Coalescence patterns of endemic Tibetan species of stream salamanders (Hynobiidae: Batrachuperus)

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Abstract

Orogenesis of topographically diverse montane regions often drives complex evolutionary histories of species. The extensive biodiversity of the eastern edge of the Tibetan Plateau, which gradually decreases eastwardly, facilitates a comparison of historical patterns. We use coalescence methods to compare species of stream salamanders (Batrachuperus) that occur at high and low elevations. Coalescent simulations reveal that closely related species are likely to have been influenced by different drivers of diversification. Species living in the western high-elevation region with its northsouth extending mountains appear to have experienced colonization via dispersal followed by isolation and divergence. In contrast, species on the eastern low-elevation region, which has many discontinuous mountain ranges, appear to have experienced fragmentation, sometimes staged, of wide-ranging ancestral populations. The two groups of species appear to have been affected differently by glaciation. High-elevation species, which are more resistant to cooler temperatures, appear to have experienced population declines as recently as the last glaciation (0.016–0.032 Ma). In contrast, salamanders dwelling in the warmer and wetter habitats at low-elevation environs appear to have been affected less by the relatively recent, milder glaciation, and more so by harsher, extensive glaciations (0.5–0.175 Ma). Thus, elevation, topography and cold tolerance appear to drive evolutionary patterns of diversification and demography even among closely related taxa. The comparison of multiple species in genealogical analyses can lead to an understanding of the evolutionary drivers.

Keywords: coalescent simulation, diversification drivers, ecological niche modelling, elevation effects, historical demography, Tibetan uplift

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Introduction

Tectonic movement, orogenesis and climatic change can drive species diversification especially via allopatric speciation. For terrestrial species, this can occur by the formation of sky islands in which dry, hot, deep valleys serve as barriers to gene flow (Knowles 2000), as well as the height of mountains forming a barrier to dispersal for species that live in the valleys (Craw et al. 2008). For aquatic species, orogenesis can divide and change the courses of rivers (Haffer 2008). Such drivers are exemplified by one region, the Tibetan Plateau (Che et al. 2010; Zhang et al. 2010). This region’s extensive biodiversity and well-studied complex geology facilitate investigations on the genetic consequence of orogenesis. For example, the eastern edge of the Tibetan Plateau contains major features that might have played important roles in the divergence of some endemic vertebrates (Yu et al. 2000; Luo et al. 2004; Peng et al. 2006). However, the processes that shaped the patterns remain to be confirmed. Are all extant populations derived from the fragmentation of a widely distributed common ancestral population, sequentially staged isolation...
events or colonization via a series of dispersals followed by isolation and divergence?

Genetic patterns can identify historical events and the associated driving processes. For example, the European alpine system is topographically complex, and the resident species have correspondingly complex evolutionary histories and patterns of divergence (Despres et al. 2002; Kropf et al. 2003). Topography can drive divergence patterns (Smith & Farrell 2005) and Pleistocene climatic fluctuations associated with cyclical glaciation events can also be involved, even in lower latitudes (Bryson et al. 2011a,b). The eastern edge of the Tibetan Plateau is extremely complicated geologically. It is composed of several nonuniform landform assemblages, yet it can be defined as having two main topographical areas: the western highland and eastern lowland. The highland area includes various northsouth extending mountain ranges, such as the Shaluli and Daxue mountains. High-elevation montane taxa might have dispersed either from north to south or vice versa along the crest of the mountain ranges during orogenesis of the Tibetan Plateau and in conjunction with associated climatic changes. In contrast, the lowland area is comprised of many isolated mountains, such as Emei, Luoji and Bailing mountains, that are separated by deep valleys. The physiographic structure would probably inhibit dispersal and promote fragmentation, especially for taxa that have limited dispersal abilities. The more continuous high-elevation habitat and ongoing orogenesis in the west would be expected to lead to a pattern of sequential isolation when compared with a more uniform early fragmentation pattern in the east. Therefore, the two topographical developments may be the drivers of contrasting patterns of genetic divergence.

The effects of Pleistocene glacial cycling on populations depend on latitude and topography. Responses to climatic oscillations appear to vary among species as a function of cold tolerance (Hewitt 1996, 2004). Whereas heavy ice is known to have covered high-latitude continental Europe and North America, glaciations occurred discontinuously on high mountains of the eastern edge of the Tibetan Plateau; low-elevation habitats are thought to have been ice free (Shi et al. 1998; Zhou et al. 2006). Climatic oscillations appear to have mitigated demographic stresses for species at lower relative to higher elevations (Qu et al. 2010).

Orogenesis of the Tibetan Plateau is known to have continued during the Late Pleistocene. A combination of uplifted terrain and lowered snowlines during this period may be responsible for previously low, glacier-free regions becoming glaciated (Shi et al. 1997; Liu et al. 1999; Zheng et al. 2002). Whereas climatic shifts in montane regions can affect the distributions of temperate species, some cold-tolerant taxa may persist in ice-free areas (Crespi et al. 2003; Smith & Farrell 2005; Shepard & Burbright 2008). Variation in lineage-specific cold tolerance complicates the analyses of elevational patterns because all taxa do not respond equally to climatic changes.

Species of *Batrachuperus* (family Hynobiidae) are primarily restricted to isolated montane regions of the Tibetan Plateau’s eastern edge. These salamanders have weak dispersal abilities, and they are likely to have experienced several orogenic events and glacial cycles (Zhang et al. 2006). The well-established phylogeny (Fu & Zeng 2008) provides a solid foundation for testing hypotheses regarding the ecological and physiographic drivers of genetic divergence. The species appear to be parapatric, yet they are separated by elevation. Elevational differences between sister species provide an opportunity to investigate responses to topographical variation and Pleistocene glaciation, the two most important factors influencing the current spatial distributions of species occurring in the Tibetan Plateau and places of similar topography (Hewitt 2000; Zhang & Jiang 2006).

Recent methodological developments facilitate the ability to infer historical processes. In particular, hypothesis tests employing coalescent-based methods allow for the statistical evaluation of the fit of divergence timing to various historical scenarios while taking into account the stochasticity of genealogical relationships (Knowles & Maddison 2002; Knowles 2004, 2009). Further, ecological modelling allows for the identification of significant differences in the environments of species and enables the prediction of suitable environmental conditions (Carstens & Richards 2007; Shepard & Burbright 2008).

Herein, we compare the patterns of diversification and demography in species of *Batrachuperus* living at high and low elevations using coalescent-based methods and a combination of historical demography and ecological niche modelling. Specifically, we test hypotheses involved in the following questions: (i) How was divergence driven in closely related, high- and low-elevation species? (ii) How did these species respond to glacial cycles as a result of elevation and cold-tolerant physiology?

Material and methods

Species sampling and lab protocols

We selected five species of *Batrachuperus* distributed in the montane eastern edge of the Tibetan Plateau (Fu & Zeng 2008) including *B. karlschmidti*, *B. londongensis*, *B. pinchonii*, *B. yenyuanensis* and an undescribed species; the undescribed species, mainly distributed in the
central and southern Shaluli Mountains, was referred to herein as ‘Daocheng species’. It corresponded to Fu & Zeng’s (2008) *Batrachuperus karlschmidtii* and Daocheng species occurred at western high elevations (3500–4300 m) such as Shaluli and Daxue mountains, while *B. londongensis*, *B. pinchonii* and *B. yenyuanensis* were mainly in eastern lower reaches (1800–3200 m), such as Emei, Luoji and Bailing mountains. A total of 418 specimens of *Batrachuperus* from 54 locations were sampled between 1999 and 2009 from throughout the species’ distributions (Fig. 1; Appendix S1, Supporting information). We also downloaded previously published sequences of *Batrachuperus tibetanus* (GenBank: DQ333817), *Pseudohynobius shuichengensis* (GenBank: FJ532060), *Hynobius quelpaertensis* (EF201847), *Liua shihi* (DQ333810), *Pachyhynobius shangchagensis* (DQ333812), *Paradactylodon mustersi* (DQ333821) and *Ranodon sibiricus* (AJ419960) for the subsequent phylogenetic analysis and age estimation.

Total genomic DNA was extracted using the high-salt method (Aljanabi & Martinez 1997). Liver or muscle tissue of alcohol-preserved specimens was minced, 20 μL 20 mg/mL Proteinase-K was added and then the mixture was incubated at 56 °C for 6 h. Three mitochondrial gene fragments were sequenced: cytochrome b (Cytb), cytochrome c oxidase subunit I (COI) and NADH dehydrogenase 5 (ND5). Slightly modified primers Bacytb1F (5′-GAACTAATGGCCCACCCAATTCGAA-3′) and Bacytb1R (5′-AAYARAAAATATCAYTCYGTTGRAT-3′) based on MVZ15 and MVZ16 were used for amplifying Cytb (Mueller et al. 2004). Using conserved portions of complete mitochondrial genomes of stream salamanders (Zhang et al. 2006), we designed two pairs of primers. Baco1-F9 (5′-CACTCGATGACATTGATTTGAC-3′) and Baco1-R943 (5′-GGATRGCAATAATTATTGTGGCGGAGTT-3′) were used for amplifying COI, and Band5-F1 (5′-ATGAATTCCGTATTTTAATTAC-3′) and Band5-R939 (5′-TAACCTTTGCGTACGTTGA-3′) were used for amplifying ND5. A reaction volume of 50 μL contained 2 mM MgCl₂, 0.2 mM of each dNTP, 0.2 μM of each primer and 2.5 U of Taq DNA polymerase (TianGen Biotech) with 20–100 ng template DNA. PCR involved 5 min at 94 °C, then 35 cycles with 94 °C for 30 s, 50 °C (Cytb) or 55 °C (COI and ND5) for 40 s and 72 °C for 1 min, followed by incubation for 10 min at 72 °C. The amplified DNA products were purified, and automated DNA sequencing was performed on an ABI3730 with an ABI PRISM BigDye terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer Biosystems). The three fragments were concatenated into one data set. All sequences were aligned using BIOEDIT 7.0.5 with the CLUSTAL W option and default multiple alignment parameters. Haplotype identification, nucleotide diversity (θ) and haplotype diversity (Hd) per lineage were estimated in DNAsp 5.10.01 (Librado & Rozas 2009) (Table 1).

**Genealogical reconstruction**

Bayesian inference analyses (BI) were performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Three partitioning strategies were applied to our data set to determine the optimal strategy. First, we defined the concatenated data set as one partition. Second, we defined a separate partition for each gene (three partitions). Third, we divided the data set into nine partitions according to each codon position in each gene. The multiple partitions controlled for heterogeneity

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**Fig. 1** Map showing collecting localities for salamanders of the genus *Batrachuperus* on the eastern edge of the Tibetan Plateau, southwestern China (see Appendix S1, Supporting information for details).
across data set, such as variation in substitution rates. The best-fitting substitution model for each partition was selected using JMODELTEST 0.1.1 (Posada 2008) (Table 2). Bayesian information criterion (BIC) was used to select a model because of its high accuracy and precision (Luo et al. 2010). For each partition strategy, Markov chains were run for 10 million generations with two parallel searches using one cold and three heated chains, each starting with a random tree. Trees were sampled every 1000 generations using split frequencies <0.01 to indicate convergence. TRACER 1.5 (Drummond & Rambaut 2007b) was used to determine when the log likelihood (lnL) of sampled trees reached a stationary distribution. In all Bayesian analyses, apparent stationarity of Markov Chain Monte Carlo (MCMC) runs was reached within 1 million generations; we conservatively discarded the first 5 million generations from each run as ‘burn-in’ and used the sampled trees from the remaining 5 million generations (5001 trees) to calculate the frequency of nodal resolution in a 50% majority-rule consensus tree, termed Bayesian posterior probabilities (BPPs). Three replicate analyses were conducted to assess whether or not individual runs failed to converge upon the optimal posterior distribution and if likelihood values, branch lengths, tree topology and posterior probabilities differed between runs. The relative fit of different partitioning strategies was evaluated using Bayes factors (BF) in TRACER. A BF > 10 was considered strong evidence for using a more-partitioned model (Kass & Raftery 1993). Maximum-likelihood (ML) analyses were performed under the BF-preferred partitioning strategy using the GTR+G model in the program RAXML 7.0.3 (Stamatakis 2006). Additional ML analyses were run using the alternative partitioning schemes to check for sensitivity to BF. A likelihood ratio test was performed to verify the BF-preferred partitioning strategy. For estimating nodal support, nonparametric bootstrap proportions (Felsenstein 1985) with 500 replicates were used. Maximum-likelihood bootstrap proportions (MLBs) $\geq 70\%$ and BPPs $\geq 0.95$ were considered strong support (Hillis & Bull 1993; Erixon et al. 2003; Huelsenbeck & Rannala 2004). The approach potentially assessed discordance among trees based on the implemented method of inference and provided a preferred topology for divergence estimates.

### Divergence time estimation

Topology, divergence dates and substitution rates were simultaneously estimated using BEAST 1.6.1 (Drummond et al. 2002; Drummond & Rambaut 2007a). The best-fitting substitution model for each data partition under the BF was chosen as in the BI analyses. Application of the optimal model avoided over-parameterization of the

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### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Lineage</th>
<th>n</th>
<th>h</th>
<th>$\pi$</th>
<th>Hd</th>
<th>SSD</th>
<th>r</th>
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<td>Daxue Mtn C</td>
<td>19</td>
<td>12</td>
<td>0.00507</td>
<td>0.924</td>
<td>0.03210</td>
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<td>Gan Zi</td>
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<td></td>
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<td>0.969</td>
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<td>9</td>
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<td>18</td>
<td>0.00952</td>
<td>0.871</td>
<td>0.07209**</td>
<td>0.06890**</td>
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<td>4</td>
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<td>0.02718</td>
<td>0.06106*</td>
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<tr>
<td></td>
<td>Total</td>
<td>77</td>
<td>40</td>
<td>0.02893</td>
<td>0.959</td>
<td>0.00938</td>
<td>0.00911***</td>
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<td>15</td>
<td>10</td>
<td>0.00201</td>
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<td>0.00947</td>
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<td>Yulongxue Mtn</td>
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<td>0.01766</td>
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<td>Total</td>
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<td>50</td>
<td>0.02320</td>
<td>0.867</td>
<td>0.04047*</td>
<td>0.02748***</td>
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<td>11</td>
<td>4</td>
<td>0.00184</td>
<td>0.745</td>
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<td>Bailing Mtn</td>
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<td>18</td>
<td>0.00445</td>
<td>0.983</td>
<td>0.00651</td>
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<td>Luoji Mtn</td>
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<td></td>
<td>Total</td>
<td>62</td>
<td>39</td>
<td>0.01675</td>
<td>0.977</td>
<td>0.01440**</td>
<td>0.00432</td>
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</tbody>
</table>

$n$, number of individuals; $h$, number of haplotypes; $\pi$, nucleotide diversity; Hd, haplotype diversity; SSD, sum of square deviation (goodness of fit to a simulated population expansion); $r$, raggedness index ($^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$).
model and reduced the trade-off between bias and unnecessary variance (Kelchner & Thomas 2007). Prior to the BEAST analysis, we ran analysis using different clock models (non-clock, strict clock and relaxed clock) in MRBAYES to determine whether our data set was evolving in a clocklike fashion and which clock model was more appropriate for our data set. For each clock model, Markov chains were run for 10 million generations. Trees were sampled every 1000 generations. BF was used to select among the models and to test the null hypothesis of a linear rate of change (Weisrock et al. 2001). For the BEAST analysis, an initial MCMC was run 20 million generations with an uncorrelated lognormal clock model and a constant population size prior to determine the optimal parameter in BEAST. TRACER was used to examine the parameter ucld.stdev to test for departures from the molecular clock. A parameter value near zero suggested that our data evolved in a clocklike manner. An additional BEAST analysis with an enforced strict clock was performed to compare the result using the relaxed clock approach. The final MCMC was run for 20 million generations while sampling every 1000 generations. Five independent runs achieved the same result. Burn-in and convergence of the chains were determined with TRACER. Further, effective sample sizes (ESS) were required to have values greater than 200.

Divergence times for Batrachuperus were estimated using two different methods: a calibration method and a mean substitution rate method. First, we used the fossil record and previously established divergence times among hynobiid genera (Zheng et al. 2011) to calibrate the clock. The split between Ranodon and Paradactylodon was constrained to be at least 2.6 Ma based on the fossil Ranodon cf. sibiricus reported from the Upper Pliocene (Averianov & Tjutkova 1995). We did not download data for Andrias davidianus because the commonly used cryptobranchid fossil (c. 145 Ma) (Gao & Shubin 2003; Marjanovic & Laurin 2007; Zhang & Wake 2009; Roe-lants et al. 2011; Zheng et al. 2011) was probably too old to be directly applicable to our younger taxa (Zhang et al. 2006), and it might have caused an overestimation of the divergence dates (Hugall et al. 2007; Zheng et al. 2011). Slowly evolving nuclear exons have been suggested to be appropriate to correct potential bias in estimating divergence dates, especially for a large-timescale phylogeny (Diego 2010; Zheng et al. 2011). Consequently, we used the divergence dates recently reported by Zheng et al. (2011) based on nuclear exons. Conservatively, we estimated priors for the divergence between Hynobius and Pseudohynobius-Liu-Batrachuperus to be 30 ± 14 Ma (mean ± 95% CI), and among Batrachuperus to 13 ± 8 Ma, which followed normal distributions. We applied all three constraints in this analysis.

Both intra- and interspecific hierarchical levels were included in our divergence time analysis. Rates of long-term (species level) substitutions have been reported to

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**Table 2** Nucleotide substitution models used in genealogical reconstruction, divergence date estimation (intra- and interspecific), demographic history estimation and coalescent simulations. The estimation of \( \theta \) and \( N_e \) was also presented

<table>
<thead>
<tr>
<th>Partition</th>
<th>Population level</th>
<th>Species level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Best model</td>
<td>Pinvar</td>
</tr>
<tr>
<td>ND5_1</td>
<td>GTR+G</td>
<td>0.241</td>
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<tr>
<td>ND5_2</td>
<td>GTR+I+G</td>
<td>0.249</td>
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<tr>
<td>ND5_3</td>
<td>HKY+G</td>
<td>1.427</td>
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<td>Cytb_1</td>
<td>SYM+G</td>
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<td>Cytb_2</td>
<td>HKY+I</td>
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<td>COI_1</td>
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<tr>
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<td>F81</td>
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<tr>
<td>COI_3</td>
<td>GTR+G</td>
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**BSPs and coalescent simulations**

<table>
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<th>Species level</th>
<th>Best model</th>
<th>Pinvar</th>
<th>Gamma</th>
</tr>
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<tbody>
<tr>
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<td>GTR+I+G</td>
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<td>0.305</td>
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<td>Batrachuperus londongensis</td>
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<td></td>
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<td>GTR+G</td>
<td>0.011</td>
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<tr>
<td>Daocheng species</td>
<td>HKY+I+G</td>
<td>0.536</td>
<td>0.305</td>
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<td>Batrachuperus yenyuanensis</td>
<td>HKY+I+G</td>
<td>0.319</td>
<td>0.018</td>
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</table>

BSP, Bayesian skyline plots; COI, cytochrome c oxidase subunit I; Pinvar, proportion of invariable sites; Gamma, gamma shape parameter; CI, confidence interval.
often strongly conflict with short-term (population level) substitutions. Thus, results based on implied rates of change across different hierarchical timescales have been suggested to be invalid and often overestimated (Ho et al. 2005). To investigate the possible effect this phenomenon might have had on our results, we also performed BEAST analyses using only species-level sampling with calibration. The Yule process was used to describe speciation under relaxed clock model. The results were used as interspecific times of divergences for Batrachuperus. The divergence dates and substitution rates estimated using this calibration method were applied to subsequent coalescent simulations.

To compare the results from the calibration methods, we calculated divergence dates using an estimated mean substitution rate (0.64% per MY per lineage) for our combined sequence data. Yoshikawa et al. (2008) reported a substitution rate of 0.68% per MY per lineage for Cytb in another hynobiid (Onychodactylus japonicas). This rate was similar to that estimated for other salamanders, for example an average rate of 0.62% per MY per lineage for plethodontids (Mueller 2006). The average net p-distance for the combined sequences vs. Cytb alone was 0.947. Therefore, the estimated mean substitution rate for the combined data was obtained by multiplying the Cytb rate of O. japonicas (0.68%) by the p-distance ratio for Cytb alone (0.947).

Coalescent simulations

Coalescent simulations were conducted in Mesquite 2.7.2 (Maddison & Maddison 2010). These were used to both investigate the possible drivers of diversification and test whether or not the timing of diversification within each species of Batrachuperus was consistent with the most recent uplift of the Tibetan Plateau and to test the climatic shift hypotheses.

Three possible models of diversification were tested: fragmented ancestor, staged fragmentation and colonization. Dates for hypotheses testing corresponded to the approximate age estimated for each species in the BEAST analysis, the most intensive and frequent uplift of the Tibetan Plateau (Li et al. 1996; Li & Fang 1999; Wu et al. 2001), and the initial Pleistocene climatic changes (2.6–3.6 Ma). These absolute times (years) were converted to coalescent times (generations) assuming a generation time of 3.5 years for the species of Batrachuperus (Han & Lu 1999). ML estimations of \( N_e \) (inbreeding effective population size) used \( \theta \)-values were calculated in the program Migrate-N 3.2.1 (Beerli & Felsenstein 1999, 2001). Using the 0.69% per lineage substitution rate produced in the BEAST analysis and a generation time of 3.5 years, the \( \theta \)-value was then converted to \( N_e \) using the formula \( \theta = N_e^2 \mu \) for mitochondrial DNA, with \( \mu = 2.42 \times 10^{-8} (3.5 \times 0.69 \times 10^{-8}) \) substitutions per site per generation (Table 2). For the coalescent simulations, the overall \( N_e \) was set to empirically estimated values. The \( N_e \) of the putative regional population was constrained to a size proportional to the overall \( N_e \) at any single point on a branch. DNA sequences were simulated under the same evolutionary model of the empirical data, as selected by JModelTest 0.1.1 (Posada 2008) (Table 2). For each simulation, 100 coalescent genealogies constrained within different hypotheses of population history were simulated under a neutral coalescent process. Paml* 4.0b10 (Swofford 2002) was used to construct trees from the simulated matrices and to generate one consensus tree representing genes for each simulation. We fit the simulated gene trees to each possible divergence model of population trees with different date hypotheses and calculated the amount of discordance between the gene trees and population trees as measured by number of deep coalescences (DC) (Maddison 1997). We then compared DC values from the empirically reconstructed gene trees to those generated from the reconstructed gene trees from simulated data. The null model was rejected when the observed DC value fell below 95% of the distribution of the expected values.

The area of origin for each species of Batrachuperus was estimated from reconstructed ancestral states using Mesquite 2.7.2 (Maddison & Maddison 2010). Each individual was assigned to its collecting location. Because no prior knowledge of dispersal rate existed, the Markov k-state 1 parameter (MK1) model with equal likelihood the rate of change among different regions was selected for estimating ancestral areas.

Historical demography

Past population dynamics of all lineages were estimated using coalescent-based Bayesian skyline plots (BSP; Drummond et al. 2005) as well as mismatch distributions (Rogers & Harpending 1992). BSPs were implemented in BEAST. They were used to estimate the dynamics of past populations through time without requiring a prespecified parametric model of demographic history. Uncertainty in the genealogy was controlled using a Bayesian approach under a coalescence model. The best-fitting model (Table 2) with a relaxed lognormal clock was selected to construct the BSP in BEAST for each lineage. Chains were run for 10 million generations, sampled every 1000 generations, and the first 10% of the trees were discarded as burn-in. The results were summarized using Tracer. Mismatch distributions were calculated using Arlequin 3.5 (Excoffier & Lischer 2010). Multimodal distributions were expected in populations at demographic equilibrium or
in decline, and unimodal distributions were anticipated in populations having experienced a recent demographic expansion (Slatkin & Hudson 1991; Rogers & Harpending 1992). The expected distributions were generated by bootstrap resampling (10 000 replicates) using a model of sudden demographic expansion. The sum of square deviations and raggedness index between the observed and the expected mismatch were used as a test statistic. P-values were calculated as the probability of simulations producing a greater value than the observed value.

Species habitat modelling

The relationship between species occurrences and the corresponding environment was estimated from a distribution model using Maxent 3.3.3a with default settings (Phillips et al. 2006; Elith et al. 2011). The 19 bioenvironment-associated variables with 30 arc-sec resolution (~1 km²) were downloaded from the high-resolution interpolated Worldclim database (http://www.worldclim.org/) (Hijmans et al. 2005). The variables were used in habitat modelling to examine for potential factors that limited the species’ distributions. Principal components analysis was used to convert a set of possibly correlated environmental variables into a set of values of independent variables. Principal components with eigenvalues >1 that explained >10% of the variation were retained. These factor scores were used in a multivariate analysis of variance (MANOVA) as implemented in SPSS version 19 to test for differences among the five species of Batrachuperus, and the multivariate tests for each principal component were performed to determine significant differences in environmental conditions between species because these may have been the drivers responsible for demographic and divergence patterns.

Results

Topology and divergence dates

We resolved 764 bp of Cytb, 848 bp of COI and 856 bp of ND5. The concatenated data set comprised 2468 aligned sites. Sequences were deposited in GenBank under accession nos JQ303644–JQ304246. Both the BF (>509) and likelihood ratio test (P < 0.001) strongly favoured the nine-partitioning strategy against the one- and three-partitioning strategies. Therefore, the nine-partitioning strategy was used for all analyses of the concatenated data using MrBayes, RAxML and BEAST.

Bayesian inference and ML produced identical overall topologies. Thus, only the ML tree [Fig. 2a] with support values was presented. However, the topology among lineages of B. pinchonii in the BEAST tree differed from those in the MrBayes and ML trees. In the BEAST tree, the lineage Qionglai Mtn NW clustered with all other lineages of B. pinchonii (BPPs, 1.0). In contrast, the lineage Qionglai Mtn NW clustered with Qionglai Mtn SE 2 [Fig. 2a; BPP = 0.96; MLB = 75%]. We then used BF comparisons to test alternative topologies among lineages of B. pinchonii. BI incorporating a topological constraint was performed with the same model. Markov chains were run for 10 million generations. Trees were sampled every 1000 generations. BFs provided strong evidence (BF > 14) for Qionglai Mtn NW and Qionglai Mtn SE 2 being sister lineages. Thus, the BI and ML topologies were preferred, and all subsequent BEAST analyses used a topological constraint. This disorder may have been caused by being trapped in local optimum of MCMC (Huelsenbeck & Ronquist 2001).

BF strongly favoured the relaxed clock model over the nonclock model (BF > 268) and the strict clock model (BF > 144), suggesting that the relaxed clock model was more appropriate for our data. Thus, an uncorrelated lognormal clock model was imposed for BEAST analyses. These estimated divergence times were then employed to get a general idea of the timescale of diversification as a reference for the subsequent coalescent simulations. However, a nearly zero ucl.sd parameter was estimated, and similar age estimations were obtained for most nodes after running BEAST with both the relaxed and strict clock models (Table 3). Therefore, the analyses failed to reject the hypothesis of a linear rate of change.

Time estimations based on calibration points indicated that the earliest intraspecific divergences within Batrachuperus occurred from the Late Miocene c. 7.2 Ma (95% CI, 5.4–9.1 Ma) to the Early Pleistocene c. 2.0 Ma (95% CI, 1.3–2.8 Ma; Fig. 2b; Table 3). These estimates were younger than those obtained using a mean substitution rate of 0.64% per MY per lineage. This rate was inferred from Cytb in the Japanese clawed salamander, and this application could not be validated independently. Thus, we did not use dates estimated by the mean rate method for subsequent coalescent simulations. The BEAST analyses using only species-level sampling estimated divergence times ranging from the Middle Miocene c. 14.9 Ma (95% CI, 11.9–18.0 Ma) to the Late Miocene c. 8.5 Ma (95% CI, 6.3–10.9 Ma). This timescale was similar to that using both intra- and interspecific hierarchical levels (Table 3). Thus, the difference between long- and short-term substitution rates probably did not heavily affect our data set (Ho et al. 2005). The mean substitution rate produced by the calibration method was 0.69% (95% CI, 0.58%–0.82%) per MY per lineage. This rate was then used in subsequent coalescent simulations.
Hypotheses testing using coalescent simulations

Results from coalescent simulations with the corresponding $N_e$ failed to reject the colonization models for $B. karlschmidti$ ($P = 0.188$) and Daocheng species ($P = 0.190$), the fragmented ancestor models for $B. lndonogensis$ ($P = 0.136$) and $B. yenyuanensis$ ($P = 0.155$), and the staged fragmentation model for $B. pinchonii$ ($P = 0.109$). Thus, the diversification of these closely related species seemed to be driven by three different causes (Fig. 3).

The coalescent framework was also used to evaluate whether or not the level of divergence within each species of *Batrachuperus* corresponded with the most recent orogenesis of the Tibetan Plateau and the corresponding glacial-induced climatic shift hypothesis. Thus, we adjusted the first divergence dates (tree depth) to 2.6 and 3.6 Ma. These two time-points were also close to or fell within the 95\% CI obtained from the BEAST dating estimates for most species (Table 3). Coalescent simulations for corresponding diversification models failed to reject the null hypotheses at 2.6 Ma for $B. karlschmidti$ ($P = 0.117$), $B. lndonogensis$ ($P = 0.159$) and $B. yenyuanensis$ ($P = 0.101$); diversification appeared to be driven by the most recent orogenesis of the Tibetan Plateau and its concomitant glacial-induced climatic shift. This hypothesis was rejected when tested for $B. pinchonii$ and Daocheng species ($P < 0.01$), which had estimated divergence dates of c. 5.0 Ma (95\% CI, 3.7–6.3 Ma) and c. 7.2 Ma (95\% CI, 5.4–9.1 Ma), respectively. Consequently, we assumed the divergences occurred in the last 3.6 Ma, the beginning of the most recent uplift of the Tibetan Plateau. Coalescent simulations were then used to test these null hypotheses. The DC values from reconstructed

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Fig. 2 Topology and divergence dates for salamanders of the genus *Batrachuperus*. (a) RAxML phylogram. Names of the non-*Batrachuperus* out-group species are removed for clarity. The branching order is the same as that in the BEAST chronogram. Maximum-likelihood bootstrap proportions (MLBs) support is shown above the node, with Bayesian posterior probabilities (BPPs) shown below. Unmarked nodes have MLBs = 100\% and BPPs = 1.0. (b) Simplified BEAST chronogram produced by calibration method under relaxed clock model. Blue bars represent 95\% highest probability densities (HPD) interval. Numbers on scale bar are millions of years before present. The mean age estimates and actual 95\% HPD are shown in Table 3. Species name abbreviations: Rs, *Ranodon sibiricus*; Pm, *Paradactylodon mustersi*; Pas, *Pachyhynobius shangchengensis*; Hq, *Hynobius quelpaertensis*; Pss, *Pseudohynobius shuichengensis*; Ls, *Liua shihii*; Bt, *B. tibetanus*; Bk, *B. karlschmidti*; DC, Daocheng species; Bp, *B. pinchonii*; Bl, *B. lndonogensis*; By, *B. yenyuanensis*. Region name abbreviations: Sll, Shaluli; Ql, Qionglai; Ylx, Yulongxue.
gene trees for *B. pinchonii* (*P* = 0.352) and Daocheng species (*P* = 0.236) were not significantly lower than those values from the gene trees simulated by neutral coalescence. Again, the null hypotheses of two main divergence events could not be rejected.

Ancestral area reconstructions assigned the highest probability for the origin of *B. karlschmidti* to the central region of Daxue Mountains (proportional likelihoods, PL = 0.61; Table 4), for Daocheng species, the southeastern region of Shaluli Mountains (PL = 0.60), and for *B. pinchonii*, the southeastern region of Qionglai Mountains (PL = 0.72). The probabilities of the three species’ origins for alternative regions were considerably lower (highest PL = 0.23). Thus, a single ancestral area of origin was identified. These results were consistent with the colonization models of *B. karlschmidti* and Daocheng species and the staged fragmentation model of *B. pinchonii*. In contrast, probabilities for all regions of *B. londongensis* and *B. yenyuanensis* were low (highest PL = 0.43). It seemed highly likely that multiple ancestral areas existed at the deeper nodes on the tree. This discovery supported results from coalescent simulations that diversification occurred via the fragmentation of a wide-ranging common ancestor.

### Historical demography

The mismatch distributions revealed distinct demographic histories for each species. Distributions of the pairwise mutation differences were unimodal for *B. karlschmidti* and *B. yenyuanensis* (Fig. S1, Supporting information), and the sum of square deviations and raggedness index suggested that the curves did not significantly differ from the distributions expected under a model of sudden demographic expansion (Table 1). In contrast, the mismatch analyses for the remaining three species of *Batrachuperus* showed a bimodal profile, which might have resulted from either a constant or declining demography. Further, the BSP analysis provided additional and sometimes different details. The population size of the two high-elevation species (*B. karlschmidti* and Daocheng species) appeared to...
remain relatively stable during the extensive glaciation period (0.5–0.175 Ma), rapidly declined in the Late Pleistocene, but increased sharply around the end of the last glacial maximum (LGM) in the eastern edge of the Tibetan Plateau (0.016–0.032 Ma; Fig. 4). The sudden expansion model for Daocheng species contradicted the results from the mismatch distributions. The population size of two low-elevation species (B. pinchonii and B. londongensis) appeared to gradually decrease at the beginning of the extensive glacial period (0.5–0.175 Ma). Substantial population growth of B. yenyuanensis started approximately 0.15 Ma, which coincided with the ending of the extensive glaciation period. The historical dynamics of the five species of Batrachuperus were also supported by the BSP analyses for each regional population within each species.

**Table 4** Proportional likelihoods for the area of origin for the oldest ancestral node in the five species of Batrachuperus

<table>
<thead>
<tr>
<th>Species</th>
<th>The area of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. karlschmidt</strong></td>
<td>Daxue Mtn C, Gan Zi, Xin Long, Shaluli Mtn C, Shaluli Mtn S</td>
</tr>
<tr>
<td><strong>B. londongensis</strong></td>
<td>Emei Mtn, Wawu Mtn, Han Yuan</td>
</tr>
<tr>
<td><strong>B. pinchonii</strong></td>
<td>Qionglai Mtn NW, Qionglai Mtn SE, Emei Mtn, Daxue Mtn S</td>
</tr>
<tr>
<td><strong>Daocheng species</strong></td>
<td>Shaluli Mtn SE, Shaluli Mtn S, Yulongxue Mtn, Shaluli Mtn C</td>
</tr>
<tr>
<td><strong>B. yenyuanensis</strong></td>
<td>Daxue Mtn S, Bailing Mtn, Luoji Mtn</td>
</tr>
</tbody>
</table>

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Ecological niche modelling

Ecological niche modelling projected that the distribution of each species of *Batrachuperus* was now restricted to a specific montane habitat. The region of highly suitable habitat was limited to and consistent with the distributions of each species (Fig. S2, Supporting information). For each distributional model, the AUC value approached one (≥0.997) indicating a better than random prediction (0.5 = random). Two principal components among the 19 possibly correlated environmental variables explained 80.64% of the total variation (Table 5). The first principal component represented precipitation and temperature (the lower the value, the colder and drier), whereas the second principal component represented climatic variability (the lower the value, the less variable). Habitats of high-elevation species appeared to be cooler, drier and more variable than those of low-elevation species (Fig. 5). The MANOVA analysis indicated that the habitats of the five species of *Batrachuperus* differed significantly (Wilk’s *λ* = 0.281, *F* = 15.07, *P* < 0.001). This result provided ecological and physiographic explanations for the distinct demographic and divergence histories of each species.

Discussion

The various drivers of species divergence associated with orogenesis seem to play roles in the evolution of *Batrachuperus*. Closely related species of *Batrachuperus* seem to have experienced different patterns of diversification driven by topography. They appear to have been affected by different glacial periods as a result of variation in elevation distributions and cold-tolerant physiology.

**Table 5** Nineteen biological environmental variables used in habitat modelling and the principal component analysis on these variables used in a comparison of climatic conditions between occurrence locations for the five species of *Batrachuperus*

<table>
<thead>
<tr>
<th>Bio-variable</th>
<th>Component 1</th>
<th>Component 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIO1 = Annual Mean Temperature</td>
<td>0.786</td>
<td>0.612</td>
</tr>
<tr>
<td>BIO2 = Mean Diurnal Range</td>
<td>−0.902</td>
<td>0.247</td>
</tr>
<tr>
<td>BIO3 = Isothermality</td>
<td>−0.666</td>
<td>0.229</td>
</tr>
<tr>
<td>BIO4 = Temperature Seasonality</td>
<td>−0.300</td>
<td>0.086</td>
</tr>
<tr>
<td>BIO5 = Max Temperature of Warmest Month</td>
<td>0.654</td>
<td>0.720</td>
</tr>
<tr>
<td>BIO6 = Min Temperature of Coldest Month</td>
<td>0.896</td>
<td>0.421</td>
</tr>
<tr>
<td>BIO7 = Temperature Annual Range</td>
<td>−0.809</td>
<td>0.174</td>
</tr>
<tr>
<td>BIO8 = Mean Temperature of Wettest Quarter</td>
<td>0.768</td>
<td>0.629</td>
</tr>
<tr>
<td>BIO9 = Mean Temperature of Driest Quarter</td>
<td>0.771</td>
<td>0.599</td>
</tr>
<tr>
<td>BIO10 = Mean Temperature of Warmest Quarter</td>
<td>0.769</td>
<td>0.625</td>
</tr>
<tr>
<td>BIO11 = Mean Temperature of Coldest Quarter</td>
<td>0.796</td>
<td>0.562</td>
</tr>
<tr>
<td>BIO12 = Annual Precipitation</td>
<td>0.829</td>
<td>−0.514</td>
</tr>
<tr>
<td>BIO13 = Precipitation of Wettest Month</td>
<td>0.737</td>
<td>−0.520</td>
</tr>
<tr>
<td>BIO14 = Precipitation of Driest Month</td>
<td>0.850</td>
<td>−0.402</td>
</tr>
<tr>
<td>BIO15 = Precipitation Seasonality</td>
<td>−0.502</td>
<td>0.051</td>
</tr>
<tr>
<td>BIO16 = Precipitation of Wettest Quarter</td>
<td>0.756</td>
<td>−0.548</td>
</tr>
<tr>
<td>BIO17 = Precipitation of Driest Quarter</td>
<td>0.849</td>
<td>−0.391</td>
</tr>
<tr>
<td>BIO18 = Precipitation of Warmest Quarter</td>
<td>0.742</td>
<td>−0.563</td>
</tr>
<tr>
<td>BIO19 = Precipitation of Coldest Quarter</td>
<td>0.847</td>
<td>−0.378</td>
</tr>
<tr>
<td>Initial eigenvalues</td>
<td>11.028</td>
<td>4.293</td>
</tr>
<tr>
<td>% of variance</td>
<td>58.042</td>
<td>22.595</td>
</tr>
</tbody>
</table>

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Distinct divergence patterns

Topographically diverse montane regions usually have a suite of species with differing and complex evolutionary histories and divergence patterns (Despres et al. 2002; Kropf et al. 2003). Closely related species of salamanders endemic to the eastern edge of the Tibetan Plateau also have distinct patterns, and these are associated with high- and low-elevation topographies. Coalescent simulations find that diversification in western high-elevation species is more consistent with a colonization model involving a series of dispersals from one mountain to another followed closely by isolation and divergence. Fragmentation of the range of a widely distributed common ancestor is not suggested. In contrast, diversification in eastern low-elevation species appears to have been driven by either fragmentation or staged fragmentation of a once wide-ranging species. Species of *Batrachuperus* generally occupy moist habitats in forested areas. Consequently, their distributions can be strongly influenced by the occurrence of mesic forests. Orogenesis of the Tibetan Plateau and Pleistocene glacial cycling are responsible for climatic and ecological shifts. The climate appears to have become gradually drier and colder; the development of glaciers in this region is associated with the contraction of broadleaf deciduous forests and a dominance of grasslands (Wu et al. 2001). Most temperate species appear to have dispersed to lower latitudes in response to Pleistocene climatic oscillations (Hewitt 2000; Rowe et al. 2004).

Under a coalescent framework, the null hypotheses states that the initial divergence within each species occurred at c. 2.6–3.6 Ma, and it cannot be rejected. This dating corresponds to the most intense uplift of the Tibetan Plateau (Zheng et al. 2000; An et al. 2001) and the beginning of the Pleistocene. Consequently, climatic changes triggered by the two events may have driven the divergence, although more evidence is needed.

The region’s north–south tending mountain ranges facilitate lower-latitude dispersal by providing favourable climate and habitats. The levels of population differentiation reflect historical isolation events. Multiple montane occurrences and drift, and not ongoing gene flow, are suggested to be the driving force. The precise colonization routes are likely to have been obscured by the accumulation of genetic differences and coalescence times. Eastern low-elevation species are highly likely to have been restricted to mesic sky island forests isolated by deep valleys with unfavourable environments. Unlike contiguous mountains in the western area, valleys appear to serve as barriers to dispersal because lineages are separated by unsuitable conditions (Wiens 2004; Wiens & Graham 2005; Wiens et al. 2006).

Ancestral area reconstructions provide additional support for the distinction between divergence patterns in high- and low-elevation species. High-elevation species are associated with a single area of origin. Their ancestors are from a relatively small area. *Batrachuperus karlschmidtii* seems to be derived from the central region of Daxue Mountains (1–4), and subsequent colonization appears to have occurred mainly along the Shaluli Mountain range eventually reaching its southern region (18, 19). Daocheng species appears to have its origin in the southeastern Shaluli Mountains (19); a series of short-distance colonizations restrict it to the central and southern Shaluli Mountains. Interestingly, the common ancestor of Daocheng species appears to have been present in the southeastern Shaluli Mountains before *B. karlschmidtii* colonized that area. Thus, *B. karlschmidtii* seems to have invaded suitable habitats previously dominated by Daocheng species. Niche modelling predicts that the two species occupy similar habitats, which may facilitate competition, as is documented to occur in other closely related species of salamanders (Hairston 1951; Crespi et al. 2003).

Our analyses cannot identify single ancestral areas of origin for most low-elevation species that exhibit...
fragmentation patterns, especially B. loudongensis and B. yenyuanensis. This finding suggests the rapid fragmentation of wide-ranging ancestral populations into several parts. The ancestral area reconstruction for B. pinchonii assigns the highest probability to southeastern Qinglai Mountains (28–32). The staged fragmentation pattern of this low-elevation species infers that its common ancestor initially fragmented into two ancestral populations (northern and southern), and subsequently both of them fragmented. The first stage may be associated with orogenesis of the Qinglai Mountains, although more powerful evidence is needed to support this scenario. Gongga Mountain, the highest (7556 m) in the region, is surrounded by more than 20 other peaks that exceed 6000 m in the central Daxue Mountains. The palaeomagnetic age of Gongga Mountain is c. 2.48 Ma (Li et al. 1991), and the first colonization event (c. 2.6 Ma) in B. karlschmidtii is closely associated with this origin. Batrachuperus karlschmidtii does not occur south of Gongga Mountain. Orogenesis in the region may have restricted ancestral populations to the north. The Late Mesozoic Yanshan movement is responsible for the initial creation of Mount Emei, and the most recent Tibetan Plateau movement (c. 3 Ma) uplifted the mountain to its present height. Concordance between dates for the fragmentation of B. loudongensis and the uplifting of Mount Emei suggests that the creation of deep valleys produced barriers to dispersion, thus reducing gene flow and promoting allopatric divergence.

**Contrasting demographic histories**

Species at high and low elevations appear to have been affected by different glacial periods. Responses to Pleistocene glacial cycles are expected to vary among species and geographical regions, in part because of differential cold tolerance (Hewitt 1996, 2004).

Bayesian skyline plots show that high-elevation species were suppressed by the last glaciation (0.016–0.032 Ma) but not by the earlier, more extensive glaciations (0.5–0.175 Ma; Shi 2002; Shi et al. 1995; Zhang et al. 2000; Zheng et al. 2002), although the mismatch distribution rejects the model of sudden demographic expansion for Daocheng species. Methods estimating population parameters based on the distribution of pairwise differences do not make full use of DNA sequence data, while methods based on coalescent theory, by incorporating information from the genealogy, may obtain better estimates of the demographic histories of populations (Felsenstein 1992; Pybus et al. 2000). Only a few mountain heights extend above the Middle Pleistocene snowline, and glaciation appears to have been limited to areas such as Gongga Mountain (Shi et al. 1990; Li et al. 1991). Glaciation is controlled by climate and geomorphology. Although the climate is documented to have been colder during the extensive glacial period than the LGM (Shi et al. 1995), mountain heights below the snowline could not be glaciated. During the LGM, if mountain heights extended above the snowline, glaciers could form and became cold sources acting as positive-feedback mechanisms through reflecting more of the sun’s energy and absorbing less; orogenesis raised mountain heights and glaciers contributed to cooler climates (Zheng et al. 2002). Current snowlines in the eastern edge of the Tibetan Plateau extend from 4200 to 5200 m (Shi et al. 1997; Liu et al. 1999; Li et al. 2009). During the LGM, the snowline descended to approximately 3300 m in many mountain ranges (Shi et al. 1997; Liu et al. 1999). The two high-elevation species currently occur above 3500 m. Their suitable habitat seems to have been heavily covered with ice during the LGM. If true, then salamanders must have dispersed to ice-free refugia, such as lower elevations or adjacent areas, only to recolonize the region via a population expansion during postglacial times.

The population sizes of the three low-elevation species seem to have been affected from the beginning of the extensive glacial period. The low-elevation species dwell below the snow line (1800–3200 m). Suitable habitat might have not been covered by ice during both the LGM and the extensive glacial period. Ecological niche modelling suggests that the low-elevation species are less cold-tolerant than the high-elevation species. If true, then low-elevation species are more likely to suffer from climatic cooling than high-elevation species. From the beginning of the extensive glaciation period, low-elevation species are predicted to have lowered population sizes even in ice-free regions. In contrast, high-elevation, cold-tolerant species are expected to be affected only if their suitable habitat is heavily covered with ice during the LGM. These predictions are consistent with the hypothesis that variation in ecological adaptations affects geographical patterns of genetic variation (Gavrilets 2003; Hewitt 2004). Glacial advances decrease temperatures while increasing aridity. These changes may have contributed to declines in population size in the less cold-tolerant species and resulted in the use of refugia by many species during the LGM (Hewitt 1996, 2000, 2004; Rowe et al. 2004). However, B. yenyuanensis, which occupies a low-elevation and low-latitude region, seems to have experienced a substantial amount of population growth after the extensive glaciation period yet did not suffer losses during the LGM. The climate during the extensive glaciation period might have been much colder and drier than that during the LGM (Shi et al. 1995; Zheng et al. 2002).
This may be an important reason why glaciation affects low-latitude species. In contrast, a cold climate without ice cover is likely to have little negative effect on more cold-tolerant species, such as *B. karlschmidtii* and Daocheng species. It remains unclear why the populations of *B. pinchonii* and *B. londongensis* did not expand after the glaciers retreated. Other factors appear to be involved.

Our study on Tibetan *Batrachuperus* underscores the various roles played by elevation, geological history and animal physiology in shaping the patterns of genetic diversity and demography among closely related species. Further studies using the rigorous testing of hypotheses are likely to yield an understanding the drivers of evolutionary change.

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B.L. is interested in understanding the genetic and evolutionary mechanisms that generate and maintain patterning variation within and between species. Y.C.Z.’s research focuses on the systematics, correlated evolution, and biogeography of amphibians. R.W.M. is broadly interested in genetics, genomics, and evolution. X.M.Z.’s general areas of interest are systematics and evolutionary biology of amphibians and reptiles. Current research activities focus on the mechanisms of speciation and more specifically to understand the role of chromosomal rearrangements in speciation.
Data accessibility

DNA sequences: GenBank accessions JQ303644–JQ304246.

Sampling information uploaded as online supplemental material.

Phylogenetic data and DNA sequence alignment for all individuals: TreeBASE Study accession no. S12425.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Mismatch distributions for five species of Batrachuperus. (a) B. karlschmidtii, (b) Daocheng species, (c) B. pinchonii, (d) B. londongensis and (e) B. yenyuanensis.

Fig. S2 The ecological niche models of the current distribution for five species of Batrachuperus. (a) B. karlschmidtii, (b) Daocheng species, (c) B. pinchonii, (d) B. londongensis and (e) B. yenyuanensis. Colours indicate predicted probability that conditions are suitable, with values ranging from 0 to 1. High values indicate high probability of suitable conditions for the species, whereas low values indicate low predicted probability of suitable conditions.

Appendix S1 Specimens of Batrachuperus used in genealogical and coalescent analyses. All vouchers are deposited in the Chengdu Institute of Biology (CIB). Locality numbers correspond to those on the map in Fig. 1.

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