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Multilocus phylogeny of the Asian Lance-headed pitvipers (Squamata, Viperidae, *Protobothrops*)

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Abstract

Despite the public health significance and potential applications to medical research, the evolutionary history of the Asian Lance-headed pitvipers (genus *Protobothrops*) remains inadequately studied. Previous research generally focused on a few selected species with no comprehensive molecular study of *Protobothrops*. Here, we conduct the first study to infer the phylogenetic relationships of all currently recognized species in this genus based on four mitochondrial DNA fragments and four nuclear genes. Bayesian Inference and Maximum Likelihood analyses show that within *Protobothrops* there are four strongly supported clades forming distinct subgroups. The first subgroup, which is sister to the other three, consists of three species, *P. himalayanus*, *P. kaulbacki*, and *P. sieversorum*. The second subgroup contains only *P. mangshanensis*. The final two subgroups, which are sister groups, include the other four and six *Protobothrops* species. Although our findings provide additional information on the phylogenetic relationships of the genus *Protobothrops,* the placement of *P. dabieshanensis* and *P. elegans* remains problematic. In addition, our molecular results indicate that *P. trungkhanhensis*, currently considered endemic to Vietnam, should be added to the species known from China. Our ancestral area estimation indicated that *Protobothrops* likely originated in southwestern China. This study improves our understanding of the evolutionary relationships among species of Asian Lance-headed pitvipers, providing a greater framework for future studies.

Key words: Asia, Bayesian, distribution, venomous snake, systematics

Introduction

Protobothrops Hoge & Romano Hoge, 1983 (Asian Lance-headed pitvipers) are venomous snakes that are important to study because of potential impacts on human health through bites. Among these terrestrial pitvipers, some species have a wide distribution (e.g. *P. jerdonii* and *P. mucrosquamatus*), while others are endemic to small areas (e.g. *P. mangshanensis* and *P. maolanensis*) (Uetz 2014; Fig. 1). Gumprecht *et al.* (2004) listed eight species under the genus *Protobothrops*. On the basis of morphological comparison and molecular phylogeny, Guo *et al.* (2007) synonymized *Triceratolepidophis* and *Zhaoermia* with *Protobothrops*, thus bringing the number of species contained in this genus to ten. In recent years, four new species have been described successively, including *P. trungkhanhensis* Orlov *et al.* 2009 from Vietnam, *P. maolanensis* Yang *et al.* 2011 from China, *P. dabieshanensis* Huang *et al.* 2012 from China, and *P. himalayanus* Pan *et al.* 2013 from China and India. Therefore, 14 species are currently recognized within this genus (Uetz 2014).

A robust phylogenetic hypothesis is a fundamental requirement for accurate taxonomic classification based on species evolution (Dayrat 2005). Recently, several independent phylogenetic analyses have been conducted including representatives of *Protobothrops* (Malhotra & Thorpe 2004; Wüster *et al.* 2008; Guo *et al.* 2006, 2009, 2011; Yang *et al.* 2011; Liu *et al.* 2012). However, these studies examined only a subset of species within this genus and sequenced only mtDNA gene to incorporate in their analyses.
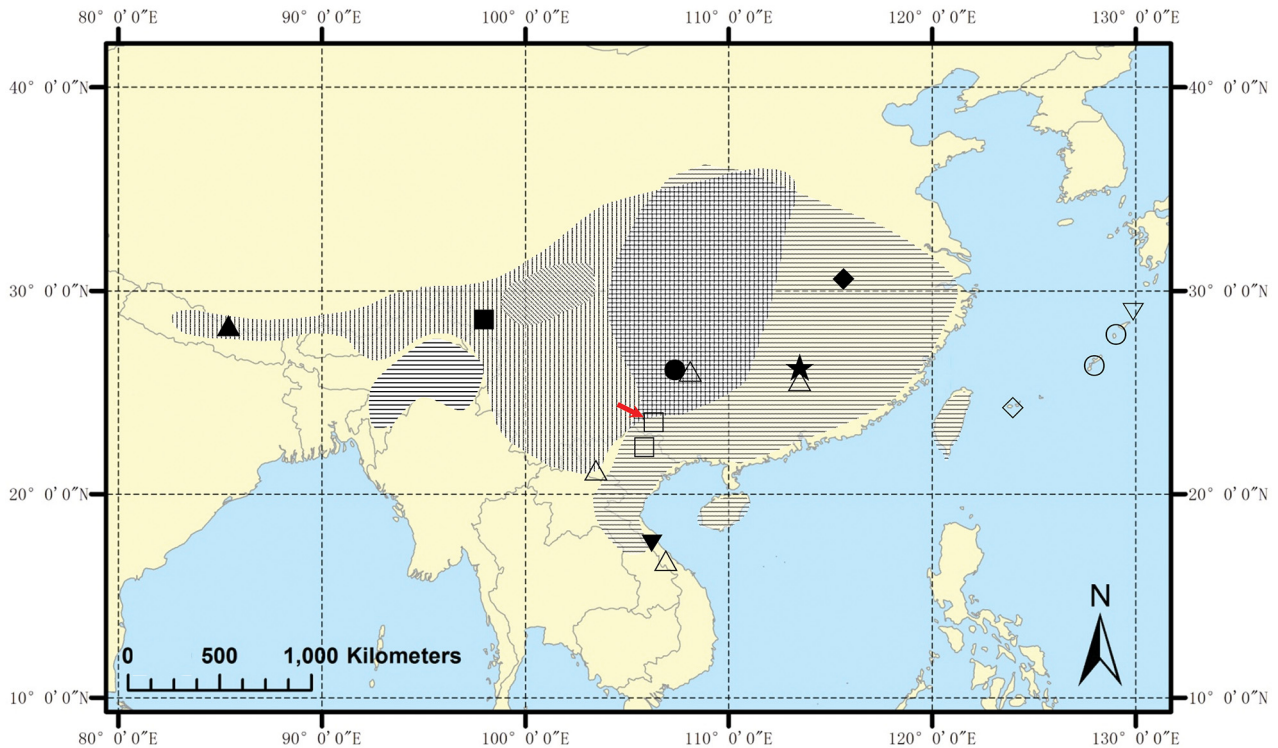
FIGURE 1. Approximate geographic distribution of *Protobothrops* species. Open circle: *P. flavoviridis*; filled circle: *P. maolanensis*; filled triangle: *P. himalayanus*; open triangle: *P. cornutus*; filled diamond: *P. dabieshanensis*; open diamond: *P. elegans*; filled square: *P. kaulbacki*; open square: *P. trungkhanhensis*; inverted filled triangle: *P. sieversorum*; inverted open triangle: *P. tokarensis*; filled star: *P. mangshanensis*; right hatching: *P. xiangchengensis*; vertical hatching: *P. jerdonii*; horizontal hatching: *P. mucrosquamatus*. The red arrow indicates the new location of *P. trungkhanhensis* in China.

In this study, we obtained tissues of all currently recognized species of *Protobothrops* providing the opportunity to explore intrageneric relationships within the genus. Based on four mtDNA gene fragments and four nuclear genes, we infer the phylogenetic relationships of *Protobothrops*. We specifically examined the phylogenetic positions of the two newly described species *P. himalayanus* and *P. trungkhanhensis*.

Material and methods

Sampling and sequencing. All samples sequenced were obtained through fieldwork or tissue loans from colleagues or museums. Thirty-three individuals representing all 14 species were sequenced and analyzed (Table 1). *Ovophis makazayazaya* was chosen as an outgroup in all phylogenetic analyses based on previous studies (Malhotra & Thorpe 2004; Liu *et al*. 2012).

Genomic DNA was extracted from liver or muscle tissues preserved in 85% alcohol using standard methods (Sambrook & Russell 2002). Four mitochondrial fragments [cytochrome *b* (cyt *b*), NADH dehydrogenase subunit 4 (ND4), 12S small subunit ribosomal RNA (12S), 16S large subunit ribosomal RNA (16S)] and four nuclear genes [oocyte maturation factor mos (c-mos), prolactin receptor (PRLR), recombination-activating gene 1 (Rag1), and ubinuclein 1 (UBN1)] were amplified by polymerase chain reaction (PCR). Primers and the cycling parameters are identical to previous studies (cyt *b*: Burbrink *et al*. 2000; ND4: Arevalo *et al*. 1994; 12S: Knight & Mindell 1993; 16S: Parkinson *et al*. 1997; c-mos: Lawson *et al*. 2005; PRLR: Townsend *et al*. 2008; Rag1: Groth & Barrowclough 1999; UBN1: Casewell *et al*. 2011).

Sequences were edited manually using Seqman in DNAstar (DNASTAR, Inc.), aligned in Mega 6 using the ClustalW algorithm with default parameters (Tamura *et al.* 2013), and checked by eye for ambiguous alignments. For the nuclear genes, heterozygous sequences were phased using the software program Seqphase (Flot 2010) and the web-server interface Phase (Stephens *et al*. 2001) and one of the phased nuclear gene copies was selected at

random to represent each individual in subsequent analyses. We translated protein-coding fragments into amino acid sequences using Mega 6 (Tamura *et al*. 2013), and aligned them with the published sequences to confirm we had not amplified pseudogenes (Zhang & Hewitt 1996). Average divergence estimates between species of interest were calculated in Mega 6 (Tamura *et al*. 2013). New sequences generated were deposited in GenBank (Table 1. Accession numbers KT220266–KT220417).

Phylogenetic inference. Bayesian Inference (BI) and Maximum Likelihood (ML) were used to infer phylogenetic relationships. Prior to analyses, four partitioning strategies were evaluated: no partition, five partitions (mtDNA fragments and four nuclear genes), eight partitions (four mtDNA gene fragments and four nuclear genes), and twelve partitions (two protein coding mtDNA fragments 1st, 2nd and 3rd position separately, 12S, 16S, and four nuclear genes). The best evolution model for each partition was selected by MrModeltest under AIC (Nylander 2004). The optimal partition scheme was chosen using Bayes Factors (BF) with the stepping-stone method as described in Ronquist *et al*. (2012). Bayesian analyses were performed in MrBayes 3.2.2 (Ronquist *et al*. 2012) executed in the CIPRES Science Gateway (http://www.phylo.org/index.php). All searches were run with two independent runs and each initiated with a random tree. Each run consists of four Markov chains (three heated chains and a single cold chain) estimated for 5 million generations and sampled every 1000 generations. Stationarity was assessed in Tracer 1.6 and the first 25% of samples were discarded as burn-in (Rambaut *et al*. 2014). Substitution parameters were unlinked and rates were allowed to vary across partitions. After confirming that three analyses reached stationarity at a similar likelihood score and the topologies were similar, the resulting trees were combined to calculate posterior probabilities (PP) for each node in a 50% majority-rule consensus tree.

ML trees were constructed in the program RaxMLv7.2.6 (Stamatakis 2006) with GTRGAMMA model under the same partition scheme as the BI analyses. Branch support was assessed by performing 1000 non-parametric bootstrap (BS) replicates of the topology.

Ancestral area estimation. The species present-day distribution was coded as follows: Xizang and Himalay Mountains (XZ), southwestern China including Vietnam (SW), southern China (SC), and Japan (JP). Two alternative reconstruction methods, both implemented in the computer software Reconstruct Ancestral States in Phylogenies (RASP) (Yu *et al*. 2010, 2015)), were used to infer the ancestral distribution of *Protobothrops*: (i) Statistical Dispersal-Vicariance Analysis (S-DIV) (Yu *et al*. 2010) and (ii) Lagrange (Dispersal-Extinction-Cladogenesis, DEC) (Ree *et al*. 2005). Ancestral area analyses were carried out on 50000 random trees selected from the posterior distribution estimated from BEAST and information on nodes were summarized and plotted as pie charts. All taxa included in the analyses were assigned to at least one of four areas.

Results

Phylogenetic relationships. The final alignment of eight gene fragments consists of 5464 base pairs (Table 2). We translated the protein-coding mtDNA sequences into amino acids and no stop codons were detected, this indicated that unintentional amplification of pseudogenes was unlikely. BF comparison indicated that the preferred partitioning scheme was five-partitions (BFs > 10 between the five-partitions scheme and the others). The optimal models of sequence evolution of each partition identified by MrModeltest are listed in Table 2. Bayesian analyses based on the combined data set recovered effective sample sizes above 200 for all parameters indicating adequate mixing.

Our BI and ML trees showed nearly complete congruence on intrageneric relationships of *Protobothrops* (Fig. 2). Within *Protobothrops* we found four strongly supported clades representing distinct subgroups. The first subgroup (A), which is sister to the other three, consists of three species (i.e. *P. himalayanus*, *P. kaulbacki*, and *P. sieversorum*). The newly described species *P. himalayanus* has a sister relationship with *P. kaulbacki*. The second subgroup (B) contains a single species *P. mangshanensis* (Fig. 2). The two most specious subgroups are the third (C) and fourth (D), which are sister to each other and contain four (i.e. *P. elegans*, *P. maolanensis*, *P. mucrosquamatus*, *P. trungkhanhensis*) and six (i.e. *P. cornutus*, *P. dabieshanensis*, *P. flavoviridis*, *P. jerdonii*, *P. tokarensis*, *P. xiangchengensis*) species, respectively. Within subgroup C, *P. trungkhanhensis* forms a clade with *P. maolanensis* with high support (100% PP), while one sample (GP 2464) previously identified as *P. maolanensis* (Chen *et al*. 2013), is nested within the clade of *P. trungkhanhensis*. Although all subgroups received high support from our ML analysis (>85 BS), subgroup D was poorly supported in our BI analysis (68% PP).

TABLE 1 General information for specimens used in this study including species, collection locality, specimen number and GenBank accession numbers. Abbreviations: IEBR: Institute of Ecology and Biological Resources, Hanoi; FK: Fred Kraus, field tag; GP: Peng Guo, sample catalogue; the samples are deposited in Yibin University; ROM: Royal Ontario Museum, Toronto; UMMZ: University of Michigan Museum of Zoology. Novel sequences generated in this study are bold.

| Species	Localities	Voucher No.	cyt. b	ND4	12S	16S	c-mos	PRLR	UBN1	Rag1
Protobothrops cornutus	Guangdong, China	GP 1631	KT220304	KT220324	KT220266	KT220285	KT220398	KT220345	KT220382	-
P. cornutus	Quang Binh, Vietnam	IEBR A2014.31	KT220306	KT220326	KT220268	KT220287	-	-	-	-
P. cornutus	Cao Bang, Vietnam	IEBR A2014.28	KT220305	KT220325	KT220267	KT220286	KT220399	KT220346	KT220383	KT220363
P. dabieshanensis	Anhui, China	GP2631	KT220307	KT220327	KT220269	KT220288	KT220400	KT220347	KT220384	KT220364
P. elegans	Ryukyu Is., Japan	UMMZI99970	AY223575	U41893	AF057201	AF057248	-	-	-	-
P. flavoviridis	Ryukyu Is., Japan	UMMZI99973	AY223574	U41894	AF057200	AF057247	-	-	-	-
P. himalayanus	Xizang, China	GP2469	KT220308	KT220328	KT220270	KT220289	KT220401	KT220348	KT220385	KT220365
P. himalayanus	Xizang, China	GP3163	KT220309	KT220329	-	-	-	-	-	KT220366
P. jerdonii	Yunnan, China	GP16	HM567474	EU810020	AY763180	AY763199	-	-	-	-
P. kaulbacki	Xizang, China	GP550	KT220310	KT220330	KT220271	KT220290	KT220403	KT220350	KT220387	KT220367
P. kaulbacki	Xizang, China	GP112	DQ666060	DQ666057	DQ666056	DQ666055	KT220402	KT220349	KT220386	-
P. mangshanensis	Hunan, China	GP2190	KT220311	KT220331	KT220272	KT220291	KT220404	KT220351	KT220388	-
P. mangshanensis	Hunan, China	GP2456	KT220312	KT220332	KT220273	KT220292	-	-	-	-
P. mangshanensis	Hunan, China	GP17	HM567537	HM567469	-	-	JQ687524	-	-	KT220368
P. maolanensis	Guizhou, China	GP 1883	JN799401	JN799409	JN799405	JN799397	KT220405	KT220352	-	KT220369
P. maolanensis	Guizhou, China	GP 1884	JN799402	JN799410	JN799406	JN799398	KT220406	KT220353	KT220389	KT220370
P. maolanensis	Guizhou, China	GP 1902	JN799403	JN799411	JN799407	JN799399	KT220407	KT220354	KT220390	KT220371
P. maolanensis	Guizhou, China	GP 1903	JN799404	JN799412	JN799408	JN799400	KT220408	KT220355	KT220391	KT220372
P. mucrosquamatus	Guizhou, China	GP2471	KT220313	KT220333	KT220274	KT220293	KT220409	KT220356	-	KT220373
P. mucrosquamatus	Guizhou, China	GP2472	KT220314	KT220334	KT220275	KT220294	KT220410	-	-	KT220374
P. mucrosquamatus	Guizhou, China	GP2473	KT220315	KT220335	KT220276	KT220295	KT220411	-	-	KT220375
P. mucrosquamatus	Vinh Phu, Vietnam	ROM31085	KT220317	KT220337	KT220277	KT220297	-	-	-	-
P. mucrosquamatus	Cao Bang, Vietnam	ROM35307	KT220318	KT220338	KT220279	KT220298	KT220413	KT220358	KT220393	KT220377
P. mucrosquamatus	Hai Duong, Vietna	ROM3529	KT220316	KT220336	KT220278	KT220296	KT220412	KT220357	KT220392	KT220376
P. tokarensis	Ryukyu Is., Japan	FK 1997	AY223576	AY223628	AF057202	AF057249	-	-	-	-
P. sieversorum	Khammuane, Laos	IEBR KM 2012.8	KT220320	KT220340	KT220281	KT220300	KT220415	KT220360	KT220395	-
P. sieversorum	Quang Binh, Vietnam	IEBR A 2014.30	KT220319	KT220339	KT220280	KT220299	KT220414	KT220359	KT220394	KT220378
P. trungkhanhensis	Cao Bang, Vietnam	ZFMK 93774	KT220323	KT220343	KT220284	KT220303	KT220417	KT220362	KT220397	KT220380
P. trungkhanhensis	Cao Bang, Vietnam	IEBR A 2014.29	KT220322	KT220342	KT220283	KT220302	KT220416	KT220361	KT220396	KT220379
P. maolanensis	Guangxi, China	GP 2464	KT220321	KT220341	KT220282	KT220301	-	-	-	-
P. xiangchengensis	Sichuan, China	GP 26	DQ666061	DQ666058	AY763188	AY763207	-	-	-	-
P. xiangchengensis	Sichuan, China	GP 27	DQ666062	DQ666059	AY763189	AY763208	-	-	-	-
Ovophis makazayazaya	Hunan, China	GP 343	HQ325172	HQ325234	HQ325299	HQ325114	-	KT220344	KT220381	-
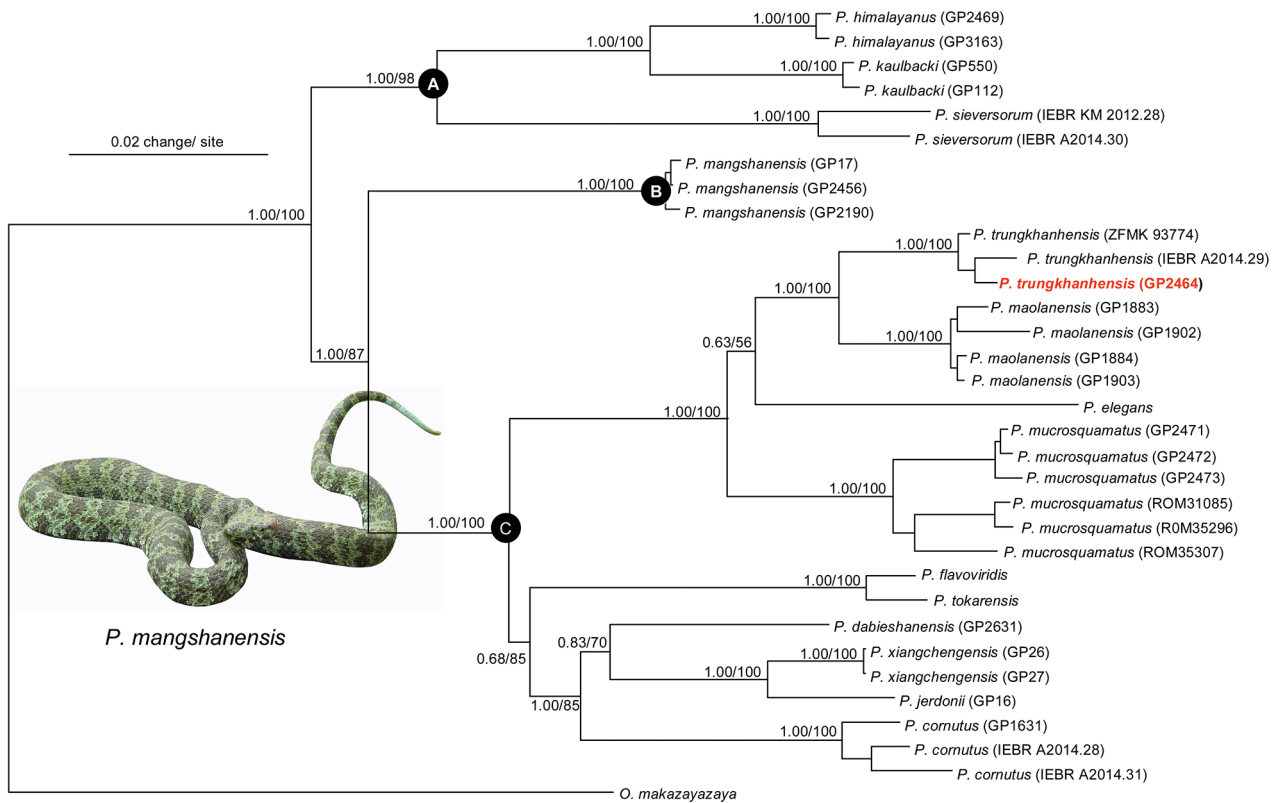
FIGURE 2. Bayesian 50% majority-rule consensus tree of *Protobothrops* inferred from the combined mitochondrial and nuclear genes dataset analyzed using the models described in the text. Posterior probabilities from Bayesian inference and bootstrap support values from maximum likelihood analysis (where >50%) are given adjacent to respective nodes for major clades.

TABLE 2. Results of AIC model selection conducted in MrModeltest for partitions of the dataset.

Partition	Size (bps)	AIC model
mtDNA	2913	GTR+I+G
cyt *b*	1084	
ND4	689	
12S RNA	662	
16S RNA	478	
c-mos	478	GTR+I
PRLR	573	GTR+G
Rag1	1005	HKY+I
UBN1	502	HKY+I

Distance measurements. The interspecific genetic divergence based on *p*-distance ranges from 0.013 to 0.154 for cyt *b* and ND4, and from 0.016 to 0.123 for cyt *b* alone (Table 3). Based on cyt *b* sequences, the largest distance was found in three species-pairs, e.g. *P. himalayanus* and *P. sieversorum*; and the smallest was found between *P. flavoviridis* and *P. tokarensis*.

Ancestral area estimation. Both S-DIV and DEC analyses consistently recovered that southwestern China was the most likely ancestral area for the genus *Protobothrops* (65% in S-DIV and 100% in DEC).

TABLE 3. Average interspecific divergence estimates (*p*-distances) calculated for cyt *b* (below diagonal) and the combined data of cyt. *b* and ND4 (above diagonal). Species abbreviations: PC: *P. cornutus*, PD: *P. dabieshanensis*, PE: *P. elegans*, PF: *P. flavorividis*, PH: *P. himalayanus*, PJ: *P. jerdonii*, PK: *P. kaulbacki*, PMan: *P. mangshanensis*, PMao: *P. maolanensis,* PMu: *P. mucrosquamatus,* PS: *P. sieversorum*, PTo: *P. tokarensis*, PTr: *P. trungkhanhensis*, PX: *P. xiangchengensis*.

Species	PC	PTr	PMan	PH	PMu	PD	PK
PC		0.114	0.112	0.128	0.114	0.081	0.133
PTr	0.087		0.124	0.111	0.05	0.107	0.122
PMan	0.099	0.106		0.105	0.13	0.113	0.099
PH	0.114	0.123	0.114		0.122	0.126	0.066
PMu	0.087	0.062	0.114	0.123		0.114	0.123
PD	0.066	0.089	0.094	0.118	0.094		0.131
PK	0.102	0.107	0.102	0.057	0.104	0.104	
PS	0.11	0.12	0.107	0.11	0.123	0.109	0.092
PE	0.088	0.066	0.102	0.116	0.067	0.094	0.101
PF	0.078	0.089	0.094	0.113	0.089	0.067	0.10
PMao	0.086	0.037	0.094	0.11	0.059	0.079	0.102
PTo	0.079	0.091	0.094	0.114	0.09	0.072	0.104
PX	0.063	0.085	0.082	0.105	0.081	0.054	0.095
PJ	0.068	0.092	0.096	0.122	0.096	0.062	0.104

continued.

Species	PS	PE	PF	PMao	PTo	PX	PJ
PC	0.138	0.108	0.104	0.119	0.097	0.081	0.077
PTr	0.141	0.081	0.107	0.045	0.111	0.109	0.107
PMan	0.119	0.122	0.109	0.128	0.103	0.114	0.12
PH	0.132	0.105	0.12	0.116	0.12	0.124	0.131
PMu	0.147	0.077	0.113	0.066	0.113	0.109	0.098
PD	0.145	0.12	0.083	0.114	0.088	0.066	0.069
PK	0.112	0.118	0.126	0.131	0.129	0.126	0.131
PS		0.139	0.152	0.145	0.154	0.149	0.144
PE	0.116		0.109	0.083	0.109	0.109	0.101
PF	0.111	0.09		0.116	0.013	0.084	0.088
PMao	0.112	0.061	0.081		0.116	0.118	0.113
PTo	0.116	0.093	0.016	0.084		0.086	0.09
PX	0.105	0.088	0.072	0.082	0.075		0.038
PJ	0.112	0.086	0.069	0.078	0.073	0.027	

Discussion

Among the four subgroups within *Protobothrops*, the first group containing *P. himalayanus*, *P. kaulbacki*, and *P. sieversorum* formed a basal split from the other taxa. In this subgroup, the three species are restricted to Qinghai-Xizang Plateau and adjacent regions, while each is endemic to a limited area (Zhao 2006; Pan *et al*. 2013). Of these, *Protobothrops himalayanus* was recently described from China (Southwest Xizang), India, and Bhutan (Pan *et al.* 2013). This species was thought to be most closely related to *P. kaulbacki* based on morphological similarity (Pan *et al.* 2013) and our molecular phylogeny confirms this inference. Although *P. kaulbacki* also occurs in China (Xizang) and India, the two species are allopatric (Uetz 2014; Fig.1). The long history of geographic isolation

across various habitats may be responsible for the great genetic divergence (5.7% based on cyt *b*) between the two species.

Protobothrops trungkhanhensis is described from Vietnam, where it is believed to be endemic (Orlov *et al*. 2009). Yang *et al*. (2010) suggested that *P. maolanensis* was phenotypically similar to this species although no specific analysis provided evidence for this suggestion. Our BI and ML analyses showed a well-supported sister relationship between *P. maolanensis* and *P. trungkhanhensis* (Fig. 2) supporting the speculation by Yang *et al*. (2010). Although genetic distance between the two species is relatively low (3.7% for cyt *b*), this is still higher than that of some other species pairs such as *P. flavoviridis* and *P. tokarensis* (1.6% for cyt *b*) or *P. jerdonii* and *P. xiangchengensis* (2.7% for cyt *b*). Chen *et al*. (2013) reported two specimens of *Protobothrops* from Nonggang, Guangxi, China (Fig. 1, indicated by a red arrow) and tentatively identified them as *P. maolanensis* based on a morphological comparison. However, our molecular phylogeny placed one of the two specimens (GP 2464; the other is unavailable for this study) with *P. trungkhanhensis* rather than *P. maolanensis* (Fig. 2). This finding represents a new record of *P. trungkhanhensis* in China. This occurrence of *P. trungkhanhensis* in Guangxi, China is unexpected due to Nonggang's proximity to the type locality of *P. trungkhanhensis* (Trung Khanh, Cao Bang, Vietnam) (Orlov *et al*. 2009).

Although our phylogeny strongly supported the relationships of most *Protobothrops*, the relationships of some species were not well resolved (Fig. 2). For example, within subgroup C the phylogenetic relationship among *P. elegans*, *P. mucrosquamatus*, and (*P. maolanensis*+*P. trungkhanhensis*) was not clear. This result was similar to a previous study (Liu *et al*. 2012). Future research with more samples and more nuclear genes may resolve this issue.

Generally, the Himalaya and adjacent regions have been suggested to be the origin for most biodiversity in Asia (Huang *et al.* 2007; Che *et al*. 2010; Guo *et al.* 2011; Yan *et al*. 2013; Zhou *et al*. 2013). Our ancestral area estimation indicates that southwestern China is the most likely ancestral area for this genus, which is consistent with previous studies to some extent. It has been assumed that the Qinghai-Xizang Plateau began its slow uplift during the late Miocene (~25–10 Mya) (Sun 1997), and a rapid uplift took place approximately 8 Mya (see Che *et al*. 2010). The estimated split within *Protobothrops* (before 20 Mya; Wüster *et al*. 2008) falls within the period of the rapid uplift of the Qinghai-Xizang Plateau, which may have resulted in different geographic and microclimatic regions, leading to speciation within *Protobothrops*.

Our study sheds new light on the evolution of the Asian Lance-headed Pitvipers. Here we not only provide the most robust phylogeny of the genus to date but also produce novel data including four nuclear loci for most species. These data and analyses will likely be a great benefit for future studies on evolution, systematics, conservation, as well as snakebite treatment.

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