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A new color pattern of the *Bungarus candidus* complex (Squamata: Elapidae) from Vietnam based on morphological and molecular data

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Abstract

Kraits with black and white bands from Nui Chua National Park, central Vietnam are morphologically similar to the Burmese Krait, *Bungarus magnimaculatus*, however, analysis of molecular data finds them to be nested within the *B. candidus* complex.

Key words: Blue Krait, *Bungarus magnimaculatus*, *COI*, genetic distance

Introduction

Kraits (*Bungarus*) are generally well known partly because of their abundance in anthropogenic settings and their venom, which is both lethal and therapeutic. Therefore, taxonomic identification of individual snakes can represent a pressing matter. With 14 species, kraits occur widely from Pakistan eastward through Asia to Indonesia (Slowinski 1994; Kuch *et al*. 2005; Uetz & Hallermann 2016). Two kraits, *Bungarus candidus* (Linnaeus, 1758) and *B. magnimaculatus* (Wall & Evans 1901), differ from the other species by the presence of distinct broad black-and-white bands on their bodies (not occurring in pairs), enlarged vertebral scales, 15 dorsal scales rows, and undivided subcaudals. They differ from each other only by the number of bands in the color pattern. Whereas *B. candidus* has 20–25 white bands on its body, *B. magnimaculatus* has 11–14 (Wall 1908; Smith 1943; Slowinski 1994). *Bungarus magnimaculatus* occurs in Myanmar and *B. candidus* occurs widely from southern Vietnam, Cambodia, Thailand to Malaysia, Indonesia, and Singapore (Uetz & Hallermann 2016).

In 2005, we collected a krait at Nui Chua National Park (hereafter Nui Chua NP), central Vietnam that had only 14 white bands. Using existing identification keys (Wall 1908; Smith 1943; Slowinski 1994) we identified it as *B. magnimaculatus*. Two additional specimens collected from Nui Chua in 2008 and 2009 have only 15 white body bands. These records suggested that the Myanmar Krait occurs in Vietnam. Alternatively, the Nui Chua material could represent a new species or a morphological variant of *B. candidus*. Because the two species are similar to each other except for the number of body bands, we used DNA sequences to aid in identification of the Nui Chua material.

DNA barcoding serves to estimate biodiversity and identify species, as well as for planning conservation (e.g. Hebert *et al*. 2003, 2004, 2009; Mabragana *et al*. 2011; Clare *et al*. 2011; Nagy *et al*. 2012). The method uses

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nucleotide sequences of the mitochondrial DNA gene Cytochrome *c* oxidase subunit I (*COI*). This gene appears to be more informative than the traditionally used 16S rRNA (Xia *et al*. 2012). Hence, we use *COI* to identify the Nui Chua kraits, access its taxonomic position, and explore variation in the number of body bands within Vietnamese *B. candidus*.

Materials and methods

Sampling. Specimens used in this study are deposited in the collections of the Institute of Tropical Biology Zoological Collection (ITBCZ), Ho Chi Minh City, Vietnam; Kunming Institute of Zoology (KIZ), Kunming, China; Hue University of Sciences (HUS), Hue, Vietnam; Museum of Vertebrate Zoology (MVZ), Berkeley, USA; and California Academy of Sciences (CAS), San Francisco, USA. Three krait specimens were collected at night within Nui Chua NP, Ninh Thuan Province, central Vietnam, including ITBCZ 12, collected in 2005, about 800 m a.s.l.; ITBCZ 900 collected on 20 November 2008, N 11°37'53.2"; E 109°09'32.1", 58 m a.s.l.; and ITBCZ 1140 collected on 22 March 2009, N 11°44'35.5"; E 109°07'50.8", 785 m a.s.l. In addition, other specimens of *B. candidus* from southern Vietnam and *B. magnimaculatus* from Myanmar were also included in our analyses (Fig. 1, Table 1, Appendix 1).

TABLE 1. List of samples used in this study. Partial *COI* sequence of *Naja naja* was extracted from its complete mitochondrial genome (Yan *et al*. 2008) without data on locality and museum code.

Taxon	Locality	Voucher	GenBank accession no.
Bungarus candidus	Virachey NP, Ratanakiri, Cambodia	MVZ Herp 258148	KY769757
B. candidus	Bu Gia Map NP, Binh Phuoc, Vietnam	KIZ 100	KY769758
B. candidus	Ta Kou NR, Binh Thuan, Vietnam	KIZ 1326	KY769759
B. candidus	Nui Chua NP, Ninh Thuan, Vietnam	ITBCZ 900	KY769760
B. candidus	Nui Chua NP, Ninh Thuan, Vietnam	ITBCZ 1140	KY769761
B. candidus	Bach Ma NP, Thua Thien–Hue, Vietnam	HUS 300	KY769762
B. fasciatus	Tam Dao NP, Vinh Phuc, Vietnam	MVZ Herp 224274	KY769763
B. fasciatus	Tam Dao NP, Vinh Phuc, Vietnam	MVZ Herp 224275	KY769764
B. fasciatus	Tam Dao NP, Vinh Phuc, Vietnam	MVZ Herp 226609	KY769765
B. fasciatus	Tam Dao NP, Vinh Phuc, Vietnam	MVZ Herp 226610	KY769766
B. fasciatus	Tam Dao NP, Vinh Phuc, Vietnam	MVZ Herp 226611	KY769767
B. magnimaculatus	Alaungdaw Kathapa, Sagaing, Myanmar	CAS 210526	KY769768
B. magnimaculatus	Sin Ma Taung, Magway, Myanmar	CAS 210696	KY769769
B. magnimaculatus	Alaungdaw Kathapa, Sagaing, Myanmar	CAS 215445	KY769770
B. magnimaculatus	Min Sone Taung, Mandalay, Myanmar	CAS 215958	KY769771
B. magnimaculatus	Min Sone Taung, Mandalay, Myanmar	CAS 215998	KY769772
B. magnimaculatus	Min Sone Taung, Mandalay, Myanmar	CAS 215999	KY769773
Naja naja			DQ343648

Tissue samples from a total of 17 specimens of *Bungarus* were used for sequencing (Table 1). The Burmese Krait, *B. magnimaculatus*, from Myanmar and *B. candidus* from Vietnam and Cambodia were compared to the kraits from Nui Chua. The Banded Krait *Bungarus fasciatus* (Schneider) and another elapid, *Naja naja* (Linnaeus) (GenBank accession number DQ343648), were used as outgroup taxa.

Laboratory methods. Tissues from liver or heart were stored in 95% ethanol and kept at room temperature or frozen at -80°C. For sequencing *COI*, total genomic DNA was extracted from tissue samples using proteinase K and the standard three-step phenol/chloroform extractions (Sambrook *et al.* 1989).

Amplification was performed in a 25 µL volume with the following procedures: initial denaturation step with 95°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for

1 min; and final extension at 72°C for 10 min. Primers used for PCR were COI-CO1, COI-CO2, COI-CO3, and COI-CO4, which were taken from Che *et al*. (2012). We combined the four sets of primers for PCR and then used the first and the third pairs for sequencing. PCR product was cleaned using a ratio of 0.55 H_20 : 0.30 ExoI : 0.15 SAP (Hanke & Wink 1994). Amplified DNA fragments were purified using BigDye™ program in a GeneAmp PCR System 9700 (Applied Biosystems) and 75% isopropyl alcohol, and then sequenced with an ABI PRISM 3730 Automated DNA Sequencer (Applied Biosystems) following the manufacturer's protocol in both directions for each sample.

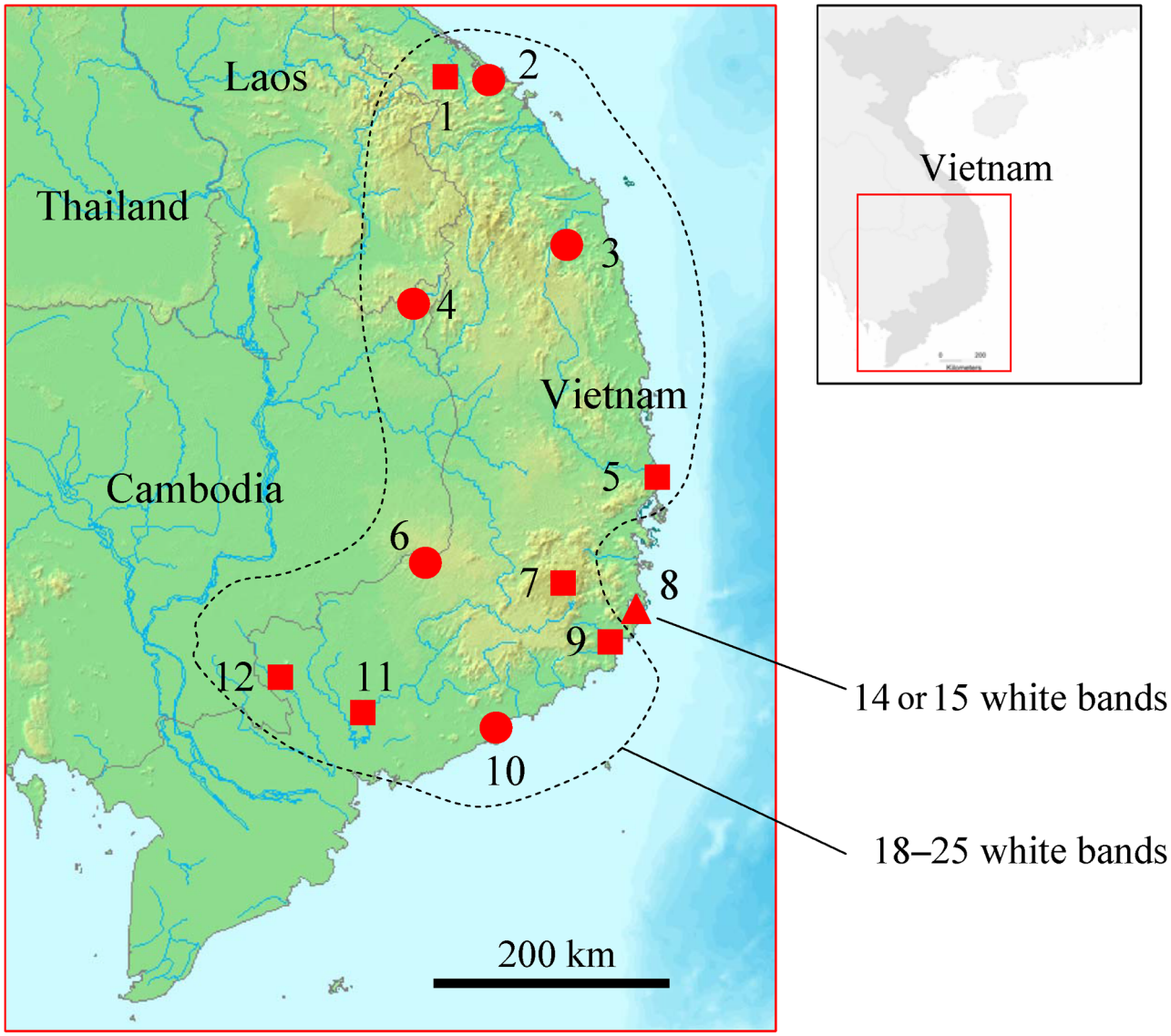

FIGURE 1. Map of sampling sites of *Bungarus candidus* in southern Vietnam. Circles indicate sample sites of *B. candidus* examined in this study, squares are localities recorded by Campden-Main (1970), and triangle shows the location of the new form of *B. candidus* from Nui Chua National Park. Localities: 1, Thua Luu, Thua Thien–Hue, Vietnam; 2, Bach Ma NP, Thua Thien–Hue, Vietnam; 3, Ba To, Quang Ngai, Vietnam; 4, Virachey NP, Ratanakiri, Cambodia; 5, Dai Lanh, Phu Yen, Vietnam; 6, Bu Gia Map NP, Binh Phuoc, Vietnam; 7, Da Lat, Lam Dong, Vietnam; 8, Nui Chua NP, Ninh Thuan, Vietnam; 9, Phan Rang, Ninh Thuan, Vietnam; 10, Ta Kou Nature Reserve, Binh Thuan, Vietnam; 11, Bien Hoa, Dong Nai, Vietnam; and 12, Tay Ninh, Vietnam.

Data analysis. Initial nucleotide sequences were verified using SeqMan (DNASTAR Lasergene 7, Madison, WI) and then aligned against a sequence from cobra in GenBank using ClustalW (Thompson *et al*. 1994) integrated in MEGA 4.1 (Tamura *et al*. 2007) with default parameters. The sequences were then translated into amino acids to check for premature stop codons, which were assumed to indicate nuclear pseudogenes (Song *et al*. 2008).

The Kimura 2-parameter distance (K2P) model of base substitution (Kimura 1980) and uncorrected *p*-distance were used to calculate genetic distances between individuals and between populations of kraits by using MEGA.

Reconstructions of the matrilineal genealogy used Bayesian Inference (BI), Maximum Parsimony (MP) and Maximum Likelihood (ML). The best-fit models of sequence evolution were determined using MrModeltest 2.3 (Nylander 2004) under the Akaike information criterion based on codon positions. The second codon position did not vary. Hence, only the first and third codon positions were used for Bayesian analyses with models of SYM+I and HKY+I, respectively. Partitioned BI was performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Bayesian posterior probabilities (BPP), i.e. the frequency of nodal resolution, were estimated using a Markov chain Monte Carlo sampling approach with one million generations, saving one tree every 100 generations based on the models chosen by MrModeltest. The runs were stopped when the average standard deviations reached 0.07. The initial 25% of the samples were discarded as burn-in. The remaining trees were combined, and a 50% majority consensus tree was generated. MP analyses were performed in TNT (Goloboff *et al*. 2008) using an implicit enumeration search with random addition sequences followed by TBR branch-swapping. Bootstrap branch support values were based on 1000 pseudoreplicates. The RAxML web server (Stamatakis *et al*. 2008) was used to search ML trees using the Gamma model of rate heterogeneity option and partitioned models. We considered bootstrap values of ≥70% for ML and MP, and BPP of ≥95% as being strongly supported (Hillis & Bull 1993, Felsenstein 2004).

For morphological analysis, we focused mainly on the number of white bands on the body because it varies among the relevant species. Scale counts (e.g., Wall 1908; Smith 1943; Slowinski 1994; Leviton *et al*. 2003), on the other hand, did not vary significantly between *B. candidus* and *B. magnimaculatus*. For a brief description of the new color pattern, the following morphological characters were used: SVL, snout to vent length: measured from the tip of the snout to the vent; TaL, tail length: measured from the vent to the tip of the tail; TL, total length: sum of SVL and TaL; HL, head length: measured from the tip of the snout to the posterior margin of the mandible; HW, head width: measured at the widest part of the head immediately posterior to the eye; HH, head height: vertical height between upper and ventral surfaces of head measured at HW; IO, interorbital distance: shortest distance between outer margins of supraoculars; ED, eye diameter: horizontal diameter of eye; SnL, snout length: distance between the tip of the snout and anterior edge of eye; EN, eye to nostril: distance between anterior margin of eye and posterior margin of nostril; SL, supralabials: number of scales on upper lip; SL-Eye: number of SL entering orbit; InL, infralabials: number of scales on lower lip; PreSubO, presubocular scale; PT, palatine teeth: number of palatine teeth on the right side of upper jaw; TP, temporal: number of scales immediately behind postocular between posteriormost SL and parietals; VS, ventral scales: counted from the ventral scale which is wider than long to the vent excluding cloacal plate; DS, dorsal scale rows at neck (number of scale rows at one head length behind the head), at midbody (number of scale rows at a position corresponding to the midpoint of the ventral scale rows, VS/2), and at one head length prior to the vent; CP, cloacal plate: number of terminal ventral scales immediately anterior to vent; SC, subcaudal scales: number of paired subcaudal scales excluding the terminal scute; WBB, number of white bands on body; and WBT, number of white bands on tail. Values of paired characters are given in order of left and right.

Measurements (in millimeters), except for SVL and TaL, were taken with a digital caliper to the nearest 0.1 mm. Teeth were counted using a zoom stereo microscope at 7X–45X. The right hemipenis of the male was forcedly everted by injecting water before fixation.

Results

Morphological variation. Medium size in adults (total length up to 801 mm); body thin and elongate; tail moderate and length up to 104 mm (Fig. 2); head faintly distinct from neck; eye small (ED/HH = 0.24–0.29), diameter shorter than distance between eye and nostril; snout obtuse; large nostril near snout and touching internasal. Rostral touching internasals, nasals, and first supralabials; supralabial 7/7, the 3th and 4th bordering eye, the 6th being largest; loreal scale absent; 1/1 preocular, longer than high; 2/2 postoculars; 1+2/1+2 temporals, anterior one elongated; presubocular absent; 7/7 infralabials, first pair in contact with each other medially, first fourth InLs in contact with anterior chin shield, the 4th largest and touching both anterior and posterior chin shields. Dorsal scales smooth in 15–15–15 rows; vertebral scales distinctly enlarged; ventral 224–226; cloacal plate undivided; subcaudal 45–47, undivided; terminal caudal scale forming a pointed cap. Palatine teeth 11–13, curved posteriorly, the posterior ones not distinctly enlarged. Hemipenis not forked; proximal part with spines and the

distal part with calyces; the largest spines are those nearest the calyces (Fig. 3). Black above with 14 or 15 broad white cross bands on body and 4 or 5 on tail; the white bands immaculate or sometime the anterior ones with black spots; head blackish above with a light indistinct chevron-shaped mark on nape; venter white; lower side of tail with black bands or blotches. Comparing to females, male has a bigger size (SVL = 801 mm vs. 760 mm), a slightly longer tail (TaL/SVL = 0.13 vs. 0.12); more ventral scales (226 vs. 224); and fewer palatine teeth (11 vs. 13). Table 2 summarizes variation in size and scalation of the Nui Chua material.

The new pattern-form of *B. candidus* differs from *B. magnimaculatus* by having immaculate white bands, or sometimes with black spots on some scales, in its anterior bands (vs. all white bands with black in centre and white in margin on each scales). The Nui Chua kraits differ from typical *B. candidus* by having fewer white bands on body (14 or 15 vs. 18–25) and on tail (4 or 5 vs. 7–10).

Our counts of the white body bands in *B. candidus* from southern Vietnam are lower than those of previous reports: 18–20 versus 20–25. Specifically, specimens HUS 300 from Bach Ma NP, Thua Thien–Hue Prov., Vietnam has 20+6 white bands, HUS 113 from Ba To, Quang Ngai Prov., Vietnam has 18+6 white bands, KIZ 100 from Bu Gia Map NP, Binh Phuoc Prov., Vietnam has 20+5 white bands, and KIZ 1326 from Ta Kou Nature Reserve, Binh Thuan Prov., Vietnam has 19+7 white bands. The gap between 15 and 18 body bands remains to be filled.

Sequence variation. The data set consists of 18 taxa with 567 characters of the *COI* fragment. Among the aligned sequences, 97 characters are potentially parsimony-informative, and 37 sites are variable. Gaps, missing data, and stop codons were absent in the data set. All sequences were deposited in GenBank with accession numbers KY769757–73 (Table 1).

FIGURE 2. Three blue kraits from Nui Chua NP with 14 or 15 white bands on body. A, ITBCZ 1140; B, ITBCZ 900; and C, ITBCZ 12.

TABLE 2. Measurements (in mm) and scalation of the new form of *Bungarus candidus*. See Materials and Methods for abbreviations.

Characters	ITBCZ 900	ITBCZ 12	ITBCZ 1140	Min-Max
Sex	Male	Female	Female	
SVL	801	511	760	511–801
TaL	104	71	90	71–104
TL	905	582	850	582–905
TaL/SVL	0.13	0.14	0.12	0.12–0.14
HL	25.8	16.2	23.9	16.2–25.8
HW	15.2	10.0	12.7	10.0–15.2
HH	11.8	7.5	9.2	7.5–11.8
IO	8.0	6.1	7.0	6.1–8.0
ED	2.8	2.2	2.4	2.2–2.8
SnL	8.21	5.1	7.0	5.12–8.2
EN	3.4	2.4	3.3	2.34–3.4
DS	15–15–15	15–15–15	15–15–15	15–15–15
VS	226	224	224	224–226
SC	45	47	45	45–47
SL	7	7	7	7
InL	7	7	7	7
SL-Eye	3+4	3+4	3+4	3+4
TP	1+2	1+2	1+2	1+2
PT	11	13	13	11–13
WBB	15	14	15	14–15
WBT	4	5	4	4–5

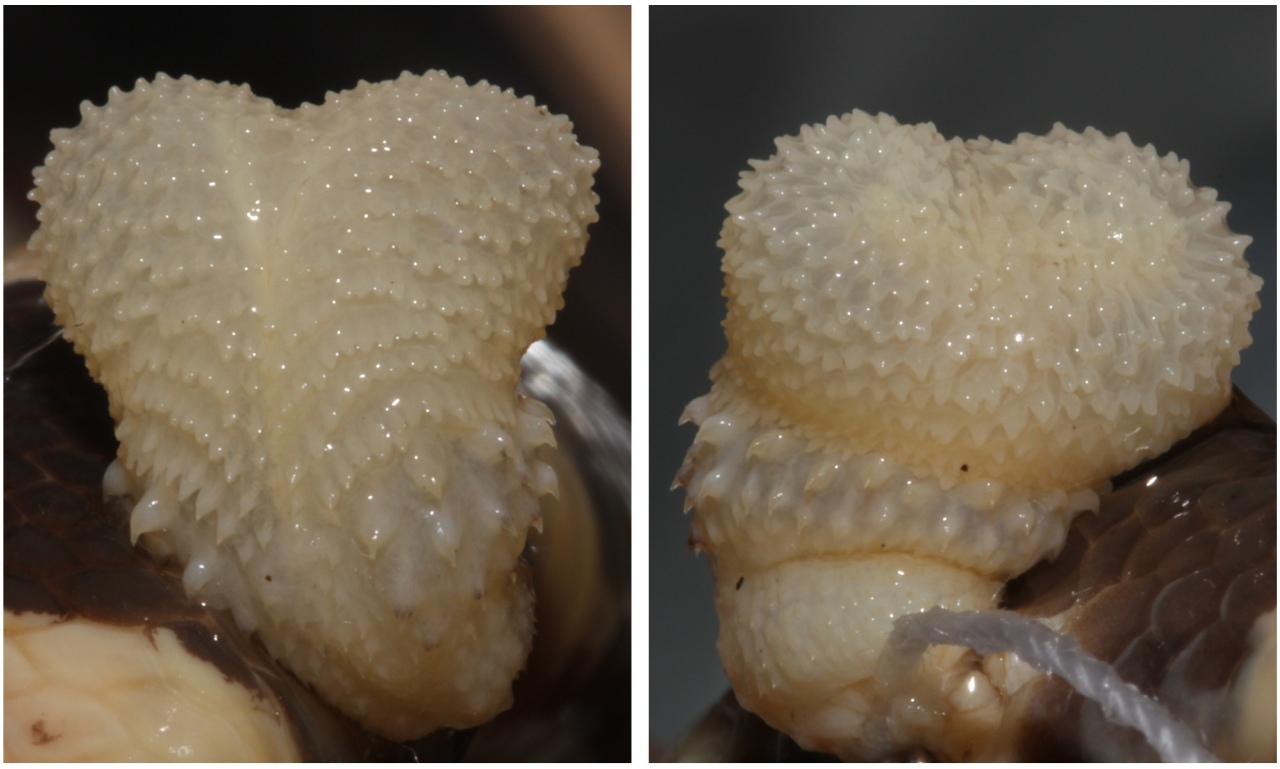

FIGURE 3. Hemipenis of *Bungarus candidus* from Nui Chua NP (ITBCZ 900).

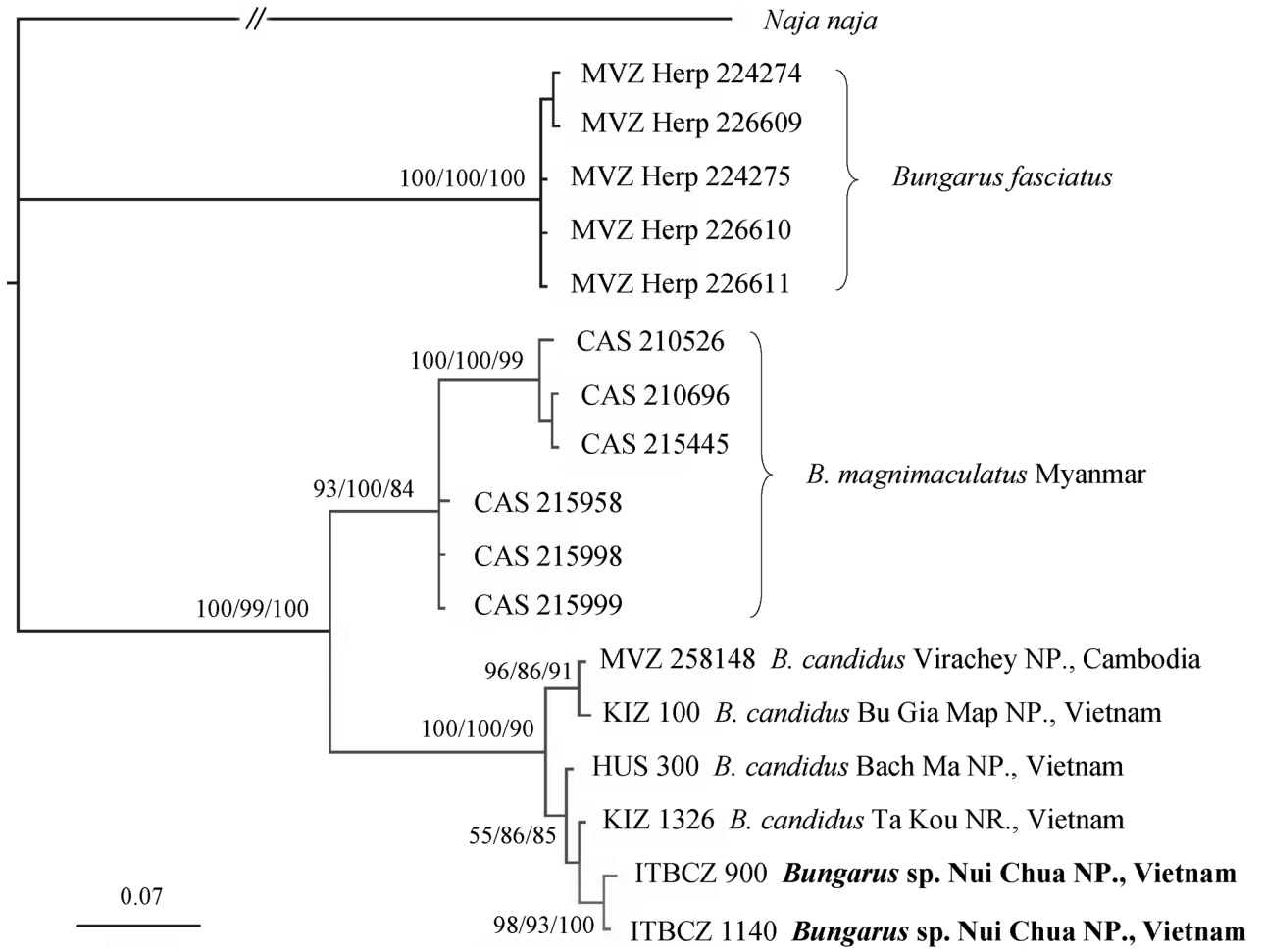

FIGURE 4. Tree from Bayesian inference for *Bungarus candidus*, *B. magnimaculatus*, and outgroup taxa. Values (%) at internal nodes are Bayesian posterior probabilities and bootstrap values from maximum parsimony and maximum likelihood, respectively.

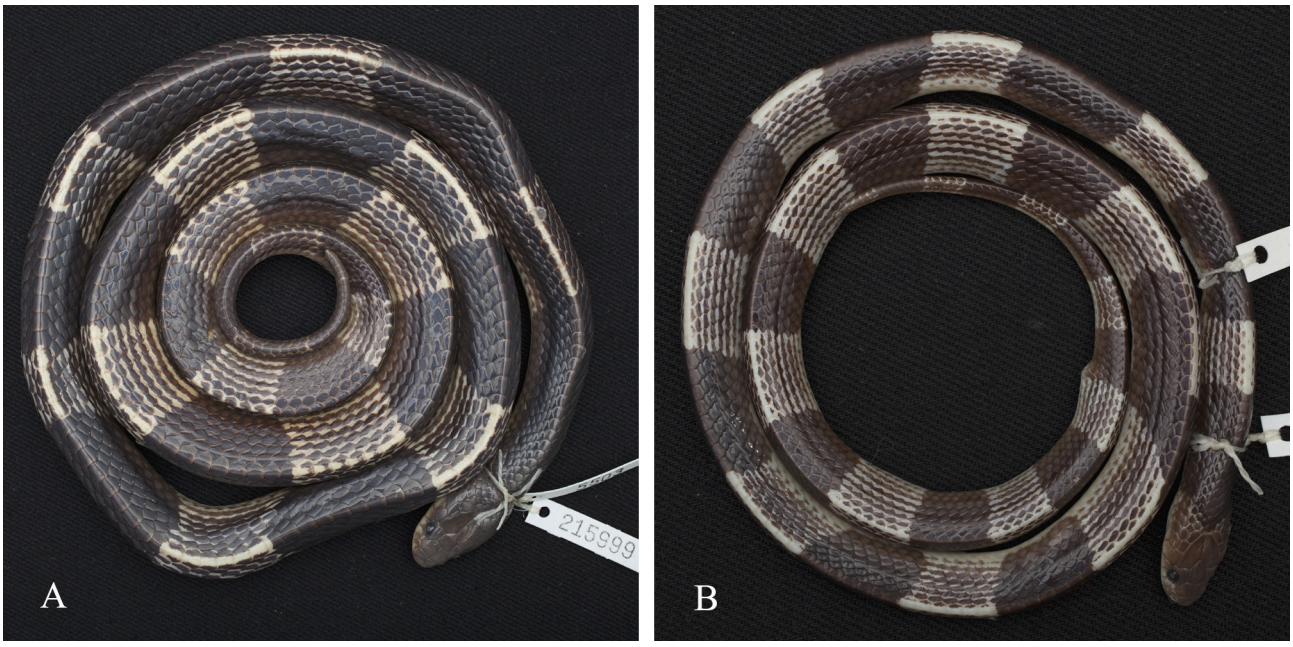

FIGURE 5. *Bungarus magnimaculatus* from Myanmar. A, specimen CAS 215999; B, specimen CAS 210696.

The K2P and *p*-distances between individuals were shown in Table 3. Accordingly, two samples from Nui Chua NP differed slightly from each other, but only by 0.2%. Krait ITBCZ 900 differed from other *B. candidus* from Vietnam and Cambodia by an average of 1.4±0.6% and ITBCZ 1140 differed by an average of 1.2±0.6%. As a group, kraits from Nui Chua NP differed genetically from Vietnamese and Cambodian *B. candidus* by about 1.3%, and from *B. magnimaculatus* by up to 7.4%.

TABLE 3. Genetic distances for *COI* (Kimura 2-parameter in lower-left and uncorrected *p*-distance in upper-right, %) between individuals of *Bungarus candidus* and *B. magnimaculatus*. Information about vouchers after species names was showed in Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12
1. *B. candidus* ITBCZ 900		0.2	0.9	0.7	1.9	1.8	6.5	7.8	7.4	7.4	6.3	6.5
2. *B. candidus* ITBCZ 1140	0.2		0.7	0.5	1.8	1.6	6.3	7.6	7.2	7.2	6.2	6.3
3. *B. candidus* HUS 300	0.9	0.7		0.2	1.1	0.9	6.0	7.6	7.2	7.2	5.8	6.0
4. *B. candidus* KIZ 1326	0.7	0.5	0.2		1.2	1.1	6.2	7.8	7.4	7.4	6.0	6.2
5. *B. candidus* KIZ 100	2.0	1.8	1.1	1.3		0.2	6.3	7.9	7.6	7.6	6.2	6.3
6. *B. candidus* MVZ 258148	1.8	1.6	0.9	1.1	0.2		6.2	7.8	7.4	7.4	6.0	6.2
7. *B. magnimaculatus* CAS 215999	6.9	6.7	6.3	6.5	6.7	6.5		2.5	2.5	2.5	0.2	0.0
8. *B. magnimaculatus* CAS 210526	8.4	8.2	8.2	8.4	8.6	8.4	2.5		0.4	0.4	2.6	2.5
9. *B. magnimaculatus* CAS 210696	7.9	7.7	7.7	7.9	8.2	7.9	2.5	0.4		0.0	2.6	2.5
10. *B. magnimaculatus* CAS 215445	7.9	7.7	7.7	7.9	8.2	7.9	2.5	0.4	0.0		2.6	2.5
11. *B magnimaculatus* CAS 215958	6.7	6.5	6.1	6.3	6.5	6.3	0.2	2.7	2.7	2.7		0.2
12. *B. magnimaculatus* CAS 215998	6.9	6.7	6.3	6.5	6.7	6.5	0.0	2.5	2.5	2.5	0.2	

Phylogenetic analysis. Topologies from the three tree-building methods differed only in their resolution of poorly supported nodes. Only the 50% majority rule consensus tree from the BI analysis was shown (Fig. 4) but with nodal support values from BI, MP, and ML, respectively. Both sequenced kraits from Nui Chua NP clustered with *B. candidus* with strong support. *Bungarus magnimaculatus* formed a highly supported independent lineage.

Discussion

Linnaeus (1758) described the Blue Krait, *Bungarus candidus*, with the type locality being "Indiis" (considered as error by Uetz & Hallermann 2016). Boulenger (1896) regarded *B. candidus*, *B. multicinctus* Blyth, and *B. caeruleus* (Schneider) as subspecies of *B. candidus* because of variation in the number of bands in the color pattern. Wall & Evans (1901) described *B. magnimaculatus* as a variety of *B. caeruleus*, which Wall & Evans (1900) described a year earlier. Wall (1908) raised *B. magnimaculatus* to a full species based on the difference in number of white broad bands. Wall (1908) also treated *B. candidus*, *B. multicinctus*, and *B. caeruleus* as full species and provided a key for 12 species of known kraits. The taxonomy and key were used by subsequent researchers (e.g., Smith 1943; Slowinski 1994). Accordingly, *B. magnimaculatus* with its 11–14 white body bands differs from *B. candidus*, which has 20–25 white bands. Several authors (Table 4) also reported these band-numbers for the two species. No intermediate forms between two these species have been recorded.

The presence of 14 or 15 white body bands suggests that the kraits from Nui Chua NP are *B. magnimaculatus* based on the keys of Wall (1908), Smith (1943), and Slowinski (1994), among others (Table 4). Molecular analyses, however, find the Nui Chua samples to be nested within the *B. candidus* complex from southern Vietnam and Cambodia.

It is possible that our kraits descended from the hybridization between male *B. magnimaculatus* (Fig. 5) and female of *B. candidus*. However, this seems unlikely. Geographically, Nui Chua does not locate at the middle of the two species' ranges in Myanmar and Vietnam, but rather at the eastern margin of Indochina (Fig. 1). Further, neither *B. magnimaculatus* nor typical *B. candidus* occur in Nui Chua NP. Finally, populations of *B. candidus* surrounding Nui Chua NP are typical of the species, and *B. magnimaculatus* remains to be recorded from southern Vietnam, Cambodia, and Thailand.

TABLE 4. Number of white bands on body of *Bungarus candidus* and *B. magnimaculatus*.

Authors	*B. candidus*	*B. magnimaculatus*
Wall & Evans (1900)	–	11–14
Wall (1908)	20–25	11–14
Smith (1943)	20–25	11–14
Taylor (1965)	20–25	–
Campden-Main (1970)	20–25	–
Slowinski (1994)	21–25	11–14
Leviton *et al*. (2003)	–	11–14
This study	14 or 15	12–15

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APPENDIX 1. Specimens examined.

Bungarus candidus: 5 specimens, HUS 300 (Bach Ma, Thua Thien–Hue Prov., Vietnam), HUS 113 (Ba To, Quang Ngai Prov., Vietnam), ITBCZ 344 (Bu Gia Map, Binh Phuoc Prov., Vietnam), KIZ 100 (Bu Gia Map, Binh Phuoc Prov., Vietnam), KIZ 1326 (Ta Kou, Binh Thuan Prov., Vietnam).

Bungarus magnimaculatus: 6 specimens, CAS 210526 (Alaungdaw Kathapa, Sagaing, Myanmar), CAS 210696 (Sin Ma Taung, Magway, Myanmar), CAS 215445 (Alaungdaw Kathapa, Sagaing, Myanmar), CAS 215958 (Min Sone Taung, Mandalay, Myanmar), CAS 215998 (Min Sone Taung, Mandalay, Myanmar), CAS 215999 (Min Sone Taung, Mandalay, Myanmar).