Journal of Biogeography (J. Biogeogr.) (2011) 38, 2326–2336

ORIGINAL ARTICLE

Molecular phylogeography of Jerdon's pitviper (*Protobothrops jerdonii*): importance of the uplift of the Tibetan plateau

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ABSTRACT

Aim To investigate the population history and demographics of Jerdon's pitviper, *Protobothrops jerdonii*, and elucidate how the unique physical conditions and heterogeneous mountain environments resulting from the uplift of the Tibetan Plateau shaped the genetic diversity and evolutionary history of the species.

Location China and Vietnam.

Methods We sequenced and analysed a total of 1752 base pairs from two mitochondrial genes, cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit (ND4), for 81 specimens sampled from 27 localities across the species' range, and a total of 464 base pairs from two nuclear genes for 28 representative samples from all mitochondrial DNA lineages. Based on these data, we constructed the genealogical relationships and estimated the divergence times of the mitochondrial DNA clades.

Results The mitochondrial DNA results revealed the existence of five distinct, strongly supported and geographically structured DNA lineages within populations of *P. jerdonii* that are paraphyletic with respect to *Protobothrops xiangchengensis*. Estimation of divergence dates suggested that *P. jerdonii* possibly evolved in the western Hengduan Mountains region *c.* 6.6 Ma in the late Miocene. Nuclear DNA data did not provide sufficient resolution to distinguish the mitochondrial DNA lineages.

Main conclusions Based on the present-day distribution and intraspecific genealogy, the evolutionary history of *P. jerdonii* can be explained by a pattern of dispersal followed by vicariance. All lines of evidence suggest that historical biogeographical factors, particularly the north–south orientation of the higher mountains, as well as low-elevation areas in western China, had the greatest influence on the population structure, lineage formation and species distribution of this snake. However, highly heterogeneous habitats and glacial cycles appear to have affected patterns of intraspecific differentiation. While our mitochondrial data provide evidence for clear phylogeographical structure, our small sampling of nuclear genes does not, suggesting that nuclear markers may not have had sufficient time to coalesce to match patterns observed in the mitochondrial data.

Keywords
Asia, China, dispersal, high-elevation biogeography, highland fauna, montane distribution, phylogeography, Tibetan uplift, venomous snake, vicariance.

http://wileyonlinelibrary.com/journal/jbi
doi:10.1111/j.1365-2699.2011.02566.x

INTRODUCTION

Despite the explosive development of phylogeography as a field utilizing DNA-based molecular technology and robust statistical analytical methods to study the geographical distribution of genealogical lineages, especially within and among closely related species (Avise, 2000, 2009; Beheregaray, 2008; Hickerson *et al.*, 2010), the number of phylogeographical studies is not evenly distributed among global regions and taxonomic groups (Beheregaray, 2008). Among vertebrates, snakes have attracted the interest of few phylogeographers, and fewer than 3% of all described snakes have been examined phylogeographically (Burbrink & Castoe, 2009). However, reptiles in general may be particularly suitable animals for studying phenomena of interest to phylogeographers, as they have lower dispersal ability and higher sensitivity to climatic fluctuations than more frequently studied endotherms (Bauer, 1989; Camargo *et al.*, 2010). In Asia, the study of reptiles (particularly snakes) is notably deficient relative to that of other organisms. This situation is exacerbated by the difficulty of sampling snakes in sufficient numbers for population-level analysis. Thus, there are only a few examples of phylogeographical studies on snakes in Asia (Karns *et al.*, 2000; Creer *et al.*, 2001; Alfaro *et al.*, 2004; Huang *et al.*, 2007).

Western China, which is characterized by the presence of many high-elevation mountains and plateaus (such as the Hengduan Mountains, Western Sichuan Plateau, Yunnan-Guizhou Plateau), exhibits unique physical conditions and abundant biodiversity. It attracts many biologists studying biogeography, biodiversity and evolution (Qu *et al.*, 2005, 2006; Zhang *et al.*, 2005; Peng *et al.*, 2006; Zhang & Jiang, 2006; Meng *et al.*, 2007; Qu & Lei, 2009). Many studies have demonstrated that the uplift of the Tibetan plateau has resulted in a unique geological history, a distinctive geomorphological configuration and diverse climates. These factors have played a vital role in shaping the present-day distribution, evolutionary history, population structure and diversity of the plants and animals native to this region (Qu *et al.*, 2005, 2006; Zhang *et al.*, 2005; Peng *et al.*, 2006; Zhang & Jiang, 2006; Meng *et al.*, 2007; Qu & Lei, 2009). An increasing number of endemic taxa have been studied with respect to their biogeography, population genetics and demography, particularly birds (e.g. Qu *et al.*, 2005, 2006; Qu & Lei, 2009) and fish (e.g. Peng *et al.*, 2006). In contrast, no study has yet been published on the phylogeographical structure of snakes inhabiting this region. Because of its montane distribution, Jerdon's pitviper, *Protobothrops jerdonii* (Günther, 1875), is an excellent model species with which to investigate how the complex eco-environment and unique geological history of this region drive species evolution and shape population structure in this area.

Protobothrops jerdonii is one of the most widespread and abundant pitvipers in Asia, occurring predominantly in China as well as in adjoining countries, including Vietnam, India, Myanmar and Nepal (Zhao *et al.*, 1998; David & Ineich, 1999; Gumprecht *et al.*, 2004) (Fig. 1). In China, it is distributed mainly from south-eastern Xizang A.R. (Linzhi) in the west to western Shanxi Province in the east, along the Hengduan Mountains, Western Sichuan Plateau and Yunnan-Guizhou Plateau. It is a high-elevation snake and is normally found at around 2000 m a.s.l. (Liu, 1940; Zhao *et al.*, 1998; Orlov *et al.*, 2001). The taxonomy of *P. jerdonii* was investigated recently using a combination of quantitative phenetics and molecular phylogeny (Guo *et al.*, 2009), and it was concluded that *P. jerdonii* should be considered a monotypic species, albeit not a monophyletic one because *Protobothrops xiangchengensis*, which appears to be a valid species, is nested within it (discussed in detail in Guo *et al.*, 2009).

We sequenced DNA sequences from two fast-evolving mitochondrial genes [cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 4 (ND4)] and two nuclear genes (anonymous locus A and locus 51 of Gibbs & Diaz, 2010) from the entire distribution of *P. jerdonii*. Our main goals were: (1) to examine the genetic diversity and population demography

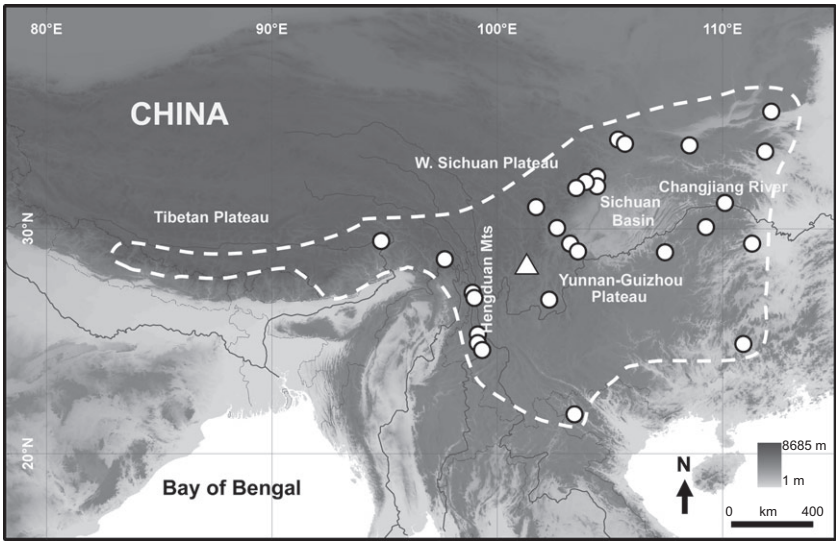

Figure 1 Topographic map of China and adjoining countries showing the distribution (dashed outline) and 27 sampling localities (circles) for *Protobothrops jerdonii.* The location of *P. xiangchengensis* samples is indicated by a triangle. Several samples included in this study but lacking detailed localities are not shown.

of *P. jerdonii*, and (2) to explore the evolutionary history and colonization routes of *P. jerdonii*.

MATERIALS AND METHODS

Samples, DNA extraction and sequencing

Eighty-one individuals of *P. jerdonii* (including several sequences retrieved from GenBank), covering most of its range, were included in the analysis. Sample localities are shown in Fig. 1, and detailed localities are given in Appendix S1 in the Supporting Information.

Genomic DNA was extracted from 85% ethanol-preserved tissues or from buffer-preserved blood using standard proteinase K and phenol-chloroform protocols (Sambrook & Russell, 2002). The entire cytochrome *b* (cyt *b*) gene and a partial NADH 4 (ND4) fragment were amplified by polymerase chain reaction (PCR) using primers L14910/H16064 (Burbrink *et al.*, 2000) and ND4/Leu (Arévalo *et al.*, 1994), respectively, using the cycling parameters given in those papers. We also amplified and sequenced two nuclear gene fragments, which were described as locus 'A' and locus '51' in Gibbs & Diaz (2010) (described hereafter as GA and G51 for convenience). Amplification and sequencing of these two fragments were performed, according to conditions described in Gibbs & Diaz (2010), for 28 specimens of *P. jerdonii*, representing all known mitochondrial DNA (mtDNA) groups. Three samples of *P. xiangchengensis* were also included. PCR products were purified using commercial kits, and double-stranded product was sequenced using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocols.

Phylogenetic reconstruction

All mtDNA sequences were aligned using the program MEGA 4.0, with default parameters (Tamura *et al.*, 2007; Kumar *et al.*, 2008). After performing data quality checks for the absence of pseudo-genes, consistency of base frequencies and partition homogeneity, we reconstructed intraspecific phylogenetic relationships using Bayesian inference (BI) and maximum-likelihood (ML) analyses. For Bayesian analysis, the sequences were partitioned by gene and codon position. The simplest best-fit model of evolution for each partition (Posada & Crandall, 1998; Posada & Buckley, 2004) was inferred using MrModeltest 2.2 (Nylander, 2004). BI analysis was performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), with three runs and four Markov chains (three heated chains and a single cold chain) using the models selected under the Akaike information criterion (AIC), and starting from a random tree. Each run was conducted with a total of 2×10^7 generations and sampled every 1000 generations; we discarded the first 5×10^6 generations as burn-in (evaluated using Tracer 1.4; Rambaut & Drummond, 2007). Trees from each run were combined to calculate posterior probabilities (PPs) for each bipartition in a 50% majority-rule consensus tree. The ML analysis was conducted with RAxML 7.2.3 using a fully partitioned model (Stamatakis *et al.*, 2008). The robustness of the trees was assessed by bootstrap resampling with 1000 random replicates. Based on previous molecular studies of Asian pitvipers, we also included three specimens of *P. xiangchengensis* within the ingroup, while *Protobothrops mangshanensis*, *P. mucrosquamatus* and *P. cornutus* were chosen as outgroups (Malhotra & Thorpe, 2004; Guo *et al.*, 2009; for details see Appendix S1). Finally, pairwise distances (*p*-distances ± SE, 1000 replicates) within and among mtDNA clades were calculated in MEGA 4.0 (Tamura *et al.*, 2007; Kumar *et al.*, 2008).

Because the nuclear sequences contained low levels of variation, and were only available from a subset of specimens, we analysed them primarily using a median-joining network (MJN) approach (Bandelt *et al.*, 1999) to depict their relationships. The MJN was estimated using NETWORK 4.1.0.7 (Bandelt *et al.*, 1999; http://www.fluxus-engineering.com), with the parameter epsilon set to 0. However, we also conducted a combined analysis, together with the mitochondrial data from the same specimens, with the nuclear data as two separate partitions, following the same procedure as described above for the mtDNA data.

Genetic diversity and population structure analysis

The number of haplotypes (*H*), haplotype diversity (*Hd*), nucleotide diversity (π) and the mean number of pairwise differences (*K*) were computed for mtDNA. The null hypothesis of neutral evolution of the mitochondrial protein-coding fragments was assessed using Fu and Li's D^* (Fu & Li, 1993) and Tajima's test (Tajima, 1989) using the program DnaSP 5.10 (Librado & Rozas, 2009). To test and assess geographical divisions and population genetic structure, analysis of molecular variance (AMOVA) of mtDNA (Excoffier *et al.*, 1992) was conducted in Arlequin 3.1.1 (Excoffier *et al.*, 2005). The variation among groups is described by F_{CT}, while F_{SC} describes the variation within groups, and F_{ST} refers to the variation within populations. The arrangement with the highest value of F_{CT} was inferred as being the most probable geographical subdivision. The significance of these *F*-statistics was determined by 1000 permutation replicates.

Divergence date estimations

The program BEAST 1.5.1 (Drummond & Rambaut, 2007) was used to estimate the date of origin of all mtDNA lineages of *P. jerdonii* under a relaxed molecular clock assumption using BI (Drummond *et al.*, 2006). Three calibrations for the tree were obtained for various groups of Serpentes: (1) the initial divergence of three South American populations of the genus *Porthidium* was 3.5 Ma (95% confidence interval, CI = 2.5–4.5 Ma) based on the uplift of the Isthmus of Panama (Wüster *et al.*, 2002); (2) the divergence between *Crotalus* and *Sistrurus* occurred before 9 Ma (Parmley & Holman, 2007; reference in Wüster *et al.*, 2008), with a lognormal prior with a standard

deviation of 1; and (3) the divergence between the sister taxa *Lampropeltis getula* and *L. extenuatum* occurred before 6.8 Ma (Prior Credible Interval, PCI = 4.75–9.94 Ma) (Holman, 2000; reference in Pyron & Burbrink, 2009). For this analysis, several sequences were retrieved from GenBank: *Porthidium lansbergii* (AF292575 + AF292613), *P. ophryomegas* (AY223580 + U41888), *Crotalus tigris* (AY223606 + AF156574), *Sistrurus catenatus* (AY223610 + AY223648), *L. getula* (AF337115 + AY739629), *L. extenuatum* (AF138776 + DQ902131). Two additional outgroups were also used in this analysis: *Boa constrictor* (AB177354) and *Acrochordus granulatus* (AB177879).

We applied the model identified by MrModeltest 2.2 (Nylander, 2004) across all genes and codon positions, and an uncorrelated lognormal tree prior with a constant population size (Drummond *et al.*, 2006). Two independent searches of 1×10^8 generations, sampling every 10,000th iteration, with 20% of the initial samples discarded as burn-in, were completed in beast 1.5.1 (Drummond & Rambaut, 2007). We compared Bayes factors in Tracer 1.3 (Rambaut & Drummond, 2007) to determine whether runs had converged on similar values.

RESULTS

Sequence information

A total of 1085 base pairs of sequence from 87 samples were aligned for cyt *b* (including 20 sequences retrieved from GenBank, which are only about 700 base pairs in length). Of these, 296 base pairs were variable (27.3% including outgroups) and 186 base pairs were parsimony-informative characters (17.1% including outgroups). The ND4 dataset consisted of 667 base pairs and included 27 sequences downloaded from GenBank. Of these, 162 base pairs were variable (24.3% including outgroups) and 96 base pairs were parsimony-informative characters (14.4% including outgroups). No indels, frameshifts or nonsense codons were found in the two fragments, indicating that we probably did not sequence nuclear pseudo-genes (Zhang & Hewitt, 1996).

The two nuclear gene fragments gave sequences from a total of 31 specimens, including two samples of *Protobothrops xiangchengensis* and one of *P. mangshanensis*. Of these, 24 sequences were obtained for GA (221 bp, 7 variable positions) and 27 for G51 (261 bp, 11 variable sites).

Phylogenetic analyses

For the BI analysis, MrModeltest 2.2 identified the following models for the data partitions: GTR + I + Γ for cyt *b* codon position 1 and position 3; HKY + I for cyt *b* codon position 2, and ND4 codon position 1 and position 2; GTR + Γ for ND4 codon position 3; and GTR + I + Γ for the combined dataset. The mtDNA phylogenetic trees from the two different methods used yielded very similar tree topologies, with five distinct and well-supported clades within *P. jerdonii* (Fig. 2). As in previous analyses (Guo *et al.*, 2009), BI and ML analysis strongly supported the inclusion of a monophyletic *P. xiangchengensis* [posterior probability (PP) and bootstrap (BS) support indices 100% and 94%, respectively] within a paraphyletic *P. jerdonii*. The first clade (clade A) includes the populations from Hongya, Tianquan, Huili, Baoxing and Markog in the west and south of Sichuan, as well as two populations from Guangxi and from Chongqing (Nanchuan). This clade is strongly supported in both methods (100% PP and 90% BS). Clade B is composed of the populations from northern Sichuan and further north, with high support indices (100% PP and 97% BS). Within this clade, two strongly supported subclades (B1 and B2) were detected. The B1 subclade contains the populations from northern Sichuan (Maoxian, Songpan, Pinwu and Anxian); the second subclade includes all representatives from Gansu, Shaanxi, Shanxi and Henan provinces. Clade C consists of populations from Hunan and Hubei with support values of 100% PP and 100% BS for BI and ML, respectively. The three clades form a very strongly supported group (100% PP and 92% BS). The fourth clade (clade D) is composed of all individuals from Yunnan (China) and two from Sapa (Vietnam), and receives very high support in each analysis (100% PP and 97% BS). Although this clade is sister to *P. xiangchengensis*, the support indices for this branch in both methods are low. Clades A to D form a strongly supported monophyletic group along with *P. xiangchengensis* in the BI analysis, but this is poorly supported in the ML analysis. The final clade (clade E) consists of the specimens from south-eastern Xizang, and receives high support in the ML tree (85% BS) as well as in the BI tree (85% PP). A concatenated mitochondrial and nuclear analysis, however, did not produce a tree that differed from the mtDNA-based tree (the resultant tree is listed in Appendix S2).

The MJNs for the two nuclear genes (Figs 3a,b) do not form distinct clades supporting the mtDNA lineages. Both networks indicate that some representatives from different mtDNA clades share haplotypes; for example, in the network for G51, haplotype 1 is observed in mtDNA clades A, C and E (Fig. 3).

Genetic diversity and population demography

Altogether, 31 haplotypes (29 for cyt *b* and 22 for ND4) were defined for the whole sample of *P. jerdonii*. No haplotype was found to be shared by individuals from different mtDNA lineage groups (Fig. 2). Overall haplotype diversity was high ($Hd = 0.9478$), with the highest within-clade haplotype diversity occurring in clade D ($Hd = 0.9643$) and the lowest in clade C ($Hd = 0$, as there is only one haplotype in this clade). Clades A ($Hd = 0.8830$) and B ($Hd = 0.8573$) have a similar diversity to clade D (Table 1). In contrast, overall nucleotide diversities (π) were low ($\pi\% = 2.33$), with similar diversities in clades A, B and D (1.39, 1.13 and 1.04, respectively; Table 1). Both Tajima's test and Fu and Li's *D*-test indicated that the DNA sequences were selectively neutral ($P > 0.1$). Because of their lower samples sizes, clades C and E were excluded from this analysis.

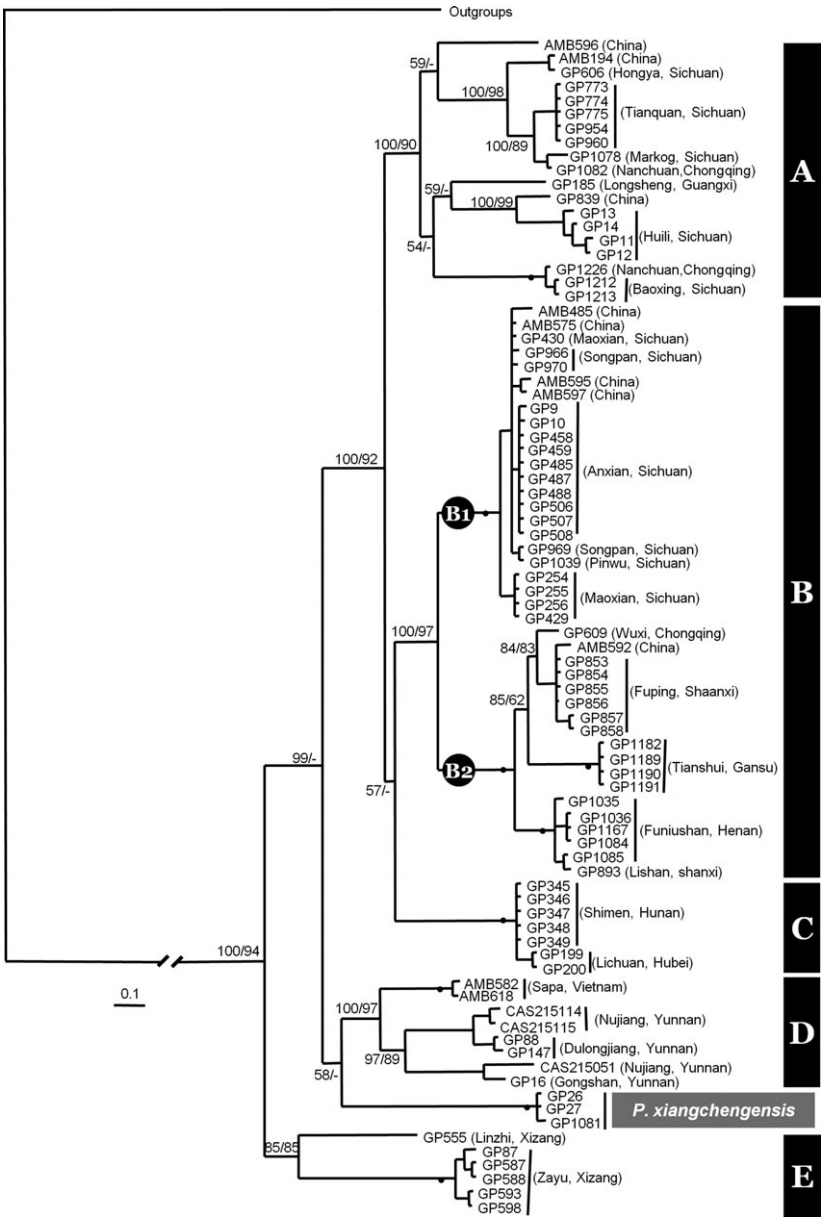

Figure 2 Bayesian 50% majority-rule consensus tree of *Protobothrops jerdonii* inferred from the combined mitochondrial dataset of cytochrome *b* and NADH dehydrogenase subunit 4 (ND4) analysed using the partitioned models as detailed in the text. Posterior probabilities from Bayesian inference and bootstrap support values from maximum likelihood analysis (where > 50%) are given adjacent to the respective nodes for major clades. Nodes receiving posterior probabilities and bootstrap support of 100% are indicated by black circles. Branch support indices are not given for most subclades, to preserve clarity.

The AMOVA based on the five mtDNA lineages defined (A to E) had the highest F_{CT} (0.3978, $P < 0.001$; Table 2), with 54.64% of the total genetic variation explained within groups. When alternative group allocations (4, 3, 2 groups) were tested, significant genetic structure was found to exist across all hierarchical levels (all $P < 0.001$), but F_{CT} was comparatively lower (Table 2). This suggests that the preferred geographical division is consistent with the geographical regions defined by the phylogenetic tree.

Sequence divergence and divergence date estimation

For the combined mtDNA data, uncorrected pairwise (p) distance divergence ranged from 9.4 to 10.2% between mtDNA clades of *P. jerdonii* and outgroups, from 3.4 to 4.8% between clades of *P. jerdonii* and *P. xiangchengensis*, and from 3.2 to 4.7% between the five major clades of *P. jerdonii* (Table 3). The largest uncorrected distances within clades were found in clade A (2.0%), and the smallest in clade C (0.1%). The largest distance between any two individuals of *P. jerdonii* was 5.3%.

Divergence date estimates based on the 95% Highest Posterior Density (HPD) indicate that *P. jerdonii* originated in the late Miocene (*c.* 6.6 Ma, 95% HPD: 2.4–13.1 Ma; Fig. 4). The first split within *P. jerdonii* occurred during the middle Pliocene (*c.* 4.4 Ma, 95% HPD: 1.3–9.3 Ma), separating clade E from the remaining clades. The divergence between clade D (together with *P. xiangchengensis*) and clades A to C is estimated to have occurred *c.* 3.6 Ma (95% HPD: 1.2–7.5 Ma). The separation of clade C from clades A and B is dated to *c.* 2.8 Ma (95% HPD: 0.9–6.0 Ma), and the divergence between clade A and clade B took place *c.* 2.6 Ma (95% HPD: 0.7–5.1 Ma). All divergences within each clade took place after *c.* 2.7 Ma.

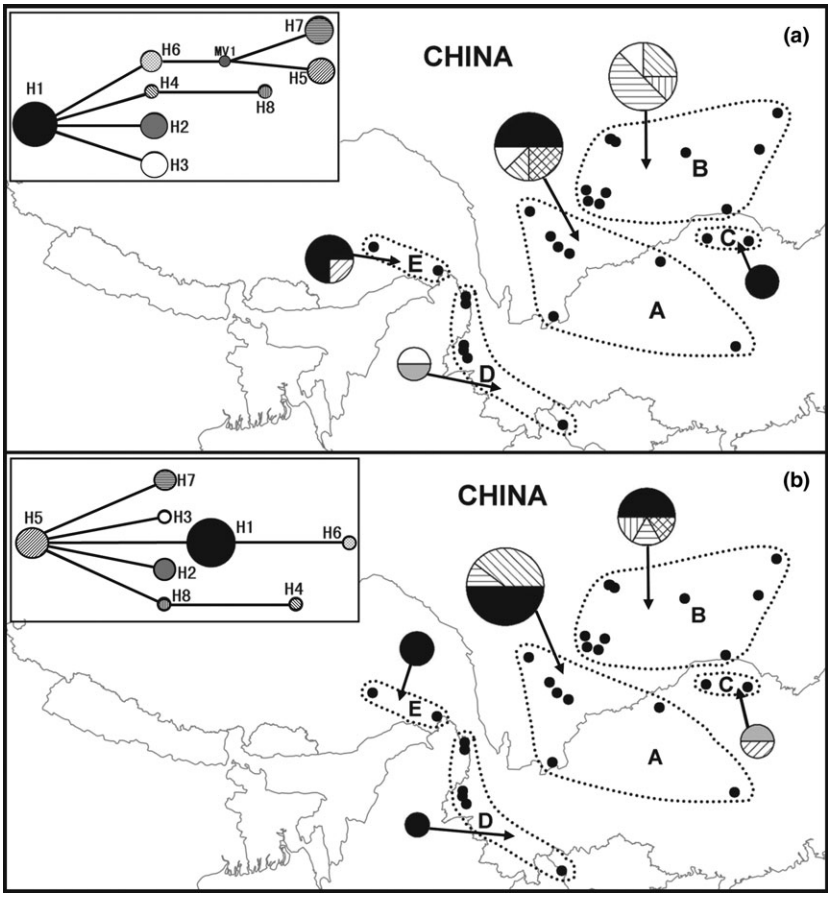

Figure 3 The distribution of the mitochondrial (mt) DNA lineages (A–E) and nuclear haplotypes of locus (a) G51 and (b) GA of *Protobothrops jerdonii*. The median-joining networks of the nuclear genes are inset (lines between the haplotypes represent one mutational step, and MV1 represents an inferred but unsampled haplotype). The clades mentioned correspond to the mtDNA lineages described in main text. The size of the circle is proportional to the number of individuals sampled for each haplotype, with H4 in (a) representing a single individual.

Table 1 Genetic diversity, average number of pairwise differences (K) and neutrality tests for each mitochondrial DNA lineage (the geographical distributions of which are illustrated in Fig. 3) and all lineages of *Protobothrops jerdonii* (A to E, Fig. 2) for the combined data.

Parameter	A	B	C	D	E	All
Sampling size	19	41	7	8	6	81
Polymorphic sites	41	31	0	24	24	111
H	9	12	1	7	2	31
Hd	0.8830	0.8573	–	0.9643	0.333	0.9478
π	0.0139	0.0113	–	0.0135	0.0104	0.0233
K	10.6491	8.666	0.952	10.25	8.000	17.8537
Fu and Li's D	−0.3763*	0.3935*	1.1781*	0.5147*	−1.4066*	−0.5343*
Tajima's D	−0.3719*	0.5468*	0.6873*	0.2037*	−1.4021*	−0.8836*

*$P > 0.1$.

H, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity.

Table 2 AMOVA analysis for groupings of populations of *Protobothrops jerdonii* based on the combined mitochondrial DNA data ($P < 0.001$).

Groups	Among groups F_{CT}	Within groups F_{SC}	Within populations F_{ST}
[A] [B] [C] [D] [E]	0.39775	0.90727	0.94416
[A] [B C] [D] [E]	0.31273	0.91971	0.94482
[A B C] [D] [E]	0.30970	0.92875	0.95082
[A B C] [D E]	0.26039	0.93115	0.94908

DISCUSSION

Genetic diversity and population demography

Protobothrops jerdonii is used here for the first time as a model species to explore how the uplift of the Tibetan plateau influenced the population structure and population evolution of montane snakes in this region. Our analysis uncovered the presence of significant genetic diversity within *P. jerdonii*, indicated by a high level of haplotype diversity and sequence divergence. For example, Jerdon's pitviper has a higher level of nucleotide diversity than the sharp-snouted pitviper, *Deinagkistrodon acutus* (2.32% in *P. jerdonii* versus 1.41% in *D. acutus* for the whole population; Huang *et al.*, 2007). Shepard & Burbrink (2008, 2009) proposed that species restricted to montane habitats commonly have high levels of inter-population genetic divergence because populations on different mountains are separated by low-elevation areas with disparate environmental conditions that act as barriers to gene flow, thereby creating a sky-island situation. Our results for the montane species *P. jerdonii* are consistent with this interpretation,

Table 3 Average sequence divergence estimates (pairwise distances, ± SE, %) between and within five major clades (A to E) of *Protobothrops jerdonii* defined by the mitochondrial DNA phylogeny, and *P. xiangchengensis* (PX). Above the diagonal: distances calculated from cytochrome *b* (above) and NADH dehydrogenase subunit 4 (ND4) (below), respectively; below the diagonal: distances calculated from all genes; on the diagonal (in italics): within-group divergence calculated from all genes.

Clades	A	B	C	D	E	PX
A	*2.0 ± 0.2*	3.4 ± 0.4	3.7 ± 0.4	3.5 ± 0.4	5.0 ± 0.5	4.8 ± 0.6
		2.8 ± 0.5	2.8 ± 0.5	3.4 ± 0.6	4.1 ± 0.6	2.8 ± 0.6
B	3.2 ± 0.3	*1.4 ± 0.2*	3.0 ± 0.4	3.2 ± 0.4	4.4 ± 0.5	4.3 ± 0.5
			2.8 ± 0.6	3.8 ± 0.6	4.1 ± 0.6	3.5 ± 0.6
C	3.3 ± 0.4	2.9 ± 0.4	*0.1 ± 0.0*	3.5 ± 0.5	4.4 ± 0.6	5.2 ± 0.6
				3.4 ± 0.6	4.7 ± 0.8	3.7 ± 0.7
D	3.5 ± 0.4	3.5 ± 0.4	3.4 ± 0.4	*1.5 ± 0.2*	4.0 ± 0.5	3.5 ± 0.5
					3.5 ± 0.6	3.1 ± 0.6
E	4.7 ± 0.5	4.3 ± 0.5	4.5 ± 0.5	3.8 ± 0.5	*1.3 ± 0.2*	5.4 ± 0.7
						3.8 ± 0.7
PX	4.0 ± 0.4	4.0 ± 0.4	4.6 ± 0.5	3.4 ± 0.4	4.8 ± 0.5	–

indicating that higher-elevation mountains as well as lower-elevation areas can represent barriers between populations.

Within *P. jerdonii*, we found five geographically distinct mtDNA lineages. These five lineages are genetically very different (the average divergence between lineages varies from 2.9 to 4.7%; Table 3). Furthermore, structure is also present within lineages (e.g. clade B). This phylogeographical pattern may be attributable to long-term extrinsic barriers to genetic exchange, as suggested by Avise (2000). No haplotype is shared among different clades or subclades, or even among some small regional populations, while many 'missing' haplotypes were detected among and within lineages. Avise (2000) proposed that extinctions of intermediate haplotypes in broadly distributed species with low dispersal and gene flow may explain the appearance of pronounced phylogenetic gaps.

In recent years, nuclear genes have increasingly been used to explore the evolutionary history of organisms and systematic relationships of taxa. However, some intraspecific phylogenetic studies found that nuclear DNA phylogenies did not support mtDNA-based phylogenies (e.g. Gonçalves *et al.*, 2007; Rato *et al.*, 2010). A plausible explanation for this could be different evolutionary rates of mtDNA and nuclear genes. Generally, mtDNA has a more rapid coalescence and a higher evolutionary rate than nuclear data. Thus, for the situation studied here, mtDNA is able to resolve fine-scale genetic structure, recovering five monophyletic groups that correspond very well with geography. In contrast, possibly as a consequence of the slower mutation rate and slower coalescence/lineage sorting of the nuclear genes (and/or the smaller amount of data used), the nuclear data are not able to resolve such groupings. Thus, the mitochondrial data support a lack of gene flow among allopatrically isolated populations, but there has been insufficient time for this to be reflected in the nuclear data. Based on the results from the mtDNA divergence dating, most clades of interest are less than 3 million years old, and nuclear genes would not be expected to undergo coalescence in this time period. The lack of population structure in nuclear genealogies in contrast to the highly differentiated mtDNA lineages has previously been detected in snakes (Rato *et al.*, 2009), lizards (Pinho *et al.*, 2007, 2008) and amphibians (Velo-Antón *et al.*, 2008).

Protobothrops jerdonii is characterized as a low-dispersing snake occurring at high elevations (Liu, 1940; Zhao *et al.*, 1998). It is possible that this lower dispersal capability, in conjunction with the large habitat heterogeneity (including numerous mountain formations and rivers) generated by the uplift of the Tibetan Plateau, led to the observed genetic

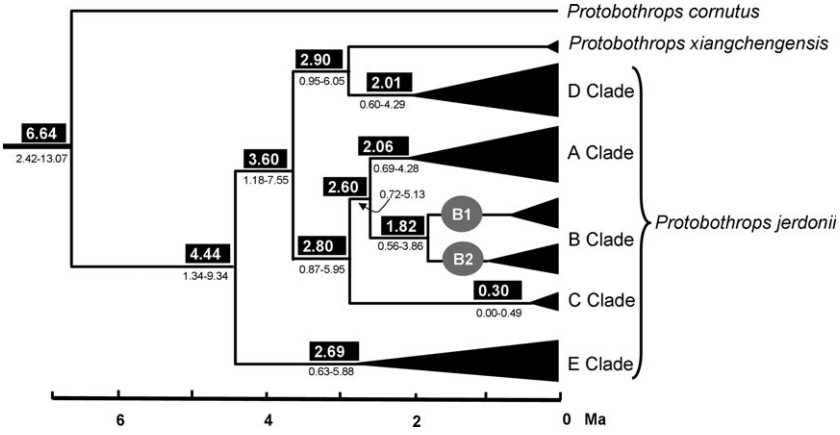

Figure 4 Bayesian estimates of mean divergence times (Ma, above the node) with 95% highest posterior densities (below the node) of *Protobothrops jerdonii* clades, computed using BEAST (Drummond & Rambaut, 2007).

differentiation among clades. Through time, the regional allopatric populations would have come to occupy recognizable and deeply separated branches in an intraspecific gene tree, as indicated in this work. In addition to the environmental cause mentioned above, the lack of human-mediated dispersal has perhaps also been an important factor in the shaping of the genetic structure of *P. jerdonii*. While medically important snakes (e.g. *Zaocys dhumnades* and *Elaphe carinata*) are likely to have undergone long-distance transport through trade (Zhou & Jiang, 2004), *P. jerdonii* is not regarded as having medical importance in traditional Chinese medicine, and has seldom been utilized as food or medicine. Thus, long-distance transport through trade would have been scarce, greatly decreasing the opportunity for gene exchange between different groups (Fu *et al.*, 2005; Huang *et al.*, 2007).

The high level of haplotype diversity, coupled with the low nucleotide diversity, implies that during the Last Glacial Maximum heterogeneous habitats would have generated multiple isolated refugia for this cold-tolerant species. Repeated range fragmentation and re-expansion of *P. jerdonii* as a result of climatic oscillations would have generated new haplotypes as well as driving some older haplotypes to extinction (observable as 'missing' haplotypes), thus facilitating intraspecific diversification and population genetic differentiation of the species.

Hewitt (2004) proposed that the dramatic climatic oscillations of the Pleistocene glacial cycles had a profound effect on the current distributions, lineage formation and genetic structure of most living organisms. This conclusion is supported by a growing number of molecular phylogeographical studies on snakes in North America and Europe (Burbrink *et al.*, 2000; Ursenbacher *et al.*, 2006a,b, 2008; Placyk *et al.*, 2007; Guiher & Burbrink, 2008; Barbanera *et al.*, 2009) indicating that glacial cycles have been a major contributor to population evolution, demographic patterns and present distributions. Other studies, however, have suggested that it was pre-Pleistocene geographical evolution that was primarily responsible for population and/or subpopulation evolution and genetic structure, while glacial cycles may have acted only as a secondary factor (e.g. Castoe *et al.*, 2009). Our findings are consistent with the work of Castoe *et al.* (2009) in suggesting that Miocene–Pliocene tectonic activity played a dominant role in generating regional highland species biodiversity.

General biogeography of *P. jerdonii*

The regions occupied by these five populations (clades) do not overlap, are located in close proximity geographically, and are distributed from west to east. This east–west distribution is strikingly congruent with the hierarchical structure of *P. jerdonii* populations displayed in the gene tree (Fig. 3). Based on the evidence presented here, we conclude that the Xizang population (clade E) is the most likely ancestral population. Although we cannot infer the centre of origin of this species in any detail with the data in hand, it was possibly in the western Hengduan Mountains. These mountains are located in the south-eastern part of the Tibetan plateau. Compared with the age of the Qinghai-Xizang plateau itself, the lower elevations of the Hengduan Mountains, along the eastern escarpment of the plateau, are much older (Li, 1953; see Fu & Zeng, 2008). It has been demonstrated that this region does not only contain many relict taxa [e.g. giant panda (*Ailuropoda melanoleuca*), red panda (*Ailurus fulgens*), Xizang hot-spring snake (*Thermophis zhaoermii*)], but also has been the centre of origin for many taxa or groups (Zheng *et al.*, 1981; Fei & Ye, 1989; Zhao & Yang, 1997; Zhang, 1999; Che *et al.*, 2010). It is therefore reasonable to suggest that the centre of origin of Jerdon's pitviper was also located here.

Combining the present-day distribution pattern with the hierarchical structure in the phylogenetic tree, we propose that the evolutionary history of *P. jerdonii* is dominated by a pattern of dispersal followed by vicariance. The Tibetan plateau began its slow uplift during the late Miocene (*c.* 25–10 Ma), and a rapid uplift occurred *c.* 3.4 Ma in the mid-Pliocene (Sun, 1997). In the late Miocene and early Pliocene, its elevation was *c.* 1000 m, and it was characterized by a warm and humid climate (Sun, 1997), which is similar to the present niche (climate and elevation) for *P. jerdonii* (Zhao *et al.*, 1998). When *P. jerdonii* originated *c.* 6.6 Ma, it may have experienced a sudden distributional range expansion by dispersing from the west to the east, and would have occupied a continuous landscape prior to the massive uplift of the plateaus. Although this interpretation is based only on cladogenetic data, and thus an alternative scenario of vicariance cannot be rejected, a similar dispersal trend from west to east has been found at various taxonomic levels in snakes (Huang *et al.*, 2007), frogs (Fu *et al.*, 2005; Che *et al.*, 2010) and birds (Qu *et al.*, 2005) in China.

The subsequent massive uplift of the plateau in the mid-Pliocene led to the current complex topology of the region, creating highly heterogeneous habitats. In particular, many north–south-oriented high-elevation mountains (e.g. Qiongnai Mountains) and low-elevation areas (e.g. the Sichuan Basin, < 800 m in elevation) were generated. These mountains and basins probably played a key role in driving population differentiation as well as acting as important barriers to gene exchange between populations. The first divergence within Jerdon's pitviper took place *c.* 4.4 Ma (forming the Xizang population); subsequent divergence occurred after 2.6 Ma, when the plateaus had greatly increased in elevation. Through time, five regional allopatric populations have come to occupy recognizable, deeply separated lineages (Fig. 2). These west–east-distributed regional populations match the orientation of the north–south-oriented mountains well. With the evidence presented here, it is difficult to conclude which of these barriers played the key role in driving inter-population differentiation, and it may be that they jointly acted as barriers for *P. jerdonii*.

ACKNOWLEDGEMENTS

We thank L. Ding, D.Q. Rao, M. Hou, J.P. Jiang, G.X. Zhu, H. Wu, D.W. Qi, G.R. Li and B.Q. Yang for kindly providing

samples; H. Chen for help with laboratory work; and Z.F. Guo for preparing the distribution map. We would also like to thank J.Z. Fu, F.T. Burbrink, A.D. McKelvy, E.A. Myers, two anonymous referees and the editors for insightful comments on the manuscript. This project is partly supported by grants from the National Natural Science Foundation of China (NSFC 30670236, NSFC 30970334, NSFC 31071892), the Program for New Century Excellent Talents in University, the Ministry of Education of the People's Republic of China (NCET-08-0908) and Sichuan Youth Sciences and Technology Foundation (08ZQ026-006). P.G. and A.M. thank the Royal Society for additional funding (International Project Grant IJP2006/R1).

REFERENCES

Alfaro, M.E., Karns, D.R., Voris, H.K., Abernathy, E. & Sellins, S.L. (2004) Phylogeny of *Cerberus* (Serpentes: Homalopsinae) and phylogeography of *Cerberus rynchops*: diversification of a coastal marine snake in Southeast Asia. *Journal of Biogeography*, **31,** 1277–1292.

Arévalo, E., Davis, S.K. & Sites, J.W., Jr (1994) Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico. *Systematic Biology*, **43,** 387–418.

Avise, J.C. (2000) *Phylogeography: the history and formation of species*. Harvard University Press, Cambridge, MA.

Avise, J.C. (2009) Phylogeography: retrospect and prospect. *Journal of Biogeography*, **36,** 3–15.

Bandelt, H.J., Forster, P. & Rohl, A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16,** 37–48.

Barbanera, F., Zuffi, M.A.L., Guerrini, M., Gentilli, A., Tofanelli, S., Fasola, M. & Dini, F. (2009) Molecular phylogeography of the asp viper *Vipera aspis* (Linnaeus, 1758) in Italy: evidence for introgressive hybridization and mitochondrial DNA capture. *Molecular Phylogenetics and Evolution*, **52,** 103–114.

Bauer, A.M. (1989) Reptiles and the biogeographic interpretation of New Caledonia. *Tuatara*, **30,** 39–50.

Beheregaray, L.B. (2008) Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. *Molecular Ecology*, **17,** 3754–3774.

Burbrink, F.T. & Castoe, T.A. (2009) Molecular phylogeography of snakes. *Snakes: ecology and conservation* (ed. by R. Seigel and S. Mullin), pp. 38–77. Cornell University Press, Ithaca, NY.

Burbrink, F.T., Lawson, R. & Slowinski, J.B. (2000) Mitochondrial DNA phylogeography of the polytypic north American rat snake (*Elaphe obsoleta*): a critique of the subspecies concept. *Evolution*, **54,** 2107–2118.

Camargo, A., Sinervo, B. & Sites, J.W. (2010) Lizards as model organisms for linking phylogeographic and speciation studies. *Molecular Ecology*, **19,** 3250–3470.

Castoe, T.A., Daza, J.M., Smith, E.N., Sasa, M.M., Kuch, U., Campbell, J.A., Chippindale, P.T. & Parkinson, C.L. (2009) Comparative phylogeography of pitvipers suggests a consensus of ancient Middle American highland biogeography. *Journal of Biogeography*, **36,** 88–103.

Che, J., Zhou, W.W., Hu, J.S., Yan, F., Papenfuss, T.J., Wake, D.B. & Zhang, Y.P. (2010) Spiny frogs (Paini) illuminate the history of the Himalayan region and Southeast Asia. *Proceedings of the National Academy of Sciences USA*, **107,** 13765–13770.

Creer, S., Malhotra, A., Thorpe, R.S. & Chou, W.H. (2001) Multiple causation of phylogeographical pattern as revealed by nested clade analysis of the bamboo viper (*Trimeresurus stejnegeri*) within Taiwan. *Molecular Ecology*, **10,** 1967–1981.

David, P. & Ineich, I. (1999) Les serpents venimeux du monde: systematique et repartition. *Dumerilia*, **3,** 3–499.

Drummond, A.J. & Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7,** 214.

Drummond, A.J., Ho, S.Y.W., Phillips, M.J. & Rambaut, A. (2006) Relaxed phylogenetics and dating with confidence. *PLoS Biology*, **4,** 88.

Excoffier, L., Smouse, P.E. & Quattro, J.M. (1992) Analysis of molecular variance inferred from metric distance among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131,** 479–491.

Excoffier, L., Laval, G. & Schneider, S. (2005) ARLEQUIN ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1,** 47–50.

Fei, L. & Ye, C.Y. (1989) Geographical distribution, origin and evolution, and centre of differentiation of high-altitude pelobatid toads in Asia (Amphibia: Peiobatidae). *Zoological Research*, **10,** 295–302.

Fu, J.Z. & Zeng, X.M. (2008) How many species are in the genus *Batrachuperus*? A phylogeographical analysis of the stream salamanders (family Hynobiidae) from southwestern China. *Molecular Ecology*, **17,** 1469–1488.

Fu, J.Z., Weadick, C.J., Zeng, X.M., Wang, Y.Z., Liu, Z.J., Zheng, Y.C., Li, C. & Hu, Y. (2005) Phylogeographic analysis of the *Bufo gargarizans* species complex: a revisit. *Molecular Phylogenetics and Evolution*, **37,** 202–213.

Fu, Y.X. & Li, W.H. (1993) Statistical tests of neutrality of mutations. *Genetics*, **133,** 693–709.

Gibbs, H.L. & Diaz, J. (2010) Identification of single copy nuclear DNA markers for North American pitvipers. *Molecular Ecology Resources*, **1,** 177–180.

Gonçalves, H., Martínez-Solano, I., Ferrand, N. & García-París, M. (2007) Conflicting phylogenetic signal of nuclear *vs* mitochondrial DNA markers in midwife toads (Anura, Discoglossidae, *Alytes*): deep coalescence or ancestral hybridization? *Molecular Phylogenetics and Evolution*, **44,** 494–500.

Guiher, T.J. & Burbrink, F.T. (2008) Demographic and phylogeographic histories of two venomous North American snakes of the genus *Agkistrodon*. *Molecular Phylogenetics and Evolution*, **48,** 543–553.

Gumprecht, A., Tillack, F., Orlov, N.L., Captain, A. & Ryabov, S. (2004) *Asian pitvipers*. Geitje Books, Berlin.

Guo, P., Malhotra, A., Li, C., Creer, S., Pook, C.E. & Wen, T. (2009) Systematics of the *Protobothrops jerdonii* complex (Serpentes, Viperidae, Crotalinae) inferred from morphometric data and molecular phylogeny. *Herpetological Journal*, **19,** 85–96.

Hewitt, G.M. (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359,** 183–195.

Hickerson, M.J., Carstens, B.C., Cavender-Bares, J., Crandall, K.A., Graham, C.H., Johnson, J.B., Rissler, L., Victoriano, P.F. & Yoder, A.D. (2010) Phylogeography's past, present, and future: 10 years after Avise, 2000. *Molecular Phylogenetics and Evolution*, **54,** 291–301.

Holman, J.A. (2000) *Fossil snakes of North America: origin, evolution, distribution, paleoecology*. Indiana University Press, Indianapolis, IN.

Huang, S., He, S.P., Peng, Z.G., Zhao, K. & Zhao, E.M. (2007) Molecular phylogeography of endangered sharp-snouted pitviper (*Deinagkistrodon acutus*; Reptilia, Viperidae) in mainland China. *Molecular Phylogenetics and Evolution*, **44,** 942–952.

Huelsenbeck, J.P. & Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17,** 754–755.

Karns, D.R., O'Bannon, A., Voris, H.K. & Weigt, L.A. (2000) Biogeographical implications of mitochondrial DNA variation in the Bockadam snake (*Cerberus rynchops*, Serpentes: Homalopsinae) in Southeast Asia. *Journal of Biogeography*, **27,** 391–402.

Kumar, S., Dudley, J., Nei, M. & Tamura, K. (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics*, **9,** 299–306.

Li, S. (1953) *Geology of China*. Zhengfeng Printing House, Beijing.

Librado, P. & Rozas, J. (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25,** 1451–1452.

Liu, C.C. (1940) Life history of *Trimeresurus jerdonii*. *Peking Natural History Bulletin, Beijing*, **14,** 245–252.

Malhotra, A. & Thorpe, R.S. (2004) A phylogeny of four mitochondrial gene regions suggests a revised taxonomy for Asian pitvipers (*Trimeresurus* and *Ovophis*). *Molecular Phylogenetics and Evolution*, **32,** 83–100.

Meng, L.H., Abbott, R.J., Miehe, G., Hu, T.H. & Liu, J.Q. (2007) Mitochondrial and chloroplast phylogeography of *Picea crassifolia* Kom. (Pinaceae) in the Qinghai-Tibetan Plateau and adjacent highlands. *Molecular Ecology*, **16,** 4128–4137.

Nylander, J.A.A. (2004) *MrModeltest v.2*. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Orlov, N.L., Ryabov, S.A., Shiryaev, K.A. & Sang, N.V. (2001) Captive breeding and conservation on the biology of pitvipers of *Protobothrops* genus (Serpentes: Colubroidea: Viperidae: Crotalinae). *Russian Journal of Herpetology*, **8,** 159–164.

Parmley, D. & Holman, J.A. (2007) Earliest fossil record of a pigmy rattlesnake (Viperidae: *Sistrurus Garman*). *Journal of Herpetology*, **41,** 141–144.

Peng, Z.G., Ho, S.Y.W., Zhang, Y.G. & He, S.P. (2006) Uplift of the Tibetan plateau: evidence from divergence times of glyptosternoid catfishes. *Molecular Phylogenetics and Evolution*, **39,** 568–572.

Pinho, C., Harris, D.J. & Ferrand, N. (2007) Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). *Biological Journal of the Linnean Society*, **91,** 121–133.

Pinho, C., Harris, D.J. & Ferrand, N. (2008) Non-equilibrium estimates of gene flow inferred from nuclear genealogies suggest that Iberian and North African wall lizards (*Podarcis* spp.) are an assemblage of incipient species. *BMC Evolutionary Biology*, **8,** 1–20.

Placyk, J.S., Jr, Burghardt, G.M., Small, R.L., King, R.B., Casper, G.S. & Robinson, J.W. (2007) Post-glacial recolonization of the Great Lakes region by the common garter snake (*Thamnophis sirtalis*) inferred from mtDNA sequences. *Molecular Phylogenetics and Evolution*, **43,** 452–467.

Posada, D. & Buckley, T.R. (2004) Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Systematic Biology*, **53,** 793–808.

Posada, D. & Crandall, K.A. (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14,** 817–818.

Pyron, R.A. & Burbrink, F.T. (2009) Neogene diversification and taxonomic stability in the snake tribe Lampropeltini (Serpentes: Colubridae). *Molecular Phylogenetics and Evolution*, **52,** 524–529.

Qu, Y.H. & Lei, F.M. (2009) Comparative phylogeography of two endemic birds of the Tibetan plateau, the white-rumped snow finch (*Onychostruthus taczanowskii*) and the Hume's ground tit (*Pseudopodoces humilis*). *Molecular Phylogenetics and Evolution*, **51,** 312–326.

Qu, Y.H., Ericson, P.G.P., Lei, F.M. & Li, S.H. (2005) Post-glacial colonization of the Tibetan plateau inferred from matrilineal genetic structure of the endemic red-necked snow finch, *Pyrgilauda ruficollis*. *Molecular Ecology*, **14,** 1767–1781.

Qu, Y.H., Ericson, P.G.P., Lei, F.M., Gebauer, A., Kaiser, K. & Helbig, A.J. (2006) Molecular phylogenetic relationship of snow finch complex (genera *Montifringilla*, *Pyrgilauda* and *Onychostruthus*) from the Tibetan plateau. *Molecular Phylogenetics and Evolution*, **40,** 218–226.

Rambaut, A. & Drummond, A.J. (2007) *Tracer v1.4*. Available at: http://evolve.zoo.ox.ac.uk.

Rato, C., Zuffi, M.A.L., Corti, C., Fornasiero, S., Gentilli, A., Razzetti, E., Scali, S., Carretero, M.A. & Harris, D.J. (2009) Phylogeography of the European Whip Snake, *Hierophis viridiflavus* (Colubridae), using mtDNA and nuclear DNA sequences. *Amphibia–Reptilia*, **30,** 283–289.

Rato, C., Carranza, S., Perera, A., Carretero, M.A. & Harris, D.J. (2010) Conflicting patterns of nucleotide diversity between mtDNA and nDNA in the Moorish gecko, *Tarentola mauritanica*. *Molecular Phylogenetics and Evolution*, **56,** 962–971.

Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19,** 1572–1574.

Sambrook, J. & Russell, D.W. (2002) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory Press, NY.

Shepard, D.B. & Burbrink, F.T. (2008) Lineage diversification and historical demography of a sky island salamander, *Plethodon ouachitae*, from the Interior Highlands. *Molecular Ecology*, **17,** 5315–5335.

Shepard, D.B. & Burbrink, F.T. (2009) Phylogeographic and demographic effects of Pleistocene climatic fluctuations in a montane salamander, *Plethodon fourchensis*. *Molecular Ecology*, **18,** 2243–2262.

Stamatakis, A., Hoover, P. & Rougemont, J. (2008) A rapid bootstrap algorithm for the RaxML web-servers. *Systematic Biology*, **75,** 758–771.

Sun, H.L. (1997) *Research of the formation, environment change on Qinghai-Xizang (Tibetan) plateau*. Hunan Science and Technology Press, Changsha, China.

Tajima, F. (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123,** 585–595.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24,** 1596–1599.

Ursenbacher, S., Carlsson, M., Helfer, V., Tegelstr, H. & Fumagalli, L. (2006a) Phylogeography and Pleistocene refugia of the adder (*Vipera berus*) as inferred from mitochondrial DNA sequence data. *Molecular Ecology*, **15,** 3425–3437.

Ursenbacher, S., Conelli, A., Golay, P., Monney, J.C., Zuffi, M.A.L., Thiery, G., Durand, T. & Fumagalli, L. (2006b) Phylogeography of the asp viper (*Vipera aspis*) inferred from mitochondrial DNA sequence data: evidence for multiple Mediterranean refugial areas. *Molecular Phylogenetics and Evolution*, **38,** 546–552.

Ursenbacher, S., Schweiger, S., Tomovic, L., Crnobrnja-Isailovic, J., Fumagalli, L. & Mayer, W. (2008) Molecular phylogeography of the nose-horned viper (*Vipera ammodytes*, Linnaeus (1758)): evidence for high genetic diversity and multiple refugia in the Balkan Peninsula. *Molecular Phylogenetics and Evolution*, **46,** 1116–1128.

Velo-Antón, G., Martínez-Solano, I. & García-Paris, M. (2008) ß-fibrinogen intron 7 variation in *Discoglossus* (Anura: Discoglossidae): implications for the taxonomic assessment of morphologically cryptic species. *Amphibia–Reptilia*, **29,** 523–533.

Wüster, W., Salomão, M.D.G., Quijada-Mascareñas, J.A., Thorpe, R.S. & BBBSP (2002) Origins and evolution of the South American pitviper fauna: evidence from mitochondrial DNA sequence analysis. *Biology of vipers* (ed. by G.W. Schuett, M.H. Höggren, M.E. Douglas and H.W. Greene), pp. 111–128. Eagle Mountain Publishing, Sandy, UT.

Wüster, W., Peppin, L., Pook, C.E. & Walker, D.E. (2008) A nesting of vipers: phylogeny and historical biogeography of the Viperidae (Squamata: Serpentes). *Molecular Phylogenetics and Evolution*, **49,** 445–459.

Zhang, D.X. & Hewitt, G.M. (1996) Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology and Evolution*, **11,** 247–251.

Zhang, F.F. & Jiang, Z.G. (2006) Mitochondrial phylogeography and genetic diversity of Tibetan gazelle (*Procapra picticaudata*): implications for conservation. *Molecular Phylogenetics and Evolution*, **41,** 313–321.

Zhang, Q., Chiang, T.Y., George, M., Liu, J.Q. & Abbott, R.J. (2005) Phylogeography of the Qinghai-Tibetan Plateau endemic *Juniperus przewalskii* (Cupressaceae) inferred from chloroplast DNA sequence variation. *Molecular Ecology*, **14,** 3513–3524.

Zhang, R.Z. (1999) *Zoogeography of China*. Science Press, Beijing.

Zhao, E.M. & Yang, D.T. (1997) *Amphibians and reptiles of the Hengduan Mountains region*. Science Press, Beijing.

Zhao, E.M., Huang, M.H. & Zong, Y. (1998) *Fauna Sinica: Reptilia, Vol. 3. Squamata Serpentes*. Science Press, Beijing.

Zheng, Z.X., Feng, Z.J., Zhang, R.Z. & Hu, S.Q. (1981) On the land-vertebrate fauna of Qinghai-Xizang plateau with considerations concerning its history of transformation. *Memoirs of Beijing Natural History Museum*, **9,** 1–21.

Zhou, Z.H. & Jiang, Z.G. (2004) International trade status and crisis for snake species in China. *Conservation Biology*, **18,** 1386–1394.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Samples used in this study, with GenBank accession numbers, locality and voucher information.

Appendix S2 Bayesian 50% majority-rule consensus tree of *Protobothrops jerdonii* inferred from the combined nuclear DNA and mtDNA using the partitioned models as detailed in the text.

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BIOSKETCH

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Editor: Brett Riddle