

## Ventral coloration differentiation and mitochondrial sequences of the Chinese Cobra (*Naja atra*) in Taiwan

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**Abstract** Differences in coloration between eastern and western populations of the Chinese cobra (*Naja atra*) in Taiwan have been noted by snake collectors, snake keepers, and users of Chinese traditional medicine, but have never been verified by scientific research. In this study, we quantified the amount of black pigment on ventral scales, and found prominent differences in ventral coloration of populations across Taiwan; populations in eastern Taiwan have black ventral scales while populations in the west are predominantly white. Previous studies have shown a similar east-west population differentiation in regards to venom components. We supplement these data with mitochondrial control region sequences, which show extremely low nucleotide diversity. Black-ventral and white-ventral snakes share major haplotypes and show no genetic differentiation. Nevertheless, moderate  $F_{st}$  and low  $N_m$  values between populations indicate low levels of gene flow. With a morphological fixation earlier than

mitochondrial sequences on a relatively short evolutionary time scale, ventral coloration is potentially a result of local adaptation. Based upon the results of this study, along with traditional observations, we strongly recommend treating each of the four populations of the Chinese cobra in Taiwan as distinct ESUs. Reintroducing confiscated snakes from the illegal trade back into the wild needs to be halted to prevent artificial gene flow.

**Keywords** Cobra · Coloration · Conservation genetics · ESU · Mitochondrial · *Naja atra*

### Introduction

Snakes, especially venomous species, are facing tremendous hunting pressure throughout Asia (Kuntz 1963; Warrell 1995; Whitaker 1997). The main reason for this is their wide use as food and traditional Chinese medicine. In countries with large amounts of snake consumption, such as China, Hong Kong, and Taiwan, overexploitation has caused serious population declines of some snake species (Warrell 1995; Whitaker 1997). An investigation of the commercial use and harvesting of snakes indicated that roughly 45,000 venomous snakes are captured and traded each year in Taiwan (Lin 1997). The Chinese cobra (*Naja atra*) constitutes the highest proportion among all snakes traded in Taiwan; more than 20,500 cobras, or 10,800 kg, are traded each year, representing 46% and 65% of the total snake trade figures, respectively (Lin 1997). Although the Chinese cobra is listed as a protected species under the *Wildlife Conservation Law* of Taiwan, little attention has been paid to its conservation due to the negative image of snakes by the general public. While this negative image is a problem for all snakes, it is particularly strong in the case

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of the cobra, due to the serious public concerns regarding snakebites (Hung 2004). Conflicts between conservationists and people who consume snakes are ongoing, but resolution of these differences are urgently needed (Dodd 1987).

Cobras with various color morphs bearing distinctly different ventral colorations have been observed in Taiwan. Our preliminary observations along with those made by the local hunters and snake dealers (H.-C. Lin, personal interviews during the 1990s) hint at geographic differentiation of color morphs. In the snake markets, “black-ventraled” cobras, harvested from eastern Taiwan, are more valuable than “white-ventraled” specimens from western Taiwan. These differences in market price are due to the traditional belief that cobras with darker ventral scales provide stronger medicinal potency. The Central Mountain Range, which separates the island into two major geographic regions, might play the major role in such geographic differentiation. The geography of Taiwan as well as observed differences in morphology and venom indicate potential for genetic differentiation across the geographic range of cobras in Taiwan (Hung 2004). However, patterns of color and genetic differentiation of cobras from both sides of the mountain range have never been quantitatively and scientifically evaluated.

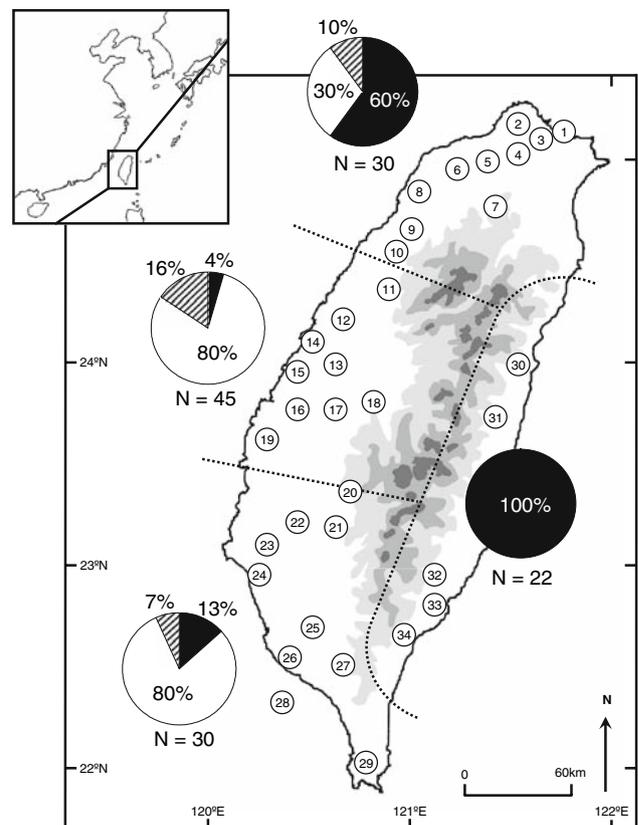
The difference seen in toxicology and potential difference in morphology between eastern and western populations show the necessity for a comprehensive survey of *N. atra* across Taiwan. Due to the severe hunting pressure, evolutionarily significant units (ESUs) of *N. atra* in Taiwan need to be clarified before suitable conservation policies can be decided (Ryder 1986; Moritz 1994a; 1994b; Crandall et al. 2000). Under these concepts, population genetic studies will provide valuable information for conservation, human consumption management, and treatment of snakebites. With conservation in particular, there is controversy surrounding the re-release of snakes illegally brought into the markets. Currently, the common practice is to release seized animals in the vicinity of confiscation. Data from this study will determine the impact, if any, of release of market animals on the natural populations of *N. atra*.

In our study, population differentiation of cobras in Taiwan is evaluated via morphological and molecular data. The objectives of this investigation are to: (1) quantify the morphological differences of the two color morphs in relation to geography; (2) identify the connection and propose hypotheses for patterns between color morph, molecular data, and geographical distribution; and (3) apply these data towards the conservation of *N. atra* in Taiwan.

## Materials and methods

### Sample collection and categorizations

From 2001–2003, a total of 127 adult snakes were collected from 34 localities throughout the island of Taiwan. All voucher specimens are now deposited at the Taipei Zoo. Samples were categorized into four geographic regions due to existing geographic barriers/features (Fig. 1). Specimens obtained in the eastern side of the Central Mountain Range were grouped into the “eastern population” (localities 30–34). The Miaoli Plateau separates the “northern population” (localities 1–10) from the “central population” (localities 11–19), while the central population is separated from the “southern population” (localities 20–29) near where the Tropic of Cancer passes through Taiwan. These biogeographic breaks were chosen because they serve as geographic barriers for the distribution of terrestrial fauna in Taiwan, including birds (Hachisuka and Udagawa 1950), frogs (Lue and Lai 1991; Lue et al. 1999), lizards (Lin et al. 2002; Lin 2003), small mammals (Fang and Lee



**Fig. 1** Sample localities and coloration compositions of the Chinese cobra (*Naja atra*) in different regions of Taiwan. The populations are divided into four regions: northern (sample localities 1–10), central (11–19) southern (20–29), and eastern (30–34)

2002), and several freshwater fishes (Tzeng 1986; Chen and Fang 1999).

Ventral coloration of each snake was defined by quantifying the overall coverage of black coloration of the ventral scales. The ventral area of a snake was first divided into 10 regions. Next, the ratio of the black area was calculated by counting the number of black scales from each region, and combined to obtain the total coverage of the ventral surface (from 0% to 100%). After evaluating all 127 individuals, we divided these snakes into 10 groups at 10% intervals. The sample sizes for each interval is represented in Fig. 2, revealing a bimodal distribution. The highest peak was located between 20% and 30% (35 individuals), representing a typical “white-ventral morph.” The second peak was located between 80% and 90% (24 individuals), representing a typical “black-ventral morph.” According to the distribution in Fig. 2, we defined snakes with ventral coverage of black scales of <40% as “white-ventral morphs,” and those with coverage exceeding 70% as “black-ventral morphs.” Individuals between 40% and 70% are categorized as “mosaic-ventral morphs.” Color composition in each sampled geographic region (Fig. 1) was evaluated and compared to each other using Chi-square tests.

Molecular techniques

One hundred five specimens were used for mitochondrial sequencing. This collection included 22–30 individuals from each geographic region, with totals of the white,

black, and mosaic color morphs from all regions being of 56:41, and 8 individuals, respectively (Table 1).

Genomic DNA was extracted from the blood or muscle tissues using a LiCl method (Gemmell and Akiyama 1996). After ethanol precipitation, DNA was suspended in 1X TE buffer and stored at –20°C. The complete mitochondrial control region, including two flanking tRNAs (tRNA-Pro and tRNA-Phe), was amplified by polymerase chain reaction (PCR). Primers were designed from the consensus sequences of several Squamata species:

PL1: 5'-CCACAAAGCATTTGTTCTTGTAACC-3' and  
PH1: 5'-GAAGCTTGCATGTATAAGTAGGG-3'.

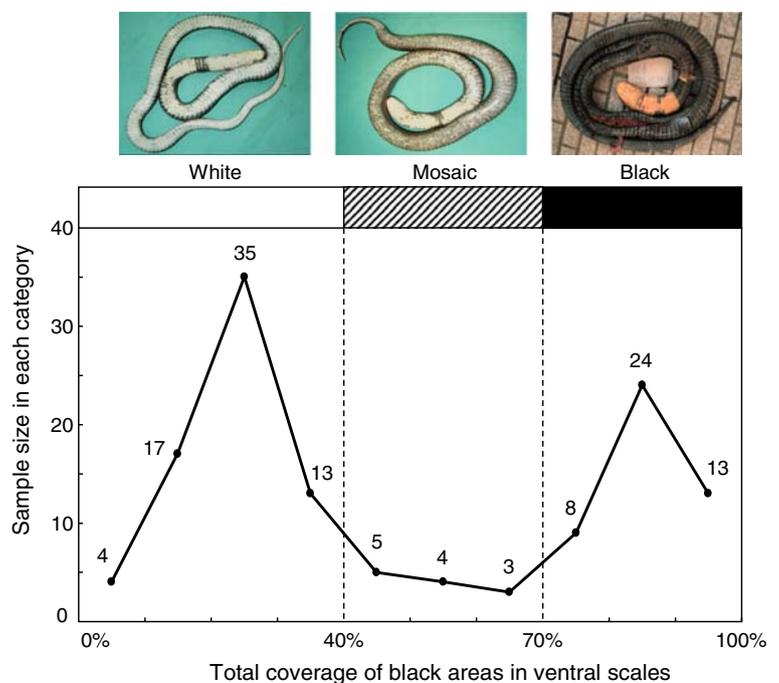
Reactions (including negative controls) were carried out in 20-µl reactions using iCycler thermal cyclers (Bio-Rad), with the following thermal cycles: 1 cycle at 94°C for 3 min; 35 cycles at 94°C for 30 s, 55°C for 40 s, and 72°C for 70 s; and 1 cycle at 72°C for 10 min. PCR products were purified with a PCR Product Pre-Sequencing Kit (USB Corporation), and subsequently used as the template for direct DNA sequencing reactions with a DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). The same primers for PCR were used for the sequencing reactions in addition to two internal primers we developed:

PL2: 5'-CCCTCAACGGCCCGGAACCATCCCTCCC-3' and

PH2: 5'-CTAAAGGTCTACCCCGCGAGGGG-3'.

Sequencing products were run on a MegaBACE 1000 automated DNA sequencer (Amersham Biosciences). Sequences were determined in both directions, and the original signals were proofread using SEQUENCHER

**Fig. 2** Evaluation of the coverage of black areas of the ventral scales from the 127 snakes in our collection, represented as percentages at 10% intervals



**Table 1** Samples sizes ( $N$ ), number of haplotypes ( $N_{hap}$ ), haplotype diversity ( $h$ ), nucleotide diversity ( $\pi$ ), and composition of ventral color types of *Naja atra* in Taiwan

Sampling Region	$N$	$N_{hap}$	$h$	$\pi$	Coloration (B/W/M)
Northern	26	8	0.695	0.00093	14/10/2
Central	27	8	0.801	0.00165	1/22/4
Southern	30	13	0.915	0.00207	4/24/2
Eastern	22	5	0.641	0.00076	22/0/0
Total	105	23	0.911	0.00180	41/56/8

B, black ventral; W, white ventral; M, mosaic ventral

software version 4.0.5 (Gene Codes Corporation). The sequences obtained were compared to those of other Elapid snakes to ensure the accuracy of the PCR amplifications.

### Data analyses

Sequences were initially assembled using SEQUENCHER 4.0.5. A hierarchical likelihood ratio test (Huelsenbeck and Crandall 1997) was performed using Modeltest 3.06 (Posada and Crandall 2001) to find the best-fit evolutionary model for *Naja* haplotypes. The HKY model with parameters for invariable sites and gamma distribution (HKY + I + G) was suggested to be the most appropriate model of evolution. This model was applied throughout all molecular calculations in this study. Haplotype diversity ( $h$ ), nucleotide diversity ( $\pi$ ), and genetic distances were calculated using MEGA 3.1 (Kumar et al. 2004). Estimations of gene flow among subpopulations were expressed by  $F_{st}$  and  $N_m$  values, computed using DnaSP 4.10.8 (Rozas et al. 2003). Furthermore, an AMOVA (analysis of molecular variance) was performed using ARLEQUIN (Schneider et al. 2000). In this analysis, samples were partitioned into (1) morphological and (2) geographic groups according to their coloration and collection sites.

Interrelationships among *Naja* haplotypes were represented by (1) a minimum spanning network constructed using MINSNET (Excoffier and Smouse 1994), and (2) an unrooted maximum-likelihood (ML) tree constructed using PAUP 4.0b10 (Swofford 2002). Parameters in the ML analysis were set based on the suggestions of Modeltest. A heuristic search was performed with a stepwise-addition pattern.

## Results

### Color differentiation of *Naja atra* in Taiwan

Cobras from different regions of Taiwan showed clear morphological differentiation in ventral coloration. From the 127 individuals evaluated, 46 snakes (36%) had

coverage of black ventral areas exceeding 70% (black-ventral morphs), while 69 snakes (54%) had coverage of <40% (white-ventral morphs). Only a minor proportion (12 snakes, 9%) was defined as having the “mosaic” form (Fig. 2).

Each geographic region in Taiwan revealed a unique composition of snake coloration (Fig. 1). The eastern population was distinctive in exclusively containing the black-ventral morph (100%). In contrast, white-ventral snakes were much more common in the central and southern populations (both 80%). In the northern population, the proportions of black and white morphs were 60% and 30%, respectively. Chi-squared tests revealed significant differences in the coloration composition between all possible pairings among the four regional populations ( $P < 0.001$ ) except for that between the central and southern populations ( $P > 0.05$ ).

### Genetic data of *Naja atra*

The obtained dataset consists of the complete sequences of tRNA-Pro (61 bp), the control region (1029 bp), and tRNA-Phe (61 bp). In total, 14 variable sites were discovered within this region. Twenty-three haplotypes were obtained from the 105 individuals (Table 1), with sequence variations ranging between 1 and 8 substitutions (with genetic distances of 0.0009–0.0070 under the HKY model). All these haplotypes have been submitted to GenBank (Accession numbers: DQ224315–DQ224337). Haplotype diversity and nucleotide diversity of each population are also presented in Table 1. The southern population exhibited the highest  $h$  and  $\pi$  values, while the eastern population exhibited the lowest.

Figure 3 shows the evolutionary relationships among the 23 haplotypes deduced from the minimum spanning network. The ML tree (not shown) exhibits almost identical topology to the network, except for some minor rearrangements among haplotype 2 and its satellites. The size of the circles is proportional to the number of individuals of each haplotype, while the shaded areas of the pie-charts show the relative proportion in terms of color morph

(Fig. 3A) and collection site (Fig. 3B). Considering the distribution of coloration, we found that haplotypes were shared among the color morphs. Sixty of the 105 snakes (57%) belonged to the four major haplotypes (haplotype 1–4), none of which are color-specific. The AMOVA analysis based on different color morphs revealed that 91.39% of the genetic variation occurred within color morphs, and only 8.61% occurred between color morphs (Table 2). Estimation of gene flow shows low  $F_{st}$  and high  $N_m$  values (Table 3A). This evidence indicates that genetic differentiation among the color morphs is limited with respect to neutral genes.

In contrast, a moderate level of genetic differentiation occurred among regional populations (Table 3B). Among these, the northern and central populations revealed a medium  $F_{st}$  value (0.1573), as did that between eastern and southern populations (0.1114). All of the other pairings indicate genetic isolation, with  $N_m$  values of  $<1$ . Although three of the four major haplotypes are still shared by different populations (Fig. 3B), the appearance of some geographic-specific haplotypes (i.e., haplotype 3) increases the  $F_{st}$  values. Conclusively, mitochondrial sequences do not show genetic differentiation among

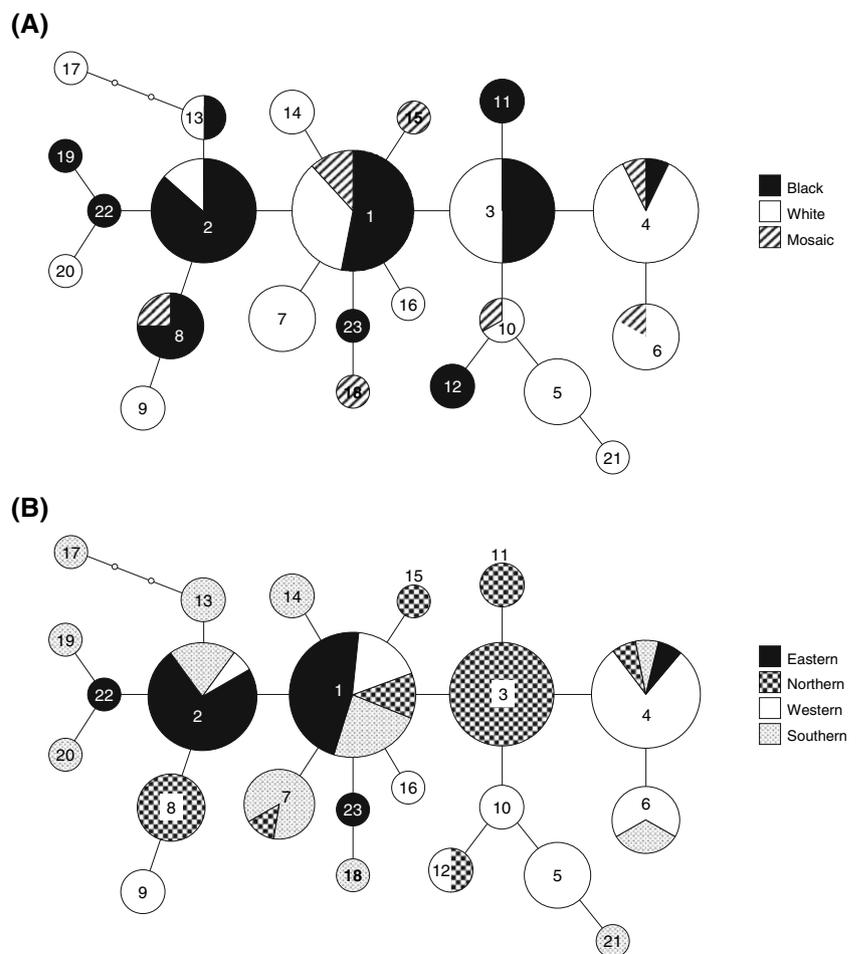
color morphs, but indicate moderate levels of differentiation among geographic regions, as seen by low estimates of gene flow.

### Discussion

#### Geographic differentiation

The morphological survey in this study verified the empirical claims of local snake collectors, traders, and commercial keepers that *N. atra* populations shows geographic differentiation based on ventral color-black in the east and white in the west. Traditional consumers, although lacking scientific training, can provide accurate and valuable information, in this case on morphology, due to their long-term observations based on financial necessity. However, we do not find any genetic differentiation between the two color morphs. Instead, genetic differentiation of cobras in Taiwan is mainly attributed to geographical factors (Table 2). As a result, we do not suggest making any taxonomic revisions based on coloration, but rather geography.

**Fig. 3** Interrelationships among the 23 cobra haplotypes represented by a minimum spanning network. The area of each pie piece indicates the proportions of (A) the different coloration patterns, and (B) the collection sites



**Table 2** AMOVA analysis of *Naja atra* among (A) color morphs, and (B) geographic regions

	Source of variation	d. f.	Sum of squares	Variance components	Percentage of variation
A	Among color morphs	2	9.353	0.11729 Va	8.61
	Within color morphs	102	126.986	1.24496 Vb	91.39
B	Among regions	3	29.422	0.33461 Va	24.02
	Within regions	101	106.917	1.05858 Vb	75.98
	Total	104	136.339	1.36225	

**Table 3** Population differentiation and gene flow of *Naja atra* among color morphs (A) and geographic regions (B). Upper-right:  $F_{st}$  values, and lower-left:  $N_m$  values

(A)				
$N_m \backslash F_{st}$	Black	White	Mosaic	
Black		0.1022	0.0326	
White	2.19		0.0433	
Mosaic	7.41	6.02		
(B)				
$N_m \backslash F_{st}$	Northern	Central	Southern	Eastern
Northern		0.1573	0.2740	0.4445
Central	1.34		0.2581	0.4322
Southern	0.66	0.72		0.1114
Eastern	0.31	0.33	2.00	

Interestingly, similar geographic patterns have been found in the venom secretion of *N. atra* in Taiwan. Toxicity per unit volume in the western populations was found to be roughly two times higher than that of the eastern populations, while the maximum envenomation of the eastern was four times higher than western populations (Liau 1991; Liau and Huang 1997). Wu (2003) found significant differences in venom composition between the eastern and western populations, especially the cardiotoxin homologues (CTXs). Analysis of populations in the northern revealed a mixed chromatogram in venom constituents of eastern and western populations, congruent with the composition of ventral coloration (Wu 2003; Chen et al. 2005; Fig. 1). These results provide explanation for the difference of medicinal potencies proposed by tradition medicine users (H.-C. Lin, personal interviews during 1990s). Furthermore, it has demonstrated that geographic differences should be carefully considered in venomous snakebite treatments.

### Demographic history

Cobras in Taiwan are characterized by relatively low levels of genetic variation across its range. Compared to other well-studied Squamata species in Taiwan, including the bamboo viper (*Trimeresurus stejnegeri*, Creer et al. 2001),

a glass lizard (*Ophisaurus harti*, Lin et al. 2002), a skink (*Sphenomorphus taiwanensis*, Guo 2002), a gecko (*Gekko hokouensis*, Tsai 1999), and several species of grass lizards (*Takydromus* spp., Lin 2003) and Agamods (*Japalura* spp., Shang 1997), *N. atra* exhibits the lowest nucleotide diversity and sequence divergence at a similar geographic scale (Table 4). *N. atra* in Taiwan are characterized as possessing high haplotype diversity ( $h$  values), but low nucleotide diversity ( $\pi$  values). Possible explanations include (1) a current small population size, or (2) a historical small population size (Rogers and Harpending 1992; Grant and Bowen 1998; Avise 2000; 2004).

As one of the more common snake species found in Taiwan, the first explanation can be discarded. The current distribution of *N. atra* is limited to lowland areas of Taiwan, and it is much more common in the southern part of the island. Furthermore, with *N. atra* being absent from the Ryukyus (Ota 2000), Taiwan is the northern distributional limit of cobras among East Asian islands. Reconstruction of palaeovegetation by ancient pollen records indicates that most regions in Taiwan have experienced drastic alteration (Harrison et al. 2001; Liew and Chung 2001; Yu et al. 2003; Hope et al. 2004), and that the climate was probably too cold for this tropical species to survive during the past glacial periods. The more probable explanation for such genetic characteristics is a population bottleneck during past glaciations. Subsequent population expansion after a glacial bottleneck would have resulted in the accumulation of singleton mutations, resulting in a star-like relationship among haplotypes, which is what we see in our data (Fig. 3; Rogers and Harpending 1992; Grant and Bowen 1998; Avise 2000; 2004).

### Is ventral coloration a selected trait?

According to our ongoing breeding experiments (Lin et al., unpublished data), the ventral coloration of *N. atra* is heritable. Hatchlings from black-ventraled individuals exhibited coloration pattern similar to their parents, as well as those reproduced by white-ventraled ones. In one case, a white-ventraled snake mated with a blacked-ventraled snake and produced mosaic-ventraled hatchlings. This

**Table 4** Genetic diversity of *Naja atra* compared to other Squamata species in Taiwan

Scientific Name	Family	DNA marker	<i>H</i>	$\pi$	Maximum Div.	References
<i>Naja atra</i>	Elapidae	Control region	0.899	0.0019	0.0070	Current study
<i>Trimeresurus stejnegeri</i>	Viperidae	Cytb	0.918	0.0197	0.0523	Creer et al. (2001)
<i>Ophisaurus harti</i>	Agamidae	Cytb	0.854	0.0100	0.0244	Lin et al. (2003)
<i>Takydromus stejnegeri</i>	Lacertidae	Cytb	0.957	0.0153	0.0352	Lin (2003)
<i>Takydromus formosanus</i>	Lacertidae	Cytb	0.977	0.0801	0.1573	Lin (2003)
		COI	0.962	0.0578	0.1013	
<i>Sphenomorphus taiwanensis</i>	Scincidae	Cytb	N/A	N/A	0.139	Guo (2002)
		COI			0.092	
<i>Gekko hokouensis</i>	Gekkonidae	12S rRNA	N/A	N/A	0.105	Tsai (1999)
		Cytb			0.181	
<i>Japalura swinhonis</i>	Agamidae	12S rRNA	N/A	N/A	0.08	Shang (1997)
<i>Japalura brevipes</i>	Agamidae	12S rRNA	N/A	N/A	0.17	Shang (1997)
<i>Japalura makii</i>	Agamidae	12S rRNA	N/A	N/A	0.19	Shang (1997)

observation supports the heritability of ventral coloration in *N. atra*. Furthermore, all hatchlings remained consistent in ventral coloration during their ontogeny. For these reasons, morphological plasticity is preliminary excluded as the reason of color differentiation.

Geographic differentiation in ventral coloration in *N. atra* may be a result of selection, since ventral coloration has become geographically fixed more quickly than mtDNA haplotypes. The extremely low genetic variation of *N. atra* in Taiwan indicates this species is relatively young in evolutionary time. Shared haplotypes are commonly found among geographic regions (Fig. 3B), indicating that the mitochondrial genome of *N. atra* in different regions has not yet reached the stage of reciprocal monophyly. Theoretically, with a higher substitution rate, along with a lower effective population size in gene copy number, mitochondrial sequences should have evolved and fixed at a faster speed than nuclear genes (Avice 2000). Without selective pressure, ventral coloration is unlikely to have formed the current pattern we see today. However, additional research needs to be done to definitively show that ventral coloration in *N. atra* is a result of local adaptation.

Founder effects or multiple invasions to eastern and western populations are alternative explanations for the color pattern observed on this island. However, mitochondrial sequences did not show evidence for multiple invasions. Furthermore, these hypotheses are excluded because ventral color polymorphism, as well as coloration differentiation, has never been reported in adjacent regions where the same species occurs (Zhao et al. 1998). According to our preliminary observation, the same species collected from south-eastern China represented exclusive black-ventral morph, congruent with those morphological descriptions in literature (Zhao et al. 1998). This

observation indicates that the white-ventral morph is probably a newly evolved form locally occurring in western Taiwan.

If ventral coloration is in fact shown to be a result of selection, we provide two potential explanations: thermoregulation and crypsis. In some cases, color variation is considered to be associated with thermoregulation (Gibson and Falls 1979; King 1988; Majerus 1998; Bittner et al. 2002). However, being distributed in tropical and subtropical regions, cobras do not seem to encounter thermal problems relative to species living in temperate regions or deserts. Furthermore, the major climate differences observed in Taiwan are latitudinal, not in an east-west direction as seen in morphological differences. With limited climate differences between the eastern and western regions, this explanation is not favored.

In other cases, coloration in snakes is attributed to natural selection by visual predators (Camin and Ehrlich 1958; Ehrlich and Camin 1960; King 1992; 1993). Snakes with suitable coloration are more cryptic against their environmental background and represent better survivorship (King 1992; 1993). The Created Serpent Eagle (*Spilornis cheela*), the most common raptor in Taiwan feeding almost exclusively on snakes, is the potential predator for *N. atra*. In fact, melanin levels influences both ventral and dorsal coloration. Dorsal coloration in black-ventraled snakes is mostly black, while the coloration of white-ventraled individuals is usually some shade of brown. In general, these dorsal colors seem to match the different soil types in eastern and western Taiwan. The majority of substrates in eastern Taiwan are composed of metamorphic rocks, usually producing dark soil substrates after weathering, where the black morphs are found. In contrast, western Taiwan is comprised of sedimentary rocks, producing red or

brownish soils. Hence, color patterns in cobras may reflect advantages in crypsis and predator avoidance.

### Implication to conservation

Re-release of captive animals back into the wild is always a serious problem when considering conservation, especially when animals are moved long distances (IUCN 2002). In the case of *N. atra*, black-ventral individuals are harvested from eastern Taiwan and transported to the densely populated western side where there is better market value (Lin 1997). Most of this trade is illegal and snakes confiscated from shipments are usually released in the local area without considering their source of collection. In addition, practitioners of Buddhism often buy large numbers of such snakes and release them to the wild for religious ceremonies. Due to the fact that we have found geographic differentiation based on coloration, venom characters, and genetics, current practices of translocated release and artificial breeding in commercial farms will likely cause serious problems of unnatural homogenization. Under this situation, proper planning for evolutionarily significant units (ESUs) for *N. atra* in Taiwan is required and should be made a high priority in the conservation and management plan.

Based on the original definitions (Ryder 1986; Waples 1991; Moritz 1994a, b), *N. atra* populations in Taiwan do not fit the criteria of ESUs due to (1) discordance between morphology and molecular data, and (2) no reciprocal monophyly for each population. However, it should be noted that mitochondrial DNA is a nearly-neutral marker, while ventral coloration is a possibly selected trait. Natural selection on morphological traits explains the discordance observed from these two datasets. Secondly, Crandall et al. (2000) have argued against the threshold of reciprocal monophyly. Such a result is highly dependent to the sensitivity of the marker that is selected; adding additional mitochondrial DNA and/or nuclear markers may provide greater resolution.

Further work with *N. atra* in Taiwan needs to be carried out to test our hypothesis of natural selection causing differentiation between local populations. Additional research directions should include molecular and ecological approaches. Molecular approaches should include the analysis of nuclear markers, including faster evolving ones like microsatellites as well the candidate gene approach investigating the role of the MC1R gene on coloration. Ecological approaches should include the use of models in the field to determine the relationship between predation and crypsis in different habitats.

In our study, the four regional *N. atra* populations in Taiwan are characterized by (1) significant coloration

differentiation; (2) reproductive isolation deduced from low gene flow estimates; (3) differences in venom characteristics, and (4) potentially different selection pressure connected with local environments. Among these, the central and the southern are the only two populations exhibiting similar color composition (0.05 in Chi-square tests). Nevertheless, mitochondrial sequences from these two populations exhibit low gene flow. These lines of evidence support the recognition of four regional populations of *N. atra* in Taiwan as different ESUs. With the delineation of ESUs for *N. atra*, in Taiwan, proper conservation and management can be undertaken. Re-release of animals has many potential problems (IUCN 2002), and we recommend that releasing animals into the wild should not be applied unless their origins could be confidently confirmed since structuring of *N. atra* populations seems to be based more on geography than ventral coloration. Releasing snakes regardless of their origins as a token of religious piety by Buddhists should be particularly forbidden.

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