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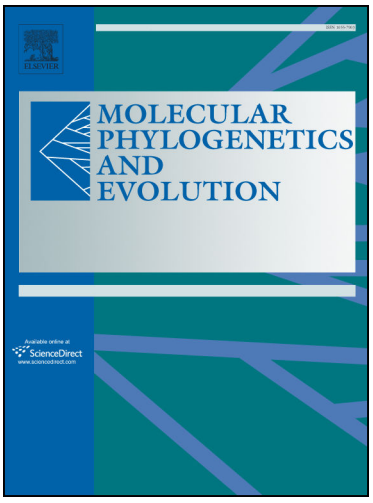
Molecular Phylogenetics and Evolution

DOI:
https://doi.org/10.1016/j.ympev.2021.107300

Published: 01/12/2021

Peer reviewed version



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PII: S1055-7903(21)00233-5
DOI: https://doi.org/10.1016/j.ympev.2021.107300
Reference: YMPEV 107300

To appear in: *Molecular Phylogenetics and Evolution*

Received Date: 8 July 2020
Revised Date: 17 August 2021
Accepted Date: 26 August 2021

Please cite this article as: Gowri Shankar, P., Swamy, P., Williams, R.C., Ganesh, S.R., Moss, M., Höglund, J., Das, I., Sahoo, G., Vijayakumar, S.P., Shanker, K., Wüster, W., Dutta, S.K., King or royal family? Testing for species boundaries in the King Cobra, *Ophiophagus hannah* (), using morphology and multilocus DNA analyses, *Molecular Phylogenetics and Evolution* (2021), doi: https://doi.org/10.1016/j.ympev.2021.107300

King or royal family? Testing for species boundaries in the King Cobra, *Ophiophagus hannah* (Cantor, 1836), using morphology and multilocus DNA analyses

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ABSTRACT

In widespread species, the diverse ecological conditions in which the populations occur, and the presence of many potential geographical barriers through their range are expected to have created ample opportunities for the evolution of distinct, often cryptic lineages. In this work, we tested for species boundaries in one such widespread species, the king cobra, *Ophiophagus hannah* (Cantor, 1836), a tropical elapid snake distributed across the Oriental realm. Based on extensive geographical sampling across most of the range of the species, we initially tested for candidate species (CS) using Maximum-Likelihood analysis of mitochondrial genes. We then tested the resulting CS using both morphological data and sequences of three single-copy nuclear genes. We used snapclust to determine the optimal number of clusters in the nuclear dataset, and Bayesian Phylogenetics and Phylogeography (BPP) to test for likely species status. We used non-metric multidimensional scaling (nMDS) analysis for discerning morphological separation. We recovered four independently evolving, geographically separated lineages that we consider Confirmed Candidate Species: 1) Western Ghats lineage; 2) Indo-Chinese lineage 3) Indo-Malayan lineage; 4) Luzon Island lineage, in the Philippine Archipelago. We discuss patterns of lineage divergence, particularly in the context of low morphological divergence, and the conservation implications of recognizing several endemic king cobra lineages.

Keywords: Species delimitation, king cobra complex, endemic, Western Ghats, phylogenetics, phylogeography.

1. Introduction

Species that were long thought to be 'widespread' and 'variable', when systematically studied, have frequently turned out to contain previously unrecognized diversity, and to comprise species complexes, typically consisting of multiple allopatric species, each inhabiting a smaller geographic area (e.g., Welton et al., 2010, 2014; Siriwut et al.,2015; Wüster et al., 2018). However, testing for species boundaries can be complicated in cryptic species that are superficially indistinguishable due to strong resemblance in morphology, behavior and ecology (Steyskal, 1972; Bickford et al., 2007). In recent years, molecular studies have improved the understanding of cryptic species across biogeographic regions (Pfenninger & Schwenk, 2007; Vodă et al., 2014).

This issue is particularly acute in the Oriental tropics, a region with diverse geological histories and heterogenous contemporaneous climate and climax vegetation types (Archbold et al., 1982; Woodruff, 2010; Lohman et al., 2011). Cryptic diversity in the Oriental tropics has been well established across many taxa including plants (Clark et al., 2009; Okuyama & Kato, 2009), invertebrates (Huelsken et al., 2013;Adler et al., 2016;), vertebrates such as fishes (Matsumoto et al., 2010; Jaafar et al., 2012), amphibians (Stuart et al., 2006; McLeod, 2010; Nishikawa et al., 2012; Liu et al., 2018), reptiles (Barley et al., 2013; Grismer et al., 2013; Guo et al., 2015; Luu et al., 2016; Klabacka et al., 2020), birds (Olsson et al., 2005; Outlaw & Voelker, 2008), and mammals (O'Brien et al., 2005;Thabah et al., 2006; Burton & Nietsch, 2010; Nater et al., 2017). Within snakes, the elucidation of several 'well-known' taxa as species complexes, including *Daboia russelii* (Wüster et al., 1992; Thorpe et al., 2007), *Naja naja* (see Wüster, 1996), *Ovophis monticola* (Malhotra et al., 2011), *Ahaetulla nasuta*

(Mallik et al., 2020), and *Cerberus rynchops* (Alfaro et al., 2004; Murphy et al., 2012) has demonstrated the need for such multi-criteria approaches.

Recent years have seen considerable conceptual, methodological and technological advances in our approaches towards species delimitation (de Queiroz 1998, 2007; Shanker et al., 2017). Similarly, there have been significant advances in species delimitation methods and integrated species delimitation methods have become increasingly used (O'Meara, 2010; Padial et al., 2010; Langa et al., 2015). It is necessary to consider different lines of evidence or operational criteria for species delimitation (de Queiroz, 2007). Over the years, the concepts, definitions, paradigms and even the approach towards 'species-delimitation' are shifting. These range from varying postulations of species concepts to the definition of species as a taxonomic rank, species as a unit in the tree of life, species boundaries, gene flow, degree of crypsis and generally about mechanisms of pre- and post-zygotic reproductive barriers that drive speciation by and large (Wiens & Servedio, 2000; Wiens, J.J. 2007; DeSalle et al., 2005; Tobias et al., 2010; Yang & Rannala, 2010; Masters et al., 2011; Carstens et al., 2013; Edwards & Knowles, 2014; Yang, 2015; Jackson et al., 2017). Integrated species delimitation methods have become used increasingly widely (O'Meara, 2010; Padial et al., 2010; Langa et al., 2015).

The king cobra, *Ophiophagus hannah* (Cantor, 1836) is the longest venomous snake in the world (up to 5 m in length), belonging to the family Elapidae. It is distributed across the Oriental tropics, subtropics and temperate areas, from the wet regions of the Western and Eastern Ghats of Peninsular India and the Himalayan foothills of northern India (Uttarakhand), east across Northeast India to southern China, and southeast across Myanmar, Thailand, Vietnam, Peninsular Malaysia, to Sumatra,

Borneo, Java, Bali, Sulawesi and with its eastern-most extent on Mindanao in the Philippines (Das, 2010; Wallach et al., 2014). Its range is discontinuous and characterized by many isolated populations restricted to the wet zones and lowland forests of the Asian mainland, and numerous island populations in southeast Asia. The disjunctions in the distribution pattern of the group members, the diverse ecological conditions in which the populations occur, and the presence of many potential geographical barriers through its range are expected to have created ample opportunities for the evolution of distinct lineages.

Geographic variation is known within this species, as presently understood, including in its morphology (Deraniyagala, 1960, 1961; Charlton, 2018). This variation is reflected in the historic descriptions of many species and subspecies across its range since its initial description in the early 19th century from "Sunderbans jungles", a deltaic region in eastern India (Cantor, 1836). Subsequent authors have not accepted those descriptions, and instead retained the king cobra as a single, widespread, variable monotypic species (Wallach et al., 2014). However, recent work has shown strong regional heterogeneity in mitochondrial DNA across Thailand (Suntrarachun et al., 2014), suggesting cryptic diversity even within this limited area. Due to the widespread distribution of the species in a biogeographically complex region, it is likely that *Ophiophagus hannah* represents a species complex, as documented in several other widely-distributed Asian taxa (read below).

Understanding the systematics of this iconic taxon matters not just for fundamental and theoretical reasons, but also for management and conservation purposes. The king cobra is categorized as 'Vulnerable' under the IUCN Red List of Threatened Species Assessment (IUCN, 2012) and listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The species is infrequently encountered in some parts of its range and population density is

strongly connected to the prevalence of undisturbed forests (Das et al., 2008). Habitat destruction, harvesting for skin, food, medicine and the pet trade are some of the threats to king cobra populations (Sodhi, 2009, Somaweera and Somaweera, 2010; IUCN, 2012). Cryptic species complexes require special consideration in conservation planning, as they may contain multiple species, each one rarer or more localized than the complex as a whole, and some potentially highly endangered by regional factors. These different species require separate, individual conservation status assessments, and may require different conservation strategies, depending on different threats (Bickford et al., 2007; Schönrogge et al., 2008; Vodă et al., 2014). Thus, understanding and delimiting evolutionarily significant units in the king cobra also has implications for its conservation.

In venomous organisms, cryptic diversity may also be reflected in their venom composition, affecting both pathology and the efficacy of anti-venom (Wüster and Thorpe, 1991; Wüster, 1996; Casewell et al., 2014). This is of significance considering that king cobras have high-yielding neurotoxic venom (Gantavorn, 1969; Pu and Wong,1995). The presence of multiple lineages within the king cobra complex will thus influence the development of anti-venom.

In order to test for cryptic diversity within the king cobra, we sampled specimens across most of the distributional range of the king cobra, from the Western Ghats to the Sunda Islands and the Philippines. We then applied an integrative approach, involving mitochondrial and single-copy nuclear gene sequences and morphology to the delimitation of species within the complex. The results support our initial expectations that *O. hannah* may comprise multiple unrecognized species. The boundaries of four recognized lineages broadly coincides with the existing biogeographic regions. We discuss the systematic and conservation implications of the results.

2. Materials and Methods

2.1. Taxon sampling

Our taxon sampling strategy for this study accounted for almost the entire distribution range of king cobras. We collected 62 samples from one of three sources: ventral scale clips from wild-caught specimens and captive individuals from zoos, shed skin from private breeders, and liver, muscle tissue, rib bones and ventral scales from preserved specimens from Muséum National d'Histoire Naturelle, Paris, as well as freshly-dead specimens (road kills or snakes killed by people). Samples were stored in molecular grade alcohol at -20°C. See Appendix I for details of samples and vouchers.

2.2. DNA amplification and sequencing

We extracted genomic DNA from the tissues obtained using standard phenol-chloroform -isoamyl alcohol extraction (Sambrook et al*.,* 1989). We estimated the amount of DNA using a Nanodrop spectrometer for each extract. PCR amplification was carried out for three mitochondrial markers: 16S ribosomal RNA (16S), Cytochrome b (Cyt b) and NADH dehydrogenase subunit4 (ND4), and three nuclear markers Neurotrophin-3 promoter (NT3), Prolactin receptor gene (PRLR) and Recombination activating gene 1 (RAG1) (see Appendix IIa for primer details). PCR reaction mixtures consisted of 10× buffer, 25 mM dNTP, 25 mM MgCl2, 10 pmol/µl of sense and antisense primers, Taq DNA polymerase and 1µl DNA template for 16S, Cyt b and ND4, whereas 3µl DNA template was used for NT3, PRLR, and RAG1.

The amplification was conducted on a thermocycler (see Appendix IIb for details) and amplified products were sequenced using an ABI 3730X sequencer at a sequencing company in Bangalore, India and Amsterdam, the Netherlands. We used both forward and reverse primers to sequence all mitochondrial markers and nuclear markers, only forward primers were used for some samples. Codon Code Aligner (CCA) (www.codoncode.com) was used initially to visualize and manually check the sequences and to assemble multiple reads into contigs. These were then edited and aligned using MEGAv5.2 (Tamura et al*.,* 2011). If heterozygous positions were found, ambiguity codes were assigned using IUPAC codes (www.bioinformatics.org). After contig assembly, contig alignment was performed using Muscle, implemented in MEGA. Protein-coding genes were checked for the presence of unexpected indels and premature stop codons. Checked contigs were then exported for further analysis.

2.3. Phylogenetic analysis

A Maximum likelihood (ML) phylogenetic tree was constructed using raxmlGUI v1.3 (Silvestro and Michalak, 2012) for the mitochondrial 16S, ND4 and Cyt b loci. The analysis was carried out using 5000 bootstrap replicates to assess branch support with GTR+GAMMA+I as a model of sequence evolution (Stamatakis 2006; Silvestro & Michalak, 2012). A sequence of *Dendroaspis angusticeps* was used as an outgroup to root the tree, based on Pyron et al.,2013, Figueroa et al.,2016 and Lee et al., 2016. A bootstrap value of above 70 was considered as well supported, and above 80 as strongly supported. Uncorrected pairwise genetic distance was determined between individuals within and among each lineage using MEGA.5.05 (Tamura et al.,2011). We treated strongly supported,

reciprocally monophyletic haplotype clades with discrete geographic distributions and reasonable sampling of their distributions as candidate species (CS).

2.4. Nuclear haplotype networks

Nuclear DNA (nDNA) sequences were imported to MEGA and aligned by Muscle. PHASE (Stephens et al., 2001; Stephens, M. and P. Donnelly, 2003) was used to reconstruct individual haplotypes from diploid nuclear sequences. Input files were generated via SeqPHASE (Stephens et al., 2001; Flot, 2010). During PHASE analysis, each locus was analysed thrice to ensure consistent proposed haplotype output. The parameters used were 1000 iterations, a thinning interval of 10 and a burn-in of 100. A different randomized four-digit seed number was used for each run. The haplotype pairs with the highest *p*-values were used for analysis.

We produced haplotype networks with PopART (Bandelt et al., 1999) for nuclear DNA using the median-joining method which has proved to be a useful statistical approach for reconstructing network from intra-specific data (Bandelt et al., 1999). This allowed visualization of haplotype sharing between candidate species and identified the individual haplotypes of individuals.

2.5. Bayesian Species delimitation

To test whether the candidate species revealed by the mitochondrial phylogeny represent independently evolving lineages, i.e., distinct species (Padial et al., 2010), Bayesian species delimitation was implemented using the reversible-jump Markov Chain Monte Carlo (rjMCMC) method of Yang & Rannala.,2010, using the software BPP Version 3 (Rannala & Yang, 2003; Yang &

Rannala, 2014), which incorporates species tree uncertainty. This method has previously been utilized for delimiting a number of squamate species (Leaché and Fujita, 2010; Chaitanya et al., 2019). Bayesian analysis may be strongly influenced by the priors used. Each combination of priors affects the probability of each OTU being recognized as a species. To determine the resilience of our conclusion to varying priors, we tested four different combinations of the key priors, which are the ancestral population size (θ) and root age (τ) (Leaché and Fujita, 2010). Both can affect the posterior probabilities for speciation models (Yang & Rannala, 2010; Sukumaran and Knowles, 2017). A11 analyses were performed for each combination of τ and θ priors, using 100,000 generations, a burn-in of 20,000 generations and a sampling frequency of two. Each analysis was run three times to confirm consistency between runs.

We considered four possible scenarios with different permutation and combinations:

(i) Large ancestral population sizes and deep divergence between species (θ = 1 10 τ = 1 10)

(ii) Small ancestral population sizes and shallow divergence between species (θ = 2 2000 τ = 2 2000)

(iii) Large ancestral population sizes and shallow divergence between species (θ = 1 10 τ=2 2000)

(iv) Small ancestral population sizes and deep divergences between species (θ = 2 2000 τ = 1 10)

Based on the mitochondrial phylogeny (see below), we tested four OTUs as candidate species:

a. Western Ghats; Indo-China (Mainland Asia, from the Eastern Ghats and Himalayan foothills to Southern China, Thailand, and Vietnam); Indo-Malayan (The Sunda Shelf, including Peninsular Malaysia, Sumatra, Borneo, Java, Bali, and the Philippine island of Mindoro); and Luzon, Philippines.

2.6. Overall patterns of genetic differentiation

Allele distance matrices were generated for each nuclear locus under the Kimura two-parameter model (K2P) (Kimura, 1980) in PAUP*4.0b10 (Swofford, 2002). We then used POFAD (Phylogeny of Organisms from Allelic Data - Joly & Bruneau, 2006) to generate a standardized multi-locus between-individual distance matrix. POFAD allows the assessment of the pattern of genetic variation across multiple loci, generating standardized multilocus distances between specimens, incorporating allelic variation and equal weighting of loci. The standardised multi-locus distance matrix produced by POFAD was analysed using MVSP (Multivariate Statistical Package - www.kovcomp.com). Principal Co-ordinate (PCO) analysis case scores were used to generate a bivariate scatter plot in PAST 3.2(Hammer et al., 2001) to visualise patterns of overall genetic differentiation across all three nuclear loci.

We tested for genetic structure in the king cobra complex using snapclust, a maximum likelihood-based method (Beugin et al., 2018). Utilizing haplotype identities from PHASE sequences of the three nuclear markers (NT3, PRLR and Rag1), we created a diploid input file that can be used to test clustering in co-dominant markers. Missing data was coded as '0' and samples with at least two nuclear markers were considered for this analysis to reduce the impact of missing data in clustering. To determine the genetic structure we used the function snapclust (Beugin et al., 2018). As a prior step, we used the function "snapclust.choose.k" (Beugin et al., 2018) to determine the optimal number of clusters (k). The function builds different models of population structure using a range of k values and the best model is chosen based on the smallest value of a summary statistic AIC (Akaike Information Criteria). The following arguments were used in snapclust: k was based on the optimal number of clusters identified using snapclust.choose.k as described above. The initial group membership was found using k-means clustering. All other parameters were set to default values

(Beugin et al., 2018). All analysis were carried out using functions within the adegenet and dependent packages (Jombart, 2008; Jombart and Ahmed, 2011) in R (R Core Team 2020). The results were visualized using inbuilt functions “scatter” and “compoplot” in adegenet (Jombart, 2008; Jombart and Ahmed, 2011).

2.7. Morphology

We identified six informative characters, namely (i) anterior dorsal scale rows (counted one head length behind head), (ii) posterior dorsal rows (counted one head length before vent), (iii) ventral count, (iv) subcaudal count, (v) supralabial count and (vi) infralabial count. Midbody scale rows count (counted at mid-SVL value) was found to be constant and hence omitted from the analysis. Scalation terminology and definition follows Whitaker and Captain (2004). These characters were counted from fluid-preserved specimens. Ventral scales were counted according to Dowling (1951) and sub-caudal counts excluded the terminal scute. In all, 47 specimens were considered for analysis (Appendix V). The final dataset was then analysed in PAST 3.2 to generate plots using non-metric multidimensional scaling (nMDS) analysis for discerning patterns of separations in the morphology of this variable species complex. Apart from the analysis elaborated above, we also considered the number of bands on the body of live snake specimens including both juveniles and adults, as well as males and females.

3. Results

3.1. MtDNA Phylogenetic analysis

We aligned a total length of 1734 base pairs of mitochondrial sequences (423 bp of 16S, 715 bp of Cyt b and 596 bp of ND4) (Table 1) for 62 individuals. The Maximum Likelihood (ML) tree generated for the mitochondrial data revealed four strongly supported lineages with cohesive distributions and adequate sampling (Fig 1), which were considered as candidate species (CS) for subsequent analyses. Each potential candidate species differed from the others by average uncorrected p-distances of 0.01 to 0.082 across 16S, ND4 and Cyt b genes (Appendix III).

The four candidate species (CS) were: CS1 (Western Ghats), CS2 (Indo-Chinese, North of the Isthmus of Kra: Eastern Ghats, Himalayas east to southern China and Vietnam, Thailand, and the Andaman Islands), CS3 (Indo-Malayan, South of the Isthmus of Kra: Peninsular Malaysia, Borneo, Bali, Sumatra. Java and Mindoro) and CS4 (Luzon, Philippines).

3.2. Nuclear haplotype network

The nuclear haplotype network analysis was carried out to examine whether the patterns of haplotype sharing were consistent with the mitochondrial candidate species and Bayesian delimitation. Figure 2 shows the haplotype networks for the four candidate species. CS4 (Luzon) displays sets of unique, exclusive haplotypes at all loci. CS3 displays a set of unique and exclusive haplotypes in PRLR, but shares some of its haplotypes with CS2 in NT3 and RAG1. CS1 and CS2 share haplotypes at all loci. mt DNA networks provide unambiguous support for these clusters (see Appendix III)

3.3. Species delimitation analyses

Species delimitation analyses using BPP3 provided strong support for the status of all four candidate species (Table 2). The posterior probability values remained consistent irrespective of the different permutations of the θ and τ priors, indicating resilience to changes in priors and high confidence in the delimited candidate species. Similarly, the posterior probabilities for the number of species remained consistent over different permutations, strongly supporting a four species model.

3.4. Overall patterns of genetic differentiation

The Principal Co-ordinates analysis of standardised between-specimen multilocus genetic distances demonstrates the distinctness of the four candidate species, all of which represent distinct, cohesive clusters (Fig 3).

The snapclust revealed four optimal number of clusters(k) (Fig 4): 1. Western Ghats (CS1), 2. Indo-Chinese (CS2), 3. Indo-Malayan (CS3), and 4. Luzon (CS4) (Fig 5). The clusters identified contained individuals with high membership probabilities (>0.99). The clustering of individuals showed a strong geographical structure and in congruence with mitochondrial phylogeny. The exception is sample 1092, from the island of Bali, which would be expected to group within CS3, shows poor assignment probabilities to the cluster. This is primarily due to its possession of unique haplotypes in both PRLR (Hap_8) and Rag1 (Hap_13).

3.5. Morphological divergence

The nMDS for 2D dimensionality analysis with Euclidean similarity index revealed four distinct, non-overlapping clusters on the scatter plot representing CS1, CS2, CS3 and CS4 (for both male and female samples pooled together) with a stress value of 0.002 (Fig 6). The four previously defined candidate species showed separation along nMDS Axis 1, but not along Axis 2. CS1 and CS2 clustered together along this axis, relative to CS3 and CS4.

3.6. Confirmed candidate species (CCS)

Taken together, the analyses suggest the presence of four confirmed candidate species (CCS – Padial et al., 2010) (Fig. 7). In the mtDNA phylogeny, CCS1 (Western Ghats) is strongly supported, and forms the sister group to the CCS2 lineage. CCS2 shows considerable mitochondrial sub-structuring, with four geographic clusters included in the CCS, albeit at low levels of genetic divergence. CCS3 is well supported with no geographical structure. CCS4 lineage is strongly supported and forms sister group to all other lineages. The distribution of each of the four lineages is provided below.

CCS1: Distributed in the low to mid-elevation moist and wet forests in the Western Ghats, in southwestern Peninsular India. Within the Western Ghats, the latitudinal range was found to extend from Agasthyamalai hill range in the south to Sindhudurg plateau in the north.

CCS2: A widely distributed lineage with its range extending from the Himalayan foothills in the northwest and eastern Peninsular India in the southwest into the northeast and southeast of mainland Asia, with isolated insular populations in the Andaman Islands in the south. Due to sparse sampling, the southern limit in mainland Asia, potentially corresponding to the Isthmus of Kra, remains unclear.

CCS3: The range extends from Peninsular Malaysia across the Greater Sunda Islands (Sumatra, Java, Borneo), southeast to Bali and northeast to Mindoro island in the Philippines.

CCS4: The range is restricted to Luzon island, in the Philippines.

4. Discussion

In recent years, many widespread species have been found to be composed of multiple deeply divergent lineages which have then been elevated to the species level (Bickford et al., 2007). This analysis of the wide-ranging king cobra, *Ophiophagus hannah*, provides evidence for at least four independently evolving lineages with unique haplotypes, but low levels of morphological divergence. The distribution of the four lineages recognized is broadly coincident with known biogeographic boundaries.

Two of the four lineages (CCS1 and CCS4) are at the geographic extremities of the range of this complex – the Western Ghats in Peninsular India and Luzon island in the northern Philippines. The lineage from the Western Ghats (CCS1) is spatially separated from its geographically and phylogenetically closest relative, the Indo-Chinese lineage (CCS2), by the arid regions of Peninsular India. The Western Ghats lineage shows strong genetic homogeneity across its range, which spans the wet zones of the escarpment (south of the Goa Gap). But for occasional occurrences in the dry leeward slopes and the tall montane peaks, its preference for low-mid elevations is consistent with a scenario of absence of genetic breaks, as seen across many lowland snake lineages (e.g. *Hypnale hypnale*, see Maduwage et al., 2009). Our findings recognize the king cobra lineage in the Western Ghats as an endemic lineage with an independent evolutionary history, and thus a likely separate species.

Sister to Western Ghats (CCS1) is an Indo-Chinese (CCS2) lineage with poorly resolved geographical boundaries. The arid zone of Peninsular India clearly separates it from the Western Ghats, but its geographic limits with the Indo-Malayan lineage remain unresolved due to our limited sampling. However, the presence of two distinct lineages, one each in the Indo-Chinese and the Indo-Malayan sub-regions, CCS2 and CCS3 respectively, accord well with the recent molecular biogeographic syntheses and investigations that reveal similar patterns in other taxa (see reviews in de Bruyn et al., 2014). Based on the mitochondrial phylogeny, CCS2 was subdivided into the following populations: a) An insular population in the Andaman archipelago. b) A cluster distributed along a narrow strip across northern parts of the Eastern Ghats in Peninsular India, extending to the eastern slopes of the Naga-Chin hills, and along the Himalayan foothills. c) A single population from eastern mainland Asia (southern China). d) A few scattered (likely a sampling artefact) populations from southeastern mainland Asia (Thailand and Vietnam).

Whether its boundary with the Indo-Malayan lineage coincides with the Isthmus of Kra, the boundary between the Indo-Chinese and Malayan biogeographic sub-regions (Wallace 1876; Hughes et al., 2003; Woodruff & Turner, 2009; Parnell, 2013), cannot be discerned in this study, but is proposed here as a working hypothesis. Additionally, the presence of multiple shallow divergent clusters within the Indo-Chinese lineage suggests additional complexity within this CCS.

The Indo-Malayan lineage (CCS3) is recovered as an independent lineage, with its northwestern boundary lying in the Malayan Peninsula and its range extending from Sumatra in the west across Borneo to Java and Bali in the southeast and Mindoro in the northeast. This lineage is predominantly

insular, with a wide distribution and, remarkably, shows no signs of geographical structuring, even in the faster-evolving mitochondrial genes used here. As we had initially hypothesized, frequent connectivity between these Islands during glacial periods (Voris, 2000; Woodruff, 2003; Hanebuth et al.,2011) most likely promoted inter-island dispersal and hence the lack of genetic divergence in this lineage.

The Luzon lineage (CCS4) represents the easternmost limit of the king cobra species complex. Despite its close proximity to the eastern limit of the Indo-Malayan lineage, it is uncovered as a divergent, independently evolving lineage. However, the systematic status and distribution of king cobras in the Philippines requires further work. *Ophiophagus* is found on most of the major islands of the Archipelago (Leviton, 1964; Leviton et al., 2018). On biogeographical grounds, it seems likely that some of the islands of this archipelago may contain additional cryptic species, as is found in a large number of other reptile species complexes (e.g., Wüster & Thorpe, 1990; Brown et al., 2013). This applies in particular to the major Pleistocene Aggregate Island Complexes (PAICs – Brown & Diesmos, 2002, 2009; Brown et al., 2013) in the Philippines that remain unsampled in this study, the Panay-Negros PAIC and the Mindanao PAIC, and also the isolated population from Sulawesi. All these island groups remained isolated from each other and the Asian mainland through phases of low sea level during the Pleistocene, due to deep marine trenches, and harbor high levels of endemism in other reptile groups (Brown et al., 2013). Given this, the presence of genetically undifferentiated populations of CCS3 on the island of Mindoro, which also remained isolated throughout the Pleistocene (Brown et al., 2013), is unexpected. Thus, assessing the taxonomic status of the king cobra populations of each of these islands individually should be a priority for further research. The high

rates of habitat loss on many of them further underscore the urgency of identifying potentially highly threatened, endemic lineages of this ecologically sensitive taxon (Brown et al., 2013).

There are some constraints to the conclusions that may be drawn from multi-species coalescent model analysis software, such as BPP3. It is often criticized for the over-estimation of genetic divergence. Sukumaran and Knowles (2017) argue that it identifies genetic structure, not species, and statistically, there is no distinction between population isolation and species boundaries, and Chambers and Hillis (2020) highlight the problems inherent with *a priori* assignment of group membership. As a result, species delimitation may be overestimated using BPP3. Moreover, geographically limited sampling may also affect coalescent-based species delimitation (Mason et al., 2020). Despite this, due to the robustness of our species delimitation to different combinations of extreme priors, the high posterior probability values of the four OTU model, there is confidence in identifying them as independent lineages. Moreover, both general multilocus distance measures and an independent maximum likelihood genetic clustering algorithm (snapclust) of the same nuclear dataset also support the four-cluster scenario suggested by our mitochondrial phylogeny, without relying on *a priori* grouping of specimens into OTUs. Thus far, in our sampling of 62 localities across most of its geographic range, in no location are two candidate species sympatric, suggesting a strong role for geographical isolation as a profound pre-zygotic reproductive barrier.

Due to limited sampling, we have not been able to investigate the nature of contact zones between the CCS2 and CCS3 (Chambers and Hillis, 2020), and therefore cannot exclude the possibility of some degree of introgression along the Isthmus of Kra. However, the clear distinction between these two species, and their relative homogeneity across thousands of kilometres of mainland southeast Asia and the Sunda Shelf, respectively, lead us to conclude that they are indeed independent evolutionary

lineages, irrespective of possible limited introgression in a very small part of their range, and not sections of a cline or reproductively continuous lineages connected by broad zones of genetic introgression (Hillis, 2019).

Though there is some external morphological variation in this complex, this was inconsistent as well as being complicated by both sexual dimorphism and ontogenetic colour shifts (i.e. intra-population variation). One external morphological character that was taxonomically informative was the number of dorsal bands. As to the number of bands on the body, the various candidate species were characterized as follows: CS1: < 40 bands; CS2: 40-70 bands; CS3: > 70 bands; CS 4 thin, obscure 'bands' that are barely discernable. However, we were unable to include this in our morphological nMDS analyses as the bands become invisible in many adults of CCS3 and CCS4. Additional morphological analyses are warranted to further test for divergence.

The refining of the species limits in the *Ophiophagus* complex provides a basis for the study of its venom variation, as shown in Russell's viper (*Daboia* Gray, 1842), which has similar biogeographic patterns (Thorpe et al*.,* 2007). There is considerable evidence supporting a reduction in antivenom efficacy because of genetic and venom variation across distributions, especially where there is evidence of isolation (Wüster, 1996; Gutiérrez et al., 2006; Casewell et al., 2014; Tan et al.,2020; Senji Laxme et al., 2019; Senji Laxme et al., 2021). Although bites from *O. hannah* are rare, they are of medical significance, producing life-threatening neurotoxic effects and releasing large doses of venom (Gantavorn*,* 1969; Pu and Wong, 1995; Tin et al.,1991). Correct identification of different species may, therefore, facilitate improved treatment of bite incidents.

Our findings highlight the presence of multiple independent lineages that need to be considered as different conservation units, which require different conservation strategies based on the particular threats facing them in each region (Bickford et al., 2007; IUCN, 2012). *Ophiophagus hannah* is currently listed as Vulnerable in the Red List (IUCN, 2012), which is largely influenced by its large geographical range. The recognition of four lineages as different species, each with different range sizes, suggests the need for a revision of their threat status. Our range estimate, based on dense taxon sampling for CCS1 shows that this lineage is endemic to the Western Ghats, a global biodiversity hotspot known for high endemism (Myers *et al*., 2000; Bossuyt et al., 2004). Based on its relatively narrow range, endemic status, and decline of populations across its current range, there may be sufficient evidence to elevate the status of this lineage. Similarly, the unique lineage from the Philippine Island of Luzon (CCS4) and the possibility of further cryptic diversity in that Archipelago deserves immediate reassessment of its conservation status. The Philippines is a key biodiversity hotspot with high rates of deforestation and habitat loss (Suarez & Sajise 2010; Brown et al., 2013) Finally, CCS2 shows evidence of genetic substructuring across mainland Asia that could indicate the existence of further cryptic diversity, again with consequences for the conservation assessment of the various subgroups. As the next phase of our study on the king cobra species complex, we aim to carry out formal taxonomic revision culminating in nomenclatural re-allocations for the candidate species within this complex.

In summary, our study has provided a new phylogenetic and systematic framework for the king cobra, with evidence for the existence of at least four species. These findings thus provide a roadmap for further study of the complex. Priorities include an assessment of the systematic status of the remaining isolated populations (different Philippine PAICs, Sulawesi), more in-depth study of population

structure within CCS2, and the elucidation of contact zones between our Confirmed Candidate Species, particularly between CCS2 and CCS3 around the Isthmus of Kra, and independent reassessments of the conservation status and needs of the four CCS.

From a larger perspective, this study provides another key example of the use of an integrated multi-criteria approach (Edwards & Knowles, 2014; Shanker et al., 2017; Zhang et al., 2013) to delimit species (Zachos, 2016; Cicero et al., 2021). This has helped uncover species diversity in one of the most iconic animals of the world – the king cobra. Integrated lineage delimitation exercises and the resulting units, whether named or unnamed, have bolstered increased lineage-specific conservation efforts, like in the crocodilian (*Paleosuchus palpebrosus)* of Brazil (Muniz et al., 2018). In this case, lineage delimitation also has a direct relevance to humans since the focal taxon is a medically important venomous snake. The presence of multiple species of king cobras is a bit surprising given the similarity in the morphology, habitat and behaviour of the species across its range. Similar cases include the recognition of the West African Forest Elephant (*Loxodonta cyclotis*) as a distinct lineage at species rank (Grub et al., 2000; Georgiadis et al., 2001), the new species of Kiwi (*Apteryx rowi*) in New Zealand (Tennyson et al., 2003), the cryptic Sunda clouded leopard (*Neofelis diardi*) in Indonesia (Kitchener et al., 2006), or the cryptic Tapanuli Orangutan (*Pongo tapanuliensis*) in northern Sumatra (Nater et al., 2017) and the recent case of Indus river dolphins *Platanista minor* (Barulik et al., 2021). In summary, our study provides the first multi-locus molecular phylogenetic study for the king cobra, with sampling throughout its global range, resulting in the delimitation of multiple lineages with important systematic, medical and conservation implications.

Highlights:

1. We performed an integrative species delimitation analysis of the iconic king cobra (*Ophiophagus hannah*) across its range in the Oriental realm
2. Mitochondrial and nuclear gene sequences and morphology support the recognition of four independently evolving lineages
3. The boundaries of four delimited lineages are broadly coincident the following biogeographic sub-regions: Western Ghats, Indo-Chinese, Indo-Malayan and Luzon island (Philippine archipelago)
4. The lineages at the western most limit (Western Ghats) and eastern most limit (Luzon) is characterized by narrow range and warrants a reassessment of their conservation status

Funding: This work was supported by ERSMUS MUNDUS:EMINTE Prog (2014-2016), Rufford Small Grants (Offer Letter: d96f84-1), IdeaWild, Kālinga Foundation, Kālinga Centre for Rainforest Ecology and the DBT- Indian Institute of Science (CES) Partnership Program.

Conflict of Interest

The authors declare that no conflict of interests exists.

Author Contributions

1. P. Gowri Shankar conceived and designed the experiments, raised funds, collected tissue samples, performed the experiments, analyzed the data, prepared figures and/or tables, authored, reviewed drafts of the paper, approved the final draft.

2. Priyanka Swamy contributed reagents/materials/analysis tools, conducted laboratory analysis, analyzed the data, authored and reviewed drafts of the paper, approved the final draft.

3. Rhiannon C. Williams contributed reagents/materials/analysis tools, conducted laboratory analysis, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.

4. S.R. Ganesh analyzed the morphological data, authored and reviewed drafts of the paper, approved the final draft.

5. Matt Moss conducted laboratory analysis.

6. Jacob Hoglund contributed reagents/materials/analysis tools, authored and reviewed drafts of the paper, approved the final draft.

7. Indraneil Das collected the morphological data, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.

8. Gunanidhi Sahoo contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

9. Seenapuram Palaniswamy Vijayakumar, analyzed the data, authored and reviewed drafts of the paper, approved the final draft.

10. Kartik Shanker raised fund, contributed reagents/materials/analysis tools, authored and reviewed drafts of the paper, approved the final draft.

11. Wolfgang Wüster performed the experiments, analyzed the data, authored and reviewed drafts of the paper, approved the final draft.

12. S.K. Dutta contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Acknowledgements

P. Gowri Shankar would like to thank Prashanth.P, Christian Künitzer Bloch, late Naveen Joseph, Colin Thomas Strine, Bartoz Nadolski, Tom Charlton, Bryan Grieg Fry, Jeevan Thapa, Jignasu Dolia, Dileep Kumar, Joy Mascarenhas, H T. Lalremsanga, Biplab Mahapatra, Bharat Bhatt, Prakash Mardaraj, Debajit Mahanta, Aaron Fernandes, Nicolas Vidal (Muséum national d'Histoire naturelle, France), Amruth Singh and Shinta Sukmawati for sample collection. Gunilla Engstrom, Aditi Jayrajan and Shree Varsha Vijay Kumar for helping with lab work. Sandesh Kadur, Sharmila Rajasegaran, P.Tattwavith, P. Vrushtivanya, J. Jagadeesh, Niladri B Kar and Shankar Subramanian for logistics and financial support. Yvonne Meyer-Lucht, Patrik Rodin Mörch, Deepak P, K.P. Dinesh, Praveen Karanth, Ashok Kumar Mallik, Varun Torsekar and R. Chaitanya helped during different stages of analysis and writing. S.R. Chandramouli, Nathan Rusli, Dr Ahmad Khaldun Ismail, Adam Francis are thanked for sharing information and photos, and Ashwini V. Mohan for support with analysis.

Indraneil Das would like to thank the curatorial staff of the following institutions for providing facilities, data on specimens and/or loans of specimens: AMNH (D. R. Frost and L. Ford), ANSP (T. Daeschler and N. Gilmore), BM (M. Wong and Hj. Bakariah), BMNH (E. N. Arnold and C. J. McCarthy), BNHM (the late N. Chaturvedi, the late J. C. Daniel and A. G. Sekar), CAS (the late J. B. Slowinski and J. B. Vindum), CIB (S. Li, C. Wang and the late E.-M. Zhao), CUZM (K. Thirathupt and P. P. van Dijk), FMNH (H. K. Voris, the late R. F. Inger, A. Resetar and C. Redhed), IMR (the late Lim Boo Liat and Rahmat Mat Sapran), LSU (D. A. Rossman and C. P. Kofron), MCBT (R. Whitaker and the late H. V. Andrews), MCZ (J. E. Cadle, the late E. E. Williams and J. P. Rosado), MNHN (P. David, A. Dubois and I. Ineich), MSPT (B. Vijayaraghavan and V. Kalaiarasan), NMP (R. Sisson), MZB (Ir. Boeadi and

M. Mumpuni), NKBP (S. K. Patnaik and S. Purohit), RMNH (M. S. Hoogmoed), SM (P. Kedit and C. Leh), NRM (E. Åhlander and S. O. Kullander), SSM (A. Wong), TNRC (the late J. Nabhitabhata), UF (D. Auth and F. W. King), UPM (the late P. Alveola and A. Diesmos), USDZ (P. K. Ng, K. K. P. Lim and C. M. Wang), USNM (W. R. Heyer, R. I. Crombie and G. R. Zug), ZMA (L. van Tuijl), ZSI (J. R. B. Alfred, S. K. Chanda, B. Dattagupta, K. Deuti, N. C Gayen, C. Murthy and D. P. Sanyal) and ZSIP (K. Chandra and P. V. Rajan).

For comments and locality information, he thanks K. Adler, D. Basu, A. M. Bauer, R. I. Crombie, P. David, C. P. Kofron, P. P. van Dijk, V. Wallach and R. Whitaker. ID was supported by Universiti Malaysia Sarawak, Universiti Brunei Darussalam, a Fulbright Fellowship at the Museum of Comparative Zoology, a Raffles Museum Fellowship at the National University of Singapore, British Council, the Centre for Herpetology, and the Smithsonian Institution.

SRG thanks the Chairman and Trustees of the Chennai Snake Park Trust for encouraging his research activities. WW, MM and RCW thank the following for help with various aspects of this study: Ashok Captain, Tom Charlton, Jennifer C. Daltry, Wendy Grail, Trinh Xuan Kiem, Gert-Jan Klootwijk, Mario Lutz, Paul D. Rowley, Kate L. Sanders, Sterrin Smalbrugge, R. David G. Theakston, Roger S. Thorpe, Romilly van den Bergh, Romulus Whitaker, Christopher Wild, the late Luke Yeomans, Matthew Yuyek, and Thomas Ziegler. KS thanks the DBT-IISC Partnership Programme for funding support.

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Table 1. Summary of mitochondrial and nuclear DNA seq alignment.

Loci	No. of Samples	Base pairs	Variable sites	Parsimony informative sites
16S	54	423	15	12
ND4	51	596	69	54
Cytb	32	714	94	80
PRLR	28	541	10	7
NT3	32	590	13	5
RAG1	36	883	7	5

Table 2. Candidate species and their posterior probabilities in BPP v3 for 4 OTUs.

	θprior = 22000 τprior = 22000 Small ancestral population, shallow divergences	θprior = 110 τprior = 110 Large ancestral population, deep divergences.	θprior = 110 τprior = 22000 Large ancestral population, shallow divergences.	θprior = 22000 τprior = 110 Small ancestral population, deep divergences.
4 OTUs				
CS1	1	1	1	1
CS2	1	1	1	1
CS3	1	1	1	1
CS4	1	1	1	1
BPP for number of species				
P[1] =	0	0	0	0
P[2] =	0	0	0	0
P[3] =	0	0	0	0
P[4] =	1	1	1	1

Table 3. Distinguishing datasets and analyses supporting species status for the four mitochondrially defined candidate species considered in this study. Above the diagonal: evidence from mitochondrial and nuclear loci; below the diagonal: evidence from morphology, nuclear loci haplotype network and snapclust. GD-Genetic distance, BPP-Bayesian Phylogenetics and Phylogeography, POFAD-Phylogeny of Organisms from Allelic Data, HPS-Haplotype sharing, NoHPS-No Haplotype sharing.

Candidate Species (CS)	CS1	CS2	CS3	CS4
CS1	-	GD BPP POFAD	GD BPP POFAD	GD BPP POFAD
CS2	HPS (NT3, PRLR and Rag1) Phenotypically distinct Snapclust	-	GD BPP POFAD	GD BPP POFAD
CS3	NoHPS Phenotypically distinct Snapclust	HPS(NT3 and Rag1) Phenotypically distinct Snapclust	-	GD BPP POFAD
CS4	NoHPS Phenotypically distinct Snapclust	NoHPS Phenotypically distinct Snapclust	NoHPS Phenotypically distinct Snapclust	-

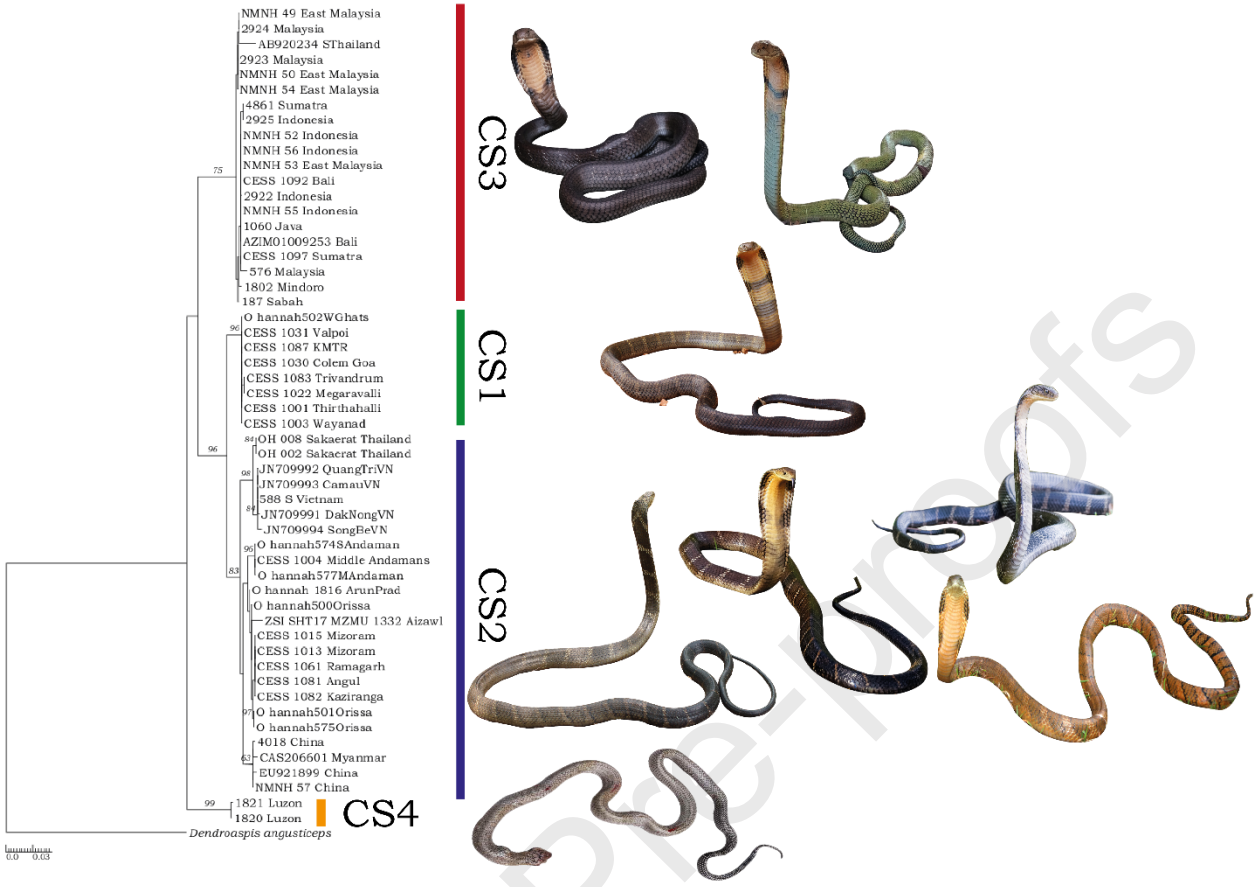


Fig 1. Maximum Likelihood (ML) mitochondrial phylogeny of the *Ophiophagus hannah* complex using 16S, Cytb and ND4 sequences. Branch support values indicate % bootstrap support; support values for the most distal branches are not shown.

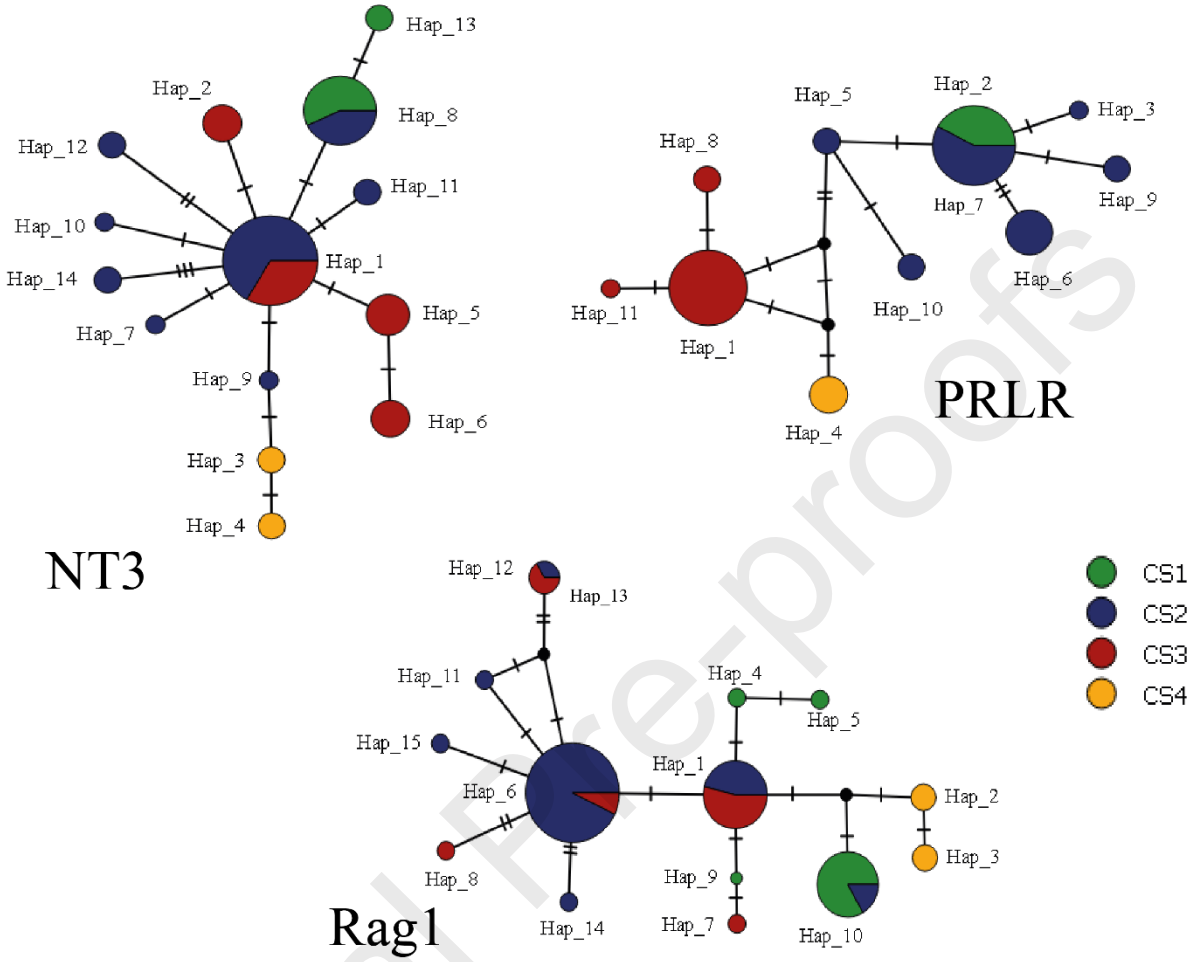


Fig. 2. Median-joining haplotype network constructed in PopART. The relative size of the circles is proportional to the number of sequences of that same haplotype. Different colours represent geographic distribution. A black circle is an inferred median, each line between haplotypes represents a single mutational step between haplotypes.

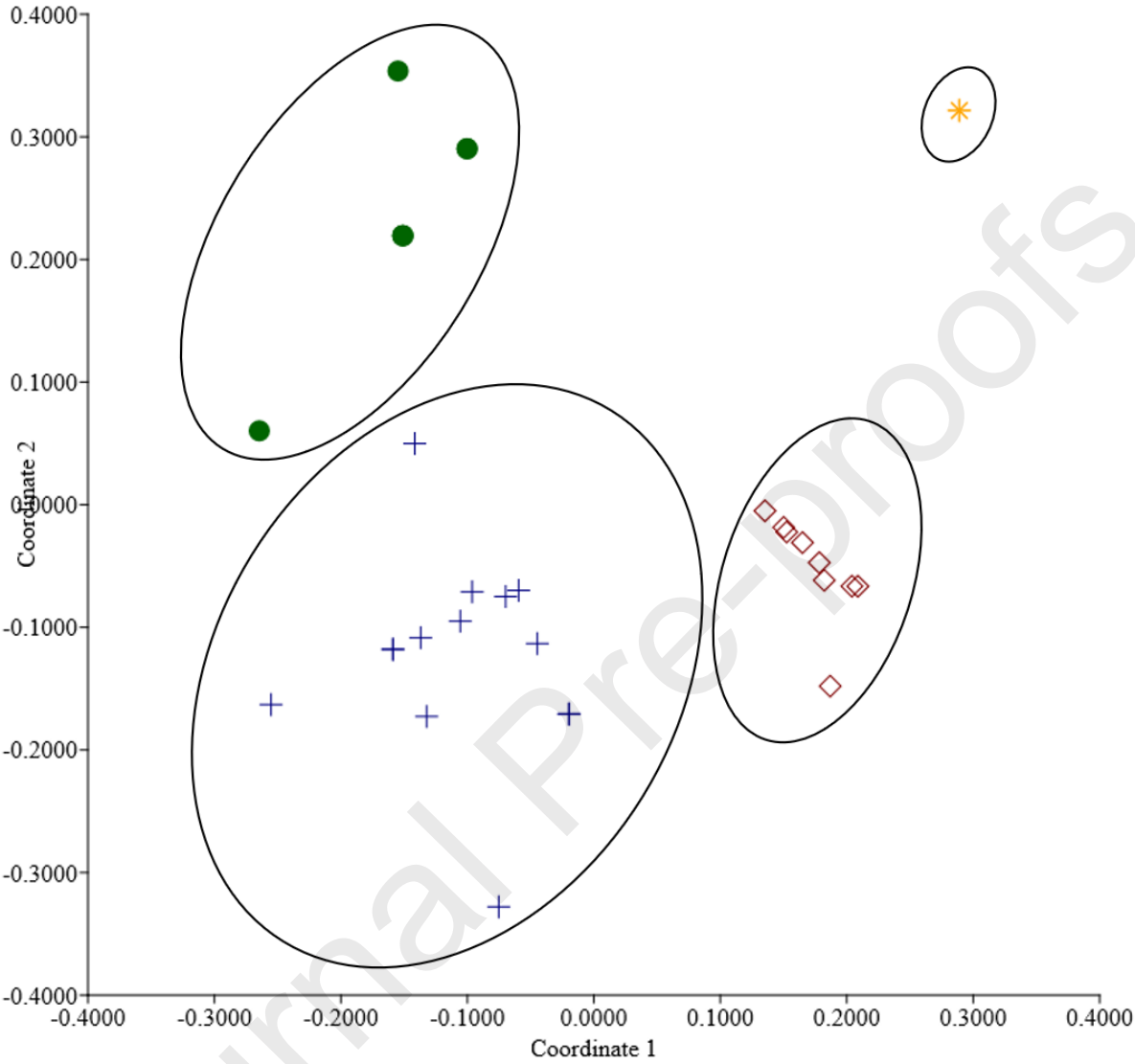


Fig 3. Ordination of individual specimens in a Principal Coordinates Analysis of standardized multilocus distances of NT3, PRLR and Rag1 sequence data. Symbols in the key indicate mtDNA lineages. Closed circle= Western Ghats (CS1), Plus= Eastern Ghats, Northeast India, Andaman Island, Vietnam and China (CS2), Open diamond = Indonesia and Malaysia (CS3) and Star= Luzon (CS4).

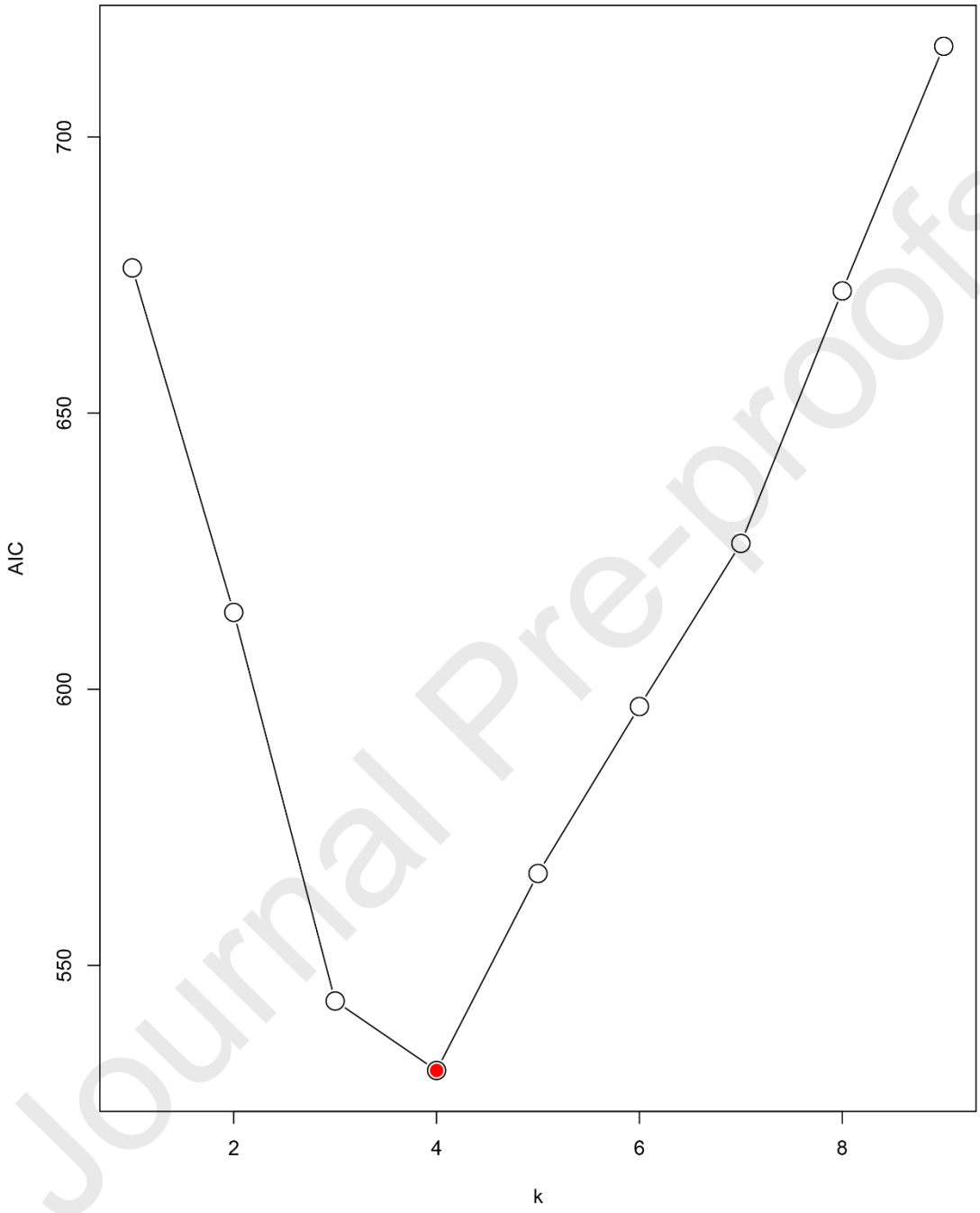


Fig.4. Optimal number of clusters identified using snapclust.choose.k. Note K=4 has the lowest AIC value.

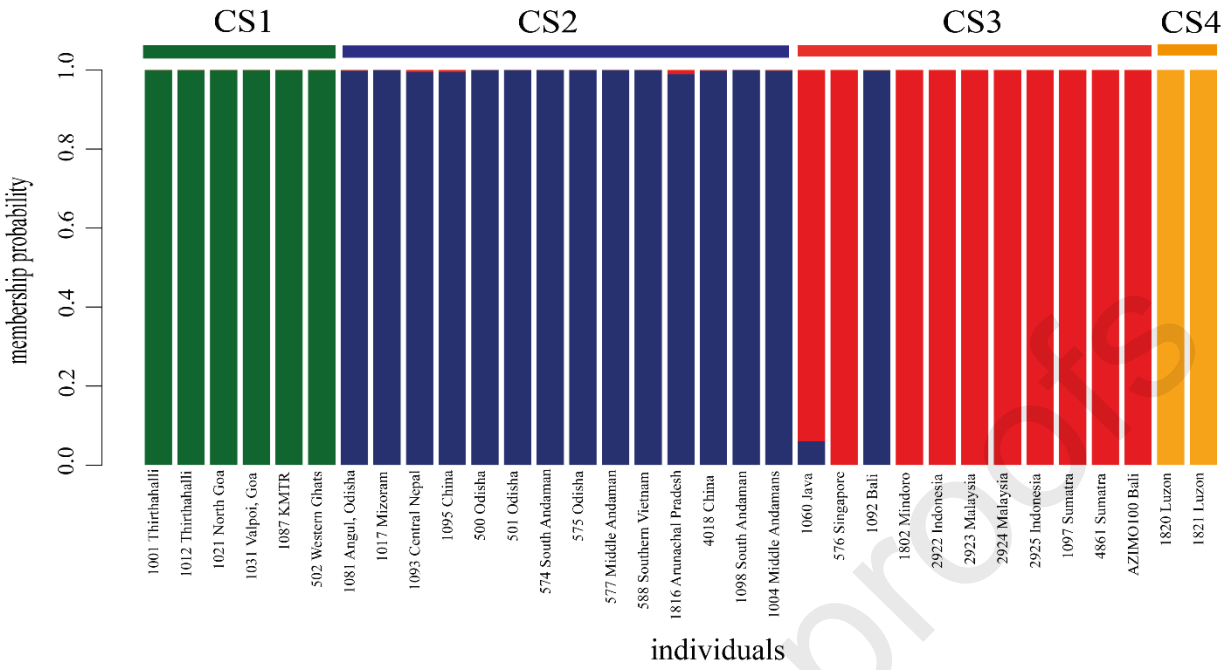


Fig 5. Assignment of individuals to the four clusters identified in snapclust. Sample 1092 showed poor assignment probabilities to the cluster. See also the map (Fig 7)

a)

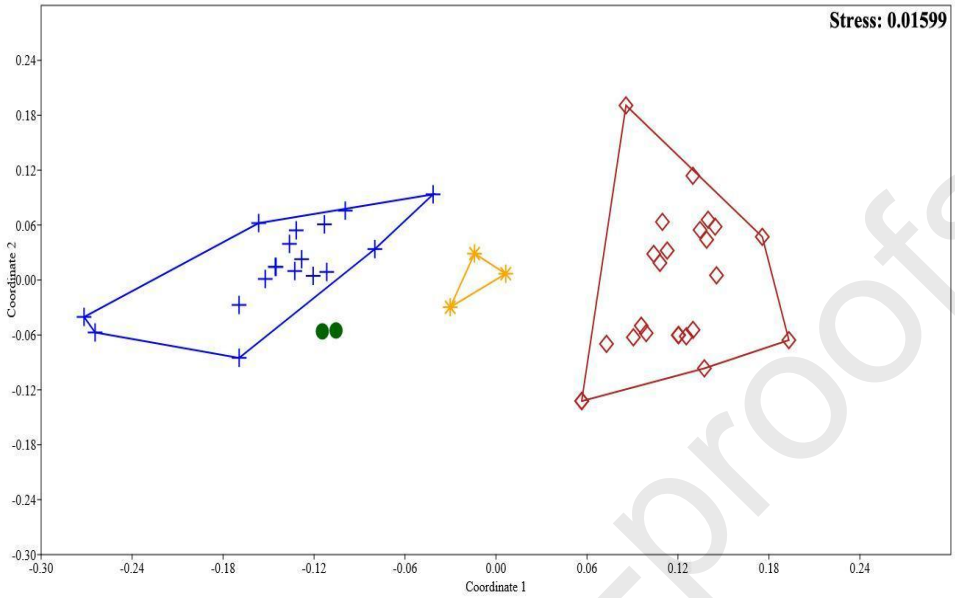


Fig 6. 2D Non-metric multidimensional scaling (nMDS) scatter plots relating to morphology for both male and female specimens of the four lineages of *Ophiophagus*. Closed circle= Western Ghats (CS1), Plus= Eastern Ghats, Northeast India, Andaman Island, Vietnam and China (CS2), Open diamond = Indonesia and Malaysia (CS3) and Star= Luzon (CS4).

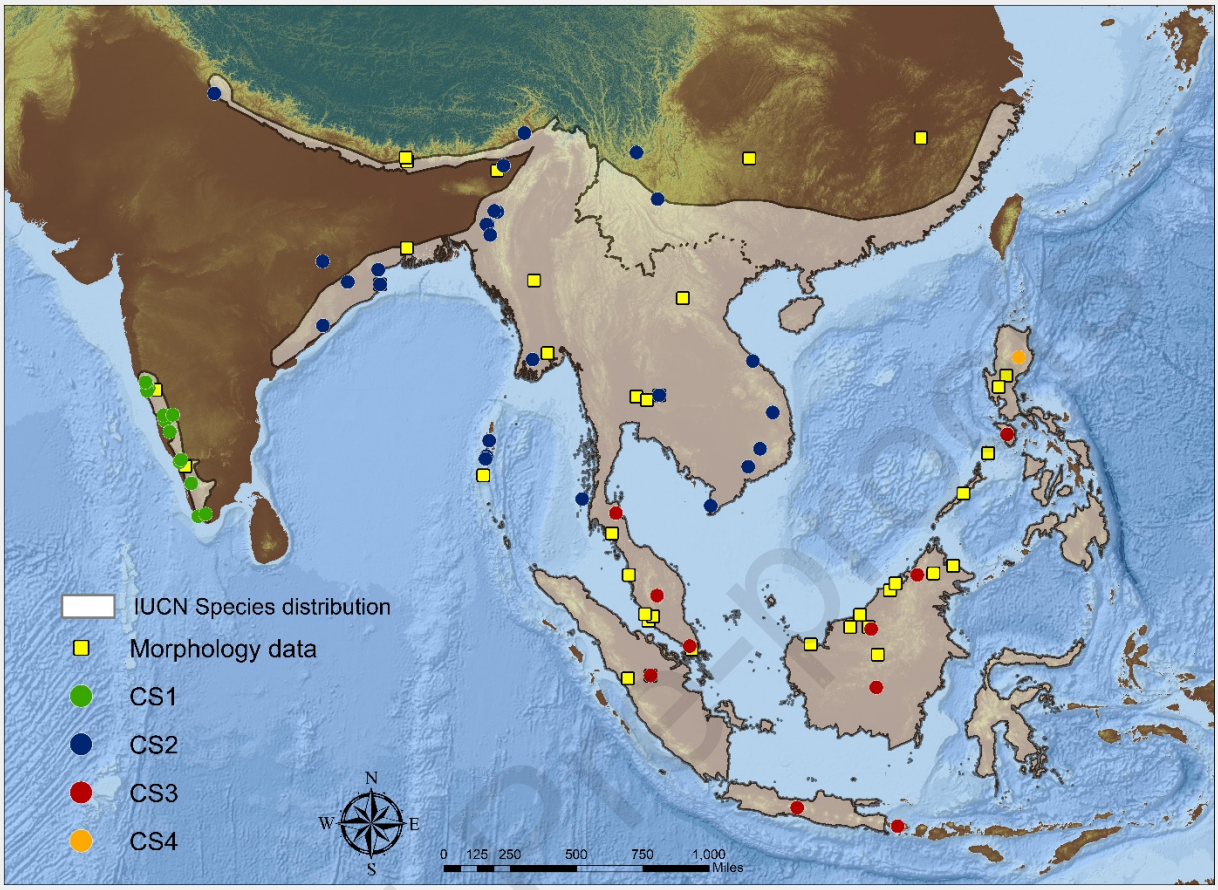


Fig.7. Distribution of the four candidate species across South and Southeast Asia.

Appendix I. Sample sizes and specimens' numbers for king cobras examined from different localities in part of the present study for molecular analysis along with Accession numbers.

Sl.No	Seq Code	Location	Lat	Long	16s	Cytb	ND4	NT3	PRLR	Rag1
1	CESS 1001	Guddekare, Thirthahalli,Karnataka	13.689	75.245	MZ087825	MZ343879	MZ343816	MZ753561	MZ753574	MZ753602
2	CESS 1003	Pookode, Wayanad, Kerala	11.541	76.022	MZ087829		MZ343848			
3	CESS 1004	Middle Andamans	11.740	92.659	MZ087840		MZ343849	MZ753562		MZ753603
4	CESS 1012	Kaimara, Thirthahalli, Karnataka	13.689	75.245				MZ753563	MZ753575	
5	CESS 1013	MZU campus, Mizoram	23.735	92.667	MZ087842	MZ343880	MZ343850	MZ753564		
6	CESS 1015	Nghalchawn, Mizoram	24.368	93.269	MZ087844	MZ343881	MZ343851			
7	CESS 1017	Darlung, Mizoram	23.458	92.599	MZ087855			MZ753565	MZ753576	MZ753604
8	CESS 1021	Naneli, North Goa	15.582	74.148				MZ753566	MZ753577	MZ753605
9	CESS 1022	Bavikere, Megaravalli, Karnataka	13.622	75.152	MZ087837	MZ343882				
10	CESS 1030	Devachowada, Panshi,Colem, Goa	15.337	74.247	MZ087834	MZ343883	MZ343854			
11	CESS 1031	Copardem, Valpoi, Goa	15.530	74.130	MZ087838	MZ343884	MZ343855		MZ753578	MZ753606
12	CESS 1033	Virdi, Dodamarg, Sindhudurga, Maharastra	15.639	74.072	MZ087830			MZ753567		MZ753607
13	CESS 1051	Yedur, Karnataka	13.689	75.245				MZ753568		
14	CESS 1061	Ramagarh, Uttarakhand	30.101	77.831	MZ087847	MZ343885	MZ343829			MZ753608
15	CESS 1081	Angul, Odisha	20.841	85.102	MZ087850		MZ343834	MZ753569		MZ753609

16	CESS 1082	Kaziranga, Assam	26.641	93.600	MZ087854					
17	CESS 1083	Trivandrum, Kerala	8.506	76.957	MZ087831	MZ343886	MZ343835	MZ753570		
18	CESS 1086	Nilgiri, Balasore, Odisha	21.462	86.768	MZ087851					MZ753610
19	CESS 1087	KMTR,Thirunelveli,Tamilnadu	8.627	77.356	MZ087833		MZ343837	MZ753571		MZ753611
20	CESS 1092	Bali	-8.330	115.091	MZ087864				MZ753579	MZ753612
21	CESS 1093	Central Nepal	27.411	85.168					MZ753580	MZ753613
22	CESS 1095	China	27.282	100.850					MZ753581	MZ753614
23	CESS 1097	Sumatra, Greater Sunda	-0.144	101.625	MZ087871			MZ753572	MZ753582	MZ753616
24	CESS 1098	South Andaman (Hut Bay)	10.633	92.600				MZ753573	MZ753583	MZ753615
25	O_hannah502	Western Ghats	13.930	75.568	MZ087836	MZ343874	MZ343844	MZ753555	MZ753589	MZ753598
26	500	Bitar Kanika, Odisha	21.884	83.739	MZ087843	MZ343872	MZ343839	MZ753553	MZ753587	MZ753596
27	501	Bitar Kanika, Odisha	21.884	83.739	MZ087852	MZ343873	MZ343840	MZ753554	MZ753588	MZ753597
28	574	Wandoor, South Andaman	11.594	92.620		MZ343875	MZ343842	MZ753556	MZ753590	MZ753599
29	575	Bitar Kanika, Odisha	20.715	86.866	MZ087853	MZ343876	MZ343838	MZ753557	MZ753591	MZ753600
30	577	Karawang, Middle Andaman	12.561	92.818	MZ087841	MZ343877	MZ343843	MZ753559	MZ753592	MZ753601
31	588	Southern Vietnam	14.058	108.277	MZ087863	MT346766	MT346939	MZ753560		MT347427
32	1816	Arunachal Pradesh	28.218	94.728	MZ087856	MZ087856	MZ343841	MZ753550	MZ753584	
33	4018	China	35.862	104.195		MT346773	MT346946	MT347190	MT347190	MZ753595
34	576	Singapore Zoo	1.457	103.753	MZ087872	MT346765	MT346938	MZ753558		MT347426
35	1060	Java	-7.328	109.614	MZ087874	MT346767	MT346940	MT347184	MT347184	MT347428

36	1802	Puerto Galera, Mindoro, Philippines (Occidental Mindoro)	12.894	121.064	MZ087875	MT346768	MT346941	MT347185	MT347185	MT347429
37	2922	Indonesia	-0.789	113.921	MZ087873	MT346769	MT346942	MT347186	MT347186	MT347430
38	2923	Malaysia	4.210	101.976	MZ087876	MT346770	MT346943	MT347187	MT347187	MT347431
39	2924	Malaysia	4.210	101.976	MZ087877	MT346771	MT346944	MT347188	MT347188	MT347432
40	2925	Indonesia	-0.789	113.921		MT346772	MT346945	MT347189	MT347189	MT347433
41	187	Sabha	5.337	116.157		MT346764	MT346937			
42	1820	Montalban, Rizal, Luzon, Philippines	16.952	121.694		MZ343862	MZ343827	MZ753551	MZ753585	MZ753593
43	1821	Montalban, Rizal, Luzon, Philippines	16.952	121.694	MZ087878	MZ343863	MZ343828	MZ753552	MZ753586	MZ753594
44	4861	Sumatra, Greater Sunda	-0.144	101.625				MT347191	MT347191	MT347434
45	NMNH_57	China	27.282	100.850	MZ087857		MZ343832			
46	NMNH_50	Malaysia (Sarawak)	2.393	113.647	MZ087866		MZ343810			
47	NMNH_54	Malaysia (Sarawak)	2.393	113.647	MZ087867		MZ343813			
48	NMNH_49	Malaysia	4.210	101.976	MZ087865		MZ343809			
49	NMNH_52	Indonesia	-0.789	113.921			MZ343811			
50	NMNH_53	Malaysia	4.210	101.976			MZ343812			
51	NMNH_55	Indonesia	-0.789	113.921	MZ087868		MZ343814			
52	NMNH_56	Indonesia	-0.789	113.921	MZ087869		MZ343815			
53	OPHA008	Sakaerat Biospheres Reserves, Thailand	14.971	102.087	MZ087861		MZ343859			MZ753618
54	OPHA002	Sakaerat Biospheres Reserves, Thailand	14.971	102.087	MZ087862		MZ343858			MZ753617
55	OPHA035	Koh Phang, Thailand	9.439	97.886						
56	AB920234	S Thailand	8.677	99.731		AB920234	AB920234			

57	AZIM010092 53	Bali	-8.330	115.091	AZIM010092 53	AZIM010092 53	AZIM01009 253	AZIM01009 253	AZIM01009 253	AZIM01009 253
58	CAS206601	Ayeyarwady, Myanmar	16.834	95.180		CAS206601	CAS206601			
59	EU921899	China	25.000	102.000	EU921899	EU921899	EU921899			
60	JN709991	DakNong, Vietman	12.129	107.587		JN709991				
61	JN709992	Quang Tri, Vietman	16.755	107.190		JN709992				
62	JN709993	Cà Mau,Vietman	9.074	104.900		JN709993				
63	JN709994	Song Be,Vietman	11.170	106.941		JN709994				
64	ZSI SHT17 MZMU 1332	Aizawl, Mizoram	23.744	92.738		ZSI SHT17 MZMU 1332				

CESS = Centre for Ecological Sciences Snakes
MNHN = Muséum national d'histoire naturelle, Paris
OPHA = Ophiophagus (Thailand samples)

Appendix IIa . Markers used in the study and their sources

Marker	Primer	Primer sequence	Reference
16S	16sar	5'-CGC CTG TTT ATC AAA AAC AT-3'	Palumbi,1996
	16sbr	5'-CTC CGG TTT GAA CTC AGA TCA-3'	Simon, 1991
ND4	ND4	5'-CACCTATGACTACCAAAAGCTCATGTAGAAGC-3'	Arévalo et al., 1994
	LEU	5'-CATTACTTTTACTTGGATTTGCACCA-3'	
cytb	Gludg	5'- TGACTTGAARAACCAYCGTTG-3'	Palumbi,1996 Burbrink *et al.* 2000
	H16064 or ATRCB3	5'- CTTTGGTTTACAAGAACAATGCTTTA-3' 5'- GAGAAGTTTTCYGGGTCRTT-3'	Harvey et al. 2000
NT3	NTF3_F1	5'-ATGTCCAATCTTGTTTTATGTGATATTT-3'	Townsend, 2008
	NTF3_R1	5'-ACRAGTTTRTTGTTYTCTGAAGTC-3'	
PRLR	PRLR F1	5'-GACARYGARGACCAGCAACTRATGCC-3'	Townsend, 2008
	PRLR r3	5'-GACYTTGTGRACTTCYACRTAATCCAT-3'	
RAG-1	AV_RAG1F	5'-AAATGTGACAGGGTCTCT-3'	Anthony von Plettenberg Laing, pers. comm
	AV_RAGR	5'-GGGCATCTCAAAACCAAATTGT-3'	

Appendix IIb. PCR conditions for nuclear and mitochondrial loci used in the study. Temperature (°C) and cycle length (min) for each step of PCR amplification. Conditions modified from Townsend *et al*., 2008; Barlow *et al.,* 2009; Maddock *et al.,* 2017 and von Plettenberg Laing *pers. comm,* 2017.

	16s	*cytb*	*nd4*	*nt3*	*prlr*	*rag1*
1 - Initial denature	94 2:00	94 2:00	94 2:00	94 2:00	94 2:00	94 2:00
2 - Denature	94 0:30	94 0:30	94 0:30	94 0:30	94 0:30	94 0:30
3 - Anneal	42 0:30	47 0:30	57 0:30	42 0:30	48 0:30	59 0:30
4 -Extension	72 0:45	72 1:00	72 1:00	72 0:45	72 0:45	72 1:00
Repeat steps 2-4	X35	X35-40	X39	X39	X39	X44
5 - Final Extension	72 5:00	72 5:00	72 5:00	72 5:00	72 5:00	72 5:00
6 - Cooling	4 15:00	4 15:00	4 15:00	4 15:00	4 15:00	4 15:00

Appendix III

Uncorrected genetic p-distance between Candidate Species

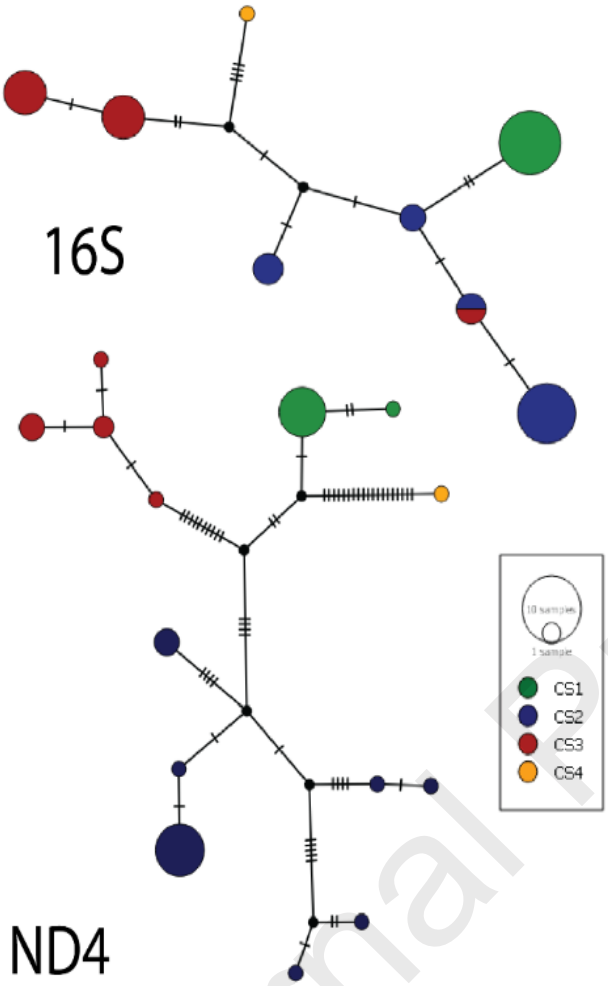
16S gene				
Candidate species	CS1	CS2	CS3	CS4
CS1	-	0.010	0.018	0.017
CS2		-	0.017	0.021
CS3			-	0.016
CS4				-

ND4 gene				
Candidate species	CS1	CS2	CS3	CS4
CS1	-	0.020	0.031	0.046
CS2		-	0.036	0.054
CS3			-	0.047
CS4				-

Cyt b gene				
Candidate species	CS1	CS2	CS3	CS4
CS1	-	0.034	0.047	0.069
CS2		-	0.056	0.077
CS3			-	0.082
CS4				-

Appendix IV

Haplotype network of Mitochondrial loci 16S and ND4



Median-joining haplotype network constructed in PopART. The relative size of the circles is proportional to the number of sequences of that same haplotype.
Different colours represent geographic distribution. A black circle is an inferred median, each line between haplotypes represents a single mutational step between haplotypes.

Appendix V. Morphological data from fluid-preserved adult specimens used for nMDS analysis

Sl no.	MUSEUM NO	Region	OTU	SVL (mm)	No. Of bands	AD	PD	SC	V	SL	IL
1	ZSIC UNREG. (USNM FIELD NO. 152882)	Western Ghats	CS1	2660	28	19	15	86	251	8	8
2	ZSIC UNREG. (USNM FIELD NO. 152884)	Western Ghats		2065	-	21	15	87	251	8	8
3	USNM 129748	Andaman		1635	36	17	15	72	243	7	8
4	ZSIP 366	Andaman		1922.8	41	19	15	87	244	7	8
5	ZSI 8292	Northeast India		2085	-	17	15	91	240	7	8
6	MCBT 152886	Northeast India		2885	41	17	15	72	245	7	8
7	BMNH 1940.6.5.63	Northeast India		2153	-	17	15	88	243	7	8
8	CAS 214017	Northeast India		2051	35	19	15	82	246	7	8
9	AMNH 29944	China		2080	41	17	13	85	244	7	8
10	CIB UNCAT.	China		2050	41	19	15	87	238	7	8
11	CIB 21-0002	China		1646	39	19	15	86	243	7	8
12	BNHM 2274	Assam, India		202.8	41	19	15	89	245	7	8
13	BNHM 2276	Tindharia, Darjeeling		121.8	37	19	15	88	245	7	7
14	ZSI 8294	Darjeeling, West Bengal		165.7	43	17	15	88	241	7	8
15	MNHN 5205 -1876.131	Laos		3276	-	17	13	99	240	7	8
16	MCZ 44699	Mt. Popa, Chin Hills, Burma		1620	35	17	15	93	244	7	8
17	CIB 21-00-02	Xhiu County, Guizhou Prov., China		164.6	39	19	15	86	243	7	8
18	BMNH 78.2.42	Siam	CS2	212		19	15	93	239	7	8
19	TNRC 1121	Thailand		191.3	0	19	11	80	251	7	8
20	USNM 72726	Pak Chong Nakhon Ratchasima, Thailand		214.5	-	21	15	89	240	7	8

21	USNM 23014	Trang Prov, Thailand		3200	-	23	15	114	264	7	8
22	UF 65488	Thailand		2481	0	19	15	114	252	7	8
23	FMNH 63566	Greater Sunda		2615	-	22	15	117	253	7	8
24	FMNH 128275	Greater Sunda		2402	0	19	15	109	261	7	8
25	ZMA 13487	Greater Sunda		2871	0	19	15	115	250	7	8
26	BM 98/6	Greater Sunda		2432	0	19	15	116	245	7	8
27	IMR 110487- 5063	Peninsular Malaysia		2980	0	17	15	114	251	7	8
28	MNHN 1899.172	Peninsular Malaysia		2097	0	17	13	108	265	7	8
29	ZSIC UNREG. (USNM 152883)	Peninsular Malaysia		2025	0	21	15	99	265	7	8
30	CAS 157468	Palawan, Philippines		1760	0	19	15	110	252	7	8
31	FMNH 53553	San Pedra, Culion, Philippines		2565	-	17	15	112	249	7	8
32	CAS 16785	Singapore, Straits Settlements		125.2	-	19	15	115	236	7	8
33	ZSIC UNREG. (FIELD NO. 152883)	Peninsular Malaysia		202.5	0	21	15	99	265	7	8
34	IMR uncataloged	Selangor, Malaysia		199.1	0	17	13	115	251	7	8
35	BMNH 1928.2.18.43	Fort-De-Kok, West Coast of Sumatra, 920 M		42.4	-	19	15	106	260	7	8
36	USDZ 2.3212	Sumatra		207	0	19	13	105	260	7	8
37	USNM 35763	Tarussan Bay, Sumatra	CS3	196.5	0	19	15	113	256	7	8
38	BM 84/80	Kota Batu, Bsb, Brunei		40.6	74	19	13	110	253	7	7
39	SM 5.45.25	Sarawak Museum,		152.5	0	19	13	108	261	7	8

		Grounds, Malaysia									
40	SM 5.45K.2K	Kuching, Sarawak, Malaysia		186	0	19	13	108	261	7	8
41	BM 224/91	Kg. Pinto Malim, Brunei		175.6	0	19	13	106	259	7	8
42	FMNH 150891	Labang Camp on Sungei Seran, Bintulu District, Sarawak, Malaysia		182.1	0	15	15	108	261	7	8
43	FMNH 71659	Baram,Borneo		208.5	0	19	15	103	260	7	8
44	SSM 0096	Sabah, Malaysia		173.5	0	19	15	111	252	7	8
45	UF 55008	Luzon	CS4	1943	0	19	15	99	247	7	8
46	CAS 61329	Luzon		2060	-	19	15	95	252	7	8
47	UPMNH 1692	Luzon		2056	0	19	15	100	250	7	9

AD-anterior dorsal scale rows (counted one head length behind head), (ii) PD-posterior dorsal rows (counted one head length before vent), (iii) V-ventral count, (iv) SC- subcaudal count, (v) SL-supralabial count, (vi) IL- infralabial count and (vii)SVL-snout vent length

Manuscript title: King or royal family? Testing for species boundaries in the King Cobra,

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Manuscript Number: MPE-D-20-00304

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I, P. Gowri Shankar the corresponding author of this manuscript certify that the contributors' and conflicts of interest statement included in this paper are correct and have been approved by all co-authors.

Regards

Highlights:

1. We performed an integrative species delimitation analysis of the iconic king cobra (Ophiophagus hannah) across its range in the Oriental realm
2. Mitochondrial and nuclear gene sequences and morphology support the recognition of four independently evolving lineages
3. The boundaries of four delimited lineages are broadly coincident the following biogeographic sub-regions: Western Ghats, Indo-Chinese, Indo-Malayan and Luzon island (Philippine archipelago)

The lineages at the western most limit (Western Ghats) and eastern most limit (Luzon) is characterized by narrow range and warrants a reassessment of their conservation status