Electronic Journal of Biotechnology 42 (2019) 23–29

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Molecular phylogenetics of Black Cobra (*Naja naja*) in Pakistan

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ARTICLE INFO

Article history:
Received 28 April 2019
Accepted 17 October 2019
Available online 24 October 2019

Keywords:
Bungarus
Cobra
Mitochondrial DNA
Naja
Nuclear DNA
Pakistan
PCR
Phylogenetics
Reptiles
Snakes
Venomous snakes

ABSTRACT

Background: Snakes are found on every continent in the world except Antarctica, and on smaller land masses. Being ecologically important, they also cause a large number of bites, leading to millions of deaths. Mitochondrial and nuclear gene sequences are being used to identify, characterize, and infer genetic biodiversity among different snake species. Furthermore, phylogenetics helps in inferring the relationships and evolutionary histories among these species. Black cobra is one of the four most venomous snakes in Pakistan. Four mitochondrial (ND4, Cytochrome b, 12S rRNA, and 16S rRNA) and four nuclear (C-mos, RAG-1, BDNF, and NT3) genes were used to trace diversity and infer the phylogenetic relationship of black cobra in Pakistan.
Results: Almost similar phylogenies were obtained through maximum likelihood and Bayesian inference, showing two species of cobra in Pakistan, namely, black cobra (*Naja naja*) and brown cobra (*Naja oxiana*). All *Naja* species were divided into three clades: black cobra (*N. naja*) and brown cobra (*N. oxiana*) cladding with different species of *Naja*; *N. naja* (Pakistan) cladding with *N. naja* from Nepal; and *N. oxiana* showed close relationship with *Naja kaouthia* from Thailand and *Naja siamensis* from Thailand.
Conclusion: It was confirmed genetically that there are two cobra species in Pakistan, i.e., black and brown cobras. This study will help in not only genetic conservation but also developing anti-venom against snake species.
How to cite: Ashraf MR, Nadeem A, Smith EN, et al. Molecular phylogenetics of Black Cobra (*Naja naja*) in Pakistan. Electron J Biotechnol 2019;42. https://doi.org/10.1016/j.ejbt.2019.10.005.

1. Introduction

Out of the total 300 species of land snakes in Pakistan, only 40 are venomous. Commonly found venomous snakes in Pakistan are cobras, kraits, and vipers [1]. The annual mortality rate estimate following envenomation soars is as high as 20,000 [2,3]. However, there is no reliable statistics regarding snake bite incidents, morbidity, and mortality except virtual statistics based on hospital cases [4,5,6].

Cobra is one of the representative animals of Pakistan and India with a high rate of mortality [7]. All the populations of cobra have now been given the status of a single species naja, but later, ten subspecies of *Naja naja* were identified. Asiatic cobras of the genus *Naja* make a complex and widespread group of venomous snakes. They have many years of controversial systematics [8,9,10,11]. There is much less confusion in Central Asia and the Indian subcontinent about the systematics of *Naja* than anywhere else. Black cobra (*N. naja*) is found throughout India, Pakistan, Nepal, and Bangladesh, whereas brown cobra (*Naja oxiana*) is found in Soviet Central Asia, Northeastern Iran, Afghanistan, Northern Pakistan, and northwestern India. *Naja kaouthia* is found in Delhi East to Assam and South to Vietnam and Northern Malaysia [12]. There are two types of cobras in Pakistan: one is black cobra (*N. naja*) and the other one is brown ox cobra (*N. oxiana*) [13]. The black cobra is found across Southern and Eastern Pakistan regions, while the brown cobra (*N. oxiana*) is only found in Northern Pakistan [14].

Phylogenetics shows relationships among organisms and genes. It gives a clearer picture of biodiversity, biogeography, and evolution of many characters in related groups [15,16]. Mitochondrial DNA studies for animal evolution have become a powerful tool in the last decade. Molecular biology has helped mitochondrial DNA studies to give an insight into the structure of population, gene flow, hybridization, biogeography, and phylogenetics [17]. Evolutionary studies provide a comparison of mitochondrial genome organization and function, while molecular studies help in the improvement of these evolutionary studies. Similarly, nuclear genes also seem to be a strong source of phylogenetic information. They can be more useful to study the

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2019.10.005

divergence of these genes whose multiple substitutions may cause destruction of the phylogenetic signal.

Pakistan has unique geographical and environmental conditions that bring diversity in local snakes. Black cobra is one of them causing significant snakebite accidents in the urban and rural areas of Pakistan. Almost no morphological and molecular data about black cobra from Pakistan are available for measuring genetic diversity and correct identification of this snake species. The present study is the first attempt from Pakistan focusing on black cobra, which would help in treating snakebites, hence saving millions of lives through effective anti-venom development strategies.

2. Materials and methods

2.1. Sampling

Samples were collected from tail tip biopsies of the black cobra. A total of 25 black cobra samples were collected from private reptile breeders in different cities of Pakistan. One tissue sample (CAS 232139) was obtained from the Department of Herpetology, California Academy of Sciences, San Francisco. Another tissue sample (MVZ Herp 248,467) was obtained from the Museum of Vertebrate Zoology, University of California, Berkeley. The samples were transferred to 70% ethanol before DNA extraction. The sampling localities were plotted on the Pakistan map obtained from (https://www.ezilon.com/maps/asia/pakistan-maps.html) as shown in Fig. 1. The coordinates of these sites are shown in Table 1.

2.2. DNA amplification

Total DNA was extracted using the phenol-chloroform-isoamyl alcohol (PCI) method [18]. The extracted DNA was dissolved in 10 mM Tris buffer (including 0.1% Tween 20) and stored in a -20°C freezer. Polymerase chain reaction (PCR) was performed to amplify the selected regions of mitochondrial and nuclear protein-coding genes. PCR primers from previous studies were used for the amplification of the mitochondrial genes ND4 [19], cytochrome b [20,21], 16S rRNA [22], and 12S rRNA [23] and the nuclear genes RAG-1 [24], C-mos [25], BDNF [26], and NT3 [26].

PCRs used 0.01% bovine serum albumin and GoTaq® Flexi DNA polymerase (Promega Corporation master mix) with Thermocycler GeneAmp® PCR System 9700. Touch-down PCR was performed with an annealing temperature range of 60–50°C with subsequent maintenance of the final temperature at 4°C. Amplicons were observed on a 1.2% agarose gel by electrophoresis. Approximately 20 μL of distilled water and 80 μL of absolute ethanol were added to the amplified DNA for precipitation. After 20 min, the tubes were centrifuged at 13,500 rpm for 10 min. The DNA pellet was obtained and air-dried for complete removal of ethanol residues. Finally, 12 μL of 10 mM Tris buffer (including 0.1% Tween 20) was added to the pellet and sent for DNA Sanger sequencing (Fig. 2 and Fig. 3).

2.3. Data analyses

All the DNA sequences were uploaded on Sequencher v5.0 (Gene Codes, Ann Arbor, Michigan, USA). Contigs of each forward and reverse sequence chromatogram were made to obtain a consensus sequence ensuring that there was no stop codon. Sequences with quality lower than 70% were not used to avoid any ambiguity. The sequences were aligned through MEGA [27] software using the ClustalW [28] multiple sequence alignment tool. Nucleotide data were translated into amino acids using the vertebrate mitochondrial and universal genetic code. DnaSP [29] was used for polymorphic DNA polymorphism

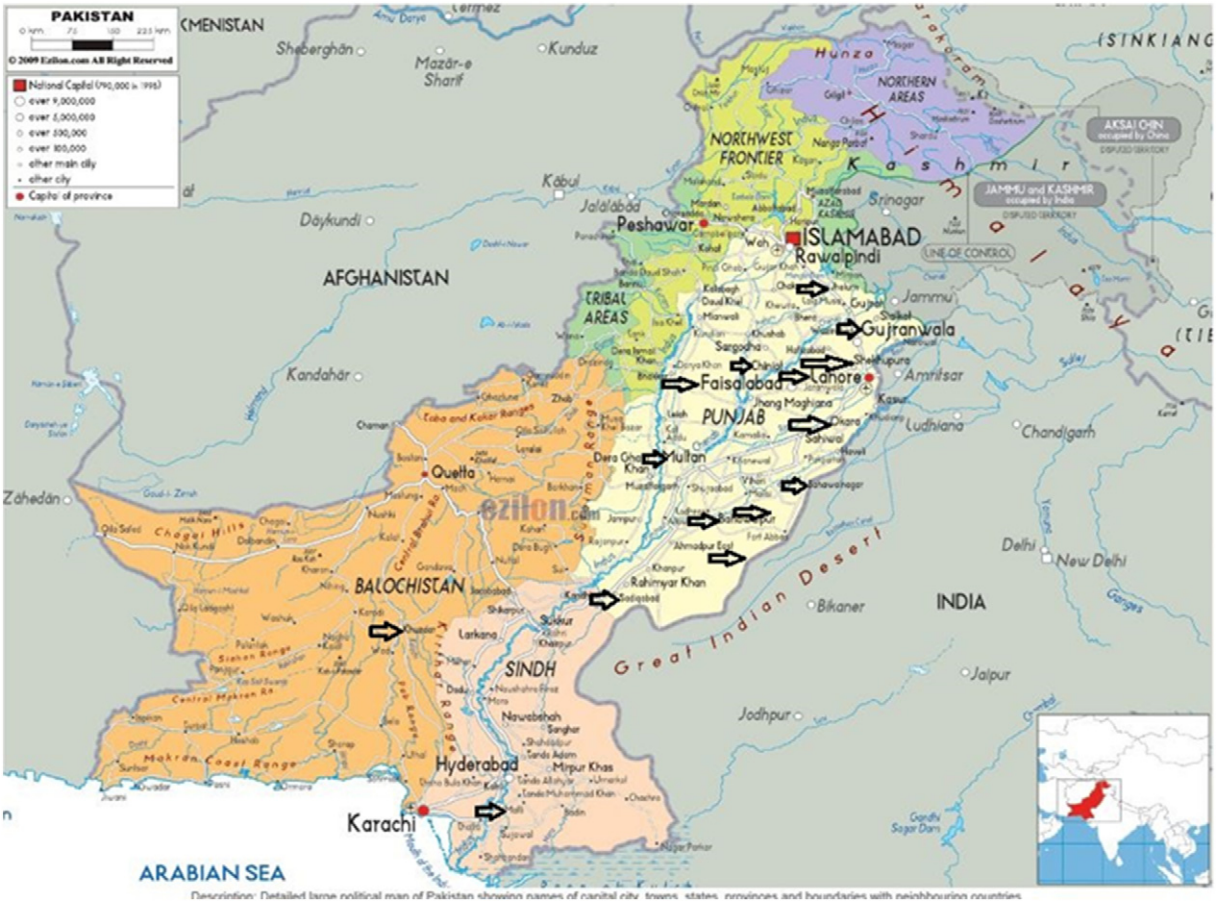

Fig. 1. Black cobra (*Naja naja*) sample collection sites in Pakistan.

Table 1
Information of black cobra (*Naja naja*) samples and their location.

Sample ID	Locality	Latitude	Longitude
NN-1	Bahawalnagar Zoo, Bahawalnagar, Punjab, Pakistan	30° 0′11.20″N	73°16′22.26″E
NN-2	Yazman Housing Society, Yazman, Punjab, Pakistan	29° 7′3.00″N	71°45′6.73″E
NN-3	Ayub National Park, Jhelum Road, Punjab, Pakistan	33°34′19.00″N	73° 4′59.00″E
NN-4	Basti Miani, Bahawalpur, Punjab, Pakistan	29°26′24.13″N	71°41′51.86″E
NN-5	Dijkot, Faisalabad, Punjab, Pakistan	31°12′55.90″N	72°59′57.12″E
NN-6	Lahore Zoo, Lahore, Punjab, Pakistan	31°33′23.78″N	74°19′33.73″E
NN-7	Fateh Pul, Hasilpur, Punjab, Pakistan	29°39′12.14″N	72°34′17.40″E
NN-8	Mian Town, Haroonabad, Punjab, Pakistan	29°36′4.41″N	73° 8′23.12″E
NN-9	Mauza Ali wala, Multan, Punjab, Pakistan	30° 5′58.03″N	71°23′39.52″E
NN-10	Hiran Minar Park, Sheikhupura, Punjab, Pakistan	31°44′34.88″N	73°57′18.64″E
NN-11	Noor Garden, Okara, Punjab, Pakistan	30°48′48.38″N	73°28′38.33″E
NN-12	Budla Sant, Multan, Punjab, Pakistan	30° 9′13.65″N	71°42′42.59″E
NN-13	Tauheedabad, Chiniot, Punjab, Pakistan	31°43′37.38″N	72°59′43.84″E
NN-14	Qadir Abad Tiba, Sadiqabad, Punjab, Pakistan	28°16′54.33″N	70° 7′45.48″E
NN-15	Chenab Park, Gujranwala, Punjab, Pakistan	30° 4′29.90″N	71°18′51.93″E
NN-16	Malkhanwala, Faisalabad, Punjab, Pakistan	31°21′21.69″N	73° 6′32.91″E
NN-17	Lahore Zoo, Lahore, Punjab, Pakistan	31°33′23.78″N	74°19′33.73″E
NN-18	Setellite Town, Hasilpur, Punjab, Pakistan	29°41′18.72″N	72°33′27.34″E
NN-19	Tiba Sharqiya, Haroonabad, Punjab, Pakistan	29°36′33.18″N	73° 8′49.67″E
NN-20	Green City, Okara, Punjab, Pakistan	30°49′23.90″N	73°28′2.54″E
NN-21	Khajiwala, Multan, Punjab, Pakistan	30° 8′32.90″N	71°22′53.94″E
NN-22	Chiniot Road, Chiniot, Punjab, Pakistan	31°43′29.21″N	73° 0′39.39″E
NN-23	Tibbi Balochan, Sadiqabad, Punjab, Pakistan	28°16′35.01″N	70° 8′6.58″E
NN-S	Makli, 4 km S, Thatta Sindh, Pakistan	24.60247″N	67.8205E
NN-B	Khuzdar, Balochistan, Pakistan	7 43.34N	66 55.24E

analyses. DNA polymorphism analyses included number of mutations, singleton variable sites, parsimony informative sites, and haplotype and nucleotide diversity. Table 2 shows the polymorphism analysis results in *Naja* species. Using multiple sequence comparison by log-expectation (MUSCLE), homology based on mitochondrial and nuclear genes was measured among the different species of the genus *Naja* from GenBank. The homology was presented as line graphs (Fig. 4 and Fig. 5). Sequence homology in the mitochondrial and nuclear genes presented gene conservation and variation in different protein coding and non-protein-coding genes. Non-protein-coding genes such as 12S

Fig. 2. Maximum likelihood (ML) phylogeny for black cobra (*Naja naja*).

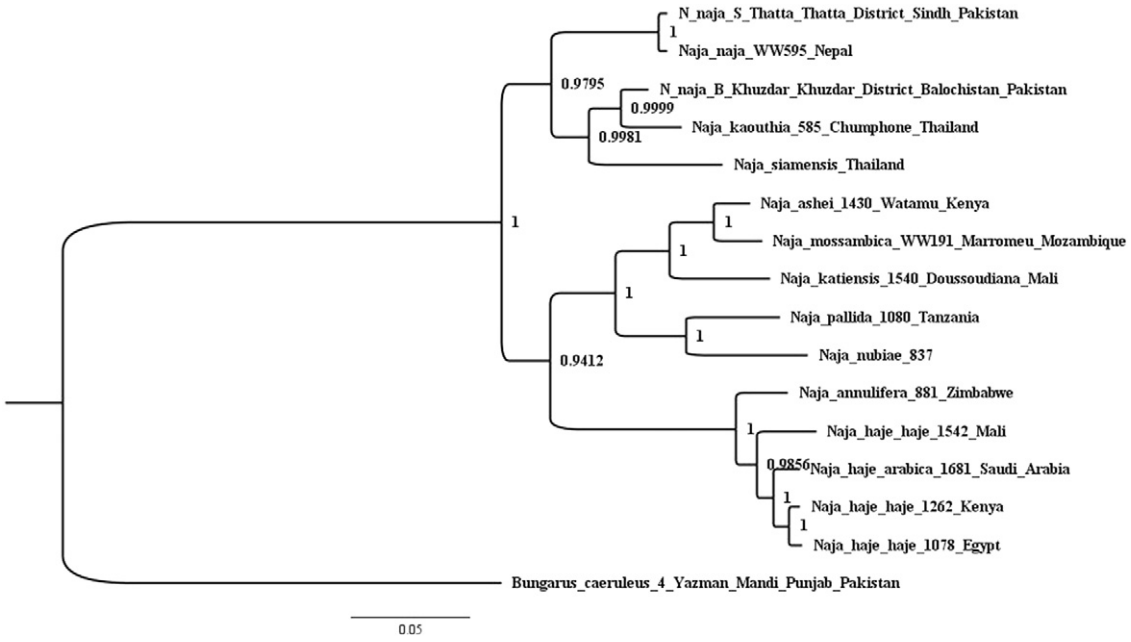

Fig. 3. Bayesian inference (BI) phylogeny for black cobra (*Naja naja*).

rRNA and nuclear protein-coding genes (C-mos, RAG-1, BDNF, and NT3) showed conservation being more homologous in the genus while mitochondrial protein-coding cytochrome b and ND4 showed sequence variation. All the sequences were concatenated using Sequence Matrix software [30].

The concatenated data were partitioned using PartitionFinder software [31] that provided the best partition scheme for partitioning the data and best models of evolution (TrN + I + G, HKY + I, and TrN) for maximum likelihood and Bayesian analyses using greedy search algorithm for examining the phylogenetic relationship of black cobra with other species already reported on the NCBI (National Center for Biotechnology Information). Maximum likelihood analyses were conducted using RaxML [32] on the CIPRES Science Gateway server [33]. Nodal support was provided by bootstrapping (BS; 1000 pseudo-replicates), and bootstrap values ≥70 or 0.7 were considered as strong supports [34]. MrBayes [35] was used for Bayesian Markov chain Monte Carlo (MCMC) phylogenetic analyses. Two simultaneous runs of four MCMC analyses with a total of four chains (one cold plus three incrementally heated chains) were run with trees for 5×10^6 total generations (sampled every 500 generations). A burn-in value of 25% was set that discarded 2500 generations. Trace plots and ESS value (>200) were used to examine stationarity on TRACER [36]. Posterior probability (PP) values ≥0.95 were considered as strong supports [37]. FigTree software was used to edit the resulting phylogeny [38].

3. Results

In the present study, mitochondrial and nuclear protein-coding genes were amplified and sequenced to infer the genetic biodiversity and phylogenetic relationship of black cobra (*N. naja)* from Pakistan. Common krait (*Bungarus caeruleus* 4) was used as the outgroup in the study from Yazman, Pakistan. Almost similar phylogenies were constructed by RaxML (InL = -8008.97556) and Bayesian analyses (LnL = -8045.494) dividing black cobra from Pakistan and other members of *Naja* from other parts of the world that were already available on the GenBank database into 3 major clades. Bayesian phylogeny showed strong support for the divergence of the three clades, but maximum likelihood did not show any support at the basal node. The first clade included *N. naja* (Punjab, Sindh, and Balochistan in Pakistan and Nepal) as well as *N. kaouthia* and *N. siamensis* (Thailand). The second clade included *Naja pallida* (Tanzania), *Naja nubiae* and *Naja ashei* (Kenya), *Naja mossambica* (Mozambique), and *Naja katiensis* (Mali). The third clade consisted of only two species *Naja haje* (Mali, Saudi Arabia, Kenya, and Egypt) and *Naja annulifera* (Zimbabwe). The second and the third clade showed strong support for sister group relationship with the first clade (ML BS = 85; BI PP = 0.94). The first clade showed strong support for sister group relationship with both the second and the third clade strongly as determined by Bayesian phylogeny (BI PP = 1) but did not have good support by maximum likelihood phylogeny. The first clade consisting

Table 2
Polymorphism in mitochondrial and nuclear genes of black cobra (*Naja naja*).

Parameters	*ND4*	*Cyt.b*	*12S rRNA*	*16S rRNA*	*C-mos*	*RAG-1*	*BDNF*	*NT3*
Total No. of Sites	619	702	650	520	556	686	696	426
Variable No. of Sites	302	325	270	191	31	15	05	03
No. of mutations	302	202	63	47	31	15	05	03
Singleton Variable sites	43	51	20	26	30	13	05	01
Parsimony Informative sites	196	151	43	21	1	2	00	02
Segregating Sites	159	151	00	00	30	15	05	03
Synonymous Changes	176	156	00	00	9	11	00	02
No. of haplotype (h)	17	17	16	15	05	04	02	04
Haplotype diversity (Hd)	0.791	0.756	0.657	0.595	0.508	0.483	0.071	0.582
Nucleotide diversity (Pi)	0.06751	0.07422	0.30998	0.03111	0.00567	0.00283	0.00054	0.00319

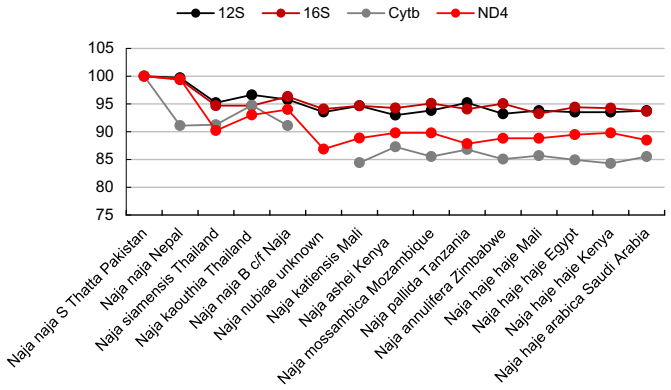

Fig. 4. Percent homology for mitochondrial genes for black cobra (*Naja naja*).

of *N. naja* from Pakistan and Nepal showed recent divergence, which is strongly supported by ML and BI (BS = 100 and PP = 1), while *N. siamensis* and *N. kaouthia* and *N. naja c/f N. oxiana* from Balochistan, Pakistan, showed deep divergence from *N. naja*.

4. Discussion

The Pakistan landscape has a wide range of variations, including fertile plains to deserts, forests, mountains, plateaus, and coastal lines. An extremely diverse bioclimatic and topographic profile has led to the creation of multifarious habitats cultivating very unique flora and fauna that have a blend of Palearctic, Indomalaya, and Ethiopian forms [39]. This blend has introduced many venomous snakes in Pakistan. These venomous snakes have great medical importance. Some of these snakes are also found in the Indian subcontinent, and some of them are unique endemic species or some overlap with Middle Eastern and Himalayan species [14].

According to Khan [14], there are two species of cobra in Pakistan. One of them is *N. naja*, also known as black cobra or spectacled cobra. The spectacled cobra is distributed across Southern and Eastern Pakistan that includes Punjab, Balochistan, and Sindh provinces. The other species of cobra, i.e., brown ox cobra (*N. oxiana*), is restricted to Northern Pakistan in areas at high elevation. In the present study, cobras from Punjab, Sindh, and Balochistan were used for phylogenetic study through mitochondrial and nuclear genes. These maximum likelihood (ML) and Bayesian inference (BI) phylogenies showed that the cobras from Balochistan were *N. oxiana,* while cobras from Sindh and Punjab were *N. naja*. One sample of black cobra (Khuzdar, Balochistan) from California

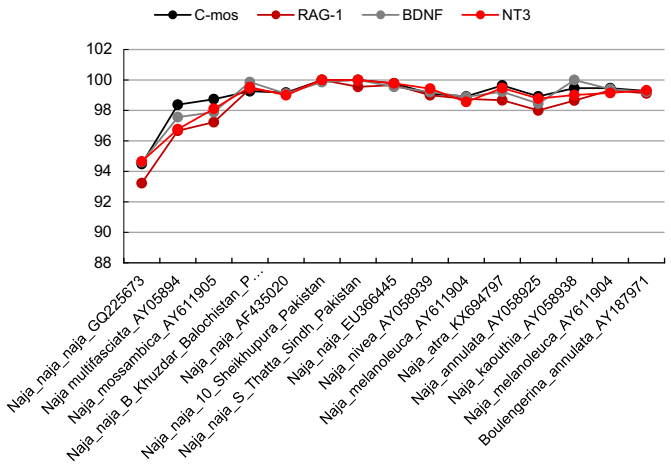

Fig. 5. Percent homology for nuclear protein-coding genes for black cobra (*Naja naja*).

Academy of Sciences, USA, was found to be the brown cobra *N. oxiana,* as it cladded with *N. kaouthia* (Thailand) and *N. siamensis* (Thailand), while *Naja* from Sindh and Punjab fell into the same clade with *N. naja* from Nepal, as they did not show difference from each other and proved to be the same species. This suggests that more diverse sampling is required from Punjab and Sindh so that the presence of *N. oxiana* may be proved to be as suggested by Khan [40]. The one that was called as black cobra by the collector and the preserving museum from Balochistan was found to clade with *N. kaouthia* and *N. siamensis*. Wuster [41] reported *N. naja naja* (spectacled or bi-nocellate cobra) to be found throughout India, Pakistan, Sri Lanka, and Bangladesh. *N. naja kaouthia* (monocellate or monoclad cobra) was reported in northeastern India (Gangetic Plain, Orissa, Bengal, and Assam) as well as from Bangladesh, Malaysia, Southern Vietnam, and Southwestern China. Central Asian cobra (*N. naja oxiana*) was reported from Kashmir and some areas of northwestern India. Furthermore, *N. naja sagittifera* (Andaman cobra) was reported in the Andaman Islands [7].

Wuster et al. [42] divided cobras of the genus *Naja* into three distinct lineages. One of them was with two major sublineages. One clade was Asiatic clade comprising *N. kaouthia*, *N. naja,* and *N. siamensis* and *Naja sputatrix*. The African non-spitting lineage was made up of two sister lineages that include *N. annulifera*, *N. haje,* and *Naja nivea*. The other one includes *Naja melanoleuca* and *Boulengerina annulata* and *Paranaja multifasciata*. The third evolutionary lineage is African spitting cobras that comprise *N. ashei*, *N. katiensis*, *N. mossambica*, *Naja nigricincta*, *Naja nigricollis*, *N. nubiae,* and *N. pallida*. Asiatic cobras of the genus *Naja* are a widespread group of snakes ranging from the Caspian Sea to China, Indonesia, and Philippines.

The absence of a hood mark had been observed in many specimens of *N. naja oxiana* from the northern and western parts of India. This absence is made a base of assigning them as *N. naja oxiana,* but in some parts of India, except Kashmir, the presence of this subspecies is not accepted universally [12]. The *Naja* species in this study (*N. naja* from Balochistan) had 68 or 69 subcaudal counts within the oxiana count. It had no hood markings above or below, like *N. naja*. The specimen had 200 ventrals (and 4 preventrals), well beyond the *N. naja* range. The others should have been *N. naja,* as the cobra from Faisalabad, Punjab, Pakistan, has 191 ventrals, and 191 is just the count you can expect for a male or a female *N. naja*. Juveniles of these are obscured in their markings many times. Minton [43] described *N. naja oxiana* as more or less uniformly jet black, dark olive, or dark brown with pale gray to butter yellow ventrals. The cobra specimen from Balochistan has a brown color, while the cobra from Sindh (NNS) and Punjab has a dark black ventral side with some pale or yellow dorsal side near the head. According to Minton (1966), the male specimen of *N. naja* has 182–192 ventrals, and the female has 183–196 ventrals with 61–68 subcaudals in male and 57–62 subcaudals in female. *N. naja* has uniformly jet black, dark olive, or dark olive brown above, while ventrally, it is pale gray to butter yellow, more or less heavily suffused with slate gray or dark brown uniformly jet black, dark olive, or dark or dark brown above. Juveniles of *N. oxiana* show a pattern of wide dark transverse bars. The adult is almost uniformly brown with no spectacles or ocellus mark on the hood. The ventral count of *N. oxiana* ranges from 195 or more. The subcaudals are 62–70 in females and 65–75 in males. Minton collected a juvenile specimen from the areas around Peshawar and two specimens from Kach in Balochistan Mountains between Quetta and Ziarat. Juvenile specimens had 198 ventrals and 66 subcaudals. The specimen from Kach had 202 ventrals and 65 subcaudals. This specimen showed almost the same counts, as it had 68 or 69 subcaudals, 200 ventrals, and 4 pre-ventrals. The *N. naja* specimen from Sindh and Punjab had 191 ventrals, which differentiates it from *N. oxiana*. Deraniyagala [44,45,46] assigned both *N. oxiana* and *N. kaouthia* as full species reviewing the systematics of Asiatic cobras. He also restricted the type locality of *N. naja* to Sri Lanka and described 5 subspecies: *N. naja* from

the Indian subcontinent, *N. naja madrasiensis* from South India, *Naja naja gangetica* from northeast, *Naja naja indusi* from Punjab, *Naja naja karachiensis* from southern Pakistan and northwestern India, *N. naja bombaya* from Maharashtra and neighboring areas. They found cobras from Central Asia and the Indian subcontinent and monocellate cobras from Indochina as three well-defined taxa. Central Asian specimens are clearly distinguishable from the other two spectacled and monocellate. Sympatry was found between the Central Asian specimen and the spectacled one, with fairly large evidence. The spectacled cobra was found to occur in Chitral Valley and Afghanistan. The Chitral Valley drains into the Kabul river valley at Jalalabad in Nangarhar province of Afghanistan. Because of the mountains around the Chitral Valley, the only route for the species to reach is through Nangarhar and Konarha provinces of Afghanistan. One can assume the species dispersal during the Pleistocene cold phase, as these valleys have high altitudes with too low temperatures to support cobra populations. Therefore, the spectacled cobra may still occur in extreme eastern Afghanistan, from where it has not been recorded before. There is evidence about cobras from Central Asia and the Indian subcontinent about their sympatric occurrence in some parts of northeastern Balochistan from a cobra from Duki. Similarly, the Central Asian cobra has been found at Quetta and Sibbi and been reported from the Sulaiman Range [12,43]. There are many reports that assign spectacled cobra as *N. naja naja* and the Central Asian taxon as *N. naja oxiana*. However, there is still a need for more careful studies, as the absence of a hood mark in some spectacled taxon populations creates confusions. Scalation and banding in juveniles can be focused.

Panagides et al. [47] tested and compared the cytotoxicity of 25 different species of Old World elapid snakes including Asian and African cobras with morphological and behavioral adaptations of hooding and spitting. According to their findings, a similar phylogenetic relationship was found among the black cobra (*N. naja*) from Pakistan and India. The present study confirmed the same relationship finding the variation between the two *Naja* species from the two countries.

Khan [48,49] also observed sympatry between the spectacled and the Central Asian cobra at Ahmed Nagar (Jhang Sadar District, Punjab, Pakistan) in some parts of Punjab and Northwest Frontier Province. This is clearer evidence that the spectacled and the Central Asian cobra (*N. oxiana*) are sympatric in many parts of Northern Pakistan. As the two cobras are clearly distinguishable and sympatric, they are accepted as two species. Linnaeus [50] found the locality of the specimen *N. naja* as India, but Deraniyagala [44] restricted its locality to Sri Lanka, and thus, Indian spectacled cobra should be assigned as *N. naja*. Von Eichwald (1831) [51] collected a Central Asian cobra from the Transcaspian area (Soviet Central Asia) and named it as *Tomyris oxiana,* which is the oldest name of Central Asian cobra. Then the correct name was assigned as *N. oxiana*. The range of *N. oxiana* from east is still not very well known. Joger in 1984 [12] mentioned Gilgit as its east side locality but did not give any clue about its occurrence in India except in only some northern Kashmir areas. Later, Murthy and Sharma [52] and Murthy et al. [53] collected specimens from the Punch Valley, Northwest Jammu. Mahajan and Agrawal [54] collected some immature specimens that had cross bands, which increased the confidence that they were *N. oxiana*.

In areas like southern Pakistan, spectacled cobras show ontogenetic change in their color, i.e., the young are gray with or without a spectacled hood mark, but as they grow older, the color darkens and develops more or less uniformly black dorsum. This ontogenetic change leads many of the workers assume that the adult belongs to different taxa than the young one. Sundersingh [55] assigned a young specimen as *N. naja naja* obtained from Pilani, Rajasthan. He assigned the other two adults as *N. naja oxiana,* as they were brown or black in color obtained from Pilani, Rajasthan. Biswas and Sanyal [56] also named the uniform brown cobra specimens from Rajasthan as *N. naja oxiana*. The scale count of these *Naja* species corresponds to those of *N. naja* sensu stricto in northern India. Thus, it shows that the adult *N. oxiana* has more or less uniformly medium or light brown color [57]. On the other hand, some specimens of spectacled cobras from southern Pakistan have uniformly black dorsum, and most *N. oxiana* specimens retain at least a trace of conspicuous juvenile banding pattern until at least the early adulthood.

Snakebites cause high mortality rates globally. In Pakistan, 20,000 annual deaths are reported due to snake envenomation [58]. Snakes belonging to the genus *Naja* are represented by two species in Pakistan: Indian cobra (*N. naja naja*) and brown cobra (*N. naja oxiana*). Black Pakistani cobra (*N. naja karachiensis*) is a subspecies of *N. naja oxiana* and found widespread in southern Pakistan [13]. Cobra envenomation includes local tissue necrosis and neuromuscular paralysis that leads to respiratory failure in victim, ultimately leading to death within several hours. Local antivenom has been used, but discrepancies in venom toxicity and therapeutic response of the antivenom from a single geographical region cannot be ignored. Venom from Pakistani *N. naja* has 30–40% alpha neurotoxins, while some studies have shown 3–7% alpha neurotoxins from Sri Lanka and India. In case of cobra envenomation in Pakistan, the cobra has adapted to many regions of Pakistan, being common in bites. The potency of antivenom against cobra venom has been reported to be low because of poor immunogenicity of the small polypeptide toxins of venom [59,60]. More diverse and a greater number of samples for these two species found in Pakistan will give more resolution about the genetic biodiversity, phylogenetics, and conservation strategies.

Acknowledgments

We are thankful to our colleagues for their fieldwork efforts for this research. We are also thankful to the reviewers who made precious efforts and provided suggestions for improving the article.

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