New evidence on the phylogenetic position of the poorly known Asian pitviper *Protobothrops kaulbacki* (Serpentes: Viperidae: Crotalinae) with a redescription of the species and a revision of the genus *Protobothrops*

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Although much systematic work has been done in recent years on the Asian pitviper genus *Protobothrops*, the phylogenetic position of *P. kaulbacki* remains poorly understood due to its rarity and the inaccessibility of its range. This species has long been regarded as morphologically close to *P. jerdonii* and therefore has been widely treated as a member of *Protobothrops*. In this paper, we evaluate the phylogenetic position of this species using skull characteristics, hemipenial, ecological and molecular data. A molecular phylogeny, based on four mitochondrial genes, shows that the species forms a very highly supported sister-group relationship with *Triceratolepidophis sieversorum*, and is distinct from all other *Protobothrops* species. We discuss the alternative systematic arrangements that could take into account these newly discovered relationships of *P. kaulbacki*, provide a redescription of the species and summarize the available information on the distribution and natural history of *P. kaulbacki*.

Key words: hemipenis, molecular systematics, morphology, natural history, *Triceratolepidophis*, *Zhaoermia*

INTRODUCTION

In recent decades, substantial attention has been directed towards the phylogeny and taxonomy of *Trimeresurus* (*sensu lato*) at different taxonomic levels, and considerable progress has been made (Malhotra & Thorpe, 2004a; Castoe & Parkinson, 2006; Creer et al., 2006). At the intraspecific level, certain widely distributed species, such as *Trimeresurus albolabris* (*sensu lato*), *Trimeresurus popeiorum* (*sensu lato*) and *Trimeresurus stejnegeri* (*sensu lato*) have been found to be paraphyletic, or to represent independent lineages (Giannasi et al., 2001; David et al., 2001, 2002; Malhotra & Thorpe, 2004b; Vogel et al., 2004; Sanders et al., 2004, 2006). At the specific level, some species have been found to be misclassified and were re-assigned to other genera (David & Vogel, 1998; Herrmann et al., 2004; Malhotra & Thorpe, 2000), while new species have been discovered and assigned to new genera (Ziegler et al., 2000). Finally, at the generic level, the taxonomy of this major radiation has been revised substantially, with several genera being revalidated and some new genera proposed on the basis of a combination of morphological comparison and molecular analysis (Malhotra & Thorpe, 2004a). Subsequently, some aspects of this latest generic rearrangement have been further confirmed by morphological (Guo & Zhao, 2006) and molecular phylogenetic (Castoe & Parkinson, 2006; Creer et al., 2006) studies. Currently, the Old World genus *Trimeresurus* (*sensu lato*, Brattstrom, 1964) is separated into a total of 11 genera, including *Protobothrops* Hoge & Romano-Hoge 1983, *Ovophis* Burger in Hoge & Romano-Hoge 1981, *Zhaoermia* Gumprecht & Tillack 2004, *Triceratolepidophis* Ziegler et al. 2000, *Cryptelytrops* Cope 1860, *Garthius* Malhotra & Thorpe 2004, *Himalayophis* Malhotra & Thorpe 2004, *Parias* Gray 1849, *Peltopelor* Günther 1864, *Popeia* Malhotra & Thorpe 2004 and *Viridovipera* Malhotra & Thorpe 2004 (see Malhotra & Thorpe, 2004a).

However, despite this taxonomic activity, controversy and/or ambiguity still surrounds the systematic position of some species (David & Ineich, 1999; Zhao et al., 1998; Malhotra & Thorpe, 2004a; Guo et al., 2006b), not only because of the political inaccessibility of their ranges, but also because many of the species are secretive in habits and are found only in remote areas. This is especially true for Kaulback's lance-headed pitviper, *Protobothrops kaulbacki*.

Protobothrops kaulbacki (Smith, 1940) was originally described as *Trimeresurus kaulbacki* based on specimens collected from Pangnamdim, north of the Triangle, Upper Burma. During the past sixty years, few studies on this species have appeared because it was known only from the type locality and no additional specimens have been found since the type specimens were collected. In their description of *Protobothrops*, Hoge & Romano-Hoge (1983) did not mention *kaulbacki* (or indeed, many of the other species that are now placed within this genus). According to a comparison of head musculature, Groombridge (1986) proposed that *kaulbacki* was closely

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related to *P. jerdonii*. Kraus et al. (1996), followed by most subsequent authors (David & Ineich, 1999; Gumprecht et al., 2004; Rao & Zhao, 2005), placed *kaulbacki* in *Protobothrops*, but without giving any reason. Obviously, the few previous studies have not provided enough data to fully understand the affinities of this species.

In recent decades, molecular phylogenetics has been shown to be a robust tool to address the systematics and evolution of pitvipers (e.g. Toda et al., 1999; Tu et al., 2000; Giannasi et al., 2001; Creer et al., 2003, 2006; Parkinson et al., 2002; Wüster et al., 2002; Herrmann et al., 2004; Malhotra & Thorpe, 2000, 2004a; Guo et al., 2006b; Castoe & Parkinson, 2006). Malhotra & Thorpe (2004b) recommended the removal of *Trimeresurus strigatus*, which had been placed in *Protobothrops* by Kraus et al. (1996), on the basis of its synapomorphies with other species in *Trimeresurus sensu stricto*. Several subsequent studies have consistently shown that *Protobothrops* is a monophyletic group (Herrmann et al., 2004; Malhotra & Thorpe, 2004a; Guo et al., 2006b; Castoe & Parkinson, 2006). However, none of these studies has included the species *kaulbacki*. This paper addresses the question of whether *Protobothrops* as currently recognized remains monophyletic after the addition of *kaulbacki*, and whether the phylogenetic position of this species is consistent with the taxonomy proposed on the basis of superficial resemblance.

In 2004, a live specimen of Kaulback's pitviper was collected from Xizang A.R., China, during a herpetological survey (Rao & Zhao, 2005), and thus made it possible to address the systematics of this species using molecular methods. In the present work, the molecular phylogeny of *kaulbacki* and related species and genera is reconstructed on the basis of four mitochondrial DNA gene fragments. In addition, a morphological (including external characters, skull and hemipenial structure) and ecological comparison is provided between *kaulbacki* and related species.

MATERIALS AND METHODS

Molecular phylogenetic analysis

The dataset analysed included 30 OTUs, representing eight genera within *Trimeresurus* (*sensu lato*): *Zhaoermia* (Gumprecht & Tillack, 2004), *Ovophis*, *Protobothrops*, *Triceratolepidophis*, *Cryptelytrops*, *Viridovipera*, *Garthius*, *Popeia* (Malhotra & Thorpe, 2004a) and representatives of other Asian pitviper genera and several New World taxa (Table 1). Thus, all major clades of pitvipers as defined by Malhotra & Thorpe (2004a) were included. *Hypnale hypnale* was chosen as the outgroup as it belongs to the basal clade of pitvipers according to previous studies (Malhotra & Thorpe, 2004a; Castoe & Parkinson, 2006; Creer et al., 2006).

Cyt *b*, ND4, 12S rRNA and 16S rRNA sequences were generated for *P. xiangchengensis* and *P. kaulbacki*, and combined with previously published data (Herrmann et al., 2004; Malhotra & Thorpe, 2004a; Guo et al., 2006b). Novel sequences were generated using primers and reaction conditions as described in Malhotra & Thorpe (2000) for cyt *b*, ND4 sequences were obtained as described in Parkinson et al. (2000), 12S rRNA as described in Knight & Mindell (1993) and 16S rRNA as in Parkinson et al. (1997).

Whole genomic DNA was extracted from 0.01–0.02 g of 90%-ethanol-preserved liver or muscle tissue with Sigma GenElute Mammalian Genomic DNA Miniprep Kits. Prior to sequencing, PCR products were incubated at 37 ºC for 50 minutes with the enzymes Exonuclease I and Shrimp Alkaline Phosphatase, in order to degrade primer dimer and remove unincorporated dNTPs. The double-stranded product was sequenced using dye-labelled terminators (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit), and subsequently run on an ABI Prism 377 DNA sequencer following manufacturer's protocols. Alignment of cyt *b* and ND4 was trivial as there were no indels. The 12S rRNA and 16S rRNA sequences were aligned following previous alignments (Malhotra & Thorpe, 2004a) using MEGA version 3.0 (Kumar et al., 2001). The cyt *b* and ND4 sequences were translated into amino acid sequences using MEGA version 3.0 (Kumar et al., 2001) to check for the unexpected occurrence of stop codons that might indicate the amplification of pseudogenes (Zhang & Hewitt, 1996). All sequences were concatenated and analysed as a single matrix because they belong to a single linkage group (mitochondrial genome), and have been found to evolve similarly (Parkinson et al., 2002). A total of 13 bp in a region of uncertain alignment (positions 290–303 of the 16S sequence) were excluded from the analysis.

The possibility of non-neutral evolution was tested using a variety of tests implemented in the program DnaSP4. 0 (Rozas & Rozas, 1999), including Fu & Li's D* and F* (Fu & Li, 1993), and Tajima's D (Tajima, 1989). Both maximum parsimony (MP) and Bayesian Markov Chain Monte Carlo (MCMC) approaches were used to reconstruct phylogenies, using PAUP* 4.0b10 (Swofford, 2003) and MrBayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) respectively. Gaps were treated as a new state (fifth character), a valid procedure in this case since no gaps included in the analysis were more than 1 bp long (Creer et al., 2006), and all characters were treated as equally weighted in MP searches. Most parsimonious trees were obtained with a heuristic search using 100 random addition replicates and TBR branch swapping. Support values for clades were calculated from 1000 bootstrap pseudo-replicates using the same settings, except that the number of random additions was reduced to 10.

Prior to Bayesian analyses, the simplest best-fit model of evolution for the combined dataset was inferred by Modeltest 3.7 (Huelsenbeck & Crandall, 1997; Posada & Crandall, 1998, 2001). Three runs were performed with four Markov chains (three heated chains and a single cold chain) starting from a random tree. Each of these runs was conducted with a total of 10 million generations and sampled every 100 generations, the first 100,000 of which were discarded as burn-in. Stationarity was confirmed by plotting the –ln *L* per generation in the program Tracer 1.2 (Rambaut & Drummond, 2003). After confirming that the three analyses reached stationarity at a similar likelihood

Table 1. Specimen details and GenBank accession numbers for the species used in this analysis. CAS: California Academy of Science, San Francisco; SCUM: Sichuan University Museum, China; SYNU: Shenyang Normal University, China; UMMZ: University of Michigan Museum of Zoology; ZFMK: Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn; AM: Anita Malhotra, own catalogue number; FK: Fred Kraus, field tag; GP: Guo Peng, own catalogue number.

Species	Localities	Voucher nos.	GenBank accession no. (cyt*b*, ND4, 12S, 16S)
Protobothrops jerdonii	Yunnan, China	CAS215051	AY294274, AY294264, AY294278, AY294269
P. jerdonii	China	AM B485	AY294273, AY294263, AY294277, AY294268
P. mucrosquamatus	Taiwan, China	AM A233	AF171897, AY294265, AY294279, AY294270
P. mucrosquamatus	Vin Phuc, Vietnam	AM B106	AY294275, AY294266, AY294280, AY294271
P. xiangchengensis	Sichuan, China	GP26/SCUM035042	DQ666061, DQ666058, AY763188, AY763207
P. xiangchengensis	Sichuan, China	GP27/SCUM035046	DQ666062, DQ666059,AY763189, AY763208
P. cornutus	Vietnam	ZFMK 75067	AY294272, AY294262, AY294276, AY294267
P. elegans	Ryukyu Is., Japan	UMMZ199970	AY223575, U41893, AF057201, AF057248
P. flavoviridis	Ryukyu Is., Japan	UMMZ199973	AY223574, U41894, AF057200, AF057247
P. flavoviridis	Ryukyu Is., Japan	AM B527	–, AY352826, AY352792, AY352730
P. tokarensis	Ryukyu Is., Japan	FK1997	AY223576, AY223628, AF057202, AF057249
P. kaulbacki	Xizang AR, China	GP112/SYNU0400II30	DQ666060, DQ666057, DQ666056, DQ666055
Triceratolepidophis sieversorum	Vietnam	AM B162	AY352753, AY352816, AY352782, AY352721
Zhaoermia mangshanensis	Hunan, China	AM B300	AY352758, AY352821, AY352787, AY352726
Ovophis monticola	China	AM B482	AY352748, AY352809, AY352775, AY352714
O. monticola	Vietnam	ROM 7798	AY223572, AY223626, AY223652, AY223665
O. monticola	Taiwan	AM A87	AF171907, AY059582, AY059545, AY059561
"O." okinavensis	Ryukyu Is., Japan	CLP 162	AY223573, U41895, AF057199, AF057246
Garthius chaseni	Sabah, Malaysia	AM B306	AY352760, AY352825, AY352791, AY352729
Gloydius shedaoensis	Liaoning, China	ROM 20468	AY223566, AY223623, AF057194, AF057241
G. blomhoffi	Teuri Isl., Japan	AM B524	AY352751, AY352814, AY352780, AY352719
G. halys	Khazakstan	—	AY223564, AY223621, AF057191, AF057238
G. brevicaudius	China	AM B525	AY352752, AY352815, AY352781, AY352720
Bothrops atrox	—	—	AF191587, AF246277, AY223659, AY223672
Lachesis muta	—	—	AF039262, U96030, AF057221, AF057268
"Trimeresurus" gracilis	Taiwan	AM A86	AF171913, AY352823, AY352789, AY352728
Cryptelytrops septentrionalis	Kathmandu, Nepal	AM B487	AY352755, AY352818, AY352784, AY352724
Hypnale hypnale	Tamil Nadu, India	AM A53	AY352750, AY352812, AY352778, AY352717
Popeia popeiorum	Sabah, Malaysia	AM B344	AY371815, AY371842, AY371736, AY371771
Viridovipera stejnegeri	Fujian, China	AM A222	AF277677, AY059594, AY059541, AY059557

score and that the topologies were similar, the resultant trees were combined to calculate posterior probabilities (PP) for each bipartition in a 50% majority-rule consensus tree.

Morphological comparison

External features were observed and recorded under a dissecting microscope. Snout–vent length (SVL) and tail length were measured using a metre ruler to the nearest centimetre, and the remaining measurements were taken using digital callipers to the nearest millimetre. For hemipenial characteristics, we largely followed the methods and terminology of Guo & Zhang (2001) and Guo et al. (2006a). External morphological characters were measured according to Malhotra & Thorpe (2004a). The specimens examined in this study and museum abbreviations are listed in Appendix 1.

To compare *Protobothrops kaulbacki* to some morphologically similar species, we carried out analysis of covariance (ANCOVA) on size-related characters (head length, head width and tail length), after having first linearized these measurements by taking the natural log of the character, with SVL as the independent variable. The remaining characters were tested for significant between-species differences using analysis of variance (ANOVA). A principal components analysis (PCA) was carried out using all characters that were significantly different between species in the ANOVA/ANCOVA, with size-related characters first being linearized as above, and size-adjusted using the pooled within-group regression slope.

RESULTS

Molecular analysis

A total of 2384 bp of sequence was obtained (806 bp of cyt *b*, 668 bp of ND4, 410 bp of 12S rRNA and 500 bp of 16S rRNA). Of these, 718 bp were parsimony informative characters. No deletions, insertions or stop codons were detected in the two protein-coding regions (cyt *b* and ND4), indicating that unintentional amplification of

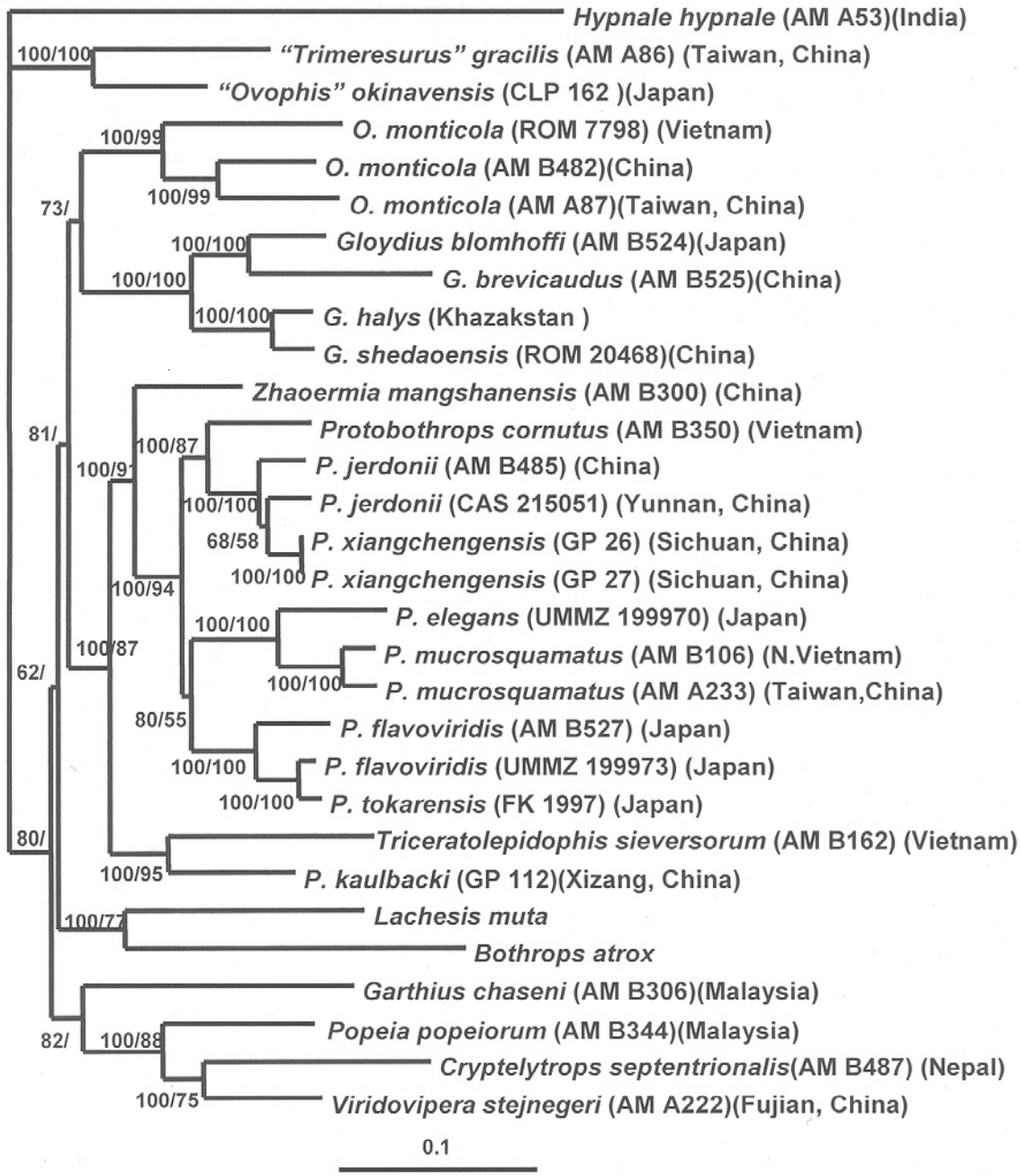

Fig. 1. Phylogenetic tree derived from Bayesian analysis of combined data from four mitochondrial genes, with posterior probabilities (preceding the slash) and bootstrap support values (after the slash) from Bayesian and maximum parsimony analyses respectively.

pseudogenes was unlikely. Base frequencies were estimated by the program Tracer 1.2 (Rambaut & Drummond, 2003) as A=0.3436, C=0.3145, G=0.1157, T=0.2262; alpha= 0.8556, and not found to be significantly different between members of the ingroup (x^2=50.594, df=87, P>0.999). None of the neutrality tests suggested a significant departure from neutral sequence evolution. Novel sequences were deposited in GenBank (Table 1, accession nos. DQ666055-DQ666062).

The parsimony analysis produced a single most parsimonious tree of length 3366 steps with CI=0.414, RI=0.460, RC=0.191. Modeltest indicated that GTR+I+Γ was the simplest best-fit model for the combined mitochondrial dataset by the Akaike Information Criterion (AIC) (Tavaré, 1986). The resulting Bayesian tree had a mean likelihood score of –17615.37.

Both parsimony and Bayesian reconstructions inferred identical topologies, although some clades in the parsimony tree have lower bootstrap (BS) support values (Fig. 1). As the focus of the present research is on the phylogenetic position of *P. kaulbacki* and the related species and genera, only the clade including the genera *Protobothrops*, *Zhaoermia* and *Triceratolepidophis*, a strongly supported region of the tree, is discussed here.

Interestingly, the species *kaulbacki*, which has been placed in *Protobothrops* by most recent authors, did not cluster with its congeneric species in either reconstruction. Instead, it formed a strongly supported sister

Table 2. Morphological comparison between *Protobothrops kaulbacki* (*P.k.*), *P. jerdonii* (*P.j.*) and *Triceratolepidophis sieversorum* (*T.s.*). Means ± SD; all sizes are in mm. Deviations are given for each character, except for size-related characters, which have been adjusted to a common SVL (the grand mean, 740.67 mm) and for which the standard error of the adjusted mean is given. **P*<0.01; ** *P*<0.05. Sample sizes given in the heading are the maximum used, with some characters having smaller sample sizes as indicated.

Characters	*P.k.* (*n*=3)	*P.j.* (*n*=17)	*T.s.* (*n*=2)
Maximum total size	1385	1020	1255
Snout–vent length*	1140 (±28.28)[a]	666 (±111.31)	973 (±122.33)
Tail length	133.66 (±12.06)[a]	149.83 (±3.15)	128.33 (±9.38)
Ventral scales*	205.67 (±2.08)	178 (±11.11)	233.5 (±4.95)
Subcaudal scales*	70.33 (±3.51)[a]	58.47 (±8.06)	80.00 (±2.12)
Head length	33.31 (±1.12)	33.91 (±1.03)	30.05 (±1.09)
Head width*	10.19 (±1.13)[a]	13.49 (±1.03)	20.37 (±1.10)
Mid-body dorsal scales*	26.00 (±1.41)[a]	21.12 (±0.49)	22.50 (±0.71)
Supralabial scales	8.00 (± 0.00)	7.18 (±0.35)	8.50 (±0.71)
Sublabial scales*	13.12 (±0.29)	10.94 (±0.79)	13.50 (±0.00)
Scales between nasal and 2nd supralabial	1.83 (±0.29)	1.47 (±1.053)	–[b]
Scales between 3rd supralabial and subocular*	1.50 (±0.87)	0.71 (±0.59)	3.00 (±0.00)[c]
Scales between 4th supralabial and subocular**	0.83 (±0.29)	0.97 (±0.87)	3.50 (±0.00)[c]
Intersupraocular scales*	7.33 (±0.58)	7.47 (±1.13)	15.00 (±0.00)[c]
Internasal scales*	0.33 (±0.58)	1.77 (±0.66)	2.50 (±0.71)
Infralabial scales touching chinshield	2.17 (±0.29)	2.62 (±0.42)	2.00 (±0.00)[c]

[a]*n*=2; [b]*n*=0; [c]*n*=1

relationship with *Triceratolepidophis sieversorum* (parsimony bootstrap [BS] = 95%, Bayesian posterior probability [PP]= 100%). *Zhaoermia* was also inferred with high support (BS=91%, PP=100%) as the sister lineage to the clade containing seven species of *Protobothrops* (itself supported by BS=94%, PP=100%), and both clades together form a sister relationship with the *Triceratolepidophis* clade (including *kaulbacki*). The general results presented here are in agreement with previous conclusions by Malhotra & Thorpe (2004a) and Castoe & Parkinson (2006) that *Zhaoermia*, *Protobothrops* and *Triceratolepidophis* form a monophyletic group. However, the present results show much stronger support for this clade (BS=87%, PP=100%).

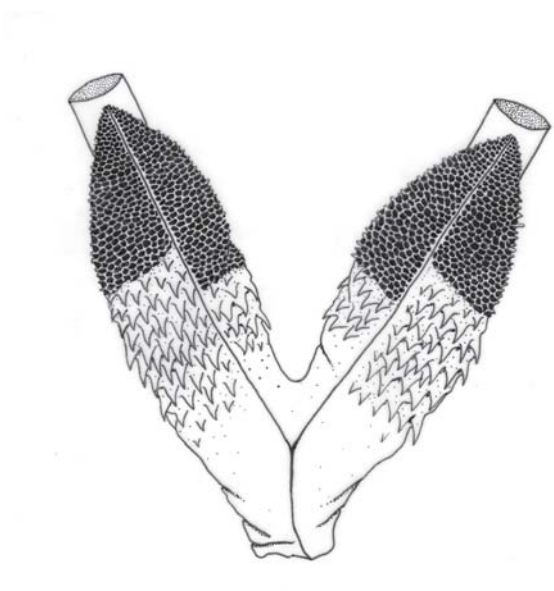

Fig. 2. The hemipenis of *Protobothrops kaulbacki* (BMNH1946.1.19.23).

Redescription of *Protobothrops kaulbacki*

Morphometric and meristic characters. The total length is 1343/1385 mm (for two males and two females respectively), with a tail length of 223/225 mm. The number of ventral scales is 205/204 (not including the anal scale, with the first ventral defined as the first undivided scale), and subcaudals are 74/70. Large internasals in contact with one other, or separated by one scale. Two enlarged scales in a line between the internasals and supraoculars, but they are fused on the right side in both specimens. Supraoculars large, entire, the minimum number of scales between them varies between 7 and 8 and there are 11 and 14–18 scales between their anterior and posterior edges. Eight supralabials, the first separated from the nasal, the second forming the anterior border of the pit cavity, and two small scales present between the second supralabial and nasal. The scales between the third supralabial and subocular vary between 1 and 3. One small scale between the fourth supralabial and subocular. Two or three postoculars. The number of scales around the supraocular varies between 9 and 12. Temporals smooth, rostral trapeziform, visible above the head. Sublabials 12–14, the first in contact with each other, and the first 2 or 3 in contact with the chinshield. Five to six scales between chinshield and the first ventral. There may be 25 or 27 scale rows at mid-body (i.e. at 50% of the ventral scale count), strongly keeled with a single keel only at midbody, except on the outer two or three rows, which are smooth.

ANOVA/ANCOVA between species showed that most characters were significantly different among *P. kaulbacki*, *P. jerdonii* and *T. sieversorum* (Table 2).

Fig. 3. The hemipenis of *Protobothrops mangshanensis* (outside lobe of right hemipenis).

While both *P. kaulbacki* and *T. sieversorum* are substantially larger than *P. jerdonii* in size, once this size difference is taken into account, the three species are not significantly different in tail length or head length, but are significantly different in head width, with *T. sieversorum* having the widest and *P. kaulbacki* having the narrowest head for a given body length. Significant differences in scalation included number of ventrals, subcaudals, mid-body dorsal scales, sublabials, internasals and intersupraoculars, as well as the number of scales between the third and fourth supralabials and the subocular scale (Table 2). The results of the PCA (not shown) were similar whether inclusion of characters or specimens was optimized, and showed no greater external resemblance between *P. kaulbacki* and *T. sieversorum* than between either of these and *P. jerdonii*.

Colour pattern. This description is based on macrophotographs of one female specimen captured alive in Medog Co., Xizang A.R., China (SYNU0400II30). The ground colour of the body is dull yellow, with a series of dark, diamond-shaped or angular vertebral spots, which cover no less than 20 scales in the mid-body in average. The spots are sometimes united to form a zig-zag band. On each side of the large mark, there are small and less distinct spots, corresponding in position with the vertebral spots. These small spots cover only about 4–5 scales. The labial scales are uniform yellow, marked or unmarked (the two type specimens examined are unmarked). Throat and anterior part of the ventral surface is mostly white, and this changes to dark with a yellow stain posteriorly. The tail is mostly dark. Top of the head black with a yellow longitudinal line, resembling an inverted "Y" shape, which extends from the tip of the snout and divides between the eyes, with the arms diverging and extending backwards to connect with the yellow postocular streak above the angle of the mouth (Fig. 1, Electronic Appendix 2).

Hemipenis (in situ, based on type specimen BMNH 1946.1.19.23). The retracted hemipenis extends to the fifteenth caudal plate, and is forked at the level of the fifth subcaudal. The distal half is calyculate, the proximal spinous. The demarcation between spinous and calyculate regions is distinct. The calyces approach closer to the fork on the sulcal side than on the asulcal side. Numerous spines, which are generally uniform in size, are present in the spinous area. Sulcus bifurcates at the level of the fourth subcaudal. Both the base of the organ and the fork are nude. *Musculus retractor penis magnus* bifurcates at the twentieth subcaudal (Fig. 2).

Distribution and natural history of P. kaulbacki. Little is known about the natural history of *Protobothrops kaulbacki* because of its rarity and secretive nature. Here, we give a brief summary of its distribution and natural history based on previous reports (Smith, 1940, 1943; Rao & Zhao, 2005).

Protobothrops kaulbacki is presently known from China (Medog Co., Xizang A.R.) and Myanmar (Pangnamdim, north of the Triangle) (Fig. 4). In China, this species was captured in open grassland within a forest (Fig. 2, Electronic Appendix 2) at an elevation of about 1000 m (Lu, pers. comm.). Taking into consideration its rarity and its appearance in Medog Co., China (in August), it is likely that this species is confined to forested parts of these localities, and may also have a limited activity period.

The species is oviparous and lays 6–32 eggs per clutch. The female has been noted to show egg guarding behaviour (Smith, 1943; Rao & Zhao, 2005).

Hemipenis of *Zhaoermia mangshanensis*

This is the first description of the hemipenis of this species, and is based on a preserved specimen at the Chengdu Zoo. The right retracted hemipenis extends to the thirteen (inside lobe) and fifteenth subcaudal (outside lobe), and is forked at the level of the fifth subcaudal. It is spinous proximally and calyculate distally; the two areas are about equal in extent. Numerous tiny spines are present in the spinous area; no enlarged spines were found. Semi-centrifugal sulcus bifurcates at the level of the fourth subcaudal and extends to the distal end of the lobe (Fig. 3). *Musculus retractor penis magnus* birfurcates at the twenty-first subcaudal.

DISCUSSION

Contrary to previous assumptions (Kraus et al., 1996; David & Ineich, 1999; Herrmann et al., 2004; Gumprecht et

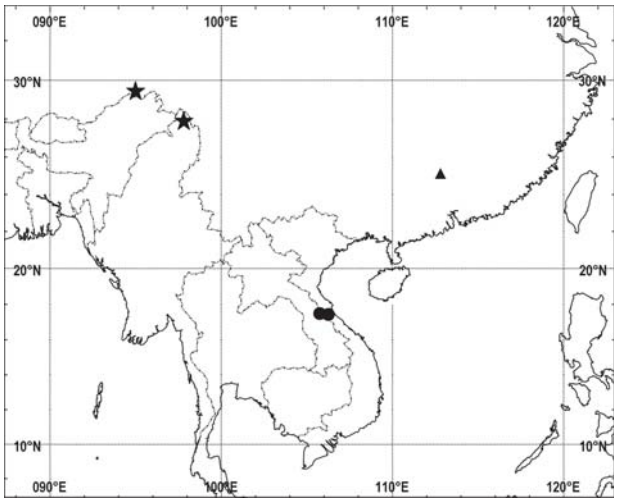

Fig. 4. Map of known distribution of *Protobothrops kaulbacki* (stars) in relation to the known range of its sister species *P. sieversorum* (circles), and *P. mangshanensis* (triangle).

al., 2004; Rao & Zhao, 2005), the results of the mtDNA molecular analyses in this study provide strong evidence that *Protobothrops kaulbacki* is more closely related to *Triceratolepidophis sieversorum* than to other *Protobothrops* species. Previous studies of nuclear genes of pitvipers have shown that mitochondrial phylogenies do appear to correspond to species trees (Creer et al., 2003, 2006). Therefore, in order to reflect these evolutionary relationships in the taxonomy of the group, *kaulbacki* must either be placed within *Triceratolepidophis*, or *Triceratolepidophis* and *Zhaoermia* must be synonymized with *Protobothrops*. The strong support for the monophyly of the clade comprising all three genera found in this study might be cited as evidence in favour of the latter view. However, if diagnosable characters can be found that warrant the retention of three genera within this clade, then this should not in itself preclude the recognition of distinct genera within a monophyletic group. Hence, we now discuss the morphological and ecological differences between species assigned to these genera (see below).

Hemipenis type has been shown to play an important role in the systematics of Asian pitvipers (Malhotra & Thorpe, 2000, 2004a; Guo & Zhang, 2001; Guo et al., 2006a; Hermann et al., 2004). *Triceratolepidophis sieversorum* shares a similar type of hemipenial morphology with all other species of *Protobothrops* (Ziegler et al., 2000; Malhotra & Thorpe, 2000, 2004a; Guo & Zhang, 2001; Herrmann et al., 2004; Guo et al., 2006a), i.e., deeply forked, calyculate distally and spinous proximally. The hemipenes of *P. kaulbacki* and *Zhaoermia mangshanensis* described here resemble that of *T. sieversorum* as well as all other *Protobothrops* species. Although both *Ovophis* and (to some extent) *Viridovipera* also exhibit a similar type of hemipenis (Guo & Zhang, 2001; Guo et al., 2006a), they can be easy discriminated from *Protobothrops*, *Zhaoermia* and *Triceratolepidophis* using skull and external characters (Guo & Zhao, 2006; Gumprecht et al., 2004; Guo et al., unpublished data).

Skull morphology has been well studied in most Asian pitvipers (Guo & Zhao, 2006; Guo et al., unpublished data.). A detailed comparison of the skulls, including a principal component analysis (PCA) (Guo et al., unpublished data), revealed that the skull of *T. sieversorum* is very phenetically similar to all other *Protobothrops* species studied, particularly to *P. elegans*, which is congruent with previous suggestions (Herrmann et al., 2002). Although *P. kaulbacki* was unavailable for skull examination in the present work, *T. sieversorum* does not exhibit any unique characters to distinguish this genus from *Protobothrops* and *Zhaoermia* (Guo & Zhao, 2006; Guo et al., unpublished data). The diagnosis of *Zhaoermia* (Gumprecht & Tillack, 2004) was based mainly on skull characters (Zhang, 1993, 1998). However, when the skulls of more species of *Protobothrops* were studied, Z. *mangshanensis* was shown to share many characters with *Protobothrops* species (Guo & Zhao, 2006; Guo et al., unpublished data). For example, the palatine teeth generally number less than three, the ectopterygoid anterior-lateral process is narrow, the shape of the parietal is triangular and no projection is present on the border of the pit cavity. These characters are synapomorphic for the clade comprising *Protobothrops* and *Zhaoermia*, as well as *P. kaulbacki* and *T. sieversorum*.

One of the main defining characteristics of the genus *Triceratolepidophis* is the unique micro-sculpturing of the body scales, which display a characteristic three-edged keel (Ziegler et al., 2000). While a detailed analysis of the microdermatoglyphic characteristics of *P. kaulbacki* is outside the scope of this paper, an examination of the scales of this species under light microscopy, which is said to be sufficient to distinguish this structure in *T. sieversorum* (Ziegler et al., 2000), failed to find any evidence that *P. kaulbacki* shares this characteristic (not shown). Thus, this character could not be a synapomorphy defining the genus *Triceratolepidophis* with the inclusion of *kaulbacki*.

Finally, several separate studies of the venoms of *Z. mangshanensis* and *T. sieversorum* additionally reported that, in many aspects, both resembled those of *Protobothrops* species (Mebs et al., 2004, 2006; Tsai et al., 2004).

Therefore, although *Zhaoermia mangshanensis* and *Triceratolepidophis sieversorum* both have several unique morphological characters (Zhang, 1993, 1998; Gumprecht & Tillack, 2004; Ziegler et al., 2000), taking all the above into consideration, *Zhaoermia*, *Triceratolepidophis* and *Protobothrops* apparently share many unique characteristics of the skull, hemipenis and venom. Hence, we suggest that *Zhaoermia* and *Triceratolepidophis* should be synonymized with *Protobothrops*, which would then contain ten species (*cornutus*, *elegans*, *flavoviridis*, *jerdonii*, *kaulbacki*, *mangshanensis*, *mucrosquamatus*, *sieversorum*, *tokarensis* and *xiangchengensis*). Further study of various characteristics of these species is highly desirable, and phylogenetic analysis of morphological data and nuclear genes are in progress.

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APPENDIX 1

Specimens examined in this study

Abbreviations: Natural History Museum, London (BMNH), Shenyang Normal University, China (SYNU), Museum für Naturkunde, Humboldt-Universität Berlin (ZMB).

Protobothrops kaulbacki (*n*=4; 2 males, 2 females). Myanmar: BMNH 1946. 1.19.23–24 (type specimens); Burma: BMNH1936.7.4.42; China: SYNU0400II30.

Protobothrops jerdonii (*n*=17; 8 males, 9 females). India: BMNH 1946.1.18.66, BMNH 1946.1.18.68 (type specimens), BMNH 1937.8.22; Burma: BMNH 1975.909–910; China: BMNH 1932.6.8.14, BMNH 1905.5.30.29–30, BMNH 1946.1.19.36–40; ZMB 28947, ZMB 28956, ZMB 28958, ZMB 48664.

Zhaoermia mangshanensis (1 male). China: uncatalogued (Chengdu Zoo, Sichuan).

ELECTRONIC APPENDIX 2

Online material available from the *Herpetological Journal*'s website at http://biology.bangor.ac.uk/~bss166/HJ/

Fig. 1. *Protobothrops kaulbacki* (Smith, 1940). A: dorsal lateral view, B: ventral view, C: dorsal lateral view of head, D: a clutch of eight eggs. Photographs by S. Lu.

Fig. 2. The habitat of *Protobothrops kaulbacki* at Ani Bridge, Medog Co., Xizang A.R., China. A: microhabit, B: macrohabit. Photographs by S. Lu.