0014-4754/94/010075-05$1.50 + 0.20

Naja siamensis, a cryptic species of venomous snake revealed by mtDNA sequencing

W. Wüster and R. S. Thorpe

School of Biological Sciences, University College of North Wales, Bangor, Gwynedd, LL57 2UW (Wales, UK)
Received 22 July 1993; accepted 13 September 1993

Abstract. Because of possible variation in venom composition, an understanding of venomous snake systematics is of great importance for the optimization of antivenom treatment of snakebite patients. Intraspecific variation in the morphology of many venomous snakes complicates the definition and identification of some species when allopatric populations are involved. Selectively neutral or near-neutral mtDNA sequences can reveal evolutionary relationships obscured by ecogenetically-caused morphological variation. We use comparative sequencing of the cytochrome oxidase subunit 1 gene to reveal the existence of a widespread, cryptic species of spitting cobra from southeast Asia. This species, *Naja siamensis*, is widely sympatric with other Asiatic cobra species. This may be of considerable medical significance, and calls for further research into venom composition in Asiatic cobras.
Key words. MtDNA; sequencing; molecular systematics; *Naja*; Serpentes; Elapidae; cryptic species; snakebite; antivenom.

It is estimated that, worldwide, between 500,000 and 1 million persons are killed or injured by venomous snakes every year[1,2]. The only specific treatment of snake venom poisoning is antivenom, i.e., animal-produced antiserum against the venom antigens. Venom composition can vary considerably even between closely related species, so that an antivenom against one species may be ineffective against another. Understanding the systematics of venomous snakes is therefore one of the fundamental prerequisites for the optimization of antivenom treatment as well as for the rationalization of venom and clinical research[3].

Asiatic cobras are responsible for a considerable proportion of the snakebites recorded in many southeast Asian countries [4–8]. Extensive geographic variation in venom effects and immunology has been documented[3,5,7,9]. The Asiatic cobra group has a long history of taxonomic confusion, which was only recently largely resolved by means of multivariate morphometrics[10–14]. However, doubts have remained regarding the interrelationships of the cobra populations of Indochina, especially Thailand[12,14]. In particular, the affinities of certain populations of spitting cobras have caused much confusion. Previous workers had assigned these populations, which exhibit considerable variation in colour pattern and scalation, to a number of southeast Asian cobra taxa[15–19]. Multivariate morphometrics suggested a southwest-northeast cline in the morphology of these forms: specimens from northeast Thailand are phenotypically more similar to the Chinese cobra (*Naja atra*) than are specimens from western central Thailand. We[12] therefore provisionally regarded these spitting cobras as conspecific with the Chinese *N. atra*. In parts of Thailand, Cambodia and Vietnam, the spitting cobras occur sympatrically with the monocellate cobra (*N. kaouthia*).

The definition and identification of morphologically variable species by morphology-based methods can be difficult, especially if allopatric populations are involved. Geographic variation in morphology can be caused both by separate evolutionary histories (phylogensis) and by current selection pressures (ecogenesis)[20,21]. The comparison of mitochondrial DNA sequences thought to be selectively neutral can recover evolutionary relationships obscured by ecogenetically caused morphological variation[22–24]. The development of the polymerase chain reaction (PCR)[25], which allows specific DNA fragments to be cloned in vitro in a matter of hours, and of direct sequencing of the PCR product using conserved primer sequences[26] have greatly increased the accessibility of DNA sequence information for systematic studies.

Asiatic cobras, like many other animals, are getting rare and difficult to obtain in some regions due to overexploitation for food, traditional medicine and leather products. It is consequently desirable to minimise the required sample size for studies involving the acquisition of these animals. The cytochrome oxidase subunit I (CO I) gene has been shown in lizards to display very low levels of sequence variation within populations and among closely related populations, while showing considerable variation between species[24]. This makes it particularly useful for studies at the species level, especially where large samples are difficult to obtain.

Here, we use PCR-based direct sequencing of the CO I gene to determine the systematic status and affinities of the different varieties of spitting cobras from Thailand, and in particular their interrelationships with the Chinese cobra (*Naja atra*).

Materials and methods

Biopsies of spitting cobras and *Naja kaouthia* were obtained in Thailand, either with the aid of local villagers or from road kills. Specimens of *N. atra* from Guangdong Province, China, were obtained in Hong Kong. This study involves four populations of spitting cobras from northern, northeastern, western central and eastern central Thailand (fig. 1), representing several previously noted varieties[17–19], one population of *N. kaouthia* from an area of sympatry with the spitting cobras in western central Thailand, and one population of *N. atra* from China. Biopsies were preserved in 75% ethanol and stored at 4 °C until used.

The samples were homogenized in TBE buffer. Proteins were removed by proteinase K digestion and phenol/chloroform extraction. A 544 bp fragment of the gene was amplified by means of the Polymerase Chain Reaction[25,26] using *Thermus aquaticus* (Taq) DNA polymerase, Direct sequencing of both strands was accomplished by the dideoxy method[27], using Sequenase version 2.0 (U.S. Biochemicals). The primer sequences used were 5′-GAATTCCCAGAGATTAGAGGGAATCAGTG-3′ and 5′-GAATTCCCTGCAGGAGGAGGAGACCC-3′.

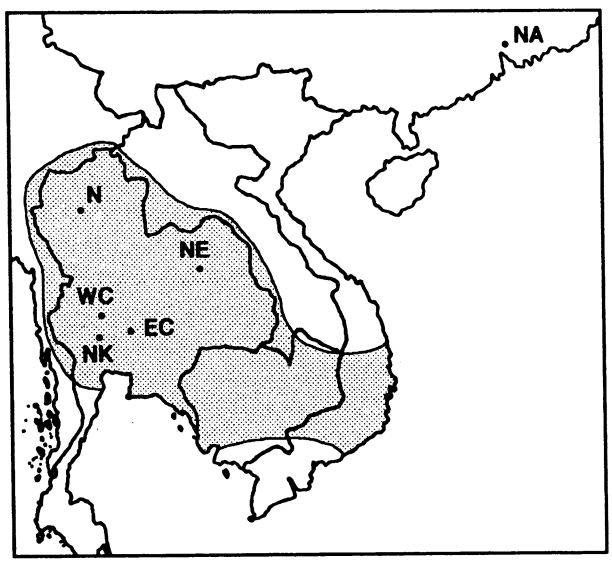

Figure 1. Approximate distribution of spitting cobras in Indochina (precise distribution limits in Burma, Laos and Vietnam unknown), and sampling localities for DNA samples. The shaded area indicates the approximate distribution of the spitting cobras, the codes the sampling localities. *Naja kaouthia* occurs sympatrically with the spitting cobras in central and southeastern Thailand, Cambodia and southern Vietnam.
WC = Chainat province, western central Thailand: black-and-white phase spitting cobra. EC = Lop Buri Province, eastern central Thailand: brown phase spitting cobra. N = Lampang province, northern Thailand: brown phase spitting cobra. NE = Khon Kaen Province, northeastern Thailand: brown phase spitting cobra. NK = Suphan Buri and Ayutthaya Provinces, western central Thailand: *Naja kaouthia*. Black-and-white phase spitting cobras are also found in this region. NA = Guangdong province, China: *Naja atra*.

The sequences were aligned against the human sequence[28]. The results presented here relate to a 330 bp region of the CO I gene. All parts of the sequence were corroborated by four specimens of *Naja kaouthia*, two specimens of *N. atra*, and at least one specimen of each geographic sample of spitting cobra. Most parts of the sequence were corroborated by further specimens of spitting cobra. The sequence runs from the equivalent of position 6720 to the equivalent of position 7049 of the human sequence[28]. Within this region of the CO I gene, there are no deletions or insertions compared to the human sequence.

Results

The CO I mtDNA sequence (fig. 2) of the spitting cobras from Thailand is as distinct from the sequence of Chinese *Naja atra* as it is from the sequence of sympatric *N. kaouthis* (table 1). Furthermore, there is no geographic variation among the Thai populations of spitting cobras, despite ample variation in colour pattern and scalation. The only case of intraspecific variation in sequence occurs at position 6987 within the sample from northern Thailand, where one specimen diverges from all other specimens of spitting cobra. We have found no sequence variation within either of the other two species. All substitutions are silent. Transitions greatly outweigh transversions, as would be expected in congeneric species. We found that C ↔ T transitions are much more common than A↔ G transitions. This substitution bias (table 2) is parallel to but higher than that found in sequence comparisons of other higher snakes and mammals in mitochondrial 12S and 16S rRNA genes[29].

Discussion

The DNA sequence data presented here define the morphologically diverse spitting cobra populations as a cohesive taxon, which is clearly distinct from the two other cobra species found in China and Indochina. If the molecular clock hypothesis is correct for the CO I gene in this group, *Naja atra* and *N. kaouthia* share a more recent common ancestor than either does with the spitting cobras. The spitting cobras occur sympatrically with *N. kaouthia*, demonstrating non-conspecificity, and it is therefore logical to regard them as a separate species from *N. atra* as well.

The spitting cobras are highly variable in colour pattern and various scalation characters. This has led to various populations being assigned to different taxa. The brown populations with a spectable mark from northeastern Thailand have been described as *Naja naja isanensis*[18], whereas the black and white populations have been assigned to a number of other taxa[17,18]. The DNA sequence data presented here provide no evidence of a separate evolutionary history for these colour forms. We therefore conclude that the recognition of subspecies would be inappropriate, and that the colour pattern

```
    Met Ile Trp Ala Met Met Ser Ile Ala Ile Leu Gly Phe Val Val Trp Ala His His
SN  ATA ATC TGA GCA ATA ATG TCT ATT GCA ATC CTA GGC TTT GTT GTA TGG GCC CAC CAC
SCE ... ... ... ... ... ..G ... ... ... ..C C.. ..C ... ... ... ... ... ..C
SCW ... ... ... ... ... ..G ... ... ... ..C C.. ..C ... ... ... ... ... ..C
SNE ... ... ... ... ... ..G ... ... ... ..C C.. ..C ... ... ... ... ... ..C
NK  ... ... ... ... ... ..A ... ... ... ..C T.. ..T ... ... ... ... ... ..T
NA  ... ... ... ... ... ..A ... ... ... ..T T.. ..T ... ... ... ... ... ..T

    Met Phe Thr Val Gly Leu Asp Ile Asp Ser Arg Ala Tyr Phe Thr Ala Ala Thr Met
SN  ATA TTC ACC GTA GGC CTT GAC ATT GAC AGC CGT GCC TAT TTC ACC GCA GCA ACA ATA
SCE ... ..C ... ... ... ... ..C ... ..C ... ..T ... ... ... ... ... ... ... ...
SCW ... ..C ... ... ... ... ..C ... ..C ... ..T ... ... ... ... ... ... ... ...
SNE ... ..C ... ... ... ... ..C ... ..C ... ..T ... ... ... ... ... ... ... ...
NK  ... ..T ... ... ... ... ..T ... ..T ... ..T ... ... ... ... ... ... ... ...
NA  ... ..C ... ... ... ... ..T ... ..C ... ..C ... ... ... ... ... ... ... ...

    Ile Ile Ala Ile Pro Thr Gly Ile Lys Val Phe Gly Trp Leu Ala Thr Leu Ala Gly
SN  ATT ATC GCC ATT CCC ACA GGA ATC/T AAA GTA TTC GGT TGA CTG GCC ACA CTA GCA GGA
SCE ... ..C ... ... ... ... ... ..C ... ... ... ..T ... ... ... ... ... ... ...
SCW ... ..C ... ... ... ... ... ..C ... ... ... ..T ... ... ... ... ... ... ...
SNE ... ..C ... ... ... ... ... ..C ... ... ... ..T ... ... ... ... ... ... ...
NK  ... ..T ... ... ... ... ... ..C ... ... ... ..C ... ... ... ... ... ... ...
NA  ... ..C ... ... ... ... ... ..C ... ... ... ..T ... ... ... ... ... ... ...

    Gly Gln Ile Lys Trp Gln Thr Pro Val Tyr Trp Ala Leu Gly Phe Ile Phe Leu Phe
SN  GGT CAA ATT AAG TGA CAA ACA CCC ATC TAC TGA GCT CTG GGG TTT ATC TTC CTA TTT
SCE ..T ... ... ..G ... ... ... ..C ... ... ... ..T ..G ... ... ... ... ... ...
SCW ..T ... ... ..G ... ... ... ..C ... ... ... ..T ..G ... ... ... ... ... ...
SNE ..T ... ... ..G ... ... ... ..C ... ... ... ..T ..G ... ... ... ... ... ...
NK  ..C ... ... ..A ... ... ... ..G ... ... ... ..C ..C ... ... ... ... ... ...
NA  ..C ... ... ..A ... ... ... ..G ... ... ... ..C ..T ... ... ... ... ... ...

    Thr Val Gly Gly Met Thr Gly Ile Val Ile Leu Asn Ser Ser Leu Asp Ile Val Leu
SN  ACT GTC GGG GGT ATA ACA GGT ATT ATT CTA GCA AAC TCG TCA CTA GAT ATC GTC CTA
SCE ... ..C ..G ... ... ... ..T ..T ... ... ... ... ... ... ..A ..T ..C ... ...
SCW ... ..C ..G ... ... ... ..T ..T ... ... ... ... ... ... ..A ..T ..C ... ...
SNE ... ..C ..G ... ... ... ..T ..T ... ... ... ... ... ... ..A ..T ..C ... ...
NK  ... ..T ..A ... ... ... ..A ..C ... ... ... ... ... ... ..A ..T ..C ... ...
NA  ... ..T ..G ... ... ... ..A ..C ... ... ... ... ... ... ..G ..C ..T ... ...

    His Asp Thr Tyr Tyr Val Val Ala His Phe His Tyr Val Leu Ser
SN  CAC GAC ACT TAC TAC GTA GTA GCA CAC TTC CAC TAT GTC CTC TCT
SCE ..C ... ..T ... ... ... ... ..A ... ... ... ... ... ..C ...
SCW ..C ... ..T ... ... ... ... ..A ... ... ... ... ... ..C ...
SNE ..C ... ..T ... ... ... ... ..A ... ... ... ... ... ..C ...
NK  ..T ... ..C ... ... ... ... ..G ... ... ... ... ... ..A ...
NA  ..T ... ..C ... ... ... ... ..G ... ... ... ... ... ..C ...
```

Figure 2. Aligned L-strand DNA sequence data of a 330 base pair region of the CO I gene of spitting cobra populations from Lampang Province, northern Thailand (SN, n = 1), Chainat Province, western central Thailand (SCW, n = 1), Lop Buri Province, eastern central Thailand (SCE, n = 2), and Khon Kaen Province, northeastern Thailand (SNE, n = 1), a monocellate cobra population from western central Thailand (*Naja kaouthia* = NK, n = 4), and a Chinese cobra population from Hong Kong (*Naja atra* = NA, n = 2). Most stretches of the sequence were corroborated by further specimens of spitting cobra.

and scalation variation which has confused their taxonomy in the past is probably ecogenetically caused.

The available scientific name for these spitting cobras is *Naja siamensis* Laurenti, 1768: this name was based on an illustration of a cobra with a spectacled hood mark originating from Thailand. In Thailand, the spitting cobra revealed in this paper is the only species with such a marking, and we conclude that the name is unambiguously applicable to this form. The species occurs throughout Thailand (except in the Peninsula), Cambodia, parts of southern Vietnam, Laos, and probably in parts of eastern Burma (fig. 1). It is widely sympatric with *N. kaouthia* in parts of Thailand, Cambodia and southern Vietnam[12, 14].

Table 1. MtDNA sequence divergence of Thai and Chinese cobra populations

	Spitting cobras (all Thai pops.)	Monocellate cobra (*Naja kaouthia*) C. Thailand	Chinese cobra (*Naja atra*) Nr. Hong Kong
Spitting cobra (all Thai pops.)	1 = 0.30%	22	21
Monocellate cobra (*Naja kaouthia*) C. Thailand	6.67%	0	12
Chinese cobra (*Naja atra*)	6.36%	3.64%	0

Differences in base pair numbers are given above the diagonal, differences as a percentage of the entire sequence below the diagonal. Out of 330 base pairs sequenced, 27 (8,18%) exhibit interspecific differences. The sequence is highly conserved within species: only one transition was found within the population of spitting cobras from northern Thailand. Another specimen from the same population displayed the same sequence as all other spitting cobra specimens. Transversions are much less frequent than transitions: transversions make up 18% of substitutions between *N. kaouthia* and the spitting cobras, 14% between *N. atra* and the spitting cobras, and 11% between *N. atra* and *N. kaouthia*.

Table 2. Observed and expected frequency of transitions.

	C↔T			A↔G		
	O	E	O/E	O	E	O/E
SC↔NK	14	9.76	1.44	4	8.24	0.49
SC↔NA	14	9.79	1.43	4	8.22	0.49
NK↔NA	9	5.95	1.51	2	5.05	0.40
Overall	37	25.50	1.45	10	21.51	0.46

Observed (O) and expected (E) L-strand transitions and observed:expected occurrence ratio for all pairwise comparisons of the sequences of Thai spitting cobras (SC), *Naja kaouthia* (NK) and *Naja atra* (NA). The expected occurrence was calculated as the product of the total number of transitions and the sum of the mean frequency of each of the two bases involved[29]. Transversions are too few in number to warrant investigation.

Naja siamensis is an important cause of snakebites in Thailand[6], and rigorous comparisons of venom composition and antivenom compatibility between this species and *N. kaouthia* are urgently required. If there are great differences between the venoms of two species, and different antivenoms are required, then we would recommend the production of a bivalent antivenom, based on the venoms of both species, for use in mainland Thailand, Cambodia, Laos and southern Vietnam. Obviously, other factors, such as age and size of the snakes, and individual, seasonal and intraspecific geographic variation also cause variation in venom composition[30]. These should also be taken into account, in order to maximise the spectrum of antigens neutralised by the antivenom.

This study confirms previous studies[24] which found very low levels of intraspecific variation in the sequence of the CO I gene. This property makes this gene particularly useful for studies around the species level, as small sample sizes for each population are sufficient.

Table 3. Distinguishing characters between *Naja siamensis* and *Naja kaouthia*

	Naja siamensis ♂♂	♀♀	*Naja kaouthia* ♂♂	♀♀
Ventrals	153–174 (usually < < 170)	162–173	170–192 (usually > > 170)	178–197
Cuneates (small scales inserted between 4th & 5th or 5th & 6th infralabials and mouth edge)	Usually 1 on each side		Often more than 1 on each side	
Shape of hood mark	Spectacle, U-, V- or H-shaped, often faint or absent		Usually O-shaped, but may vary (see Cox 1991[19])	
Behaviour	Spits readily; often very irritable		Never or practically never spits; often relatively placid	
Appearance of scales	Usually dull		Usually shiny	
Adult size	Small, usually < 120 cm total length		large, often ≫ 120 cm	
Frontal shield	Usually slightly elongated, posterior end pointed.		Usually short and squarish, posterior ends in obtuse angle	
Dorsal scale rows just ahead of midbody	19–21 (Usually 19 in area of sympatry)		19–23 (Usually 21–23 in area of sympatry)	

Most single characters show occasional overlap between the two species. In practice, characters such as the number of ventral scales, the shape of the hood mark and the presence or absence of spitting behaviour will reliably identify practically all specimens.

Acknowledgements. This study was funded by NERC and the Leverhulme Trust. We thank A. M. Cumming, W. C. Jordan and D. P. McGregor for provision of laboratory facilities and other assistance, M. J. Cox, P. Jintakune, J. Nabhitabhata and L. Trutnau for their help in Thailand, and H. M. Smith, R. D. G. Theakston, G. L. Underwood and D. A. Warrell for many suggestions and discussions.

1 Swaroop, S., and Grab, B., Bull. Wld Hlth Org. *10* (1954) 35.
2 Minton, S. A., and Minton, M. R., Venomous Reptiles. Scribners, New York 1980.
3 Warrell, D. A., in: Natural toxins – Animal, plant and microbial, p. 25. Ed. J. B. Harris. Clarendon, Oxford 1986.
4 Looareesuwan, S., Viravan, C., and Warrell, D. A., Trans. R. Soc. trop. Med. Hyg. *82* (1988) 930.
5 Viravan, C., Veeravat, U., Warrell, M. J., Theakston, R. D. G., and Warrell, D. A., Am. J. trop. Med. Hyg. *35* (1986) 173.
6 Viravan, C., Looareesuwan, S., Kosakarn, W., Wuthiekanun, V., McCarthy, C., J., Stimson, A. F., Bunnag, D., Harinasuta, T., and Warrell, D. A., Trans. R. Soc. trop. Med. Hyg. *86* (1992) 100.
7 Sawai, Y., Koba, K., Okonogi, T., Mishima, S., Kawamura, Y., Chinzei, H., Abu-Bakar, I., Devaraj, T., Phong-Aksara, S., Purana nanda, C., Salafranca, E. S., Sumpaico, J. S., Tseng, C., Taylor, J. F., Wu, C., and Kuo, T., Jap. J. exp. Med. *42* (1972) 283.
8 Watt, G., Padre, L., Tuazon, M. L., and Hayes, C. G., Am. J. trop. Med. Hyg. *37* (1987) 636.
9 Watt, G., Padre, L., Tuazon, M. L., Theakston, R. D. G., and Laughlin, L. W., Am. J. trop. Med. Hyg. *39* (1988) 306.
10 Wüster, W., and Thorpe, R. S., Biol. J. Linn. Soc. *36* (1989) 391.
11 Wüster, W., and Thorpe, R. S., in: Vertebrates in the tropics, p. 333. Eds G. Peters and R. Hutterer. Museum A. Koenig, Bonn 1990.
12 Wüster, W., and Thorpe, R. S., Experientia *47* (1991) 205.
13 Wüster, W., and Thorpe, R. S., Herpetologica *48* (1992) 69.
14 Wüster, W., and Thorpe, R. S., Herpetologica *48* (1992) 424.
15 Smith, M. A., Fauna of British India: Reptilia and Amphibia, vol. III, Serpentes. Taylor and Francis, London 1943.
16 Taylor, E. H., Kans. Univ Sci. Bull. *45* (1965) 609.
17 Lingenhöle, S., and Trutnau, L., Herpetofauna *11* (1989) 6.
18 Tumwipat, B., and Nutaphand W., [The treatment of poisonous snakebite victims and the poisonous snakes of Thailand] (in Thai). Peekhanet Print Shop, Bangkok 1982.
19 Cox, M. J., The Snakes of Thailand and Their Husbandry. Krieger, Malabar 1991.
20 Thorpe, R. S., Boll. Zool. *54* (1987) 3.
21 Thorpe, R. S., Brown R. P., Malhotra, A., and Wüster, W., Boll. Zool. *58* (1991) 329.
22 Wilson, A. C., Cann, R.L., Carr, S. M., George, M., Gyllensten, U. B., Helm-Bychowski, K. M., Higuchi, R. M., Palumbi, S. R., Prager, E. M., Sage, R. D., and Stoneking, M., Biol. J. Linn. Soc. *26* (1985) 375.
23 DeSalle, R., and Templeton, A. R., Evolution *42* (1988) 1076.
24 Thorpe, R. S., McGregor, D. P., Cumming, A. M., and Jordan, W. C., Evolution (1993) in press.
25 Saiki, R.K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A., Science *239* (1988) 487.
26 Kocher, T. D., Thomas, W.K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X., and Wilson, A. C., Proc. natl Acad. Sci. USA *86* (1989) 6196.
27 Sanger, F., Nicklen, S., and Coulson, A. R., Proc. natl Acad. Sci. USA *74* (1977) 5463.
28 Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G., Nature *290* (1981) 457.
29 Knight, A., and Mindell, D. P., Syst. Biol. *42* (1993) 18.
30 Meier, J., Toxicon *24* (1986) 41.

SCIENTIFIC CORRESPONDENCE

EXPERIENTIA welcomes letters concerning articles which have appeared in our journal. Letters will be sent to the authors concerned to allow them the opportunity to reply. The correspondence will be published as rapidly as possible.