A phylogeny of Chinese species in the genus
Phrynocephalus (Agamidae) inferred from mitochondrial
DNA sequences

Junfeng Pang, Yuezhao Wang, Yang Zhong, A. Rus Hoelzel, Theodore J. Papenfuss, Xiaomao Zeng, Natalia B. Ananjeva, and Ya-ping Zhang

Abstract

We investigated the phylogenetic relationships among most Chinese species of lizards in the genus Phrynocephalus (118 individuals collected from 56 populations of 14 well-defined species and several unidentified specimens) using four mitochondrial gene fragments (12S rRNA, 16S rRNA, cytochrome b, and ND4-tRNALEU). The partition-homogeneity tests indicated that the combined dataset was homogeneous, and maximum-parsimony (MP), neighbor-joining (NJ), maximum-likelihood (ML) and Bayesian (BI) analyses were performed on this combined dataset (49 haplotypes including outgroups for 2058bp in total). The maximum-parsimony analysis resulted in 24 equally parsimonious trees, and their strict consensus tree shows that there are two major clades representing the Chinese Phrynocephalus species: the viviparous group (Clade A) and the oviparous group (Clade B). The trees derived from Bayesian, ML, and NJ analyses were topologically identical to the MP analysis except for the position of P. mystaceus. All analyses left the nodes for the oviparous group, the most basal clade within the oviparous group, and P. mystaceus unresolved. The phylogenies further suggest that the monophyly of the viviparous species may have resulted from vicariance, while recent dispersal may have been important in generating the pattern of variation among the oviparous species.

Keywords: Phrynocephalus; Mitochondrial DNA; Reproduction; Phylogeny

1. Introduction

The sand lizards of the genus Phrynocephalus (Family Agamidae; Kaup, 1825) include over 40 species distributed from north-western China to Turkey, and are one of the major components of the central Asian desert fauna. Their range includes the north-western Qinghai-Xizang (Tibetan) plateau, southwest Asia and western Russia (53°N, 55°E) (Zhao, 1999). Because of their variable morphology (Arnold, 1999; Moody, 1980; Wermuth, 1967; Zhao and Alder, 1993), life history and chromosomal structure (Peters, 1984; Sokolovsky, 1974; Wang and Macey, 1993; Zeng et al., 1997; Zhao, 1997), and their cosmopolitan distribution, species in the genus Phrynocephalus have long been a subject of study by systematists (Ananjeva and Tuniyev, 1992; Arnold, 1999; Bediaga, 1909; Carevskij, 1929; Zhao and Alder, 1993). However, the phylogenetic relationships among the 18 Phrynocephalus species distributed in China (Fig. 1) are still poorly understood. In China these species are found from below sea level (~42 m) up to an elevation of 5300 m (Wang and Macey, 1993; Zhao, 1999).
There are two reproductive modes in *Phrynocephalus*: viviparity and oviparity. All six viviparous species are endemic to China, and are mainly restricted to high elevations in the Qinghai-Xizang (Tibetan) plateau (>2200 m). One viviparous species, *P. forsythii*, is also sympatrically distributed with the oviparous species *P. axillaris* in the south-western Xinjiang Uygur Autonomous Region (see Fig. 1). Wang and Macey (1993), in an attempt to explain reproductive bimodality, divided the Chinese *Phrynocephalus* into two major ecogeographical groups: the high-elevation-cold-arid species group (6 viviparous species), and the low-elevation-arid species group (12 oviparous species), and predicted that the high-elevation-cold-arid species group would be mono-phyletic. Although this prediction seems obvious with respect to morphological, ecological, and behavioral similarity, it had not been supported by a robust phylogenetic reconstruction (Zhao, 1997). Using allozyme markers, Macey et al. (1993) assigned the viviparous *P. forsythii* to the lowland oviparous Chinese species clade on the basis of phylogenetic relationships among 8 species (12 populations) in China and 7 species (7 populations) in the former USSR. Later Zeng et al. (1997) showed that 11 Chinese species (27 populations) exhibit three kinds of karyotypes. All viviparous species have $2n = 48$ (12 large and 12 small pairs of chromosomes), *P. mystaceus* has a unique type with $2n = 48$ (11 large and 13 small pairs of chromosomes), and all oviparous
species have $2n = 46$ (11 large and 12 small pairs of chromosomes). Based on this, Zeng et al. (1997) concluded that the viviparous species are monophyletic. However, further phylogenetic information from karyotopic analysis was limited due to the high similarities among most chromosomes within each group. Recently, Arnold (1999) indicated that *P. forsythii* was not closely related to other viviparous species based on a parsimony analysis of 25 *Phrynocephalus* species (including 10 Chinese species), using 46 morphological characters involving 54 derived states. Yet the phylogenetic relationships among the Chinese species are still poorly resolved.

Mitochondrial DNA genes such as ND4-tRNA	extsubscript{LEU}, 12S ribosome RNA (12S rRNA), 16S ribosome RNA (16S rRNA), and cytochrome *b* (Cyt *b*) have been widely used for elucidating interspecific phylogenetic relationships among reptiles, including lizards (Malone et al., 2000; Mausfeld et al., 2000; Parkinson et al., 2000; Sites et al., 1996). More importantly, large molecular datasets under appropriate phylogenetic analyses have shown the potential to resolve longstanding controversies in systematics (Murphy et al., 2001). Therefore, we sequenced these four mitochondrial gene fragments in the Chinese *Phrynocephalus* in order to address two key questions: Do the viviparous species form a monophyletic lineage, and more generally, what are the phylogenetic relationships among the Chinese *Phrynocephalus* species?

2. Materials and methods

2.1. Sample collection

A total of 118 individuals from 56 populations of 14 well-defined species and 10 unidentified populations were examined, including at least two individuals for each species whenever possible (see Appendix A). We included samples from five viviparous species (*P. forsythii*, *P. vlangalii*, *Phrynocephalus theobaldi*, *P. zetangensis*, and *P. hongyuanensis*) and nine oviparous species (*P. acutirostris*, *P. albolineatus*, *P. guttatus*, *P. przewalskii*, *P. frontalis*, *P. versicolor*, *P. axillaris*, *P. mystaceus*, and *P. helioscopus*). For most species, individuals from different localities were used to increase the reliability of the phylogenetic analyses (see Fig. 1). Our samples cover a broad geographic range, including most parts of north-western China and the Qinghai-Xizang (Tibetan) plateau (Fig. 1). *Trachelus sanguinolentus* and *Laudakia caucasia* were selected as outgroup taxa based on current understanding of the phylogenetic relationships among agamid lizards (Arnold, 1999; Joger, 1991; Macey et al., 2000; Moody, 1980). Muscle tissues were used, preserved in either 80% ethanol or 10% formalin. Voucher specimens are held in the Chengdu Institute of Biology and the Kunming Institute of Zoology, Chinese Academy of Sciences.

2.2. DNA extraction

Total DNA was extracted following the method of Shedlock et al. (1997) with some modifications: (1) tissues were washed for five, rather than three, successive 24-h periods (rotary shaker, 4°C) in 1 ml fresh 1× GTE (100 mM glycine, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0); (2) 2 mg proteinase K was used for each sample at the beginning of digestion; and (3) standard 3-step phenol/chloroform extractions were performed. A negative control sample (extract without tissues) was treated identically through both the extraction procedure and the following PCR amplifications.

2.3. PCR amplification and DNA sequencing

The four genes chosen as target fragments were amplified with published primers (Arevalo et al., 1994; Kocher et al., 1989; Rassmann, 1997). For Cyt *b*, 12S rRNA and 16S rRNA, PCR amplifications were accomplished with one pair of primers for all samples: L14841-H15149 (Kocher et al., 1989) for Cyt *b*, L1091-H1478 (Kocher et al., 1989) for 12S rRNA, and L2510-H3063 (Rassmann, 1997) for 16S rRNA. For the ND4-tRNA	extsubscript{LEU} fragment (>800 bp), PCR amplifications were accomplished with one pair of primers (ND4-LEU, Arevalo et al., 1994) for ethanol-fixed samples, whereas various primer combinations (Arevalo et al., 1994) were used to facilitate amplification from formalin-fixed samples. Reactions were performed in a total volume of 25 µl and contained 2.5 µl 10× reaction buffer (Sino-American), 1.5 mmol MgCl\textsubscript{2}, 0.2 mM dNTPs (Amresco), 0.2 µM each primer, 0.8 units Taq DNA polymerase (Sino-American), and approximately 60 ng genomic DNA. After a pre-denaturing step of 4 min at 95°C, each cycle had a 1 min denaturing step at 94°C. Annealing was for 45 s per cycle, with a touchdown step from 60°C to 45°C. Once 45°C was reached, 20 further cycles were performed. Extension was at 72°C for 1 min per cycle. Post-extension at 72°C was performed for 10 min. The profiles of PCR amplification for the four target fragments were identical.

The PCR products were gel-purified (Zhang et al., 1999) and directly sequenced using the BigDye Terminator Kit (Perkin–Elmer) according to manufacturer’s protocols with the same primers as used for PCR amplification. Unincorporated reagents were removed from the sequencing products with CentriSep columns (Princeton Separations), and then analyzed on an ABI Model 377XL Automated Sequencer (PE Biosystems). All PCR products were sequenced from both directions.

DNA sequences were edited and aligned using DNASTAR (DNASTAR Inc) and checked manually.
The cytochrome \( b \) gene and the coding region of the ND4 gene were aligned based on the putative amino acid sequence, and the 12S rRNA gene, the 16S rRNA gene, and the tRNA genes were aligned based on their secondary structures to facilitate proper alignments.

### 2.4. Phylogenetic analyses

Base compositional information for the four genes was estimated from aligned sequences using MEGA 2.1 (Kumar et al., 2001). Phylogenetic analyses were performed using PAUP 4.0b8a (Swofford, 2001). Data from the four genes were initially analyzed separately with maximum-parsimony (MP) analyses. Prior to combining the four datasets, congruence between them was examined with a partition-homogeneity test (Farris et al., 1995; Swofford, per. comm.), implemented in PAUP 4.0b8a (Swofford, 2001). Each nucleotide was treated as an unordered character with four alternative states, and gaps were considered as missing data in all analyses. Data were treated with equal weight for all analyses (Allard and Carpenter, 1996; Cibois et al., 1999; Kjer, 1995). For all phylogenetic analyses, both \( T. sanguinolentus \) and \( L. caucasia \) were used as outgroups.

Most parsimonious trees (MPTs) were generated using heuristic search routines with 100 random-addition sequences and TBR branch swapping. Support for the resulting nodes was assessed using bootstrapping with 1000 replications (Felsenstein, 1985a). Decay indices (Bremer, 1994) were calculated using Autodecay 4.0 (Eriksson, 1998) with the same search options as mentioned previously.

Modeltest 3.06 (Posada and Crandall, 1998) was used to select the substitution model for maximum-likelihood (ML) and neighbor-joining (NJ) analyses. Likelihood ratio tests indicated that GTR + G was the most appropriate for subsequent analyses. Settings for the GTR + G model were as follows: \( R \)-matrix = (1.4779, 15.5037, 1.8496, 0.8132, 23.9771, and 1.0000); base frequencies = (A = 0.3735, C = 0.2750, G = 0.1301, and T = 0.2214); proportion of invariant sites = 0; and the shape parameter of the gamma distribution = 0.2031. NJ trees were generated using PAUP 4.0b8a (Swofford, 2001) with the GTR + G model. Support for the NJ tree was assessed using bootstrapping with 1000 replications (Felsenstein, 1985a). A heuristic ML search with 10 random additional sequences and TBR branch swapping was performed with the GTR + G model. Tests for clocklike behavior of the combined data were performed with a molecular clock likelihood ratio test.

Bayesian inference (BI) was carried out using MrBayes2.0 (Huelsenbeck and Ronquist, 2001). Two separate runs were performed with four Markov chains, one starting from a random tree and another from the ML tree. The Markov chains were run for 2,000,000 generations. Sampling every 50 generations thinned the data to 40,000 sample points each run. The first 1000 samples from each run were discarded as burn-in, and the remaining samples analyzed using the “sumt” command (contype = allcompat) in MrBayes. Both independent runs found essentially identical tree topologies and posterior probabilities, indicating that the sample number was sufficient to permit the algorithm to converge on a global solution (Huelsenbeck et al., 2001).

### 2.5. Hypothetical testing and the mantel test

Alternative hypotheses (different MP and ML topologies) were compared by the nonparametric two-tailed Wilcoxon signed-ranks test (Felsenstein, 1985b; Templeton, 1983) and the Shimodaira–Hasegawa test (Goldman et al., 2000; Shimodaira and Hasegawa, 1999) implemented in PAUP 4.0b8a (Swofford, 2001). The alternative phylogenetic topologies (Appendix B) were reconstructed using MacClade (Maddison and Maddison, 1992).

The pairwise sequence divergences of the combined dataset were also calculated with the GTR + G model. Finally, for the well-supported group (Clade B1) within Clade B (the oviparous species group that excludes \( P. axillaris \), \( P. mystaceus \), and \( P. helioscopus \)), a Mantel test was performed using MANTEL (available from http://life.bio.sunysb.edu/morph/) to determine possible associations between geographic distance (km) and genetic distance among the sampling regions. The significance of the correlation was tested using a Monte Carlo approach based on 10,000 random permutations.

### 3. Results

#### 3.1. Authenticity of the mitochondrial sequences

All sequences are deposited in Genbank (Accession Nos. **AY053643–AY054119**). After alignment the four datasets were formed: 12S rRNA gene (367 bp), 16S rRNA (510 bp), Cyt \( b \) (307 bp), and ND4-tRNA\(^{LEU} \) (874 bp). Neither of the two protein-coding genes (Cyt \( b \) and ND4) had premature stop codons or ambiguous nucleotides in translation, suggesting that these sequences are functional genes. The dynamics of nucleotide substitutions and indels in the tRNA, 12S rRNA, and 16S rRNA genes were constrained to preserve the stable secondary structure, indicating functional genes. Furthermore, the compositional bias of the second codon position for T or C (T2 or C2; see Naylor et al., 1995) of the protein encoding genes (ND4: T2 = 39.6%, C2 = 31.0%; Cyt \( b \): T2 = 44.0%, C2 = 20.7%), and the strong bias against guanine on the light strand (\( G = 12.2–18.9% \), \( A = 32.2–37.6% \), \( C = 22.8–26.0% \), \( T = 20.1–30.6% \)), are all characteristic of the mitochondrial genome, but not the nuclear genome. Therefore, the
DNA sequences analyzed here should represent true mitochondrial genes and not nuclear transpositions (Macey et al., 2001; Zhang and Hewitt, 1996).

### 3.2. MP analysis for individual genes

Table 1 summarizes indices for the MP analyses of the four datasets. ND4-tRNA\textsuperscript{LEU} has the most parsimony-informative sites, and 16S rRNA has the least. Although the Cyt b locus did not amplify for 11 samples (MVZTP22375, KIZ-Rdq101, KIZ-Rdq102, KIZ-Rdq121, KIZ-Rdq122, KIZ-Rdq141, KIZ-Rdq142, KIZ-Rdq160, CIB-01337, CIB-01258, and CIB-01248), there are 104 parsimony-informative sites in the Cyt b dataset, based on the remaining samples. Each dataset supports the monophyly of the viviparous species, although there are slight differences in the relationship among oviparous species (not shown).

The partition-homogeneity test showed no significant incongruence among the four genes (P = 0.09), as is expected since the genes in the mitochondrial genome are inherited as a single entity without recombination (Nei, 1991). Thus, it is logical to combine and analyze all four genes as a single dataset.

### 3.3. Combined analysis

MP analysis of the combined dataset (2058 nucleotide characters with 796 variable sites and 580 parsimony-informative sites) generated 24 MPTs (CI = 0.563, RI = 0.837, RC = 0.471, L = 1877), and the strict consensus tree of these 24 MPTs is shown in Fig. 2A. Within the Chinese Phrynocephalus, two major clades are found: one containing all viviparous species (Clade A), and a second containing all oviparous species (Clade B). Clade A is strongly supported (BP = 100, DI = 26), and sister to a poorly supported Clade B (BP = 49, DI = 5).

Within Clade A, P. forsythii forms a well-supported monophyletic lineage, basal to the remaining viviparous species. The next most basal lineage is of the polyphyletic P. vlangalii. Samples collected from Tianzhu (Gansu Province) and Guide (Qinghai Province) form a monophyletic lineage (BP = 100, DI = 57). The more derived P. vlangalii haplotypes (1–4) form a well-supported clade which also contains the P. hongyuansensis haplotype (BP = 100, DI = 21). P. theobaldi is polyphyletic, with a well-supported group containing haplotypes 1–3 (BP = 100, DI = 23). The sister group contains P. theobaldi haplotypes 4–5 and P. zetangensis (BP = 100, DI = 12).

Clade B contains all nine oviparous species sampled in this study. There are four distinct groups within Clade B, three of which are well-supported (Fig. 2A). The most basal and least well-supported clade contains P. axillaris, P. mystaceus, and P. helioscopus. There is good support for P. axillaris, and P. helioscopus as monophyletic lineages (BP = 100, DI = 42 and BP = 100, DI = 57, respectively). However the position of P. mystaceus is not well-supported within this group.

All other sampled oviparous species, P. albolineatus, P. acutirostris, P. sp (Fuhai), P. guttatus, P. sp (Kaytun), P. frontalis, P. przewalskii, P. versicolor, and the remaining haplotypes from unidentified taxa, are contained in a well-supported clade (BP = 100, DI = 26) (Clade B1 in Fig. 2). The basal subclade contains P. albolineatus, P. acutirostris, P. sp (Fuhai), P. guttatus, and P. sp (Kaytun) (BI = 87, DI = 5). The P. frontalis/P. przewalskii clade is well-supported (BI = 100, DI = 13) and sister to a clade containing P. sp (Ganhezi) and P. versicolor (BP = 99, DI = 11). As current taxonomy stands, P. frontalis is paraphyletic with respect to P. przewalskii, P. versicolor with P. sp (Ganhezi) and P. sp 1–4 form two well-supported monophyletic groups (BP = 100, DI = 22, BP = 100, DI = 14, respectively).

The ML analysis of the combined data under the GTR + G model resulted in a ML tree with −ln = 11561.845 (not shown). The ML tree was then used as a starting tree for the BI analysis. Both independent BI runs found essentially identical tree topologies and posterior probabilities. The BI tree (congruent with the ML tree, Fig. 2B) shows higher resolution than the MP tree for some lineages, but in most cases shows the same topology. The BI tree indicated the following

<table>
<thead>
<tr>
<th>Comparison</th>
<th>ND4-tRNA\textsuperscript{LEU}</th>
<th>12SrRNA</th>
<th>16SrRNA</th>
<th>Cyt b</th>
<th>Combined data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of characters</td>
<td>874</td>
<td>367</td>
<td>510</td>
<td>307</td>
<td>2058</td>
</tr>
<tr>
<td>Number of variable characters</td>
<td>399</td>
<td>141</td>
<td>121</td>
<td>135</td>
<td>796</td>
</tr>
<tr>
<td>Number of parsimony-informative characters</td>
<td>305</td>
<td>90</td>
<td>81</td>
<td>104</td>
<td>580</td>
</tr>
<tr>
<td>Number of most parsimonious trees</td>
<td>56</td>
<td>444</td>
<td>12</td>
<td>48</td>
<td>24</td>
</tr>
<tr>
<td>Tree length</td>
<td>1038</td>
<td>273</td>
<td>209</td>
<td>324</td>
<td>1877</td>
</tr>
<tr>
<td>Consistency index (CI)</td>
<td>0.5241</td>
<td>0.6667</td>
<td>0.6842</td>
<td>0.5802</td>
<td>0.5631</td>
</tr>
<tr>
<td>Retention index (RI)</td>
<td>0.8252</td>
<td>0.8748</td>
<td>0.8986</td>
<td>0.8431</td>
<td>0.8366</td>
</tr>
<tr>
<td>Rescaled consistency index (RC)</td>
<td>0.4325</td>
<td>0.5832</td>
<td>0.6087</td>
<td>0.4892</td>
<td>0.4711</td>
</tr>
</tbody>
</table>

Tree length, CI, RI, and RC include uninformative characters.
Fig. 2. (A) The strict consensus tree derived from the MP analysis based on the combined mitochondrial DNA sequence dataset. The number above the branches is the bootstrap value; the decay index is indicated below the branches. (B) A Bayesian tree generated from the allcompat rule showing a consensus of 39,000 trees. The numbers on nodes are posterior probabilities. We defined two major clades (Clade A and Clade B) and one subclade (Clade B1) to facilitate discussion.
relationships: (P. vlangalii 2, (P. hongyuanensis, (P. vlangalii 4, (P. vlangalii 1, P. vlangalii 3)))) and (P. sp 3, (P. sp 1, (P. sp 2, P. sp 4))). The main exception is in the placement of P. mystaceus, which clusters with P. axillaris in the MP tree, but is basal to most basal clade within Clade B, and supported in the Bayesian tree (namely Clade B, the poorly supported nodes from Fig. 2A are also poorly helioscopus in the BI tree. It is noteworthy that the placement of P. mystaceus forms the most basal lineage in the NJ tree (not shown).

Although a likelihood ratio test rejects the hypothesis of a molecular clock in Clades A and B (−lnLno clock = 11561.845, −lnLclock = 11597.947, df = 47, P = 0.010506), estimating the approximate time of divergence can provide a crude indication of the formation process of the Chinese Phrynocephalus. The separation of Clades A and B was dated based on net average distance (p-distance) between two groups, i.e., 6.34% for ND4-tRNALEU and 4.54% for 12S rRNA + 16S rRNA, excluding P. axillaris, P. mystaceus, and P. helioscopus because of their uncertain phylogenetic position. Since the Cyt b locus did not amplify in 11 samples, only the rates derived from ND4-tRNALEU (1.13–2.04% per million years, estimated from the data of Malone et al., 2000, and Rassmann, 1997) and 12S rRNA + 16S rRNA (0.73–1.32% per million years) were used in the molecular calibration (Zamudio and Green, 1997). All estimated divergence times were within the last 6.2 million years (5.6–3.1 million years for ND4-tRNALEU and 6.2–3.4 million years for 12S rRNA + 16S rRNA).

Within Clade B (excluding P. axillaris, P. mystaceus, and P. helioscopus), pairwise genetic distances between haplotypes from different species or populations (as calculated with the GTR + G model) ranged from 0.0045 (P. sp (Kuytun) vs P. guttatus) to 0.0864 (P. sp (Fuhai) 2 vs P. frontalis 1 (Lanzhou)). There was a statistically significant positive correlation between increasing geographic distance and genetic distance (Mantel r = 0.708 (= normalized Mantel statistic Z); approximate Mantel t test: t = 14.49; ProbrandomZ < ProbobsZ: p = 1.0000), suggesting a recent history of dispersal for these oviparous species.

4. Discussion

4.1. Monophyly of viviparous species

Earlier studies have shown that all viviparous species share the following two characteristics: (1) a fontanel between the frontal and prefrontal bones in their skull (Wang and Macey, 1993); and (2) a karyotype with 2n=48 (12 large and 12 small pairs of chromosomes; Zeng et al., 1997). Using the DNA sequences from four mitochondrial genes, our phylogenetic analyses of the Chinese Phrynocephalus species strongly support the hypothesis of Wang and Macey (1993), who anticipated the monophyly of the viviparous lineage. Furthermore, these reconstructions indicate that P. forsythii is the basal taxon in the monophyletic viviparous group. The placement of P. forsythii into the lowland oviparous Chinese species clade, as suggested by the earlier allozyme study (Macey et al., 1993), is statistically rejected (Table 2).

Our reanalysis of Arnold’s (1999) data showed that the two characters recognized by this author: (1) no dark pigment in the mid-line area of belly in adults, and (2) oviparity, are not phylogenetically informative for P. forsythii (Wang and Macey, 1993; Zhao, 1997).

4.2. Phylogenetic relationships of the Chinese Phrynocephalus

Our phylogenetic reconstructions reveal that all viviparous species form a strongly supported monophyletic group (Clade A), which are mainly restricted to the Qinghai-Xizang (Tibetan) plateau (Fig. 1). On the other

<table>
<thead>
<tr>
<th>Tree</th>
<th>No. of extra steps</th>
<th>Different − ln</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1a</td>
<td>15</td>
<td>19.5453</td>
<td>0.0003</td>
</tr>
<tr>
<td>T2b</td>
<td>22</td>
<td>31.3784</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>T3c</td>
<td>23</td>
<td>49.76999</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>T4d</td>
<td>48</td>
<td>115.0388</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

Constrained topologies were evaluated against the optimal topology with Templeton (1983; MP) and Shimodaira–Hasegawa (1999; ML) tests. Associated probabilities are given.

a P. frontalis monophyly (Zhao, 1997).

b P. vlangalii monophyly (Pope, 1935; Zhao, 1997).

c P. theobaldi is sister to P. zetangensis (Wang et al., 1999).

d Paraphyly of viviparous species clade (Macey et al., 1993; Macey, per.comm.).
hand, the MP, ML, and BI trees show that all oviparous species examined here form a poorly supported monophyletic group (Clade B), and the basal relationships within Clade B are not resolved. The NJ tree shows that *P. mystaceus* forms the most basal lineage. In all analyses, *P. albolineatus, P. acutirostris, P. sp (Fuhai), P. guttatus, P. sp (Kuytun), P. frontalis, P. przewalskii, and P. versicolor*, and the remaining haplotypes from unidentified taxa, are included in a well-supported clade (Clade B1), which are distributed in the northern deserts of China along the northern slope of Tianshan Mountain (Fig. 1). *P. axillaris* is sympatrically distributed with viviparous *P. forsythii* in the south-western Xinjiang Uygur Autonomous Region (Fig. 1). *P. mystaceus* and *P. helioscopus* are only found in the Ili Prefecture of the Xinjiang Uygur Autonomous Region and the north-western Xinjiang Uygur Autonomous Region, respectively (Fig. 1).

*Phrynocephalus mystaceus* has a high level of anatomical variability (Ananjeva, 1986), which together with its unique karyotype (Zeng et al., 1997) has led to its uncertain taxonomic classification. In fact, it was once classified as a monotypic species in the proposed genus *Megalochilus* (Eichwald, 1831). In our phylogenetic analyses based on several tree constructing methods, the position of *P. mystaceus* is uncertain, and further study will be required to resolve its position within this lineage. The lack of phylogenetic resolution of *P. axillaris, P. helioscopus*, and *P. mystaceus* (Fig. 2) may be attributed to limited sampling, as our primary focus was on the Chinese *Phrynocephalus* species. Further sampling of the remaining species within this genus, including a broader-based sampling from each of these three species, may facilitate resolution at the more basal nodes (Murphy, per. comm.).

The classifications of a number of other species also remain uncertain on the basis of our reconstructions and hypothetical group tests (Table 2). For example, *P. vlangali* is clearly polytypic and *P. hongyuanensis* (Wang and Jiang, 1992) clusters within one of the *P. vlangali* lineages. *P. frontalis*1 (Lanzhou) (Pope, 1935) clusters within the *P. przewalskii* lineage, and *P. zetangensis* (Wang et al., 1996) clusters within one of two well-defined *P. theobaldi* lineages. In addition, a ((*P. sp (Ganhezi), P. sp 1–4), *P. versicolor*) relationship is highly supported in all of our analyses. These findings suggest that the speciation of the Chinese *Phrynocephalus* is probably more complex than indicated by our present knowledge, and could only be illuminated by further combined field, morphologic and molecular studies.

4.3. Biogeographical implications

The sand lizards (*Prynocephalus* sp.) have been shown to have high fidelity to their inhabited areas (sand dunes or the Gobi desert), and unsuitable environment prevents dispersal among habitats (Wang and Macey, 1993; Murphy, per. comm.). Therefore, some biogeographical implications can be drawn from combining data on the geologic history and the phylogenetic pattern of the Chinese *Phrynocephalus* species. Our phylogenetic analyses suggest that these species show evidence of evolution by both vicariance and dispersal.

Many studies on squamate reptiles have shown that viviparity often evolved in cold climates (Andrews, 2000; Blackburn, 2000; Heulin et al., 1991; Shine, 1985, 1995; Surget-Groba et al., 2001). An earlier hypothesis proposed that the origin of the viviparous species group was the result of a vicariance event associated with the uplifting of the Qinghai-Xizang (Tibetan) plateau (Wang and Macey, 1993). This original hypothesis has been recently refined by Zeng et al. (1997) and Wang et al. (1999) based on karyotypic studies and a morphological clustering of the *P. theobaldi* species group (including *P. theobaldi, P. zetangensis*, and *P. erythrurus*), respectively.

Wang and Macey’s (1993) hypothesis also helps to explain the monophyly of Clade A. Recent geologic studies of the Qinghai-Xizang (Tibetan) plateau reveal that during the late Miocene (10 MYBP) the plateau gradually became arid, which may have been the result of rain shadowing caused by the uplifting of the Himalayan mountains (see Shi et al., 1998). In the late Pliocene, the plateau underwent 3000 m of additional uplifting to reach an average 5000 m in elevation, and during this period the climate on the plateau cooled (Shi et al., 1998). This event could have led to the evolutionary divergence of the viviparous and oviparous species (assuming that the ancient and modern populations of the viviparous species have similar distributions). During this phase considerable strike-slip faulting and volcanic activity took place that resulted in the creation of mountainous regions, together with the flat valley plains now present on the plateau (Shi et al., 1998). Therefore, most speciation of the viviparous species could have occurred after the topography of the Qinghai-Xizang (Tibetan) plateau diversified (Wang et al., 1999). It is known that the Qinghai-Xizang (Tibetan) plateau was not completely glaciated during the Quaternary Glaciations (Shi et al., 1998), which provided a good opportunity for the differentiation of ancestral viviparous species. The estimated divergence times between Clades A and B (all less than 6.2 million years) were consistent with the third phase of uplifting of the the Qinghai-Xizang (Tibetan) plateau, i.e., in the Pliocene. However, these rough divergence timings should be interpreted with caution, as they are based on calibrations derived from other species (a strategy necessitated by the lack of fossil data) (Zhang and Ryder, 1995).
Zeng et al. (1997) proposed that the uplifting of the Kunlun Mountain isolated ancient *P. forsythii* on the northern slope of the mountain, separating it from the other viviparous species. The ancient anatomical characteristics of *P. forsythii*, such as a long tail and long appendages, support this hypothesis (Wang et al., 1999). All of our analyses show that *P. forsythii* is the most basal and ancient species within the monophyly for viviparous species, supporting the hypothesis proposed by Zeng et al. (1997). This result suggests that *P. forsythii* dispersed into lowland habitats after splitting from the remainder of Clade A.

For Clade B, the species in the well-supported group (Clade B1) are distributed allopatrically, but also sympatrically in some contact zones in the northern deserts of China. The current deserts of north China are almost connected and there are few geographic barriers to dispersal. Comparatively low genetic distances (not shown), the results of the Mantel’s test, and the shape of the trees (many short branch lengths in the NJ, ML, and BI trees (Fig. 2B)) reflect the geologic history of a recent radiation in this group. In addition, geological studies indicate that the formation of the Chinese desert is recent (Xia and Hu, 1993). Therefore, the most plausible explanation for this pattern is a recent history of dispersal for these species. Recent dispersal of *P. axillaris* is also suggested by the low level of nucleotide divergence among those samples. Less than 1.3% nucleotide divergence is found among four haplotypes of *P. axillaris*, while the geographic separation is more than 1000 km.

**Acknowledgments**

This work was supported by grants from the State Key Basic Research and Development Plan (G2000046806), the Chinese Academy of Sciences (KSCX2-1-05 and KSCX2-1-06A), the Program for Key International S&T Cooperation Project of China (2001CB711103) and the NSFC. We thank Professor Guo Li (Inner Mongolia University), Rao Dingqi (Kunming Institute of Zoology, Chinese Academy of Sciences), Wei Yusheng (Lanzhou University), and Liu Zhijun (Chengdu Institute of Biology, Chinese Academy of Sciences) for their help in collecting samples.

**Appendix A**

The species examined, sample sizes, localities, and museum numbers are listed. The taxonomy used is after Zhao and Alder (1993) and Fauna Sinica (Zhao, 1999). Acronyms are CIB, Chengdu Institute of Biology, Chinese Academy of Sciences; KIZ Kunming Institute of Zoology, Chinese Academy of Sciences; MVZ, Museum Vertebrate Zoology, University of California at Berkeley. CIB-XM represents Xiaomao Zeng’s field number for uncatalogued specimens that were deposited at the Chengdu Institute of Biology, Chinese Academy of Sciences. KIZ-P and KIZ-W represent field numbers from Junfeng Pang for uncatalogued specimens that were deposited at the Kunming Institute of Zoology, Chinese Academy of Sciences. KIZ-Rdq represents a field number from Dingqi Rao for uncatalogued specimens that were deposited at the Kunming Institute of Zoology, Chinese Academy of Sciences. MVZ-RM represents field numbers from Robert J. Macey for uncatalogued specimens that were deposited at the Museum Vertebrate Zoology, University of California at Berkeley. MVZ-TP represents a field number from Theodore J. Papenfuss for uncatalogued specimens that were deposited at the Museum Vertebrate Zoology, University of California at Berkeley.

*P. hongyuuanensis*, *n* = 4. Hongyuan Prefecture, Sichuan Province, P.R. China, CIB-Xm276, CIB-Xm277, CIB0714, CIB0541.

*P. viangalli*, *n* = 15. Guide Prefecture, Qinghai Province, P.R. China CIB0771-3; Dachaigou of Tianzhu Prefecture, Gansu Province, CIB0709, CIB0769; Golmud Prefecture, Qinghai Province, KIZ-Rdq1-3; Xiangrige Farm, Qinghai Province CIB0796-7; Dulan Prefecture, Qinghai Province, CIB0792-3; Suhai Lake, Qinghai Province, CIB0970, CIB0972; Aksay Prefecture, Gansu Province, CIB0740.


*P. theobaldi*, *n* = 10. Lhasa, Tibet Autonomous Region, P.R. China, CIB0070-1, KIZ-Rdq160; Mailing Prefecture, Tibet Autonomous Region, CIB7495; Saga Prefecture, Tibet Autonomous Region, KIZ-Rdq101-2; The 20th road control from Saga to Ngamring, Tibet Autonomous Region, KIZ-Rdq121-2; Maquan Lake, Tibet Autonomous Region, KIZ-Rdq141-2.

*P. forsythii*, *n* = 5. Jamtai of Wensu Prefecture, Xinjiang Uygur Autonomous Region, P.R. China, CIB01037-8; The Airport of Aksu, Xinjiang Uygur Autonomous Region, CIB01097, CIB01099; Kashi, Xinjiang Uygur Autonomous Region, CIB0004.

*P. mystaceus*, *n* = 1. Ili Prefecture, Xinjiang Uygur Autonomous Region, P.R. China, MVZ-TP22375.

*P. acutirostris*, *n* = 2. 216 road (Urumqi–Fuyun), 170 km far from Urumqi, Xinjiang Uygur Autonomous Region, P.R. China, CIB01041-2.

*P. albolineatus*, *n* = 2. 222 road, south of Tacheng, Xinjiang Uygur Autonomous Region, P.R. China, CIB01000.

*P. frontalis*, *n* = 6. Bailing Temple, Inner Mongolia Autonomous Region, P.R. China, CIB01337; Baotou, Inner Mongolia Autonomous Region, CIB01258; Yulin Prefecture, Shaanxi Province, CIB01248; Lanzhou, Gansu Province, KIZ-W1-3.
Appendix B


The MPT tree or ML tree derived by constraining P. frontalis to form a monophyletic group (T1 in Table 2): MPT.(1,(((2, ((14,15),16)),(17,32)),3),(((24,26),25),(10,((11,12),13)),(((27,28,29),31),((38,39,40))),(((4,7),5,6),23),((18,20,21),19)),((8,9),((22,(44,44,45),(43,33,35),34),((36,((37,47,48))))),(41,46))),49);ML.(1,(((((((2, ((14,15),16)),(17,32)),3),(((24,26),25),(10,((11,12),13)),)(((27,28,29),31),((38,39,40))),(((4,7),5,6),23),((18,20,21),19)),((8,9),((22,(44,44,45),(43,33,35),34),((36,((37,47,48))))),(41,46))),49).

The MPT tree or ML tree derived by constraining P. vlangalii to form a monophyletic group (T2 in Table 2): MPT.(1,(((2, ((14,15),16)),(17,32)),3),(((10,24,26),25),((11,12),13)),(((27,28,29),31),((38,39,40))),(((4,7),5,6),23),((18,20,21),19)),((8,9),((22,(44,44,45),(43,33,35),34),((36,((37,47,48))))),(41,46))),49);ML.(1,(((((((2, ((14,15),16)),(17,32)),3),(((10,24,26),25),((11,12),13)),)(((27,28,29),31),((38,39,40))),(((4,7),5,6),23),((18,20,21),19)),((8,9),((22,(44,44,45),(43,33,35),34),((36,((37,47,48))))),(41,46))),49).

The MPT tree or ML tree derived by constraining monophyletic P. theobaldi is sister to monophyletic P. zetangensis (T3 in Table 2): MPT.(1,(((2, ((14,15),16)),(17,32)),3),(((10,24,26),25),((11,12),13)),(((27,28,29),31),((38,39,40))),(((4,7),5,6),23),((18,20,21),19)),((8,9),((22,(44,44,45),(43,33,35),34),((36,((37,47,48))))),(41,46))),49);ML.(1,(((((((2, ((14,15),16)),(17,32)),3),(((10,24,26),25),((11,12),13)),)(((27,28,29),31),((38,39,40))),(((4,7),5,6),23),((18,20,21),19)),((8,9),((22,(44,44,45),(43,33,35),34),((36,((37,47,48))))),(41,46))),49).

The MPT tree or ML tree derived by constraining paraphyly of viviparous species clade (T4 in Table 2): MPT.
References


Kumar, S., Tamura, K., Jakobsen, I., Nei, M., 2001. MEGA2: Moleculer Evolutionary Genetics Analysis Software. Arizona State University, Tempe, Arizona, USA.


