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Research Article

Taxonomic assessment of *Bungarus caeruleus* (Schneider, 1801) in Kasur and Lahore Districts, Punjab, Pakistan: A morphological and phylogenetic analysis

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Abstract

Habitat fragmentation, inbreeding, and genetic drift are responsible for the loss of genetic variability. Advancement in molecular techniques is key to understanding species identification, species relationships, and evolution. The present was designed to collect information about the morphological variations and genetic diversity of *Bungarus caeruleus* (Schneider, 1801) from two districts (Kasur and Lahore) of the Punjab Province of Pakistan. Thirteen specimens of *Bungarus caeruleus* (Schneider, 1801) were collected from different locations in both districts. Data related to habitat, species, age, sex, location (village/tehsil/district), urbanicity global positioning system (GPS) coordinate, and sampling date were noted in the field. First, we study morphological variations in individuals. Then mtDNA (cytochrome-b) sequence of *B. caeruleus* was used to compare the phylogenetic status among inter and intra-species. Significant morphological variations are seen among the samples. The body measurement results show that area in the length and weight of the individual. There was no difference genetically between the thirteen samples of *B. caeruleus*.

Keywords: Diversity, Common Krait, Snake, Pakistan

Introduction

Molecular techniques have become one of the leading tools for the characterization of morphologically conservative species. Many studies have revealed that phylogenetic isolation occurs according to geographic division as opposed to natural history or morphology, revealing discordance with taxonomic concepts based on phenetic similarity. Advancements in molecular sciences through the last era have proven attractive to conservation biologists seeking to determine genetic distinctions at both intra-population and inter-population levels in a study species. Several multivariate techniques currently used widely to quantify genetic variability at many levels of taxonomic classification have been utilized for studies of genetic variability at the population level (Burbrink et al., 2000).

Snakes are critical models for research in ecology and evolution, and much of the recent work in this field has been conducted in a phylogenetic framework. These studies include sexual-size dimorphism (Sahlean et al., 2015), functional morphology (Passos et al., 2016), population genetics (Levine et al., 2016; Folta et al., 2018), genetic diversity (Madsen et al., 2000; Clark et al., 2009), conservation genetics (Austin et al., 2010), species diversification and morphological disparity (Marin et al., 2013), venom variability (Chippaux et al., 1991), forensic investigation (Ciavaglia et al., 2014), morphology and taxonomy (Khan, 1985; Murphy et al., 2013) and hematology (Dissanayake et al., 2015). Within this body of research, snakes in Pakistan are underrepresented, though some research has been conducted in diversity and taxonomy, geographic distribution, envenomation, phylogenetics, conservation genetics, and biology (Madsen et al., 2000; Wong et al., 2004; Khan, 2006; Dubey et al., 2009; Dubey et al., 2010).

In Pakistan, 77 snake species and subspecies are represented by 34 genera belonging to 8 families. Four of these families, the Elapidae, Hydrophidae, Viperidae, and Crotalidae, are venomous, while the remaining four families, Leptotyphlopidae, Typhlopidae, Boidae, and Colubridae are non-venomous (Khan, 2006). Despite their relatively equal representation, venomous snakes are usually poorly represented in studies of conservation genetics, and particularly, some medically important species such as *B. caeruleus* have not been studied much before in Pakistan. *B. caeruleus* is a proteroglyphous elapid snake commonly found in Pakistan (Khan, 2006) and by its nature, *B. caeruleus* bites occur less frequently than viper bites in Pakistan, but cause significant morbidity and mortality (Mahmood et al., 2010). The composition, biological activity, and antigenic properties of snake venoms could vary significantly between species (Chippaux et al., 1991), and studying the phylogenetic status of a snake can therefore have crucial implications for understanding key evolutionary processes leading to speciation and venom variability (Hartl and Clark, 2007; Hughes et al., 2008; Casewell et al., 2011). In practical terms, this study of venomous snakes could be of major medical significance to Pakistan. Many mitochondrial markers have been considered reliable tools for species identification and phylogenetic construction. The mitochondrial cytochrome b (Cyt-b) gene has been extensively used to study phylogenetic relationships, genetic variation, and taxonomy in many vertebrates and invertebrates. In this study, our primary goal was to use a combination of molecular techniques and traditional morphological taxonomy to elucidate the phylogenetic status of *B. caeruleus*. Focusing on the district of Kasur and Lahore as a local population, we sequenced part of the Cyt-b gene and performed morphological and morphometric measurements of *B. caeruleus*. With an emphasis on this observation, we want to provide new insights into the phylogeographic processes leading to speciation in venomous snake taxa, which may be necessary for a better understanding of evolution.

Material and methods

Study species

The *B. caeruleus* is an elapid terrestrial snake and one of the most medically important and widely distributed throughout all pen plains in Pakistan (Khan, 2006). The *B. caeruleus* is an average-sized snake, with no distinction between head and neck, round pupils black eyes, and black to blue color (Khan, 2006). They are found in multiple habitats like agriculture fields, grasslands, farms, scrublands, along water courses, and near human habitations. They live in crevices, rodent holes, old buildings, graveyards, under piles, and bricks (Khan, 2006).

Study area and sample collection

The study was conducted in the district Lahore and district Kasur in Punjab, Pakistan. The district Lahore (N 31025`E 74020`) is the capital of Punjab province. Add more Snake samples (dead) were collected from different locations in both districts. Data related to the sample; habitat, species, age, sex, global positioning system (GPS) coordinates, location (village/tehsil/district), urbanicity, and date were noted in the field survey form. To avoid the degradation of the sample, they were preserved in 70% ethanol and brought to the Wildlife Epidemiology and Molecular Microbiology Laboratory, the University of Veterinary and Animal Sciences, Lahore, Ravi Campus, Pattoki.

Morphological identification and measurement

Each sample was identified and measured using taxonomic identification keys (Khan, 2006). Morphometric data taken from the specimens in the type series were: snout to vent length (SVL), length from the tip of snout to the posterior margin of the anal plate; tail length (TAL), length from posterior margin of the anal plate to tip of tail; head width (HW), the widest distance between lateral margins of the quadrates; head length (HL), the length between the anterior medial margin of the rostral plate and posterior margin of the parietals; the trunk length is measured by the subtraction of head length from the snout to vent length. The sum of SVL and TAL was used to obtain the total length (TOTL). Measurements were taken using a digital Vernier caliper (Mitutoyo vernier calipers) to 0.01 mm. SVL and TAL were measured using a measuring tape. Sex was determined by dissecting specimens to examine the reproductive organs.

DNA extraction, amplification, and sequencing

Total DNA was extracted from tissues preserved in 70% ethanol. DNA extraction was performed in the post-graduate Laboratory, Institute of Biochemistry and Biotechnology, UVAS, Lahore using standard DNA extraction protocol from tissue (Sambrook & Russell, 2001). The quality and quantity of DNA were measured by Spectrophotometer (Nanodrop 2000) and Agarose gel electrophoresis. A 239bp fragment of the cytochrome b gene was amplified using the polymerase chain reaction, Forward 5’-CCATCCAACATCTCAGCATGATGAAA-3’ and Reverse 5’-GCCCCTCAGAATGATATTTGTCCTCA-3’ (Kocher et al., 1989) using a hot-start method in a thermal cycler with 3 minutes denaturing step at 94°C followed by 39 cycles of denaturing for 45 sec at 94°C, primer annealing for 45 sec at 45-50°C, and elongation for 1.5 min at 72°C, with a final 5 min elongation step at 72°C.

Sequencing and bioinformatics analysis

Sanger sequencing was performed on all of the samples using a DNA analyzer from Malaysia. The ambiguous bases are trimmed from obtained DNA sequences and aligned using BioEdit 7.0. Phylogenetic analysis of *Bungarus caeruleus* from Pakistan was conducted using mitochondrial genes. A model was used to infer the phylogenetic relationships of *Bungarus caeruleus* from local and outgroup with other members of the genus *Bungarus* around the world. The closely matched sequences of *Bungarus caeruleus* and other members of the elapidae family were acquired from the NCBI database.

Results

The snake samples of *B. caeruleus* were collected from district Kasur and Lahore. The field data related to samples collected from different sites are shown in Table 1. During the study period, 13 dead specimens of *B. caeruleus* were collected: 8 females and 5 males. Based on the physical characteristics of the specimen, eleven were identified as adults and two young ones. Maximum numbers of samples were collected from Kasur (61.5%) as compared to district Lahore (38.5%). However, the maximum diversity of *B. caeruleus* was observed in rural areas (76.9%) as compared to urban (23%). Ecologically, 7 (53.8%), 3 (23%), 2 (15.3%), and 1 (7.6%) samples were collected from agricultural land, human settlements, barren land, and near water bodies, respectively (Table 1).

Table 1. Descriptive information for the snake collected during the study period

ID	Locality	Sex	Age	Landscape	GPS coordinate	Date
1	Lahore	M	Adult	Human settlements	31.489672N, 74.243175E	11/6/2017
2	Kasur	F	Young	Agriculture land	31.024069N, 74.521255E	13/6/2017
3	Lahore	F	Adult	Human settlements	31.328923N, 74.370569E	15/7/2017
6	Kasur	M	Young	Water body	31.010767N, 73.893779E	1/8/2017
7	Kasur	F	Adult	Barren land	31.043012N, 73.874966E	2/8/2017
8	Kasur	F	Adult	Agriculture land	31.010767N, 73.893779E	3/8/2017
9	Lahore	M	Adult	Human settlements	31.519558N, 74.327441E	3/9/2017
10	Kasur	F	Adult	Agriculture land	31.059845N, 73.860278E	7/9/2017
11	Kasur	M	Adult	Barren land	31.043012N, 73.874966E	9/9/2017
14	Kasur	F	Adult	Agriculture land	30.985812N, 73.820613E	12/9/2017
15	Lahore	M	Adult	Agriculture land	31.407385N, 74.389391E	23/09/2017
16	Lahore	F	Adult	Agriculture land	31.407385N, 74.389391E	23/09/2017
17	Kasur	F	Adult	Agriculture land	31.046178N, 74.380812E	24/9/2017

Seven morphometric parameters i.e. SVL (mm), TAL (mm), length (HL), Head width (HW), Body width (BW), Trunk length (T), Total length (TL), and body weight are described using descriptive statistics in Table 2 & 3. The body measurement results show that area influences the length and weight of the individual. The average total lengths (727.50±302.17) of samples that were collected from district Kasur were more than the average length (711.00±192.89) of district Lahore. The weight of district Kasur (113.70±90.09) samples was slightly larger than district Lahore (98.60±40.13).

Table 2. District vise morphological characteristics of snakes

Parameters	Kasur	Lahore

	Mean±SD	**Mean±SD**
SVL (mm)	624.88±261.62	613.20±157.67
TAL (mm)	102.63±42.26	97.60±36.07
HL (mm)	23.13±8.43	20.00±6.48
HW (mm)	19.00±7.96	15.80±5.50
BW (mm)	39.38±24.94	33.00±9.11
W (g)	113.70±90.09	98.60±40.13
TL (mm)	727.50±302.17	711.00±192.89
T (mm)	601.75±254.15	593.20±151.55

Table 3. Male and female morphological measurements

Body parameters	**Male Mean±SD**	**Female Mean±SD**	**Male & Female Combined**
SVL (mm)	500.80±494.96	695.13±227.35	U=13, P=0.02, Z=1.90
TAL (mm)	76.00±26.59	116.13±37.80	U=8, P=0.007, Z=2.43
HL (mm)	17.00±7.04	25.00±6.55	U=9.5, P=0.0084, Z= 2.34
HW (mm)	12.00±3.67	21.38±6.23	U=9, P=0.009, Z= 2.32
BW (mm)	28.80±10.62	42.00±23.37	U= 18.5, P=0.93, Z=1.32
W (g)	82.78±59.76	123.59±79.78	U=20, P=0.12, Z=1.16
TL (mm)	577.00±184.85	811.25±262.76	U=13, P=0.02, Z=1.90
T (mm)	483.80±152.11	670.13±221.96	U=13, P=0.02, Z=1.90

Table 4. District Lahore and Kasur male female comparison

Body Parameters	**Kasur Mean±SD**		**Lahore Mean±SD**	
	Male	**Female**	**Male**	**Female**
SVL (mm)	469±260	676.83±263.29	522±122	750±84.85
TAL (mm)	76±43.84	111.5±41.68	76±21.28	130±28.28

HL (mm)	17.5±10.61	25±7.75	16.67±6.51	25±0.00
HW (mm)	11.5±4.95	21.50±7.34	12.33±3.79	21±1.41
BW (mm)	27±16.97	43.5±27.05	30±8.72	37.5±10.61
W (g)	70.45±88.18	128.12±93.87	91±54.78	110±2.83
TL (mm)	545±304.06	788.33±302.62	598.33±142.86	880±113.14
T (mm)	4511.5±249.61	651.83±256.76	505.33±115.69	725±84.85

The measurements based on sexual dimorphism indicate that the female's snout-to-vent length (695.13±227.35) and TL (811.25±262.76) were greater as compared to males SVL (500.80±494.96) and TL (577.00±184.85)], respectively. The average tail length of females (670.13±221.96) was higher than males (483.80±152.11). The heads of males (17.00±7.04) were shorter than the female head length (25.00±6.55). The heads of females (21.38±6.23) were wider than the males (12.00±3.67). The female (42.00±23.37) mid-body was wider than the opposite sex (28.80±10.62). The males (82.78±59.76) were light in weight as compared to females (123.59±79.78). The female's average (654.13±220.40) trunk length was higher than males (483.80±152.11) Table 3.

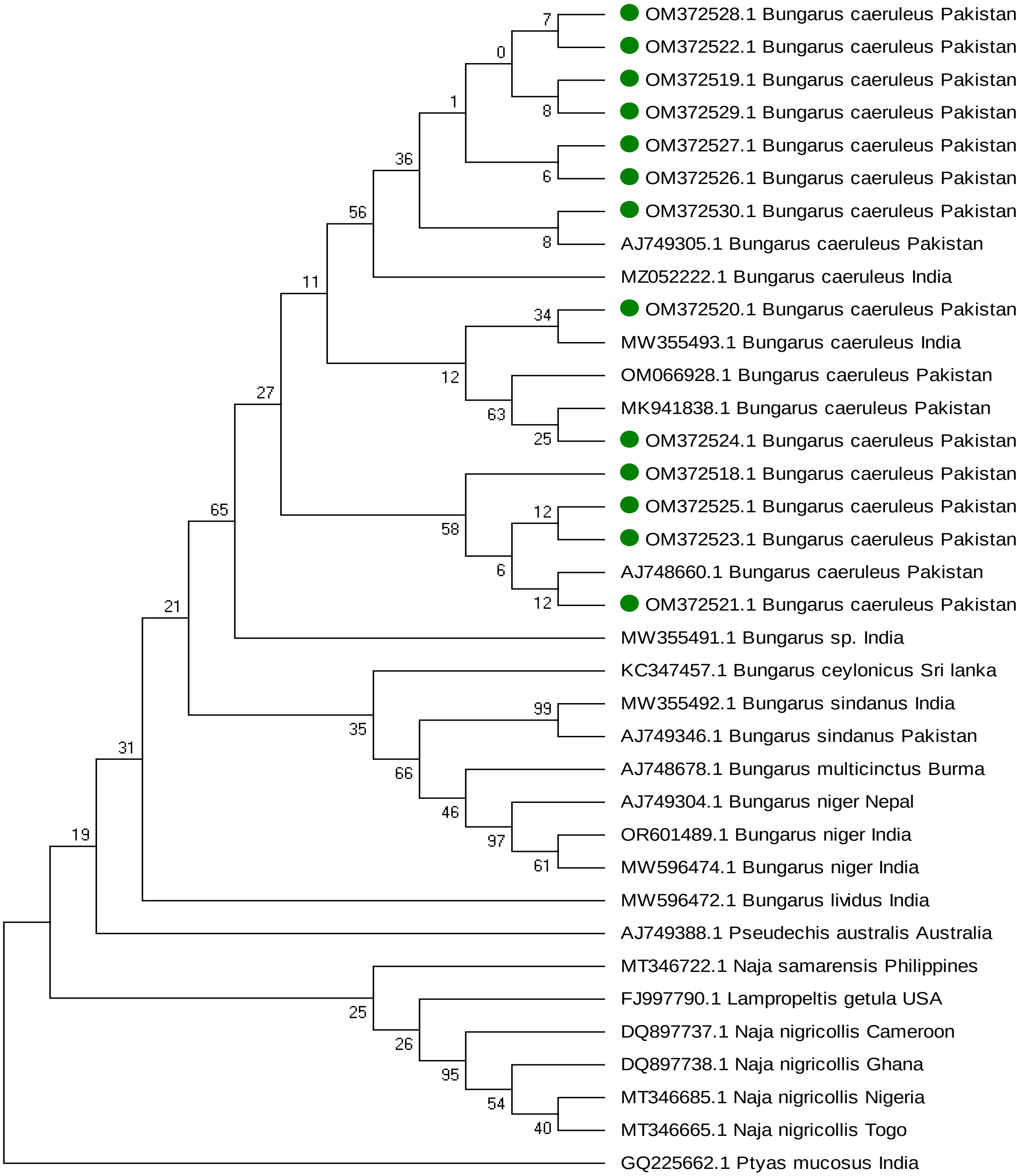

Figure 1. Mitochondrial Cyt-b gene maximum likelihood phylogenetic tree for *B. caeruleus* based on Tamura-Nei model with 1000 Bootstraps consensus.

A few DNA barcoding investigations of *B. caeruleus* have been conducted recently. The mitochondrial coding genes were used in a phylogenetic analysis of the Common Krait (*B. caeruleus*) from Pakistan.

The Cyt-b sequences of all specimens were used to search the National Center for Biotechnology Information (NCBI) database for mitochondrial gene homologies using the BLAST programs, and the results were deposited in the GenBank (OM372519-OM372531). MEGA 6 was used to align sequences. The partial mitochondrial Cyt-b sequences of *B. caeruleus* were aligned using the default parameters of MEGA 6 software (Kumar et al. 2004). All unalignable bases and gaps were wisely deleted from these data sets.

Discussion

Pakistan is a country that has rich wild fauna and flora. Among these wild fauna, multiple snake species are reported from Pakistan. Most of them are venomous like the Indian rock python, but some are poisonous i.e. Common krait, Brown cobra, saw-scaled viper, and Russell viper. These snake species face many threats especially when they are inhabited by human vicinity. Comparatively non-poisonous snakes are not frequently killed by humans; when they accidentally have an encounter with a human being. But poisonous snakes like *B. caeruleus* are killed by humans more frequently to ensure their safety from snake biting, which could be lethal otherwise.

One of the major causes of the decline of snakes is the degradation of natural habitats occupied by humans for agriculture, roads, and other infrastructure (Gibbons JW, 2000). Several environmental factors are present that have the potential to treat reptiles. Some metals accumulate in organs. Agricultural chemicals, herbicides, pesticides, and fertilizers have effects on snake's health. Radioactive waste also has a direct and indirect influence on reptiles (Bickford et al., 2010). An isolated population is going into inbreeding and is near to probable destruction due to genetic erosion (Frankham et al., 2002). Snakes are more affected by the roads and highways that divide the natural habitat into patches that decrease the population correlation. Conservationists claim that the influence of roadsides on population is a highly persistent conservation dispute (Clark et al., 2009).

A lot of work has been done on different aspects of snake species including ecology, breeding, conservation, threats, snake bite incidence, and medical importance in most countries (Singh et al., 2001; Keogh et al., 2003; Fornasiero et al., 2007; Neuman-Lee et al., 2011; Levine et al., 2016; Sharma et al., 2016). However, the snake species are poorly studied in Pakistan. Due to the scarcity of scientific literature on *B. caeruleus* in the country, the literature from other snake species was used for the comparison of different morphological and genetic parameters. The common krait is an elapid terrestrial snake generally recorded in Sri Lanka, Pakistan, Bangladesh, and India. In Sri Lanka, it is usually found in the arid region (Kularatne, 2002).   It’s seen all over Pakistan. They are found in multiple habitats like agricultural fields, grasslands, farms, scrublands, along water courses, and near human habitations. They live in cervices, rodent holes, old buildings, graveyards, and under piles and bricks (Khan, 2006).

The scientific data related to common krait from Pakistan is limited but no detailed recent information on morphological and genetic diversity is available in the country. We have informed above human death due to the biting of common krait or killing of snakes by humans in the study area. The present study was an initiative to collect the genetic differences in said snake species using dead samples. Damage to genetic variability caused by genetic drift and inbreeding is directly associated with the actual population size. The large constant populations with random breeding lead to genetic drift and inbreeding is insignificant and genetic diversity is conserved through generations. Amphibians habitually have very low effective population sizes (Beebee et al., 2005), with just a small percentage of the individuals who can produce new ones and next contribute to the gene pool during mating season (Rowe & Beebee, 2004).

On the basis of the evolutionary perspective, several species of snakes modify their characteristics (Shine et al., 2000). This influence is also seen in the external morphology of the *B. caeruleus* had distinct differences among genders, with females considerably larger as compared to males. A similar gender base difference in body length was reported in blotched snakes (*Elaphe sauromates*) (Sahlean et al., 2016). Foransiero et al (2007), reported size base sexual dimorphism in whip snake (*Hierophis viridiflavus*); males with larger body size and larger relative head size than females in the Central Mediterranean region.

The environmental factors put more pressure on the species' phenotype. We have observed intra-population morphological variation in the individuals that were collected from the populated or urban areas that have generally small body sizes due to congested range and availability of limited resources. On the other hand, the species found in the rural sites are overall large. However, there is a vast difference in the weight of the urban and rural site snakes. Similar site base intra-population physiological differences like soft anatomy, hemisphere, cephalic glands, osteology, and external morphology have been observed in the *Atractus ptoschi* in Brazil (i.e. northeastern region) (Passos et al., 2016). According to the data, the head size (HL) and head width (HW) of females are larger than the males showing that females are more sensitive to evolution than the opposite sex. The reasons behind the head size of females are increasing due to the availability of plenty of food and intake of bulky and large-size prey to succeed in their breeding season, While males are short in length (TL) and as well as in weight to females.

Areas have a great impact on the wildlife of that place; animals advance themselves according to their surrounding environment. This study also gives information about different areas of morphological differences between individuals of the same species. District Lahore is more populated than district Kasur, human interference with wildlife puts pressure on them, and limits resources (food, shelter, etc.). The *B. caeruleus* related to urban areas suppressed their size for survival as compared to individuals related to rural areas who have greater morphological characteristics.

Wild animals have traditionally been overlooked in Pakistan, despite their importance as a component and value of any ecosystem (Zafar et al., 2020). Khan (1985), documented two species of the genus *Bungarus* from Pakistan, *B. caeruleus* from Punjab province and *B. sindanus* from Sindh province. Knowing the determined structure of genetic variation is crucial for figuring out how current genetic diversity within and among species may be influenced by past landscape dynamics. This is essential as genetic variability can influence the course of evolution and the possibility of adaptation (Cox et al., 2018). This study aimed to evaluate the species delimitation by using phylogenetic analysis. A total of thirteen samples were sequenced for the cyt-b gene to construct maximum likelihood cladograms. The resultant contigs were checked by Bioedit 7.2 (Informer Technologies, Inc.) to reduce the amount of low-quality sequence reads and gaps. To get accession numbers, the generated sequences were submitted to the GenBank database. The DNA sequences of *B. caeruleus*, which were gathered from various parts of Pakistan, are almost similar and revealed no genetic distances. They produced a similar supported monophyletic clade of the sequences belonging to the same species obtained from the Genbank database and this study. This shows the diagnostic capability of Cyt-b to acceptably identify species. *B. caeruleus* DNA sequences are similar and indicate negligible genetic distances. The sequence generated from the study (OM372519-OM372531) shows 100% similarity with previously reported sequences (AJ749305.1 and AJ48660.1) from Pakistan and 99.6% with *B. caeruleus* from India (MZ052222.1) that fall in the same clade. A similar study on *B. caeruleus* from Pakistan can justify our phylogenetic results (Ashraf et al., 2019). While other species of the genus *Bungarus*; *B. sindanus* (India), *B. lividus* (India), *B. ceylanicus* (Sri Lanka), and *B. multicinctus* (Burma) are from common ancestors the genetic distance is shown by the separate taxon. The species from the other genera such as *Naja*, Diadophis, Elaphe, and others act as outgroups.

Conclusion

The demography of the population can influence how a species evolves. Where populations have existed for a long time, there may be genetic variations that can help populations adapt to changing environmental conditions. On the other hand, the limited genetic diversity at the inception of a territorial expansion can hinder the possibility of adaptability. This study successfully summarized the phylogenetic status of *B. caeruleus* from Pakistan. More morphological and genomic research that used a wider variety of samples from the region is necessary to infer a higher level of resolution in phylogenetic relationships and accurate identification.

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