

ORIGINAL ARTICLE

Molecular phylogeny found the distribution of *Bungarus candidus* in China (Squamata: Elapidae)

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Abstract Based on molecular phylogeny of the Chinese white cross-band krait, *Bungarus multicinctus*, several individuals from southwestern and southern China were found to be nested within putative species *B. candidus* (Linnaeus, 1758) with high support values. Morphological examination also indicated these specimens were identical to *B. candidus*. We concluded that the Chinese white cross-band krait, previously identified as *B. multicinctus* in southwest and south China, should be recognized as *B. candidus*, a new record in China, and *B. m. wanghaoti* should be invalid. *B. multicinctus* is recurrently limited to southern China within China.

Key words Kraits, *Bungarus multicinctus*, Cytb, new record.

1 Introduction

The kraits of the Elapid genus *Bungarus* Daudin, 1803, are proteroglyphous venomous snakes with moderate to large body size occurring in Southeast Asia from Pakistan, India, and Afghanistan in the west, and eastwards into tropical Southeast Asia (Slowinski, 1994; Kharin *et al.*, 2011). Presently, there are 15 species within this genus (Uetz & Hošek, 2017). To date, three species have been recorded in China, including *B. fasciatus* Schneider, 1801, *B. bungaroides* Cantor, 1839, and *B. multicinctus* Blyth, 1861 (Zhao, 2006). Of these species, *B. bungaroides* is restricted to Medóg, Xizang; *B. fasciatus* occurs in southern China, and *B. multicinctus* is widely distributed in southwestern and southern China (Zhao, 2006). Morphologically, *B. multicinctus* is very similar to its congener *B. candidus* (Linnaeus, 1758) occurring in Cambodia, Indonesia, Malaysia, Singapore, Thailand, Vietnam (Uetz & Hošek, 2017), and Laos (Kuch & Stuart, in press).

Based on a phylogenetic study of *B. multicinctus* from China and Vietnam, 43 out of 63 specimens, previously identified as *B. multicinctus*, were re-identified as *B. candidus* based on molecular and morphological data.

2 Materials and methods

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2.1 Molecular phylogenetics

Sixty-three samples of kraits previously identified as *B. multicinctus* (62) and *B. fasciatus* (1) were sequenced and analyzed against 53 previously published congener sequences (Table 1). All samples, collected from China and Vietnam, were preserved in 95% alcohol. *Laticauda semifasciata*, *Elapsoidea sundevallii*, and *Naja naja* were chosen as outgroups (Pyron *et al.*, 2013).

Total DNA was extracted using a commercial tissue DNA isolation kit (Omega, USA). One mitochondrial gene fragment was amplified by polymerase chain reaction (PCR). Cytochrome b (Cytb) sequences were obtained using primers L14910/H16064 (Burbrink *et al.*, 2000). Sequences were edited manually using SeqMan in DNASTAR (DNASTAR, Inc.), aligned in Mega 6.0 using the ClustalW algorithm with default parameters (Tamura *et al.*, 2013), and checked by eye for ambiguous alignments.

Table 1. Detailed information of samples used in this study.

Taxa	Voucher numbers*	Locality	GenBank accession nos.
<i>Bungarus candidus</i>	UK-B P4	Java, Indonesia	AJ565001
<i>Bungarus candidus</i>	UK B19	Java, Indonesia	AJ749343
<i>Bungarus candidus</i>	UK BP3	Java, Indonesia	AJ749342
<i>Bungarus candidus</i>	UK B21	Java, Indonesia	AJ749341
<i>Bungarus candidus</i>	UK BX3	Java, Indonesia	AJ749340
<i>Bungarus candidus</i>	Bcba	Bali, Indonesia	AJ749339
<i>Bungarus candidus</i>	FMNH 255260	Laos	AJ749338
<i>Bungarus candidus</i>	FMNH 255259	Laos	AJ749337
<i>Bungarus candidus</i>	UK BT8	Thailand	AJ749336
<i>Bungarus candidus</i>	UK BT2	Thailand	AJ749335
<i>Bungarus candidus</i>	UK BT1	Thailand	AJ749334
<i>Bungarus candidus</i>	UK BT9	Thailand	AJ749333
<i>Bungarus candidus</i>	UK BT4	Thailand	AJ749332
<i>Bungarus candidus</i>	UK BT6	Thailand	AJ749331
<i>Bungarus candidus</i>	Bmnam	Vietnam	AJ749330
<i>Bungarus candidus</i>	UK B39	Java, Indonesia	AJ749329
<i>Bungarus candidus</i>	QSMI	Thailand	AJ749328
<i>Bungarus candidus</i>	Bcnam2	Vietnam	AJ749326
<i>Bungarus candidus</i>	LACM FS 873	Vietnam	AJ749325
<i>Bungarus candidus</i>	ROM 30843	Vietnam	AJ749324
<i>Bungarus candidus</i>	ROM 30063	Vietnam	AJ749323
<i>Bungarus candidus</i>	ROM 25676	Vietnam	AJ749322
<i>Bungarus candidus</i>	ROM 25677	Vietnam	AJ749321
<i>Bungarus candidus</i>	ROM 35257	Vietnam	AJ749320
<i>Bungarus candidus</i>	ROM 25674	Vietnam	AJ749319
<i>Bungarus candidus</i>	ROM 30844	Vietnam	AJ749318
<i>Bungarus candidus</i>	ROM 25675	Vietnam	AJ749317
<i>Bungarus candidus</i>	ROM 35268	Vietnam	AJ749316
<i>Bungarus candidus</i>	ROM 35260	Vietnam	AJ749315
<i>Bungarus candidus</i>	ROM 35249	Vietnam	AJ749314
<i>Bungarus candidus</i>	ROM 35258	Vietnam	AJ749313
<i>Bungarus candidus</i>	ROM 35020	Vietnam	AJ749312
<i>Bungarus candidus</i>	ROM 35259	Vietnam	AJ749311
<i>Bungarus candidus</i>	ROM 35251	Vietnam	AJ749310
<i>Bungarus candidus</i>	ROM 35256	Vietnam	AJ749309
<i>Bungarus candidus</i>	ROM 35250	Vietnam	AJ749308

Table 1 (continued)

Taxa	Voucher numbers*	Locality	GenBank accession nos.
<i>Bungarus candidus</i>	ROM 31062	Vietnam	AJ749307
<i>Bungarus candidus</i>	GP 3375	Xishuangbanna, Yunnan, China	KY952766
<i>Bungarus candidus</i>	GP 2420	Rongjiang, Guizhou, China	KY952767
<i>Bungarus candidus</i>	GP 2387	Congjiang, Guizhou, China	KY952768
<i>Bungarus candidus</i>	GP 4463	Cangwu, Guangxi, China	KY952769
<i>Bungarus candidus</i>	GP 3473	Port of Fangcheng, Guangxi, China	KY952770
<i>Bungarus candidus</i>	GP 2207	Maoming, Guangdong, China	KY952771
<i>Bungarus candidus</i>	GP1004	Pingnan, Guangxi, China	-
<i>Bungarus candidus</i>	GP3187	Zhongshan, Guangdong, China	-
<i>Bungarus candidus</i>	GP4849	Hezhou, Guangxi, China	-
<i>Bungarus candidus</i>	GP4869	Liuzhou, Guangxi, China	-
<i>Bungarus candidus</i>	GP1011	Dinghu District, Guangdong, China	-
<i>Bungarus candidus</i>	GP2388	Congjiang, Guizhou, China	-
<i>Bungarus candidus</i>	GP1882	Maolan, Guizhou, China	-
<i>Bungarus candidus</i>	GP4456	Cangwu, Guangxi, China	-
<i>Bungarus candidus</i>	GP4595	Guangdong, China	-
<i>Bungarus candidus</i>	GP1012	Dinghu District, Guangdong, China	-
<i>Bungarus candidus</i>	GP4440	Cangwu, Guangxi, China	-
<i>Bungarus candidus</i>	KIZ01584	Hong Kong, China	-
<i>Bungarus candidus</i>	GP4478	Dinghu District, Guangdong, China	-
<i>Bungarus candidus</i>	GP4452	Cangwu, Guangxi, China	-
<i>Bungarus candidus</i>	GP4420	Cangwu, Guangxi, China	-
<i>Bungarus candidus</i>	KIZ018314	Huizhou, Guangdong, China	-
<i>Bungarus candidus</i>	GP2210	Guangxi, China	-
<i>Bungarus candidus</i>	GP4834	Cangwu, Guangxi, China	-
<i>Bungarus candidus</i>	GP4850	Hezhou, Guangxi, China	-
<i>Bungarus candidus</i>	GP4851	Hezhou, Guangxi, China	-
<i>Bungarus candidus</i>	GP4876	Liuzhou, Guangxi, China	-
<i>Bungarus candidus</i>	KIZ011559	Vietnam	-
<i>Bungarus candidus</i>	KIZ09931	Fujian, China	-
<i>Bungarus candidus</i>	CAS25675	Vietnam	-
<i>Bungarus candidus</i>	CAS30843	Vietnam	-
<i>Bungarus candidus</i>	CAS30840	Vietnam	-
<i>Bungarus candidus</i>	CAS35250	Vietnam	-
<i>Bungarus candidus</i>	CAS35258	Vietnam	-
<i>Bungarus candidus</i>	GP4208	Shangsi, Guangxi, China	-
<i>Bungarus candidus</i>	CAS35256	Vietnam	-
<i>Bungarus candidus</i>	GP2224	Guangdong, China	-
<i>Bungarus candidus</i>	GP3491	Port of Fangcheng, Guangxi, China	-
<i>Bungarus candidus</i>	YPX26585	Dongzhong, Guangxi, China	-
<i>Bungarus candidus</i>	KIZ01542	Mengla, Yunnan, China	-
<i>Bungarus candidus</i>	GP4686	Gengma, Yunnan, China	-
<i>Bungarus candidus</i>	GP4690	Jingdong, Yunnan, China	-
<i>Bungarus candidus</i>	GP4697	Jingdong, Yunnan, China	-
<i>Bungarus multicinctus</i>	Bm1	China	AJ749344
<i>Bungarus multicinctus</i>	UK Bm9204	Taiwan, China	AJ565002

Table 1 (continued)

Taxa	Voucher numbers*	Locality	GenBank accession nos.
<i>Bungarus multicinctus</i>	LSU 4243	China	AJ749327
<i>Bungarus multicinctus</i>	GP 877	Nanfeng, Jiangxi, China	KY952773
<i>Bungarus multicinctus</i>	GP 2208	Guangxi, China	KY952774
<i>Bungarus multicinctus</i>	GP 3984	Guilin, Guangxi, China	KY952775
<i>Bungarus multicinctus</i>	GP 3775	Yichang, Hubei, China	KY952776
<i>Bungarus multicinctus</i>	GP 1649	Conghua, Guangdong, China	KY952777
<i>Bungarus multicinctus</i>	GP 2432	Dehua, Fujian, China	KY952778
<i>Bungarus multicinctus</i>	GP 4481	Dinghu District, Guangdong, China	KY952779
<i>Bungarus multicinctus</i>	GP4584	Qingtian, Zhejiang, China	-
<i>Bungarus multicinctus</i>	KIZ03651	Jiangxi, China	-
<i>Bungarus multicinctus</i>	KIZ06171	Guilin, Guangxi, China	-
<i>Bungarus multicinctus</i>	KIZ07174	Shicheng, Jiangxi, China	-
<i>Bungarus multicinctus</i>	KIZ011979	China	-
<i>Bungarus multicinctus</i>	GP2209	Guangxi, China	-
<i>Bungarus multicinctus</i>	GP3760	Yichang, Hubei, China	-
<i>Bungarus multicinctus</i>	GP4914	Shuangpai, Hunan, China	-
<i>Bungarus multicinctus</i>	KIZ047146	Longyou, Zhejiang, China	-
<i>Bungarus multicinctus</i>	KIZ014732	Qionghai, Hainan, China	-
<i>Bungarus multicinctus</i>	KIZ09930	Yangjiang, Guangdong, China	-
<i>Bungarus multicinctus</i>	KIZ01792	Meizhou, Guangzhou, China	-
<i>Bungarus fasciatus</i>	GP 915	Guizhou, China	KY952772
<i>Bungarus fasciatus</i>	-	Australia	EU547086
<i>Bungarus fasciatus</i>	UK B24	Java, Indonesia	AJ749350
<i>Bungarus fasciatus</i>	UK H9	Java, Indonesia	AJ749349
<i>Bungarus fasciatus</i>	UK B2	Java, Indonesia	AJ749348
<i>Bungarus fasciatus</i>	Bfas T	Thailand	AJ749347
<i>Bungarus ceylonicus</i>	RS-135	Kandy, Sri Lanka	KC347457
<i>Bungarus flaviceps</i>	JAM 1946	Perak, Malaysia	AJ749351
<i>Bungarus sindanus</i>	Bsin1	Pakistan	AJ749346
<i>Bungarus caeruleus</i>	UK H7	Pakistan	AJ749305
<i>Bungarus niger</i>	Bnig	Nepal	AJ749304
<i>Bungarus sp.</i>	CAS221526	Kachn State, Burma	AJ749345
<i>Bungarus slowinskii</i>	IEBR 1172	Vietnam	AJ749306
<i>Bungarus bungaroides</i>	KIZ 98R0186	China	AY973270
<i>Laticauda semifasciata</i>	KUZ (JPN) R67763	Ryukyu Islands, Japan	AB701344
<i>Elapsoidea sundevallii</i>	HLMD RA-2211	Madagascar	AY188016
<i>Naja naja</i>	-	India	GQ225657

*Abbreviation. CAS—California Academy of Science, San Francisco; GP—P. Guo's catalogue number, the samples are deposited in Yibin University; HLMD—Hessisches Landes Museum Darmstadt; IEBR—Institute of Ecology and Biological Resources, National Center for Natural Science and Technology of Vietnam, Hanoi, Vietnam; KIZ—Kunming Institute of Zoology, the Chinese Academy of Science; LACM—Los Angeles County Museum; ROM—Royal Ontario Museum; RS—R. Somaweera's catalogue number; UK—U. Kuch's catalogue number. No detail information for other abbreviation. Missing data indicated by “-”.

Prior to phylogenetic reconstruction, the best-fit model for the sequences was selected in MrModeltest 2.3 under AIC (Nylander *et al.*, 2004). We used MrBayes 3.2.2 (Ronquist *et al.*, 2012) to construct Bayesian phylogenetic relationships. All searches were run with three independent runs and each was begun with a random tree. Every run consisted of four Markov chains (three heated chains and a single cold chain) estimated for 5 million generations and sampled every 1000 generations with 25% initial samples discarded as burn in. Convergence was assessed by plotting the likelihood plots through time in

TRACER v1.5 (Rambaut *et al.*, 2009). After confirming that the analyses were stationary at similar topologies and likelihood scores, the resultant trees were combined to calculate posterior probabilities (PP) for each node in a 50% majority-rule consensus tree. We also constructed maximum likelihood (ML) phylogenetic relationships using PhyML 3.1 (Guindon *et al.*, 2010) based on the model selected in MrModeltest 2.3.

2.2 Morphological examination

Fifteen representative specimens previously identified as *B. multicinctus*, which showed close genetic relationships to *B. candidus* in analysis (Table 2), were morphologically examined. All specimens are persevered at Yibin University (YBU). The *B. candidus* and *B. m. wanghaioti* data were from Das (2010) and Yang (2008).

Total length (tip of snout to tip of tail) was measured with a ruler to the nearest 1 cm. Symmetrical head characters were measured on both sides and given in left/right order; ventral scales were counted according to Dowling (1951). The number of dorsal scale rows were given at two head-lengths posterior to the head, at mid-body (corresponding to half the total ventral number), and at two head-lengths anterior to the anus. Coloration pattern was based on preserved specimens.

3 Results

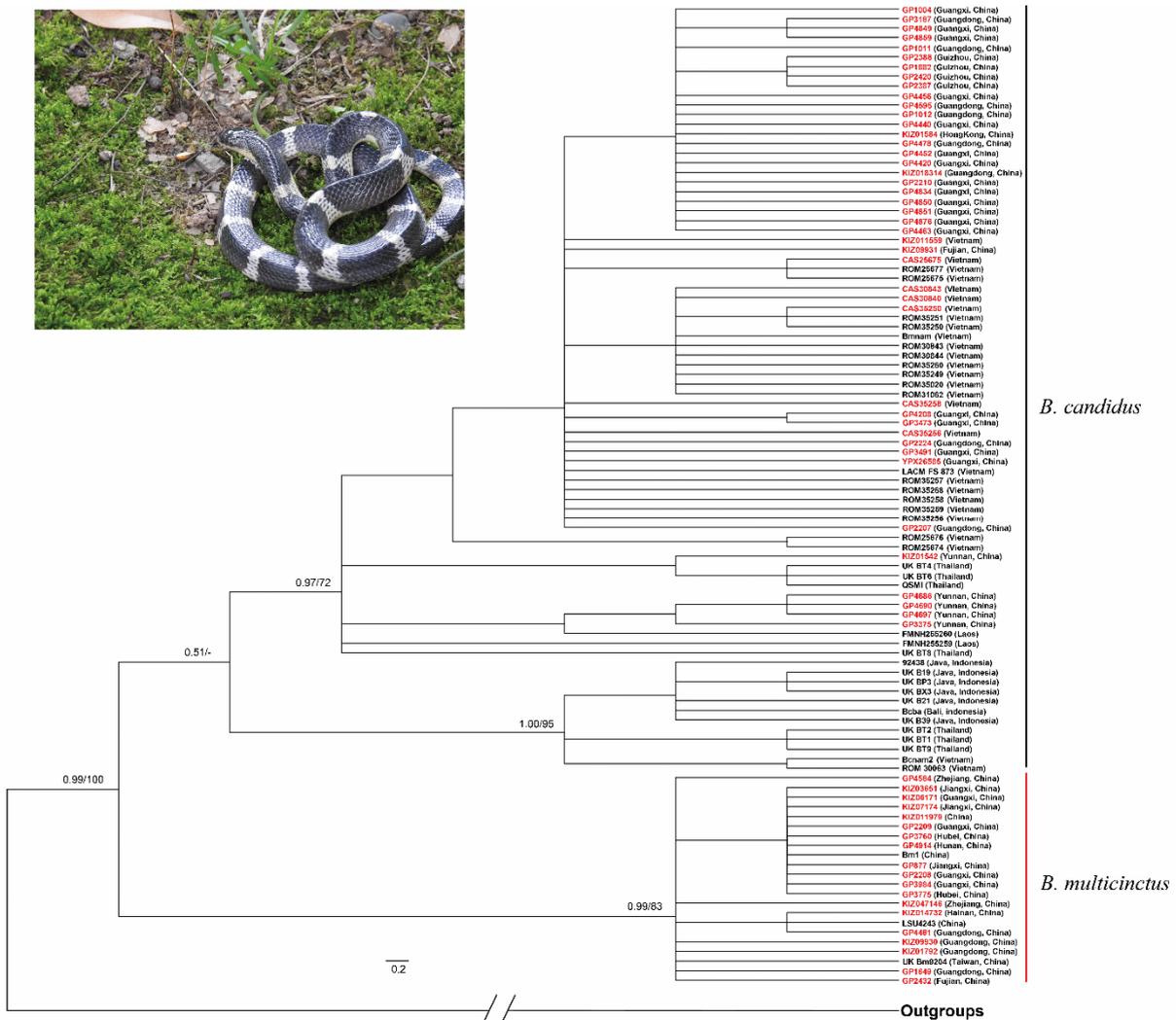


Figure 1. Bayesian 50% majority-rule consensus tree inferred from cytb based on the GTR+I+G model. Posterior probabilities and bootstrap values for the clades are shown adjacent to the nodes which they refer to in order. Support values less than 50% are indicated by “-”. Samples indicated by red belong to our laboratory. Samples indicated by black represent the sequences obtained from GenBank.

Table 2. Morphological characters of the examined specimens.*

Specimens/Taxon	Localities	Sex	TL	TA	Pr	Po	Sup	SO	In	IS	MS	Ve	Sub	Anus	WB	WT	Reference
YBU16093/ GP4463 (A)	Guangxi: Cangwu	Male	489	68	1/1	2/2	7/7	3-4	7/7	4/4	15	209	46	Single	33	10	This study
YBU14140/ GP3375 (J)	Yunnan: Xishuangbanna	Female	685	94	1/1	2/2	7/7	3-4	7/7	4/4	15	237	51	Single	22	9	This study
YBU12176/ GP2387 (J)	Guizhou: Congjiang	Female	318	44	1/1	2/2	7/7	3-4	7/7	4/4	15	207	45	Single	38	11	This study
YBU14469/ GP3473 (A)	Guangxi: Fangchenggang	Male	1025	125	1/1	2/2	7/7	3-4	7/7	4/4	15	214	46	Single	30	9	This study
YBU12119/ GP2420 (A)	Guizhou: Pingyang	Male	677	88	1/1	2/2	7/7	3-4	7/7	4/4	15	209	47	Single	30	9	This study
YBU091085/ GP1004 (A)	Guangxi: Pingnan	Male	1024	119	1/1	2/2	7/7	3-4	7/7	4/4	15	215	43	Single	35	9	This study
YBU11156/ GP1882 (J)	Guizhou: Maolan	Male	463	54	1/1	2/2	7/7	3-4	7/7	4/4	15	203	44	Single	38	14	This study
YBU16149/ GP4595 (A)	Guangdong	Female	600	76	1/1	2/2	7/7	3-4	7/7	4/4	15	214	46	Single	35	12	This study
YBU16071/ GP4420 (A)	Guangxi: Cangwu	Male	934	120	1/1	2/2	7/7	3-4	7/7	4/4	15	209	48	Single	35	12	This study
YBU17161/ GP4834 (A)	Guangxi: Cangwu	Male	773	96	1/1	2/2	7/7	3-4	7/7	4/4	15	211	46	Single	35	9	This study
YBU17241/ GP4876 (A)	Guangxi: Luzhai	Male	826	100	1/1	2/2	7/7	3-4	7/7	4/4	15	208	48	Single	32	10	This study
YBU15127/ GP4208 (J)	Guangxi: Shangsi	Female	324	43	1/1	2/2	7/7	3-4	7/7	4/4	15	216	47	Single	33	13	This study
YBU14487/ GP3491 (J)	Guangxi: Fangchenggang	Female	302	36	1/1	2/2	7/7	3-4	7/7	4/4	15	219	48	Single	31	11	This study
YBU17053/ GP4686 (A)	Yunnan: Gengma	Female	710	87	1/1	2/2	7/7	3-4	7/7	4/4	15	225	44	Single	23	7	This study
YBU17061/ GP4697 (J)	Yunnan: Jingdong	Male	350	54	1/1	2/2	7/7	3-4	7/7	4/4	15	216	55	Single	27	12	This study
<i>B. candidus</i>	-	-	1600	-	1/1	2/2	7/7	3-4	7/7	3-4/3-4	15(rarely 17)	194-237	37-56	Single	20-35	7-10	Das, 2010
<i>B. m. wanghaoti</i>	-	-	1332	152	1/1	2/2	7/7	3-4	7/7	4/4	15	218-233	47-56	Single	21-31	7-15	Yang, 2008

*Abbreviations. A—Adult; In—Infralabials; IS—Infralabials contacting anterior chin shields; J—Juvenile; MS—Midbody scale rows; Po—Postocular; Pr—Preocular; SO—Supralabial contacting orbit; Sup—Supralabial; Sub—Subcaudals; TA—Tail length; TL—Total length; Ve—Ventrals; WB—White bands on body; WT—White bands on tail.

3.1 Molecular phylogenetic analysis

The final aligned sequence data of *cytb* consisted of 1131 bp. The best-fit model selected by MrModeltest 2.3 was GTR+I+G. The BI tree indicated that all samples identified as *B. multicinctus* and *B. candidus* formed a strong supported monophyletic lineage (100% PP) (Fig. 1). Within this lineage, two sublineages were recovered. The first sublineage was composed of all putative *B. multicinctus* samples and 19 samples from southern China (99% PP); the second consisted of all putative samples of *B. candidus* and 43 samples previously identified as *B. multicinctus* from southwestern and southern China and Vietnam. However, the second sublineage was poorly supported (Fig. 1). The ML tree showed several areas of agreement with the BI tree, but also some areas of disagreement mainly in support values in several nodes (Fig. 1).

3.2 Morphological studies

Total length varied from 350 mm to 1025 mm in males and 302 mm to 710 mm in females. Tail length ranged from 54 mm to 125 mm in males and 36 mm to 94 mm in females. The body was robust, with head indistinct from neck. The top of the head was black or light brown, with juvenile specimens exhibiting a large white spot on both sides of the head, except individuals YBU17061 and YBU14140. White bands were found on the body/tail, and ranged from 27–38/9–14 and 22–38/7–13 in males and females, respectively. The distances between white bands decreased from the fore- to hind-part of the body. There were many black bands or spots on the tails, except for YBU14140 whose tail was faint yellow. The eyes were small. Preocular 1 and Postoculars 2/2. Supralabials 7/7, third and fourth enter the orbit. Infralabials 7/7, fourth contacts anterior chin shields. Dorsal scales 15-15-15 in rows. Ventrals ranged from 203 to 216 in males and 207 to 237 in females. Subcaudals ranged from 43–55 in males and 44–51 in females. Single anus.

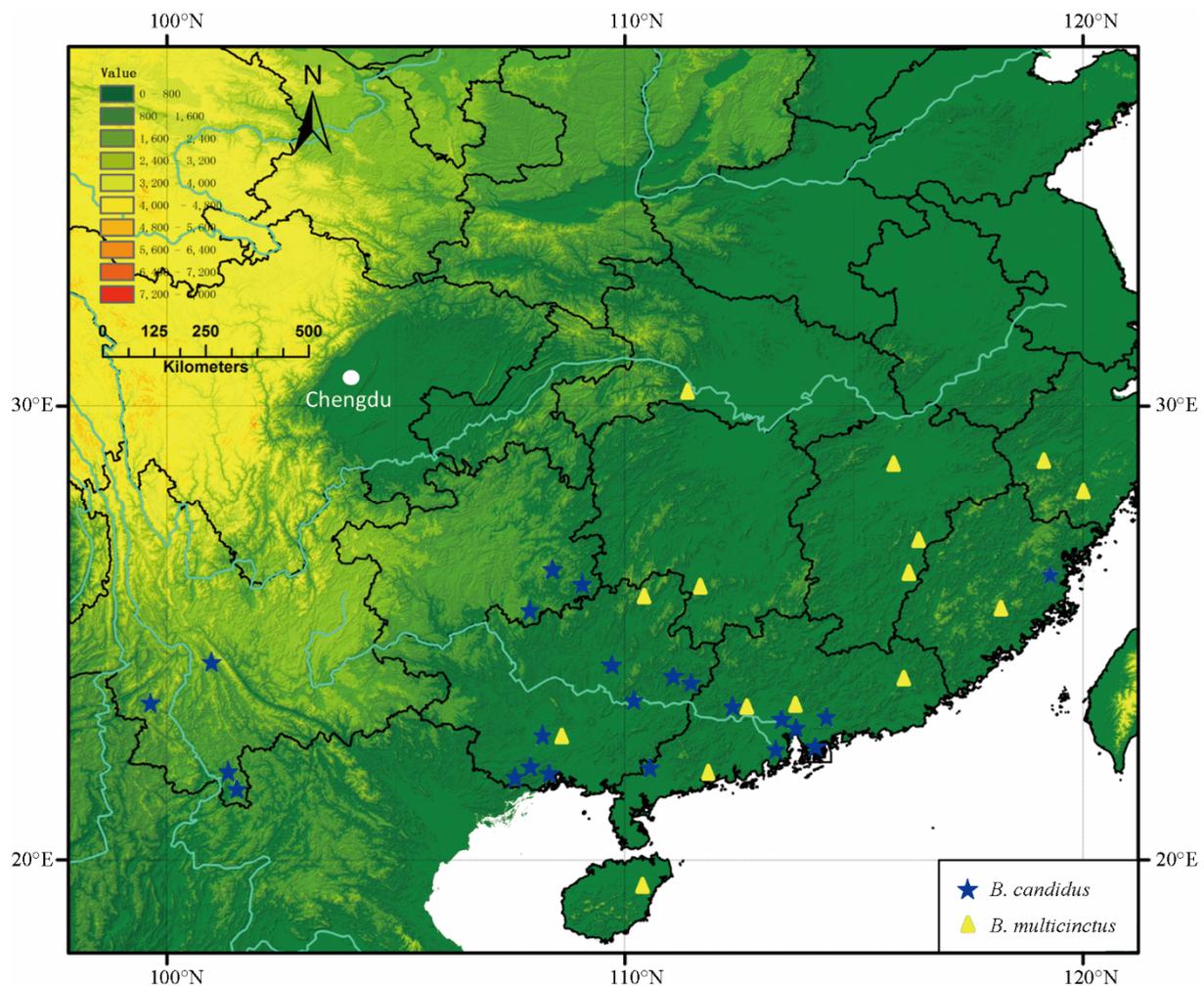


Figure 2. Sample localities of *B. candidus* and *B. multicinctus* in China.

4 Discussion

Kraits with white cross-bands have long been identified as *Bungarus multicinctus* in China (Zhao, 2006). Our molecular phylogenetic study demonstrated that 43 samples previously identified as *B. multicinctus* were genetically close to putative *B. candidus*, with another 19 specimens nested within putative *B. multicinctus* (Fig. 1). Morphologically, 15 specimens were also similar to *B. candidus* in scalation characters and body cross-bands (Table 2). Thus, based on morphological comparison and molecular phylogenetics, we concluded that the 43 individuals previously identified as *B. multicinctus* should be designated as *B. candidus*, which is a national record new to China.

Bungarus multicinctus was initially described based on specimens from Fujian, China (Blyth, 1861), and is widely recorded in Myanmar, Laos, Vietnam, Thailand, and China (Uetz & Hošek, 2017). Two subspecies (n nominate form and *B. m. wanghaoti*) are currently recognized (Zhao, 2006). In China, kraits with white cross-bands are consistently identified as *B. multicinctus*. Except for a population in southwest Yunnan, which is recognized as *B. m. wanghaoti*, all others have been identified as *B. m. multicinctus* (Zhao, 2006). Our molecular phylogenetic analyses indicated that the 43 kraits with white cross-bands from southwestern and southern China (including Yunnan, Guizhou, Guangxi, Guangdong, and Fujian) were nested within *B. candidus*, with another 19 from southern China (including Fujian, Jiangxi, Hubei, Hunan, Hainan, Zhejiang, Guangdong, and Guangxi) clustered with putative *B. multicinctus* (Fig. 1). We concluded, therefore, that *B. candidus* occurs in southwestern and southern China, including Yunnan, Guizhou, Fujian, Guangxi, and Guangdong, thus expanding its distribution eastward, and *B. multicinctus* is restricted to southern China within China (Fig. 2). *Bungarus m. wanghaoti*, which was described from southwestern Yunnan (Zhao, 2006), is invalid.

Bungarus candidus has been previously recorded in Southeast Asia, including Thailand, Vietnam (Uetz & Hošek, 2017), and Laos (Kuch & Stuart, in press), and thus its occurrence in China is not surprising. In addition to China, *B. multicinctus* has also been recorded in Vietnam, Thailand, and Myanmar (Uetz & Hošek, 2017). Based on our present data, we are not sure whether kraits previously identified as *B. multicinctus* in Vietnam, Thailand, and Myanmar are true *B. multicinctus* or a different species. Our molecular phylogenetic study, together with previous independent studies (Kuch *et al.*, 2005; Kuch & Mebs, 2007; Pyron *et al.*, 2013), consistently revealed that the examined *B. candidus* samples formed two distinct monophyletic sublineages with high support values. It is possible that there is cryptic diversity in this wide-ranged species, and therefore further study is necessary. Future studies with more extensive sampling in these countries will clarify these issues.

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