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Geographic differentiation and cryptic diversity in the monocled cobra, Naja kaouthia (Elapidae) from Thailand

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Geographic differentiation and cryptic diversity in the monocled cobra, *Naja kaouthia* (Elapidae) from Thailand

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Abstract: South-east Asia has an exceptionally high diversity of snakes, with more than 250 snake species currently recorded from Thailand. This diversity likely reflects the diverse range of geographical and climatic conditions under which they live, but the evolutionary history and population genetics of many snake species in South-east Asia has been little investigated in comparison with morphological studies. Here, we investigated genetic variation in the monocled cobra, *Naja kaouthia*, Lesson, 1831, across its distribution range in Thailand using mitochondrial-DNA (Cytochrome b, control region) for ~100 individuals, and the nuclear DNA gene (C-mos) for a small subset. Using population genetic and phylogenetic methods, we show high levels of genetic variation between regional populations of this non-spitting cobra, including the north-eastern, north-central, and southern regions, in addition to a population on Pha-ngan Island, 150 km offshore from the southern peninsula. Moreover, inclusion of the north-eastern population renders *N. kaouthia* paraphyletic in relation to other regional *Naja* species. The north-eastern population is therefore probably specifically distinct. Given that these cobras are otherwise undifferentiated based on colour and general appearance to the 'typical' cobra type of this region; they would represent a cryptic species. As has been shown in other animal groups from Thailand, it is likely that the geographic characteristics and/or tectonic alteration of these regions have facilitated high levels of population divergence of *N. kaouthia* in this region. Our study highlights the need for dense sampling of snake populations to reveal their systematics, plan conservation, and facilitate anti-snake venom development.

Keywords: Monocled cobra, snakes, molecular phylogenetics, phylogeography, cryptic species

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Introduction

South-east Asia has an exceptionally high diversity of snakes (Jitakune, 2004), with more than 250 snake species currently recorded from Thailand (Cox *et al.*, 2013). This diversity likely reflects the diverse range of geographical and climatic conditions under which they live (Mott, 2010), but the evolutionary history and population genetics of many snake species in South-east Asia has been little investigated in comparison with morphology-based studies. For example, phenotypic polymorphisms at the population level have been found in snakes living between different geographical regions of South-east Asia e.g. Asiatic cobra species in the genus *Naja* (Wüster *et al.*, 1995), the monocled cobra, *Naja kaouthia*, the King cobra, *Ophiophagus hannah* (Jitakune, 2004), and Kruki snakes, *Oligodon* (Cox *et al.*, 2013), but their genetic variation is generally lacking. Such morphology-based studies have formed the basis for understanding biodiversity, but where species occur over a wide range, with distinct populations, cryptic species may be missed.

The monocled cobra (*Naja kaouthia*) is reportedly the only species of non-spitting cobra that occurs throughout South-east Asia and some parts of China and India (Uetz and Hošek, 2014), in which the type locality is listed as "Bengale" (Lesson 1932: 314), India. Due to the large distribution area of *N. kaouthia*, variation in adult characteristics and morphological divergence in populations have repeatedly been found (e.g. Wüster *et al.*, 1995; Wallach *et al.*, 2014). The adult variation may reflect environmental (e.g. tropical savanna, rain forest) and geophysical differences resulting from mountain, river, and land-ocean partitions. In Thailand, *Naja kaouthia* shows high levels of variation among regional populations in features such as colouration (Cox, 1991; Wüster *et al.*, 1995; Jitakune and Chanhome, 1996; Jitakune, 2004) and toxin components (e.g. Tan *et al.*, 2017). Field observations also supported that the colour and pattern of *N. kaouthia* (north: black; central: black and brown; south: olive), and timing of their reproductive period (north-central: Oct/Nov - Mar/Apr and south: Apr – Sep) vary

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among regions in Thailand. However, these differences have not been investigated from a genetic perspective to test if phenotypic variation is reflected by genetic variation.

The relationship between environmental conditions and population genetic diversification is well documented in *Naja atra*, the closest relative of *N. kaouthia*. The genetic study of Lin *et al.* (2014) revealed three distinct populations of *N. atra* across East Asia (*n* = 285) and suggested that lineage diversification was caused by the development of the Nanling and Luoxiao mountain ranges (the ranges run from west to east in Southern China, and continue north into Central China). Wüster and colleagues (1989, 1992, 1995) examined the systematics of the genus *Naja* in Asia, and variation of *N. kaouthia* across its range, and these studies have formed the basis of subsequent discussions on the evolution of cobras in South-east Asia. Analysis of *N. kaouthia* based on meristic and linear measurements found no differentiation (Wüster *et al.*, 1995), while 'typical' and Suphan (white) phase cobras were found to be genetically identical, although only four samples of *N. kaouthia* were included. Other investigations of genetic differentiation in snake taxa have generally yielded inconclusive results such as *Elapidae* in Asia, Africa, and America, (Slowinski and Keogh, 2000), sequences of Caenophidian snakes from GenBank (Kelly *et al.*, 2003; Vidal *et al.*, 2007), sequences of Colubroidea snakes from GenBank (Lawson *et al.*, 2005; Yan *et al.*, 2008), and many snake species in Thailand (Laopichienpong *et al*., 2016).

As shown for *N. atra* (Lin *et al.*, 2014), our prediction was that geographically separated regions (e.g. Woodruff, 2003; Hughes *et al.*, 2003) and temporal geological changes had the potential to facilitate divergence of *N. kaouthia* in Thailand (e.g. Buddhachat and Suwannapoom, 2018). The northern region is a mountainous area covered by many high, parallel mountain ranges that extend from the north, alternating with many deep valleys and steep rivers. It is also drained by rivers that unite in the lowlands of the central region. Further south, the Himalayan-Tanoasri mountain range stretches from the central region into the southern peninsula region. Similarly, the north to south Phetchabun, Dong Phaya

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Yen, and San Kamphaeng mountain range forms a potential barrier between the central region and the high plateau of the north-eastern region. Major tectonic organizations in Thailand > 60 Million years ago (Mya) (Department of Mineral Resources, Thailand, 2007) occurred before the arrival of ancestral cobras into Asia (11-16 Mya) (Slowinski and Keogh, 2000; Wüster *et al.*, 2007) but the rise in sea level after the Late Pleistocene (8,000-6,000 years ago) (Sinsakul, 1992; Voris, 2000) may have restricted the distribution ranges of the cobra population.

Although high levels of morphological variation have been reported among populations of *N. kaouthia* (e.g. Jitakune, 2004), there remains the question as to whether this variation reflects genetic diversification (Ursenbacher *et al.*, 2008) or local ecological/environmental adaptations resulting from phenotypic plasticity (Forsman, 2015; Lin *et al.*, 2008). Given the limited data on the interrelationships of *N. kaouthia*, this study investigated genetic differentiation among populations across 12 localities (provinces) and four regions, with collecting sites covering different environments in Thailand. Mitochondrial DNA (mtDNA) for Cytochrome b (Cyt *b*) and the control region (CR) was generated for over 100 individuals and analysed using phylogenetic inference, and population genetics. As mtDNA trees are not necessarily the same as species trees (e.g. Taggart *et al*. 2001), we also generated nuclear DNA (C-mos) data for a subset of these samples to validate results from the mitochondrial genome.

Our findings demonstrated a high degree of population differentiation among the sampled *N. kaouthia* populations from Thailand. These populations are genetically divergent among regions, and revealed that the north-eastern population should be elevated to a separate species to render *N. kaouthia* monophyletic in relation to other cobras. Moreover, these data can help to explain the eco-evolutionary history of these lineages across Thailand.

Material and methods

Taxon sampling

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Samples from scales and shed skin were collected from 102 wild caught *Naja kaouthia*. The collecting sites were selected across the range of *N. kaouthia* within Thailand, and designed to cover all regions including north, south, central, and north-east. Within each region there are multiple provinces from which the snakes were sampled (Fig. 1, see also supporting information, Table S1 for precise sampled locality). However, within the north-eastern region, it was only possible to sample *N. kaouthia* in one of the provinces (Nakhon Ratchasima: NR). For outgroup comparison, two samples of *N. siamensis* were also collected, based on its reported close relationship with *N. kaouthia* (Slowinski and Wüster, 2000), together with a single sequence each of *N. atra* and *N. naja* from Genbank (see supporting information, Table S2).

All snakes collected from the wild were handled by experienced regional collectors. The fresh scale samples were clipped at the venter (belly scale) and the shed body skins were taken at the Bangkok snake farm, QSMI, to which the wild cobras had been moved under official permit by the snake farm (Document no. 1/2015). Scale samples were subsequently stored in 70% EtOH and kept in a freezer at -20°C. Archival tissues from this study are stored in a -20°C freezer at the Molecular laboratory, Chulalongkorn University, Bangkok, Thailand.

Genetic markers and sequences

Two mitochondrial DNA markers: Cytochrome *b* (Cyt *b*) and the Control Region (CR) were amplified from all samples in this study as these markers have been shown to be useful for resolving genetic divergences within snake species (e.g. Wüster *et al.*; 2007; Hofmann, 2012; McCartney-Melstad, 2012). As there are only a small number of published mtDNA sequences of *N. kaouthia* which are mostly partial, primers were newly designed using complete mtDNA sequences of *N. naja* and *N. atra* from Genbank (Ref. No.: NC_010225.1; Yan *et al.*, 2008 and NC_011389.1; Chen and Fu, 2008) in BioEdit software version 7.0 (Hall, 1999).

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The location of the novel designed primers was based on a preliminary comparison of those *Naja* sp. sequences that show low/high polymorphism (see supporting information, Table S3 for sequence of primers). Based on the surprising placement of the north-eastern samples in the phylogenetic analysis, a subset of samples were also amplified for the nuclear DNA (nuDNA) marker: Oocyte maturation factor (C-mos) (n = 20) using the primers from Lawson *et al.*, (2005). Amplification of these samples resulted in a total of twelve samples (n = 2 for each of the following locality codes: BK, SB, PNG, PL, NR, and n = 2 for *Naja siamensis*) being successfully sequenced after several attempts.

DNA extraction, PCR amplification, and sequencing

Prior to DNA extraction, tissue samples were washed twice using 70% ethyl-alcohol and diluted water to remove any contamination (Graziano *et al.*, 2013). Each sample was then cut into small pieces (~1-2 mm^2). Extractions were carried out using a Favorgen genomic DNA kit. PCRs were performed in 20.0 µl reactions including EmeralAmp Max solution (10.0 µl), the mtDNA (4.0 µl), ddH_2O (4.0 µl), forward and reverse primers (1.0 µl each). Conditions for PCR were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 40 seconds, an annealing at temperatures between 49-55°C (Cyt *b*), 47-62°C (CR), and 56°C (C-mos) for 40 seconds, then an extension step of 72°C for 120 seconds. The PCR product was then transferred to a column for DNA purification following Favorgen kit protocols. A final concentration of DNA (80.0-100.0 µl) was checked using gel-electrophoresis and stored at -20°C before sequencing. Sequencing was carried out at Bioneer Ltd. (Daejeon, South Korea, *www.bioneer.com*) using both forward and reverse primers of all markers.

Sequence alignment and data matrix

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Following visual checking of electropherograms, correction of base comparison of complementary strands was performed in the BioEdit. Contigs were generated from the forward and reverse primers. The resulting sequences were subsequently submitted to BLAST searches for comparison with sequences in GenBank and aligned. Sequences of Cyt *b* and CR were of good quality with the former markers consisting of 603 bp (Cyt *b*), the first CR domain (CR1) 599 bp (two insertion bases) in north-eastern and out-group populations, while all other populations were of 597 base pairs; and the second CR domain (CR2) consisted of 470 bp, in which an indel of 1 bp was present in the north-east populations, and outgroups. The nuDNA marker C-mos was 480 bp for all samples.

A concatenated mtDNA matrix of 1,673 bp of CR and Cyt *b* sequences (CR: 1-1,070 + Cyt *b*: 1,071-1,673) from 104 samples and two *N. naja* and *N. atra* samples was initially generated. However, the flanking tRNAs from the CR and Cyt *b* sequences were deleted as these regions were too small to apply evolutionary models, resulting in a concatenated matrix of 1,531 base pairs for all downstream genetic analyses. All data matrices were aligned using ClustalW (Thompson *et al.*, 1994) using default settings in the programme BioEdit version 7, and visually inspected. Sequences for all samples in this study are available from Genbank (see supporting information, Table S1 for Genbank Accession Numbers).

Haplotype analyses

The concatenated mtDNA matrix of 102 samples of *N. kaouthia* and 4 outgroup samples of *N. siamensis*, *N. atra*, and *N. naja* were imported into the programme DnaSP 5.0 to analyse DNA polymorphisms for haplotype analysis (Betrán *et al.*, 1997; Librado and Rozas, 2009; Rozas *et al.*, 2017). The programme Network version 5.0.0.1 (Fluxus Technology Ltd.) was used to generate a full haplotype network of all 106 mtDNA and 12 nuDNA samples using a median joining algorithm (Bandelt *et al.*, 1995, 1999) with epsilon equal to zero. The final network was

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reconstructed using image editing software (e.g. Paint and ACDSee Pro 4). The same analysis was also implemented for the nuDNA data.

Phylogenetic analyses

To obtain the best-fit partitioning scheme and models of nucleotide evolution, the combined mtDNA dataset was partitioned by markers, and for the protein-coding Cyt *b* gene only by codon position, giving a total of four partitions. The nuDNA C-mos dataset was analysed separately and was not partitioned. The datasets were executed in Partition-Finder version 1.0 (Lanfear *et al.*, 2012) using the Bayesian Information Criterion (BIC) as a metric of model selection with a heuristic algorithm.

For phylogenetic analyses, we used MrBayes version 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist *et al.*, 2012) which uses Bayesian Markov chain Monte Carlo (MCMC) methods. Two simultaneous analyses (nruns = 2) were run for 5,000,000 generations, sampling every 100 generations (number of chains = 4, heating schemes = 0.2) with an initial burn-in equal to 12,000. Analyses were run ensuring chains were stationary (assessed using Tracer version 1.6) with effective sample size (ESS) values > 200 (Drummond *et al.*, 2006). FigTree version 1.4.3 (Rambaut *et al.*, 2014) was used to view the summarized tree which was generated in TreeAnnotator version 1.8.4 (Drummond *et al.*, 2012). Branch support was calculated using Bayesian Posterior Probabilities (BPP). Individual markers (CR and Cyt *b*) were also analysed to determine if the resulting trees were congruent with the concatenated mtDNA tree, using the same settings as described above.

Molecular diversity

Molecular genetic diversity based on nucleotide difference between all samples, including haplotype diversity (*h*), nucleotide diversity (π), Tajima's D (*D*), and Fu and Li's *F* (*Fs*), were calculated using DnaSP and Arlequin version 3.5.2.2

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(Excoffier *et al.*, 1992; Excoffier and Lischer, 2010). To confirm the population differentiation grouped by locality (province), Analysis of hierarchical Molecular Variance (AMOVA) was conducted in Arlequin for Φ-$_{values}$ (genetic structure based on sequence differences between samples) using pairwise differences (Excoffier *et al.*, 1992), and for conventional $F_{-values}$ (bases on haplotype frequency). For all analyses the settings were left as default, allowing missing data and a significance level equal to 0.05, as well as permutations equal to 10,000.

Results

Phylogeny of N. kaouthia *in Thailand*

The best partitioned models for the phylogenetic inference were K81uf+I for CR, Cyt *b* codon 1 and 2; HKY for Cyt *b* codon 3; and F81 for C-mos. Bayesian analysis of the concatenated mtDNA marker supported four main clades (0.97-1.00 BPP) of *Naja kaouthia* which are composed of different geographical regions of Thailand (Fig. 2). These clades are: 1) a northern (Sukhothai - SK and Nakhon Sawan - NW) and central (Saraburi - SB, Bangkok - BK, Samutprakarn - SMP, with the exception of the Prachuabkirikhan [PJ]) population that is sister to 2) a southern population (Ranong [R], Phattalung [PL], and Trang [T]) combined with one from Prachuabkirikhan (a province between the central and southern regions). This grouping of samples (0.97 BPP) is sister to the cobra population from 3) Pha-Ngan Island (PNG) (1.0 BPP), a small (125 Km^2) offshore island close to the south of Thailand (50 Km). A fourth geographic clade, Nakhon Ratchasima (NR) in the north-eastern region is also identified. However, a major finding of this study is that *N. kaouthia* is currently non-monophyletic. As shown in Fig. 2, both *N. naja* and *N. atra* are more closely related to the geographic north-central + southern + island clades of *N. kaouthia* than is the population from the north-east.

Separate phylogenetic analyses of the CR and Cyt *b* markers gave similar results to the concatenated mitochondrial tree in that the geographic clades were

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largely recovered, but they differed in their position within the tree. Most notably, the monophyly of the ingroup differs between analyses. Within the CR tree, three clades were identified (see supporting information, Fig. S1), in which, as in the combined analyses, the north-east clade is distinct and is the sister group to other *N. kaouthia*, but *N. kaouthia* is monophyletic in relation to the outgroup species. The southern population is not separated from the north-central population but nests within it. The Cyt *b* tree is more similar to the concatenated tree except that the north-eastern population is sister to the spitting cobra (*Naja siamensis*) and the island clade is sister to the north-central clade (see supporting information, Fig. S2). A single sample (PL_NK_03) was resolved in alternative positions: nesting within the Southern clade within the concatenated tree and the CR tree, but was found to be the sister group to the Island clade in the Cyt *b* tree. Support for the latter relationship was not strong, and therefore additional data is needed to clarify its position.

Although the phylogeny based on the nuclear marker C-mos does not support or contradict the non-monophyly of *N. kaouthia*, it supports the mtDNA data by indicating the separation of the north-eastern (NR) clade (1.00 BPP) from all other regional samples of *N. kaouthia* (supporting information, Fig. S4A). The southern (PL) clade was also supported, but no unique bases were identified in any of the other regional clades, which is unsurprisingly given generally slowly evolving nature of the C-mos gene region (e.g. Jesus *et al.*, 2005).

Haplotype network

A haplotype network based on the concatenated mtDNA matrix showed ten haplotypes (H1-10) of *N. kaouthia* across its regional distribution in Thailand, supporting the results of the mtDNA concatenated phylogenetic analysis in that four main groups are identified. These included: north and central (H1-2: red), southern (H3-5: blue), island (H6: grey), and north-eastern (H7-10: green), with outgroups denoted as (H11-14) (Fig. 3). There is no haplotype sharing among the

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four main groups of *N. kaouthia*. One haplotype (H1) is found in all five provinces (SK, SB, SMP, BK, and NW) in northern and central regions (Fig. 3). One unique haplotype (H6) occurs in the island *N. kaouthia* only, and four haplotypes were found only in the north-eastern region (NR) (Fig. 3 and Table 1). In agreement with the concatenated mtDNA phylogenetic tree (Fig. 2), the island population is distinct from that of the mainland, and the north-eastern population is highly isolated from other regions (Fig. 3). A haplotype network for C-mos also supported the north-eastern population being highly divergent from all other regions and the outgroup species *Naja siamensis* (see supporting information, Fig. S4B). The haplotype network obtained from analysis of nuDNA loci (Fig. S4B) is consistent with some results of the mtDNA analysis (Fig. 3). Cobras from the central region (BK), Pha-ngan island (PNG), and the spitting cobra outgroup (NS) were grouped together (H1), but samples from Phattalung (PL) in the southern region (H2) and from Nakhon Ratchasima (NR) in the north-eastern region (H3) (Fig. S4B), had distinct haplotypes, supporting the result of mtDNA network (Fig. 3). The four main groups of *N. kaouthia* and the shared haplotypes displayed in the separate CR and Cyt *b* haplotype networks (see supporting information, Fig. S3A-B) correspond to the concatenated network (Fig. 3) and the phylogenetic trees (Fig. 2). With the exception of the outgroups, the Cyt *b* and CR networks exhibit five (H1-5, Fig. S3A for Cyt *b* network) and nine (H1-9, Fig. S3B for CR network) haplotypes, respectively.

Molecular diversity

Genetic diversity indices supported the geographical divergence of *N. kaouthia* into four groups. Over the entire concatenated dataset, there are 142 polymorphic sites (*S*) (67 in CR and 76 in Cyt *b*). Haplotype diversity (*h*) of the concatenated data among all samples is high (0.773), and, unsurprising, the rapidly evolving CR (0.770) has higher values than Cyt *b* (0.571). Within each population group, the *h*

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values range from 0.47 to 0.60, but are zero in the island population (Table 1) indicating that the island population have their own unique haplotype.

High nucleotide diversities (π) were detected for the whole population i.e. the concatenated data = 0.973% (CR = 0.665% and Cyt *b* = 1.415%) but values within each population group were low = 0.030-0.057% (Table 1). The values of *h* and π corresponding to haplotype numbers suggest that genetic variation is higher in the north-eastern population (11 samples) than in other populations, while the genetic sequence of all island samples is identical (only eight samples were tested).

Population differentiation indices

Analysis of hierarchical Molecular Variance (AMOVA), based on sequence divergence of the concatenated mtDNA between samples (Φ), demonstrated a high level of genetic variation between cobras from different geographic regions, Φ_{CT} (97.57%, *p*-value < 0.05) but low levels of variation within provinces, Φ_{ST} (1.43%, *p*-value < 0.05), and between provinces within regions, Φ_{SC} (1.00%, *p*-value < 0.05). The same analysis for the separate CR and Cyt *b* datasets produced similar results to the concatenated data (CR = 94.81% and Cyt *b* = 99.77%, *p*-value < 0.05). The AMOVA based on variation of haplotype frequencies (conventional *F*-test) displayed values for concatenated data = 42.99%, CR = 46.29%, and Cyt *b* = 96.77% (*p*-value < 0.05). These confirmed the population differentiation of *N. kaouthia* into four clearly separated regions, as supported by the clustering in the phylogenetic trees (e.g. Fig. 2) and haplotype networks (e.g. Fig. 3).

The pairwise comparison of Φ_{CT} also confirmed a high level of population differentiation between regions (Table 2). The Φ_{CT} results are also supported by F_{CT}. Although the values are lower, they still indicated significant levels of regional difference in *N. kaouthia*. A similar pattern is also shown in the results of the separate analyses of the CR (81.26-98.72% for Φ_{CT} and 50.35-80.16% for F_{CT})

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and Cyt *b* genes (99.31-100.0% for Φ_{CT} and 81.18-100.0% for F_{CT}). Thus, all genetic results suggested that the populations of *N. kaouthia* in all four regions are highly distinct from each other.

Discussion

In this study, substantial genetic diversity was found among populations of *N. kaouthia* from the different areas of Thailand, in which the north-eastern region population was shown to be highly distinct, lying outside of the ingroup populations. Additional nuclear data (C-mos) supported this hypothesis, and it is likely that the north-eastern population represents a new species of cobra. The island population was also found to be isolated from mainland populations, but this finding requires more samples from neighbouring islands to determine if this genetic lineage is endemic.

Sequence diversity

This study found high levels of genetic diversity between samples of *N. kaouthia* (see Table 1). Similar values for a species have been reported in other snake studies (although some of these studies have used different mtDNA markers), such as *Deinagkistrodon acutus* (π = 1.409% - NADH dehydrogenase (ND2) subunit 2; Huang *et al*., 2007), *Epicrates subflavus* (*h*=0.79, π = 0.76% - Cyt *b*; Tzika *et al*., 2009), *Philodryas chamissonis* (*h* = 0.97, π = 1.51% - CR; Sallaberry-Pincheira *et al*., 2010), and *Naja atra* (*h* = 0.868, π = 0.827% - Cyt *b*; Lin *et al*., 2014). A few studies that calculated the same indices for several populations of a single species showed low π as in this study, for example *Aipysurus laevis* (*h* = 0.55–0.63, π = 0.12–0.52% - *ND4*, Lukoschek *et al.*, 2008) and *Vipera latastei/monticola* group (*h* = 0.678–1.000, π = 0.8–4.1% - ND4 and Cyt *b*; Velo-Antón *et al.*, 2012). As in other studies, the high π value for the *N. kaouthia* samples generated here, is evidence for a high level of nucleotide divergence between samples. However, the

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low π values calculated for populations within regional groups (0.030–0.057%) represent short nucleotide distances between haplotypes (de Jong *et al.*, 2011). All of these values support the genetic variation among the regional populations of *N. kaouthia* in Thailand.

Regional diversification of Naja kaouthia

Four population groups of *N. kaouthia* are recognised in Thailand. In contrast to Wüster *et al.* (1995) who found no differentiation among populations of *N. kaouthia* based on their multivariate analysis of morphology (n = 132) and mtDNA data (n = 4), our study revealed strong regional differentiation in *N. kaouthia*, based on our genetic data (Fig. 2) and also by some colour and patterning from our fieldwork observations (Ratnarathorn, 2019). However, although the genetically differentiated northern+central population and southern population differed in colouration (black, and plain olive respectively), this is not the case for the island and the north-eastern populations. While the mtDNA data revealed that the island and the north-eastern populations are not closely related, their colour and patterns are barely distinguishable by visual inspection from the lowland basin cobras (north-central), which are regarded as 'typical cobras' (Wüster *et al.*, 1995). On the other hand, we found no genetic difference between the typical cobras and two samples of 'Suphan' phase or the white cobra (SB_NK_08 and SB_NK_05), supporting the result of Wüster *et al.* that there appears to be no divergence of the cobras within the north-central region (1.00%, p-value < 0.005). These data show that the relationship between phenotypic and genetic variation of *N. kaouthia* are ambiguous.

The separation of monocled cobra populations is likely attributed to natural barriers limiting genetic exchange (Lin *et al.* 2014). The mountain ranges of Phetchabun, Dong Phayayen, and Sankamphaeng form a barrier between the north-eastern and central regions in Thailand (Fig. 1). The southern region is characterized as a peninsular and is partitioned from the central region by the

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Himalayan-Tanoasri mountain range (Fig. 1). Stuart and Wogan (2014) note that *N. kaouthia* could be found at 1,000 meters above mean sea level although this is likely very rare as *N. kaouthia* prefers to live close to lowland basins, in habitats such as swamps and paddy fields (Cox *et al.*, 2013). However, there are more than ten peaks along the Himalayan-Tanoasri mountain range and all of them extend over 1600 meters above mean sea level. This region is also known as a transition zone and plays an important role in diversity in other species (Hughes *et al.*, 2003; Woodruff, 2003). Based on genetic data, comparisons among populations of the mainland Asian tree frog, *Polypedates leucomystax* (Buddhachat and Suwannapoom, 2018) gave similar results to our study in that the frogs were also divided into north-central, north-eastern, and southern populations. The differentiation between northern and southern populations has also been reported in the king cobra (*Ophiophagus hannah*) using phylogenetic analyses of ND2 and CR (Suntrarachun *et al.*, 2014), and other amphibian and reptile species (Inger and Voris, 2001). Population phylogenetic studies of other snake species, such as *Deinagkistrodon acutus* (Huang *et al*., 2007), *Aipysurus laevis* (Lukoschek *et al.*, 2008), *Philodryas chamissonis* (Sallaberry-Pincheira *et al.*, 2010), *Gloydius brevicaudus* (Ding *et al.*, 2011), *Vipera latastei*/*monticola* group (Velo-Antón *et al.*, 2012), and *Naja atra* (Lin *et al*., 2014) showed similar results to those of this study in finding that, for example, geography, climate, and/or distance correlate with regional genetic variation.

Cryptic speciation in N. kaouthia

Based on the molecular phylogenetic analyses reported herein, snakes from the genetically distinct north-eastern population (from NR province) do not nest within *N. kaouthia* and are as distinct from *N. kaouthia* as are members of the other cobra species sampled (*N. naja*, *N. atra,* and *N. siamensis*). This result is supported by the haplotype network (Fig. 3) and high values of AMOVA population indices (0.98-0.99, *p*-value < 0.005, in Table 2). This placement was unexpected and novel,

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possibly because previous studies did not include samples from this part of the range of *N. kaouthia* (Wüster and Thorpe, 1994; Wüster *et al.*, 1995). These novel genetic data strongly support the designation of a new cobra species in the north-eastern region of Thailand. However, despite the strong genetic evidence that this lineage is highly distinct, our observations during fieldwork revealed neither major morphological traits nor a difference in reproductive timing between north-eastern cobras and typical monocled cobras from the lowland basin. Many of the north-eastern cobras have lighter black spots on the ventral scales (see supporting information, Fig. S4), but this trait is absent in some individuals and was rejected as a fixed regional trait by Lin *et al.* (2008). Another potential character is head length when the hood expands. Based on personal observation (see supporting information, Fig. S4), the head of north-eastern cobras appears to be flatter and wider posteriorly and longer at nasal area than in typical monocled cobras, but it can be limited by their posture, age, health or orientation (e.g. Bonnet *et al.*, 2001). A detailed morphological investigation is required to assess whether there are traits that differ between the NE lineage and 'typical' monocled cobras and is currently in progress. Nonetheless, the deep genetic divergence between the NE clade with respect to other populations of the monocled cobra, indicates cryptic speciation within this group of snakes. Our finding supports Laopichienpong *et al.*, (2016) who indicated that *N. kaouthia* samples from Bangkok (n = 3) were genetically divergent from those from Bangladesh (KM521202) + Myanmar (AF217835) using CO1 and Cyt *b*.

The negative values in both Tajima'*D* (-1.32) and Fu and Li's *F* (-1.02) of the north-eastern *N kaouthia* (Table 1) indicate that they are currently experiencing different demographic processes (excess of rare alleles or population expansion) compared to other regional cobra populations (Tajima, 1989; Fu, 1997).

Species divergence of the north-eastern cobra may be due to geographic and/or climatic differences. This region differs from other regions of Thailand (Wittayarat *et al.*, 2001), and is known as a 'tropical savanna', experiencing a complete drought from October-May. Most of this region is represented by a high

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plateau surrounded by mountain ranges that form a closed environment. These features greatly restrict animal movement between regions, particularly for species with typically low vagility such as terrestrial snakes (Brito, 2003; Pyron and Burbrink, 2009; Breininger *et al.*, 2011). Similar findings of diversification within species have been reported for various taxa including the fighting fish, *Betta smaragdina* (Sriwattanarothai *et al.*, 2010), the cardamom mountain horned agamid *Acanthosaura cardamomensis* (Wood Jr *et al.*, 2010), the bamboo pit viper, *Trimeresurus macrops sensu stricto* (Mrinalini *et al.*, 2015), and species of the Asian tree frog, *Polypedates leucomystax* (Buddhachat and Suwannapoom, 2018).

Geographical history also supports the possibility of population divergence or speciation for the north-eastern cobra group. In relation to *Betta*, Sriwattanarothai *et al.* (2010) suggested that changes in tectonic plate and mineral deposition during the Mesozoic (Smith and Stokes, 1997) led to allopatric speciation of fish populations within this highland. However, this would not explain the speciation of north-eastern cobras. The ancestor of Asiatic cobras is estimated to have split from its African sister group and dispersed to South-east Asia during the early to mid-Miocene (Wüster *et al.*, 2007), which was long after the major geological changes in this region during the Mesozoic. Similar to the divergence and dispersal of Asiatic cobras to South-east Asia, other examples include the pit viper *Trimeresurus* (Mrinalini *et al.*, 2015), mammalian faunas (Ducroq *et al.*, 1994), and floral assemblages (Songtham *et al.*, 2003).

It is possible that divergence of the north-eastern cobra occurred around the beginning of the glacial period (from Late Pliocene to Early Pleistocene). The development of a cooler and drier climate than in preceding periods (Udomchoke, 1989; Penny, 2001) may have resulted in the extinction of most of this warm-restricted species, and led some populations to diverge in allopatry (refugial area) (Hewitt, 2001; Hamilton *et al.*, 2001; Lin *et al.*, 2014), eventually promoting genetic differentiation (Hewitt, 2001). A possible refugium exists in the south of the north-eastern province and is dominated by tropical forest (where the focal cobras are

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found) rather than the deciduous forest or savanna found in most other parts of the north-eastern region. A similar suggestion with respect to refugia was made for *Vipera latastei*/*monticola* (Velo-Antón *et al.*, 2012) and *Naja atra* (Lin *et al*., 2014), but has not been confirmed for *Naja kaouthia*. The only evidence in our study comes from the high sequence variation (4 haplotypes) within the north-eastern samples, with expansion of the current population (high *h* and low π [Avise, 2000] and negative values of *D* and *Fs* [Lin *et al*., 2014]) after the end of glaciation. However, the *D* and *Fs* values were not significant ($p > 0.05$).

Endemic island N. kaouthia *and population history*

In Thailand, the Isthmus of Kra is considered to be a buffer zone that facilitates species divergence between the southern archipelagos and northern mainland (Woodruff, 2003; Hughes *et al.*, 2003). As we report in this study, sampled *N. kaouthia* from one small island (125km^2) in the Gulf of Thailand, Pha-ngan (known as 'Snake Island'), which is about 50 km from the mainland, showed a signal suggesting isolation of this island population. This divergence is supported by phylogenetic, network, and population statistics (e.g. AMOVA test (0.95-0.99, *p*-value < 0.05 in Table 2). The isolation of the monocled cobra population on Pha-Ngan island was likely due to a massive rise of sea level in this region around the late Pleistocene (8,000-6,000 B.P.) (Sinsakul, 1992; Voris, 2000). Similar patterns (island isolation in this region) have been recorded in insects such as the Varroa mites, *Varroa destructor* (Warrit *et al.*, 2006), the Eastern honey bee, *Apis cerana* (Warrit *et al.*, 2006; Rueppell *et al.*, 2011), and the tephritid fruit fly, *Zeugodacus cucurbitae* (Boontop *et al.*, 2017). Observations from fieldwork indicated that the island cobras are approximately 50% smaller than those on the mainland (Ratnarathorn, pers. obs), although it was not possible to take measurement data under our permits. They also do not have the olive colouration of the southern mainland snakes and are instead coloured black and brown. The smaller size of the island cobras could be due to limited resources, leading to selection for a

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reduction in body size and, if confirmed, could represent an example of insular dwarfism in snakes (e.g. Boback, 2003; Keogh *et al.*, 2005; Luiselli *et al.*, 2015; Card *et al.*, 2016). However, a much larger data set based on measurement and body weight data is needed to confirm this observation, as the sample from Pha-ngan was limited to eight small individuals whose maturity was not determined.

To explore the genetic diversity found in the Pha-ngan Island population requires further sampling, particularly from other islands situated between Pha-ngan Island and the mainland, such as Samui (~20 km from the mainland, ~252 km² in size) and/or Phaluai Islands (~20 km, ~16 km²), as well as the 42 islands in the region. Some unobserved intermediate haplotypes are suggested on the network branch between the mainland and island populations (Fig. 3), so that additional samples may yield the missing haplotypes and thus act as 'stepping stones' for dispersal, as opposed to the alternative hypothesis of vicariance, caused by the rise in sea level that split this population from the mainland (e.g. Michaelides *et al.*, 2015). Moreover, the distance between the island and the mainland could be important. Studies of *N. kaouthia* on Phuket Island (distance: ~0.5 km only) did not reveal any phenotypic difference between the island and mainland samples (Wüster and Thorpe, 1989, 1992; Wüster *et al.*, 1995), and the tree frog species, *Polypedates leucomystax* on Phuket Island displayed haplotypes shared with mainland populations (Buddhachat and Suwannapoom, 2018). However, no haplotypes of the Pha-ngan Island cobras were found to be shared with the mainland populations in this study (Fig. 3). This suggests that the island cobra may be independent, and that the population is endemic to this offshore island.

Demographic and ecological interpretation

A preliminary signal suggesting current demographic expansion was demonstrated in every population except that on Pha-ngan (due to the small sample size). Population expansion is supported by the high haplotype diversity (h = 0.471 -

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0.600) and low nucleotide diversity (π = 0.030-0.057%) (Avise, 2000; de Jong *et al.*, 2011), and may reflect the abundance of *N. kaouthia* in Thailand (Chaitae, 2011). The hypothesis of a bottleneck in the last glacial period (Udomchoke, 1989; Penny, 2001) is well-supported by our current data on *N. kaouthia*. A field study by Chaitae (2011) revealed high survivorship in juvenile (47%) and adult (93%) cobras suggesting an ability to maintain large populations and the possibility of population expansion. However, the study represented only the central population and was carried out over a short time period (1-2 years).

Limitations of current sampling and markers

Although we suggest that the north-eastern population should be recognised as a separate species to maintain monophyly of the other *N. kaouthia* populations, alternative possibilities are that the other populations sampled could be transferred to a new species or that neither the north-eastern nor other populations sampled are *N. kaouthia*. However, the geographical separation of the north-east region makes it unlikely that this population is *N. kaouthia*. To test between these alternative scenarios more samples of *N. kaouthia* are needed across its range, including samples from the type locality (Bengal, India, Lesson 1932). Additional outgroups should also be included in future studies to investigate the relationships between *Naja* species in this region, and to test whether the nesting of several outgroups included in this study within the ingroup (i.e. *N. kaouthia sensu lato*) is real or an artefact of sampling.

To give a clearer picture of the population structure of cobras in Thailand, and ultimately across the range of *N. kaouthia*, we also recommend that future studies should harness nuclear markers such as SNPs from Restriction site-Associated DNA markers (RAD-seq), combined with comprehensive field work (e.g. Lin *et al*., 2014). As our work is largely based on mtDNA data, it may therefore be prone to the recognised issues of such data (e.g. incomplete lineage sorting and/or introgression).

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The insignificant values (p >0.05) generated regarding some of the population indices (see Table 1) could be because the demographic process is evolving neutrally (without natural selection) (Subramanian, 2016), but may be due to insufficient sample numbers. An increase in sample numbers and collecting locations across each region would help to resolve this and provide a more complete picture of the phylogeography of *N. kaouthia*.

The monocled cobra in Thailand may be undergoing a neutral evolutionary process driven by genetic drift. In particular for the lowland basin cobras (northern and central regions), annual flooding (May-October) and the clearance of paddy fields (October-November) can lead to cobras being present in larger numbers in some areas, where they are killed or harvested for commercial reasons (Chaitae, 2011; Stuart and Wogan, 2014). A generalist lifestyle, combined with high reproductive success and a high growth rate in surviving cobras (Chaitae, 2011), would facilitate population rebound. These traits could lead to sustainable natural populations and gene flow between north and central provinces (shared haplotypes – lowest h and π) along river networks. A similar demographic pattern was also suggested in spiny rats, *Proechimys* sp. (Matocq *et al*., 2000).

Conclusion

The relationships between populations of *Naja kaouthia* in Thailand have not been investigated since the genetic and morphological study of Wüster (1995) who reported a similar level of trait variation across their distribution. Our study is the first to show differentiation among populations of the monocled cobra between geographical regions in Thailand, and also reveals a likely cryptic species in the north-eastern region. The divergence of four regional cobra groups (north and central, south, island, and north-east) is also supported by the geographical, demographic, and geological-history information in this study. The results not only lead to a better understanding of monocled cobra diversity but also pave the way

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for further applications e.g. new species identification, anti-snake venom improvement, and conservation.

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References

Avise, J. C. (2000). *Phylogeography: the history and formation of species*. Cambridge, Massachusetts: Harvard University Press.

Bandelt, H. J., Forster, P., & Rohl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, 16(1), 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Bandelt, H. J., Forster, P., Sykes, B. C., & Richards, M. B. (1995). Mitochondrial portraits of human populations using median networks. *Genetics*, 141(2), 743–753.

Betrán, E., Rozas, J., Navarro, A., & Barbadilla, A. (1997). The estimation of the number and the length distribution of gene conversion tracts from population DNA sequence data. *Genetics*, 146(1), 89-99.

Boback, S. M., (2003). Body size evolution in snakes: evidence from island populations. *Copeia*, 2003(1), 81-94. https://doi.org/10.1643/0045-8511(2003)003[0081:BSEISE]2.0.CO;2

Review Copy

Bonnet, X., Shine, R., Naulleau, G., & Thiburce, C. (2001). Plastic vipers: influence of food intake on the size and shape of Gaboon vipers (*Bitis gabonica*). *Journal of Zoology*, 255(3), 341-351. https://doi.org/10.1017/S0952836901001443

Boontop, Y., Kumaran, N., Schutze, M. K., Clarke, A. R., Cameron, S. L., & Krosch, M. N. (2017). Population structure in *Zeugodacus cucurbitae* (Diptera: Tephritidae) across Thailand and the Thai–Malay peninsula: natural barriers to a great disperser. *Biological Journal of the Linnean Society*, 121(3), 540–555. https://doi.org/10.1093/biolinnean/blx009

Breininger, D. R., Bolt, R. M., Legare, M. L., Drese, J. H., & Stolen, E. D. (2011). Factors influencing home-range sizes of Eastern Indigo Snakes in central Florida. *Journal of Herpetology*, 45(4), 484–490. https://doi.org/10.1670/10-176.1

Brito, J. C. (2003). Seasonal variation in movements, home range, and habitat use by male *Vipera latastei* in northern Portugal. *Journal of Herpetology*, 37(1), 155-160. https://doi.org/10.1670/0022-1511(2003)037[0155:SVIMHR]2.0.CO;2

Buddhachat, K., Suwannapoom, C. (2018). Phylogenetic relationships and genetic diversity of the *Polypedates leucomystax* complex in Thailand. *PeerJ*, 16, e4263. https://doi.org/10.7717/peerj.4263

Card, D. C., Schield, D. R., Adams, R. H., Corbin, A. B., Perry, B. W., Andrew, A. L., … Castoe, T. A. (2016). Phylogeographic and population genetic analyses reveal multiple species of *Boa* and independent origins of insular dwarfism. *Molecular Phylogenetics and Evolution*, 102, 104-116. https://doi.org/10.1016/j.ympev.2016.05.034

Carfagno, G. L. F., & Weatherhead P. J. (2006). Intraspecific and interspecific variation in use of forest-edge habitat by snakes. *Canadian Journal of Zoology*, 84(10), 1440–1452. https://doi.org/10.1139/z06-124

Chaitae, A. (2011). *Demography of the monocled cobra (Naja kaouthia) in the central region of Thailand* (Master's thesis). Retrieved from https://doi.org/10.18297/etd/228

Chen, N., & Fu, X. Y. (2008). *Nucleotide: Naja atra voucher CIB093931 mitochondrion, complete genome*. Retrieved from https://www.ncbi.nlm.nih.gov/nuccore/194739362

Cox, M. J. (1991). *The snakes of Thailand and their husbandry*. Malabar, Florida: Krieger Public Company Limited.

Cox, M. J., Hoover, M. F., Chanhome, L., & Kumthorn, T. (2013). *The Snakes of Thailand*. Bangkok, Thailand: Chulalongkorn University Printing Press.

de Jong, M. A., Wahlberg, N., Van Eijk, M., Brakefield, P. M., & Zwaan, B. J. (2011). Mitochondrial DNA signature for range-wide populations of *Bicyclus anynana* suggests a rapid expansion from recent refugia. *PLoS ONE*, 6(6), e21385. https://doi.org/10.1371/journal.pone.0021385

Ding, L., Gan, X. N., He, S. P., & Zhao, E. M. (2011). A phylogeographic, demographic and historical analysis of the short-tailed pit viper (*Gloydius brevicaudus*): evidence for early

Review Copy

divergence and late expansion during the Pleistocene. *Molecular Ecology*, 20(9), 1905–1922. https://doi.org/10.1111/j.1365-294X.2011.05060.x

Drummond, A. J., Ho, S. Y., Phillips, M. J., & Rambaut, A. (2006). Relaxed phylogenetics and dating with confidence. *PLoS Biology*, 4(5), e88. https://doi.org/10.1371/journal.pbio.0040088

Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, 29(8), 1969-1973. https://doi.org/10.1093/molbev/mss075

Ducroq, S., Chaimanee, Y., Suteethorn, V., & Jaeger, J. J. (1994). Ages and paleoenvironment of Miocene mammalian faunas from Thailand. *Palaeogeography Palaeoclimatology Palaeoecology*, 108(1-2), 149-163. https://doi.org/10.1016/0031-0182(94)90027-2

Dupuis, J. R., Roe, A. D., & Sperling, F. A. (2012). Multi-locus species delimitation in closely related animals and fungi: one marker is not enough. *Molecular Ecology*, 21(18), 4422–4436. https://doi.org/10.1111/j.1365-294X.2012.05642.x

Excoffier, L., & Lischer, H. L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10(3), 564-567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131(2), 479-491.

Forsman, A. (2015). Rethinking phenotypic plasticity and its consequences for individuals, populations and species. *Heredity*, 115(4), 276–284. https://doi.org/10.1038/hdy.2014.92

Fu, Y. X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, 147(2), 915–925.

Godinho, R., Crespo, E. G., Ferrand, N., & Harris, J. D. (2005). Phylogeny and evolution of the green lizards, *Lacerta* spp. (Squamata: Lacertidae) based on mitochondrial and nuclear DNA sequences, *Amphibia-Reptilia* 26(3), 271-285. https://doi.org/10.1163/156853805774408667

Graziano, M. U., Graziano, K. U., Pinto, F. M., Bruna, C. Q., de Souza, R. Q., & Lascala, C. A. (2013). Effectiveness of disinfection with alcohol 70% (w/v) of contaminated surfaces not previously cleaned. *Revista Latino-Americana de Enfermagem*. 21(2), 618-623. https://doi.org/10.1590/S0104-11692013000200020

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.

Hamilton, A., Taylor, D., & Howard, P. (2001). Hotspots in African forests as Quaternary refugia. In: W. Weber, L. J. White, A. Vedder, & L. Naughton-Treves, (Eds.), *African Rain Forest Ecology and Conservation: an Interdisciplinary Perspective* (pp. 183-206). New Haven and London: Yale University Press.

Review Copy

Hewitt, G. M. (2001). Speciation, hybrid zones and phylogeography or seeing genes in space and time. *Molecular Ecology*, 10(3), 537-549. https://doi.org/10.1046/j.1365-294x.2001.01202.x

Hofmann, S. (2012). Population genetic structure and geographic differentiation in the hot spring snake Thermophis baileyi (Serpentes, Colubridae): Indications for glacial refuges in southern-central Tibet. *Molecular Phylogenetics and Evolution*, 63(2), 396-406. https://doi.org/10.1016/j.ympev.2012.01.014

Huang, S., He, S., Peng, Z., Zhao, K., & Zhao, E. (2007). Molecular phylogeography of endangered sharp-snouted pitviper (*Deinagkistrodon acutus*; Reptilia, Viperidae) in Mainland China. *Molecular Phylogenetics and Evolution*, 44(3), 942-952. https://doi.org/10.1016/j.ympev.2007.05.019

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17(8), 754-755. https://doi.org/10.1093/bioinformatics/17.8.754

Hughes, J. B., Round, P. D., & Woodruff, D. S. (2003). The Indochinese–Sundaic faunal transition at the Isthmus of Kra: an analysis of resident forest bird species distributions. *Journal of Biogeography*, 30(4), 569-580. https://doi.org/10.1046/j.1365-2699.2003.00847.x

Inger, R. F., & Voris, H. K. (2001). The biogeographical relations of the frogs and snakes of Sundaland. *Journal of Biogeography*, 28(7), 863–891. https://doi.org/10.1046/j.1365-2699.2001.00580.x

Jesus, J., Brehm, A., & Harris, J. D. (2005). Phylogenetic relationships of *Hemidactylus* geckos from the Gulf of Guinea islands: patterns of natural colonizations and anthropogenic introductions estimated from mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*. 34(3), 480-485. https://doi.org/10.1016/j.ympev.2004.11.006

Ji, X., & Wang, Z. -W. (2005). Geographic variation in reproductive traits and trade-offs between size and number of eggs of the Chinese cobra (*Naja atra*). *Biological Journal of the Linnean Society*, 85(1), 27-40. https://doi.org/10.1111/j.1095-8312.2005.00470.x

Jitakune, P. (2004). งูพิษในประเทศไทย 2 (Venomous Snakes in Thailand 2). Bangkok, Thailand: Matichonbook Publishing.

Jitakune, P., & Chanhome, L. (1996). งูพิษในประเทศไทย (Venomous Snakes in Thailand). Bangkok, Thailand: Pachachon Co., Ltd.

Kelly, C. M., Barker, N. P., & Villet, M. H. (2003). Phylogenetics of advanced snakes (Caenophidia) based on four mitochondrial genes. *Systematic Biology*, 52(4), 439-459. https://doi.org/10.1080/10635150390218132

Keogh, S. J., Scott, I. A., & Hayes, C. (2005). Rapid and repeated origin of insular gigantism and dwarfism in Australian tiger snakes. *Evolution*, 59(1), 226-233. https://doi.org/10.1111/j.0014-3820.2005.tb00909.x

Review Copy

Lanfear, R., Calcott, B., Ho, S. Y., & Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, 29(6), 1695–1701. https://doi.org/10.1093/molbev/mss020

Laopichienpong, N., Muangmai, N., Supikamolseni, A., Twilprawat, P., Chanhome, L., Suntrarachun, S., … Srikulnath, K. (2016). Assessment of snake DNA barcodes based on mitochondrial COI and Cytb genes revealed multiple putative cryptic species in Thailand. *Gene*, 594(2), 238-247. https://doi.org/10.1016/j.gene.2016.09.017

Lawson, R., Slowinski, J. B., Crother, B. I., & Burbrink , F. T. (2005). Phylogeny of the Colubroidea (Serpentes): new evidence from mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution*, 37(2), 581-601. https://doi.org/10.1016/j.ympev.2005.07.016

Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25(11), 1451-1452. https://doi.org/10.1093/bioinformatics/btp187

Lin, H. -C., Li, S. -H., Fong, J., & Lin, S. -M. (2008). Ventral coloration differentiation and mitochondrial sequences of the Chinese cobra (*Naja atra*) in Taiwan. Conservation Genetics, 9(5), 1089-1097. https://doi.org/10.1007/s10592-007-9418-8

Lin, L. H., Mao, L. X., & Ji, X. (2012). Genetic structure and demographic history should inform conservation: Chinese cobras currently treated as homogenous show population divergence, *PLoS ONE*, 7(4), e36334. https://doi.org/10.1371/journal.pone.0036334

Lin, L. -H., Hua, L., Qu, Y. -F., Gao, J. -F., & Ji, X. (2014). The phylogeographical pattern and conservation of the Chinese cobra (*Naja atra*) across its range based on mitochondrial Control Region sequences. *PLoS ONE*, 9(9), e106944. https://doi.org/10.1371/journal.pone.0106944

Luiselli, L. M., Petrozzi, F., Mebert, K., Zuffi, M. A. L., & Amori, G. (2015). Resource partitioning and dwarfism patterns between sympatric snakes in a micro-insular Mediterranean environment. *Ecological Research*, 30(3), 527-535. https://doi.org/10.1007/s11284-015-1250-x

Lukoschek, V., Waycott, M., & Keogh, J. S. (2008). Relative information content of polymorphic microsatellites and mitochondrial DNA for inferring dispersal and population genetic structure in the olive sea snake, *Aipysurus laevis*. *Molecular Ecology*, 17(13), 3062–3077. https://doi.org/10.1111/j.1365-294X.2008.03815.x

Matocq, M. D., Patton, J. L., & da Silva, M. F. (2000). Population genetic structure of two ecologically distinct Amazonian spiny rats: separating history and current ecology. *Evolution* 54(4), 1423-1432. https://doi.org/10.1111/j.0014-3820.2000.tb00574.x

McCartney-Melstad, E., Waller, T., Micucci, P. A., Barros, M., Draque, J., Amato, G., & Mendez, M. (2012). Population structure and gene flow of the Yellow Anaconda (*Eunectes notaeus*) in Northern Argentina. *PLoS ONE*, 7(5), e37473. https://doi.org/10.1371/journal.pone.0037473

Review Copy

Meyer, C.P., & Paulay, G. (2005). DNA barcoding: error rates based on comprehensive samplings. PLoS Biolology, 3(12), e422. https://doi.org/10.1371/journal.pbio.0030422

Michaelides, S., Cornish, N., Griffiths, R., Groombridge, J., Zajac, N., Walters, G. J., Aubret, F., … Uller, T. (2015). Phylogeography and conservation genetics of the common wall lizard, *Podarcis muralis*, on islands at its northern range. *PLoS ONE*, 10(2), e0117113. https://doi.org/10.1371/journal.pone.0117113

Mott, C. L. (2010). Environmental Constraints to the Geographic Expansion of Plant and Animal Species. *Nature Education Knowledge* 3(10), 72.

Mrinalini, M., Thorpe, R. S., Creer, S., Lallias, D., Dawnay, L., Stuart, B. L., & Malhotra, A. K. (2015). Convergence of multiple markers and analysis methods defines the genetic distinctiveness of cryptic pitvipers. *Molecular Phylogenetics and Evolution*, 92, 266-279. https://doi.org/10.1016/j.ympev.2015.06.001

Penny, D. (2001). A 40,000 year palynological record from north-east Thailand; implications for biogeography and palaeo-environmental reconstruction. *Palaeogeography Palaeoclimatology Palaeoecology*, 171(3-4), 97-128. https://doi.org/10.1016/S0031-0182(01)00242-5

Pyron, A. R., & Burbrink, F.T. (2009). Lineage diversification in a widespread species: roles for niche divergence and conservatism in the common kingsnake, *Lampropeltis getula*. Molecular Ecology, 18(16), 3443–3457. https://doi.org/10.1111/j.1365-294X.2009.04292.x

Rambaut, A., Suchard, M. A., Xie, D., & Drummond, A. J. (2014). *FigTree*. Retrieved from http://tree.bio.ed.ac.uk/software/figtree/

Ratnarathorn, N. (2019). *Regional variation of the Monocled Cobra, Naja kaouthia Lesson, 1831 (Squamata: Elapidae) in Thailand: Development, Temperature Effects, Environment, and Phylogeny* (Doctoral Dissertation). Retrieved from http://discovery.ucl.ac.uk/10064939/

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61(3), 539-542. https://doi.org/10.1093/sysbio/sys029

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sánchez-Gracia, A. (2017). DnaSP v6: DNA sequence polymorphism analysis of large datasets. *Molecular Biology and Evolution*, 34(12), 3299-3302. https://doi.org/10.1093/molbev/msx248

Rueppell, O., Hayes, A., Warrit, N., & Smith, D. (2011). Population structure of *Apis cerana* in Thailand reflects biogeography and current gene flow rather than Varroa mite association. *Insectes Sociaux*, 58(4), 445-452. https://doi.org/10.1007/s00040-011-0161-2

Sallaberry-Pincheira, N., Garin, C. F., González-Acuña, D., Sallaberry, M. A., & Vianna, J.A. (2011). Genetic divergence of Chilean long-tailed snake (*Philodryas chamissonis*) across latitudes:

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conservation threats for different lineages. *Diversity and Distributions*, 17, 152–162. https://doi.org/10.2307/41058148

Sinsakul, S. (1992). Evidence of quarternary sea level changes in the coastal areas of Thailand: a review. *Journal of Southeast Asian Earth Sciences*, 7(1), 23-37. https://doi.org/10.1016/0743-9547(92)90012-Z

Slowinski, J. B., & Keogh, S. J. (2000). Phylogenetic relationships of elapid snakes based on Cytochrome b mtDNA sequences. *Molecular Phylogenetics and Evolution*, 15(1), 157-164. https://doi.org/10.1006/mpev.1999.0725

Smith, P. F., & Stokes, R. B. (1997). Geology and petroleum potential of the Khorat Plateau basin in the Vientiane area of Lao P.D.R. *Journal of Petroleum Geology*, 20(1), 27–50. https://doi.org/10.1111/j.1747-5457.1997.tb00754.x

Songtham, W., Ratanasthien, B., Mildenhall, D. C., Singharajwarapan, S., & Kandharosa, W. (2003). Oligocene-Miocene climatic changes in northern Thailand resulting from extrusion tectonics of Southeast Asian landmass. *ScienceAsia*, 29, 221-233.

Spicer, J. I. (2006). *Biodiversity: A Beginner's Guide*. Oxford, UK: Oneworld Publications,

Sriwattanarothai, N., Steinke, D., Ruenwongsa, P., Hanner, R., & Panijpan, B. (2010). Molecular and morphological evidence supports the species status of the Mahachai fighter *Betta* sp. Mahachai and reveals new species of *Betta* from Thailand. *Journal of Fish Biology*, 77(2), 414-424. https://doi.org/10.1111/j.1095-8649.2010.02715.x

Stuart, B., & Wogan, G. (2014). *Naja kaouthia*. Retrieved from http://www.iucnredlist.org/details/177487/0

Subramanian, S. (2016). The effects of sample size on population genomic analyses – implications for the tests of neutrality. *BMC Genomics*, 17, 123. https://doi.org/10.1186/s12864-016-2441-8

Suntrarachun, S., Chanhome, L., & Sumontha, M. (2014). Phylogenetic analysis of the king cobra, *Ophiophagus hannah* in Thailand based on mitochondrial DNA sequences. *Asian Biomedicine*, 8(2), 269-274. https://doi.org/10.5372/1905-7415.0802.289

Taggart, T. W., Crother, B. I., & White, M. E. (2001). Palm-Pitviper (*Bothriechis*) phylogeny, mtDNA, and consilience, *Cladistics*, 17(4), 355-370. https://doi.org/10.1006/clad.2001.0183

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123(3), 585–595.

Tan, K. Y., Tan, C. H., Chanhome, L., & Tan, N. H. (2017). Comparative venom gland transcriptomics of *Naja kaouthia* (monocled cobra) from Malaysia and Thailand: elucidating geographical venom variation and insights into sequence novelty. *PeerJ*, 5(5), e3142. https://doi.org/10.7717/peerj.3142

Review Copy

Thailand Department of Mineral Resources, Ministry of Natural Resources and Environment (2007). *Geology of Thailand*. Bangkok, Thailand: Dokbia Printing House.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22(22), 4673-4680.

Tzika, A. C., Remy, C., Gibson, R., & Milinkovitch, M. C. (2009). Molecular genetic analysis of a captive-breeding program: the vulnerable endemic Jamaican yellow boa. *Conservation Genetics*, 10(1), 69–77. https://doi.org/10.1007/s10592-008-9519-z

Udomchoke, V. (1989). Quaternary stratigraphy of the Khorat Plateau area, Northeastern Thailand. In N. Thiramongkol. *Proceedings of the Workshop on Correlation of Quaternary Successions in South, East, and Southeast Asia*, Bangkok, Thailand (pp. 69-94). Department of Geology: Chulalongkorn University.

Uetz, P., & Hošek, J. (2014). *Naja kaouthia* LESSON, 1831. Retrieved from: http://reptiledatabase.reptarium.cz/species?genus=Naja&species=kaouthia&search_param=%28%28taxon%3D%27Elapidae%27%29%29

Ursenbacher, S., Schweiger, S., Tomovic´, L., Crnobrnja-Isailovic´, J., Fumagalli, L., & Mayer, W. (2008). Molecular phylogeography of the nose-horned viper (*Vipera ammodytes*, Linnaeus (1758)): Evidence for high genetic diversity and multiple refugia in the Balkan peninsula. *Molecular Phylogenetics and Evolution*, 46(3), 1116–1128. https://doi.org/10.1016/j.ympev.2007.11.002

Velo-Antón, G., Godinho, R., Harris, D. J., Santos, X., Martínez-Freiria, F., Fahd, S., … Brito, J. C. (2012). Deep evolutionary lineages in a Western Mediterranean snake (*Vipera latastei*/monticola group) and high genetic structuring in Southern Iberian populations. *Molecular Phylogenetics and Evolution*, 65(3), 965-973. https://doi.org/10.1016/j.ympev.2012.08.016

Vidal, N., Delmas, A. S., David, P., Cruaud, C., Couloux, A., & Hedges, S. B. (2007). The phylogeny and classification of caenophidian snakes inferred from seven nuclear protein-coding genes. *Comptes Rendus Biologies*, 330(2), 182-187. https://doi.org/10.1016/j.crvi.2006.10.001

Voris, H. K. (2000). Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and time durations. *Journal of Biogeography*, 27(5), 1153–1167. https://doi.org/10.1046/j.1365-2699.2000.00489.x

Wallach, V., Williams, K. L. & Boundy, J. (2014). *Snakes of the World. A Catalogue of Living and Extinct Species*. Boca Raton/London/New York: CRC Press.

Warrit, N., Smith, D. R., & Lekprayoon, C. (2006). Genetic subpopulations of Varroa mites and their *Apis cerana* hosts in Thailand. *Apidologie*, 37(1), 19-30. https://doi.org/10.1051/apido:2005051

Review Copy

Wittayarat, P., Worakawin, K., Mūnsin, W., Kaewmoung, D., Amaranan, K., & Parithan, V. (2001). *Atlas แผนที่ภูมิศาสตร์-ประวัติศาสตร์*. Bangkok, Thailand: Thai Watana Panich Press.

Wood, P. L., Grismer, L. L., & Thy, N. (2010). A new cryptic species of *Acanthosaura* Gray, 1831 (Squamata: Agamidae) from Thailand and Cambodia. *Zootaxa*, 2488(1), 22–38. https://doi.org/10.11646/zootaxa.2488.1.2

Woodruff, D. S. (2003). Neogene marine transgressions, palaeogeography and biogeographic transitions on the Thai–Malay Peninsula. *Journal of Biogeography*, 30(4), 551-567. https://doi.org/10.1046/j.1365-2699.2003.00846.x

Wüster , W. (1998). The cobras of the genus *Naja* in India. *Hamadryad*, 23(1), 15-32

Wüster, W., & Thorpe, R. S. (1989). Population affinities of the Asiatic cobra (*Naja naja*) species complex in south-east Asia: reliability and random resampling. *Biological Journal of the Linnean Society*, 36(4), 391–409. https://doi.org/10.1111/j.1095-8312.1989.tb00503.x

Wüster, W., & Thorpe, R. S. (1992). Asiatic Cobras: Population Systematics of the *Naja naja* species complex (Serpentes: Elapidae) in India and Central Asia. *Herpetologica*, 48(1), 69-85.

Wüster, W., & Thorpe, R. S. (1994). *Naja siamensis*, a cryptic species of venomous snake revealed by mtDNA sequencing. *Experientia*, 50(1), 75–79.

Wüster, W., Crookes, S., Ineich, I., Mane´, Y., Pook, C. E., Trape, J. -F., & Broadley, D. G. (2007). The phylogeny of cobras inferred from mitochondrial DNA sequences: evolution of venom spitting and the phylogeography of the African spitting cobras (Serpentes: Elapidae: *Naja nigricollis* complex). *Molecular Phylogenetics and Evolution*, 45(2), 437-453. https://doi.org/10.1016/j.ympev.2007.07.021

Wüster, W., Thorpe, R. S., Cox, M. J., Jintakune, P., & Nabhitabhata, J. (1995). Population systematics of the snake genus *Naja* (Reptilia: Serpentes: Elapidae) in Indochina: multivariate morphometrics and comparative mitochondrial DNA sequencing (cytochrome oxidase I). *Journal of Evolutionary Biology*, 8(4), 493–510. https://doi.org/10.1046/j.1420-9101.1995.8040493.x

Yan, J., Li, H., & Zhou, K. (2008). Evolution of the mitochondrial genome in snakes: gene rearrangements and phylogenetic relationships. *BMC Genomics*, 9, 569. https://doi.org/10.1186/1471-2164-9-569

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Figures

Fig. 1. Map of collection localities and numbers of specimens from each site in Thailand. In the figure, brightly coloured areas indicate where *N. kaouthia* is commonly found. Collection sites for the central region (blue) consist of Bangkok (BK), Samutprakarn (SMP), the boundary between Saraburi and Phra-Nakhon-Si-Ayutthaya (SB), and Prachuabkirikhan (PJ); for the northern region (pink): Sukhothai (SK) and Nakhon Sawan (Bueng Boraphet) (NW); for the southern region (green): Ranong (R), Phattalung (PL), Pha-Ngan Island (PNG), and Trang (T); for the north-eastern region (orange): Nakhon Ratchasima (Sakaerat) (NR). The outgroup specimens of *Naja siamensis* were collected from Phetchabun (PB) and Nan (NN).

Fig. 2. **A)** The mtDNA (concatenated CR and Cyt *b*) phylogeny of *N. kaouthia* from different provinces of Thailand generated from a Bayesian analysis. Support values are shown on branches are Bayesian Posterior Probabilities (BPP). Images show examples of the colour variation within and between each lineage grouped by colour (top to bottom): north-east (green), island (grey), south (blue), and north-central (red). Only branches with >50% BPP support are shown; **B)** Inset: Estimated distribution of the four inferred clades of *Naja kaouthia* in Thailand including the north-central population (red), the north-eastern population (green), southern population (blue), and island population (grey).

Fig. 3. Median-joining haplotype network of concatenated mtDNA (Cyt *b* and CR): 10 haplotypes were recovered (H1-10) from *N. kaouthia* across its distribution in Thailand and another four from the outgroups (H11-14). Based on the network analysis, colours represent the collection province (locality) while the colour tone indicates the four main groups (populations) of *N. kaouthia*; North and Central (red), Southern (blue), Island (grey), and North-eastern (green). The outgroups are shown in black, and include the non-spitting cobra species, *Naja naja* and *Naja atra* and a spitting cobra species, *Naja siamensis*. A different colour shade indicates collection site (e.g. Sukhothai (SK) = yellow, Nakhon Sawan (NW) = orange, Bangkok (BK) = brown, etc.) White dots on branches represent inferred missing haplotypes. Short transverse lines inferred mutational steps (one base difference)

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Tables

Population	*n*	*nh*	*H*	*π (%)*	*D*	*Fs*
North + Central	63	2	0.471 ± 0.034	0.0308 ± 0.0302	1.547 (0.947)	1.936 (0.751)
South	20	3	0.542 ± 0.104	0.0396 ± 0.0366	0.173 (0.662)	0.153 (0.488)
Island	8	1	0.000	0.000	0.000 (1.000)	-
North-east	11	4	0.600 ± 0.159	0.0571 ± 0.0488	-1.322 (0.092)	-1.026 (0.104)
All samples	102	14	0.773 ± 0.028	0.9375 ± 0.4691	0.503 (0.748)	18.524 (0.997)

Table 1. Genetic diversity and population indices measured within each population and between all samples of *N. kaouthia* (using the concatenated gene from partial Cytochrome *b* and the Control Region): *n*, sample size; *nh*, number of haplotypes; *h*, haplotype diversity; *π*, nucleotide diversity; *D*, Tajima´s *D*; *Fs*, Fu and Li´s *F*. Standard deviations for *h* and *π* follow the indices. Significant *p*-values for *D* and *Fs* are in brackets.

Regions	North + Central	South	Island	North-east
North + Central		0.50352*	0.63563*	0.48977*
South	*0.93458**		0.64116*	0.43354*
Island	*0.96621**	*0.95175**		0.66132*
North-east	*0.99068**	*0.98666**	*0.99037**	

** Significance test (p-value) < 0.05*

Table 2. Degrees of *Phi (Φ)*- (bottom left-italics) and *F*-statistics (top right) between regional *N. kaouthia* based on the concatenated mtDNA gene data.

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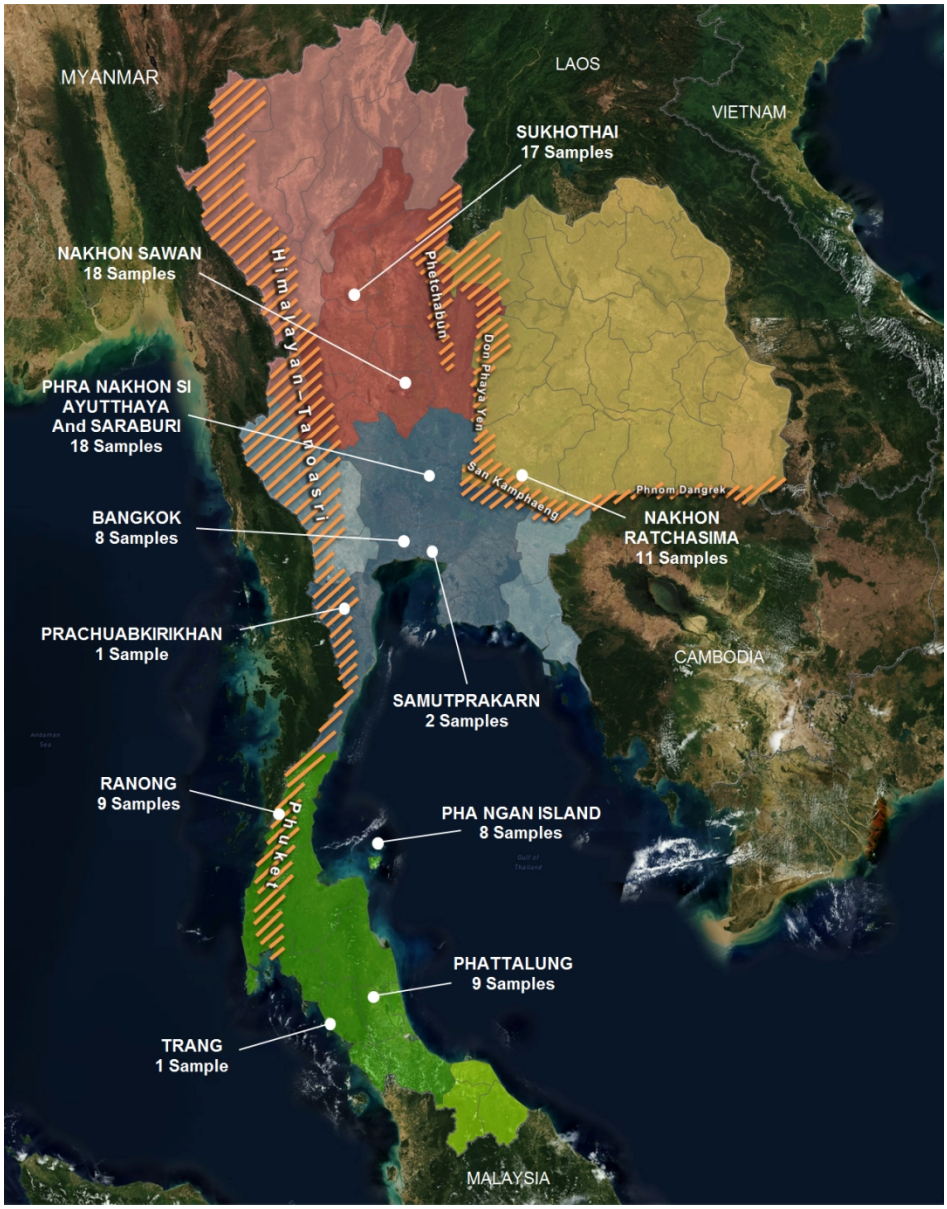
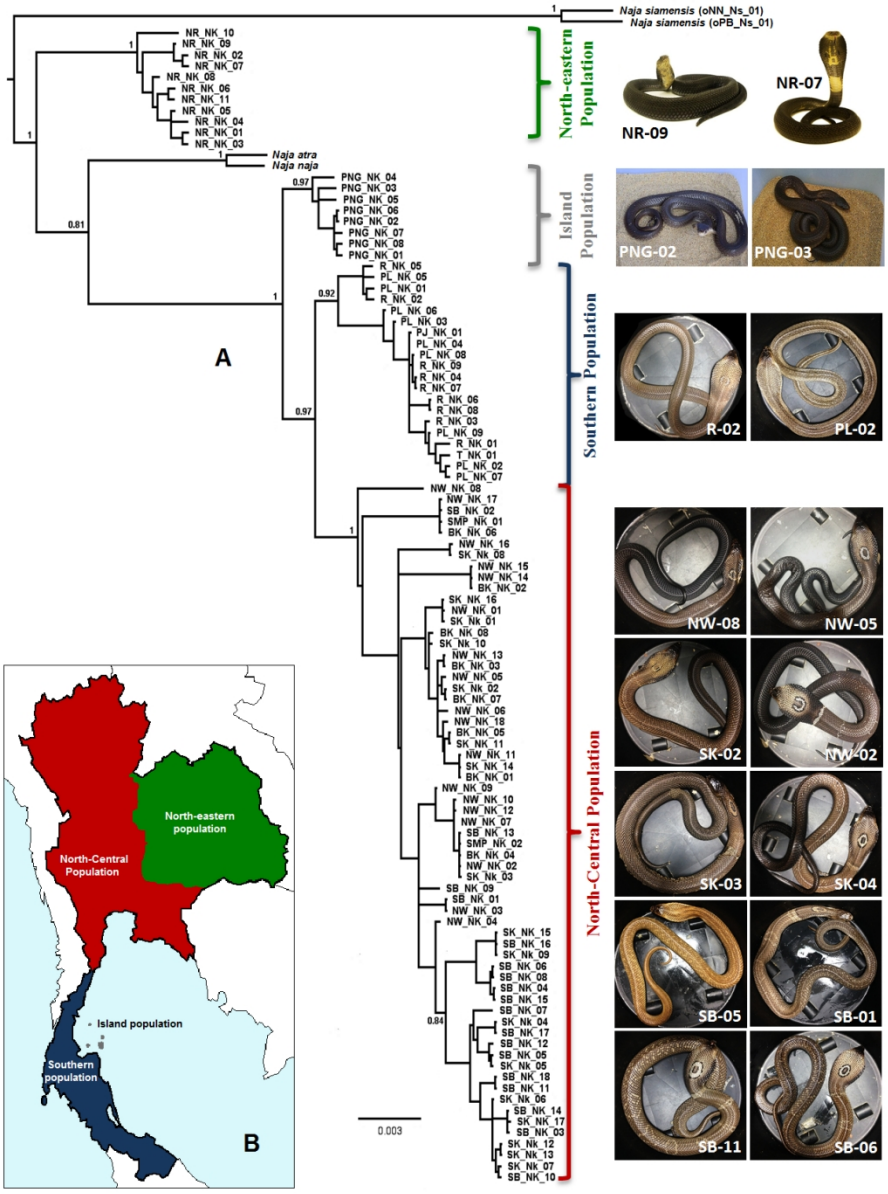


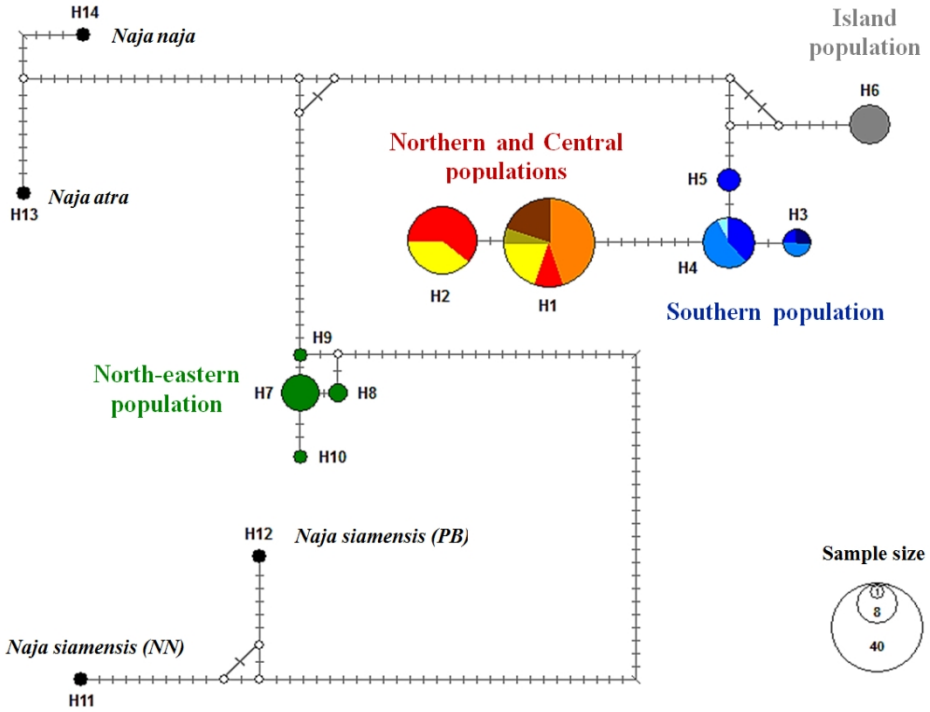
Map of collection localities and numbers of specimens from each site in Thailand. In the figure, brightly coloured areas indicate where N. kaouthia is commonly found. Collection sites for the central region (blue) consist of Bangkok (BK), Samutprakarn (SMP), the boundary between Saraburi and Phra-Nakhon-Si-Ayutthaya (SB), and Prachuabkirikhan (PJ); for the northern region (pink): Sukhothai (SK) and Nakhon Sawan (Bueng Boraphet) (NW); for the southern region (green): Ranong (R), Phattalung (PL), Pha-Ngan Island (PNG), and Trang (T); for the north-eastern region (orange): Nakhon Ratchasima (Sakaerat) (NR). The outgroup specimens of Naja siamensis were collected from Phetchabun (PB) and Nan (NN).

336x429mm (96 x 96 DPI)



A) The mtDNA (concatenated CR and Cyt b) phylogeny of N. kaouthia from different provinces of Thailand generated from a Bayesian analysis. Support values are shown on branches are Bayesian Posterior Probabilities (BPP). Images show examples of the colour variation within and between each lineage grouped by colour (top to bottom): north-east (green), island (grey), south (blue), and north-central (red). Only branches with >50% BPP support are shown; B) Inset: Estimated distribution of the four inferred clades of Naja kaouthia in Thailand including the north-central population (red), the north-eastern population (green), southern population (blue), and island population (grey).

342x449mm (96 x 96 DPI)



Median-joining haplotype network of concatenated mtDNA: 10 haplotypes were recovered (H1-10) from N. kaouthia across its distribution in Thailand and another four from the outgroups (H11-14). Colours represent the collection province (locality) while the colour tone indicates the four main groups (populations) of N. kaouthia; North and Central (red), Southern (blue), Island (grey), and North-eastern (green). The outgroups are shown in black, and include the non-spitting cobra species, Naja naja and Naja atra and a spitting cobra species, Naja siamensis. A different colour shade indicates collection site (e.g. Sukhothai (SK) = yellow, Nakhon Sawan (NW) = orange, Bangkok (BK) = brown, etc.) White dots on branches represent inferred missing haplotypes.

321x240mm (96 x 96 DPI)

Supplementary Information

Geographic differentiation and cryptic diversity in the monocled cobra, *Naja kaouthia* (Elapidae) from Thailand

Napat Ratnarathorn, Pongchai Harnyuttanakorn, Lawan Chanhome,

Susan E. Evans, and Julia J. Day

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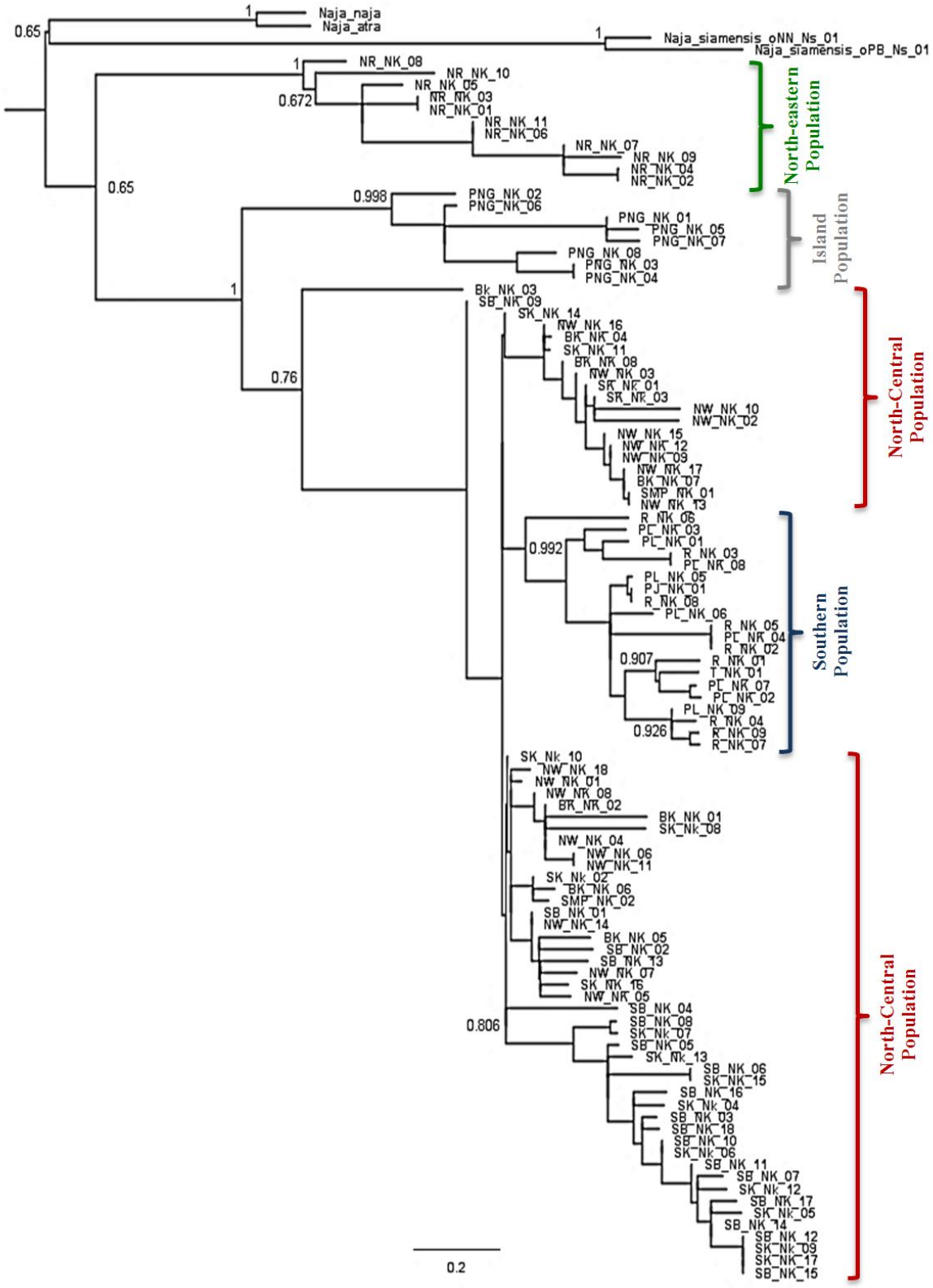

Fig. S1. Phylogenetic tree based on the Control Region of *Naja kaouthia* from different provinces of Thailand. BPP >50% shown below branches. Bayesian Posterior Probability (BPP) values support three clades of *N. kaouthia*: north-eastern (green), island (grey), and mainland (red) populations.

Fig. S2. Phylogenetic tree of partial Cytochrome b data of *Naja kaouthia* from different provinces of Thailand. Only branches with >50% are shown. BPP values supported four distinct clades of the *N. kaouthia* across Thailand: north-east (green), southern population (blue), island (grey), and northern & central populations (red).

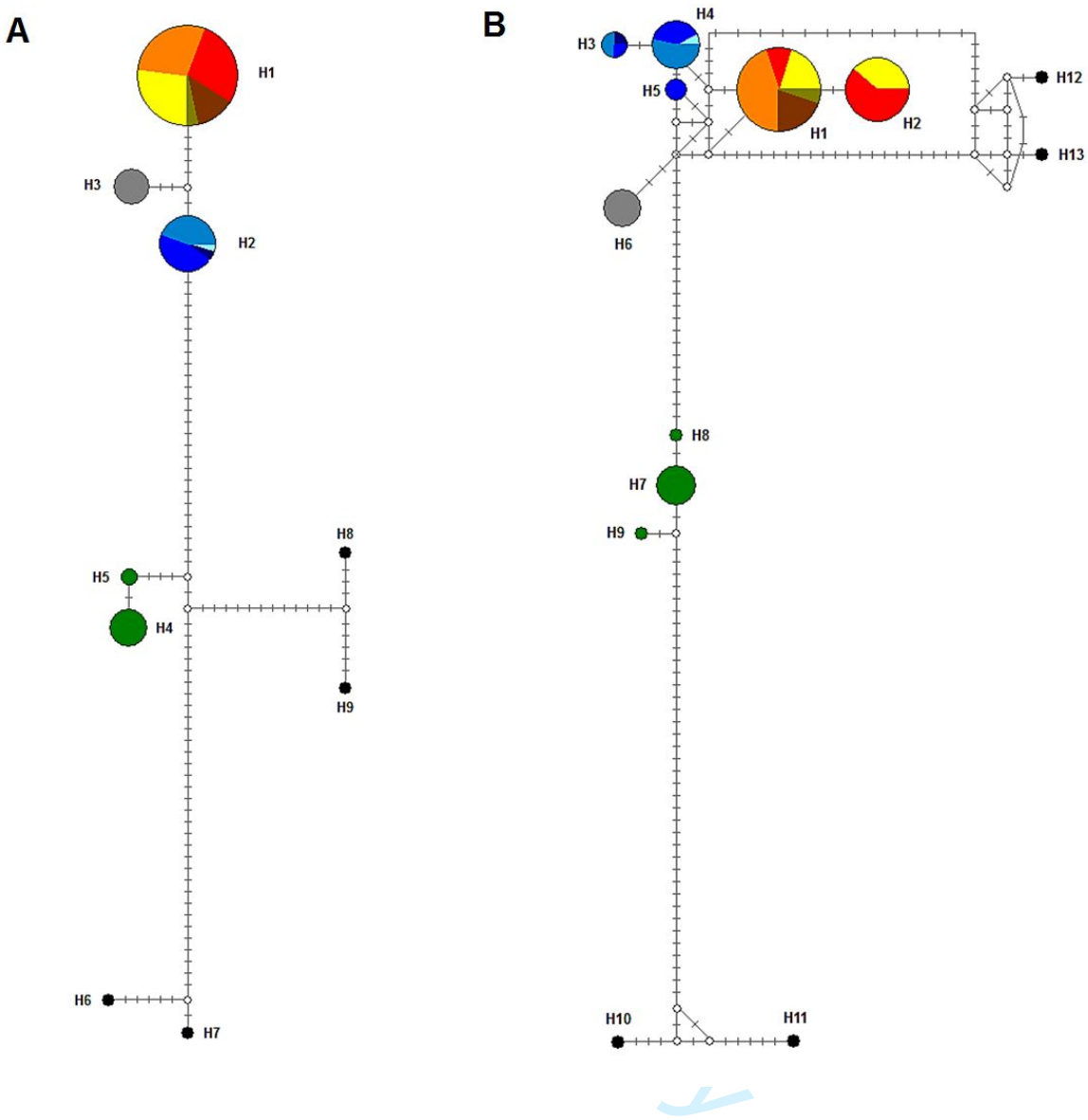

Fig. S3. A) Haplotype network of partial Cytochrome b identifies five main groups of *N. kaouthia* (H1-5) and four outgroup samples (H6-9); **B)** haplotype network of Control Region identifies nine main groups (H1-9) and four outgroup samples (H10-13).

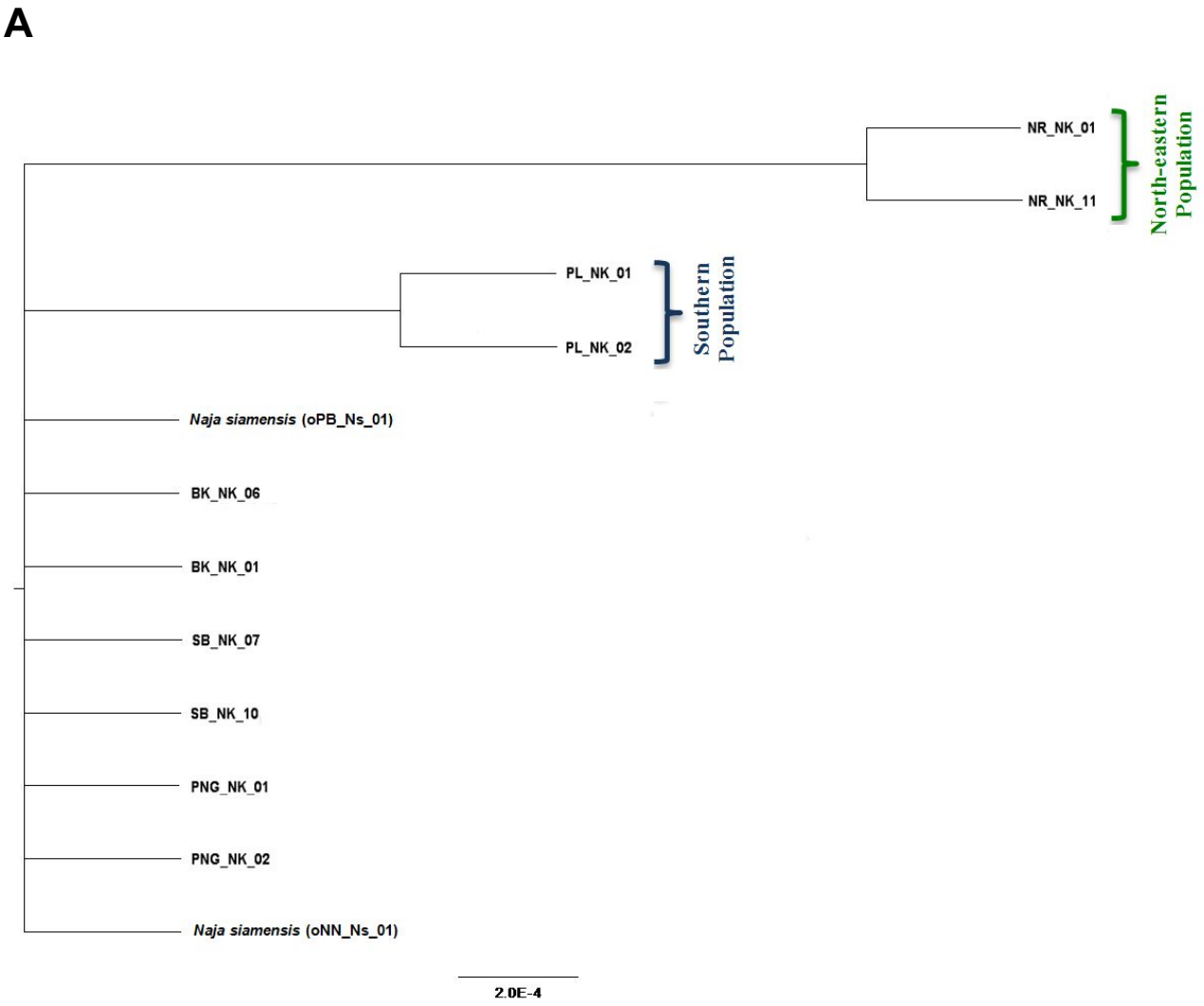

Fig. S4. A) Phylogenetic tree and **B)** haplotype network based on the nuclear loci *C-mos* for 12 samples of *N. kaouthia*. In Fig S4a support values are represented by BPP. Both analyses identify the north-east samples as divergent from all others, including the outgroup *Naja siamensis*.

Table S1. Details of the localities, area sizes, and numbers of cobras sampled

Population	Location	Sample No.	Genbank Accession Number			Sample No.	Genbank Accession Number		
			Cytb	CR	Cytb		Cytb	CR	Cytb
Central + North	**Provinces:** *Bangkok (BK)* **Location:** Jomthong and Bangkae Districts **Coordinates**: 13°42'20.0"N 100°25'21.0"E **Radius from the coordinates:** 9 km **Collector:** Donation from local resident to the snake farm	BK_NK_01 BK_NK_02 BK_NK_03 BK_NK_04 BK_NK_05	MK721266 MK721267 MK721268 MK721269 MK721270	MK721410 MK721411 MK721412 MK721413 MK721414	MK721317 - - -	BK_NK_06 BK_NK_07 BK_NK_08	MK721271 MK721272 MK721273	MK721415 MK721416 MK721417	MK721318 - -
	Provinces: *Samutprakarn (SMP)* **Location:** Bangphli District **Coordinates:** 13°37'29.3"N 100°44'00.6"E **Radius from the coordinates:** 11 km **Collector:** Donation from local resident to the snake farm	SMP_NK_01 SMP_NK_02	MK721274 MK721275	MK721418 MK721419	- -				
	Provinces: *Boundary between Saraburi and Phra-Nakhon-Si-Ayutthaya (SB)* **Location:** Angthong-Tharuea Street, Don Phut Sub-district, Don Phut District, 18210 **Coordinates:** 14°35'10.7"N 100°36'56.6"E **Radius from the coordinates:** 5 km **Collector:** Local snake catcher	SB_NK_01 SB_NK_02 SB_NK_03 SB_NK_04 SB_NK_05 SB_NK_06 SB_NK_07 SB_NK_08 SB_NK_09	MK721248 MK721249 MK721250 MK721251 MK721252 MK721253 MK721254 MK721255 MK721256	MK721392 MK721393 MK721394 MK721395 MK721396 MK721397 MK721398 MK721399 MK721400	- - - - - - MK721319 - -	SB_NK_10 SB_NK_11 SB_NK_12 SB_NK_13 SB_NK_14 SB_NK_15 SB_NK_16 SB_NK_17 SB_NK_18	MK721257 MK721258 MK721259 MK721260 MK721261 MK721262 MK721263 MK721264 MK721265	MK721401 MK721402 MK721403 MK721404 MK721405 MK721406 MK721407 MK721408 MK721409	MK721320 - - - - - - - -
	Provinces: *Sukhothai (SK)* **Location:** Sukhothai-Kamphaeng Phet Street, Ban Kluai Sub-district, Mueang District, 64000 **Coordinates:** 16°58'25.9"N 99°47'47.6"E **Radius from the coordinates:** 6 km **Collector:** Local snake catcher	SK_NK_01 SK_NK_02 SK_NK_03 SK_NK_04 SK_NK_05 SK_NK_06 SK_NK_07 SK_NK_08 SK_NK_09	MK721213 MK721214 MK721215 MK721216 MK721217 MK721218 MK721219 MK721220 MK721221	MK721329 MK721330 MK721331 MK721332 MK721333 MK721334 MK721335 MK721336 MK721337	- - - - - - - - -	SK_NK_10 SK_NK_11 SK_NK_12 SK_NK_13 SK_NK_14 SK_NK_15 SK_NK_16 SK_NK_17	MK721222 MK721223 MK721224 MK721225 MK721226 MK721227 MK721228 MK721229	MK721338 MK721339 MK721340 MK721341 MK721342 MK721343 MK721344 MK721345	- - - - - - - -
	Provinces: *Nakhon Sawan (Bueng Boraphet) (NW)* **Location**: Moo 8, Nakhon Sawan-Chaiyaphum Street, Thap Krit Tai Sub-district, Chum Saeng District, 60250 **Coordinates:** 15°44'55.9"N 100°14'55.8"E **Radius from the coordinates:** 8 km **Collector:** Local snake catcher	NW_NK_01 NW_NK_02 NW_NK_03 NW_NK_04 NW_NK_05 NW_NK_06 NW_NK_07 NW_NK_08 NW_NK_09	MK721230 MK721231 MK721232 MK721233 MK721234 MK721235 MK721236 MK721237 MK721238	MK721346 MK721347 MK721348 MK721349 MK721350 MK721351 MK721352 MK721353 MK721354	- - - - - - - - -	NW_NK_10 NW_NK_11 NW_NK_12 NW_NK_13 NW_NK_14 NW_NK_15 NW_NK_16 NW_NK_17 NW_NK_18	MK721239 MK721240 MK721241 MK721242 MK721243 MK721244 MK721245 MK721246 MK721247	MK721355 MK721356 MK721357 MK721358 MK721359 MK721360 MK721361 MK721362 MK721363	- - - - - - - - -
South	**Provinces:** *Prachuabkirikhan (PJ)* **Location:** n.a. **Collector:** Field-work of the snake farm staff	PJ_NK_01	MK721295	MK721383	-				
	Provinces: *Ranong (R)* **Location:** Bang Rin Sub-district, Mueang District, 85000 **Coordinates:** 9°54'51.5"N 98°37'41.2"E **Radius from the coordinates:** 15 km **Collector:** Local snake catcher	R_NK_01 R_NK_02 R_NK_03 R_NK_04 R_NK_05	MK721276 MK721277 MK721278 MK721279 MK721280	MK721364 MK721365 MK721366 MK721367 MK721368	- - - - -	R_NK_06 R_NK_07 R_NK_08 R_NK_09	MK721281 MK721282 MK721283 MK721284	MK721369 MK721370 MK721371 MK721372	- - - -
	Provinces: *Phattalung (PL)* **Location:** Moo 8, Thale Noi Sub-district, Khuan Khanun District, 93150 **Coordinates:** 7°48'11.8"N 100°06'24.7"E **Radius from the coordinates:** 12 km **Collector:** Local snake catcher	PL_NK_01 PL_NK_02 PL_NK_03 PL_NK_04 PL_NK_05 PL_NK_06	MK721285 MK721286 MK721287 MK721288 MK721289 MK721290	MK721373 MK721374 MK721375 MK721376 MK721377 MK721378	MK721323 MK721324 - - - -	PL_NK_07 PL_NK_08 PL_NK_09	MK721291 MK721292 MK721293	MK721379 MK721380 MK721381	- - -
	Provinces: *Trang (T)* **Location:** Moo 5, Chao Mai beach, Mai Fat Sub-district, Sikao District, 92150 **Coordinates:** 7°26'15.8"N 99°20'45.2"E **Collector:** Road-kill	T_NK_01	MK721294	MK721382	-				
Island	**Provinces:** *Pha Ngan Island (PNG)* **Location:** Ko Pha-ngan, Ko Pha-ngan District, Surat Thani, 84280 **Coordinates:** 9°43'13.7"N 99°59'47.3"E **Radius from the coordinates:** Within Island (12 km) **Collector:** Mr Stefan Pullitzky	PNG_NK_01 PNG_NK_02 PNG_NK_03 PNG_NK_04 PNG_NK_05 PNG_NK_06	MK721296 MK721297 MK721298 MK721299 MK721300 MK721301	MK721384 MK721385 MK721386 MK721387 MK721388 MK721389	MK721321 MK721322 - - - -	PNG_NK_07 PNG_NK_08	MK721302 MK721303	MK721390 MK721391	- -
Northeast	**Provinces:** *Nakhon Ratchasima (Sakaerat) (NR)* **Location:** Udom Sap Sub-district, Wang Nam Khiao District, 30370 **Coordinates:** 14°30'36.4"N 101°55'51.2"E **Radius from the coordinates:** 10 km **Collector:** Dr Bartosz Nadol	NR_NK_01 NR_NK_02 NR_NK_03 NR_NK_04 NR_NK_05 NR_NK_06	MK721304 MK721305 MK721306 MK721307 MK721308 MK721309	MK721420 MK721421 MK721422 MK721423 MK721424 MK721425	MK721325 - - - - -	NR_NK_07 NR_NK_08 NR_NK_09 NR_NK_10 NR_NK_11	MK721310 MK721311 MK721312 MK721313 MK721314	MK721426 MK721427 MK721428 MK721429 MK721430	- - - - MK721326
Out-Group	**Nan (NN):** *Naja siamensis*	oNN_Ns_01	MK721315	MK721431	MK721327				
	Phetchabun (PB): *Naja siamensis*	oPB_Ns_01	MK721316	MK721432	MK721328				

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Table S2. Details of *Naja* species used as outgroups in this study

Species	Vouchers	Genbank Accession number (Cytb and CR)	Publication
Naja atra	CIB093931	NC_011389.1 (mitochondrial DNA genome) (Location: Cytb = 14,941-16,057, and CR = 16,187-17,214)	Chen and Fu, 2008
Naja naja	n.a.	NC_010225.1 (mitochondrial DNA genome) (Location: Cytb = 14,939-16,055, and CR = 16,185-17,213)	Yan *et al.*, 2008

Table S3. Details of primers used in this study

Primer	Primer name	Primer sequence	Tm°	%GC	Length (bp)
Cyt *b*	NkParCytb-F01	5'- TTT GCT CCA ATA GAG TCC TGC GGC CTG - 3'	76.9	55.5	~665
	NkParCytb-R01	3'- TGA TGG TTA TCC TTC TTT GAT GTT CTG G - 5'	69.2	39.2	(partial)
CR	NkParCR-F01	5'- TCC CCC AGA ACA TCA AAG AAG GAT AAC C - 3'	72.3	46.4	~645
	NkPar1CR-R01	3'- TAA CGG GCA TAC CTG TGT CAG GTG AAA GT - 5'	73.5	48.2	(partial)
	Nk2ParCR-F02	5'- CAA GGT TGA GCT CGA TTC TTG GTC TGG C - 3'	75.9	53.5	~489
	Nk2ParCR-R02	3'- CTT GTG CTG TCA GGC ATG GCC GTC TTA GC - 5'	79.3	58.6	(partial)
C-mos	NkParCmos-F01	5'- CAT GGA CTG GGA TCA GTT ATG - 3'	n.a.	47.6	~567
	NkParCmos-R01	5'- CCT TGG GTG TGA TTT TCT CAC CT - 3'	n.a.	47.8	(partial)

For CR, the same pairs of primers were always used i.e. NkPCR-F01and NkP1CR-R01, and Nk2PCR-F02 and Nk2PCR-R02 for PCRs and sequencing.

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