	6.394 ± 0.1647	9.02 ± 0.154	9.10 ± 0.21
Standard length (cm)	5.421 ± 0.095	8.26 ± 0.078	8.29 ± 0.09
Head length (cm)	1.542 ± 0.59	1.74 ± 0 .113	1.70 ± 0.02
Body depth (cm)	1.499 ± 0.06	1.491 ± 0.023	1.54 ± 0.12
Eye diameter (cm)	0.269 ± 0.036	0.28 ± 0.81	0.3 ± 0.01
Snout length (cm)	2.8 ± 0.078	2.53 ± 0.045	2.5 ± 0.02
Weight (gram)	3.21 ± 0.48	3.6 ± 0.51	4.5 ± 0.5

Table 4: Difference in morphological characters among the fishes of *Triplophysa* complex comparison of fin rays counted during identification of genus *Triplophysa* in the fresh water of river Swat Malakand division Khyber Pakhtunkhwa, Pakistan.

Characters	*Triplophysa naziri*	*Triplophysa microps*	*Triplophysa choprai*
Dorsal fin rays	2/7	2/10 – 12	17
Anal fin rays	2/5	2/5	7
Pectoral fin rays	10	11	13-14
Pelvic fin rays	8	8	17
Caudal fin rays	14	17	21

illumination before Sanger Sequencing. The pure clarified 3 samples were carefully chosen for sequencing. The Sanger sequencing was performed bi-directionally for discrimination of cryptic species identification.

2.7. Bioinformatic Analysis. Obtained sequence files of *COI* genes were edited and aligned by using Bio Edit software and Clustal W [25]. The *COI* sequences from each specimen were blasted against the GenBank and BOLD databases to find the range of variation of cryptic species. For Phylogenetic analysis, the Neighbor Joining was used to calculate the pairwise genetic distance by the help of software MEGA version x [26].

2.8. Results. The current study was designed to explore Genetic diversity and cryptic species identification of the genus *Triplophysa* from River Swat in Malakand Division, Khyber Pakhtunkhwa, Pakistan. In the present study three species of the genus *Triplophysa* (*Triplophysa naziri, Triplophysa microps,* and *Triplophysa choprai)* were identified (as shown in Figures 2–4) by the help of a dichotomous taxonomic key (as shown in Table 1).

Figure 2: *Triplophysa naziri.*

2.9. Morphological Study

2.9.1. Morphometric Measurement. Morphological measurements of the species showed that the largest species among the collected species were *Triplophysa choprai* as shown in Tables 2 and 3.

2.9.2. Meristic Parameter Triplophysa Complex. The comparison of fish fin rays was made to find the difference between the fishes of the genus *Triplophysa. Triplophysa naziri* has Dorsal Fin Rays 2/7, Anal Fin Rays 2/5, Pectoral Fin Rays 10, Pelvic Fin Rays 8 and Caudal Fin Rays are 14.

Triplophysa microps has Dorsal Fin Rays 2/10 – 12, Anal Fin Rays 2/5, Pectoral Fin Rays 11, Pelvic Fin Rays 8 and Caudal Fin Rays are 17. *Triplophysa choprai has* Dorsal Fin rays 17, Anal Fin Rays 7, Pectoral Fin Rays 13-14, Pelvic Fin Rays 17 and Caudal Fin Rays 21 as shown in Table 4.

2.10. Molecular Study. The current investigation was based on mitochondrial gene *COI* to explore genetic diversity and cryptic species identification of the genus *Triplophysa*. Prior to molecular identification, the obtained samples had first been examined for morphological features in saving time and sequencing costs.

2.11. Phylogenetic Study

2.11.1. Phylogenetic Study Query Samples of Triplophysa Species from River Swat. The nucleotide sequence of COI gene of each species was blast and each blast sequence showed similarity with the sequences of the GenBank, which ranged from 97–100%. So, it was confirmed that these are valid species of the genus *Triplophysa*. All query samples of *Triplophysa* species were analyzed along with highly similar seventeen (*COI*) database reference sequences retrieved from GenBank. The 20 nucleotide sequences were aligned through Clustal W and Muscle alignment was done with the help of MEGA software. The Neighbor Joining method was applied to conclude the evolutionary history of all query samples of *Triplophysa species* in River Swat Malakand Division. The Phylogenetic tree was gained. The phylogenetic trees are seen next branches where related taxa are clustered together. The starting tree for systematic search was obtained by using a matrix of pairwise distances projected using the Maximum Composite Likelihood (MCL) technique. The analysis involved 30 nucleotide sequences *COI, and* all query samples of *Triplophysa species* lie in Clade I Showing similarity with the species of this clade as shown in Figure 5.

2.12. Genetic Diversity. A total of 39 fish specimens of genus *triplophysa* were identified morphologically from this study area.For the genetic diversity of most clarified three species were analysed genetic Pairwise distance for three species (*Triplophysa naziri, Triplophysa microps, and Triplophysa choprai* through MEGA software as shown in Table 5. The fish of genus *Triplophysa* species nucleotide discrimination revealed varied GC% (Guanine + Cytosine) and AT% (Adenine + Thiamine) contents of mitochondrial gene (*COI*). Among 3 freshwater fish of the genus *Triplophysa* detected nucleotide base composition analyzed sequences through MEGA software. The results of these freshwater fish of the genus *Triplophysa* species show that the total nucleotide composition contained more AT% than GC% as shown in Figure 6.

3. Disscussion

Fish are vertebrate animals belonging to the phylum Chordata species found in salt and fresh waters of the world and highly diverse groups of chordates are found in each niche of the hydrosphere. The molecular approach is considered backbone

Table 5: The genetic Pairwise distance for three species (*Triplophysa naziri, Triplophysa microps, and Triplophysa choprai* through MEGA software.

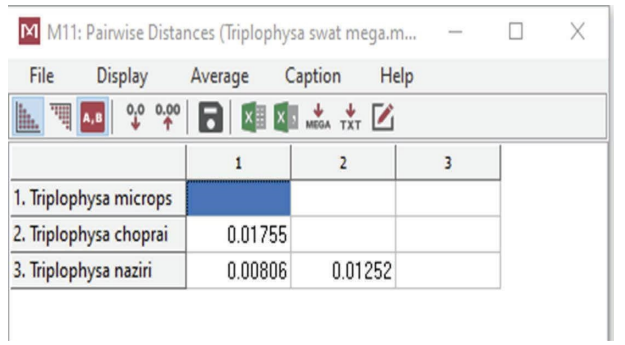

Figure 3: *Triplophysa microps.*

for evaluating fish species evolutionary variation and conservation process for biological resources [27]. Unfortunately, as far as the rivers system of Pakistan is concerned, no confirmed data about the genetic diversity of the genus *Triplophysa* is available. Therefore, in the present study attempts were made to identify and estimate the genetic diversity and cryptic species identification of the genus *Triplophysa* in the fresh waters of River Swat Malakand Division. This current investigation was based on mitochondrial *COI* gene sequences for the three studied species (*Triplophysa naziri*, *Triplophysa microps,* and *Triplophysa choprai)* of the genus *Triplophysa*. The genetic Pairwise distance for three species (*Triplophysa naziri, Triplophysa microps* and *Triplophysa choprai) was done* through MEGA software. Nucleotide Discrimination among River Panjkora fish of genus Triplophysa as shown in Figure 3. Phylogenic analysis was performed using neighbor-joining, maximum likelihood. Nucleotide composition and genetic pairwise distance recommend that the calculated species of subfamily Nemacheilinae are organically dissimilar [6]. The fish diversity study was conducted to evaluate the genetic diversity and cryptic species identification of the genus *Triplophysa* in the fresh waters of Malakand Division through a molecular and morphological approaches [28]. In the present study three species (*Triplophysa naziri*, *Triplophysa microps* and *Triplophysa choprai) of* genus *Triplophysa* were recorded from River Swat in Malakand Division, Khyber Pakhtunkhwa, Pakistan. In this study the morphometric and meristic parameters were subjected to statistical analysis. A total of 39 fish specimens of genus *Triplophysa* were identified morphologically from the study area. It was found that the three species differ significantly in morphometric and meristic

Figure 4: *Triplophysa choprai*.

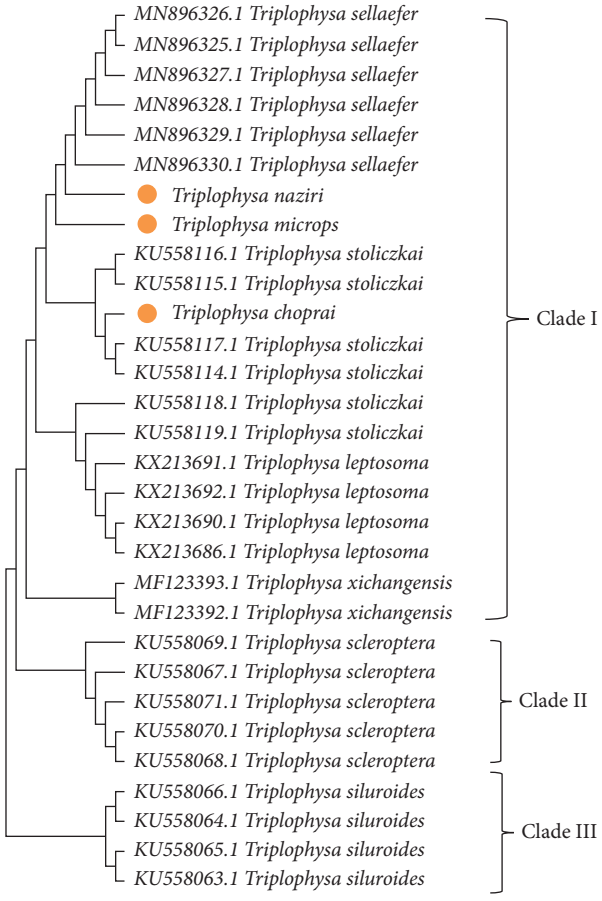

Figure 5: Construct phylogenetic tree of *COI* sequences of all query samples of *Triplophysa* species from river Swat in Malakand division, Khyber Pakhtunkhwa, Pakistan through MEGA software.

parameters like Total Length, Forked Length, Standard Length, Head Length, Body Depth, Eye Diameter, Snout Length, Weight, and meristic counts such as Dorsal Fin Rays, Anal Fin Rays, Pectoral Fin Rays, Pelvic Fin rays and Caudal Fin Rays. So, the morphometric and meristic parameters are necessary to treat these three species as valid in the genus *Triplophysa*. Further study of molecular identification is required to determine the identification issues of all fish species in Pakistan. The Molecular identification of fish is brilliance and shorthand procedure to identified doubt fish species of Pakistan. This type of data is more necessary for the purposes of development of cryptic diversity in Pakistan.

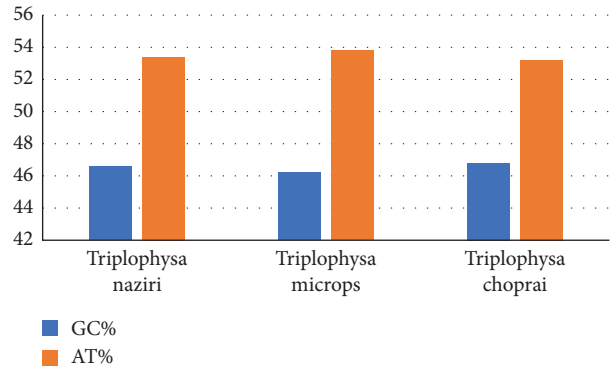

Figure 6: Nucleotide discrimination among freshwaters fish of genus *Triplophysa species* of *cytochrome COI* gene.

4. Conclusion

The current investigation was conducted to study the genetic diversity and cryptic species identification of the genus *Triplophysa* in River Swat can be concluded that large ichthyofauna diversity present in fresh waters of River Swat. Molecular identification is not a conversant exercise in Pakistan. Molecular identification is one of the important approaches compared to various old-style identification methods. The three species identified of genus *Triplophysa (Triplophysa naziri, Triplophysa microps,* and *Triplophysa choprai)*. The genetic diversity, morphometric, and meristic *parameters* analysis will be significant in the future for proper identification of fish fauna.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Omer Dad, Muhammad Fiaz Khan, and Mohammad Attaullah collected the fish specimens. Muhammad Fiaz Khan did Morphometric identification of fishes. Omer Dad and Naveed Akhtar performed the molecular biology work and sequence analysis. Akhtar Rasool performed phylogenetic and genetic analysis. Omer Dad and Naveed Akhtar wrote the manuscript.

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