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Range extension and extended diagnosis of *Lycodon pictus*: First country record from China

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Abstract: The Painted Wolf Snake, *Lycodon pictus*, was recently described based on the type series from Trung Khanh and Ha Lang districts in Cao Bang Province, northern Vietnam. Herein, we report new findings and a range extension of the recently described species. One individual was collected close by the type locality, in Ha Lang District, Cao Bang Province, Vietnam and another one in Nonggang National Nature Reserve, Longzhou County, Guangxi Zhuang Autonomous Region, China, approximately 60 km apart from the type locality. Both specimens generally accorded with the morphological diagnosis of *L. pictus* provided in the original description. Molecular analyses supported the morphological findings: the newly collected specimens were approximately 0.3-0.9% (cyt *b*) genetically divergent from those of the type series. Molecular analyses also revealed that the holotype of *L. pictus*, for which no sequences were available so far, showed, based on the results of a formalin protocol, about 0.9-1.3% differentiation from the remaining type series, 0.5% from the new record from Cao Bang and 0.9% from the new record from China. Based on the morphological and molecular findings, we herein present the first country record of *L. pictus* from China, with a detailed morphological description of the specimen from Nonggang National Nature Reserve and slightly extend the original diagnosis of the species.

Keywords: New record, *Lycodon pictus*, China, karst forest, morphology, phylogeny, taxonomy.

INTRODUCTION

The genus *Lycodon* Boie, 1827 is one of the most diverse genera of colubrid snakes, with 64 currently recognized species (Uetz *et al.,* 2020; Wang *et al.,* 2020b). Recent phylogenetic studies showed that the genera *Dinodon*, *Dryocalamus* and *Lepturophis* are nested within *Lycodon* and suggested the taxa to be placed into the genus *Lycodon* (Guo *et al.*, 2013; Siler *et al.*, 2013; Figueroa *et al.*, 2016). The members of *Lycodon* have a broad distribution from eastern Iran to southern China and Japan, southward to the Philippines as well as to the Indo-Australian Archipelago (Lanza, 1999; Siler *et al.*,

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2013; Neang *et al.*, 2014). From Vietnam, sixteen species of *Lycodon* have been reported to date, comprising *L. capucinus* (Boie, 1827), *L. cardamomensis* (Daltry & Wüster, 2002), *L. davisonii* (Blanford, 1878), *L. fasciatus* (Anderson, 1879), *L. flavozonatus* (Pope, 1928), *L. futsingensis* (Pope, 1928), *L. laoensis* Günther, 1864, *L. meridionalis* (Bourret, 1935), *L. namdongensis* Luu, Ziegler, Ha, Le & Hoang, 2019, *L. paucifasciatus* Rendahl in Smith, 1943, *L. pictus* Janssen, Pham, Ngo, Le, Nguyen & Ziegler, 2019, *L. rosozonatus* (Hu & Zhao, 1972 [1975]), *L. rufozonatus* Cantor, 1842, *L. ruhstrati abditus* Vogel, David, Pauwels, Sumontha, Norval, Hendrix, Vu & Ziegler, 2009, *L. septentrionalis* (Günther, 1875) and *L. subcinctus* Boie, 1827 (Uetz *et al.*, 2020).
The last description from Vietnam was that of *Lycodon pictus* based on a type series collected from Cao Bang Province, northern Vietnam. Herein, we report new findings of the species, supported by morphological and molecular analyses, resulting in an extended diagnosis and range extension of *L. pictus*.

MATERIAL AND METHODS

Sampling: Field surveys in Vietnam were conducted in Duc Quang Commune, Ha Lang District, Cao Bang Province (22.42'.48''N; 106.39'.55'' E, 510 m a.s.l.) on 16 May 2019 by Tao Thien Nguyen, and in China in Nonggang National Nature Reserve, Longzhou County, Guangxi Zhuang Autonomous Region (106°57'18.77" E, 22°28'3.92" N, 233 m a.s.l.) on 8 July 2019 by Jin-Long Ren and Fan-Xing Zeng. The collected specimens were photographed alive, euthanised with ethyl-acetate, fixed in approximately 85% ethanol for 10 hours, and subsequently transferred to 70% ethanol for permanent storage. Liver tissue samples were preserved separately in 95% ethanol. The specimens are deposited in the collections of the Vietnam National Museum of Nature, Vietnam Academy of Science and Technology (VNMN), Hanoi, Vietnam and of Herpetological Museum, Chengdu Institute of Biology, Chinese Academy of Sciences (CIB), Chengdu, China. The holotype, deposited in the Institute of Ecology and Biological Resources, Hanoi, Vietnam, (IEBR 4166) was also included in the molecular analysis.

Morphological analysis: Identification of sex was performed by dissection (inspection of gonads and presence of hemipenes). Maxillary teeth were counted by dissecting the right maxilla for teeth / sockets. Scalation and maxillary teeth number were examined with a binocular dissecting microscope. Measurements were taken following Ziegler *et al.* (2018) with a measuring tape to the nearest 1 mm.
Abbreviations of morphological characters are as follows: SVL - Snout-vent length (from tip of snout to vent); TaL - tail length; TaL/TL - ratio of tail length / total length; TL - total length; DSR - dorsal scale rows number at one head length posterior to the head – number of dorsal scale rows at midbody – number of dorsal scale rows at one head length anterior to the vent; SL - supralabials (counted on upper lips); SL/orbit - number of supralabials entering orbit; IL - infralabials (counted on lower lips); Lor - loreals; Lor/eye - loreal scale touching the eye (yes or no); PreOc - preoculars; PostOc - postoculars; Atem - number of anterior temporals; Ptem - number of posterior temporals; BodySc - scalation of the body (keeled or smooth); PreVen - number of preventral scales; Ven - number of ventral scales; SubC - number of subcaudal scales; PreC - precloacal (or cloacal) plate (single or divided); Teeth max - number of maxillary teeth / alveoli. Scale counts were taken following Vogel *et al.* (2009). Ventral scales (Ven) were counted according to Dowling (1951). Bilateral scale counts were given as left / right.

Molecular analysis: A tissue sample was collected from the holotype of *Lycodon pictus* (IEBR 4166) then extracted following formalin protocol developed by Friedman & Desalle (2008) using GTE and

Table 1. Primers used in this study

Primer	Sequence	Reference
L14910	5' – GACCTGTGATMTGAAAACCAYCGTTGT –3'	Burbrink *et al.* (2000)
H16064	5' – CTTTGGTTTACAAGAACAATGCTTTA –3'	Burbrink *et al.* (2000)
Ly_R1	5' – GATGAAAAAGCAAGGTTGATGTT – 3'	This study
Ly_F1	5' – CTACAAACCGTAACCGGATTCTT – 3'	This study
Ly_R2	5' – GGATAAATAGTAGGGTGGTTCCTGAT – 3'	This study
Ly_F2	5' – GGAACCACCCTACTATTTATCCTCAT – 3'	This study
Ly_R3	5' – GGATGAAGTGTAAGGCAAAGAAT – 3'	This study
Ly_F3	5' – GGTTTCTCAATTAATGACCCAA – 3'	This study
Ly_R4	5' – CATTGAATATGTTTGGGGTAAATGAT – 3'	This study
Ly_F4	5' – CACCCTAATAATAACTATTATATT – 3'	This study
Ly_R5	5' – GTTATAGATCGTATGTGGGATGTGTGA – 3'	This study

Puregene Tissue Kit (Qiagen, Germany) following the manufacturer's instructions to allow the efficient recovery of even small amounts of residual undegraded DNA. We designed nine new internal cytochrome *b* primers to optimize the amplification of the degraded DNA sample (Table 1). The sample was cut into small pieces using a sterile razor blade and placed in 1.5 ml centrifuge tube then removed formalin by incubation in GTE (100 nM Glycine, 10 MM Tris-HCl, pH 8.0, 1 mM EDTA) at 55 °C for 72 h. During this step, the extraction was checked, and GTE was replaced every 24 h. A negative control was used in the extraction. Sample VNMN 011227 was extracted following Janssen *et al.*'s (2019) protocol.

Extracted DNA was amplified by HotStar Taq PCR Mastermix (Qiagen, Germany) with 21 µl volume (10 µl of mastermix, 5 µl of water, 2 µl of each primer at 10 pmol/ml and 2 µl of DNA). PCR condition was: 95 °C for 15 minutes to active the taq followed by 40 cycles at 95 °C for 30 s, 45 °C for 45 s, 72 °C for 60 s and the final extension at 72 °C for 6 minutes. The PCR products were then used as a template for the new PCR reactions by DreamTaq Mastermix (ThermoFisher Scientific, Lithuania) with the same volume and PCR conditions. Negative controls were used in all amplifications to check for possible contamination. PCR products were visualized using electrophoresis through a 2% low melting-point agarose gel stained with ethidium bromide. Successful amplifications were purified to eliminate PCR components using GeneJET™ PCR Purification kit (ThermoFisher Scientific, Lithuania). Purified PCR products were sent to FirstBase (Malaysia) for sequencing in both directions.

Genomic DNA of sample CIB 115609 was extracted from macerated liver tissue samples using an Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech, China), according to the manufacturer's protocol. Mitochondrial cytochrome *b* (cyt *b*) was targeted and amplified using primers L14910 and H16064 (Burbrink *et al.,* 2000) (Table 1). Polymerase chain reactions (PCR) were performed with 25 µl volume, and the PCR condition was initial denaturing for 7 min at 94 °C, 41 cycles of denaturation for 40 s at 94 °C, annealing for 30 s at 46 °C, extension for 1 min at 72 °C, and final extension for 8 min at 72 °C. PCR products were purified using a commercial kit and sequenced in both directions by an ABI 3730xL sequencer (Applied Biosystems,

Table 2. Locality, sex and scalation of the newly collected specimens of *Lycodon pictus* from Vietnam and China.

	VNMN 011227	CIB 115609
Locality	Ha Lang, Cao Bang, Vietnam	Nonggang, Guangxi, China
Sex	Female	Female
TL	572	591
SVL	450	467
TaL	125	124
TaL/TL	0.219	0.210
Teeth max	12	13
SL	8	8
SL/orbit	3-5	3-5
IL	10	10/9
PreOc	1	1
PostOc	2	2
Lor	1	1
Lor/eye	no	yes/no
Atem	1(2)	1
PTem	3	3
DSR	17-17-15	17-17-15
PreVen	1	1
Ven	215	218
Prec	single	single
Subc	85	90
BodySc	smooth	smooth
Dark bands on body	29	29
Light bands on body	29	29
Dark bands on tail	15	14
Light bands on tail	16	14

Foster City, CA, USA). Sequence editing was performed in Geneious Pro 4.8.4 (Kearse *et al.*, 2012).
After sequences were checked and aligned by Sequencher v5.4 (Gene Codes Corp, Ann Arbor, MI, USA), they were compared with those generated by Janssen *et al.* (2019) using BLAST (Basic Local Assignment Search Tool) (McGinnis & Madden, 2004) on GenBank. For sample IEBR 4166, after removing the primers, the cytochrome *b* fragments, which overlapped by approximately 50 bps, had an average of approximately 200 bps in length. The final sequence was 1117 bps in length. Other two sequences both contained 1056 bps in length. The newly obtained sequences were uploaded on Genbank under accession numbers MT845093-MT845095.

RESULTS

Both specimens, one from the site close to the type locality in Cao Bang Province, Vietnam (VNMN 011227), and the other one from Nonggang, Longzhou County, Guangxi Zhuang Autonomous Region, China (CIB 115609, field no. GX2019069), generally accorded with the morphological diagnosis of *Lycodon pictus* provided by Janssen *et al.* (2019) (see Table 2). Molecular analyses supported the morphological data. Sequences of the new specimens from Nonggang National Nature Reserve, Longzhou County, Guangxi Zhuang Autonomous Region, China (CIB 115609) and from Cao Bang Province, Vietnam were approximately 0.5-0.9% and 0.3-0.9% (cyt *b*) genetically divergent from those of the type series, respectively. Molecular analyses also revealed that the holotype IEBR 4166 (Field number CB.2012.97) of *Lycodon pictus* Janssen, Pham, Ngo, Le, Nguyen & Ziegler, 2019 showed about 0.9-1.3% differentiation from the remaining type series and 0.5% from VNMN 011227. The Guangxi's sequence is roughly 0.9% differentiated from the holotype sequence. Nucleotide substitutions were distributed randomly over the sequence derived from the type specimen (IEBR 4166).

TAXONOMIC ACCOUNT

Lycodon pictus Janssen, Pham, Ngo, Le, Nguyen & Ziegler, 2019

Figs 1-3

New record for Vietnam (n = 1): VNMN 011227 (Fig. 1), Ha Lang, Cao Bang, Vietnam, Coordinates 22°42'50'' N, 106°40'74'' E, 960 m a.s.l., collected by Nguyen Quoc Huy on 18 May 2019.

Fig. 1. The new record of *Lycodon pictus* (VNMN 011227) from Vietnam in life. Photograph by Tao Thien Nguyen.

New record for China (n = 1): CIB 115609 (field no. GX2019069; Figs 2-3), Nonggang National Nature Reserve, Longzhou County, Guangxi Zhuang Autonomous Region, China, 106°57'18.77" E, 22°28'3.92" N, 233 m a.s.l., collected by Ren Jin-Long and Zeng Fan-Xing on 8 July 2019.

Description of the Chinese specimen: Head elongate, moderately distinct from neck, rather flattened, longer than wide, snout narrow, distinctly shorter than head width; nostril large, lateral, located in the middle of the nasal; eye large, pupils vertically elliptic; rostral triangular, much broader than high, hardly visible from above; nasal divided, narrowed medially; two internasals, anteriorly round, slightly wider than high, bordered by two large, hexagonal prefrontals posteriorly; frontal single, enlarged, pentagonal to hexagonal, narrowed posteriorly; parietals large, longer than wide, in contact with each other medially, with upper anterior and posterior temporals, paraparietal laterally and four nuchal scales posteriorly; paraparietals elongated, anterior part widened, about equal in length or slightly longer than posterior temporals; loreal 1/1, elongate, not entering orbit on right side, whereas the posterior corner entering orbit on the left side (Fig. 3C and D); supralabials 8/8, first and second in contact with nasal, third to fifth entering orbit, sixth largest, slightly higher than third on left side; infralabials 10/9, first pair in broad contact with each other, first to fifth/first to fourth in contact with anterior pair of chin shields; anterior and posterior pairs of chin shields elongate, second pair not meeting in midline; preocular 1/1, located on antero-upper part of eye; postoculars 2/2, lowermost smaller, bordering anterior temporals; anterior temporal 1/1, in contact with sixth supralabial; posterior temporals 3/3, upper one thinner than lower one. Left maxilla arched, with an angular apex, distinctly bent inwards anteriorly. A total of 13 maxillary teeth or teeth alveola, with the following formula: five small anterior teeth, slightly enlarged posteriorly + three strongly enlarged teeth, thick, and not much curved + a distinctly wide gap + three small teeth + a small gap + two enlarged posterior teeth.
Body elongate, TL 591 mm; SVL 467 mm; TaL 124 mm; preventral 1, ventrals 218, from behind neck region distinctly notched laterally; subcaudals 90, paired; precloacal plate single; DSR 17-17-15, all smooth; the vertebral scales not enlarged.

Coloration in preservative: Dorsal surface of head and neck with pigmentation, paler on lateral side of head, the lower part of supralabials yellowish cream, speckled

Fig. 2. New country record of *Lycodon pictus* (CIB 115609) from China (Nonggang, Guangxi), adult female in life. Photograph by Jin-Long Ren.

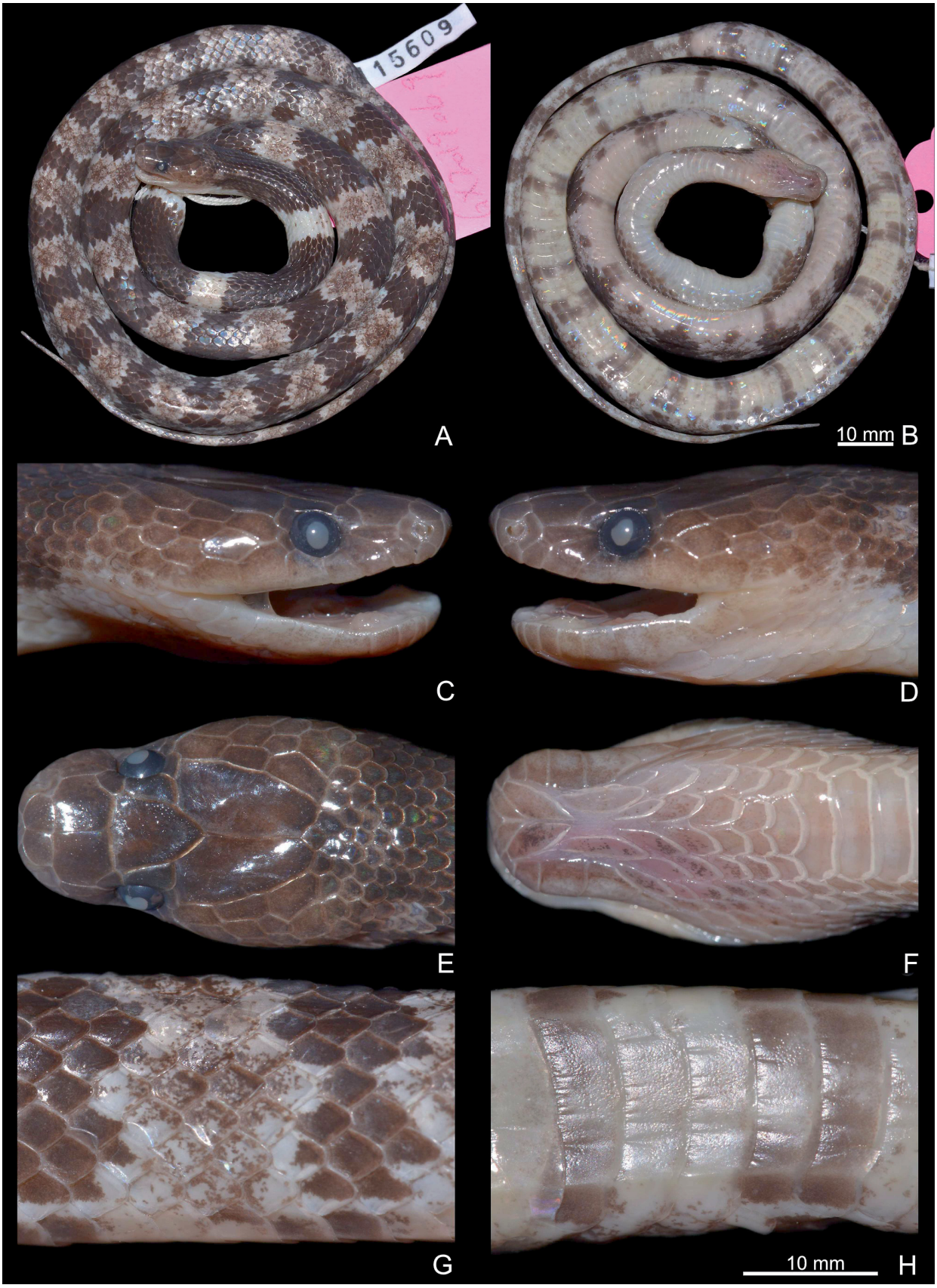

Fig. 3. Adult female of *Lycodon pictus* (CIB 115609; Guangxi, China) in preservative. (A) Dorsal general view; (B) Ventral general view; (C) Lateral head view, right side; (D) Lateral head view, left side; (E) Dorsal view of head; (F) Ventral view of head; (G) Lateral body view; (H) Ventral scale view. Scale bar 5 mm. Photographs by Jin-Long Ren.

with pale brownish blotches. Body brownish black, light body bands beginning after 1.5 times the head length behind the head, in total 29 transverse light bands on body and 14 light bands on tail; the first four body bands yellowish cream, and distinctly widened towards the venter, increased in size posteriorly; a dark mottling in the vertebral region more prominent posteriorly; the subsequent light body bands with two distinct indentations on each side, fused in the middle in the last third of the body.

Ventral surface of head and neck yellowish cream, belly cream and greyish cream in the last third part of body and on lower tail surface; the dark dorsal bands in part extending towards the venter, forming complete dark bands around the posterior body and on the tail; lateral side of the head cream below, with the lighter pattern beginning in the supralabial region; tip of lower jaw and infralabial region in part greyish brown; dorsal surface of the head and upper head sides a bit paler than the remaining head dorsum.

Variation of the new specimens: The Chinese specimen (CIB 115609) largely matches the morphological data of the original description of *Lycodon pictus*, except for having (1) a slightly shorter tail, TaL/TL 0.210 vs 0.215 in the single female paratype; (2) fewer infralabials 9/10 vs 10; (3) fewer anterior temporals 1 vs 2; however, Janssen *et al.* (2019) already documented the condition "2*" each for one side of both paratypes, meaning two anterior temporals with the lowermost not touching the postocular, thus referred to as posterior temporal herein, resulting in only one anterior temporal in afore mentioned cases in the type series already.

The Vietnamese specimen (VNMN011227) generally agrees with the morphological data of the original description of *Lycodon pictus*, except for having (1) a slightly longer tail, TaL/TL 0.219 vs 0.211 to 0.215 in the single juvenile and the single female from the type series for which TaL/TL ratio was available, (2) slightly fewer maxillary teeth (12 vs 13 or 14) and (3) fewer subcaudals 85 vs 90 or 91.

Both specimens showed more bands on the tail; 14 dark bands in CIB 115609 and 15 dark bands in VNMN011227 (vs 9 and 13); 14 light bands in CIB 115609 and 16 light bands in VNMN011227 (vs 9+ and 13).

Extended diagnosis: *Lycodon pictus* can be differentiated from its congeners by the following morphological characters: dorsal scales in 17–17–15 rows, all smooth; supralabials eight (rarely nine); infralabials ten (rarely nine); one elongated loreal on each side, either in contact with the eye or separated from it; precloacal plate single; ventral scales 212-218 (plus one or two preventral scales); subcaudals 85-91; a total length of 597+ mm in males and 543-591 mm in females; tail / total length ratio 0.210-0.219 in females;

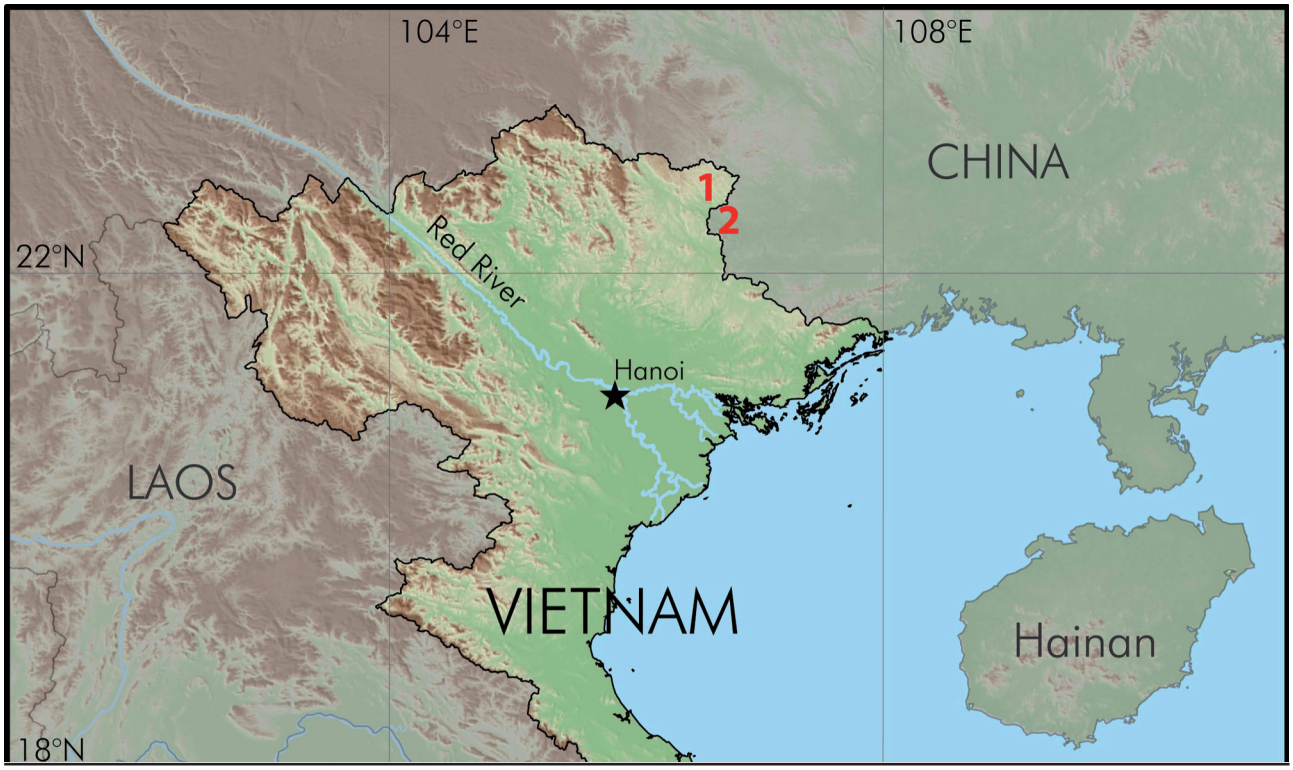

Fig. 4. Map showing the new findings of *Lycodon pictus*. 1: VNMN 011227, from closeby the type locality in Cao Bang Province, northern Vietnam. 2: CIB 115609, the new country record of *L. pictus* from Nanggong, Guangxi, southern China.

maxillary teeth 12 to 14; dorsal surface of body with 28 or 29 light body bands; dorsal surface of tail with 9 to 15 dark bands, and 13 to 16 cream bands forming a distinct blotch in the vertebral region; ventral surface of body and tail mostly cream with the dark body bands in part extending towards the venter, sometimes forming complete dark bands around the body.

Distribution: *Lycodon pictus* is currently known from the type locality in Cao Bang Province, northern Vietnam and Longzhou County in Guangxi, southern China (Fig. 4).

Etymology: The specific name of the species, "*pictus*", meaning painted or decorated in Latin, refers to the unique dorsal color pattern of this species (Janssen *et al.*, 2019). Because this species is herein reported as a new member of the Chinese snake fauna, we suggest "Jin Bai Huan She (锦白环蛇)" as its Chinese common name, deriving from its scientific name.

Natural history: *Lycodon pictus* inhabits karst environment in between elevations of 233 m (Nonggang, Guangxi, China, this study, Fig. 5) and 701 m a.s.l. (type locality; Janssen *et al.,* 2019). In southern China, the species was found at night between 00:00 and 1:00 am after light rain, active in a drainage ditch along a forest path, covered with dead leaves. The surrounding habitat was secondary karst forest, consisting of short hardwood, shrubs and vines. The snake did not attempt to bite when it was handled, neither aggressive nor defensive behavior was observed. In terms of the herpetofauna, several other amphibians and reptiles were observed during the survey period in 2019 in the same microhabitat, including *Kurixalus odontotarsus* (Ye & Fei in Ye *et al.*, 1993), *Rhacophorus kio* (Ohler & Delorme, 2006), *Draco maculatus* (Gray, 1845), *Acanthosaura lepidogaster* (Cuvier, 1829), *Goniurosaurus luii* Grismer, Viets & Boyle, 1999, and *Psammodynastes pulverulentus* (Boie, 1827).

DISCUSSION

The holotype of *Lycodon pictus* (IEBR 4166) could be genetically confirmed. The newly discovered specimen from the site close to the type locality in northern

Fig. 5. Macrohabitat of *Lycodon pictus* in Nonggang National Nature Reserve, Longzhou, Guangxi, China. Photograph by Jin-Long Ren.

Vietnam only differed slightly from the original diagnosis of *Lycodon pictus* in a somewhat lower number of subcaudals (85 vs 90 or 91 in the original diagnosis), a tail / total length ratio of 0.219 (vs 0.211-0.215 in the original diagnosis), more tail bands (16 vs 13 light bands, and 15 vs 9 to 13 dark bands) and 12 or 13 (vs 13 or 14) maxillary teeth. The new record from China deviates from the original description in having fewer infralabials (9/10 vs 10) and a slightly smaller TaL / TL ratio (0.210 vs 0.211-0.215 in the original diagnosis). The slight morphological differences are evaluated by us as being within infraspecific variation, in particular given the low number of specimens of *L. pictus* known so far. The high level of molecular similarity between the new records and the type series affirm their conspecific status. In the original description the loreal was mistakenly given as in contact with the eye both in the abstract and in the diagnosis. This study confirms that the loreal both can be in contact or not with the eye in this species. This represents not only an extended morphological diagnosis of *L. pictus* but also the first country record of *Lycodon pictus* from China, which increases the known species number from China to 17 (Wang *et al.*, 2020a, b). The location of the new record of *L. pictus* from China is approximately 60 km apart from the type locality in Vietnam.

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