

Is the horned pitviper *Ceratrimeresurus shenlii* Liang and Liu, 2003 from China a valid *Protobothrops*?

Shiping Gong¹, Ellen Hitschfeld², Anna K. Hundsdörfer², Markus Auer², Fumin Wang^{1,3},
Lihua Zhou¹, Uwe Fritz^{2,*}

Abstract. Previous records of horned pitvipers from Vietnam and China are reviewed and the phylogenetic placement of four snakes from two sites in Tianjingshan Forest, China (Ruyan County, Guangdong Province; 24°43'N, 113°03'E, 563 m a.s.l.; 24°43'N, 113°02'E, 585 m a.s.l.) is examined. Using mitochondrial DNA sequence data (12S, 16S, ND4, *cyt b*; 2306 bp) and Bayesian and Maximum Likelihood analyses, the Tianjingshan pitvipers are revealed as sister to *Protobothrops cornutus* with a differentiation resembling those of *P. flavoviridis* and *P. tokarensis*. This indicates a close relationship with *P. cornutus* and suggests that *Ceratrimeresurus shenlii* Liang and Liu, 2003, previously considered a junior synonym of *P. cornutus* (Smith, 1930), could be a valid subspecies of *P. cornutus* or a recently split distinct species. However, further studies and samples from intermediate localities are needed to decide whether the observed differentiation reflects a pattern of isolation-by-distance or a phylogeographic, and thus perhaps taxonomically relevant, break.

Keywords: *Ceratrimeresurus shenlii*, China, morphology, phylogeny, *Protobothrops cornutus*, Vietnam.

Until recently, the Chinese pitviper *Ceratrimeresurus shenlii* Liang and Liu, 2003 was overlooked by most authors (e.g., Gumprecht et al., 2004; Hermann et al., 2004; Zhao, 2006). This taxon was described as a new genus and a new species based on the first “horned pitviper” recorded from the People’s Republic of China, being characterized by horn-shaped supraocular scutes. The holotype of this taxon was collected in Wuzhishan Forest, Ruyuan County, Guangdong Province, China (24°30'-24°48'N, 112°56'-113°4'E) in July 1996. Comparing the holotype and original description of *C. shenlii* with other Asian horned pitvipers, David et al. (2008) suggested that this taxon should be synonymized with *Protobothrops cornutus* (Smith, 1930), a pitviper previously known

only from Vietnam (Hermann et al., 2004; Ziegler et al., 2006). In summer 2005 another horned pitviper was recorded from the Shimentai Nature Reserve (24°22'-24°31'N, 113°05'-113°31'E), about 60 km southeastwards from the type locality of *C. shenlii*. This second specimen was found in the home of a local villager, having been preserved in alcohol as medicinal liquor, and was assigned to *P. cornutus* as well (David et al., 2008). Four further specimens of horned pitvipers were then collected between April and July 2009 from two localities in Tianjingshan Forest, Ruyan County, Guangdong Province, China (24°43'N, 113°03'E, 563 m a.s.l.; 24°43'N, 113°02'E, 585 m a.s.l.). Their external morphology and collection sites were described by Gong et al. (2010).

David et al. (2008) and Gong et al. (2010) concluded that the Chinese snakes did not differ in scalation and pattern from Vietnamese *P. cornutus*. However, Chinese and Vietnamese specimens have never been compared by molecular genetic means before. In the present paper, we compare mitochondrial DNA sequence data of the Tianjingshan pitvipers with previously published sequences of a Vietnamese *P. cornutus*

1 - Guangdong Provincial Public Laboratory on Wild Animal Conservation and Management, South China Institute of Endangered Animals, Guangzhou 510260, China

2 - Museum of Zoology (Museum für Tierkunde), Senckenberg Dresden, A. B. Meyer Building, D-01109 Dresden, Germany

3 - Guangdong Provincial Wildlife Rescue Center, Guangzhou 510520, China

* Corresponding author; e-mail: uwe.fritz@senckenberg.de

(12S, 16S, ND4, *cyt b*; Herrmann et al., 2004) and other *Protobothrops* species.

For genetic analyses, tissue (muscle or liver) was removed from the freshly killed Tianjingshan Forest pitvipers prior to ethanol fixation. The complete voucher specimens are housed in the collection of the South China Institute of Endangered Animals, Guangzhou (SCIEA-R-S-2009-001 to SCIEA-R-S-2009-004) and alcohol-preserved tissues are stored in the Museum of Zoology, Senckenberg Dresden (MTD T 6034-6037).

Total genomic DNA was extracted using the DTAB method (Gustinich et al., 1991) and the innuPREP DNA Mini Kit for tissue (Analytik Jena). The same four mitochondrial genes as used by Herrmann et al. (2004) were amplified with the pairs primer L1091 + H1557 for 12S (Parkinson et al., 2000), L2510F + H3059R for 16S (Parkinson et al., 2000), ND4 + LEU for ND4 (Arevalo et al., 1994), and L14841 + MVZ16 for *cyt b* (Malhotra and Thorpe, 2004). The temperature protocol for 12S and 16S was 5 min at 94°C for initial denaturation, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and 10 min at 72°C for final elongation; for ND4, 5 min at 94°C (initial denaturation), then 30 cycles of 1 min at 94°C, 2 min at 58°C and 2 min at 72°C, and for final elongation 7 min at 72°C; for *cyt b*, 5 min at 94°C (initial denaturation), followed by 35 cycles of 1 min at 94°C, 2 min at 42°C and 2 min at 72°C, and for final elongation 7 min at 72°C. PCR products were purified using the ExoSAP-IT enzymatic cleanup. The forward and reverse primers used for PCR were also applied for sequencing on an ABI 3130xl genetic analyzer (Applied Biosystems); cycle sequencing reaction products were purified by salt/ethanol precipitation. GenBank accession numbers for sequences produced in the present study are FR695490-FR695505.

GenBank sequences of the same genes and ingroup species as used by Herrmann et al. (2004) were downloaded and supplemented by homologous GenBank sequences of two further pitvipers now assigned to the genus *Pseudobothrops* (*P. mangshanensis*, *P. sieversorum*; Malhotra et al., 2010) and the closely allied *Ovophis monticola*. *Gloydus blomhoffi* and *Trimeresurus trigonocephalus* were chosen as outgroups and trees were rooted with *T. trigonocephalus*. *Gloydus blomhoffi* and *T. trigonocephalus* belong to two successive sister groups of a clade comprising *O. monticola* and all *Protobothrops* species (Malhotra et al., 2010). Accession numbers for *G. blomhoffi* sequences are: AY352780, AY352719, U41867, AY352719; for *P. mangshanensis*: AY352787, AY352726, AY352821, AY352758; for *P. sieversorum*: AY352782, AY352721, AY352816, AY352753; for *O. monticola*: AY763191, AY352714, AY223626, AF182549; and for *T. trigonocephalus*: AY059549, AY059565, AY059597, AF171890; for other accession numbers see Herrmann et al. (2004). For *P. tokarensis*, no sequence data were available for the ND4 gene.

Sequences were aligned in BIOEDIT 7.0.5.3 (Hall, 1999). Only the ND4 fragment of one of the four Tianjingshan pitvipers (MTD T 6034) differed slightly (by two substitutions) from the sequences of the three other snakes (MTD T 6035-6037), while all the other sequences were

identical. For phylogenetic analyses the complete dataset of one Tianjingshan pitviper was used (MTD T 6037) and sequences of the four genes were concatenated, resulting in an alignment of 2306 bp (including gaps). The partial 12S rRNA gene contributed 426 bp; the partial 16S rRNA gene, 517 bp; the partial ND4 gene, 694 bp; and the partial *cyt b* gene, 669 bp. The best evolutionary models (12S, 16S: TIM2 + G; ND4, *cyt b*: HKY + G) were determined using JMODELTEST (BIC; Posada, 2008). The concatenated sequences were partitioned by gene and analyzed using the maximum likelihood (ML) approach with the on-line interface RAXML (<http://phylobench.vital-it.ch/raxml-bb/index.php>); the robustness of the branching pattern was tested by bootstrapping. Moreover, Bayesian reconstructions were performed using MRBAYES 3.1.2 (Ronquist and Huelsenbeck, 2003; settings ngen = 1000000 nchains = 4 nrun = 2 sample = 500; unlinked model parameters for every gene fragment).

The horned pitviper from Tianjingshan Forest is with 100% support sister to *P. cornutus* from Vietnam. Yet, branch lengths of the Tianjingshan pitviper and *P. cornutus*, reflecting their evolutionary distances, resemble those of the species pair *P. tokarensis* and *P. flavoviridis*, indicating some differentiation. The sister taxon of the Tianjingshan pitviper + *P. cornutus* is *P. jerdonii*. This clade occurs together with two other clades, *P. elegans* + *P. mucrosquamatus* and *P. flavoviridis* + *P. tokarensis*, in a well-supported more inclusive clade to which *P. mangshanensis* and *P. sieversorum* are the successive sister taxa (fig. 1).

Protobothrops cornutus was first recorded from Vietnam and considered for a long time to be endemic there (Smith, 1930; Herrmann et al., 2004), until David et al. (2008) suggested that *Ceratrimeresurus shenlii* from two sites in Guangdong Province represents the same species. Further horned pitvipers were then reported by Gong et al. (2010) from Tianjingshan Forest in Guangdong. All Chinese records are separated from the nearest *P. cornutus* locality in northern Vietnam by about 760 km (fig. 2). David et al. (2008) considered it likely that *P. cornutus* occurs also in elevated regions of Guangxi Zhuang Autonomous Region and Guangdong Province, and that the great distance between the Vietnamese and Chinese records results only from a lack of appropriate collecting efforts. According to Herrmann

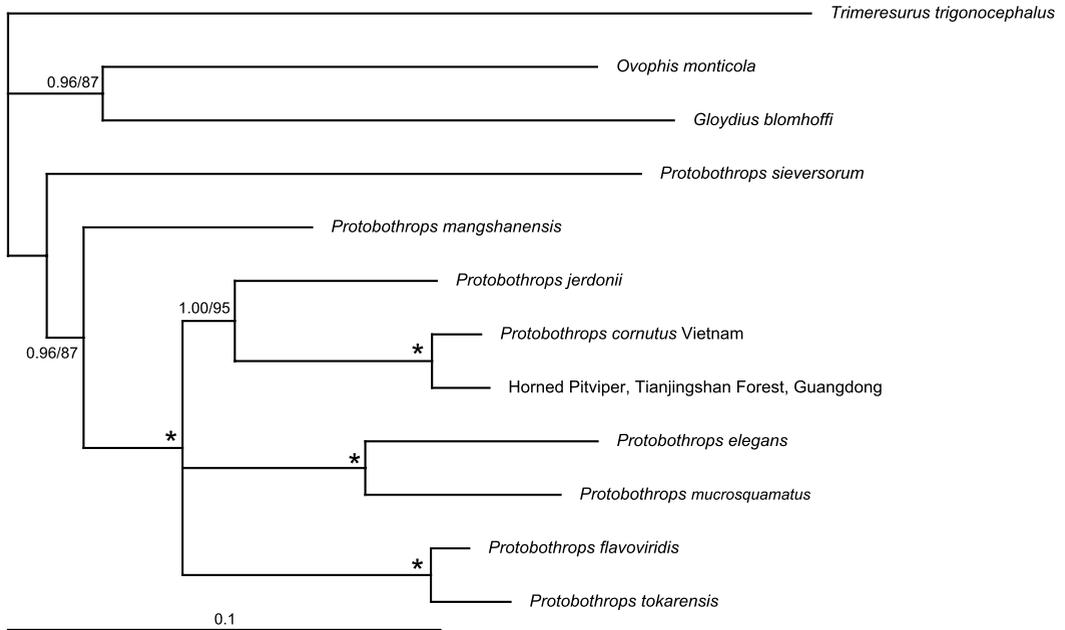


Figure 1. Phylogenetic placement of the Tianjingshan pitviper within *Protobothrops* (Bayesian tree). Numbers along branches are Bayesian posterior probabilities and ML bootstrap values $\geq 0.95/50$. Asterisks indicate 100% support under both methods.

et al. (2004), *P. cornutus* also inhabits the lowlands of Central Vietnam, whereas David et al. (2008) write that the species is absent from the dry lowland areas, which separate the hilly or mountainous ranges of southern China in a subtropical humid climate with high annual amounts of rainfall. The morphological (Gong et al., 2010) and molecular data of the Tianjingshan pitviper suggest that these snakes are closely related to, but slightly different from *P. cornutus* from Vietnam. This implies that *C. shenlii* could be a valid subspecies of *P. cornutus* or a recently split distinct species. However, for the time being we cannot exclude that the observed molecular differences between the Vietnamese specimen studied by Herrmann et al. (2004) and our Tianjingshan pitvipers reflect rather a pattern of isolation-by-distance and not a phylogeographic, and thus perhaps taxonomically relevant, break resulting from a former vicariant event. To disentangle this situation, further efforts are necessary to procure additional material from intermediate localities. Also an in-depth comparison of

the external and hemipenis morphology of Chinese and Vietnamese specimens should be conducted.

Acknowledgements. The authors would like to thank Changteng Yang, Chunyu Yu, Bing He, and Haijing Wang for their help during fieldwork. This study was supported financially by Guangdong Provincial Public Laboratory on Wild Animal Conservation and Management, Conservation Leadership Program.

References

- Arevalo, E., Davis, S.K., Sites, J.W. (1994): Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico. *Syst. Biol.* **43**: 387-418.
- David, P., Tong, H., Vogel, G., Tian, M. (2008): On the status of the Chinese pitviper *Ceratrimeresurus shenlii* Liang and Liu in Liang, 2003 (Serpentes, Viperidae), with the addition of *Protobothrops cornutus* (Smith, 1930) to the Chinese snake fauna. *Asiat. Herpetol. Res.* **11**: 17-23.
- Gong, S.P., Wang, H.J., Yang, C.T., Yu, C.S., Pan, D.S., Wang, F.M. (2010): *Protobothrops cornutus* (Smith, 1930) discovered in Tianjingshan Forest, Guangdong

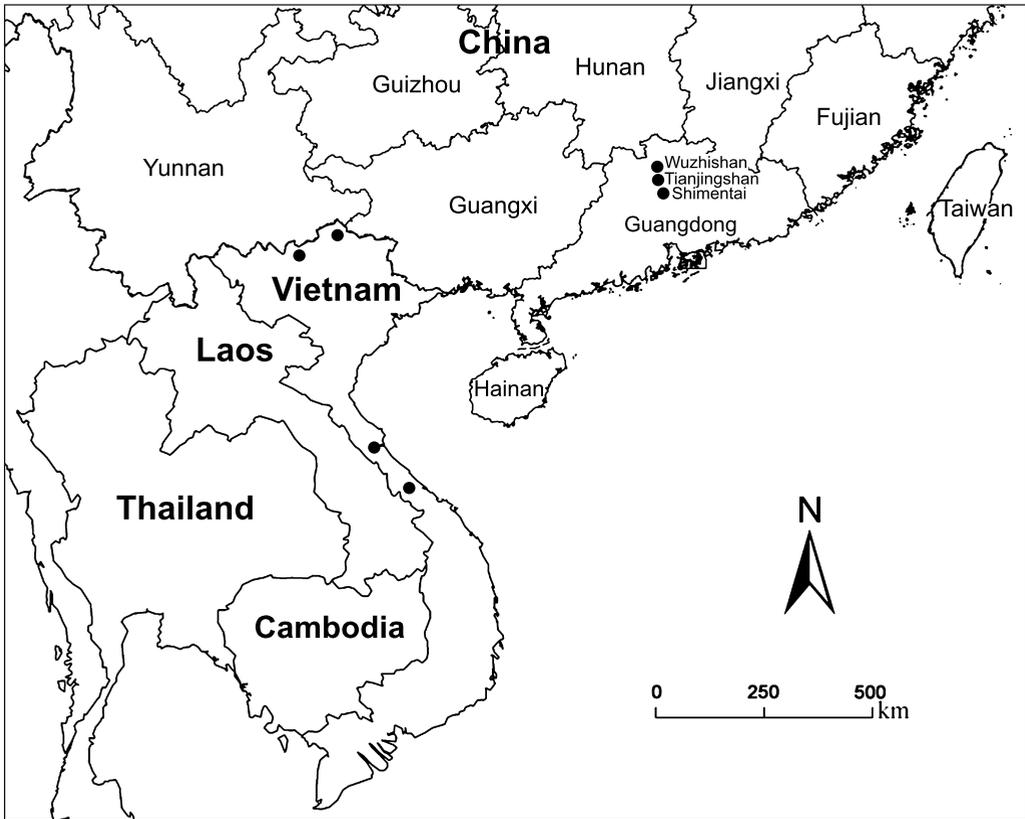


Figure 2. Locality records for horned pitvipers in Vietnam and Guangdong, China (solid circles).

- Province, China. *Chin. J. Zool.* **45**: 170-173 [in Chinese].
- Gumprecht, A., Tillack, F., Orlov, N., Captain, A., Ryabov, S. (2004): Asian Pitvipers. Geitje Books, Berlin, Germany.
- Gustincich, S., Manfioletti, G., del Sal, G., Schneider, C., Carninci, C. (1991): A fast method for high-quality genomic DNA extraction from whole human blood. *BioTechniques* **11**: 298-302.
- Hall, T.A. (1999): BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* **41**: 95-98.
- Herrmann, H.-W., Ziegler, T., Malhotra, A., Thorpe, R.S., Parkinson, C.L. (2004): Redescription and systematics of *Trimeresurus cornutus* (Serpentes: Viperidae) based on morphology and molecular data. *Herpetologica* **60**: 211-221.
- Malhotra, A., Thorpe, R.S. (2004): A phylogeny of four mitochondrial gene regions suggests a revised taxonomy for Asian pitvipers (*Trimeresurus* and *Ovophis*). *Mol. Phylogenet. Evol.* **32**: 83-100.
- Malhotra, A., Creer, S., Pook, C.E., Thorpe, R.S. (2010): Inclusion of nuclear intron sequence data helps to identify the Asian sister group of New World pitvipers. *Mol. Phylogenet. Evol.* **54**: 172-178.
- Parkinson, C.L., Zamudio, K.R., Greene, H.W. (2000): Phylogeography of the pitviper clade *Agkistrodon*: historical ecology, species status, and conservation of cantils. *Mol. Ecol.* **9**: 411-420.
- Posada, D. (2008): JMODELTEST: phylogenetic model averaging. *Mol. Biol. Evol.* **25**: 1253-1256.
- Ronquist, F., Huelsenbeck, J.P. (2003): MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572-1574.
- Smith, M.A. (1930): Two new snakes from Tonkin, Indo-China. *Ann. Mag. Nat. Hist. (Ser. 10)* **6**: 681-683.
- Zhao, E.M. (Ed.) (2006): Snakes of China. Anhui Science and Technology Press, Hefei, China [in Chinese].
- Ziegler, T., Vu, N.T., Le, K.Q., Nguyen, Q.T., Hallermann, J., Le, V.K., Thach, M.H. (2006): Neue Verbreitungsnachweise einiger wenig bekannter vietnamesischer Amphibien und Reptilien. *Sauria* **28** (2): 29-40.

Received: August 25, 2010. Accepted: October 6, 2010.