Official journal website:
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Amphibian & Reptile Conservation
13(2) [General Section]: 203–211 (e205).

Phylogenetic analysis of the Common Krait (*Bungarus caeruleus*) in Pakistan based on mitochondrial and nuclear protein coding genes

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***Abstract.*—Pakistan has more than 40 species of venomous snakes. One of them, the Common Krait (*Bungarus caeruleus*), is responsible for most of the reported snake bites followed by Russel's Viper, Saw-scaled Viper, and Black Cobra. Molecular studies not only help in correctly identifying organisms but also in finding the phylogenetic relationships and diversity among and between them. Morphological studies can be supplemented with confirmatory molecular data to make them more authentic and accurate. This study is the first to characterize the genetic diversity and phylogenetic relationships of Common Kraits from Pakistan, which will help in developing effective strategies for managing snake bites through effective antivenom development. Tail tip biopsies of 25 Common Kraits were collected from different cities in Pakistan. The whole DNA was extracted. Four mitochondrial (ND4, Cytochrome b, 12S rRNA, and 16S rRNA) and three nuclear protein coding (C-mos, RAG-1, and NT3) gene fragments were amplified using specific PCR primers. The amplified DNA was sequenced by Sanger di-deoxy sequencing. Forward and reverse sequences were cleaned and contiged using Sequencher 5.0 software. DNA data were aligned and concatenated using MEGA 6.0 and SequenceMatrix software, respectively. Partition Finder software was used for obtaining the best partitioning scheme and evolutionary models. Concatenated maximum likelihood and Bayesian phylogenetic trees were constructed using RaxML and MrBayes software. The same alignments were used to perform DNA polymorphism analysis using DnaSP 5.0 software. A percent identity matrix was created for all sequences using the online bioinformatics tool, MUSCLE. Homology was presented in tabular form, showing the similarity among different species of genus *Bungarus*. All *Bungarus* species were differentiated into four groups. Common Krait (*B. caeruleus*) from Pakistan showed close relationships with *B. sindanus* and *B. ceylonicus*, as one monophyletic group. The first clade included *B. candidus* (Indonesia, Thailand, Vietnam, and Laos), *B. multicinctus* (China, Taiwan, and Burma), and *B. niger* (Nepal). The second clade included *B. sindanus* and *B. caeruleus* (Pakistan), and *B. ceylonicus* (Sri Lanka). The third clade included *B. fasciatus* (Thailand and Indonesia), while the fourth clade included B. *bungroides* (China) and *B. flaviceps* (Malaysia and Indonesia). This study traces the diversity and phylogenetic relationships of the Pakistani elapid, Common Krait, showing the considerable inter- and intra-specific variations from different geographical regions of the world.**

Keywords. Asia, Elapidae, PCR, polymorphism, Serpentes, venomous snakes

Citation: Ashraf MR, Nadeem A, Smith EN, Javed M, Smart U, Yaqub T, Hashmi AS, Thammachoti P. 2019. Phylogenetic analysis of the Common Krait (*Bungarus caeruleus*) in Pakistan based on mitochondrial and nuclear protein coding genes. *Amphibian & Reptile Conservation* 13(2) [General Section]: 203–211 (e205).

Received: 25 January 2019; **Accepted:** 24 September 2019; **Published:** 17 December 2019

Introduction

Snakes are legless carnivorous reptiles of suborder Serpentes, and their lack of eyelids and external ears distinguish them from legless lizards (Reeder et al. 2015). Except Antarctica and some other large islands, like Ireland, Iceland, Greenland, the Hawaiian archipelago, and New Zealand islands, snakes are found everywhere in the world (Roland 1994). South Asia is the region most affected with venomous snake bites. For example, the World Health Organization reports 35,000 to 50,000 deaths annually in India (Chippaux 1998; Pyron et al. 2013), and Pakistan reports 40,000 snake bites every year that result in 8,200 fatalities (Pyron et al. 2013).

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Fig. 1. Sample collection sites in Pakistan for Common Krait (*Bungarus caeruleus*).

Venomous snakes in Southeast Asia belong to families Elapidae (cobras and kraits) and Viperidae (typical vipers and pit vipers). A study of hospital-admitted snakebite cases in Pakistan revealed less than 5% neurotoxic snakebites, and the rest were viper bites (Nisar et al. 2009).

Kraits, genus *Bungarus*, are identified by alternating black and white cross-bands across the body, and are found in all South Asian countries except the Philippines. Members of genus *Bungarus* are moderate to large sized elapids distributed in Pakistan and eastward through southern Asia to Indonesia (Smith 1943). Currently, 12 species of kraits are recognized (Yulin et al. 2018), including three in Pakistan. In Pakistan, Common Kraits (*Bungarus caeruleus*) are reported throughout Punjab, Khyber Pakhtoonkhwa (KPK), Azad Kashmir, Sindh, and Southern Balochistan. Common in the Indus valley, this is the only species of kraits found in Rawalpindi and Islamabad (Khan 2002a; Oh et al. 2019). Sindhi Krait (*B. sindanus*) is prevalent in Tharparkar, Bahawalnagar, and Bahawalpur. Northern Punjab Krait (*B. razai*) is reported from Mianwali (Khan 2002b). Determining the relationships among the members of Elapidae can help in understanding their distribution and diversity. Many studies have focused on the evolutionary relationships of kraits.

Before the widespread use of DNA sequencing, systematics and taxonomy were used to infer phylogenies among species in order to explain their relationships. Now many fields in biology are using phylogenies for a wide variety of purposes, such as examining paralogous relationships, population histories, dynamics of pathogens with respect to their evolution and epidemiology (Zhou et al. 2018; Blanquart 2019), the ontogeny of body cells during development, and the differentiation of tumors (Kester and van Oudenaarden 2018). Variations in nucleotide sequences can construct phylogenies for inferring relationships among the compared sequences. The topology of phylogeny gives some estimates about the mutation rates, time-scales of evolutionary events, and prehistoric movement among different geographical regions (Soroka and Burzyński 2018). Phylogenetics shows relationships among organisms and genes (Friberg et al. 2019), and can give a clearer picture of the biodiversity, biogeography, and evolution of many characters in related groups (Pilfold et al. 2019; Grismer and Davis 2018; Silva et al. 2019).

The use of mitochondrial DNA data for studying animal evolution has become a powerful tool in the last decade. Molecular biology has helped in these mitochondrial DNA studies to give insights into population structure, gene flow, hybridization, biogeography, and phylogenetics (Chandrasekaran et al. 2019; Soroka and Burzyński 2018). Evolutionary studies give comparisons of mitochondrial genome organization and function while molecular studies help to improve these evolutionary studies (Ng et al. 2019). Nuclear encoded genes seem to be a strong source of phylogenetic information. They can be more useful for showing the divergence of those genes whose multiple substitutions may obscure clear phylogenetic signals.

This is the first study from Pakistan focusing on genetic characterization, biodiversity, and molecular phylogenetics of the Common Krait (*Bungarus caeruleus*).

Materials and Methods

A collection of 25 Common Kraits (*Bungarus caeruleus*) was obtained from reptile breeders in different cities in Pakistan (see Fig. 1, Table 1). Scalation patterns and

Table 1. Common Krait (*Bungarus caeruleus*) samples used in this study, and their location information.

Sample ID	Locality	Latitude	Longitude
BC-1	Jallo Park, Lahore, Punjab, Pakistan	31°34'17.29"N	74°28'36.78"E
BC-2	Balochanwali, Bahawalpur, Punjab, Pakistan	29°28'56.90"N	71°59'41.86"E
BC-3	Qila Ram Qaur, Hafiz Abad, Punjab, Pakistan	32°4'57.12"N	73°40'49.02"E
BC-4	Yazman Housing Society, Yazman, Punjab, Pakistan	29°7'3.00"N	71°45'6.73"E
BC-5	Changa Manga Forest, Kasur, Punjab, Pakistan	31°4'54.19"N	73°59'53.49"E
BC-6	Lal Suhanra National Park, Bahawalpur, Punjab, Pakistan	29°19'1.36"N	71°54'16.43"E
BC-7	Rahim Yar Khan Zoo, Rahim Yar Khan, Punjab, Pakistan	28°24'14.30"N	70°15'32.63"E
BC-8	Chak Risalwala, Faisalabad, Punjab, Pakistan	31°22'4.90"N	73°1'24.80"E
BC-9	Qila Ram Qaur, Hafizabad, Punjab, Pakistan	32°4'57.12"N	73°40'49.02"E
BC-10	New City Housing Society, Jaranwala, Punjab, Pakistan	31°19'16.60"N	73°23'21.44"E
BC-11	Chak 126 GB Pind Janjua, Jaranwala, Punjab, Pakistan	31°21'38.48"N	73°25'28.74"E
BC-12	Rahim Yar Khan Zoo, Rahim Yar Khan, Punjab, Pakistan	28°24'14.30"N	70°15'32.63"E
BC-13	Yazman Housing Scheme, Yazman, Punjab, Pakistan	29°6'54.39"N	71°45'17.40"E
BC-14	Ayub National Park, Jehlam Road, Punjab, Pakistan	33°34'19.00"N	73°4'59.00"E
BC-15	Tibbi Balochan, Sadiqabad, Punjab, Pakistan	28°16'35.01"N	70°8'6.58"E
BC-16	Maraghzar Colony, Lahore, Punjab, Pakistan	31°30'8.71"N	74°14'55.48"E
BC-17	Lahore Zoo, Punjab, Pakistan	31°33'23.78"N	74°19'33.73"E
BC-18	Lahore Zoo, Punjab, Pakistan	31°33'23.78"N	74°19'33.73"E
BC-19	Raza Garden Phase 1, Sargodha, Punjab, Pakistan	32°2'51.23"N	72°37'31.68"E
BC-20	Pir wala, Jhang, Punjab, Pakistan	31°1'42.61"N	72°16'45.51"E
BC-21	Noor Garden, Okara, Punjab, Pakistan	30°48'48.38"N	73°28'38.33"E
BC-22	Chenab Park, Multan, Punjab, Pakistan	30°4'29.90"N	71°18'51.93"E
BC-23	Kalarwala, Chiniot, Punjab, Pakistan	31°28'26.21"N	72°33'56.11"E
BC-24	Qadir Abad Tiba, Sadiqabad, Punjab, Pakistan	28°16'54.33"N	70°7'45.48"E
BC-25	Chenab Park, Gujranwala, Punjab, Pakistan	30°4'29.90"N	71°18'51.93"E

tail tip biopsies were obtained from each specimen. After DNA extraction (Sambrook and Russel 2001), the Polymerase Chain Reaction (PCR) primers of representative mitochondrial genes (ND4, Cytochrome b, 12S rRNA, and 16S rRNA) and nuclear genes (C-mos, RAG1, and NT3) from previous studies were used for the amplification of selected regions through PCR (see Table 2). After amplification, amplicons were sequenced bi-directionally by Big DyeTM Terminator on an ABI 3130XL Genetic analyzer. Forward and

Table 2. Mitochondrial and nuclear protein coding gene primers for Common Krait (*Bungarus caeruleus*).

Sr. No	Gene Name	Primer Sequence	Source
1	Cyt.b	5'-TGACTTGAARAACCAYCGTTG-3'	Palumbi 1996
		5'-TGAGAAGTTTTCYGGGTCRTT-3'	Parkinson et al. 2002
2	16S rRNA	5’-CGCCTGTTTAYCAAAAACAT-3	Vences et al. 2005
		5’-CCGGTCTGAACTCAGATCACGT-3’	Vences et al. 2005
3	12S rRNA	5’GTACACTTACCTTGTTACGACTT 3’	Knight and Mindell 1993
		5’ AAACTGGGATTAGATACCCCACTAT3’	Knight and Mindell 1993
4	ND4	5’-CATTACTTTTACTTGGATTTGCACCA-3’	Arevalo 1994
		5’-CACCTATGACTACCAAAAGCTCATGTAAGC-3’	Arevalo 1994
5	RAG-1	5’AGCTGCAGYCARTAYCAYAARATGTA3’	Chiari et al. 2004
		5’AACTCAGCTGCATTKCCAATRTCA3’	Chiari et al. 2004
6	NT3	5’ATATTTCTGGCTTTTCTCTGTGGC3’	Townsend et al. 2008
		5’GCGTTTCATAAAAATATTGTTTGACC3’	Townsend et al. 2008
7	C-mos	5' CATGGACTGGGATCAGTTATG 3'	Lawson et al. 2005
		5'CCTTGGGTGTGATTTTCTCACCT 3'	Lawson et al. 2005

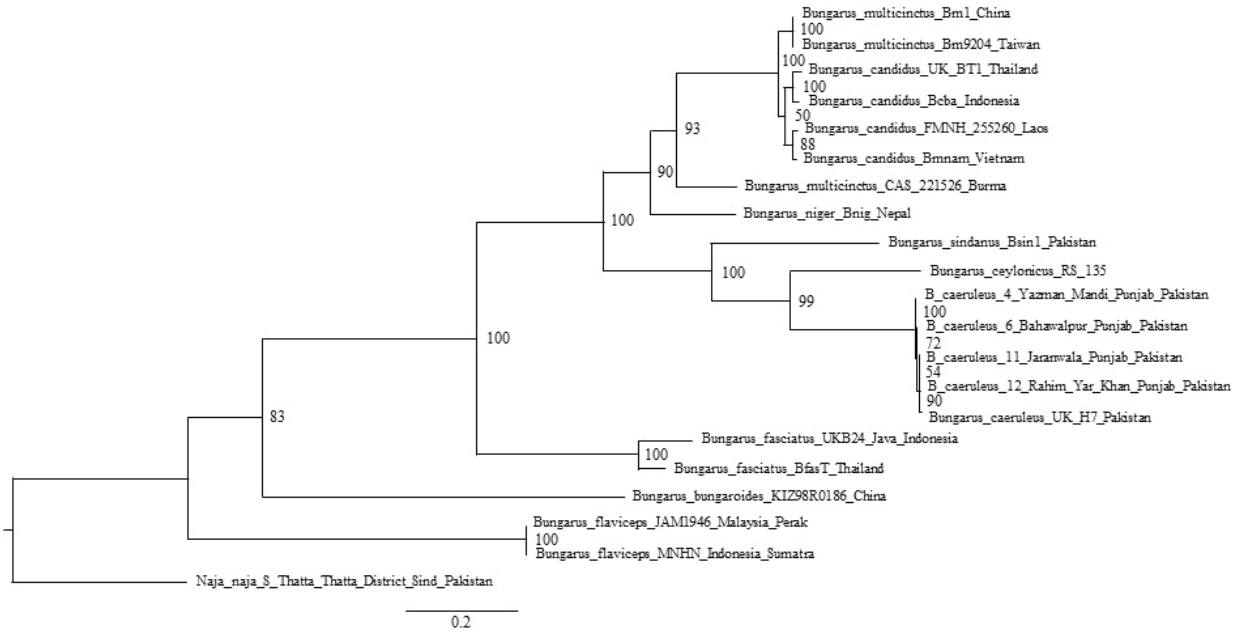

Fig. 2. Mitochondrial and nuclear genes (ND4, Cyt. b, COI, 12S rRNA, 16S rRNA, C-mos, RAG-1, NT3, and BDNF) based Maximum Likelihood phylogeny for Common Krait (*Bungarus caeruleus*).

reverse sequences were assembled through Sequencher 5.0 software. The resulting contigs (sequences) were given specific identities. These contigs were then aligned with other reported sequences obtained from the NCBI database through MEGA (v 6.0, Tamura et al. 2013) using the ClustalW tool for further data analyses. The nucleotide data for each gene were concatenated using SequenceMatrix (v1.7.8, Vaidya et al. 2011) software. The concatenated data were partitioned through PartitionFinder (v1.1.1, Lanfear et al. 2012) to give the best partition scheme and evolutionary models for phylogenetic analyses.

Two types of phylogenetic analyses, i.e., Maximum Likelihood (ML) and Bayesian inference (BI), were performed through RaxML (v8.0, Stamatakis 2014) and MrBayes (v3.2, Ronquist and Huelsenbeck 2012) software. The resulting phylogenetic trees were visualized and saved using Figtree (v1.4.3, http://tree.bio.ed.ac.uk/software/figtree/) software. DnaSP (v5.0, Librado and Rozas 2009) was used for analyzing polymorphic sites and DNA polymorphism, to determine the variation and genetic biodiversity in Common Krait (*Bungarus caeruleus*) in relation to other species of the genus *Bungarus*. Percent identity matrices were also constructed by comparing different species of every snake genus using online tool MUSCLE (available from the European Molecular Biology Laboratory, https://www.ebi.ac.uk/Tools/msa/muscle/).

Results

DnaSP software was used for analyzing the polymorphism of the mitochondrial and nuclear genes as shown in Table 3. The ribosomal RNA coding genes showed the least variation, with lower numbers of variable sites, mutations, and parsimony informative sites. The polymorphism data show the variations in different mitochondrial and nuclear genes with their conservation among Common Krait and other species of genus *Bungarus*. In addition, no significant variations were found among Common Kraits from different cities in Pakistan.

The online MUSCLE tool was used to find relationships among *Bungarus* species on the basis of homology in the mitochondrial and nuclear genes (Table 4). This table shows the conservation patterns in mitochondrial and nuclear protein coding genes of various *Bungarus* species.

Phylogenetic analysis of Common Krait (*Bungarus caeruleus*) from Pakistan was conducted using mitochondrial and nuclear protein coding genes. In this study, Black Cobra (*Naja naja*) from Thatta Sindh was used as the outgroup for constructing maximum likelihood and Bayesian phylogenies. The best partition scheme and evolutionary models were used to infer the phylogenetic relationships of Common Krait in Pakistan with other members of genus *Bungarus* around the world. Concatenated Maximum likelihood and Bayesian Inference results gave very similar phylogenies (Figs. 2–3). All *Bungarus* species were divided into four main clades. The first clade included *B. candidus* (Indonesia, Thailand, Vietnam, and Laos), *B. multicinctus* (China, Taiwan, and Burma), and *B. niger* (Nepal). The second clade included *B. sindanus* and *B. caeruleus* (Pakistan), and *B. ceylonicus* (Sri Lanka). The third clade included *B. fasciatus* (Thailand and Indonesia), while the fourth clade included *B. bungroides* (China) and *B. flaviceps* (Malaysia and Indonesia). The first and second clades showed a sister clade relationship with strong support (ML BS = 100, BI PP = 1). *Bungarus candidus* and

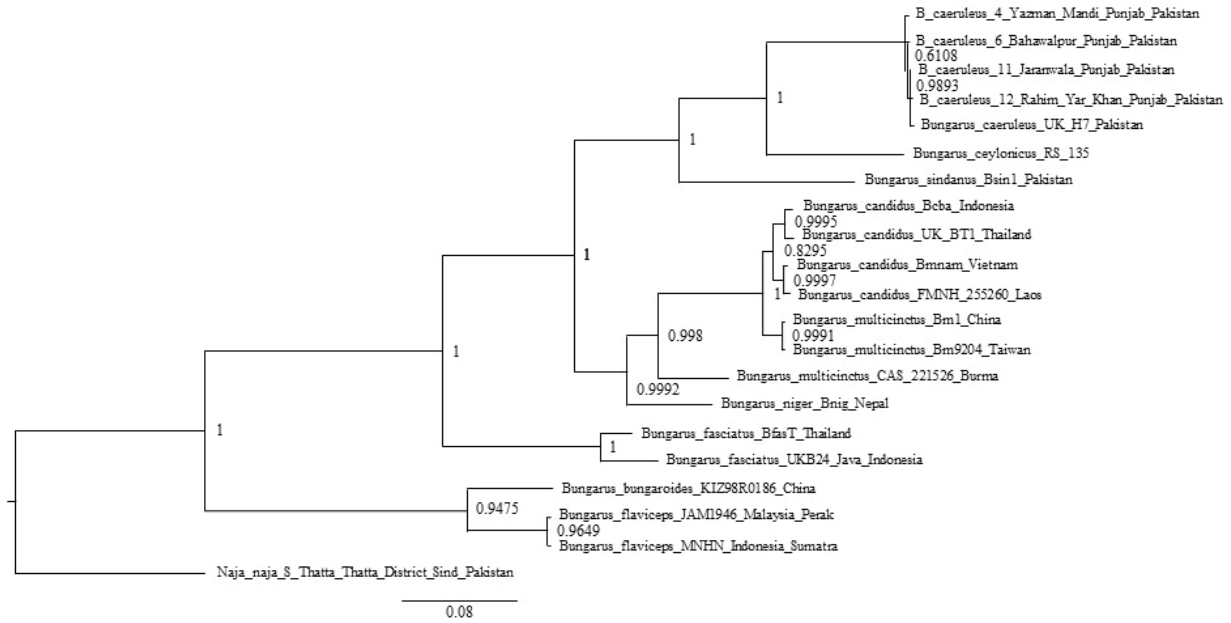

Fig. 3. Mitochondrial and nuclear genes (ND4, Cyt b, COI, 12S rRNA, 16S rRNA, C-mos, RAG-1, and NT3) Bayesian phylogeny for Common Krait (*Bungarus caeruleus*).

B. multicinctus probably diverged as separate species only recently. *Bungarus candidus*, *B. multicinctus*, and *B. niger* showed highly supported sister relationships with a complex pattern of divergence (BI PP = 1.0 ML BS = 90). In the second clade *B. sindanus* and *B. caeruleus* have been reported from Pakistan, thus they are sympatric species, while *B. ceylonicus* (from Sri Lanka) also showed a significant difference with strong support through Maximum likelihood and Bayesian inference phylogenies (PP = 1.0 and BS = 100). Pyron et al. (2012) also revealed the same relationships between *B. caeruleus*, *B. sindanus*, and *B. ceylonicus.*

In addition to the molecular data obtained, examination of the 25 *B. caeruleus* specimens showed varying numbers of ventral scales (207–218), 15 rows of mid-body scales, and average numbers of subcaudals of 41–47.

Discussion

Elapids comprise 300 of the 2,500 known species of snakes (Leviton et al. 2018). The Southern Asian elapids include cobras (*Naja* and *Ophiophagus*), kraits (*Bungarus*), long-glanded snakes (*Maticora*), and Asian coral snakes (*Calliophis*) [Sanz et al. 2019]. The uncertain phylogenetics of elapids has been a major factor for the varying numbers of identified species of elapids in the past (Mirtschin et al. 2017).

This study aimed to characterize the genetic biodiversity and phylogenetic relationships of Common Krait (*Bungarus caeruleus*) as there is a great deal of unpublished data on this species. Here, mitochondrial and nuclear protein coding genes were used to construct the phylogeny of Common Krait from Pakistan along with some morphological characterization. One variable character is the number of ventral scales that ranges from 207–218 among the 25 specimens in this study. Khan (1985) wrote a note on the taxonomic status of Common Krait and Sindh Krait (*B. sindanus*), and by comparing 46 specimens, Khan noted an almost similar range of 207–218 ventral scales. The *B. caeruleus* in this study had 15 rows of mid-body scales which is the same as described by Khan (1985). *Bungarus caeruleus* and *B.*

Table 3. Polymorphism in mitochondrial and nuclear protein coding genes of Common Krait (*Bungarus caeruleus*).

Parameters	ND4	Cytochrome b	12S rRNA	16S rRNA	C-mos	RAG-1	NT3
Total number of sites	619	702	650	520	586	802	425
Variable number of sites	302	325	270	191	419	665	325
Number of mutations	302	202	63	47	12	20	32
Singleton variable sites	43	51	20	26	09	19	03
Parsimony informative sites	196	151	43	21	03	01	28
Segregating sites	159	151	00	00	12	19	31
Synonymous changes	176	156	00	00	06	03	23
Number of haplotypes	17	16	08	10	04	03	05
Haplotype diversity	0.866	0.615	0.686	0.521	0.333	0.145	0.754
Nucleotide diversity	0.08243	0.09528	0.03617	0.03451	0.00288	0.00226	0.03389

Table 4. Percent homology of mitochondrial and nuclear genes for Common Krait (*Bungarus caeruleus*) from Pakistan among various *Bungarus* species and countries, sorted by decreasing Cytb homology. The asterisk (*) indicates the sequence from this study, all others are based on GenBank sequences.

		Homology percentages of nine representative genes								
Species	**Country**	**Cytb**	**ND4**	**12S**	**16S**	**COI**	**C-mos**	**RAG-1**	**NT3**	**BDNF**
*B. caeruleus**	Pakistan	100	100	100	100	100				
B. caeruleus	Pakistan	99.84	99.51	100	100	99.99				
B. ceylonicus	NA	89.21	87.46	93.68	96.62		100	99.4		
B. candidus	Indonesia	89.19	84.53	90.57	95.08					
B. niger	Nepal	86.31	84.36							
B. sindanus	Pakistan	86.31	85.5							
B. candidus	Thailand	85.35	83.88							
B. multicinctus	Burma	85.19	84.34			85.65				
B. multicinctus	China	85.19	85.18							
B. candidus	Vietnam	85.19	85.67							
B. multicinctus	Taiwan	85.02	85.5	91.73	90.09	86.11				
B. fasciatus	Thailand	83.74	83.55	90.79	91.91					
B. fasciatus	Indonesia	83.57	83.39	91.38	95.5	85.03	99.38	99.62	96.63	99.76

sindanus had 15 and 17 rows of mid-body scales, with the central larger row being hexagonal and white in color. Boulenger (1897) also observed 15 rows of mid-body scales in Indo-Pakistan Common Krait while 17 mid-body scale rows were reported in *B. sindanus* from Indus Basin. The average number of sub-caudals observed here is 41–47 which is within the range observed by Khan (1985): 40–54 in males and 30–54 in females. Boulenger (1897) reported small eyes with round pupil which is similar to those observed in this study.

There are also reports about the distribution of *B. caeruleus* in Indo-Pakistan subcontinent. Eastward it is found in Assam and Bengal (Jamal et al. 2018; Ganesh and Vogel 2018); westward to the Pakistan-Iran Border; Shockley (1949) and Kral (1969) reported it in Afghanistan; Smith (1943) reported it southward in Peninsular India and the Andaman Islands; and de Silva (1981) also reported it in Sri Lanka. This study is one attempt to infer the phylogenetics of *B. caeruleus* in Pakistan, but suggests more studies from the above-mentioned parts of the *B. caeruleus* distribution are needed, as there are almost no studies from other parts of of the Indo-Pakistan subcontinent on the phylogenetics of the Common Krait *B. caeruleus*.

Determining the relationships among the members of Elapidae can help in understanding the distribution and diversity of elapids. Many studies have focused on evolutionary relationships of elapids, and this study examined the molecular phylogenetics of *B. caeruleus* from Pakistan. The second clade (Figs. 2–3) includes *B. sindanus* and *B. caeruleus* (Pakistan), and *B. ceylonicus* (Sri Lanka). *Bungarus sindanus* and *B. caeruleus* are sympatric species, while *B. ceylonicus* also showed a significant difference with strong support through the Maximum likelihood and Bayesian inference phylogenies (PP = 1.0 and BS = 100). Pyron et al. (2012) also revealed the same relationships between *B. caeruleus*, *B. sindanus*, and *B. ceylonicus*. They presented a large-scale phylogeny of squamate reptiles for future comparative studies, and a revised classification of squamates at the family and subfamily levels so that taxonomy might be brought in a line with data from the new phylogenetic studies. Their phylogeny shows the same relationship (ML BS = 100, BI = 1) between *B. caeruleus*, *B. sindanus*, and *B. ceylonicus* as is shown in this study through Maximum Likelihood and Bayesian phylogenies.

Conclusions

Most of the currently recognized krait species (genus *Bungarus*) are poorly understood. This study characterized the genetic biodiversity and phylogenetic relationships of Common Krait (*Bungarus caeruleus*) from Pakistan showing inter- and intra-specific variations among different geographical regions of the world. More diverse sampling and a larger number of samples with more genomic data could help to further resolve the taxonomic status of the *Bungarus* species in Pakistan. This study also provides guidance for the correct identification of these snakes with authentication using molecular biology tools which will be helpful in the development of effective and region-specific antivenoms for such venomous snakes.

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