Systematics of the *Protobothrops jerdonii* complex (Serpentes, Viperidae, Crotalinae) inferred from morphometric data and molecular phylogeny

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Although the systematics of *Protobothrops* is well resolved at the interspecific level, the intraspecific taxonomy and geographical boundaries of *P. jerdonii* are still controversial. In the present work, based on a combination of multivariate morphometric analysis and molecular phylogeny, the intra-specific relationships among lineages of *P. jerdonii*, as well as the relationship between *P. jerdonii* and *P. xiangchengensis*, were explored. Both parsimony and Bayesian inference showed that *P. xiangchengensis* was nested within *P. jerdonii*. Specimens of *P. jerdonii* fell into three distinct clades, but relationships among these three clades, and with *P. xiangchengensis*, were not well resolved. Morphometric trait analysis could not reveal distinct clusters among *P. jerdonii*, but *P. xiangchengensis* was shown to be significantly different from all *P. jerdonii* specimens. Colour pattern differences among *P. jerdonii* populations do not correspond to mitochondrial clades. Taking the results of the morphological comparison and molecular analysis into consideration, we affirm the validity of *P. xiangchengensis*, and suggest that *P. jerdonii* should be considered a monotypic species.

Key words: Asian pitvipers, morphometric trait analysis, *Protobothrops jerdonii*, *P. xiangchengensis*, taxonomy

INTRODUCTION

Pitvipers of the genus *Protobothrops* are among the most common venomous snakes in most of eastern and southern Asia (Zhao et al., 1998; David & Ineich, 1999; McDiarmid et al., 1999; Gumprecht et al., 2004). Considerable progress has been made in addressing the taxonomic problems with this genus in the last decade. Firstly, the validity of *Protobothrops* has been strengthened by morphological comparison and/or molecular data (Kraus et al., 1996; Parkinson et al., 2002; Malhotra & Thorpe, 2000, 2004a; Herrmann et al., 2004; Guo & Zhao, 2006; Guo et al., 2006; Creer et al., 2006; Castoe & Parkinson, 2006). Secondly, the phylogenetic relationships of several species have also been re-evaluated and confirmed (Herrmann et al., 2004; Guo et al., 2006). More recently, a molecular phylogenetic study based on four mtDNA fragments disclosed that *P. kaulbacki*, widely recognized as a member of *Protobothrops*, formed a clade with *Triceratolepidophis sieversorum*. When combined with morphological comparisons, this led to the synonomization of both *Triceratolepidophis* and *Zhaoermia* with the genus *Protobothrops* (Guo et al., 2007). Therefore, the genus *Protobothrops* currently contains ten species. However, all of the above work concerned higher-level relationships (interspecific or intergeneric), and so far research on this genus has neglected intraspecific taxonomy.

Protobothrops jerdonii Günther, 1875 is widely distributed in southwestern China, as well as northeastern India, Nepal, northern Myanmar and northern Vietnam (Zhao et al., 1998; David & Ineich, 1999; McDiarmid et al., 1999; Gumprecht et al., 2004; Fig.1). The high level of variation in scale counts, coloration and habitat has led to a controversial intraspecific taxonomic classification. Some authors (Pope, 1935; Smith, 1943; Tian et al., 1986; Zhao et al., 1998; David & Ineich, 1999; Zhao, 2006) consider it to be monotypic and do not recognize any subspecies. However, most authors suggest that *P. jerdonii* is a polytypic species (Mell, 1929; Maslin, 1942; Welch, 1988; Hoge & Romano-Hoge, 1983; Golay et al., 1993; Orlov et al., 2001; Gumprecht et al., 2004), but the subspecific division adopted varies, depending on particular authors. Over the past one hundred years of taxonomic effort, five species or subspecies have been nominated in relation to the *P. jerdonii* complex: *jerdonii* Günther, 1875; *xanthomelas* Günther, 1889; *melli* Vogt, 1922; *meridionalis* Bourret, 1935; and *bourreti* Klemmer, 1963. All of these proposals were based on conventional taxonomic techniques, using a small number of characters, with differences in the number of subcaudal and ventral scales being the main diagnostic criteria. Maslin (1942) recognized four subspecies: *jerdonii*, *melli*, *xanthomelas* and *meridionalis*, while Welch (1988) accepted an alternative taxonomy, including *jerdonii*, *bourreti*, *xanthomelas* and *meridionalis*. Because *meridionalis* (=*Trimeresurus jerdonii meridionalis*) is a primary homonym of *Trimeresurus monticola meridionalis* (Bourret, 1935), it was unavailable. Several authors, including Hoge & Romano-Hoge (1983), Golay et al. (1993), Orlov et al. (2001) and Gumprecht et al. (2004) have recognized only *jerdonii*, *bourreti* and *xanthomelas*. However, the geographic boundaries of these three proposed subspecies differ considerably among these authors.

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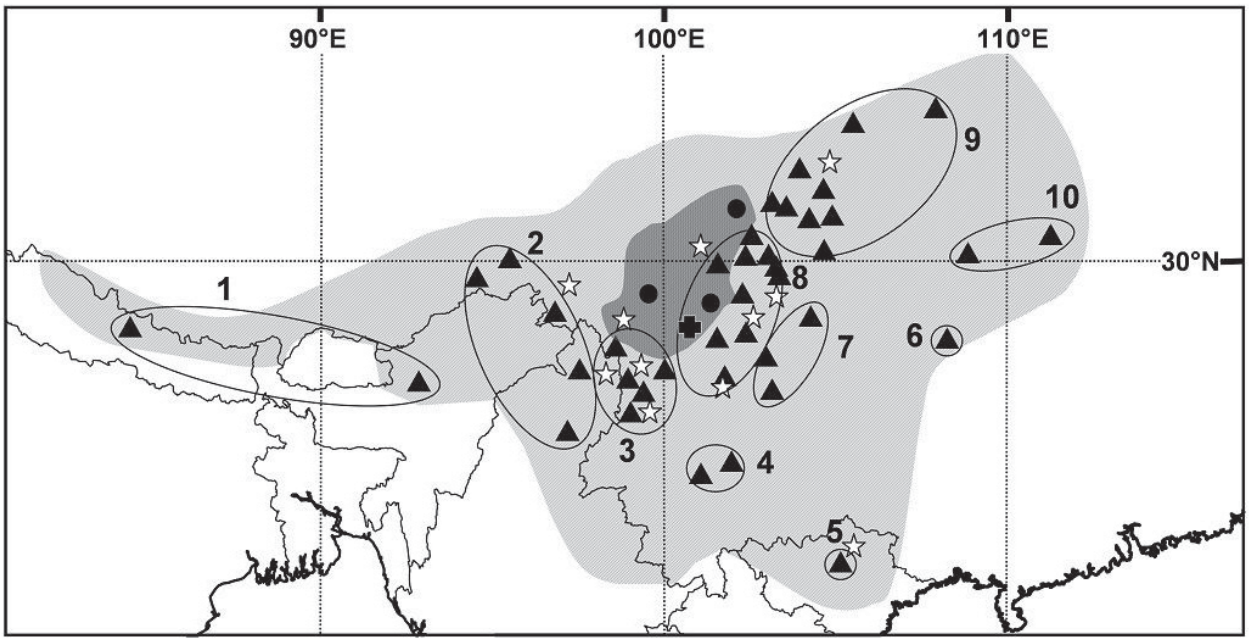

Fig. 1. The current distribution of *P. jerdonii* (hatching) and *P. xiangchengensis* (stipples) according to Zhao (2006). Stars and triangles represent DNA samples and morphological specimens of *P. jerdonii*, and circles and crosses indicate DNA samples and morphological specimens of *P. xiangchengensis*, respectively. Several specimens that are available from unknown localities in China are not represented on the map. The numbered composite localities correspond to those listed in Figure 2.

Protobothrops xiangchengensis Zhao, Jiang & Huang, 1977 is endemic to China and restricted to a small area over 3000 m in altitude in western Sichuan and northwestern Yunnan (Zhao, 2006; Fig.1). Recently, a study of the molecular phylogeny of the genus *Protobothrops* showed that *P. xiangchengensis* was nested within *P. jerdonii*, raising questions about the validity of *P. xiangchengensis* (Guo et al., 2006). Based primarily on morphological comparison, Guo et al. (2006) suggested that *P. xiangchengensis* should be considered a valid species. However, due to the limited sample size, Guo et al. (2006) did not make any recommendations about the systematics of *P. jerdonii*.

Species delimitation is fundamental in studying ecology, evolution, systematics and conservation biology. However, various methods are available to delimit species (de Queiroz, 1998; Puorto et al., 2001; Sites & Marshall, 2004; Strand & Sundberg, 2005). Sites & Marshall (2004) reviewed twelve of these methods that were organized into tree- and non-tree-based approaches. They suggested that researchers need to make qualitative judgements when using these methods, because all methods would sometimes fail to delimit species boundaries properly or give conflicting results. In this study, we conducted a combination of molecular phylogenetic inference and morphometric analysis with the largest set of specimens and sample localities for *P. jerdonii* and *P. xiangchengensis* to date. Our main aims were to explore the intraspecific taxonomy of *P. jerdonii*, and to test the validity of *P. xiangchengensis* further.

MATERIALS AND METHODS

Morphological methods

One hundred and sixty preserved specimens (20 *P. xiangchengensis* and 140 *P. jerdonii*) were obtained for examination from a number of museums in Europe, North America and China (Appendix 1). A large number of characters relating to scalation, colour pattern and body proportions were recorded in most specimens (Appendix 2). Measurements of body and tail lengths were taken with a ruler to the nearest 1 cm; the others were taken with a slide caliper to the nearest 0.1 mm. Symmetric mensural head characters were taken only on the right side unless they were unavailable (e.g. damaged); meristic characters were recorded on both sides and the average used in the analysis. Because some specimens had been preserved for a long time, and had a faded or faint colour pattern, body colour was described from either freshly preserved specimens or colour photos (Gumprecht et al., 2004; Zhao, 2006; personal photos); these data were excluded from the quantitative analysis, but used in a detailed qualitative comparison. All data were collected by PG to avoid inter-observer bias.

For analysis, specimens were grouped by locality (sample localities are illustrated in Fig. 1), and the geographical boundaries of *P. jerdonii* subspecies proposed previously were also taken into account. All characters were first checked for significant between-locality variation using one-way analysis of variance (ANOVA), and only characters showing significant between-locality

Table 1. Details of samples used in the molecular phylogenetic analysis. CAS: California Academy of Science, San Francisco; ROM: Royal Ontario Musuem; UMMZ: University of Michigan Museum of Zoology; ZFMK: Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn; CLP: CL Parkinson, field tag; FK: Fred Kraus, field tag; AM & GP: authors' catalogue number.

Species	Voucher nos.	Locality	GenBank accession no. (cyt *b*, ND4, 12S and 16S)
P. jerdonii	GP9	Anxian, Sichuan	AY763212, EU810015, AY763174, AY763193
	GP10	Anxian, Sichuan	AY763213, EU810016, AY763175, AY763194
	GP11	Huili, Sichuan	EU809995, EU810017, AY763176, AY763195
	GP12	Huili, Sichuan	EU809996, EU810018, AY763177, AY763196
	GP13	Huili, Sichuan	EU809990, EU810010, AY763178, AY763197
	GP14	Huili, Sichuan	EU809997, EU810019, EU809961, EU809978
	GP16	Gongshan, Yunnan	EU809998, EU810020, EU809962, EU809979
	GP86	Ebian, Sichuan	EU809991,EU810011, EU809957, EU809974
	GP87	Zayu, Xizang	EU809992, EU810012, EU809958, EU809975
	GP88	Dulongjiang, Yunnan	EU809993, EU810013, EU809959, EU809976
	GP147	Dulongjiang, Yunnan	EU809994, EU810014, EU809960, EU809977
	GP162	Puxiong, Sichuan	–, EU810001, EU809948, EU809965
	AM B194	China (trade)	EU809982, EU810002, EU809949, EU809966
	CAS 215051	Nujiang, Yunnan	AY294274, AY294264, AY294278, AY294269
	CAS 215114	Nujiang, Yunnan	EU809999, EU810021, EU809963, EU809980
	CAS 215115	Nujiang, Yunnan	EU810000, EU810022, EU809964, EU809981
	AM B485	China	AY294273, AY294263, AY294277, AY294268
	AM B575	China (trade)	EU809983, EU810003, EU809950, EU809967
	AM B582	Sapa, Vietnam	EU809984, EU810004, EU809951, EU809968
	AM B592	China (trade)	EU809985, EU810005, EU809952, EU809969
	AM B595	China (trade)	EU809986, EU810006, EU809953, EU809970
	AM B596	China (trade)	EU809987, EU810007, EU809954, EU809971
	AM B597	China (trade)	EU809988, EU810008, EU809955, EU809972
	AM B618	Sapa, Vietnam	EU809989, EU810009, EU809956, EU809973
P. xiangchengensis	GP26	Sichuan, China	DQ666061, DQ666058, AY763188, AY763207
	GP27	Sichuan, China	DQ666062, DQ666059, AY763189, AY763208
P. cornutus	ZFMK 75067	Central Vietnam	AY294272, AY294262, AY294276, AY294267
P. sieversorum	AM B162	Central Vietnam	AY352753, AY352816, AY352782, AY352721
P. mangshanensis	AM B300	Hunan, China	AY352758, AY352821, AY352787, AY352726
P. mucrosquamatus	AM A233	Taiwan, China	AF171897, AY294265, AY294279, AY294270
	AM B106	Vietnam	AY294275, AY294266, AY294280, AY294271
P. tokarensis	FK 1997	Japan	AY223576, AY223628, AF057202, AF057249
P. flavoviridis	UMMZ 199973	Japan	AY223574, U41894, AF057200, AF057247
P. elegans	UMMZ 199970	Japan	AY223575, U41893, AF057201, AF057248
P. kaulbacki	GP112/SYNU0400II30	Xizang, China	DQ666060, DQ666057, DQ666056, DQ666055
Ovophis monticola	AM B482	China	AY352748, AY352809, AY352775, AY352714
	ROM 7798	Vietnam	AY223572, AY223626, AY223652, AY223665
	AM A87	Taiwan	AF171907, AY059582, AY059545, AY059561
"O." okinavensis	CLP 162	Japan	AY223573, U41895, AF057199, AF057246
"Trimeresurus" gracilis	AM A86	Taiwan	AF171913, AY352823, AY352789, AY352728
Gloydius halys		Khazakstan	AY223564, AY223621, AF057191, AF057238
G. shedaoensis	ROM 20468	Liaoning, China	AY223566, AY223623, AF057194, AF057241

variation were selected for further analysis. Size-correlated characters were adjusted using the pooled within-group regression coefficient against snout–vent length (SVL) to remove size-related bias. Male and female specimens were treated separately because these taxa are reported to be sexually dimorphic (Malhotra & Thorpe, 2004b).

Because canonical variate analysis (CVA) has been shown to be sensitive to heteroscedasticity in the data, and principal component analysis (PCA) does not require a priori grouping of the specimens (Malhotra & Thorpe, 2004b), the multivariate analyses were performed in two steps. First, a principal component analysis (PCA) was performed on all *P. jerdonii* samples, then a separate PCA

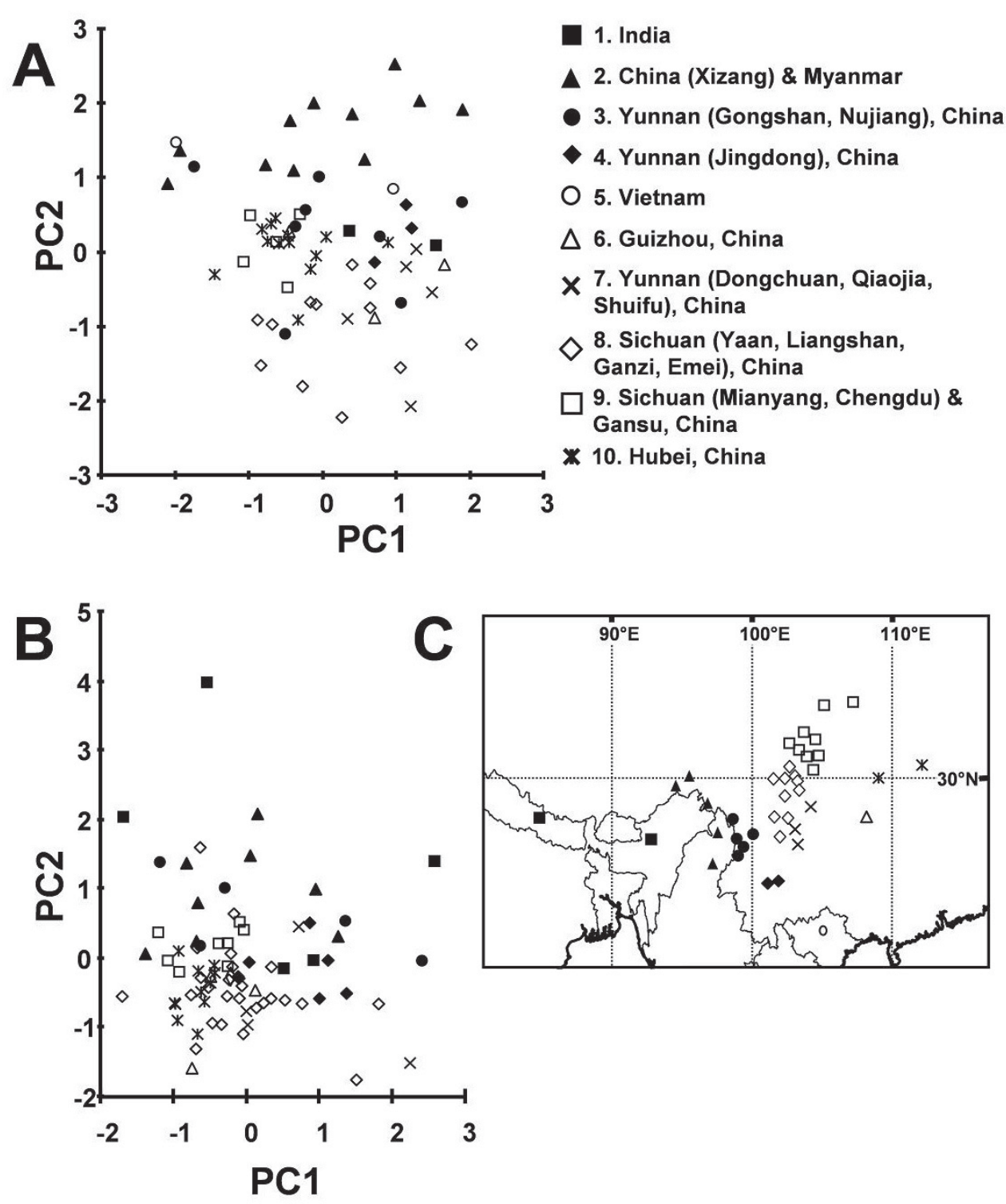

Fig. 2. Plots of the first two principal components for *P. jerdonii*. A) Males; B) females; C) map showing the geographic location of the symbols representing individuals on the PCA plots.

and a canonical variate analysis (CVA) were conducted for both *P. jerdonii* and *P. xiangchengensis* to test their morphological distinctness. Some characters that are highly correlated with each other among localities (r>0.7) may not provide independent information, and in PCA they may result in over-emphasis of the correlated variables (Thorpe, 1976); thus only one of the characters from the correlated character sets was used. However, in CVA these correlations are taken into consideration during the analysis and no variables were eliminated. All analyses were performed using SPSS 11.5 (SPSS Inc., Chicago).

Molecular methods

Sampling and sequencing. Liver or muscle tissues, preserved in 80% ethanol, were collected by ourselves, obtained from museum collections or donated from private collections (Table 1 and Fig. 1). Whole genomic DNA was extracted with Sigma GenElute Mammalian Ge-

nomic DNA Miniprep Kits. Four mitochondrial gene fragments were amplified. Cytochrome *b* (cyt *b*) sequences were obtained as described in Malhotra & Thorpe (2000), NADH dehydrogenase subunit 4 (ND4) sequences as in Arévalo et al. (1994), 12S rRNA as in Knight & Mindell (1993) and 16S rRNA as in Parkinson et al. (1997). PCR products were cleaned prior to sequencing using shrimp alkaline phosphatase (SAP) and Exonuclease I to dephosphorylate residual deoxynucleotides and degrade excess primers (Werle et al., 1994). Single stranded product was then sequenced using dye-labelled terminators (ABI PRISMTM BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit) and run on an ABI 3730XL automated sequencer.

Sequence analysis. Alignment of cyt*b* and ND4 was straightforward as there were no indels. Coding genes were translated into amino acid sequences using MEGA 3.0 (Kumar et al., 2004) to check for the unexpected occurrence of stop codons, which might indicate the inadvertent amplification of pseudogenes (Zhang & Hewitt, 1996).

Both parsimony and Bayesian Markov chain Monte Carlo (MCMC) approaches were used to reconstruct phylogenies. Maximum parsimony (MP) trees were obtained using PAUP* 4.0b10 (Swofford, 2003) from unweighted characters with a heuristic search using 1000 random sequence addition replicates and TBR (tree bisection–reconnection) branch swapping. Support values (BS) for clades were calculated from 1000 bootstrap pseudoreplicates (Felsenstein, 1985). For Bayesian analysis, the sequences were partitioned into 8 partitions (12S, 16S, cytb codon position 1, cytb pos 2, cytb pos 3, ND4 pos 1, ND4 pos 2 and ND4 pos 3). The best-fit model of evolution for each partition was inferred by Modeltest 3.7 (Huelsenbeck & Crandall, 1997; Posada & Crandall, 1998, 2001). The Bayesian MCMC approach was implemented in the program MrBayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Three runs were performed, each 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 5 million generations and sampled every 100 generations. Stationarity was confirmed in the program Tracer 1.2 by inspecting plots of ln(*L*) against generation (Rambaut & Drummond, 2003) and the first 50,000 generations were discarded as burn-in. Posterior probabilities (PP) for nodes were assembled from all post burn-in sampled trees.

Based on interspecific pitviper phylogenies (Malhotra & Thorpe, 2004a; Castoe & Parkinson, 2006; Creer et al., 2006), *Gloydius shedaoensis* and *G. halys* were chosen as outgroups. Some other Asian pit vipers related to the focal species were also included in the phylogenetic analysis, using sequences retrieved from Genbank (Table 1).

RESULTS

Morphometric trait analysis

Fifty-one characters, including one qualitative character, 31 meristic characters and 19 mensural characters, were recorded for most specimens. A one-way ANOVA revealed that several characters, including SUPLAB, SL3POC, SUPO2, DS20, DV6TO4, HROS and LINNAS, were not significantly different between localities. These characters were thus not used in subsequent analyses.

PCA results (Fig. 2) showed that *P. jerdonii* did not form distinct clusters in either sex, but a clearer pattern of geographical variation was present in males than in females. The first two components explained a cumulative variance of 33.2% in males (19.2% and 14.0% in PC1 and PC2 respectively) and 28.9% in females (15.0% and 13.9% in PC1 and PC2 respectively). In males, several characters (DNOSEY, LHEAD, W3SUPL, H3SUPL, DS30, DS40, DS50 and H4SUPL) make a relatively large contribution to component 1. BORSUPOC and WROSB have a relatively higher loading on component 2. In females, component 1 is dominated mainly by body dimensions such as LHEAD, DNOSEY, H3SUPL, W3SUPL, W4SUPL and WROSB, and component 2 is dominated by aspects of scale numbers and size (DS40, DS50, DS80, DS10, WSUPOC and LSUPOC). In males (Fig. 2A), the specimens from Xizang AR, China and Myanmar are clearly separated from the remaining samples on PC2, with the exception of two specimens from northwestern Yunnan, China, and one from Vietnam, which overlap with these specimens. The specimens from Yichang (Hubei), Anxian (Sichuan) and Qinling (Shaanxi) are in closer proximity than those from Yunnan, China (Fig. 2A). In the analysis of females, most samples from different localities overlapped each other and were not easy to separate (Fig. 2 B).

In the PCA (not shown) and CVA (Fig. 3) including both *P. xiangchengensis* and *P. jerdonii*, two distinct clusters corresponding to the species were detected for both sexes. The CVA successfully discriminated between *P. jerdonii* and *P. xiangchengensis* with high success for both males and females (Fig. 3).

In males, *P. xiangchengensis* is distinguished from *P. jerdonii* by several scalation characters. In *P. xiangchengensis*, the numbers of dorsal scale rows at several ventral scale positions (DS10, DS20, DS40, DS50, DS60, DS70, DS80) are higher than in *P. jerdonii*. The males of *P. xiangchengensis* also have more postocular scales (POSTOC), more supralabials (SUPLAB), more scales between the internasal and the second largest scale on the dorsal surface of the head (INTN2), more scales between the nasal and the second supralabial (INTNA2SUP) and more scales between the anterior edges of the supraoculars (SUPOCA) than males of *P. jerdonii* (Table 2).

Female *P. xiangchengensis* are distinguished from *P. jerdonii* in having more dorsal scale rows at several ventral scale positions (DS10, DS20, DS40, DS50, DS60, DS70). Female *P. xiangchengensis* also have more supralabials (SUPLAB), more sublabials (SUBLAB), more postocular scales (POSTOC), more scales between internasal and the second larger scale above head (INTN2) and more scales between the nasal and the second supralabial (INTNA2SUP) than female *P. jerdonii* (Table 2). Female *P. xiangchengensis* also have a longer head (LHEAD) and a relatively shorter tail (TAIL) than female *P. jerdonii* (Table 2).

Molecular phylogenetic analysis

No deletions, insertions or stop codons were found in the two protein-coding regions, indicating that paralogous nuclear insertions had not been amplified. A total of 2383 bp of sequence were obtained (806 bp of cyt *b*, 668 bp of ND4, 409 bp of 12S rRNA and 500 bp of 16S rRNA). Of these, 603 characters were parsimony-informative. Novel sequences were deposited in Genbank (accession numbers EU809948–EU810022; Table 1). The optimal model was selected under the ACI in Modeltest. GTR + I + Γ was for 12S, 16S; TrN+I+Γ, GTR + Γ and TVM + I + Γ were for the three codon position of cyt *b*; TVM + I+ Γ, TVM + I and TrN+I+Γ were for the three codon positions of ND4 respectively.

In the parsimony analysis, 30 equally parsimonious trees, of length 2109 steps, were found. The 50% majority-rule tree is mostly identical to the Bayesian inference (BI) tree (Fig. 4A,B). *Protobothrops xiangchengensis* and *P. jerdonii* together formed a very highly supported clade (100% PP, 99% BS). Four different well-supported clades (BS=69–100%, PP=98–100%) could be defined within this group (*P. jerdonii* clades A, B and C, and *P. xianchengensis*). Clade A includes only one specimen from Xizang AR, China, while clade B (supported by 100% PP and 91% BS) consists of all specimens studied from Sichuan and several samples with no detailed localities but that were identified as belonging to the subspecies *xanthomelas* by the collector. Two subclades could be detected within clade B, one comprising specimens from southwestern Sichuan, the other including specimens from northern Sichuan. Both subclades are highly supported (100% PP, 93–95% BS). Clade C includes specimens from Yunnan (China) and Vietnam. This clade received 100% PP, but only 69% BS. The most striking difference between two trees is in the position of clade A. The MP tree indicates a sister relationship between clade A and clade B, although the support value is not high (69% PP), while the BI tree did not. However, the relationships among clades A to C, and with *P. xiangchengensis*, are not strongly supported, and collapse into an unresolved polytomy if 95% PP is used as the cut-off for the inference of strong support (Fig. 4A, B).

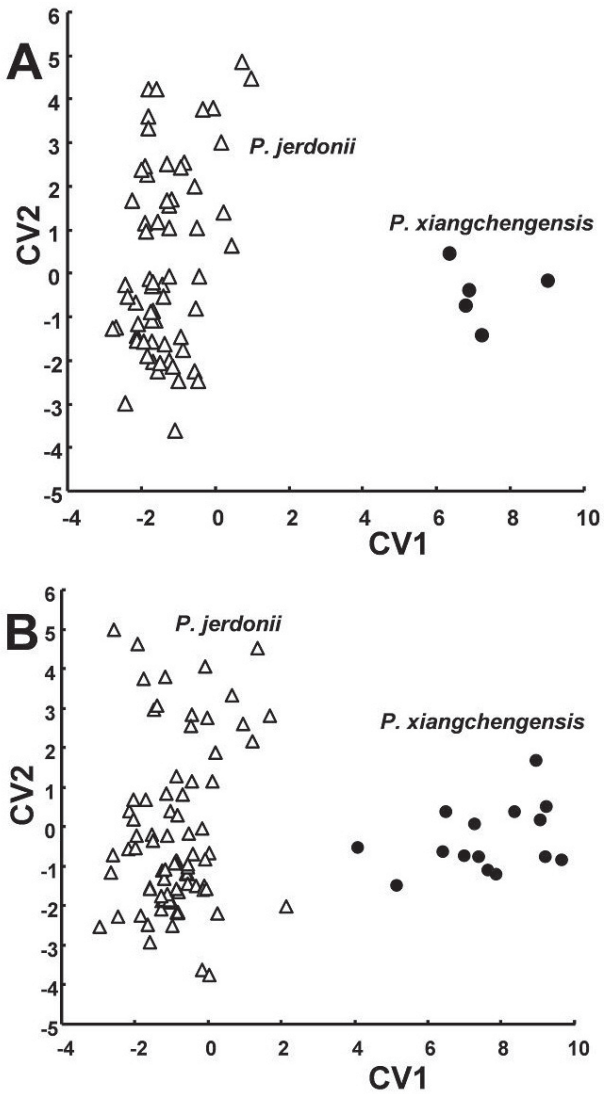

Fig. 3. Plot of first two canonical variates for *P. jerdonii* and *P. xiangchengensis* in A) males and B) females.

Body colour

There are three primary body colour modes in the *Protobothrops jerdonii* populations studied. The first consists of yellow, black and red. The yellow and black are basal body colours, and a series of transverse, rhomboidal or irregularly shaped reddish-brown dorsal patches with black margins are present (Fig. 1 in Electronic Appendix). The specimens from most of Sichuan, Hubei, Shaanxi, Hunan, Chongqing, Guizhou and Shanxi belong to this type. The second mode includes only black and yellow, with red being absent. The patches of the body are either black, or are mostly absent (Fig. 2 in Electronic Appendix). This mode is found in populations from China (southwestern Sichuan, Yunnan, Guangxi) and Vietnam. However, specimens collected from Zayu County, Xizang Autonomous Region (AR), China also have this type of body coloration, but differ slightly in having very faint yellow pigmentation (Fig.3 in Electronic Appendix). The last colour mode consists of black, white and red, but no yellow. The patches are red with a black margin (Fig. 4 in Electronic Appendix). Specimens from Linzhi, Xizang AR, China, Myanmar and India could be grouped into this mode. The correspondence of these colour modes with the three clades or three proposed subspecies is not perfect. Clade A displays both modes II and III, clade B displays both modes I and II, while clade C displays only mode II (Fig. 4).

DISCUSSION

The morphological method is one of the non-tree-based methods identified by Sites & Marshall (2004) for delimiting species. One application of this method is the use of the phenetic cluster criterion. This criterion distinguishes species by their allocation to separate clusters in multivariate analyses, which can summarize information on variation in a number of characters simultaneously and can thus reveal subtle patterns of variation in the general phenotype (Sokal & Sneath, 1963). This method has been

Table 2. Mean values, standard deviation and range (in parentheses) of morphological characters important in multivariate discrimination between *Protobothrops xiangchengensis* and *P. jerdonii.* Size-related characters are adjusted to the grand mean size of SVL (661.13 mm). Characters are listed in order of magnitude of their contribution to the discriminant function, and their abbreviations are explained in Appendix 1.

A) Males	*P. jerdonii* (*n*=63)	*P. xiangchengensis* (*n*=5)
DS40	21.21±0.70 (19.00–23.00)	24.80±0.45 (24.00–25.00)
DS50	21.17±0.75 (19.00–23.00)	24.40±0.89 (23.00–25.00)
POSTOC	2.01±0.33 (0.50–3.00)	3.10±0.55 (2.50–4.00)
DS20	21.24±0.61 (21.00–23.00)	23.00±0.00 (23.00–23.00)
SUPOCM	7.45±1.39 (4.00–11.00)	8.80±1.01 (7.00–11.00)
DS80	16.95±0.40 (15.00–18.00)	17.80±0.84 (17.00–19.00)
INTN2	0.631±0.47 (0.00–1.50)	1.53±0.34 (1.00–2.00)
DS10	22.61±1.17 (21.00–25.00)	24.80±0.45 (24.00–25.00)
SUPOCA	11.48±1.85 (7.00–16.00)	13.00±1.07 (11.00–15.00)
SUPLAB	7.17±0.36 (6.00–8.00)	7.80±0.45 (7.0–8.00)
DS70	17.59±.91 (17.00–20.00)	19.00±0.00 (19.00–19.00)
DS60	20.28±1.00 (17.00–22.00)	21.80±0.84 (21.00–23.00)
INTNA2SUP	1.71±0.80 (0–3.50)	2.80±0.45 (2.00–3.00)
B) Females	*P. jerdonii* (*n*=75)	*P. xiangchengensis* (*n*=15)
DS40	21.17±0.60 (21.00–24.00)	24.20±0.94 (23.00–25.00)
DS50	21.09±0.55 (19.00–23.00)	23.93±1.03 (22.00–25.00
SUPLAB	7.28±0.40 (7.00–8.50)	8.67±0.70 (8.00–10.50)
DS20	21.16±0.57 (21.00–24.00)	22.93±0.80 (21.00–25.00)
POSTOC	2.18±0.42 (1.00–3.50)	3.17±0.36 (3.00–4.00)
INTN2	0.63±0.47 (0.00–1.50)	1.53±0.40 (1.00–2.00)
INTNA2SUP	1.76±0.78 (0.00–3.00)	3.23±0.68 (2.00–4.50)
DS70	17.53±0.90 (16.00–21.00)	19.00±1.00 (17.00–21.00)
SUBLAB	10.85±0.87 (9.00–12.50)	12.17±0.59 (11.00–13.00)
DS10	22.61±1.17 (21.00–25.00)	24.80±0.45 (24.00–25.00)
DS60	20.28±1.00 (17.00–22.00)	21.80±0.84 (21.00–23.00)
LHEAD	29.81±3.92 (22.34–42.61)	34.48±1.63 (32.54–37.77)
TAIL	129.79±14.07 (89.43–159.30)	113.36±6.31 (105.25–125.17)

used previously in snake systematics, including for pitvipers (e.g. Malhotra & Thorpe, 2004b). However, due to the extreme morphological conservativeness displayed by some pitvipers, not all previous work has been able to clearly define distinct phenetic clusters for all the samples studied (e.g. Wüster et al., 1996).

Reciprocal monophyly is a widely-used tree-based criterion in delimiting taxa (Sites & Marshall, 2004). However, there are two difficulties associated with this criterion (Fu & Zeng, 2008; references therein): firstly, the actual level at which species should be recognized is not clear, because monophyly exists at many levels in a phylogeny. Secondly, this criterion is not applicable in the case of ancestral species that retain plesiomorphic characters (Funk & Omland, 2003). This is likely to occur when peripheral populations speciate from widespread ancestor species (Talbot & Shields, 1996; Fu & Zeng, 2008). In recent years, increasing recognition of paraphyletic species poses a strong challenge to the monophyly criterion (Funk & Omland, 2003; Fu & Zeng, 2008).

The results of the multivariate analysis presented here clearly show that *P. xiangchengensis* is significantly different from *P. jerdonii* in several scale count characters and in body dimensions; the two species could be separated by several non-overlapping or discrete characters (Table 2). These characters include DS20, DS40, DS50, DS80, SUBLAB and POSTOC in both sexes. In addition, although *P. xiangchengensis* is very closely related to *P. jerdonii* in a molecular phylogeny, it formed a distinct clade. Thus we conclude that on several of the above criteria, *P. xiangchengensis* should be considered a valid species. The low genetic distance between *P. xiangchengensis* and *P. jerdonii* (the lowest distance is about 0.025 between clade C and *P. xiangchengensis*) and their lack of reciprocal monophyly could be explained by the relatively recent divergence of *P. xiangchengensis* from *P. jerdonii* by "budding speciation" (Funk & Omland, 2003; Fu & Zeng, 2008; references therein). However, the specialized habitat experienced by *P. xiangchengensis* (higher altitudes, colder weather and stronger seasonal and diurnal variation in temperature) has probably led to rapid and relatively large morphological divergence between the two species.

For *Protobothrops jerdonii*, the results presented by morphometric trait analysis show a lack of strong morphological or geographic structure. Most specimens from different localities overlapped in multivariate phenetic

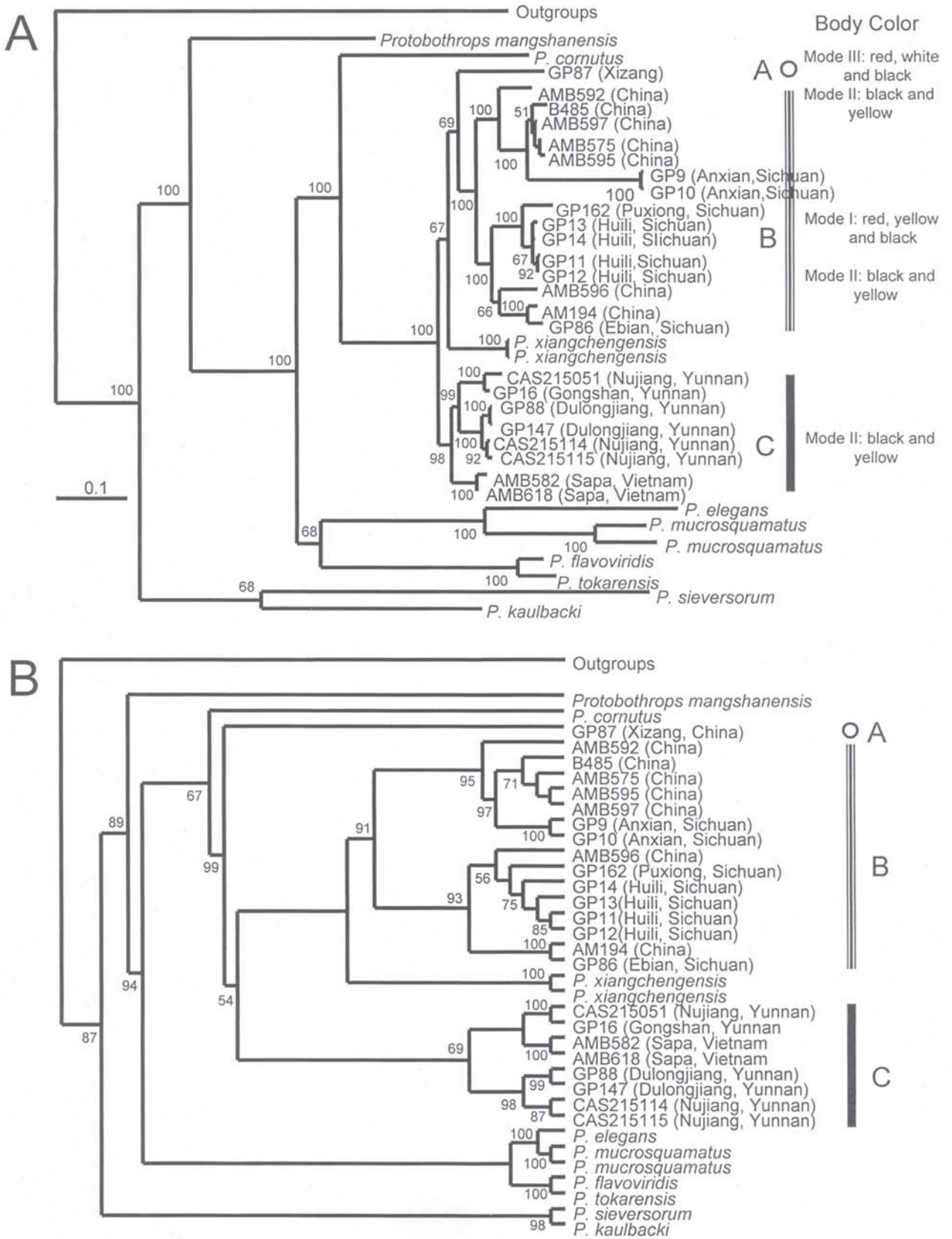

Fig. 4. A) 50% majority rule BI phylogram and B) 50% majority rule consensus tree of 30 equally parsimonious trees for *Protobothrops jerdonii* and *P. xiangchengensis*. Posterior probabilities (PP) and bootstrap support (BS) from the parsimony analysis for the clades are shown adjacent to the node to which they refer. A node with no associated values has both BS and PP <50%. For a description of the colour pattern modes, see text.

space and could not be separated clearly. Body colour did not correspond with clades identified in the DNA phylogeny of this species. Recognition of subspecies based predominantly on body colour has repeatedly been shown to be misguided in snakes (Burbrink et al., 2000; Gardner & Mendelson, 2004; Manier, 2004, Allsteadt et al., 2006) and other reptiles (Malhotra & Thorpe, 1991;Thorpe & Stenson, 2003). In addition, although three distinct clades were detected in the molecular phylogenetic tree (Fig. 4), only a few fixed nucleotide differences and low genetic distances (0.028–0.035) exist between these clades. Therefore, taking the molecular and morphometric results together, we recommend that *P. jerdonii* should be recognized as a monotypic and paraphyletic species.

Although the DNA samples did not cover the entire range of the species in our present work, and some conclusions may need to be revised once samples are obtained from populations from the extreme west of the range, this study represents a significant step forward in the exploration of the systematics of *P. jerdonii* and its relationships with *P. xiangchengensis*.

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APPENDIX 1

Specimens examined

Museum abbreviations: CAS: California Academy of Science, San Francisco; FMNH: Field Museum of Natural History, Chicago; USNM: Smithsonian Institution, National Museum of Natural History, Washington; BMNH: Natural History Museum, London; NMW: Natural Museum Wien, Austria; ZMB: Museum fur Naturkunde, Humboldt-Universität, Berlin; KIZ: Kunming Institute of Zoology, the Chinese Academy of Sciences; CIB: Chengdu Institute of Biology, the Chinese Academy of Sciences, AFS: Field references.

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P. xiangchengensis (20). Jiulong, Sichuan: CIB 13924, 13926–30, 13932–35, 13939, 13942–44, 13946–49; Xiaojing, Sichuan: CIB 72442; Xiangcheng, Sichuan: CIB 72443.

APPENDIX 2

Quantitative traits recorded, and their abbreviations

A) Scalation (18)

All bilateral meristic characters are an average of the left and right sides.

SUPLAB: the average number of supralabials on the left and right hand side.

SUBLAB: the average number of sublabials on the left and right hand side.

POSTOC: number of postocular scales.

BORSUPOC: the number of scales bordering the supraocular scales (average of right and left), not counting pre- and post-oculars.

SUPOCA: the minimum number of scales between the anterior edge of the supraoculars.

SUPOCM: the minimum number of scales between the middle of the supraoculars.

SUPOCP: the number of scales between the posterior edge of the supraoculars.

INTNAS: the number of scales separating the internasal scales.

INTNA2SUP: the number of scales between the nasal and the second supralabial.

VS: the number of ventral scales.

SC: the number of subcaudal scales.

INTERA: internasal touching rostral (1) or not (0).

TOUCH: the number of sublabials touching the enlarged chinshields (average of right and left).

SL4SUBO: the number of scales between the fourth supralabial and subocular.

SL3POC: the number of scales between the third supralabial and third pre-ocular.

VCH1: the minimum number of scales between the first ventral and enlarged chin shields.

INTN2: the number of scales between the internasal and the second largest scale on the dorsal surface of the head.

SUPO2: the number of scales between the second largest scale on the dorsal surface of the head and the supraocular.

B) Scale reduction formula (14)

DS10: number of dorsal scale rows in the position corresponding to 10 percent of ventral scales.

DS20, DS30, DS40, DS50, DS60, DS70, DS80, DS90, DS100: as above.

SC8TO6: subcaudal scale position of the reduction from 8 to 6 scale rows.

DV8TO6: dorsoventral position of reduction from 8 to 6 scale rows.

SC6TO4: subcaudal scale position of the reduction from 6 to 4 scale rows.

DV6TO4: dorsoventral position of reduction from 6 to 4 scale rows.

C) Body dimensions (19)

All measurements were made on the right side of the head only unless this was damaged, in which case they were done on the left.

SVL: distance between the tip of the snout and the cloaca.

TAIL: distance between the cloaca and the tip of the tail.

WHEAD: width of the head measured between the outer edges of the supraoculars.

LHEAD: length of the head measured between the tip of the snout and the posterior edge of the lower jawbone.

DEYE: maximal diameter of the eye measured between the edges of the scales surrounding it.

DNOSEYE: distance between posterior edge of nostril and anterior edge of eye.

DNOSPIT: distance between posterior edge of nostril and anterior edge of pit.

WSUPOC: maximal width of the supraoculars measured in mm.

LSUPOC: maximal length of the supraoculars measured in mm.

H3SUPL: height of third supralabial.

W3SUPL: width of third supralabial.

H4SUPL: height of fourth supralabial.

W4SUPL: width of fourth supralabial.

WROST: width of the top edge of the rostral.

WROSB: width of the bottom edge of the rostral.

HROS: height of the rostral.

WMENT: width of the mental.

HMENT: height of the mental.

LINNAS: length of the internasal.

ELECTRONIC APPENDIX 1

Fig. 1. *Protobothrops jerdonii* from Hunan Province, China, showing body colour consisting of yellow, black and red (corresponding to Mode I in text).

Fig. 2. *Protobothrops jerdonii* from Huili Co., Sichuan Province, China, showing body colour consisting of yellow and black (corresponding to Mode II in text).

Fig. 3. *Protobothrops jerdonii* from Linzhi Co., Xizang AR, China, showing body colour consisting of faint yellow and black (also corresponding to Mode II in text).

Fig. 4. *Protobothrops jerdonii* from Zayu Co., Xizang AR, China, showing body colour consisting of black, white and red (corresponding to Mode III in text).