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Sexual Dimorphism, Intrapopulation Phenotypic Variation and Matrilineal Genetic Diversity of *Naja kaouthia* Lesson, 1831 (Reptilia: Squamata: Elapidae) from Mizoram, Northeast India

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Naja kaouthia Lesson, 1831 is a common and widely distributed venomous snake in South and Southeast Asia. The species is regarded as having a high degree of phenotypic plasticity and variability in venom composition across its distributional range. Limited information is available on this complex species in the context of population studies and systematics, and population studies, particularly in northeast India, are scarce. In this study, we explore sexual dimorphism and potential intraspecific morphological variation within the population of *N. kaouthia* from Mizoram, northeast India, while also aiming to enrich the systematic data and reappraise the matrilineal genetic diversity across various geographical locations. Sexually dimorphic traits are identified in both meristic and morphometric characters. We hypothesise that male-biased sexual selection for success in male-male rivalry may be the cause of sexual dimorphism in head size and shape in this species. Our mitochondrial phylogeny (*16s*, *cytb* and *cox1*) recovered two well-supported South Asian and Southeast Asian clades within *N. kaouthia* with a wide genetic divergence (4.4%) between them. Our study also revealed intraspecific variation in the hood markings from the examined population, which warrant more studies to determine whether these variants are randomly distributed or reflect geographic structure and lineage differentiation.

Keywords: Genetic diversity, Intraspecific variation, Molecular phylogenetics, Monocled cobra, Morphometric analysis

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BACKGROUND

The elapid snake genus *Naja* (true cobras) comprises 35 currently recognised species (Shi et al. 2022; Uetz et al. 2025). The Asian representatives had been regarded as a single species *N. naja* sensu lato, until systematic reassessment (Wüster 1996; Wüster et al. 2007 2018) and nomenclatural re-allocation of the extant true cobras (Wallach et al. 2009). *Naja* is currently partitioned under four subgenera, and includes 12 species of Asiatic cobras in the subgenus *Naja*, namely *N. naja* (type species); *N. oxiana*, *N. kaouthia*, *N. fuxi*, *N. sagittifera*, *N. atra*, *N. mandalyensis*, *N. siamensis*, *N. sumatrana*, *N. philippinensis*, *N. samarensis*, and *N. sputatrix*. In India, four species of *Naja* (*N. oxiana*, *N. naja*, *N. sagittifera*, and *N. kaouthia*) have been recorded so far (Wüster 1996; Aengals et al. 2018; Shi et al. 2022), and in the study region (Mizoram, northeast India), *Naja* is solely represented by *N. kaouthia* (Lalremsanga et al. 2011). Systematic uncertainty in Asiatic cobras is mainly attributed to extreme within-population phenotypic plasticity, which complicates species identification. Consequently, it can be difficult to interpret older literature because of the uncertainty regarding what species is actually being referred to (*sensu* Wüster 1996). The name-bearing type material of *N. kaouthia* is a 2,475 mm specimen, which has been lost (fide Wallach et al. 2014). The type locality was given as “Inde continentale” (= Indian subcontinent) (Wallach et al. 2014). However, a recent study designated a neotype and restricted the type locality to 24 Parganas, West Bengal, India (Balchan et al. 2025). In India, *N. kaouthia* is found throughout northeast Indian states, westward through the Indian states of West Bengal, Odisha, Chhattisgarh, Bihar, Jharkhand, and as far as Haryana, which likely represents the westernmost extreme of its range in the country, and as far north as Uttar Pradesh and Uttarakhand (Wüster 1996; Stuart and Wogan 2012; Wallach et al. 2014). Within its known range, the species is found at elevations of 40–1,500 m a.s.l. (Lalremsanga et al. 2011), occupying a wide range of natural and artificial environments, including paddy fields, swamps, mangroves, grasslands, shrublands, forests, and human settlements (Stuart and Wogan 2012). *Naja kaouthia* is presently categorised as a Least Concern (LC) species in the IUCN Red List (Stuart and Wogan 2012), and is listed in Schedule I under the Wildlife (Protection) Amendment Act (2022) in India.

Sexual selection, as postulated by Darwin (1871), is an evolutionary mechanism affecting traits that provide an advantage in reproductive success (Hosken and House 2011). Sexual selection is the most accepted mechanism driving the evolution of sexual dimorphism (Hedrick and Temeles

1989; Abouheif and Fairbairn 1997). Moreover, sexual selection within sex, driven by competition for matings has been proposed as a strong evolutionary force behind speciation events (West-Eberhard 1983; Price 1998; Seddon et al. 2013). Sexual dimorphism often represents the most common and striking aspect of intraspecific variation among species with sexual reproduction (Bolnick et al. 2011; Start and De Lisle 2018), and is widely seen in the animal kingdom, including in reptiles (Butler and Losos 2002; Antonini et al. 2023). Only a few studies have investigated the degree of sexual dimorphism among certain African and Asian cobras and have found that cobras generally attained similar adult body sizes in both sexes, while males typically had longer tails and larger heads than females with similar body length (Boeadi et al. 1998; Luiselli et al. 2002; Shine et al. 2007; Chaitae 2011). However, very limited data is available for *N. kaouthia* to ascertain whether the species follows similar trends in sexual dimorphism, and whether this trait is found in potentially informative taxonomical characters (Chaitae 2011). Despite the inclusion of *N. kaouthia* in multiple phylogenetic studies of elapid snakes (Slowinski and Wüster 2000; Wüster et al. 2007; Wallach et al. 2009; Pyron et al. 2013), only a few studies on venomics (Tan et al. 2016; Ryabinin et al. 2019) and systematics (Ratnarathorn et al. 2019; Kundu et al. 2020; Balchan et al. 2025) have suggested intraspecific or geographical variability among *N. kaouthia* populations, some of which may be attributable to the recently recognised *N. fuxi* Shi, Vogel, Ding, Rao, Liu, Zhang, Wu, and Chen, 2022, which was previously identified as *N. kaouthia*. Although multilocus-based phylogeographic approaches have been increasingly utilised for robust systematic study, especially among cryptic taxa (Wüster et al. 2018), mitochondrial DNA has also been widely implemented for detecting putative cryptic species (Niemiller et al. 2022). Mitochondrial genes are considered to evolve under neutral (or nearly-neutral) selection (Ballard and Kreitman 1995); consequently, they have also been widely used in population genetic studies (Consuegra et al. 2015). Prompted by the limited availability of biological samples and systematic studies of *N. kaouthia* populations from northeast India, and the high degree of phenotypic plasticity across the species distributional range (Balchan et al. 2025), this work aims to explore sexual dimorphism and the potential intraspecific morphological variation within *N. kaouthia* from Mizoram, northeast India. We anticipate that this study helps augment the available systematic data, and provide new insights into the matrilineal genetic diversity of the species.

MATERIALS AND METHODS

Sampling

In this study, a total of 44 live and preserved specimens (28 adult and 16 juvenile) were examined. Of these, 33 are preserved specimens housed in the collections of the Departmental Museum of Zoology, Mizoram University (MZMU). Liver tissue was dissected from three mutilated specimens (MZMU1426, MZMU2044, MZMU2415), and stored in 95% ethanol at -20°C for molecular analyses. Morphological examination was also performed for two of the aforesaid specimens (MZMU1426 and MZMU2415). MZMU1426 corresponds to one of the six previously sequenced specimens in Kundu et al. (2020). The three genetically sampled specimens were stored in 70% ethanol, while the majority of the other museum specimens examined were preserved in 10% unbuffered formalin. In addition to preserved specimens, a total of 11 newly collected live individuals were examined, which were subsequently released after taking morphological data. Comparison between preserved specimens (formalin/ethanol) and live individuals was conducted using the morphometric data of adults, and one-way analysis of covariance (ANCOVA) using snout-vent length (SVL) as a covariate showed no statistically significant difference between them in all characters ($p > 0.05$). A separate test for juveniles was not feasible as only one live juvenile was examined. We subsequently combined data from preserved specimens and live individuals for further morphological analyses.

DNA extraction and PCR amplification

Genomic DNA was extracted from stored liver tissue using DNeasy (Qiagen™) blood and tissue kits following the manufacturer's instructions (DNeasy® Blood & Tissue Handbook 2023). Fragments of two mitochondrial markers, 16S rRNA (*16s*) and cytochrome b (*cytb*) were amplified through Polymerase Chain Reaction (Mullis et al. 1986). Target gene sequences were amplified in a 20 µL reaction volume, containing 1X DreamTaq PCR Buffer, 2.5 mM MgCl2, 0.25 mM dNTPs, 0.2 pM of each gene primer pair, approximately 3.0 ng of extracted DNA, and 1U of Taq polymerase under the PCR conditions provided in Table S1. PCR products were checked using gel electrophoresis on a 1.5% agarose gel containing ethidium bromide. The PCR products were cleaned using ThermoFisher ExoSAP-IT PCR product cleanup reagent and subsequently sequenced using Sanger's dideoxy method using the ABI 3730xl DNA Analyzer at Barcode BioSciences, Bangalore, India. The newly generated partial gene sequences were then screened through ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) and subsequently deposited in the NCBI repository (GenBank accession numbers are given in Table S2). In this work, a total of three *16s* and three *cytb* sequences were generated and were combined with published *16s*, *cox1* and *cytb* sequences of *Naja* species obtained from GenBank. Because six *cox1* sequences of *N. kaouthia* from Mizoram, northeast India (MZMU982, MZMU998, MZMU1163, MZMU1170, MZMU1195, and

MZMU1426) have already been published by Kundu et al. (2020), we did not generate a new *cox1* sequence in this study. Instead, we generated *16s* and *cytb* sequences from one of the previously sampled specimens (MZMU1426) by Kundu et al. (2020).

Molecular phylogenetics

DNA sequences were aligned with the default parameter settings of the MUSCLE algorithm (Edgar 2004) in MEGA 11 (Tamura et al. 2021); for the *16s* dataset, the ambiguously aligned sites were removed under the heuristic method in trimAL software (Capella-Gutiérrez et al. 2009). The three mitochondrial gene alignments were concatenated and were partitioned by gene and codon positions, where appropriate. Using the untrimmed datasets for each gene (1,478 bp for *16s*; 1,117 bp for *cytb*; 1,602 bp for *cox1*), uncorrected p-distances were estimated in MEGA 11 (Tamura et al. 2021) using the complete deletion option for the treatment of gaps/missing data. The symmetric p-distance matrices were standardised and utilised to visualise the mitochondrial gene-based genetic differentiation among the target taxa through Principal Coordinate Analysis (PCoA) in PAST 4.13 (Hammer et al. 2001). PartitionFinder v2.1 (Lanfear et al. 2017) was utilised to search for the optimal partitioning schemes and nucleotide evolutionary models using the Bayesian Information Criterion (BIC). *Bungarus fasciatus* (MZMU1883) was used as the outgroup because it is a closely related yet clearly distinct elapid genus that diverged earlier than *Naja* (Lee et al. 2016). A Bayesian inference (BI) phylogeny was reconstructed by applying the best partitioning schemes and nucleotide evolutionary models in Mr.Bayes v3.2.5 (Ronquist et al. 2012). The MCMC was run with four chains (one cold and three heated chains) for 20 million generations and sampled every 5000 generations. Tracer v1.7 (Rambaut et al. 2018) was used to check the convergence of likelihood and determine the burn-in cut-off. The first 25% of trees from the BI analysis were discarded as burn-in. A maximum likelihood (ML) phylogenetic tree was inferred using the IQ-TREE webserver (Nguyen et al. 2015) by selecting FreeRate heterogeneity (Soubrier et al. 2012) and 10,000 Ultrafast Bootstrap (UFB) replicates (Minh et al. 2013), using the partitions identified by PartitionFinder v2.1 (Lanfear et al. 2017), and the models selected based on BIC values by ModelFinder (Kalyaanamoorthy et al. 2017) integrated into the IQ-TREE (Nguyen et al. 2015). The phylogenetic trees were viewed and illustrated using web-based tree annotator iTOL software v5 (Letunic and Bork 2021). The best partitioning schemes and nucleotide evolutionary models selected for the two types of phylogenetic inferences are provided in table S3.

Mitochondrial haplotype networks

The three newly obtained mitochondrial DNA sequences were utilised to screen the matrilineal genealogy among populations through mitochondrial DNA-based haplotype estimation. The three datasets were imported to MEGA 11 (Tamura et al. 2021), trimmed to the same length at 465 bp for *16s*, 561 bp for *cytb* and 621 bp for *cox1* datasets. The aligned datasets were utilised for estimating haplotype diversity in DnaSP v.6 (Rozas et al. 2017), and generating the trait matrix corresponding to the locality. The haplotype networks were plotted in PopArt v.1.7 (Leigh and Bryant 2015) using the Median-Joining method, which is a useful statistical approach for intra-species haplotype networks (Bandelt et al. 1999). Because *N. fuxi* was only erected as a new species during the processing of this work, they are included in the haplotype estimation to further corroborate the identity of the aforesaid taxon.

Morphology

External morphological (mensural and meristic) data were obtained from the 44 individuals examined from the *N. kaouthia* population of Mizoram. Except SVL and tail length (TaL) which were measured with a measuring tape, the following mensural characters were measured to the nearest millimetre with a Mitutoyo digital calliper : eye diameter (ED); nostril diameter (ND); eye-nostril length (E-N); eye-snout length (E-S); snout-nostril length (S-N); head length (HL); head width (HW); height of rostrum (RH); width of rostrum (RW); internarial space (INS); interorbital space (IOS); internarial length (INL); internarial width (INW); prefrontal length (INL); frontal length (FL); frontal width (FW); parietal length (PL); parietal width (PW). Meristic characters were taken as follows: supralabials (SL); supralabials touching eye (SLe); infralabials (IL); temporals (Tem), postoculars (PoO); preoculars (PrO); anal scute (As); dorsal scale rows (DSR) is counted around the body from one side of ventrals to the other in three positions, on one head length behind the neck i.e anterior dorsal scale rows (ADSR), at midbody i.e mid dorsal scale rows (MDSR), and at one head length before cloacal plate i.e posterior dorsal scale rows (PDSR); when counting the number of ventral scales (Ve), the values were scored following Dowling (1951). Subcaudal scales (Sc) were counted from the first subcaudal scale meeting its opposite to the scale before the tip of the tail, excluding the terminal scute.

The sex of the preserved specimens was identified by examining everted hemipenes or by ventral tail dissection to detect the retractor muscles and inverted hemipenes. In the case of live individuals, sex was determined either by a metal sexing probe or manually everting the hemipenes by gently pressing the ventral tail base towards the cloaca in smaller-sized individuals. Values for bilateral head characters are given in left/right order. Other than the conventional taxonomic

characters, relevant morphological parameters that were utilised by Wüster et al. (1995) were adopted and tested for sexual dimorphism.

Statistical analyses

The morphological information was obtained from various localities in Mizoram state, India. Prior to conducting any further statistical analyses, missing values for the variables were calculated and maintained below 30% by removing specimens with a high amount of missing data. To avoid the effect of life stages in the analyses, the samples were broadly categorised into two life stage groups: individuals with SVL ≥ 1,000 mm were grouped as adult, while those with SVL < 1,000 were grouped as juvenile. In the presence of reproductive state data, the SVL threshold for sexual maturity in both males and females have been established for some species of snakes (Masunaga et al. 2003). In African cobras, it was found that males attained smaller minimum sizes of sexual maturity in *Aspidelaps lubricus* and *Hemachatus hemachatus*, but no significant difference was seen between the sexes of the congeneric species *N. melanoleuca* (Shine et al. 2007). A study from Thailand provided the SVL for the smallest reproductively active wild caught female *N. kaouthia* as 1,110 mm with 690 g in body mass, where individuals ≥ 1,000 mm and body mass ≥ 0.7 kg were grouped as adult in their study. The author further emphasised that sexual maturity is believed to be reached at around 1,000 mm during their third year of age in captive rearing (see Chaitae 2011 and references therein). Therefore, we applied this SVL threshold as a proxy for sexual maturity for both male and female to standardise analyses, despite the fact that we do not have body mass and reproductive state data, and this threshold may not reflect a definitive life stage criterion for the species. The meristic data were standardised first to zero mean and unit standard deviation. For mensural characters (TaL, HL, HW, HD, ED, ND, E-S, N-S, N-E, RH, RW, INS, IOS, INL, INW, PFL, PFW, FL, FW, PL, and PW), the best allometric adjustment model was determined based on the linear regression model that gives the highest within-group correlation (Thorpe 1975). Subsequently, one-way ANCOVA was carried out with SVL as a covariate and sex as a factor.

The meristics data (Ve and Sc) of adults and juveniles were tested for sexual dimorphism utilizing a separate one-way analysis of variance (ANOVA) for both life stages using sex as a factor along with Levene's test (Levene 1961) for testing homogeneity of variances. Brown- Forsythe test (Brown and Forsythe 1974) was also tested as an alternative approach in case the assumptions of ANOVA were violated.

The sexually dimorphic characters identified through the univariate analyses were ordinated through Principal Component Analysis (PCA). The correlation matrices between all pairs of the morphological variables, the variance explained by each eigenvalue, and the correlations of each

variable to the first two components are explored. All statistical analyses and graphical representations were performed using the free access statistical packages PAST 4.13 (Hammer et al. 2001) and PSPP v.1.6.2 (GNU Project 2015).

RESULTS

Phylogenetic relationships

The concatenated untrimmed datasets for *16s*, *cox1*, and *cytb* corresponded to 4,197 bp in length; the flanking gaps created by shorter nucleotide sequences were treated as missing data. The BI and ML trees were largely congruent in their topology (Fig. 1A). The average standard deviation of split frequencies among the four Bayesian runs was 0.020811 with the combined values of ESS = 11660.1; the Bayesian posterior probability (PP) values are interpreted as the nodal support in the BI tree. The concatenated phylogenetic analyses depicted two well-supported (PP = 0.99; UFB = 93) lineages among *N. kaouthia* populations: a South Asian clade containing populations from northeast India (Mizoram) and Bangladesh; and the Southeast Asian Clade which accommodates the populations from Myanmar, China, Vietnam, Thailand, and a sample from an unknown locality. Based on the known range of *N. kaouthia* (Wüster et al. 1995; Stuart and Wogan 2012; Wallach et al. 2014), our estimated range for the two putative independent lineages of the species recovered in this study is mapped in figure 2.

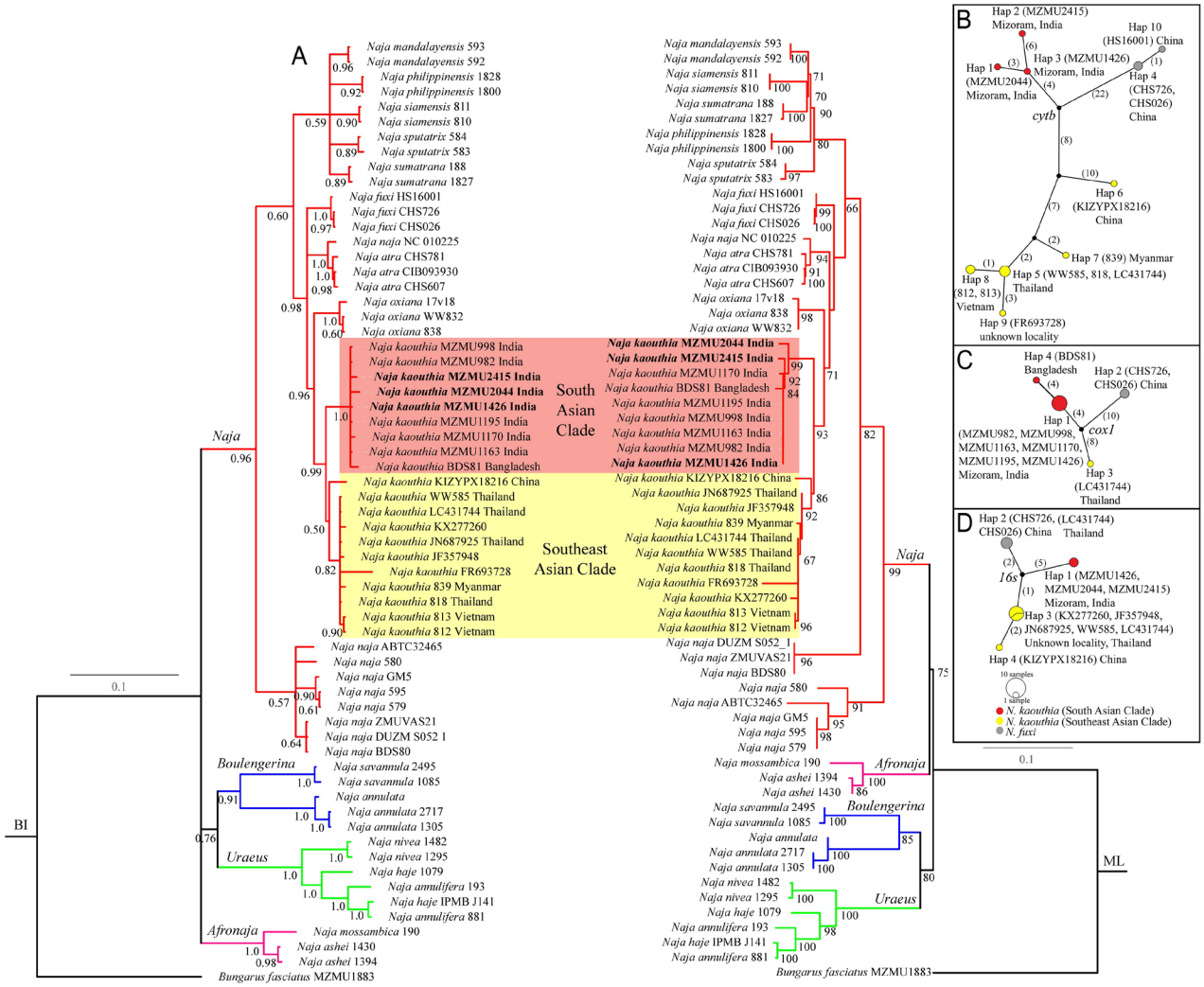

Fig. 1. A. Bayesian Inference (left) and Maximum likelihood (right) phylogenetic trees of *Naja* species inferred based on the concatenated *16s*, *cox1* and *cytb* genes. Bayesian posterior probabilities > 0.50 from the BI analysis and ultrafast bootstrap values > 50 from the ML analysis are shown at the nodes. The newly sequenced specimens are indicated in bold. The branch colours depict the distinct subgenera of *Naja* as partitioned in Wallach et al. (2009): red for *Naja*; blue for *Boulengerina*; green for *Uraeus*; pink for *Afronaja.* Mitochondrial gene-based median-joining haplotype network among populations of *Naja kaouthia*: B. Cytochrome *b* (*cytb*); C, Cytochrome c oxidase subunit 1 (*cox1*); D, 16S rRNA (*16s*). The number of samples present in each haplotype corresponds to the relative size of the circles. Numbers at the branch represent mutational steps found between haplotypes, and a black dot at the branch is an inferred median. Each haplotype is specified with the corresponding specimen voucher (or GenBank Accession nos. for specimens with no vouchers) and localities are presented.

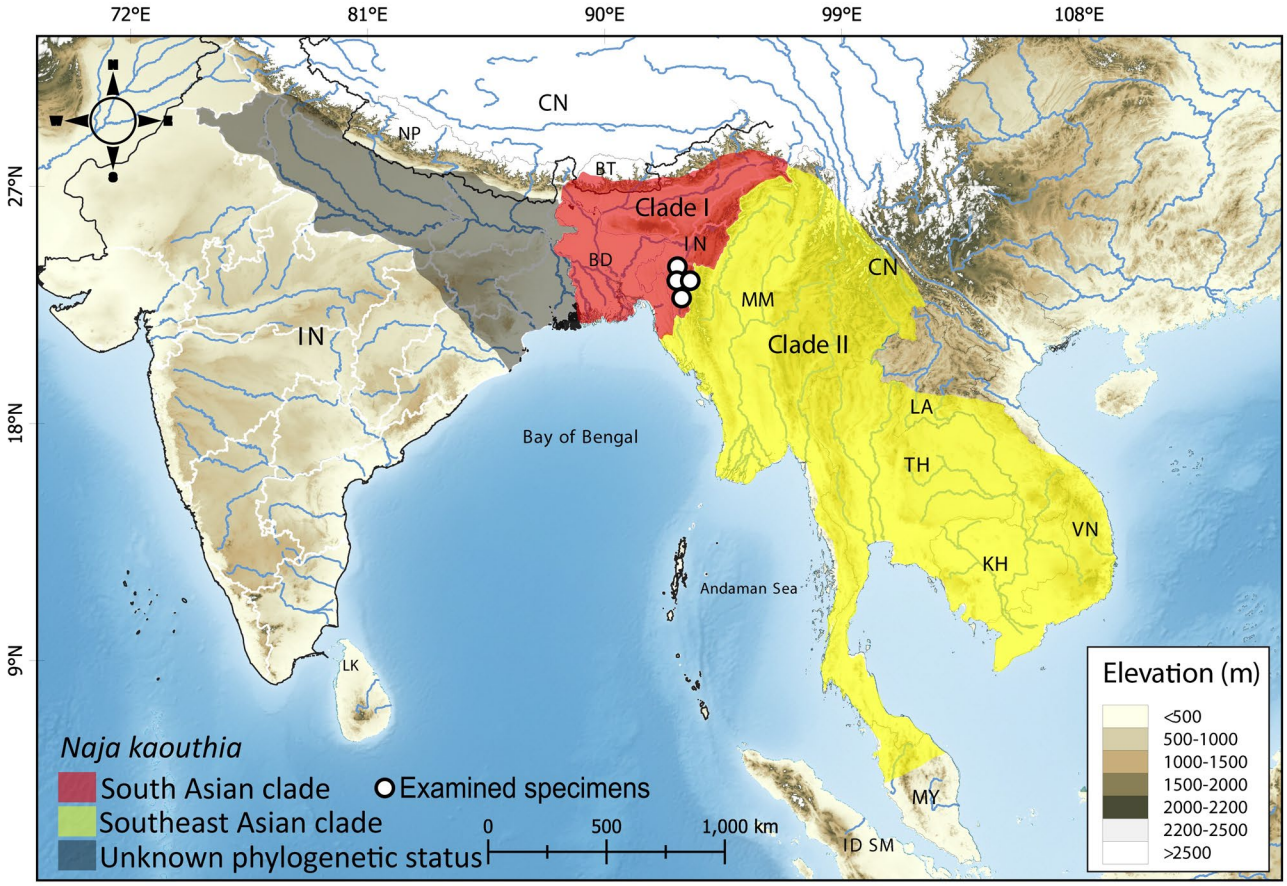

Fig. 2. Map showing the known distribution range of *Naja kaouthia,* based on the distributional range provided in published works (Wüster et al. 1995; Stuart and Wogan 2012; Wallach et al. 2014); the colours correspond to the distinct clades recovered in the phylogenetic analyses. Clade I and II represents South Asian and Southeast Asian clades, respectively. The locations for the examined specimens are indicated in white dots. Abbreviations for countries are: IN, India; NP, Nepal; BT, Bhutan; CN, China; BD, Bangladesh; MM, Myanmar; LA, Laos; TH, Thailand; KH, Cambodia; VN, Vietnam; MY, Malaysia; ID SM, Indonesia (Sumatra).

Genetic divergence and haplotype estimation in *N. kaouthia*

The gathered dataset of 14 *cytb* sequences identified a total of 65 variable sites with 10 haplotypes (Hap), and a haplotype diversity (Hd) = 0.9451. The three samples from the studied population (Mizoram) accounted for three distinct haplotypes (Hap 1–3), separated by 3–6 mutations with genetic divergence of 0.5–1.6% among them. However, all Mizoram specimens shared a single haplotype for *cox1* (Hd = 0.6444) and *16s* (Hd = 0.7455) (Fig. 1B–D; Tables S4–S6).

The mean genetic distance between Clade I (South Asian) and II (Southeast Asian) of *N. kaouthia* is 4.4% in *cytb* (Table S7). The PCoA ordinations of genetic divergence in the three genes also showed the discrete clustering of Asian and African cobras in *16s* and *cytb*, while *cox1* is lacking comparable sequence for African cobras during the processing of this work to see the similar clustering of the aforesaid two genes. However, the PCoA ordinations showed the discrete clustering of *N. kaouthia* from Southeast Asia from the other congeneric samples from South Asia,

while a single specimen from China is seen as distant from both South Asia and Southeast Asian samples (Fig. 3).

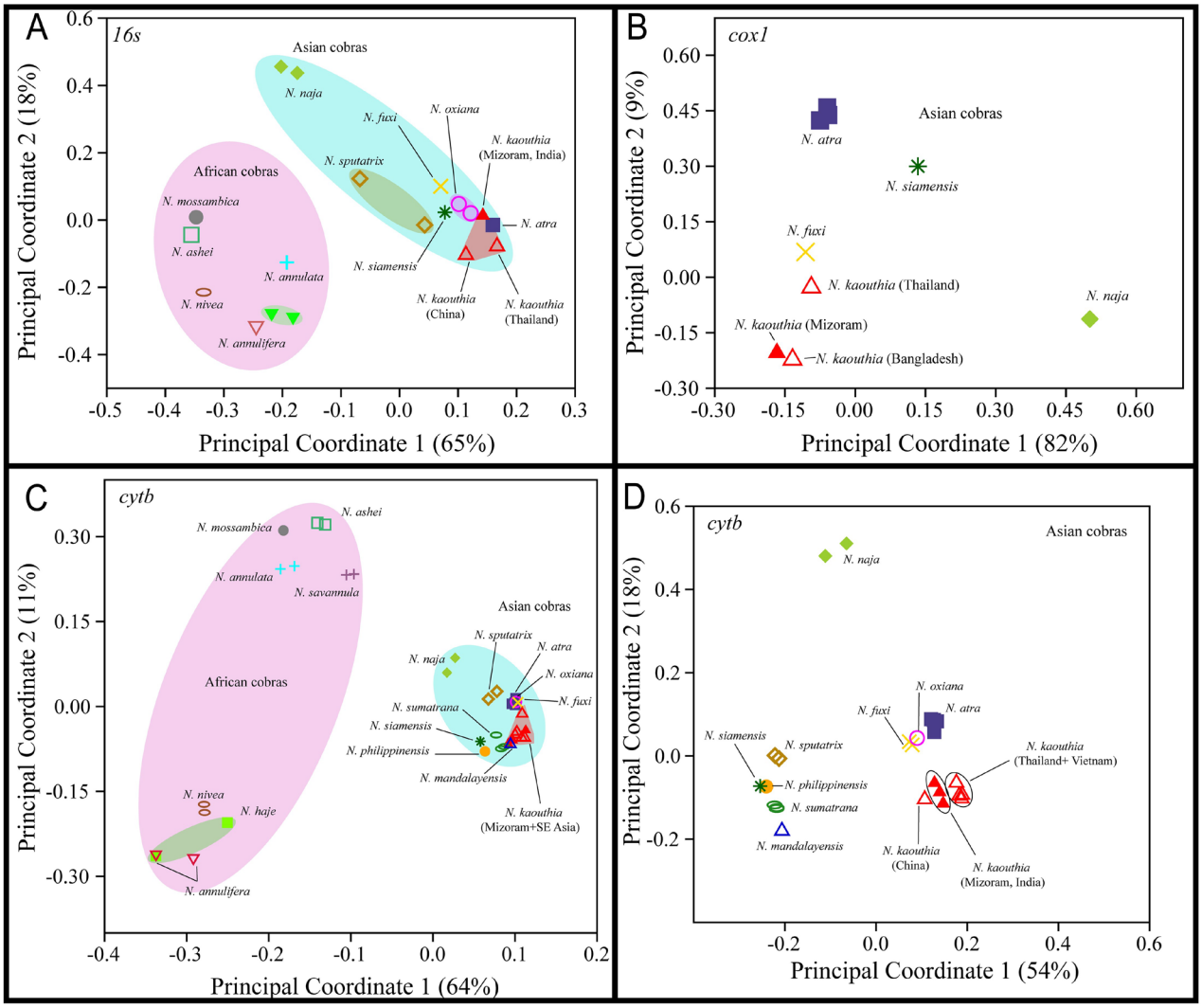

Fig. 3. Ordination of *Naja* species in a Principal Coordinate Analysis of standardised *p*-distance of three mitochondrial genes, where a total percentage of variance captured by the first and second principal coordinates are given in the x and y axes, respectively. A, 16S rRNA (*16s*) among African and Asian cobras where the first two axes explained 65% and 18% of the total variation, respectively; B, Cytochrome c oxidase subunit 1 (*cox1*) among Asian cobras where the first two axes explained 82% and 9% of the total variation, respectively; C, Cytochrome *b* (*cytb*) among African and Asian cobras where the first two axes explained 64% and 11% of the total variation, respectively; D, *cytb* among Asian cobras with the exclusion of African cobras for better visualization of the clustering, where the first two axes explained 54% and 18% of the total variation, respectively. The distinct shape and colour of each dot correspond to the different species of cobras; oval shapes tinted red and blue (plate A and C) correspond to African and Asian cobras, respectively.

Morphology

The studied population of *Naja kaouthia* from Mizoram is represented by a total of 33 museum specimens and 11 live individuals. The raw morphological data for the Mizoram specimens are given in Table S8 while comparative meristic data originating from this study, along with data from other populations derived from published taxonomic literature and unpublished data, are given in table 1. Based on the conventional taxonomical features the species was diagnosed in having oval-shaped smooth dorsal scales with ADSR 23–29, MDSR 19–23, PDSR 13–17; SL 7/7 (rarely 6/7 or 8/7); SLE 3–4/3–4 (rarely 3/3–4 or 3–3/3–3); IL mostly 9/9 or 8/8 (sometimes 8/9, 9/8, 9/10, 10/9, or 10/10); PrO 1/1; PoO 3/3 (rarely 2/3, 2/2, or 1/3); Te mostly 2+3/2+3 (sometimes 2+3/2+4, 2+4/2+3, 2+4/2+4, and rarely 2+3/1+4); As undivided; Ve (sex pooled) 179–200; Sc (sex pooled) 50–61. The inverted hemipenis extends to 11 to 15 Sc, and the organ is bilobed with small spines throughout that are slightly larger in the basal region of the organ (Fig. 4).

Table 1. Comparative meristic data of *Naja kaouthia* originating from this study, together with published and unpublished data from Indo-Myanmar region

Populations	Sex	Ve	Sc	ADSR	MDSR	PDSR	Hood mark	Sources
Mizoram (India)	Male (*n* = 26)	179–198	51–61	23–29	19–21	13–17	Typical and variant	This study
	Female (*n* = 18)	188–200	50–59	24–29	20–23	15		
Meghalaya (India)	Male (*n* = 3)	183–191	55	23–25	19–21	13–15	Typical	Yashpal Singh Rathee (unpubl. data)
	Female (*n* = 3)	189–191	52–58	24–25	17–21	14–15		
Sumhpawng Mada, Mahtum, Kajitu, Sumpra (Upper Myanmar)	Male (*n* = 2)	187–188	53–55	25–31	21	15	Typical and variant	Smith (1940)
	Female (*n* = 2)	189–191	54–55	25–27	21	15		
Indo-Myanmar	Sex pooled	164–196	43–58	25–31	19–21	15–17	Typical	Smith (1943); Leviton et al. (2008)

Various phenotypic forms of hood markings were observed in the studied specimens, such as individuals with a typical monocellate mark (either rounded or rhomboid shaped), and variations such as broken monocellate (either at the anterior or both anterior and posterior edges of the mark), rounded monocellate (with a prominent dot within one or both lateral sides of the mark), a complete absence of hood mark, the mark extended at the lateral flanks to form a mask-like marking, or widely open at the anterior end, forming a spectacle-like mark (Fig. 5).

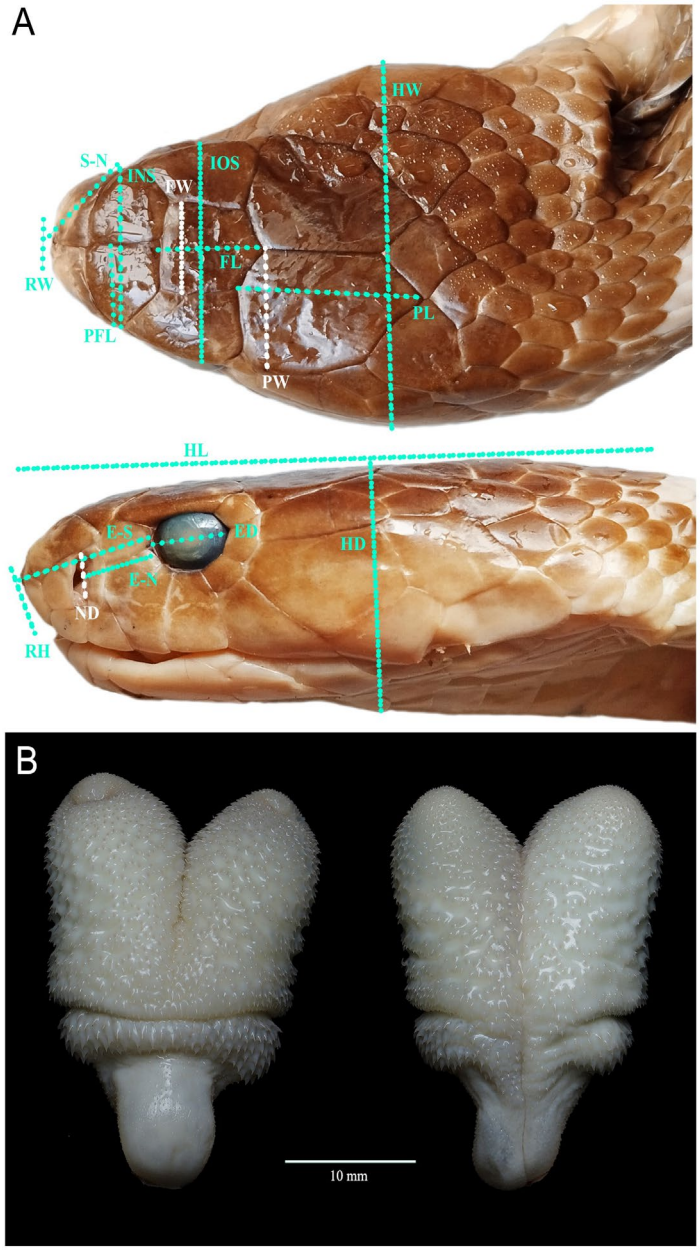

Fig. 4. A. Description of head morphometrics used in *Naja kaouthia* from this study. B. Hemipenis showing the asulcate (left) and sulcate (right) views of *Naja kaouthia*. The organ is bilobed with small spines throughout that are slightly larger at the basal region.

Fig. 5. *Naja kaouthia* specimens from Mizoram, northeast India in life (A) and preserved specimens. (B–D) The dorsal views of the hood with typical monocellate mark in adult. (E) Preserved juvenile specimen with the typical monocellate hood mark. (F–I) The dorsal views of adult anterior body showing a broken monocellate mark either at the anterior end (F, H, I) or at both anterior and posterior sides (G). (J) Preserved juvenile with a prominent dot present within the left lateral side of the mark. (K) Complete absence of hood mark. (L) The monocellate extended at the lateral flanks to form a mask-like pattern. (M) The monocellate mark widely open at the anterior end forming a spectacle-like mark (this particular specimen was killed by local people in the northern part of Mizoram not far from the Assam border, and was preserved as a museum specimen).

Sexual dimorphism

We adjusted the mensural data against SVL using the equation Character+(grand mean SVL-SVL)*Regression coefficient, where 1261 is the grand mean of SVL in adults and 441.63 is the grand mean SVL in juveniles. The characters compared between males and females showed significant dimorphism in the following characters: in adult (n = 23), Ve (p < 0.01), Sc (p < 0.05), TaL (p < 0.01), ED (p < 0.01), S-E (p < 0.05); in juvenile (n = 16), HL (p < 0.05), and ND (p < 0.05) (Table 2; see Table S9 for the complete table). In adults, there are more ventrals in females, a longer tail length and more subcaudals in males, larger horizontal eye diameter in males, longer snout to eye distance in males; in juveniles, a longer head is present in males, while females have a larger nostril diameter.

Table 2. Test for sexual dimorphism on the meristic and mensural characters using 44 (14 adult males; 15 females; 13 juvenile males; 3 juvenile females) *Naja kaouthia* individuals from Mizoram, northeast India, including mean, standard deviation, minimum and maximum values. Individuals with SVL ≥ 1,000 mm were grouped as adults, while those below this threshold were grouped as juveniles. Standardised meristic data were analysed through one-way ANOVA with sex as a factor. Adjusted mensural data were analysed through one-way ANCOVA using SVL as a covariate and sex as a factor. Only the characters with statistically significant variations at the alpha level of 0.05 are shown in this table. After removal of specimens with high number of missing data, a total of 13 adult males, 10 adult females are retained, while all the juvenile specimens are retained because the estimated percentage of missing value is < 30%

Characters	Life stage	Male		Female		Sexual dimorphism	
		Mean ± SD	Range	Mean ± SD	Range		
Ve	Adult	188.85 ± 3.44	184–197	192.80 ± 3.05	190–200	$F_{1,21} = 8.24$	$p = 0.009$
Sc	Adult	56.42 ± 2.57	52–61	54.10 ± 2.02	52–59	$F_{1,20} = 5.33$	$p = 0.032$
TaL	Adult	233.00 ± 27.36	174–288	210.20 ± 23.97	176–252	$F_{1,19} = 12.90$	$p = 0.002$
HL	Juvenile	17.63 ± 3.77	14.30–27.25	16.87 ± 2.96	13.72–19.58	$F_{1,13} = 8.36$	$p = 0.013$
ED	Adult	5.85 ± 0.72	5.06–7.30	5.10 ± 0.58	4.20–5.80	$F_{1,20} = 9.07$	$p = 0.007$
ND	Juvenile	1.34 ± 0.26	1.06–1.80	1.81 ± 0.39	1.58–2.26	$F_{1,12} = 7.30$	$p = 0.019$
S-E	Adult	11.38 ± 1.74	9.38–13.80	9.92 ± 1.79	7.74–13.12	$F_{1,14} = 5.20$	$p = 0.039$

In an exploratory analysis (PCA), all of the identified sexually dimorphic characters in adults (Ve, Sc, TaL, ED, and S-E) were utilised. Multivariate analysis (PCA) was not conducted in juveniles because only two variables are identified as sexually dimorphic. The first two components accounted for 78% of the total variation of the data, with PC1, PC2 and PC3 representing 52%, 26% and 11%, respectively. The loadings of TaL, ED, and S-E are highly positive on the first axis, while Ve loaded highly negatively, with Sc dominating the second axis. The ordination of the first two components depicts evident separation between males and females on the first axis (PC1) (Fig. 6; Table S10).

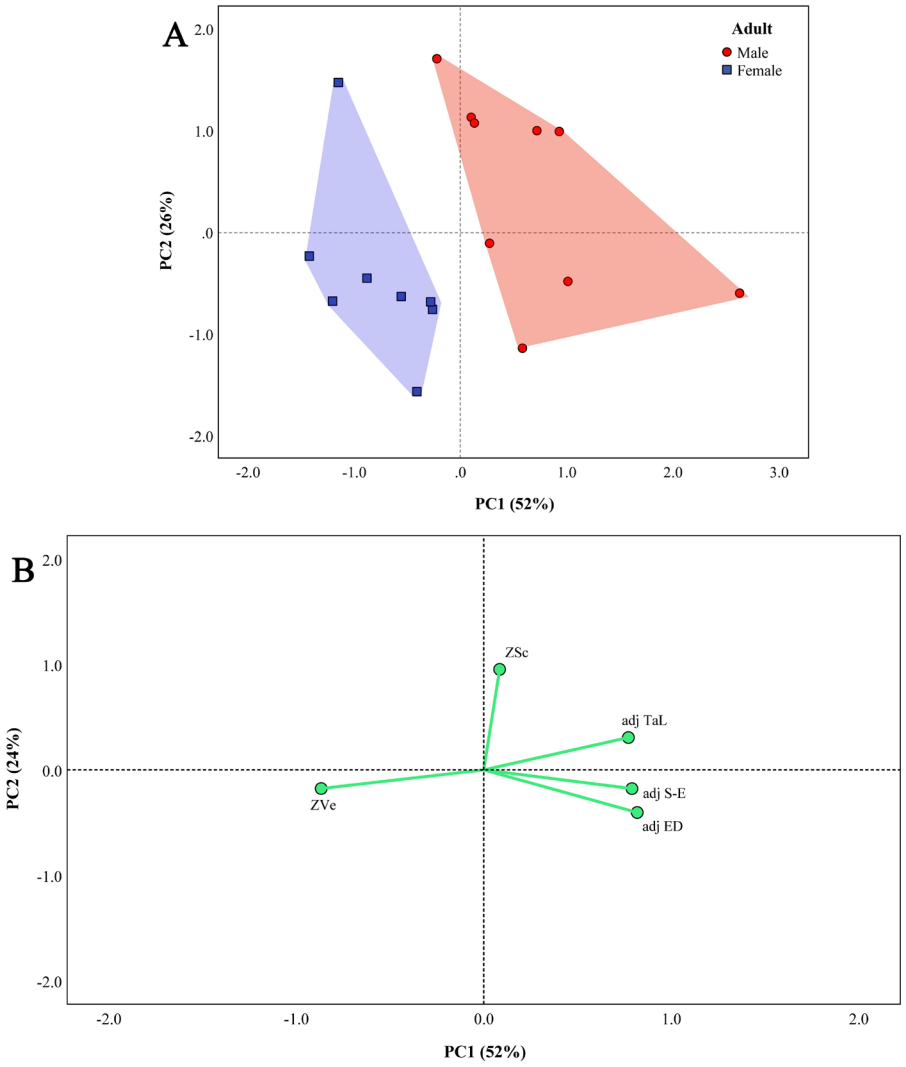

Fig. 6. (A) Ordination of *Naja kaouthia* from Mizoram along the first two principal components based on a PCA of the standardised ventrals (Ve), subcaudals (Sc), and adjusted tail length (TaL), snout to eye distance (S-E) and eye diameter (ED) of adult specimens. Total variance summarised by PC1 and PC2 are 52% and 26%, respectively. (B) PCA loading plot showing the distribution of the analysed variables along the first two principal components.

DISCUSSION

Although the examined specimens from Mizoram, northeast India, conform to the conventional taxonomic characters of the species, our study reveals sexually dimorphic traits and substantial phenotypic variability, particularly in the hood markings, which may have taxonomic implications in future assessments. Sexual dimorphism in snakes is common and well-studied, but intersexual difference in head shape and size is apparently neglected (Borczyk et al. 2024). A study on *N. melanoleuca* found that females have significantly larger heads, even though males and females show nearly identical diets. Because this sexually dimorphic trait could not be explained by feeding ecology, the authors suggested that other factors might be involved, such as male preference for females with larger heads (Luiselli et al. 2002). However, Shine et al. (2007) disagreed with this, because their own analysis appeared to indicate smaller head sizes in females, and snakes appear to rely more upon pheromonal than visual cues for male mate choice. Thus, it was hypothesised that the larger heads of male cobras may enhance success in male-male rivalry, because male cobras were documented to bite each other vigorously during combat bouts, unlike many other venomous snake species (see Shine et al. 2007 and references therein). Shine (1994) considered the occurrence of male-male combat to be phylogenetically conservative, and his study scored male-male combat as likely present if the congeneric species showed this behaviour, with an exception among the subspecies of the python *Morelia spilota*. To the best of our knowledge, no existing literature to date has documented male-male combat in *N. kaouthia*, while it has been documented in its phylogenetic sister lineage *N. oxiana* (Kudrjavtsev and Mamet 1989; also see Shine 1994), as well as the other congeneric species like *N. naja* (Shine 1994), *N. annulifera* (Carpenter 1986; Shine et al. 2007), and *N. melanoleuca* (Branch 1998). Therefore, we consider that male-male combat likely to be present in *N. kaouthia*, and hypothesise that the larger horizontal eye diameter and longer snout-to-eye distance in adult males, as well as greater head length in juvenile males, of *N. kaouthia* may be driven by male-biased sexual selection for success in male-male rivalry (Shine et al. 2007). In another perspective, male-male combat is possibly either absent completely for the species or at least among the urban populations due to the effect of urbanisation, a factor known to alter sexual selection pressure on sexual traits, which was even regarded influencing sexual selection to operate differently between some rural and urban animals (Cronin et al. 2022). We further speculate that the sexual dimorphic head morphology could be due to intersexual niche divergence associated

with different diets, as has also been reported in many snake species (Shetty and Shine 2002; Borczyk et al. 2024). Due to the fact that our study lacks evidence on reproductive, dietary preferences or foraging behaviour, this hypothesis requires further behavioural and ecological studies.

Accounting for sexual dimorphism in comparative morphological study is critical to avoid potential biases and misinterpretation of the analyses (Biakzuala et al. 2023a 2023b; Huang et al. 2025). Overall, our findings on sexual dimorphism follow those of previous studies: tail length, head length and body mass were found to be greater in males of *N. kaouthia* from Thailand (Chaitae 2011), the Asiatic cobra *N. sputatrix* was documented to show larger relative head sizes in males (Boeadi et al. 1998), and a study on eight African cobra species in the genera *Aspidelaps*, *Hemachatus*, and *Naja* also found that males typically have larger heads and longer tails than conspecific females (Shine et al. 2007).

Even though a recent study highlighted the high degree of phenotypic variation in overall body colouration and hood markings, as well as in venom composition, across the species distributional range (Balchan et al. 2025), the wide range of hood-mark phenotypes documented from the relatively small sample size in Mizoram, northeast India, also underscores the need for further quantitative analyses on this variation. This should include tests of association with sex, ontogeny, geography, and evolutionary lineages, to evaluate whether these phenotypic variants are randomly distributed or potentially informative for geographic structuring and lineage differentiation.

This work not only supports the inter-population genetic variability that has been mentioned in earlier work (Ratnarathorn et al. 2019; Kundu et al. 2020; Balchan et al. 2025), but also complements the phylogeny of *Naja*. The existence of two putatively distinct evolutionary lineages, namely the South Asian and Southeast Asian clades, was previously detected also by Shi et al. (2022) but without further taxonomic recommendation for the two clades. So far, the two distinct clades of *N. kaouthia* i.e South Asian clade is represented by populations from northeast India (Mizoram), Nepal, Bhutan, Tibet (China), and Bangladesh; while the Southeast Asian clade is represented by populations from Southern Myanmar, central and southern Thailand, Cambodia, central and southern Laos, southern Vietnam, and the Malay Peninsula. Aligning with our findings and Shi et al. (2022), Kundu et al. (2020) also detected cryptic diversity among *N. kaouthia* populations in China, India, Thailand and Bangladesh. Thus, extensive population and systematic studies are needed for precise determination of the species limits across and within different biogeographic boundaries. In addition, the systematic status of the Chinese specimens of *N. fuxi* (CHS026, CHS726,

HS16001) was reaffirmed, and they formed a highly supported sister lineage of *N. atra* in both ML and BI trees. The outlying sequence of *N. naja* (NC010225) from GenBank also appears to be a misidentified specimen of *N. atra*, which would need taxonomic confirmation.

Given that the overall genetic sampling (new + database) for *N. kaouthia* in our analyses is limited to 20 specimens originating from a wide geographic range, we encourage future studies incorporating larger sample sizes with the integration of multiple lines of evidence for more comprehensive phylogeographic and taxonomic reassessments. Particularly, incorporating multiple nuclear genes will be crucial for more robust species delimitation and evolutionary inference, because mitochondrial DNA is a maternally inherited single locus, therefore species delimitation and phylogeographical studies solely based on mitochondrial genes may not accurately reflect species boundaries (Ballard and Whitlock 2004; Wüster 2025). Consequently, the PCoA clustering observed in our analyses should also be interpreted as a preliminary insight rather than conclusive evidence of population or taxonomic boundaries. Moreover, defining the population structure is critical for delimiting the evolutionary conservation units (Xuereb et al. 2022). For this, high-resolution molecular markers like single nucleotide polymorphisms (SNP) and microsatellites have increasingly been used because they are inferred to have advantages in detecting recent population divergence and speciation (Guichoux et al. 2011; Benestan et al. 2021; Wenne 2023). Hence, we also recommend an extensive population genetics study to accurately delimit the population structure and identify the underlying genetic differentiation driving the high degree of intrapopulation phenotypic variability in *N. kaouthia.*

CONCLUSION

In this paper, we substantially contribute new data to the genetic diversity and morphology of *N. kaouthia* from Mizoram, northeast India. Our morphological inferences recommend extensive studies on the systematics and population genetics to determine whether the phenotypic variations in hood marking and body colouration, also reported in Balchan et al. (2025), will have future taxonomic implications. Further reproductive and ecological studies with specifically formulated experimental designs to test the hypotheses for the evolutionary drivers of the sexually dimorphic traits are also recommended. Considering the matrilineal phylogenetic evidence and wide inter-clade mean genetic divergence (4.4%), we emphasise the need for an integrative taxonomic reassessment for the

two distinct clades of *N. kaouthia* with sufficient evidence to show that any proposed species is a distinct evolutionary lineage based on extensive morphological and molecular analysis (Wüster and Kaiser 2023). The generation of additional morphological and molecular evidence, including data from the neotype and topotypical specimens, would be imperative for further taxonomic re-evaluation of *N. kaouthia*, a medically significant snake in South and Southeast Asia (Mukherjee 2020).

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Supplementary materials

Table S1. Information on PCR thermal conditions used in the amplification of two mitochondrial genes of *Naja kaouthia*. (download)

Table S2. Details of three mitochondrial genes utilized in this study. Sequences generated in this study are indicated by bold. Morphologically examined specimens are indicated with asterisk. (download)

Table S3. The best partitioning schemes and nucleotide substitution models. (download)

Table S4. Uncorrected *p*-distance estimate among the different haplotypes recovered in *cytb* gene fragment. (download)

Table S5. Uncorrected *p*-distance estimate among the different haplotypes recovered in *cox1* gene fragment. (download)

Table S6. Uncorrected *p*-distance estimate among the different haplotypes recovered in *16s* gene fragment. (download)

Table S7. Uncorrected *p*-distance estimate between the two clades of *Naja kaouthia* along with other congeneric sequences based on *cytb* dataset. The mean of within clade distance for each clade are given under the column marked with "#". (download)

Table S8. Raw morphological data and sampling sites of *Naja kaouthia* population in Mizoram, Northeast India. Measurements in mm. (download)

Table S9. Complete table for the test of sexual dimorphism on the meristic and mensural characters using 44 (14 adult males; 15 females; 13 juvenile males; 3 juvenile females) *Naja kaouthia* individuals from Mizoram, Northeast India, including mean, standard deviation, minimum and maximum values. Individuals with SVL ≥ 1,000 mm were grouped as adults, while those below this threshold were grouped as juveniles. Standardized meristic data were analysed through one-way ANOVA with sex as a factor. Adjusted mensural data were analysed through one-way ANCOVA using SVL as a covariate and sex as a factor. The characters with statistically significant variations at the alpha level of 0.05 are shown in bold. After removal of specimens with high number of missing data, a total of 13 adult males, 10 adult females are retained, while all the juvenile specimens are retained because the estimated percentage of missing value is <30%. (download)

Table S10. Information on Principal Component Analysis (PCA) and loadings for *Naja kaouthia* population in Mizoram, northeastern India between the adult male and female specimens. (download)