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Research Article

Molecular identification of Herpetofauna from Punjab, Pakistan, using mtDNA genes

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Abstract

The herpetofauna diversity of Pakistan is underestimated due to the country's lack of molecular-based identification. Field surveys were conducted from August 2018 through July 2022 to collect as many as possible specimens from Punjab, Pakistan. A total of 21 species were collected and initially identified by morphological characteristics. The three gene fragments in four amphibian species and seven reptile species were successfully amplified and sequenced. A total of 18 DNA sequences of 11 species representing nine genera and five families were deposited in GenBank, and accession numbers were obtained. Furthermore, phylogenetic analysis was performed through the Neighbor-joining method using 100 bootstrap pseudo-replicates in MEGA X. Closely related toad species, namely Duttaphrynus melanostictus and Duttaphrynus stomatitis, were clearly separated in the tree inferred from Cytb gene sequences. Similarly, conspecific sequences were analyzed for multiple individuals of *Platyceps rhodorachis* clustered together in the tree inferred from COI gene sequences. In our findings, 16S rRNA appears to be more reliable in identifying amphibian species, while COI has a better success rate in reptile species identification. In our recommendations, molecular-based identification of herpetofauna is necessary nationwide to document any new subspecies.

Keywords: Mitochondrial DNA, phylogenetic analysis, genetic divergence, species identification, COI

Introduction

Amphibians and reptiles are facing serious threats, and their populations are declining at an unprecedented rate globally. Hence, they have received considerable attention from researchers around the globe and the scientific community, making efforts to conserve these environmentally friendly taxa (Valenzuela et al., 2019). Their decline is due to overexploitation, habitat loss, and climate change (Longo et al., 2019). Likewise, the reptilian populations are also declining globally, although they are less affected than amphibians (Ortiz-Santaliestra et al.,2018). Their decline causes are similar to amphibians; however, overexploitation and illegal trade are more challenging (Van et al., 2018).

The limitations of morphology-based identification urge researchers to a new approach to identify higher-ranked taxa (Ali et al., 2020 a,b,c). Molecular-based identification is an exceptional method used to identify biological diversity through small segments of the genome (Coates et al., 2018). Species identification through DNA sequences forms the foundation of DNA taxonomy and barcoding. There is significant emphasis on employing a mitochondrial marker, specifically a fragment derived from the cytochrome oxidase subunit I (COI) gene, for this purpose (Hebert et al.,2003; DeSalle & Goldstein, 2019). There are increasing numbers of publications on vertebrate identification by DNA barcoding (Bingpeng et al., 2018; Goldstein et al., 2019; Zangl et al., 2020). However, identifying species based on DNA sequences takes work as the molecular evolution rate varies in different genome fragments across the taxa (Zhao et al., 2016). Previously, phylogenetic systematics focused on mitochondrial genes such as ribosomal 12S and 16S rDNA, but the occurrence of insertions and deletions in comprehensive taxonomic studies is a major constraint (Vences et al., 2005; Vences & Wake, 2005). Meanwhile, the mitochondrial cytochrome *b* (Cytb) gene has been successfully applied in species identification in diverse vertebrates (Branicki et al., 2002; Parson et al., 2000). Identifying amphibians and reptiles on morphology is still considered authentic; however, molecular analysis of these taxa led to the discovery of many new morphologically cryptic species (Ali et al., 2020).

There are several publications regarding the herpetofauna diversity of Pakistan, and most of the studies are limited to Sindh, Baluchistan, and KPK provinces, or very few species have been explored extensively (Ali et al.,2017; Khan, 2010). Overall, 24 amphibians and 195 reptiles species have been reported so far. Out of these, nine amphibians and 13 reptile species are endemic to Pakistan (Ali et al., 2018a, b). Amphibians and reptiles are considered fearsome animals in Pakistan and are poorly studied by the scientific community (Ali et al., 2016). The taxa remain to be explored in most parts of Pakistan, and data regarding diversity and distribution

is outdated (Ali et al., 2017). The present study, therefore, plans to identify amphibians and reptiles of Punjab, Pakistan, using 16S rRNA, Cytb, and COI genes.

Material and methods

Specimen's collection

Field surveys were extended from August 2018 through July 2012 to collect specimens from selected sites of Punjab Province, namely Bahawalpur, Bahawalnagar, Kasur, Lahore, Mianwali, Jhelum, and Rawalpindi districts (Fig. 1). Information about each collected specimen such as GPS coordinates, date and time of collection and species was noted. Each captured specimen was tagged with a specific voucher number and identified using morphological keys (Khan, 2006). A few specimens (n = 5) of each species were euthanized, preserved in 75% alcohol, and brought to the lab for further identification through molecular analysis. The voucher specimens were deposited in the Zoological Museum, UVAS, Lahore, Pakistan. The UVAS Animal Care and Use Committee approved all procedures.

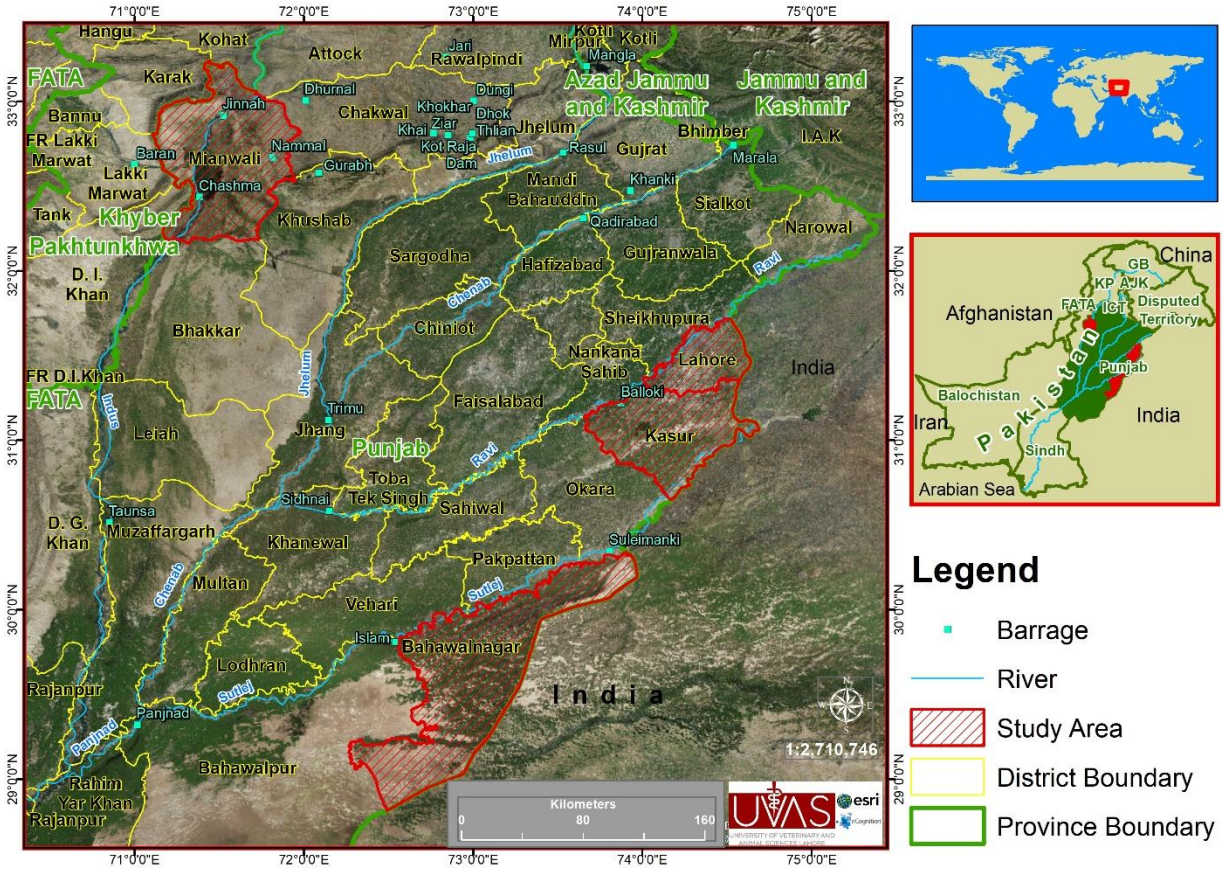

Figure 1. Map illustrating the study area

DNA extraction, PCR amplification, and sequencing. Total Genomic DNA was extracted from preserved tissues by phenol-chloroform method and quantified using NanoDrop-ONE in the post-graduate lab, Department of Wildlife and Ecology, UVAS, Pakistan. DNA samples were taken to the PC2 lab at the School of Animal and Veterinary Sciences, The University of Adelaide, Australia, for PCR and Sanger sequencing. Three different mitochondrial DNA fragments were amplified using one set of 16S rRNA, Cytb, and COI primers for amphibians, while one set of Cytb and four sets of COI primers were used for reptiles (Table 1). PCR amplification was done following Ali et al. (2020a). Unsuccessful amplified and sequenced samples were re-amplified up to twice. According to standard protocols, all the samples were sequenced on an ABI3730XL DNA Analyzer (Applied Biosystems) from AGRF, Australia.

Table 1. List of primer sets used in the study

Taxa	Marker	Primer Pair	Sequence (5′–3′)	Annealing condition	Source
Amphibians	16S rRNA	16SA-L	CGCCTGTTTATCAAAAACAT	50 °C for 30 seconds	Vences et al. 2005
		16SB-H	CCGGTCTGAACTCAGATCACGT		
	Cytb	L14850	TCTCATCCTGATGAAACTCTTGGCTC	50 °C for 30 seconds	Tanaka-Ueno et al. 1998
		H15502	GGATTAGCTGGTGTGAAATTGTCTGGG		
	COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	42°C for 30 seconds	Folmer et al 1994
		LCO1490	GGTCAACAAATCATAAAGATATTGG		
Reptiles	Cytb	L14910	GACCTGTGATMTGAAAAACCAYCGTTGT	48 or 50 °C for 30 seconds	de Queiroz et al. 2002
		H16064	CTTTGGTTTACAAGAACAATGCTTTA		
	COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	42°C for 30 seconds	Folmer et al 1994
		HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		
	COI	C1J1718	GGAGGATTTGGAAATTGATTAGTTCC	42°C for 30 seconds	Simon et al 1994
		C1J2191	CCCGGTAAAATTAAAATATAAACTTC		
	COI	RepCOI-F	TNTTMTCAACNAACCACAAAGA	48.5 for 30 seconds	Nagy et al 2012
		RepCOI-R	ACTTCTGGRTGKCCAAARAATCA		
	COI	L-turtCOI-F	TACCTGTGATTTTAACCCGTTGAT	56 °C for 30 seconds	Zhao et al 2016
		H-turtCOI-R	TGGTGGGCTCATACAATAAAGC		

*The forward primer is above, and the reverse primer is below for each primer pair.

Data analysis

The obtained DNA sequences were analyzed and trimmed in MEGA X (Kumar et al., 2018). Each DNA sequence was BLAST at NCBI to download closely matched sequences. The neighbor-joining (NJ) tree was constructed in MEGA X under the p-distance model. The nodes ' bootstrap support (Felsenstein, 1985) was assessed with 1000 pseudo-replicates. Genetic divergence within and between each species was calculated using MEGA 10 with the uncorrected p-distances.

Results

PCR Amplification and sequencing

The DNA of 21 species was included in this study; however, only the DNA of 4 amphibians and 7 reptile species were successfully amplified. Table 2 summarizes the successfully amplified DNA and their GenBank accession numbers. After trimming ambiguous bases, the obtained 16S rRNA, Cytb, and COI fragments of amphibians were 560 bp, 672 bp, and 660 bp, respectively. Similarly, Cytb and COI fragments of reptiles were 670 bp and 652 bp, respectively. The 16S rRNA, Cytb, and COI fragments aligned with DNA sequences retrieved from GenBank comprised 530 bp, 650 bp, and 610 bp, respectively.

Table 2. List the successfully amplified DNA of the species and the GenBank accession numbers of voucher specimens.

Family	Genus	Species	GenBank accession and voucher number		
			16S	Cytb	COI
Class Amphibia					
Bufonidae	Duttaphrynus	*Duttaphrynus stomaticus*	MK910158 (ZMUVAS4)	MK941837 (ZMUVAS9)	MK947909 (ZMUVAS14)
		Duttaphrynus melanostictus	-	MK941836 (ZMUVAS7)	-
Dicroglossidae	Euphlyctis	*Euphlyctis kalasgramensis*	MK881165 (ZMUVAS1) MK920114 (ZMUVAS5)	-	-
	Hoplobatrachus	*Hoplobatrachus tigerinus*	MK922123 (ZMUVAS3)	-	-
Class Reptilia					
Varanidae	Varanus	*Varanus bengalensis*	-	-	MK947910 (ZMUVAS16)
Colubridae	Platyceps	*Platyceps rhodorachis*	-	MK941835 (ZMUVAS12)	MK936174 (ZMUVAS22) MK941839 (ZMUVAS24)
	Oligodon	*Oligodon arnensis*	-	MK941834 (ZMUVAS10)	-
		Oligodon formosanus		-	MK941840 (ZMUVAS19)
	Ptyas	*Ptyas mucosa*	-	-	MK947911 (ZMUVAS20)
Elapidae	Bungarus	*Bungarus caeruleus*		MK941838 (ZMUVAS11)	-
	Naja	*Naja naja*	-	MK936173 (ZMUVAS8)	MK941841 (ZMUVAS21)

As shown in Table 2, the 16S rRNA, Cytb primers had a 75% success rate of amplifying amphibians DNA, while only 25% for amplification of COI. Cytb primers set did not amplify successfully *Euphlyctis cyanophlyctis* and *Euphlyctis kalasgramensis* while the COI primers set did not amplify successfully *Duttaphrynus melanostictus, Hoplobatrachus tigerinus, Euphlyctis cyanophlyctis* and *Euphlyctis kalasgramensis*. However, COI primers were 71% successful as compared to Cytb (57%) in amplifying reptiles DNA. Cytb and COI primers set (Table 1) did not successfully amplify *lissemys punctata, Calotes versicolor, Amphiesma stolatum, Echis carinatus, Ablepharus grayanus, Eryx johnii, Lycodon aulicus, Uromastyx hardwickii* and *Hemidactylus flaviviridis.* It should be noted that the fixation and DNA extraction methods of the samples may have affected amplification success.

Genetic diversity and variation. Table 3 compares the uncorrected p-distances of 16S rRNA, Cytb, and COI sequences. The mean intraspecific divergences of 16S rRNA, Cytb, and COI in frogs were 0.001, 0.04, and 0, while those in toads were 0, 0.09, and 0, respectively. The mean intraspecific divergences of Cytb and COI in lizards were 0 and 0.02, while those in snakes were 0.22 and 0.17, respectively—phylogenetic analysis. The study obtained 18 DNA sequences of 11 species representing nine genera and five families. The specimens (voucher nos. ZMUVAS1, ZMUVAS5) of morphology-identified "*Euphlyctis cyanophlyctis*" were confirmed as *Euphlyctis kalasgramensis* by using rRNA sequences (Fig. 2).

Table 3. Comparison of 16S rRNA, Cytb, and COI-based genetic divergence of amphibians and reptiles using p-distance.

Taxa	16S rRNA	Cytb	COI
Class Amphibia			
Frogs	0.01	0.04	0
Toads	0.00	0.09	0.00
Class reptilian			
Snakes	-	0.22	0.17
Lizards	-	-	0.02

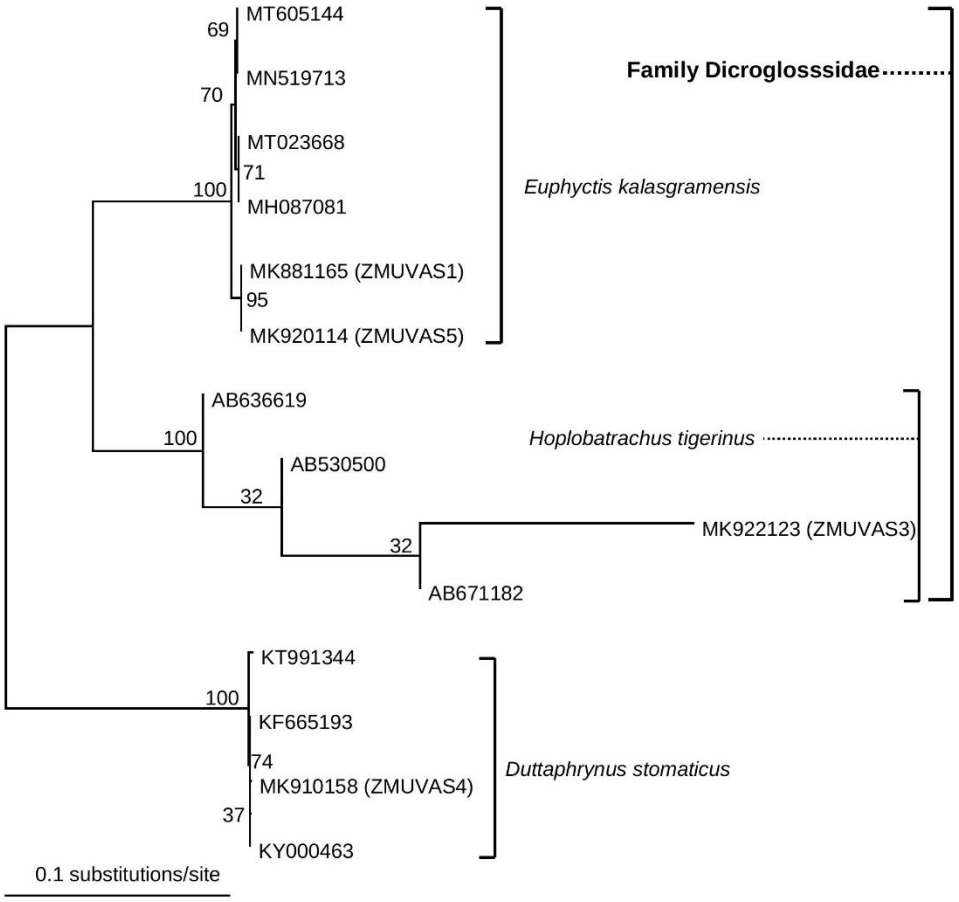

Figure 2. Neighbor-joining tree of amphibians inferred from 16S rRNA sequences. Bootstrap values are given above the nodes.

Recently, molecular-based identification of Asian amphibians and reptiles has been done, and DNA sequences of related amphibians and reptile species have been available at NCBI. Closely related DNA sequences of 16S rRNA and COI were retrieved from NCBI in BLAST searches but Cytb was less responsive. Neighbor-joining trees based on obtained DNA sequences are shown in Figures 2-4. Two closely related toad species, namely *D. melanostictus* and D. stomaticus, clearly separated in the Cytb tree (Fig. 3). Conspecific sequences analyzed for multiple individuals clustered together, *D. stomaticus* (BS = 100%) and *P. rhodorachis* (BS = 70%) in COI based NJ tree (Fig. 4).

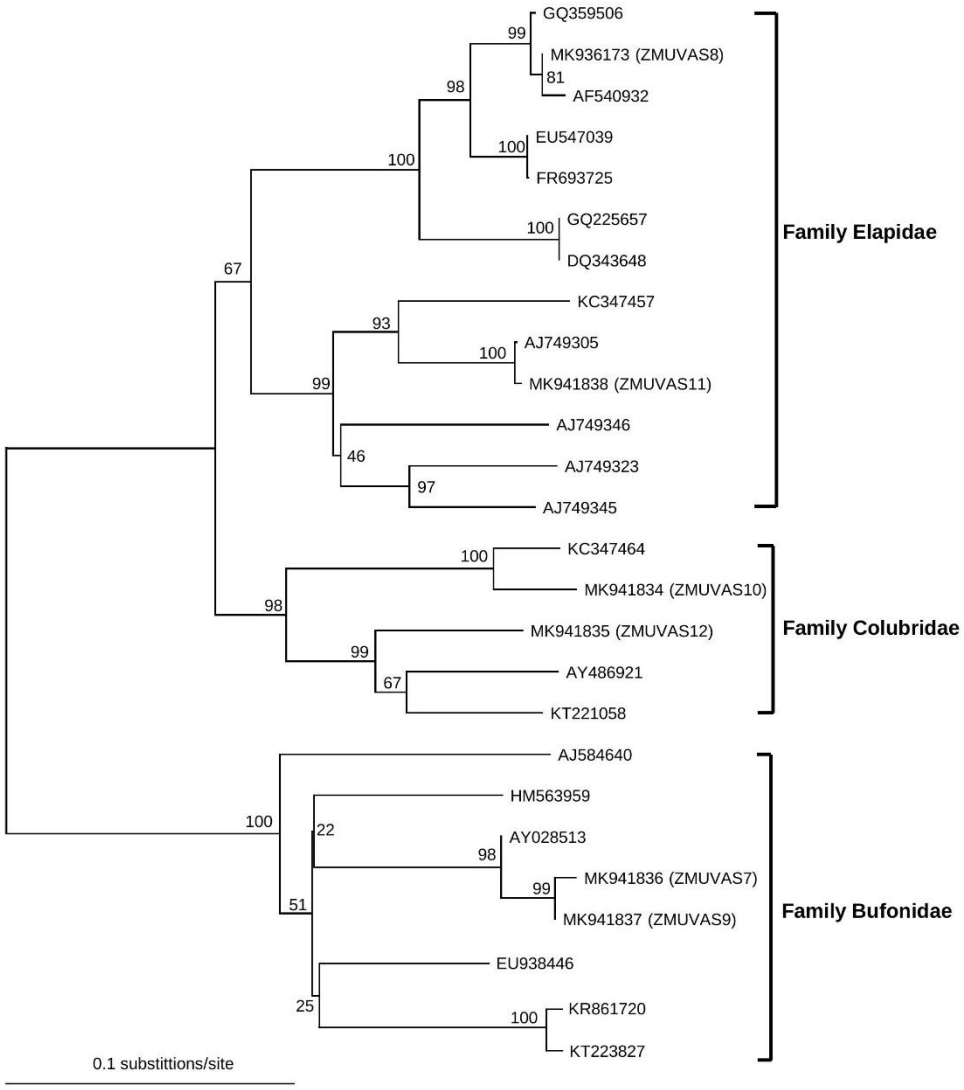

Figure 3. Neighbor-joining tree of amphibians and reptiles inferred from Cytb sequences. Bootstrap values are given above the nodes.

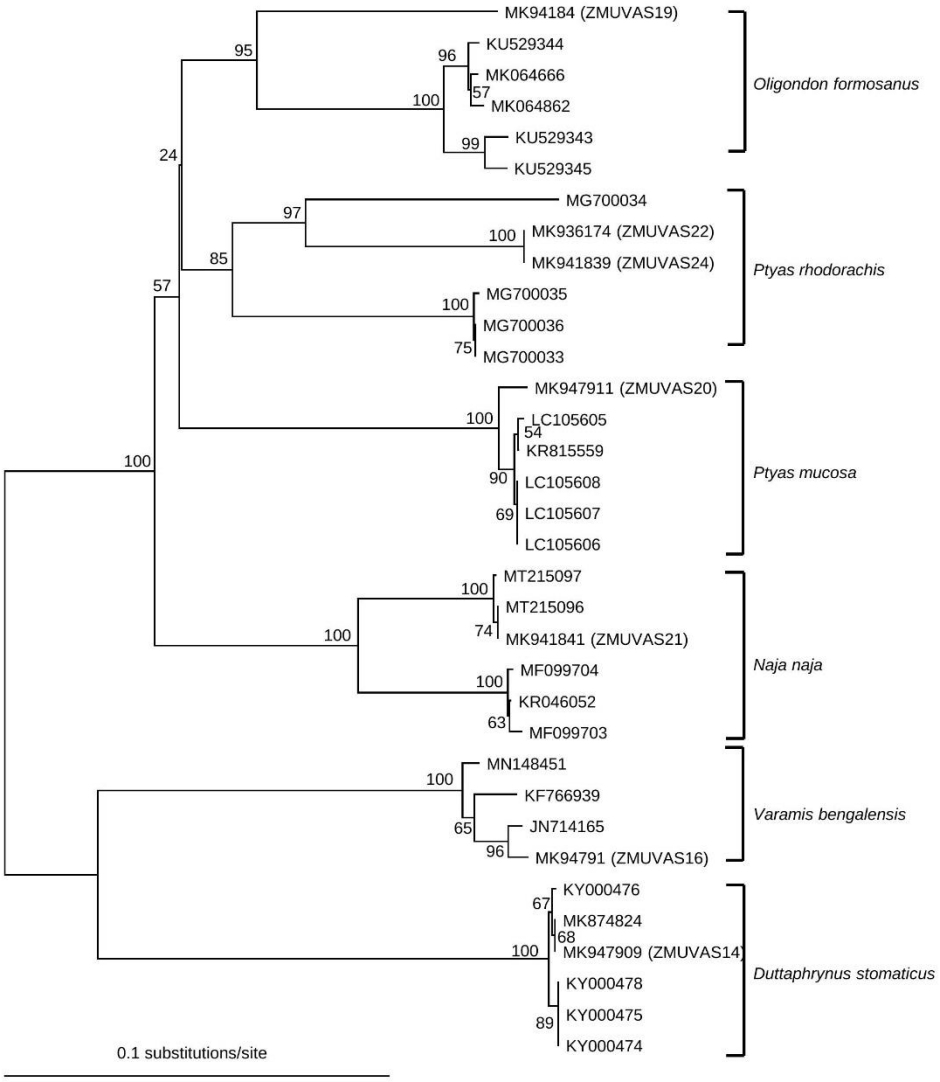

Figure 4. Neighbor-joining tree of amphibians and reptiles inferred from COI sequences. Bootstrap values are given above the nodes.

Discussion

In the recent past, globally, herpetofauna has drawn considerable attention to wildlife biologists due to the decline in species due to many anthropogenic hazards. Overall, at least 43% of amphibians globally are threatened with extinction (Valenzuela et al., 2019). Although reptiles are also facing a high rate of decline, significantly less than amphibians, overexploitation, and illegal trade are causing significant problems (Nijman et al., 2012; Ali et al., 2018a). The DNA barcoding-based identification of species was started in 2009; however, DNA-based identification of amphibians and reptiles is relatively new (Che et al., 2012; Xia et al., 2012).

Vences et al. (2005) reported that one mtDNA marker cannot identify all the amphibians and reptile species successfully. According to recent studies, 1851 CO1 and 37637 sequences of 16S rRNA of amphibians are available in GenBank (Benson, 2014). In this study, we have used three different mtDNA markers, i.e., 16S rRNA, Cytb, and COI, to identify amphibians and reptile species from Punjab, Pakistan.

Five amphibian species, namely Duttaphrynus stomatitis, *D. melanostictus*, Hoplobatrachus trigeminus, *Euphlyctis cyanophlyctis*, and *E. kalasgramensis*, were included in this study. Amphibians are challenging to identify precisely using Cytb and COI primers due to the high overlap rate of intraspecific and interspecific variation (Fouquet et al., 2007). The 16S rRNA marker is proposed as an alternative DNA-based identification in amphibian systematics and taxonomy (Menegon et al., 2017). In addition, recent molecular studies revealed that 16S rRNA sequence data can easily differentiate between cryptic frog species. During this study, specimens of *Euphlyctis kalasgramensis* were morphologically identified as '*E. cyanophlyctis*', but their identity was confirmed using 16S rRNA. *Euphlyctis kalasgramensis* has recently been reported from Bangladesh and Pakistan, while its range is expected to extend into India (Ali et al., 2020; Howlader, 2015).

During the study, mean intraspecific divergences of 16S rRNA, Cytb, and COI in frogs were 0.001, 0.04, and 0, respectively, while in toads were 0, 0.09, and 0, respectively. Recent amphibians' DNA barcoding studies documented 5% threshold variation for 16S rRNA and 9% for CO1 (Xia et al., 2012). Amaral et al., (2019) demonstrated that 3% divergence in 16S rRNA sequences is enough to identify amphibians. The COI has a high success rate as compared to Cytb in the identification of many taxa, including birds, fish, and invertebrates (Crandall et al., 2019). In addition, CO1 has a higher success rate in the identification of cryptic species, sexual dimorphism, and high phenotypic variations in reptiles (Smith et al., 2013). A total of 7 species, namely *Varanus bengalensis*, *Platyceps rhodorachis*, *Oligodon arnensis*, *Oligodon formosanus*, Ptyas mucosa, *Bungarus caeruleus*, and Naja naja, were successfully amplified and sequenced using Cytb and COI primers set listed in table 1. However, the amplification failure of Cytb was limited to specific taxa as compared to COI. Overall, COI and Cytb revealed good performance in separating closely related species. During the study, Cytb clearly separated two closely related toad species, namely *D. melanostictus* and *D. stomaticus*, while *D. melanostictus* was not amplified using COI primers set. Similarly, conspecific sequences of multiple individuals of *D. stomaticus* and *P. rhodorachis* clustered together in the COI tree. The mean intraspecific

divergences of Cytb and COI in lizards were 0 and 0.02, respectively; while 0.22 and 0.17 in snakes, respectively. However, molecular variation thresholds of herpetofauna are still arbitrary and there are not enough studies to support the information. However, in the case of reptiles COI and Cytb, 10% variation threshold is considered reliable (Dinh et al., 2019). Our results also echo those of Pons et al. (2006), who documented that DNA taxonomy for a given taxa is based on more than one region of mtDNA.

Conclusion

The amphibians and reptiles are generally considered fearsome creatures in Pakistan and have taken less concern to the scientific community. The country's diversity and distribution of herpetofauna are underestimated due to the lack of an integrative framework for combining molecular data with morphological traits. This study provides reference data based on molecular-identification of herpetofauna from Punjab, Pakistan. The study obtained 18 DNA sequences of 11 species representing nine genera and five families. In our findings, the 16S rRNA gene is more reliable in identifying amphibians, while the COI gene has a better success rate in reptile identification. In our recommendations, sequence-based identification of herpetofauna is required nationwide to report any new species or subspecies.

Acknowledgments

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