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IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF CHANNA SPECIES FROM RIVERINE SYSTEM OF PAKISTAN USING COI GENE AS A DNA BARCODING MARKER

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ABSTRACT

Channa are the freshwater and important food fish species in Pakistan belonging to family Channidae. However, identification and phylogenetic analysis based on molecular tools of these species in Pakistan was not well known. Herein, the current investigation was conceptualized, which dealt with mitochondrial DNA sequences from three geographically distinct populations of this species from Pakistan's water system. DNA from fin tissues was extracted. COI region of mtDNA was amplified using universal primers for fish. PCR products were sequenced. Phylogenetic analysis conducted in the present study, i.e. neighbor-joining (NJ) cladogram, maximum likelihood, K2P genetic divergence and histogram suggests that the studied species of family Channidae are genetically different. The K2P intraspecific divergences were lower than interspecific divergences. The clades in the evolutionary tree for three species were clearly separated.

Keywords: Channidae, COI, phylogeny, barcode gap, molecular identification.

INTRODUCTION

A better understanding of the taxonomy of freshwater fish species has become crucial today because of the serious conservation concerns as well as the importance of fish as a major source of protein diet available to society (Lakra et al., 2016; Afridi et al., 2019). Traditionally, morphology-based taxonomy has been used for identification of species which might not be reliable. Recently, molecular techniques are being used as a reliable and precise approach for systematics (Ward et al., 2009), especially DNA barcoding for the purpose of identification of species as well as species discovery (Hajibabaei et al., 2006). The efficacy of this approach is well tested and can be considered relatively reliable for both vertebrates and invertebrates (Hebert et al., 2004; Hajibabaei et al., 2006; Lakra et al., 2016). Several successful studies concerning fish fauna have been reported using this method particularly for freshwater fisheries (Ward et al., 2005; Lakra et al., 2011; Landi et al., 2014; Yaqub et al., 2019). Additionally, many of these studies have contributed to building a large-scale collection of barcoding data available at BOLD (Costa et al., 2007; Kamran, 2017). However, for a complete and comprehensive database, data still needs to be uploaded to the barcode reference library from various regions of the world, particularly from Pakistan.

In Pakistan, although a key of freshwater fishes based on morphological characteristics is available (Rafique and Khan, 2012; Yaqub et al., 2019). However, there are significant problems for accurate species identification of snakehead (Serrao et al., 2014) because updated data based on molecular taxonomy is missing.

Family Channidae is one of the importer families in ichthyofauna. To date, in Pakistan four Channa species are found, which includes *Channa striata*, *Channa punctata*, *Channa marulius* and *Channa gachua* (Pervaiz et al., 2018). Snakeheads are not only commercially important fish, but also its population in the wild is at threat (Courtenay and Williams, 2004). Channa species have the unusual capability to stay alive, potentially even out of water for months, if buried in the moist soil (Zhu et al., 2013a). Snakeheads have evolutionary importance because of their unique behavior (Adamson et al., 2010). Thus, correct identification of snakehead fishes is necessary, as they differ in their potential ability and ecological requirements (Courtenay et al., 2004; Serrao et al., 2014).

For long, the taxonomic position of snakeheads found in Pakistan has been creating trouble for taxonomists. Due to ambiguities, taxonomy is not resolvable through morphology-based identification. Existing morphological keys provide insufficient identification for the Channidae (Serrao et al., 2014; Conte-Grand et al., 2017). Hence, this issue has received incredible consideration with new species being described day by day (Musikasinthorn, 1998; Geetakumari and Vishwanath, 2010). There is an urgent need to investigate and update the taxonomic status of these fishes as the clear picture of snakehead diversity in Pakistan remains elusive. Interspecies and intraspecies analysis of genetic distance and barcode gap, can significantly help in taxonomic resolution of the family Channidae (Lakra et al., 2009; Lakra et al., 2010; Benziger et al., 2011).

Current investigation focuses on the identification of snakeheads using molecular techniques called DNA barcoding. DNA Barcoding is used for identification and delimitation of new species (Hebert et al., 2004; Smith et al., 2008; Kerr et al., 2009). In DNA Barcoding, a fragment of mtDNA, commonly COI gene (cytochrome oxidase I gene) is used for the purpose of identification. COI is a bio-identical marker because it has good discrimination power for almost all animal phyla due to the following important characteristics: it is easy to isolate, high copy number (Cywinska et al., 2006), free of recombination, insertion and deletion is uncommon, (Daravath et al., 2013), having minimal variations within species, it seems to have sequence uniqueness that is adequate to separate firmly related species (Hebert et al., 2003; Hebert et al., 2004) and can be recovered from degraded as well as small samples (Waugh, 2007).

In the current investigation, identification, genetic diversity and phylogeny pattern of three species of family Channidae belonging to Pakistani region were approached using mtDNA sequence of COI region, which might be helpful for effective management and conservation strategies of Channa species in Pakistan.

METHODOLOGY

Sampling

Specimens of snakehead fish were collected and identified using available fish identification key (Rafique and Khan, 2012) representing three populations throughout different geographical areas of Pakistan (Table 1). Caudal fin tissues were taken from each specimen (50–100 mg each) for subsequent molecular studies.

Table 1: Sampling location and geographical coordinates of snakehead population.

Population	Collection site	Latitude/ longitude	Sample size
Channa punctata	Head Taunsa bridge	30° 42' 20.0052" N / 70° 39' 27.9936" E	03
	Baloki Head works	31°13'20.96"N/ 73°51'59.05"E	03
	Head Marala	32° 29' 33.65" N/ 74° 31' 52.82" E	03
Channa marulius	Head Taunsa bridge	30.705557/ 70.657776	03
	Baloki Head works	31°13'20.96"N/ 73°51'59.05"E	03
	Head Marala	32° 29' 33.65" N/ 74° 31' 52.82" E	03
Channa striata	Head Taunsa bridge	30° 42' 20.0052" N / 70° 39' 27.9936" E	03
	Baloki Head works	31°13'20.96"N/ 73°51'59.05"E	03
	Head Marala	32° 29' 33.65" N/ 74° 31' 52.82" E	03

DNA extraction and Amplification

Modified slat extraction method was used to extract the DNA**.** Amplification of 655bp barcode region of the mitochondrial DNA was performed with reported primers (Ward et al., 2005). Primers were synthesized from MACROGEN Inc., Seoul, Korea.

Primer	Primer sequence (5'- 3')
Fish F1	TCAACCAACCACAAAGACATTGGCAC
Fish R1	TAGACTTCTGGGTGGCCAAAGAATCA

PCR reaction was carried out using Q cycler using following reaction conditions; an initial denaturing for 4 minutes, 35 cycles of amplification (60 seconds denaturation at 94 °C, 45 seconds annealing at 46.3 °C, 60 seconds extension at 72 °C), and final extension at 72 ºC then storage at 4 °C. PCR was followed by confirmation of product at 1.5 % agarose gel. Bands showing good quality of PCR were sent to MACROGEN, Korea for sequencing using forward and reverse primers.

Bioinformatics Analysis

Obtained sequences were edited and aligned by using Bioedit software and ClustalW respectively (Thompson et al., 1997). The range of difference of sequence among the different species of family Chandidae were calculated. For phylogenetic analysis, the Neighbor joining (NJ) and Maximum likelihood (ML) cladogram as well as to calculate the pair-wise genetic distance based on Kimura-2-parameter (K2P) model the MEGA version X was used

(Kumar et al., 2018). Tajima's Neutrality Test was also performed using above mentioned tool. Automatic Barcode Gap Discovery and Barcode Gap analysis software was used to calculate the histogram of genetic and ranked distances (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html).

RESULTS

Barcode sequences having region of 655bp for 27 specimens from family Channidae were generated during the study. BLAST results showed that sequences of studied specimen were previously uploaded and available at Gene Bank data. But there were no barcode records from Pakistan for Snakeheads in Gene Bank or BOLD.

The constructed Neighbor Joining (NJ) tree based on K2P model for twenty-seven specimens belonging to three species of Chandidae family is shown in Figure 1. NJ tree revealed three clades, which is clearly showing the three separate species. In the present study, Maximum likelihood based on K2P model was also constructed (Figure 2). Both the approaches (NJ and ML) showed that there is an identical phylogenetic relationship among the studied species. The analyses of histogram of genetic and ranked distances revealed a clear gap between conspecific and congeneric species (Figure 3).

Based on K2P model, the intraspecific divergence for three species ranged from 0.00-0.023 and interspecific divergence 0.182-0.195 (Table 2). The highest genetic distance was observed between *C. striata* and *C. marulius* and the lowest distance was observed between *C. punctata* and *C. marulius* (Table 3). Results from Tajima's Neutrality Test are presented in Table 4. The estimated transition/transversion bias (*R*) (2.62) was calculated based on K2P Model.

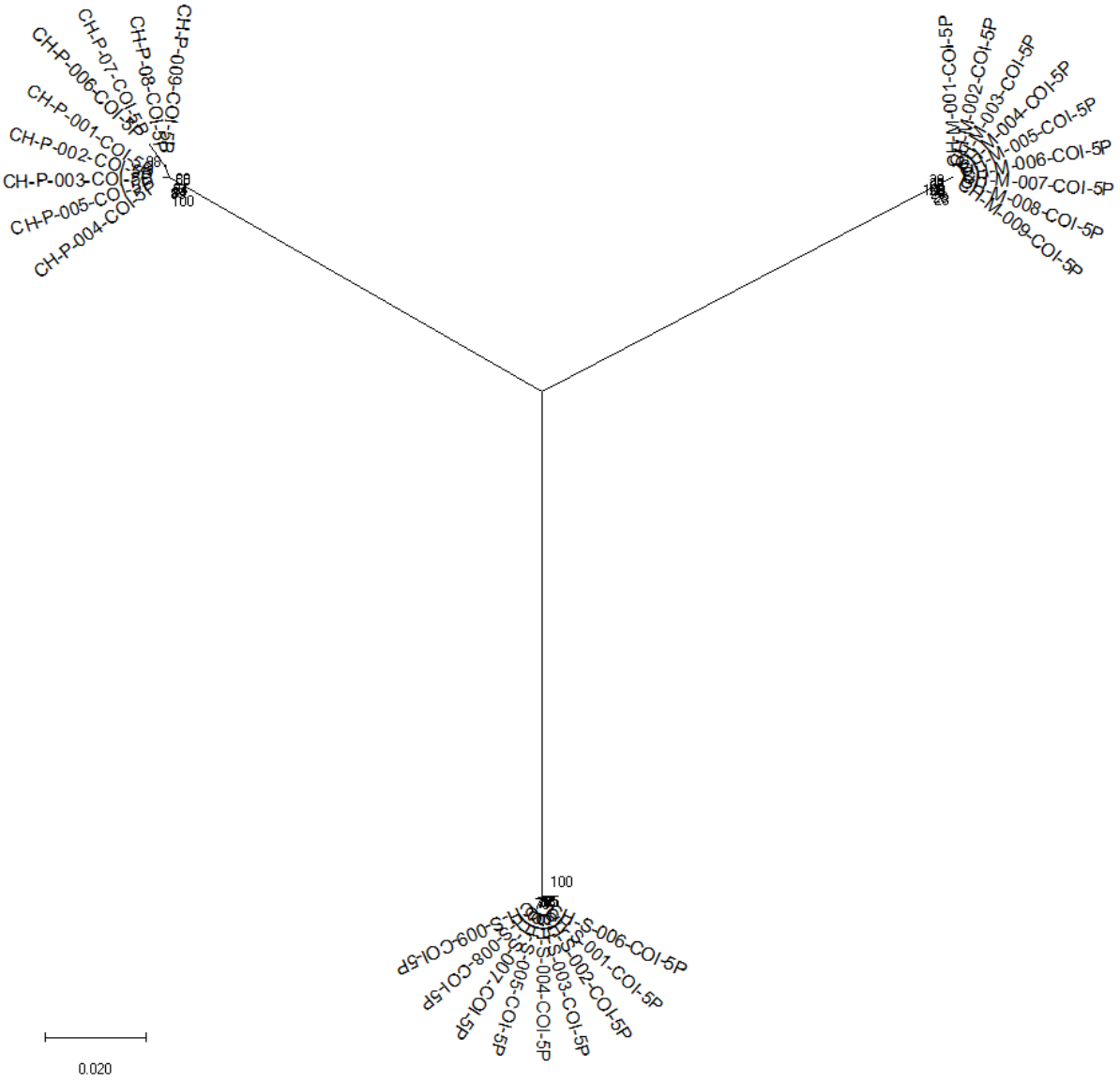

Figure 1: Neighbor joining Phylogenetic tree of COI gene sequences derived from snakehead based on the K2P distances.

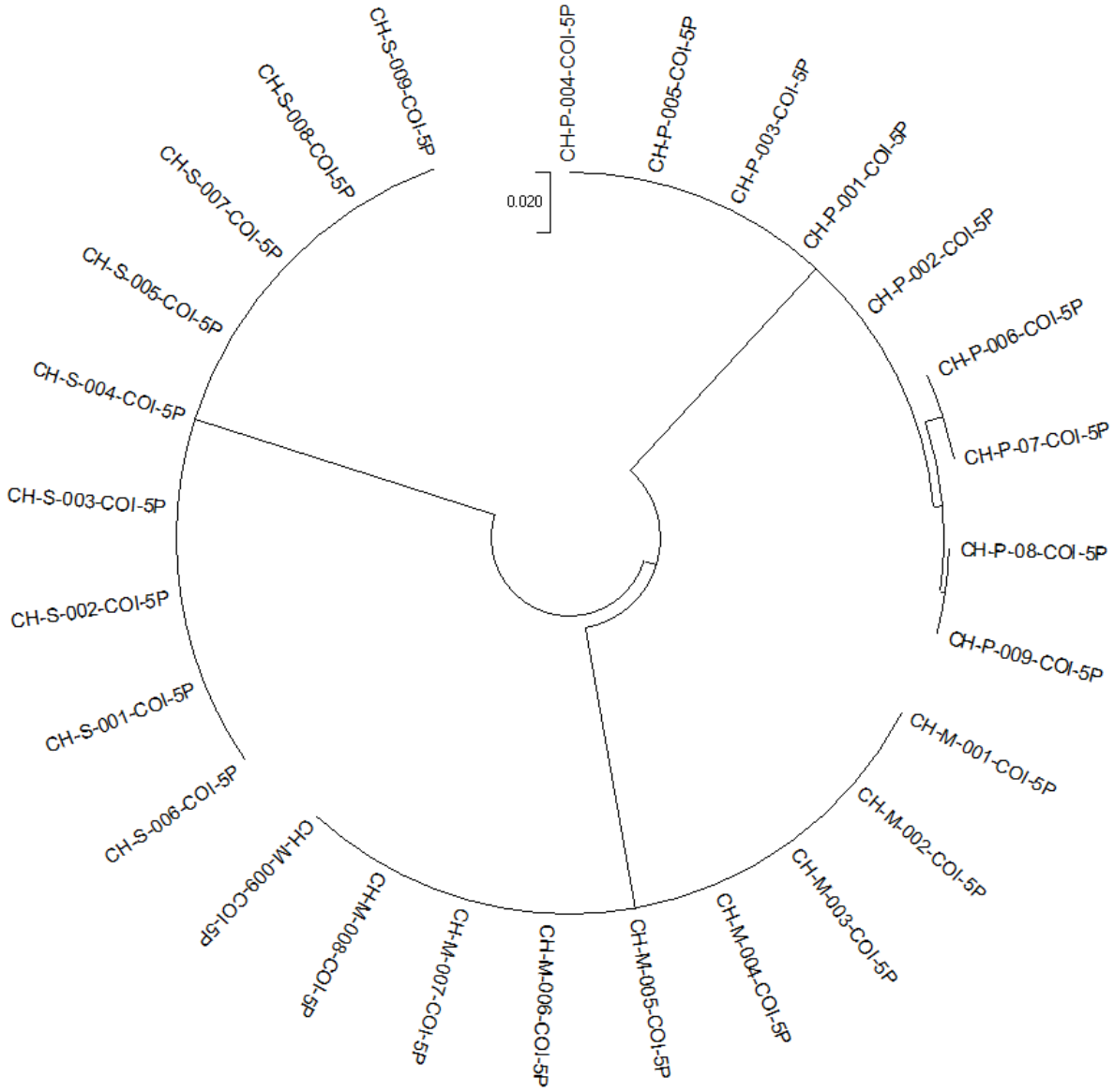

Figure 2: Maximum likelihood Phylogenetic tree of COI gene sequences derived from snakehead based on the K2P distances.

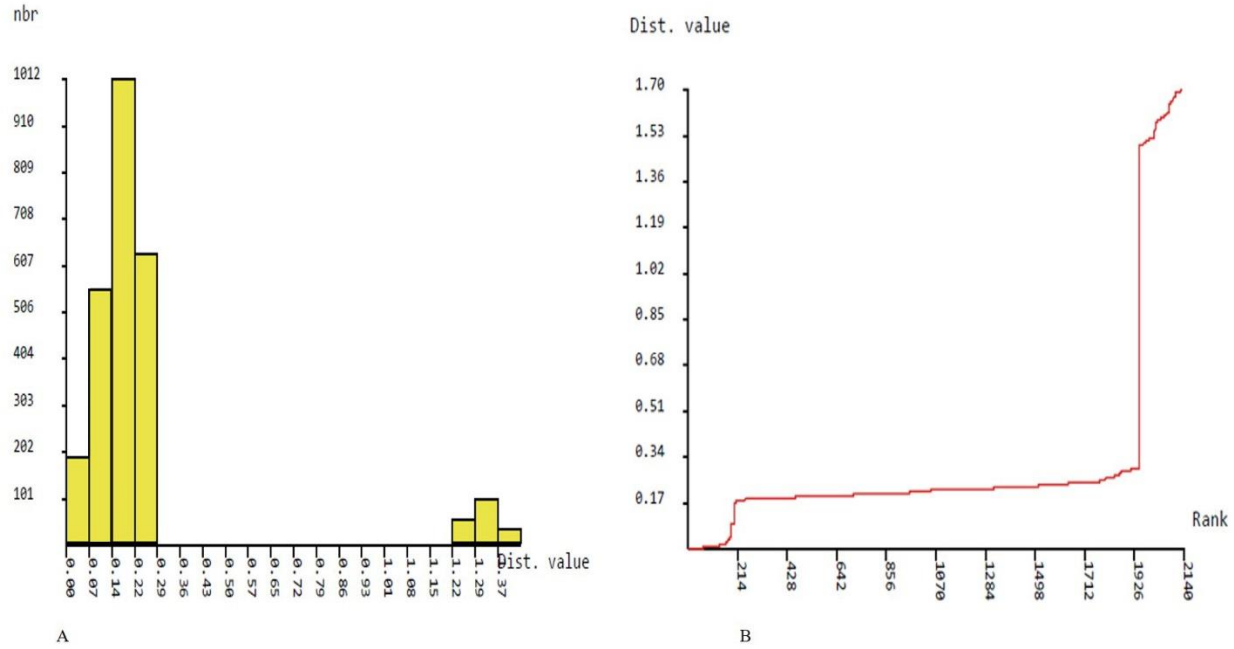

Figure 3: Histogram of genetic distances for Family Channidae from Pakistan (A) and ranked distances (B) created using ABGD and BGA.

Table 2: Kimura-2-parameter (K2P) distances between Species of family Channidae

Comparison within species				Comparison between species			
Min.%	Max. %	Mean%	S.E %	Min.%	Max. %	Mean%	S.E %
0.0	0.023	0.0017	0.00325	0.182	0.195	0.189	0.0055

Table 03: Estimates of Evolutionary Divergence over Sequence Pairs between Groups

	Channa punctata	***Channa marulius***	***Channa striata***
Channa punctata	0.023		
Channa marulius	0.182	0.012	
Channa striata	0.192	0.195	0.009

Table 04: Results from Tajima's Neutrality Test.

m	***S***	***Ps***	***Θ***	***π***	***D***
27	137	0.235395189	0.061071499	0.114048227	3.39422347

m = number of sequences, n = total number of sites, S = Number of segregating sites, ps = S/n, Θ = ps/a1, π = nucleotide diversity, and D is the Tajima test statistic.

DISCUSSION

For evolutionary and ecological process, the phylogeny of species is playing an important role due to its fitness in ecological function (Baisvar et al., 2019). The genetic diversity is an important element in species conservation (Almeida et al., 2003; Baisvar et al., 2019). Unfortunately, as far as aquatic ecosystem of Pakistan is concern, no validated data about genetic variability of Family Channidae is available. Therefore, in current investigation, efforts were made to identify and evaluate the genetic distance/phylogeny of species within family Channidae based on COI sequences.

In the current investigation, based on the COI sequences, for three studied species of family Channidae, a total of 27 individuals were identified and their genetic diversity was calculated. There was no overlap detected between the interspecies and intraspecies distance and current study was in agreement with the previously reported findings (Barrett and Hebert, 2005; Zhu et al., 2013b). K2P divergence within species was calculated from 0.00-0.027 and the divergence between species ranged from 0.182-0.195. K2P divergence in this study were showed a high degree of resemblance with similar observations reported by previous studies (Zhu et al., 2013b; Serrao et al., 2014). Our results were similar to previously reported study by Herbert et al. (2004) that the species can be defined on the basis of intraspecific and interspecific divergence. They suggested that intraspecific dissimilarity ought to be lower than interspecific similarity.

COI gene based phylogenetic analysis revealed that the twenty-seven sequences belonged to three different species of family Channidae. The NJ and ML cladogram showed the three species separately. The branch length of species tends to be much deeper than within species leading to gap between snakeheads based on the K2P distances as shown in figure 1 and 2. As shown in NJ tree the *C punctata* found closely with *C. marulius,* the findings of this study corroborate with previously reported studies (Serrao et al., 2014; Conte-Grand et al., 2017). This may be due to their nucleotide divergence as different studies show that congeneric species were found clustered together in NJ tree (Ward et al., 2009; Lakra et al., 2016).

Figure 3 is showing the genetic and ranked distances based on barcode gap. The barcode gap in DNA barcoding is considered an important parameter for accurate species declination. In DNA barcoding the barcode gap is considered effective when interspecific distances are higher than intraspecific distances derived from COI gene (Austerlitz et al., 2009; Puillandre et al., 2012). Our results clearly showed considerable barcode gap between the genetic distances from intra and interspecific. Taxonomists have been using barcode gap as a first set of species hypotheses (Puillandre et al., 2012).

Further study of the barcode is needed in resolving the identification issues of all the fish species in Pakistan. The genetic strategy for an identification purposes are an excellent shorthand to build a database of fish species of Pakistan. This type of genetic data is essential for the purpose of improvement in fish biodiversity of Pakistan.

CONCLUSION

The current scenario has shown effectiveness of COI gene region of mtDNA in molecular identification of the freshwater fish species of Pakistan. The undertaken study is useful for broadening of the Linnaean framework and can be used to improve the conservation strategies of Channa species in Pakistan.

CONFLICT OF INTEREST

All authors have no conflict of interest.

ETHICAL APPROVAL

All the research work was carried out after the approval of Bioethical committee of Faculty of Science and Technology, GC University Lahore. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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