

## **Population systematics of the snake genus *Naja* (Reptilia: Serpentes: Elapidae) in Indochina: Multivariate morphometrics and comparative mitochondrial DNA sequencing (cytochrome oxidase I)**

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### **Abstract**

We analyze the population systematics of Asiatic cobras in Indochina, China and the Andaman Islands by means of comparative sequencing of the cytochrome oxidase subunit I gene of the mitochondrial DNA molecule and multivariate analysis of morphological characters. Canonical variate analysis and mtDNA sequence information reveal that the cobras of this region comprise four distinct species: *Naja atra* from China and northern Vietnam, *Naja kaouthia* from Burma, central Thailand, Cambodia and southern Vietnam, *Naja siamensis* from Thailand, Cambodia and southern Vietnam, and *Naja sagittifera* from the Andaman Islands. The subspecies *N. kaouthia suphanensis* Nutaphand 1986 shows no mtDNA sequence difference from typical *N. kaouthia* from central Thailand, and multivariate analysis does not reveal differences in general phenotypic profile; the subspecies is therefore synonymised with *Naja kaouthia*. The cytochrome oxidase subunit I gene, little used in molecular taxonomy, is shown to be well suited for studies at the species level, as it shows taxonomically useful levels of interspecific divergence but low levels of intraspecific variation; this is particularly relevant for studies of rare

species, where sample size is a problem. The combination of multivariate morphometrics and molecular systematics can be particularly powerful in resolving systematic problems in such cases.

## Introduction

The systematics of the cobras of SE. Asia have been a source of considerable confusion for many decades. For many years, all Asiatic cobra populations were assigned to various subspecies of the single species *Naja naja* (e.g., Klemmer, 1963; Harding and Welch, 1980; Golay, 1985; Welch, 1988). In China and Indochina (here defined as Thailand, Cambodia, Vietnam and Laos), all populations were assigned to the two subspecies *Naja naja atra*, supposedly found in southern China and northern Vietnam, and *Naja naja kaouthia*, reported to occur throughout Burma, Thailand, Cambodia and in southern Vietnam. This classification predicts the existence of only one cobra form in any one part of this region.

However, a number of authors noted and discussed the existence of a variety of different cobra “forms” in Indochina, and in particular the occurrence of several “forms” of spitting cobra in parts of Thailand (Taylor, 1965; Nootpand, 1972; Nutaphand, 1986; Tumwipat and Nutaphand, 1982; Warrell, 1986; Lingenhöle and Trutnau, 1989; Cox, 1991).

Wüster and Thorpe (1989, 1990, 1991, 1992a) used multivariate analysis of morphological characters in order to elucidate the systematics of the Asiatic cobra group, and these findings have been followed by a number of other workers (e.g., Viravan et al., 1992; Golay et al., 1993; Welch, 1994). Wüster and Thorpe (1991) split the Asiatic cobra complex into 8 separate species. In Indochina, they noted the widespread sympatric occurrence of two species: the monocellate cobra (*Naja kaouthia*), and a group of highly variable spitting cobras. As a provisional arrangement, these spitting cobras were at first regarded as conspecific with the Chinese cobra (*Naja atra*). However, a preliminary analysis of the affinities of these highly variable spitting cobras using comparative mtDNA sequencing demonstrated that they should be regarded as a separate species, and they were assigned to the taxon *Naja siamensis* Laurenti, 1768 (Wüster and Thorpe, 1994).

Two further taxonomic problems remain unresolved within *N. kaouthia* sensu Wüster and Thorpe (1991, 1992a): one concerns the status of the cream-coloured subspecies *N. k. suphanensis*, described from central Thailand by Nutaphand (1986), and the other the cobra population of the Andaman Islands, which was described as *Naja tripudians* var. *sagittifera* by Wall (1913). *Naja k. suphanensis*, the “Suphan cobra”, is a relatively rare, pale yellowish coloured cobra, which is superficially quite distinct from the much more common “normally” coloured, monocellate *N. kaouthia*. This form, which has received very little attention in the literature outside Thailand, has been reported from the provinces of Suphan Buri, Ayutthaya, Ang Thong, Sing Buri and Ratchaburi in central Thailand (see Fig. 3). The Andaman cobra was widely recognised as a subspecies of *N. naja* before the recent revision of the Asiatic cobra species complex, although Whitaker (1978)

assigned it to *N. naja kaouthia*. Wüster and Thorpe (1991, 1992a, b), Golay et al. (1993) and Welch (1994) regarded the Andaman Islands cobra as part of *N. kaouthia*.

The analysis of population affinities based on morphological characters alone may be inadequate, because morphological characters can exhibit variation both as a result of phylogenesis (separate ancestry) and ecogenesis (current natural selection for environmental conditions). The sequencing of selectively neutral portions of the mitochondrial DNA molecule can recover evolutionary relationships obscured by ecogenetically caused morphological variation (Thorpe et al., 1994a). The development of the polymerase chain reaction (Saiki, 1988) and direct DNA sequencing using conserved primer sites (Kocher et al., 1989) have greatly eased the acquisition of mtDNA sequence information.

When working with relatively rare or difficult-to-obtain taxa, a number of compromises are necessary. In many cases, obtaining sufficient numbers of suitable tissue samples of all relevant species from a potentially wide distribution covering many countries, and often in the face of considerable legal and logistic obstacles, remains problematic. This situation is likely to worsen as conservation legislation is tightened further in many countries. In some cases, useful DNA can be obtained from preserved museum material, but in herpetology, this is often very difficult due to the practice of fixing specimens in formalin before storage in alcohol. Consequently, morphological studies, which can be carried out on museum material collected over past decades or centuries, will retain much of their usefulness. On the other hand, sequence information from a gene showing low within-group variability from a very few specimens can settle taxonomic problems which are difficult to resolve with multivariate morphometrics due to insufficient sample size. A combination of morphological and molecular techniques is therefore likely to be the most promising approach to any systematic problem where access to sufficient samples presents difficulties.

In this paper, we use a combination of multivariate analysis of morphological characters recorded from museum specimens and comparative sequencing of the cytochrome oxidase subunit I (CO I) gene to further investigate the population affinities of the cobras of Indochina. This gene has been used by few systematic workers (e.g., Brown et al., 1994; Yokobori et al., 1994), but has been shown to exhibit taxonomically useful levels of differentiation at the species level (Sperling and Dickey, 1994; Thorpe et al., 1994b).

## Materials and methods

### *Materials and character choice*

Specimens of *Naja* from Indochina, China and the Andaman Islands were obtained from a number of museums in Europe, the United States, and India, as well as from private sources in Thailand. A list of specimens is provided in Appendix 1. A large number of morphological characters relating to scalation,

**Table 1.** List of morphological characters used for multivariate analysis.

- 
1. No. of ventral scales
  2. No. of subcaudal scales
  3. %CS number of undivided subcaudal scales
  4. No. of cuneate scales
  5. No. posterior temporal scales
  6. No. of nuchal scale
  7. No. of dorsal scales at the 10th ventral scale
  8. No. of dorsal scales at 20% VS length
  9. No. of dorsal scales at 40% VS length
  10. No. of dorsal scales at 60% VS length
  11. No. of dorsal scales at 80% VS length
  12. No. of dorsal scales at 100% VS length
  13. %CS tail segments with two scale rows
  14. %CS position of reduction from 6 to 4 scale rows on tail
  15. %CS position of reduction from 8 to 6 scale rows on tail
  16. %CS position of reduction from 10 to 8 scale rows on tail
  17. %VS position of the anterior edge of the thyroid
  18. %VS position of the posterior tip of the heart
  19. %VS position of the systemic junction
  20. %VS position of the anterior tip of the liver
  21. %VS position of the posterior tip of the liver
  22. %VS position of the anterior tip of the pancreas
  23. %VS position of the junction between the cystic duct and the intestine
  24. %VS length of the cystic duct
  25. %VS position of the anterior tip of the right testis
  26. %VS position of the posterior tip of the right testis
  27. %VS position of the anterior tip of the left testis
  28. %VS position of the posterior tip of the left testis
  29. %VS position of the anterior tip of the right kidney
  30. %VS position of the posterior tip of the right kidney
  31. %VS position of the anterior tip of the left kidney
  32. %VS position of the posterior tip of the left kidney
  33. No. of lateral throat spots
  34. Length of frontal scale
  35. Width of frontal scale
  36. Distance between anterior edge of frontal scale and posterior edge of rostral scale
  37. Mean length of supraocular scales
  38. Length of suture between prefrontal scales
  39. Length of suture between parietal scales
  40. Mean length of parietal scales
  41. Head width
  42. Distance from snout tip to posterior end of interparietal suture (mean of both sides)
  43. Distance from snout tip to posterior end of lower jaw (mean of both sides)
  44. Head depth across the middle of the supraoculars
  45. Head depth from labial edge of supralabials to the top surface of the supraoculars
-

colour pattern, internal anatomy and body proportions were recorded from each specimen (Wüster, 1990).

The ventral scales were numbered according to Dowling (1951), and the positions of internal organs were recorded as the number of the ventral scale opposite which they are situated. In order to correct for variation in the number of ventral scales in different specimens, this was then converted into percent ventral scale (%VS) position (see Thorpe, 1975). Similarly, the subcaudals were numbered from the anal scale to the tail tip, and the positions of scale reductions along the tail was recorded as percent caudal scale (%CS) position. The snout-vent length was recorded with a piece of string to the nearest mm, and other body proportions characters to the nearest 0.01 mm with the aid of digital callipers. The characters used are listed in Table 1.

#### *Construction of operational taxonomic units (OTUs)*

Because the localities of most museum specimens used were widely scattered, it was necessary to pool specimens from several localities into one OTU in order to obtain statistically representative samples. In such situations, it is important to avoid the formation of compound localities with geographic variation or two sympatric species within them. OTUs were therefore defined a priori on the basis of collecting gaps and potential physiographic distribution barriers. These groups were then tested for geographic heterogeneity or the presence of several taxa by means of principal components analysis (PCA). Where such an analysis revealed geographic heterogeneity of the sample or the possible existence of two sympatric forms, the proposed OTUs were split into separate units for each phenotype. Populations from central Thailand were split a priori into specimens with "typical" monocellate marks, specimens conforming to literature descriptions of *N. k. suphanensis*, and specimens of the highly variable spitting cobra assemblage. All PCAs were run separately for each sex, to avoid difficulties due to sexual dimorphism. The OTUs established are listed in Table 2.

#### *Multivariate analysis techniques*

Since this study involves a mixture of meristic and linear measurements, the effect of ontogenetic growth was removed from the linear measurements by regressing them to the mean snout-vent length of 665 mm, using the pooled within-group regression coefficient obtained by means of analysis of covariance.

The population affinities of the OTUs were investigated by means of canonical variate analysis (CVA). This technique maximises the separation between groups relative to the within-group variance, taking into account the within-group correlation between characters (Thorpe, 1976, 1980), and is the technique of choice for the investigation of population differentiation.

**Table 2.** List of the OTUs

The numbers of the OTUs of *Naja kaouthia* are in agreement with Wüster and Thorpe (1992), others follow in sequence.

		♂♂	♀♀
<i>Naja kaouthia</i>			
19.	Central Thailand	23	15
20.	Phuket Island, Thailand	6	0
21.	Eastern slope of Malayan Peninsula	3	5
22.	Western slope of Malayan Peninsula	5	6
23.	Northern India and Bangladesh	17	15
24.	Rangoon area, southern Burma	6	2
25.	Southern Vietnam	3	2
26.	Northern Burma	5	3
27.	Sikkim area, India	4	0
28.	Hue area, Vietnam	3	0
29.	Assam, India	0	3
30.	Yongde, Yunnan, China	0	1
31.	Central Cambodia	0	1
"Naja kaouthia suphanensis"			
32.	Central Thailand	2	2
Andaman Islands			
33.	Andaman Islands	5	1
<i>Naja atra</i>			
34.	Northern Vietnam	9	8
35.	Hong Kong and Guangzhou area, China	9	6
36.	Eastern China	7	6
37.	Hainan Island, China	2	3
38.	Taiwan	8	3
39.	Guangxi Province, China	0	1
40.	Chusan Island, Fujian, China	1	0
<i>Naja siamensis</i>			
41.	Central Plain of Thailand	10	9
42.	Northeastern Thailand	12	0
43.	Northern Thailand	5	5
44.	Southeastern Thailand	4	1
45.	Khok Samrong, Lop Buri Prov., Thailand	5	2
46.	Southern Vietnam	2	2
47.	Trapeang Chan, Cambodia	0	5

The following CVAs were run: CVA 1 used the male specimens of OTUs 19 – 28, 32 – 38, and 40 – 46, using characters 1 – 12 and 17 – 45. CVA 2 was run on female specimens of OTUs 19, 21 – 26, 29 – 39, 41 and 43 – 47, using characters 1 – 24, 29 – 35, and 37 – 45. In order to further resolve the status of the Andaman cobras, CVA 3 was run on OTUs 19 – 28 and 32 – 33, using the same characters as CVA 1. The female equivalent is not included due to insufficient numbers of

female specimens from the Andaman Islands. Discrepancies in character and OTU use between the two analyses reflect missing data (due to the state of preservation of crucial specimens) and different specimen availability.

### *Molecular techniques*

Living cobra specimens for molecular work were obtained in Thailand with the help of local villagers and snake catchers. Large-scale dealers were not used, as locality records furnished by them would be difficult to authenticate. Biopsies were obtained from specimens of spitting cobra from four localities in Thailand (western Central Plain [Manorom District, Chainat Province, and Suphan Buri Province], eastern Central Plain [Khok Samrong District, Lop Buri Province], North [Hang Chat District, Lampang Province] and Northeast [Nam Phong District, Khon Kaen Province] – see Wüster and Thorpe, 1994), two specimens of *Naja kaouthia* from Suphan Buri Province, central Thailand, two specimens of “*N. k. suphanensis*” from Ayutthaya Province, central Thailand, and two specimens of *Naja atra* from the Chinese mainland near Hong Kong.

The biopsies were homogenised in STE buffer. Proteins were extracted by overnight proteinase K digestion at 37° C and phenol, phenol chloroform and chloroform extraction. The DNA was purified by ethanol precipitation. A 544 base pair segment of the cytochrome oxidase subunit I gene was amplified by means of the polymerase chain reaction (PCR) (Saiki, 1988). The primer sequences were adapted from Kessing et al. (1989):

5'-GAATCCCAGAGATTAGAGGGAATCAGTG-3' and

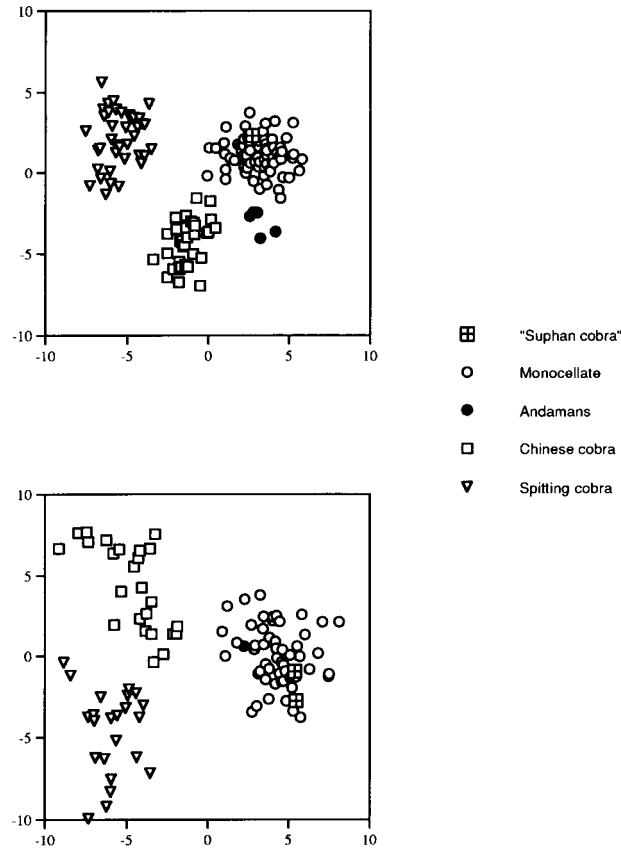
5'-GAATCCCTGCAGGAGGAGGAGACCC-3'.

Direct sequencing (Kocher et al., 1989) of both strands of the amplified segments was accomplished by the dideoxy method (Sanger et al., 1977). The sequences were aligned against the human mtDNA sequence (Anderson et al., 1981), against which there are no deletions or insertions. The sequence runs from the equivalent of position 6660 to 7064 of the human mtDNA sequence.

## **Results**

### *Multivariate morphometrics*

CVA 1 (Fig. 1) shows four principal groups within the male cobra specimens from Indochina: the spitting cobras, from Thailand and southern Vietnam; the Chinese cobras, from southern China and northern Vietnam; the monocellate cobras from northern India, Burma, Thailand and southern Vietnam; and the cobras from the Andaman Islands. The “Suphan” cobras are not distinct from the monocellate cobras. CVA 2 (Fig. 1) shows a very similar picture in the females,



**Fig. 1.** Ordination of the male specimens of all OTUs included in the study along the first two canonical variates of CVA 1 (top) and the female specimens of all OTUs included along the first two canonical variates of CVA 2 (bottom).

except in that the OTU from the Andaman Islands is not shown to be very distinct (however, it is difficult to draw definitive conclusions from multivariate analyses involving single-specimens OTUs), and that the separation between the spitting cobras and the Chinese cobras is less clear than in CVA 1.

CVA 3 (Fig. 2) shows more clearly the distinct nature of the Andaman sample, while re-emphasising the lack of phenetic differentiation between the “Suphan cobra” and the monocellate specimens.

#### *MtDNA sequence information*

The 408 base pair region of cytochrome oxidase subunit I gene sequenced for all 4 putative taxa (Tab. 3) shows a very clear pattern of differentiation (Tab. 4): the

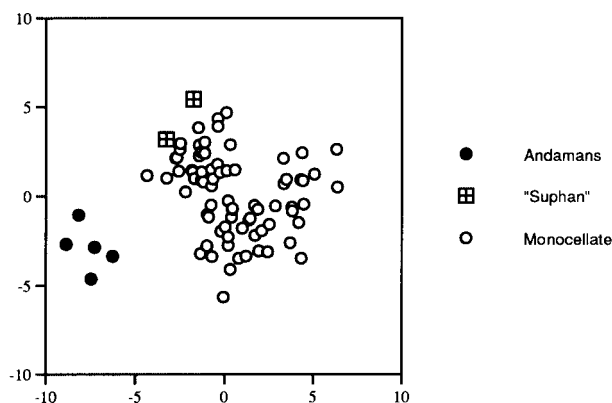


Fig. 2. Ordination of the male specimens of *Naja kaouthia*, "*N. k. suphanensis*" and the Andaman cobra along the first two canonical variates of CVA 3. Note the highly distinct nature of the specimens from the Andaman Islands.

spitting cobras, the Chinese cobra (*Naja atra*) and the Monocellate cobra (*Naja kaouthia*) are clearly distinct. The spitting cobras are almost as distinct from the Chinese cobras, with which they were regarded as conspecific by previous workers (Lingenhöle and Trutnau, 1989; Wüster and Thorpe, 1991), as they are from the sympatric monocellate cobras; the monocellate and Chinese cobras are less distinct from each other in their mtDNA sequence than either is from the spitting cobras. The sequence from the "Suphan" cobra shows no differences whatsoever from the sequence of the monocellate cobra. There is no geographic variation in sequence between the different geographical samples of spitting cobra, and there is only one single case of intraspecific variation in sequence at the equivalent of position 6987 of the human mtDNA molecule (Anderson et al., 1981) within the sample of spitting cobra from northern Thailand.

## Discussion

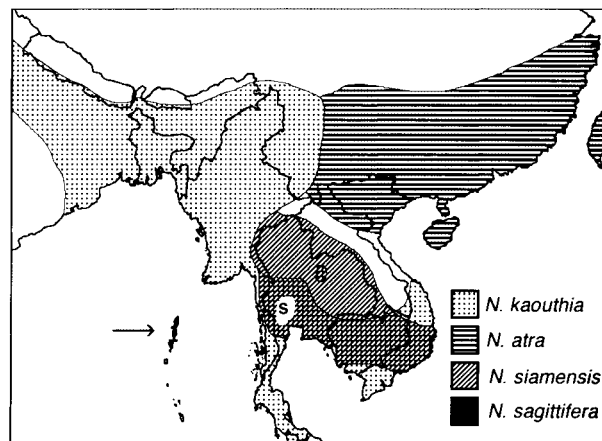
The results of this combined morphological and molecular taxonomic study clarify a number of systematic problems concerning the genus *Naja* in China and Indochina, which were hitherto unresolved. The multivariate analyses reveal the existence of four distinct cobra taxa in China, Indochina and the Andamans: the monocellate cobras (*Naja kaouthia*), the Chinese cobra (*Naja atra*), the Indochinese spitting cobra and the Andaman population. The distinctness of these taxa is fully supported by the mtDNA sequence information, where available (not for the Andamans). The distribution of the four taxa is mapped in Fig. 3.

**Table 3.** Aligned L-strand sequence data for a 408 base pair region of the cytochrome oxidase subunit I gene. Abbreviations: KS: Spitting cobras from Khok Samrong, Lop Buri Province, Thailand ( $N = 2$ ); NE: Spitting cobra from Nam Phong District, Khon Kaen Province, Thailand ( $N = 1$ ); N: Spitting cobras from Hang Chat District, Lampang Province, Thailand ( $N = 2$ ); WCP: Spitting cobras from Suphan Buri and Chainat Provinces, Western Central Plain, Thailand ( $N = 2$ ); MO: Monocellate cobra, Suphan Buri Province, Thailand ( $N = 2$ ); SU: "Suphan cobra", Bang Pa-in District, Ayutthaya Province, Thailand ( $N = 2$ ); HK: Chinese cobra, Chinese mainland near Hong Kong ( $N = 2$ ).

	Ile	Val	Ser	Ser	Ile	Ile	Thr	Phe	Tyr	Ser	Gly	Lys	Lys	Asn	Thr	Phe	Gly	Tyr	Thr	Ser
KS	ATT	GTG	TCT	AGT	ATC	ATC	ACC	TTT	TAT	ACT	GGG	AAA	AAA	AAC	ACC	TTT	GGC	TAC	ACA	AGC
NE	...	..G	...	..T	...	...	...	..T	...	...	..G	...	..A	...	..C	...	...	...	...	...
N	...	...	...	...	...	...	...	...	...	...	..G	...	..A	...	..C	...	...	...	...	...
WCP	...	..G	...	..C	...	...	...	..C	...	...	..G	...	..G	...	..T	...	...	...	...	...
MO	...	..G	...	..C	...	...	...	..C	...	...	..G	...	..G	...	..T	...	...	...	...	...
SU	...	..G	...	..C	...	...	...	..C	...	...	..G	...	..G	...	..T	...	...	...	...	...
HK	...	..C	...	..T	...	...	...	..T	...	...	..A	...	..A	...	..T	...	...	...	...	...
	Met	Ile	Trp	Ala	Met	Met	Ser	Ile	Ala	Ile	Leu	Gly	Phe	Ile	Val	Trp	Ala	His	His	Met
KS	ATA	ATC	TGA	GCA	ATA	ATG	TCT	ATT	GCA	ATC	CTA	GGC	TTT	GTT	GTA	TGG	GCC	CAC	CAC	ATA
NE	...	...	...	...	...	..G	...	...	...	...	..C	..C	...	...	...	...	...	...	..C	...
N	...	...	...	...	...	..G	...	...	...	...	..C	..C	...	...	...	...	...	...	..C	...
WCP	...	...	...	...	...	..G	...	...	...	...	..C	..C	...	...	...	...	...	...	..C	...
MO	...	...	...	...	...	..A	...	...	...	...	..C	..T	..T	...	...	...	...	...	..T	...
SU	...	...	...	...	...	..A	...	...	...	...	..C	..T	..T	...	...	...	...	...	..T	...
HK	...	...	...	...	...	..A	...	...	...	...	..T	..T	..T	...	...	...	...	...	..T	...
	Phe	Thr	Val	Gly	Leu	Asp	Ile	Asp	Ser	Arg	Ala	Tyr	Phe	Thr	Ala	Ala	Thr	Met	Ile	Ile
KS	TTC	ACC	GTA	GGC	CTT	GAC	ATT	GAC	AGC	CGT	GCC	TAT	TTC	ACC	GCA	GCA	ACA	ATA	ATT	ATC
NE	..C	...	...	...	...	..C	...	...	...	..T	...	...	...	...	...	...	...	...	..C	...
N	..C	...	...	...	...	..C	...	..C	...	..T	...	...	...	...	...	...	...	...	..C	...
WCP	..C	...	...	...	...	..C	...	..C	...	..T	...	...	...	...	...	...	...	...	..C	...
MO	..T	...	...	...	...	..T	...	..T	...	..T	...	...	...	...	...	...	...	...	..T	...
SU	..T	...	...	...	...	..T	...	..T	...	..T	...	...	...	...	...	...	...	...	..T	...
HK	..C	...	...	...	...	..T	...	..C	...	..C	...	...	...	...	...	...	...	...	..C	...
	Ala	Ile	Pro	Thr	Gly	Ile	Lys	Val	Phe	Gly	Trp	Leu	Ala	Thr	Leu	Ala	Gly	Gly	Gln	Ile
KS	GCC	ATT	CCC	ACA	GGA	ATC	AAA	GTA	TTC	GGT	TGA	CTG	GCC	ACA	CTA	GCA	GGA	GGT	CAA	ATT
NE	...	...	...	...	...	..C	...	...	...	..T	...	...	...	...	...	...	...	...	..T	...
N	...	...	...	...	...	..C	...	...	...	..T	...	...	...	...	...	...	...	...	..T	...
WCP	...	...	...	...	...	..C	...	...	...	..T	...	...	...	...	...	...	...	...	..T	...
MO	...	...	...	...	...	..C	...	...	...	..C	...	...	...	...	...	...	...	...	..C	...
SU	...	...	...	...	...	..C	...	...	...	..C	...	...	...	...	...	...	...	...	..C	...
HK	...	...	...	...	...	..C	...	...	...	..T	...	...	...	...	...	...	...	...	..C	...
	Lys	Trp	Gln	Thr	Pro	Val	Tyr	Trp	Ala	Leu	Gly	Phe	Ile	Phe	Leu	Phe	Thr	Val	Gly	Gly
KS	AAG	TGA	CAA	ACA	CCC	ATC	TAC	TGA	GCT	CTG	GGG	TTT	ATC	TTC	CTA	TTT	ACT	GTC	GGG	GGT
NE	..G	...	...	...	..C	...	...	...	..T	..G	...	...	...	...	...	...	...	..C	..G	...
N	..G	...	...	...	..C	...	...	...	..T	..G	...	...	...	...	...	...	...	..C	..G	...
WCP	..G	...	...	...	..C	...	...	...	..T	..G	...	...	...	...	...	...	...	..C	..G	...
MO	..A	...	...	...	..G	...	...	...	..C	..C	...	...	...	...	...	...	...	..T	..A	...
SU	..A	...	...	...	..G	...	...	...	..C	..C	...	...	...	...	...	...	...	..T	..A	...
HK	..A	...	...	...	..G	...	...	...	..C	..T	...	...	...	...	...	...	...	..T	..G	...
	Met	Thr	Gly	Ile	Ile	Leu	Ala	Asn	Ser	Ser	Leu	Asp	Ile	Val	Leu	His	Asp	Thr	Tyr	Tyr
KS	ATA	ACA	GGT	ATT	ATT	CTA	GCA	AAC	TCG	TCA	CTA	GAT	ATC	GTC	CTA	CAC	GAC	ACT	TAC	TAC
NE	...	...	..T	..T	...	...	...	...	...	...	..A	..T	..C	...	..C	...	...	..T	...	...
N	...	...	..T	..T	...	...	...	...	...	...	..A	..T	..C	...	..C	...	...	..T	...	...
WCP	...	...	..T	..T	...	...	...	...	...	...	..A	..T	..C	...	..C	...	...	..T	...	...
MO	...	...	..A	..C	...	...	...	...	...	...	..A	..T	..C	...	..T	...	...	..C	...	...
SU	...	...	..A	..C	...	...	...	...	...	...	..A	..T	..C	...	..T	...	...	..C	...	...
HK	...	...	..A	..C	...	...	...	...	...	...	..G	..C	..T	...	..T	...	...	..C	...	...
	Val	Val	Ala	His	Phe	His	Tyr	Val	Leu	Ser	Met	Gly	Ala	Val	Phe	Ala				
KS	GTA	GTA	GCA	CAC	TTC	CAC	TAT	GTC	CTC	TCT	ATG	GGG	GCA	GTA	TTC	GCC				
NE	...	...	..A	...	...	...	...	...	..C	...	...	..G	..	...	...	...				
N	...	...	..A	...	...	...	...	...	..C	...	...	..G	..	...	...	...				
WCP	...	...	..A	...	...	...	...	...	..C	...	...	..G	..	...	...	...				
MO	...	...	..G	...	...	...	...	...	..A	...	...	..A	...	...	...	...				
SU	...	...	..G	...	...	...	...	...	..A	...	...	..A	...	...	...	...				
HK	...	...	..G	...	...	...	...	...	..C	...	...	..G	...	...	...	...				

**Table 4.** Pairwise mtDNA sequence divergences between the spitting, monocellate, "Suphan" and Chinese cobra samples used in this study. Figures above the diagonal indicate percentage base pair differences, figures below the diagonal the absolute number of base pairs.

	Spitting cobra	Monocellate cobra	"Suphan cobra"	Chinese cobra
Spitting cobra	0.25% 1/408 bp	6.62%	6.62%	5.64%
Monocellate cobra	27/408 bp	-	0	4.41%
"Suphan cobra"	27/408 bp	0	-	4.41%
Chinese cobra	23/408 bp	18/408 bp	18/408 bp	



**Fig. 3.** Distribution of the four cobra species revealed by this study. The arrow indicates the Andaman Islands, which constitute the range of *Naja sagittifera*. The unshaded area in central Thailand, labelled with the letter "S", indicates the reported range of "*Naja kaouthia suphanensis*", which was shown to be a colour variety of *Naja kaouthia* in this paper. The precise distribution limits of *Naja kaouthia*, *Naja atra* and *Naja siamensis* in Laos, southwestern China, central Vietnam and parts of Burma are still unclear.

#### *The status of the spitting cobras*

Using multivariate analysis of much fewer specimens than available in the present study, Wüster (1990) and Wüster and Thorpe (1991) regarded the Indochinese spitting cobras as conspecific with the Chinese *Naja atra*, based on an apparent phenotypic cline from southwestern Thailand to southern China. The more extensive spitting cobra material available in this study clarified the distinct nature of these populations, especially in the case of the male specimens (CVA 1). In the case of the female specimens (CVA 2), the distinction is less clear. The mtDNA sequence analysis confirms their status as a distinct species: the spitting cobras are as distinct from the Chinese cobras as they are from the sympatric (and therefore by definition

non-conspecific) monocellate cobra, and it is therefore logical to regard them as a distinct species. This is particularly so in view of the lack of intraspecific variation in the sequence of this gene: the morphologically distinct populations of spitting cobra from 4 different localities in Thailand show no among-population variation in their CO I sequence. The valid scientific name for the spitting cobras is *Naja siamensis* Laurenti, 1768 (Wüster and Thorpe, 1994).

#### *The status of the Andaman cobra*

CVA 1 shows that the Andaman Island population is clearly differentiated from all other *Naja* populations in Indochina, and CVA 3 demonstrates its very clear distinctness from *Naja kaouthia*, with which it was hitherto regarded as conspecific. There are therefore ample reasons to give this form some level of taxonomic recognition. The question of whether such allopatric populations should be given species or subspecies rank has been a vexing question for many decades. Since the Andaman Island population is allopatric with respect to all other *Naja* species, the biological species concept, which uses reproductive isolation as the sole criterion for species status, is inapplicable in this case.

In recent years, there has been an increasing trend away from the biological species concept and the related recognition of subspecies, and towards species concepts employing historical definitions of species (Frost and Hillis, 1990; Frost et al., 1992). Under the phylogenetic and evolutionary species concepts, allopatric populations showing clear evidence of a separate evolutionary history are regarded as separate species rather than as subspecies. We feel that in the case of the Andaman cobra, the evidence presented here supports the elevation of this form to the rank of a full species. The very clear morphological differentiation shown by the specimens of this population provides firm evidence that this form is on a different evolutionary trajectory than the mainland *Naja kaouthia*. We therefore feel that the evidence provided here justifies elevating the Andaman population to species rank, and we therefore raise the Andaman cobra to the rank of a full species, *Naja sagittifera* Wall, 1913.

#### *The status of Naja kaouthia suphanensis Nutaphand 1986*

The status of this form has been clearly resolved by this study. Multivariate analyses of morphological characters (CVAs 1, 2 and 3) have all failed to show any differentiation between specimens assigned to this form and typical *N. kaouthia*. However, because of the small sample size available for this taxon, the evidence based on multivariate morphometrics alone would not necessarily be conclusive. However, the comparative sequencing of the CO I gene showed a complete absence of sequence differentiation between *N. kaouthia* and *N. k. suphanensis*.

Since "*Naja kaouthia suphanensis*" is sympatric with typical *N. kaouthia* in Thailand, it has to be either a full species, or a taxonomically irrelevant colour

morph. The CO I gene has been shown to exhibit taxonomically useful levels of differentiation between closely related species or well differentiated groups of conspecific populations (Thorpe et al., 1994b). The absence of sequence divergence between “*N. k. suphanensis*” and typical *N. kaouthia*, coupled with an absence of morphological divergence, therefore constitutes strong evidence of an absence of a separate evolutionary history. We therefore formally synonymise *N. k. suphanensis* Nutaphand, 1986 with *N. kaouthia* Lesson, 1831.

#### *The systematic value of the CO I gene*

The CO I gene sequenced in this study has received relatively little attention in the literature, when compared to more popular genes such as cytochrome b, 12s and 16s rRNA and CO II. This gene is known to exhibit a slow rate of evolution, and to be subject to considerable selective constraint, as the rate of amino acid substitutions is very low even between very distantly related taxa, even belonging to different phyla (Jacobs et al., 1988; Simon, 1991). All base pair substitutions in the three cobra species examined here are silent, reflecting the selective constraint on the structure of the CO I protein. In this study, we have been able to confirm the results of Thorpe et al. (1994b), in which the CO I gene was shown to exhibit taxonomically useful levels of variation between species and well differentiated intraspecific lineages, while showing very low levels of variation within populations, and among relatively closely related populations. This combination of characteristics makes it very useful in studies such as this, where sample size can be a limiting factor due to various restrictions.

This study also clearly demonstrates the value of a combined morphological and molecular approach to problems of species-level systematics. A purely multivariate approach to the problems of the systematics of the Indochinese populations of *Naja* resolved only some of the problems of the genus in the area, but left others, such as the interrelationships of the Chinese and spitting cobra unresolved. Due to the small number of specimens available of this rare form, the status of “*Naja kaouthia suphanensis*” would also have been difficult to resolve by means of multivariate morphometrics. On the other hand, the use of multivariate morphometrics, using material collected over the last 150 years from localities where collecting tissue biopsies would today be logistically, administratively and financially difficult, allowed the resolution of the problem of the status of the Andaman cobra, allowed a much clearer definition of the distribution of the taxa concerned, and was responsible for generating many of the questions addressed through the use of the molecular methods.

#### *Practical implications of this study: snakebite, venom research and conservation*

The results of this study have a number of implications in fields other than systematics, especially concerning public health and conservation. Cobras are an

important cause of snakebite mortality and morbidity in many parts of SE Asia (Reid, 1964; Looareesuwan et al., 1988; Viravan et al., 1986, 1992). Antivenoms are produced principally against *Naja kaouthia*. However, recent studies in Thailand have indicated that *Naja siamensis* is in fact responsible for a far higher number of bites than *Naja kaouthia* (Viravan et al., 1992). The question of antivenom efficacy against bites by *Naja siamensis* is so far unresolved. Comparative studies on the venoms of these forms are urgently required.

The results of this study also have implications for the conservation of these snakes. *Naja sagittifera* is known only from very few specimens, and appears to be relatively uncommon in its range. Since this form has here been shown to be a separate species from *Naja kaouthia*, the problem of its conservation needs to be addressed separately from that concerning *Naja kaouthia*. The latter has a very wide range, and adapts well to habitat changes associated with agricultural activities. Although it has become locally rare or even extinct due to excessive hunting for food, traditional oriental medicines and leather products, its survival as a species is unlikely to become imperiled in the short or medium term. Furthermore, it is one of the most commonly captive-kept and bred cobra species, although often under erroneous identification. *Naja sagittifera* has a very restricted range, and appears to be a rare form there, and has not, to our knowledge, been kept or bred in captivity. Due to its small range and apparent rarity, it must be regarded as vulnerable. Fieldwork in the Andamans is urgently required to investigate the current status of this form, and to identify potential threats to its continued survival. On the other hand, the "Suphan" cobra had been regarded as a rare taxon with a restricted distribution (Cox, 1991). This study has shown that it is in fact just a colour variety of a common taxon, and as such does not warrant any special conservation measures.

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## Appendix 1

Specimens examined for analysis of variation in morphology. Museum acronyms according to Leviton et al., 1985. WW = Wolfgang Wüster, personal collection;

WW-TISTR = uncatalogued voucher specimens deposited in Thailand Institute of Scientific and Technological Research, Bangkok (with WW field numbers).

*Naja kaouthia*: BURMA – CHIN; Falam (BNHM 2262). IRRAWADDY; Bassein (ZMH R 03117). KACHIN; Bhamo (ZMH R 3105), Myitkyina (AMNH 58524), Sumprabum (BMNH 1974.905), Triangle (BMNH 1940.6.5.64–6). MAN-DALAY; Pyawbwe (BNHM 2263). PEGU; Rangoon (CAS 12418; NHRM 1935.3.19.3293, NHRM NNN 1975.999.3056A, 1975.999.3056B, 1975.999.3056C, 1975.999.3057B), Taikkyi (UF 48842), Tharawaddy (BMNH 1987.710). SARGAING; Mogok (BMNH 1900.9.20.17–8). CAMBODIA – Kampot (FMNH 11545), Snoc Trou (MNHN 1963.735). CHINA – YUNNAN; Yongde (UF 63902). INDIA – ASSAM; “Assam” (ZMUC 65318, 65344), Dibrugarh (USNM 118974). BIHAR; Bettiah (BMNH 1940.3.7.33). HARYANA; Sonipat (UF 20521). SIKKIM; Sikkim (FMNH 15822–3). UTTAR PRADESH; Rampur (BNHM 2251), Sonaripur (BMNH 1940.3.7.34). WEST BENGAL; “Bengal” (RMNH 1319; SMF 20618; UMZC R 9.177/11, 9.177/14, 9.177/16; ZMUC R 65316–7, 65487, 65495), Burigoalni (MCZ 58407), Calcutta (BMNH 60.3.19.1339, CM 91864; MHNG 1328.17–8, 1328.21–2; NHRM 1828.168.4602; NMW 27755:1; ZMUC R 6513, 6536–7, 65315, 65320, 65323), Canning Thana (FMNH 165076, 165080–3, 165085), Darjeeling (BNHM 2247), Dum-Dum (LACM 104329–30), Howrah (BNHM 2244, 2296), Jalpaiguri (BNHM 2245), Tarda Thana (FMNH 165075, 165077, 165079). MALAYSIA – KEDAH; Alor Setar (BMNH 98.9.22.56), Kulim (BMNH 95.10.7.22). KELANTAN; “Kelantan” (BMNH 1905.2.7.9), Kota Bharu (BMNH 1913.7.24.6). PENANG; Georgetown (FMNH 118998), Kapala Batas (ZFMK 16544). THAILAND – AYUTTHAYA; Ayutthaya (BMNH 1974.5499). BANGKOK; Bangkok (BMNH 97.10.8.33, 98.11.8.34, 1921.4.1.25; MCZ 8386; MHNG 1328.19–20; NMBE 351A, 351B, 351C, 351D; NHRM 1914.989.3528–9, 1914.989.4550, 1914.989.5531, 1914.989.5550; NMW 22800:2, 27780:2, 27794; UMMZ 65343; USNM 94762; ZMH R 02885, 02896; ZMUC R 65354). CHACHOENGSAO; Chachoengsao (BMNH 1987.647). CHON BURI; Siracha (BMNH 1968.834–5). KANCHANABURI; Kanchanaburi (BMNH 1987.640), Sai Yok (BMNH 1987.638–9), Tong Pha Phum (BMNH 1987.641). KRABI; Krabi (BMNH 1987.652–3; ZFMK 16678). NAKHON PATHOM; Nakhon Pathom (BMNH 1987.655). NAKHON RATCHASIMA; Sakaerat, Amphoe Pak Thong Chai (FMNH 180603). NAKHON SAWAN; Bung Borapet (USNM 81843), Nakhon Sawan (FMNH 60960). NAKHON SI THAMMARAT; Nakhon Si Thammarat (BMNH 1987.654); PHANGNGA; Phangnga (BMNH 1987.642). PATHUM THANI; Pathum Thani (BMNH 1987.686). PATTANI; Na Pradoo (FMNH 179120). PHATTALUNG; Phattalung (BMNH 1987.645; FMNH 191096). PHITSANULOK; Phitsanulok (BMNH 1987.643), Tha Law (NHRM 1912.169.5394). PHUKET; Phuket (BMNH 1902.12.12.5, 1977.2027–2031). SAMUT PRAKAN; Bang Phli (BMNH 1987.646, 1987.656). SATUN; Satun (BMNH 1987.637). SONGKHLA; Songkhla (FMNH 179124). SUPHAN BURI; Suphan Buri (BMNH 1987.694). TRANG; Trang (BMNH 1987.628–9). YALA; Biserat (UMZC R 9.177/5). VIETNAM – “Cochinchina” (FMNH 11546–7, MNHN 1892.93), Da Lat (BMNH 1921.4.1.43), Ho Chi Minh City/Saigon (MTKD D

24167), Quang Tri (USNM 165072), Thua Luu, Hue (NHRM BJÖ 1939.989.3096A, 1939.989.3096B).

“*Naja kaouthia suphanensis*”: THAILAND – AYUTTHAYA; Bang Pa-in (WW 51); RATCHABURI (TNRC, 2 uncatalogued specimens); UNKNOWN (TNRC, 1 uncatalogued specimen; WW, 1 unnumbered specimen).

*Naja atra*: CHINA – ANHUI; Chin Hua Shan, Ching Yang Hsien (NHRM JGA 1920.180.5610). FUJIAN; Fuzhou (BMNH 1940.3.19.19–20, USNM 67695–6), “Northern Fujian” (MHNG 2165.7), No specific locality (ZMH R 03118). GUANGDONG; Guangzhou (SMF 20626–7, CAS 74503), Tung Kun (MHNG 675.86, 1464.77, 1465.29), Shantou (CAS 14929). GUANGXI; Guangxi Province (MHNG 1406.27). HAINAN DAO; Nodda (FMNH 6634), No specific locality (FMNH 6633, 6636, NMW 27806:3, SMF 20628). HUNAN; Southwestern Hunan (USNM 63190). ZHEJIANG; Tung Lu (MCZ 28830–3), Zhoushan Island (BMNH 92.12.12.12). HONG KONG (BMNH 1931.8.4.8, 1956.1.13.11–12, 1983.271–2). TAIWAN; Taitung (USNM 142460, 142467), No specific locality (BMNH 1953.1.2.82–3, CAS 19028, 19031–2, MHNG 1166.71, NMW 27807). VIETNAM; Bac Giang (FMNH 15275), Bao Lac (MHNP 1904–406), Blan Son Mountains (BMNH 1912.3.25.3), Gia Lam (MHNP 1935.106–7), Ngan Son (MHNP 1935.109), Phu Lang Thuong (CAS 16704), “Tonkin” (BMNH 1912.3.25.2–3, FMNH 124107, MHNP 5518, 1897.314, 1908.215, 1911.21–21A, MTKD D 11629).

*Naja sagittifera*: INDIA – ANDAMAN ISLANDS; South Andaman (ZMUC R 65324), Port Blair (BNHM 2222), No specific locality (BMNH 1940.3.9.12, 1940.3.9.14, NMW 27781, BNHM 1378).

*Naja siamensis*: CAMBODIA – Trapeang Chan (MHNP 1970.575–8, 580). THAILAND – BURIRAM; Khu Muang District (BMNH 1987.670). CHAINAT; Manorom (BMNH 1987.657; WW-TISTR 26, 29). CHANTHABURI; (BMNH 1987.633–4); Khao Saming (BMNH 1987.632); Laem Sing (USNM 84802). CHIANG MAI; (USNM 101532). CHIANG SAEN; Ban Malva (BMNH 1969.1928). CHON BURI; Bang Saen (FMNH 191122); Siracha (BMNH 1968.833). KAMPHAENG PHET; (BMNH 1987.695–6). KANCHANABURI; Sai Yok Camp (BMNH 1987.704–5). KHON KAEN; (BMNH 1987.636, BMNH 1987.683–4); Ban Phai (BMNH 1987.678); Nam Phong District (WW 48). LAMPANG; Hang Chat District (WW 37, 39; WW-TISTR 32); Mae Tae District (BMNH 1987.672). LOP BURI; Khok Samrong District (WW 21, WW-TISTR 1, 4, 8, 19, 22). MAE HONG SON (BMNH 1987.677). NAKHON PHANOM; Nakhon Phanom (USNM 279013). NAKHON RATCHASIMA; Sakaerat, Amphoe Pak Thong Chai (FMNH 180150). NAKHON SAWAN (BMNH 1987.699). PHETCHABUN; Lom Sak (BMNH 1987.679). PHITSANULOK; Phitsanulok City District (BMNH 1987.673–6). PHRAE; Den Chai (BMNH 1938.8.7.61). ROIET; Tawatchaburi District (BMNH 1987.635). SAKON NAKHON (BMNH 1987.688–9). SING BURI (WW-TISTR 5). SISAKET; (BMNH 1987.690). SUPHAN BURI; (BMNH 1987.697; WW BW2; WW-TISTR 10, 13, 15). UDON THANI; (FSM 65737); UTHAI THANI; (BMNH 1987.702–3). UTTARADIT; (1987.671). YASOTHON; Yasothon (BMNH 1987.687). VIETNAM – “Cochinchina” (NHRM 1987.989.3605); Phu Bon Province (MHNP 1973.145); Saigon (MHNP 1974.1342–3); Unknown (Choray Hospital collection, unnumbered).