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Conservation Genetics of the Chinese Cobra (*Naja atra*) Investigated with Mitochondrial DNA Sequences

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We collected Chinese cobras (*Naja atra*) from one island (Dinghai) and four mainland (Huangshan, Lishui, Quanzhou, and Baise) populations in southeastern China, and used sequence data derived from the ND2 (1032 bp) and cytochrome *b* (1117 bp) genes and molecular variance estimates to investigate the population genetic structure of the species. Our sequence data show that: (1) the three eastern (Dinghai, Huangshan, and Lishui) populations are genetically segregated from the two southern (Quanzhou and Baise) populations; (2) the Quanzhou and Baise populations consist of two well-defined subclades, suggesting that the two populations have been well differentiated; (3) *N. atra* from the Huangshan population do not differ from those from the Lishui population, and lineage sorting in the northeastern part of the cobra's distributional range has not yet been completed because of the young age of Zhoushan Islands. The three eastern populations, the Quanzhou population, and the Baise population should be regarded as different management units (MUs). For these MUs, we suggest that in-situ protection measures should be taken because of their genetic uniqueness. Re-introductions or translocations are required to protect or re-establish natural populations of *N. atra*, but great care should be taken to enhance or retain local genetic variation.

Key words: Elapidae, *Naja atra*, conservation genetics, mitochondrial DNA, management unit

INTRODUCTION

Cobras of the genus *Naja* are among the most eye-catching snakes in the world because of their unique defensive behavior and their highly venomous nature. Of the 19 species of *Naja* cobras recognized worldwide, eleven can be found in Asia, and two [*N. atra* (Chinese cobra) and *N. kaouthia* (monocellate cobra)] in China (Wüster, 1996; Wüster et al., 1997). The Chinese cobra is mainly distributed in the southeastern provinces of China, including Taiwan and Hainan, northeastward to the mouth of the Yangtze River and southward to northern Vietnam and northern Laos (Huang, 1998). The cobra uses a variety of habitats in the hilly countryside, and was one of the most commonly found snakes in China some thirty years ago. Largely because local people have overhunted the cobra for meat, skin, medicine, and handiwork, this species is currently regarded a highly vulnerable, according to a recently published volume of the China Red Data Book of Endangered Animals (Zhao, 1998). There are realistic threats of local extinction in some southern provinces such as Guangdong and Hainan (Zhao, 1998). Therefore, measures should be taken immediately to protect the cobra.

The quantification of genetic variability and population genetic structure is crucial for improved management and conservation (Avise, 1989; Marmi et al., 2006). Because of the nature of fast evolution and maternal inheritance, mitochondrial DNA has been widely used to investigate genetic differences and evolutionary history among and within species (Avise, 1989). Comparisons of control region, cytochrome *b* (cyt *b*), ND2, ND4, or other mitochondrial gene sequences have provided a powerful technique to resolve intraspecific phylogenies for reptiles (e.g., Heulin et al., 1999; Brown et al., 2002; Poulakakis et al., 2003; Pinho et al., 2007). Nonetheless, sequence data on *Naja* cobras are scarce. Broadley and Wüster (2004) analyzed the systematics of African *Naja* cobras by comparing partial cyt *b* sequences. In a more recent study of *N. atra* from Taiwan, Lin et al. (2008) provided data derived from mitochondrial control region sequences. Until now, there have been no comparable sequence data on *N. atra* from widely separated populations, although such data are urgently needed to elucidate whether among-population differences in life-history traits found in *N. atra* correspond to genetic differences and to develop an appropriate management and conservation strategy for the species. Descriptions of life-history traits have made some contributions to the biogeography of *N. atra* (Ji and Wang, 2005), but population surveys of mitochondrial DNA markers may provide additional advantages

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by providing higher resolution to detect population differentiation (Brown et al., 2002).

In the present study, we sequenced ND2 and cyt *b* to examine the geographical pattern of genetic differences in *N. atra*. The aims of this study were to: (1) describe the genetic variation in *N. atra* across its distributional range in China; (2) define phylogeographic groups; and (3) discuss possible management and conservation strategies for *N. atra*.

MATERIALS AND METHODS

Samples and DNA extraction

We collected 15 adults (snout-vent length >90 cm; Ji and Wang, 2005) in June 2004 from each of five localities (populations) in two eastern (Zhejiang and Anhui) and one southern (Guangxi) provinces of China (Fig. 1). Efforts were made to avoid collecting more than one cobra from the outskirts of the same village in each locality. One locality is in Dinghai (the Zhoushan population), Zhoushan Islands, eastern Zhejiang, which is approximately 25 km away from the nearest coastline. The other four localities are situated on the mainland: Huangshan (the Anhui population), southern Anhui; Lishui (the Lishui population), central Zhejiang; Quanzhou (the Quanzhou population), northern Guangxi; Baise (the Baise population), southwestern Guangxi. The five localities cover almost all of the cobra's distributional range in mainland China (Fig. 1).

All cobras were transported to our laboratory in Hangzhou, where the tail tip of each was clipped off, and DNA was extracted from fresh tail muscle using the standard phenol-chloroform extraction method (Sambrook et al., 1989) with some modifications (Rao et al., 2001). All samples used in the present study were deposited at Hangzhou Normal University under voucher numbers identified by locality-haplotype numbers.

DNA sequencing of the mitochondrial ND2 and cyt *b* genes

We used primers L4437b (5'-CAG CTA AAA AAG CTA TCG GGC CCA TAC C-3') (Kumazawa et al. 1996) and Trna-trpR (5'-GGC TTT GAA GGC TAC TAG TTT-3') to amplify the entire ND2 gene (1032 bp) (Ashton and de Queiroz, 2001). Amplification conditions were 1 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C; and 3 min at 72°C. After removal of primers and unincorporated nucleotides with spin columns containing Sepacry S-400 (Amersham Bioscience AB, Uppsala, Sweden), the purified amplified products were sequenced using both forward and reverse primers on an ABI-PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems, USA).

We used primers L14910 (5'-GAC CTG TGA TMT GAA AAA CCA YCG TTG T-3') and H16064 (5'-CTT TGG TTT ACA AGA ACA ATG CTT TA-3') to amplify the entire cyt *b* gene (1117 bp) (Burbrink et al., 2000; de Queiroz et al., 2002). PCR reactions were performed in 100-μl volumes, using a hot start method; PCR conditions were 7 min at 94°C; 40 cycles of 40 sec at 94°C, 30 sec at 46°C, and 1 min at 72°C; and 7 min at 72°C. For cycle sequencing, we used primers L14761 (5'-MTC HAA CAA CCC AAY MGG-3'), H14892 (5'-TGC NGG KGT RAA KTT TTC-3'), and H15149 (5'-CCC TCA GAA TGA TAT TTG TCC TCA-3') (Busack et al., 2005).

Data analysis

Sequences were compiled and aligned by using MEGA version 3.1 (Kumar et al., 2004). Measures of population genetic parameters, including haplotype diversity and nucleotide diversity (Jukes and Cantor, 1969), were estimated from mitochondrial DNA sequence data by using DNASP version 4.0.6 (Rozas et al., 2003). To reconstruct phylogeny, we performed maximum-likelihood (ML) analysis implemented in PAUP* 4.0 (Swofford, 2002). We used MRMODELTEST version 2.2 (Nylander, 2004) to test several partitioning strategies for analysis: (1) each gene separately; (2) the combined dataset by codon position; and (3) each gene separately by codon position. We used *N. kaouthia* as the outgroup. Bootstrap analyses were performed with 100 full heuristic replicates for maximum likelihood. Bayesian posterior probabilities were estimated in MRBAYES 3.1 (Ronquist and Huelsenbeck, 2003). Two independent runs

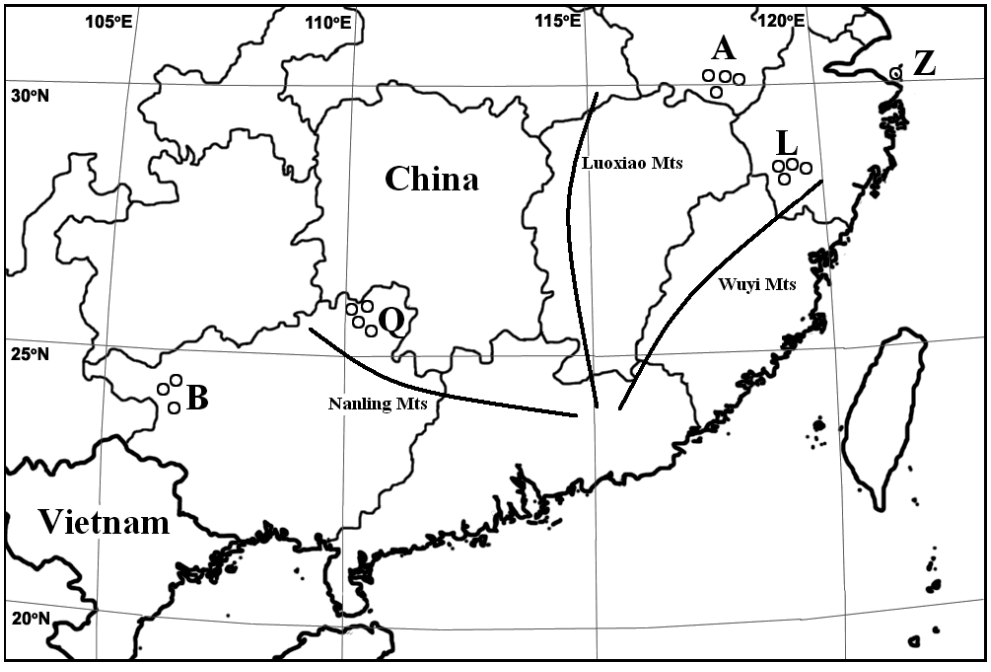

Fig. 1. Sample localities for the Chinese cobra (*N. atra*) in different regions of mainland China. Open dots indicate where cobras were collected. Z, Dinghai, Zhoushan Islands, eastern Zhejiang; L, Lishui, central Zhejiang; A, Huangshan, southern Anhui; Q, Quanzhou, northern Guangxi; B, Baise, southwestern Guangxi.

Table 1. Polymorphic sites, geographic distribution, and frequency of the four ND2 and cyt *b* haplotypes found in the Chinese cobra. Dots indicate identity with the base in haplotype ALZ. See Fig. 1 for explanations of Z, L, A, Q, and B.

Haplotype	ND2									Cytochrome *b*																Population				
	3	3	5	5	6	6	8	9	9		1	4	4	4	4	5	5	5	5	6	7	7	8	9	9	1 0	Eastern			Southern	
	2	3	6	7	1	5	9	1	3	9	9	2	5	7	8	4	5	7	8	0	2	9	7	0	7	8					
	1	3	1	3	7	4	4	2	3	3	5	6	0	4	0	3	0	1	1	0	3	2	6	7	8	3	A	L	Z	Q	B
ALZ	T	C	A	A	T	C	C	C	A	T	A	C	T	G	T	T	C	T	A	C	G	G	C	T	C	C	15	15	6		
Z	●	●	G	●	●	●	●	●	●	●	●	●	●	●	●	●	T	●	●	●	●	●	●	●	●	●			9		
Q	C	T	G	G	●	A	T	●	●	C	G	T	C	A	●	C	C	C	G	T	A	●	T	C	T	T				15	
B	C	T	G	G	C	A	T	T	G	C	G	T	C	A	C	C	●	C	G	T	A	A	●	C	T	T					15

using four Markov chains and temperature profiles at the default setting of 0.2 were conducted for ten million generations, sampling every 1000 generations. Random trees were used to begin each Markov chain. The initial 10% of trees were discarded as 'burn-in' to ensure stationarity after examination of the posterior probability. We confirmed that we had a sufficient sample from the posterior probability distribution by examining the potential scale-reduction factors (Gelman and Rubin, 1992) produced by MRBAYES for all parameters, and these were very close to one (to the second decimal), indicating that the runs had adequately converged. We used the program TCS version 1.13 (Clement et al., 2000) to construct a haplotype network. To estimate gene flow between populations, the fixation index (F_{ST}) and number of female migrants per generation (N_m) were calculated in DNASP version 4.0.6 (Rozas et al., 2003), according to Hudson et al. (1992). To assess whether there was significant geographical differentiation, the partitioning of total genetic variation was hierarchically examined by an analysis of molecular variance (AMOVA) in ARLEQUIN version 3.0 (Excoffier et al., 2005).

RESULTS

Sequence variation and haplotype frequency

ND2 gene sequences (1032 bp) from the 75 individuals yielded four distinct haplotypes (Table 1). One haplotype (ALZ; GenBank, DQ302759) is shared by the Anhui, Lishui, and Zhoushan populations. The Zhoushan population has two haplotypes (ALZ and Z), whereas the other four populations all have only one hapolotype (Table 1). There were nine polymorphic sites, five of which were parsimony informative (Table 1). The mean base frequencies for A, C, G, and T were 38.1%, 29.7%, 9.0%, and 23.3%, respectively. There were eight transitions and one transversion. No insertions or deletions were observed.

Cyt *b* gene sequences (1117 bp) from the 75 individuals yielded four distinct haplotypes (Table 1). One haplotype (ALZ; GenBank, EF206656) is shared by the Anhui, Lishui, and Zhoushan populations. The Zhoushan population has two haplotypes (ALZ and Z), whereas the other four populations all have only one hapolotype (Table 1). There were 17 polymorphic sites, 13 of which were parsimony informative (Table 1). The mean base frequencies for A, C, G, and T were 30.3%, 29.7%, 11.3%, and 28.6%, respectively. There were 17 transitions but no transversion. No insertions or deletions were observed.

Analysis of genetic structure within and among regions

Under all partitioning strategies, HKY was identified by using MRMODELTEST as the best-fitting substitution model, with the parameter values differing to some extent. All phylogenetic analyses resulted in almost identical tree topologies (Fig. 2), identifying two main haplogroups: (1) an Eastern clade (including the three populations in Anhui and Zhejiang), and (2) a Southern clade (including the two populations in Guangxi). The partition homogeneity test (PHT), implemented in PAUP* version 4.0b10, was used to test the incongruence between the ND2 and cyt *b* datasets. The test is based on the incongruence-length difference test of Farris et al. (1995). The null hypothesis is that the two loci are no more incongruent than two randomly generated partitions of equal size. One hundred replicates were generated and the *P* value obtained was 1.0, which indicates congruence between the data sets. Therefore, the two gene sequences were combined for all subsequent analyses.

The haplotype distribution (Table 1) and haplotype net-

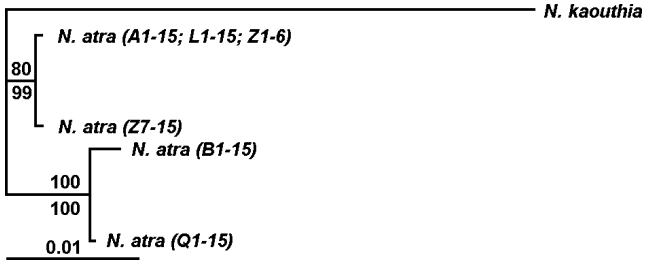

Fig. 2. Phylogenetic tree of *N. atra* based on the combined ND2 and cyt *b* data set, with *N. kaouthia* as the outgroup. Numbers above/below the branches represent bootstrap values for the maximum likelihood (ML) analysis and posterior probability for the Bayesian inference (BI) analysis, respectively. Letters after the species name in parenthesis denote individuals from different sampling localities. Scale bar indicates substitutions per site. See Fig. 1 for explanation of Z, L, A, Q, and B.

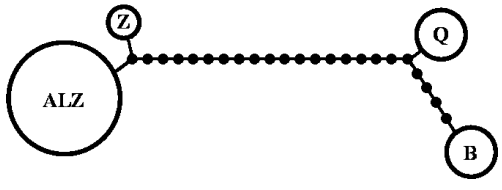

Fig. 3. Haplotype network for the mitochondrial DNA sequences, constructed with TCS version 1.13 (Clement et al. 2000). Refer to Table 1 for information on haplotype names. Circles represent haplotypes, and their size is proportional to the frequency observed. Lines connecting circles are proportional to the number of mutations, indicated by black dots.

Table 2. Intrapopulation variability in the mitochondrial DNA phylogroups of *N. atra* in China. Standard deviations are in parentheses.

Region	Sample size	Number ofhaplotypes	Number of polymorphic sites	Haplotype diversity	Nucleotide diversity (%)
Eastern	45	2	2	0.327 (0.072)	0.030 (0.007)
Southern	30	2	6	0.517 (0.024)	0.144 (0.007)
Total	75	4	26	0.684 (0.036)	0.532 (0.030)

Table 3. Analysis of molecular variance (amova) among *N. atra* populations from the two major groups.

Source of variation	Degrees of freedom	Variance components	Percentage of variation
Among groups	1	9.607	88.44
Among populations within group	3	1.153	10.62
Among individuals within population	70	0.103	0.95
Total	74	10.863	100

work (Fig. 3) show clearly differentiated sequences from two geographic regions: eastern China and southern China. This geographic pattern was also supported by a major phylogenetic tree based on the combined ND2 and cyt *b* gene data and inferred from ML (maximum-likelihood) and BI (Bayesian approach) analyses (Fig. 2).

The haplotype diversity and the nucleotide diversity within the Zhoushan population were 0.514±0.069 and 0.00048±0.00006, respectively, whereas the two diversities within the other four populations were all zero. Pairwise differences between haplotypes, based on the ND2 and cyt *b* sequences, ranged from 0.1 to 1.1% (average 0.8%). The number of polymorphic sites within the eastern (two sites in Zhejiang and Anhui) and southern regions (six sites in Guangxi) was smaller than that within the whole region sampled (26 sites), as was also the case for haplotype diversity and nucleotide diversity (Table 2). The F_{ST} value between the eastern and southern phylogroups and the N_m value calculated from this F_{ST} were 0.915 and 0.02, respectively. Except for the Anhui and Lishui populations, which according to our sequence data could be regarded as the same population, N_m values were very low between populations, ranging from 0 to 0.19. The AMOVA partitioned 88.44% of the total genetic variation between the two geographic regions and 10.62% of the total within the regions (Table 3), indicating that most of the variation occurred between the two regions.

DISCUSSION

Overexploitation and illegal trade of *N. atra* are currently so serious in China that it is always not easy to collect cobras from the field in many places of the country. It is likely that the five localities involved in the present study were the last opportunity to collect a sufficient number of cobras native to their own populations with relatively little effort. The five localities cover almost all of the cobra's distributional range in mainland China, and thus our data can provide a relatively accurate overview of genetic variability within the species.

The number of polymorphic sites within the eastern and southern regions was smaller than that within the whole region sampled (26 sites), as was also the case with haplotype diversity and nucleotide diversity (Table 2). There has been on average one female migrant between the two regions about every 50 generations (N_m=0.02). Moreover, the AMOVA partitioned 88.44% of total genetic variation between the two geographic regions and 10.62% of the total within the regions (Table 3), indicating that most of the variation occurred between the two regions. Therefore, taken together, our data show that *N. atra* in the eastern region are genetically segregated from those in the southern region. This genetic divergence primarily resulted from the lack of gene flow between the two regions, partly because they are far from each other and partly because a series of mountains, the Nanling and Luoxiao Mountains, have served as barriers to animal dispersal between the northern and southern parts of southeastern China since the Pleistocene (Zhou, 1984; Zhang, 2002).

Our sequence data show that *N. atra* from the Anhui population do not differ at all from those from the Lishui population. This observation is not so surprising because dispersal, and thus gene flow, may be facilitated by the similarities in vegetation and climate, and the lack of dispersal barriers, between the two localities (ca. 300 km apart). The Baise and Quanzhou populations (ca. 500 km apart), however, consist of two well-defined subclades (Fig. 2). This spatial pattern is also supported by the haplotype network showing that the two populations have their own haplotypes and are separated by five mutational steps (Fig. 3). The population differentiation occurring in Guangxi presumably resulted from limited animal dispersal due to the existence of the west-to-east trending Nanling Mountains between Baise and Quanzhou (Fig. 1). Of the 15 cobras from the Zhoushan population, six shared haplotype ALZ with those from the Anhui and Lishui populations. Moreover, haplotype Z (only found in the Zhoushan population) and haplotype ALZ differ by only two base pairs, one in cyt *b* and the other ND2 (Table 1). These observations suggest that lineage sorting has not yet been completed in the northeastern part of the cobra's distributional range, because of the young age of Zhoushan Islands, which were separated from the mainland for the last time some 10,000 years ago (Wang and Wang, 1980).

Results from life-history studies show that *N. atra* from geographically separated populations differ in morphology and female reproductive traits. For example, females from the Zhoushan population have longer tails and produce more and larger eggs than females from the Lishui population (Ji and Wang, 2005), and females from the Baise population have larger body size (SVLs) and produce more but

smaller eggs than females from the Quanzhou population (Lin, 2005). However, the spatial pattern of female morphological and reproductive traits is not consistent with the pattern revealed by our sequence data. For example, females from the Lishui and Quanzhou populations, although separated by a distance of approximately 1600 km, are similar to each other in all traits examined (Ji and Wang, 2005). What can be inferred from this inconsistence is that biogeography revealed by life-history data may not necessarily correspond to that revealed by sequence data.

Management units (MUs) are identified by significant differences in allele frequency distributions and significant divergence in mitochondrial or nuclear loci (Moritz, 1994). Accordingly, populations with genotypes that are closely related to, but not shared with, other populations, should be considered as separate MUs. Results from AMOVA suggest that [Anhui, Lishui, Zhoushan], [Quanzhou] and [Baise] is the most parsimonious geographical subdivision, and no haplotype is shared between among these three units (Table 3). We therefore suggest that the three eastern populations, the Quanzhou population, and the Baise population should be regarded as three different MUs. The design of an integrated conservation program for a species should take into account the genetic isolation of populations so that local genetic variability can be enhanced or retained (Castilla et al., 1998). Therefore, for the three MUs, we suggest that in-situ protection measures should be taken because of their genetic uniqueness.

Because we found geographic genetic differentiation, current practices of translocated release and artificial breeding in commercial farms will likely cause serious problems due to unnatural homogenization. We recommend that animals should not be released into the wild unless their origin can be determined with confidence. Re-introductions and translocations have been considered for many organisms to be effective measures to raise populations to minimum viable size or to facilitate gene flow. Such measures are, of course, required to protect or re-establish natural populations of *N. atra*, but great care should be taken to ensure that individuals are re-introduced to or translocated between appropriate populations or localities.

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Scale bar indicates substitutions per site

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