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Significance of pre-Quaternary climate change for montane species diversity: Insights from Asian salamanders (Salamandridae: *Pachytriton*)

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ABSTRACT

Despite extensive focus on the genetic legacy of Pleistocene glaciation, impacts of earlier climatic change on biodiversity are poorly understood. Because amphibians are highly sensitive to variations in precipitation and temperature, we use a genus of Chinese montane salamanders (Salamandridae: *Pachytriton*) to study paleoclimatic change in East Asia, which experienced intensification of its monsoon circulation in the late Miocene associated with subsequent Pliocene warming. Using both nuclear and mitochondrial DNA sequences, we reconstruct the species tree under a coalescent model and demonstrate that all major lineages originated before the Quaternary. Initial speciation within the genus occurred after the summer monsoon entered a stage of substantial intensification. Heavy summer precipitation established temporary water connectivity through overflows between adjacent stream systems, which may facilitate geographic range expansion by aquatic species such as *Pachytriton*. Species were formed in allopatry likely through vicariant isolation during or after range expansion. To evaluate the influence of Pliocene warming on these cold-adapted salamanders, we construct a novel temperature buffer-zone model, which suggests widespread physiological stress or even extinction during the warming period. A significant deceleration of species accumulation rate is consistent with Pliocene range contraction, which affected *P. granulosus* and *P. archospotus* the most because they lack large temperature buffer zones. In contrast, demographic growth occurred in species for which refugia persist. The buffer-zone model reveals the Huangshan Mountain as a potential climatic refugium, which is similar to that found for other East Asian organisms. Our approach can incorporate future climatic data to evaluate the potential impact of ongoing global warming on montane species (particularly amphibians) and to predict possible population declines.

1. Introduction

Compared to the extensively studied Quaternary climatic fluctuations and associated genetic legacy on extant organisms (reviewed in Hewitt, 2000), the impacts of more ancient environmental changes as far back as the Pliocene and late Miocene, which witnessed remarkable shifts in global climate and continental ecosystems (Cerling et al., 1997; Zachos et al., 2001), are poorly known. Yet, those early climatic transitions may have had crucial roles in promoting diversification within radiations of Cenozoic organisms (Kozak et al., 2006; Vieites et al., 2007; Lo Presti and Oberprieler, 2009; Zheng et al., 2009; Dasmahapatra et al., 2010; Miralles and Carranza, 2010; Patel et al., 2011).

During the Quaternary glacial period, East Asia was largely not glaciated due to dry winters (Hewitt, 2000; Li et al., 2004). Species from this region may not have experienced the extreme demographic fluctuations that characterize biota from glaciated areas. Old gene lineages are more likely to be preserved, which allows us to study the evolutionary forces that acted upon organismal diversity before the glacial period. Studies have demonstrated that glacial climatic oscillations are too recent to explain some of the deep genetic divergences observed today (Dasmahapatra et al., 2010; Miralles and Carranza, 2010; Patel et al., 2011). East Asia experienced a distinctive climatic history since the late Miocene, which is characterized by development of the unique East Asian monsoon system accompanied by the accelerating uplift of the Tibetan Plateau (An et al., 2006; Harris, 2006). Seasonality increased, with most precipitation occurring in summer (An et al., 2001). The monsoon is strongly associated with East Asian terrestrial ecosystem turnover (Jia et al., 2003). On the other hand, East Asia was affected by global warming in the Pliocene (Zachos et al., 2001; Ravelo et al., 2004; Salzmann et al., 2011). This

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warm and wet period contrasts with the otherwise prevailing trend towards global cooling that existed since the late Miocene, and it could have devastated cold-adapted organisms in ways similar to current global warming (Pounds et al., 2006; Milanovich et al., 2010). Because these climatic shifts greatly altered precipitation patterns and environmental temperatures, we might expect to find signals in extant East Asian amphibians, whose life history and species richness are governed by precipitation and temperature (Buckley and Jetz, 2007). Nevertheless, few phylogeographic studies have focused on East Asia, and our knowledge of the genetic consequences of historical climatic change in this region remains sparse.

The stout newts (*Pachytriton*, also known as paddle-tailed newts) from southeastern China are an ideal system with which to investigate organismal response to historical climatic change. Earlier studies suggest that *Pachytriton* diverged from its sister genus *Laotriton* as early as the middle Miocene (Zhang et al., 2008b; Vieites et al., 2009). Such long-term persistence is essential to be able to capture climatic signatures over the last 10 million years (Ma). Species in this genus are restricted to montane habitats at high elevations (i.e., on sky islands), which were uplifted by major orogenies before the middle Eocene (Guo, 1998). Therefore, the phylogeographic pattern in *Pachytriton* evolved too recently to be explained by mountain orogeny. However, ranges of individual populations likely expanded and contracted along elevational gradients that changed as the result of climatic fluctuations, in a manner similar to that recorded in North American montane salamanders (Shepard and Burbrink, 2008). Salamanders generally have low dispersal ability and exhibit philopatry; thus, they are more sensitive than are endotherms to changes in precipitation and temperature (Buckley and Jetz, 2007; Vieites et al., 2009). Adult *Pachytriton* permanently live in small, shallow and clean montane streams (usually 1–3 m wide) and have morphological and osteological specializations associated with their aquatic habitat (Özeti and Wake, 1969; Zhao et al., 1994; Fei et al., 2006; Wu et al., 2012a). They are adapted to live in cold water, and a small increase of ambient temperature may be fatal (Zhao et al., 1994).

In this paper, we use *Pachytriton* to understand the likely impact of late-Miocene monsoon intensification and Pliocene warming on montane species diversity in East Asia. First, we evaluate the hypothesis that the substantial intensification of the East Asian summer monsoon in the late Miocene (7–10 Ma) triggered range expansion in *Pachytriton*, which subsequently promoted initial speciation in the genus. By sampling throughout the known geographic range of *Pachytriton*, we employ a coalescent method to generate a species tree along with divergence times and test whether speciation occurred after monsoon intensification. During the monsoon season, we have encountered *Pachytriton* in temporary waterways after heavy rains or in puddles with signs of a recent flood (unpublished personal observations). Given that this genus is the most aquatic in the family Salamandridae (Özeti and Wake, 1969; Titus and Larson, 1995), floods that connect neighboring stream systems could facilitate colonization of new habitats and lead to range expansion. Recent studies suggest a critical role for range expansion in promoting speciation (Moyle et al., 2009; Van Bocxlaer et al., 2010). Speciation could occur followed by vicariance that reduces gene flow across a barrier formed after expansion. Second, we follow the rationale of ecological niche-modeling to examine likely physiological constraints on *Pachytriton* and the extent to which Pliocene warming (3–5 Ma) may have affected their survival. Because these salamanders are sensitive to high temperatures and restricted to specific montane habitats, they may experience range contraction toward higher elevations or even habitat collapse under a climatic warming scenario (Wake and Vredenburg, 2008; Wake, 2009, 2012). Third, we reconstruct historical demography using a multi-locus coalescent model to assess how population size may have fluctuated under different climatic conditions. We predict no population growth in species that were most stressed by Pliocene warming and stable demographic growth in species for which areas of suitable habitat persisted. Fourth, we test the tempo of diversification in the genus against a null model of constant rate of lineage accumulation. A detrimental impact of Pliocene warming would favor rate deceleration over constancy.

2. Material and methods

2.1. Taxon sampling and sequence preparation

We conducted extensive fieldwork throughout southeastern China and acquired specimens and tissues from 6 of the 8 species in *Pachytriton*: 4 populations of *P. archospotus*, 6 populations of *P. brevipes*, 7 populations of *P. granulosus*, 1 population of *P. feii*, 5 populations of *P. inexpectatus* and 1 population of *P. xanthospilos* (Fig. 1; Supplementary Table S1). Samples of the recently described *P. moi* from Guangxi province and *P. changi* (described from two salamanders obtained from a Japanese pet store with unknown origin) were unavailable for this study.

Total genomic DNA was extracted from ethanol-preserved liver or muscle tissue by using a QIAGEN DNeasy Blood and Tissue Kit (Valencia, CA, USA). We amplified from all specimens two mitochondrial fragments (*ND2* and the *cytb* with flanking tRNAs, each with ~1200 base pairs, bp) and two nuclear fragments that cover the 3′ end of the *RAG-1* (~1200 bp), as well as a non-coding region of the tyrosinase gene (*NCRT*, 600 bp). The first three markers are widely used in phylogenetic studies; *NCRT* is used for the first time and developed following the anonymous-loci development method described in Espregueira Themudo et al. (2009). The *NCRT* fragment was amplified using the forward primer TYR-F (5′AGC GGT AGA CCT GTG GCT TC3′) and reverse primer TYR-R (5′TGC ACA CTG ATG GGG TTG GT3′). Other PCR primers and thermal cycle protocols are the same as Wu et al. (2010). Nucleotide sequences were manually aligned in Se–Al 2.0 (Rambaut, 1995). Sequences are deposited in GenBank (Supplementary Table S1).

2.2. Individual gene-trees estimation

We inferred separate gene genealogies from *RAG-1*, *NCRT* and the concatenated mitochondrial DNA (mtDNA). We utilized the Bayesian criterion in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and the maximum-likelihood (ML) criterion in RAxMLv7.4.3 (Stamatakis, 2006). The concatenated mtDNA was partitioned by codon positions, but nuclear DNAs were not partitioned due to limited variable sites. Substitution models in Bayesian inference were determined by the Akaike Information Criterion (AIC) in MRMODELTEST 2.2 (Nylander, 2004). Bayesian Markov Chain Monte Carlo (MCMC) was run for 10-million generations sampled every 100 generations. In the ML analysis, the GTRGAMMA model was used for each gene partition, and 1000 nonparametric bootstrap replicates were performed to calculate node support.

2.3. Species-tree and divergence-time estimation

It is a common practice to assume that a species phylogeny equals a single-gene genealogy (including trees derived from gene concatenation). However, a gene tree represents only one realization of the stochastic process embedded inside the species tree (Ho and Shapiro, 2011). Discrepancies between species trees and gene trees can arise if lineage sorting is incomplete among recently diverged species (Pamilo and Nei, 1988). Therefore, we used a multispecies coalescent model to estimate the species tree based on multi-locus data under the Star BEAST (*BEAST) option implemented in BEAST v1.6.1 (Drummond and Rambaut, 2007; Heled and

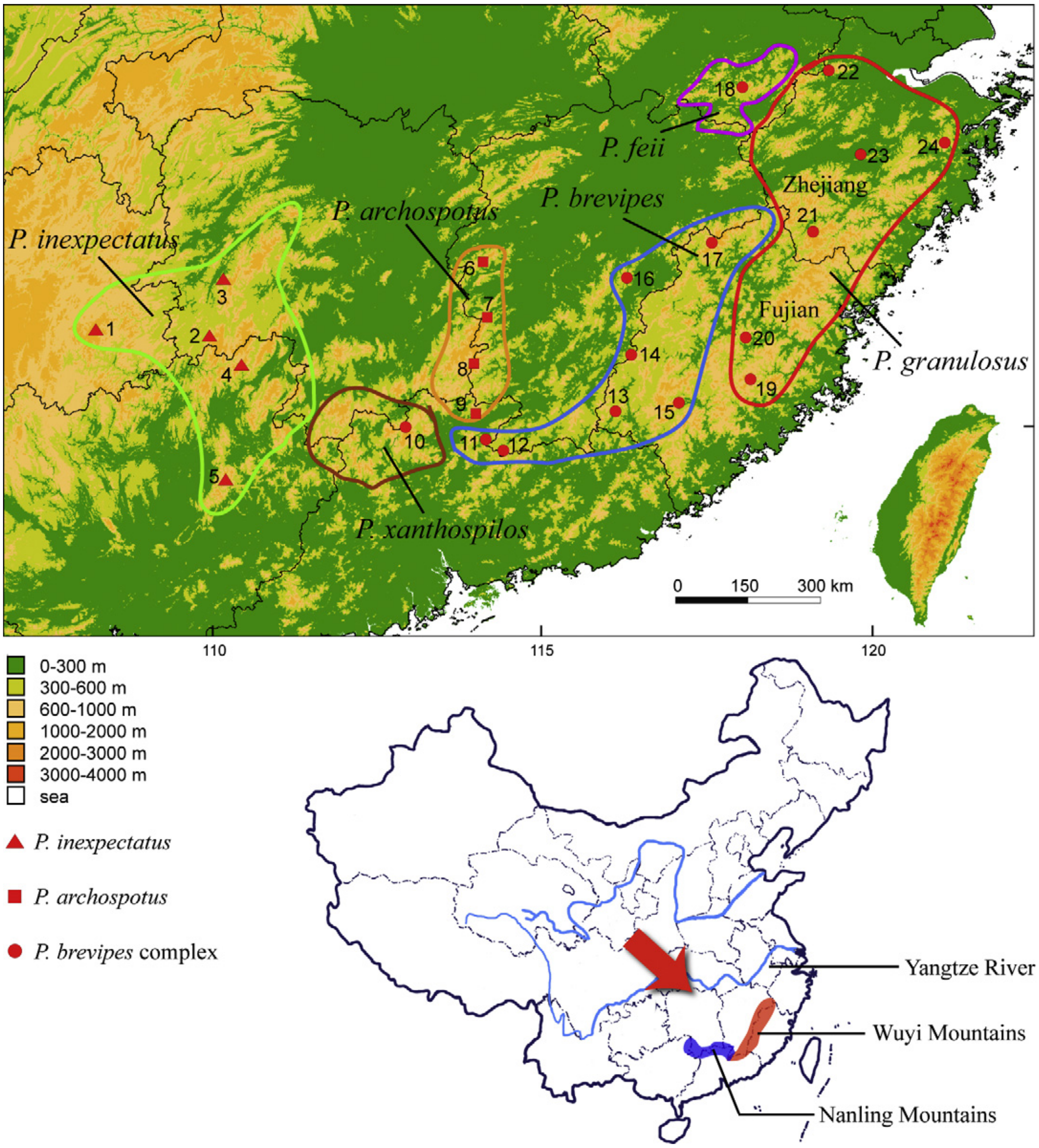

Fig. 1. Top: geographic distribution of the genus *Pachytriton* in southeastern China and sampling localities (Supplementary Table S1). Bottom: country map showing the focal study region (red arrow) and location of the Nanling and Wuyi mountain ranges.

Drummond, 2010). This approach outperforms gene-concatenation methods in both species-tree topology inference and speciation-time estimation (Heled and Drummond, 2010). Sequence data were tri-partitioned into mtDNA, *RAG-1* and *NCRT*. Substitution models were unlinked across partitions and determined by AIC in MRMODELTEST 2.2 (Nylander, 2004). Star BEAST does not require outgroup taxa (Heled and Drummond, 2010). The root of the species tree and speciation times were estimated through an uncorrelated relaxed-clock model, in which evolutionary rate in each branch is drawn independently from a lognormal distribution. This relaxed-clock model performs well with both clocklike and non-clocklike data (Drummond et al., 2006). Due to the paucity of the fossil record for East Asian salamandrids, we imported calibration dates from Zhang et al. (2008b), who propose two sets of divergence times within Salamandridae based on mitogenomic data. The younger set is more compatible with independent biogeographic evidence and congruent with corresponding dates from another study that uses both mitochondrial and nuclear genes with different fossil calibrations (Roelants et al., 2007). This set of divergence times likely approximates the true ages of salamandrids and is consistent across data types (mtDNA vs. nuclear DNA). Following Zhang et al. (2008b), we constrained the age of the genus *Pachytriton* between 3.9 and 18.3 Ma with a uniform prior distribution. This interval includes the oldest estimate for the split between *Pachytriton* and its sister genus *Laotriton* as well as the youngest divergence time within *Pachytriton*. A second calibration prior was placed at the split between *P. granulosus* and *P. xanthospilos* at 7.5 Ma (Zhang et al., 2008b) with a normal-distributed 95% confidence interval (CI) between 3.9 and 11.2 Ma. Bayesian MCMC was performed for 100-million generations with a sample frequency at every 1000 generations. Burn-in period was determined by Tracer 1.4 (Rambaut and Drummond, 2007). Node ages were calculated as mean node heights from the maximum clade credibility tree summarized from the posterior distribution trees, which were displayed with DensiTree (Bouckaert, 2010).

2.4. Influence of Pliocene warming

It is difficult to assess the impact of Pliocene warming on montane-adapted *Pachytriton* when relevant physiological data do not exist. By assuming that physiological constraints are reflected in habitat selection, which also is the key assumption of the ecological niche-modeling approaches (Kozak et al., 2008), we can use environmental temperature at a population's lowest elevational limit as a proxy for its maximum heat tolerance. Because there is only one collecting locality on each mountain and no historical collection data available, standard ecological niche-modeling approaches (e.g., Maxent) cannot be applied to obtain a mountain-specific response to climatic change. Given that we know only the temperature increase during Pliocene warming and have no information on quantitative precipitation change, our evaluation of the impact is limited to temperature-related variables. Although temperature and precipitation are coupled in driving amphibian diversity, changes in either constraint influence the distributional limit of amphibians (Buckley and Jetz, 2007).

When global temperature rises, the elevational limits of montane salamanders are pushed upwards and eventually there may no longer be suitable habitat, and the population, or even species, goes extinct (Wake and Vredenburg, 2008; Wake, 2009, 2012). Montane salamanders are less likely to acclimate because small increases in temperature can induce significant metabolic depression (Bernardo and Spotila, 2006). In southeastern China, summer and winter temperatures of the late Miocene are about equal to present values (Utescher et al., 2011). The subsequent Pliocene, however, witnessed a global warming of 2–5 °C (Novacek, 1999; Ravelo et al., 2004; Salzmann et al., 2011). This provides a comparable scenario to the current path of climatic warming projected to the end of the 21st century (IPCC, 2007; Salzmann et al., 2009). Therefore, we proposed a temperature buffer zone for *Pachytriton*, calculated as the contemporary temperature difference between the top of the mountain (the theoretical refugium) and the population's lowest distributional limit. If the buffer zone does not or only slightly exceeds the 2–5 °C window, suitable environmental conditions would not exist locally during the Pliocene warming period. *Pachytriton* at this local area would have declined or even been extirpated. We used seven climatic variables to calculate seven buffer zones that address different aspects of the environmental temperature: annual mean temperature (AMT), maximum temperature of the warmest month, minimum temperature of the coldest month, and mean temperatures of warmest, coldest, wettest and driest quarters. Our method assumes that the ancestral *Pachytriton* were adapted to a cool climate, which is consistent with the reconstruction of climatic envelopes of Salamandridae through geological time (Vieites et al., 2009).

All sampled *Pachytriton* were collected in cold, montane streams. The exact lowest distributional limit of most samples is unknown. However, in many cases the lowest collecting locality should be close to the limit because we collected from downstream to upstream. Consequently the same locality was treated as the upper bound of the lowest distribution, which yields a conservative (minimum) estimate of the buffer zone. We also sampled five random localities 200 m lower than the lowest collecting locality, and used those localities, where *Pachytriton* are absent based on previous fieldwork, as the lower bound of the lowest distribution limit. The arithmetic mean of temperatures from the five absence locations was used to yield a more liberal (maximum) estimate of the temperature buffer zone. In each case there are at most minimal differences in either stream availability or forest composition between occurrence and absence locations. No other aquatic organism has been recorded to compete with or prey on *Pachytriton*. Thus, environmental temperature difference is likely the primary factor that determines the occurrence and absence of *Pachytriton* at these sites.

Temperature variables for East Asia were obtained from the WorldClim contemporary database (Hijmans et al., 2005) at 30 arc-sec resolution ($\sim$1 km^2). Country-level elevational data at the same resolution were downloaded from the DIVA-GIS website (http://www.diva-gis.org/). Both data sets were imported into the geographic analysis program DIVA-GIS 7.1.7. For each of the 24 sampling locations, values of the seven temperature variables were extracted at the mountain top, the collecting site and the five corresponding absence locations (110 absence locations in total). The temperature buffer zone for each population was established as the temperature difference between the mountain top and the lower and upper bound of the lowest distribution and was compared to the 2–5 °C temperature increment of the Pliocene.

2.5. Historical demographic analyses

To estimate population-size dynamics through geological time, we reconstructed Extended Bayesian Skyline Plots (EBSPs, Heled and Drummond, 2008) implemented in BEAST v1.6.1 for four species for which we sampled more than one locality: *P. archospotus*, *P. brevipes*, *P. granulosus* and *P. inexpectatus*. This coalescent-based, non-parametric Bayesian MCMC algorithm incorporates multi-locus data to reduce estimate errors associated with single genes (e.g., traditional Bayesian Skyline Plots) and increases the power to detect demographic dynamics (Ho and Shapiro, 2011). Sequence partitions, substitution models and the clock model were the same as those used in the species-tree analysis. Divergence time was converted from number of substitutions to millions of years by inputting the geometric mean of substitution rates of genes. Population growth was depicted as piecewise linear, allowing population size to change continuously through the genealogical history (Heled and Drummond, 2008). Bayesian MCMC was performed for 50-million generations with a sampling frequency of every 1000 generations. The first 10% of the sample was discarded as burn-in. Skyline plots were visualized using EXCEL. We further used non-genealogical summary statistics including Tajima's D (Tajima, 1989) and Fu and Li's D* (Fu and Li, 1993) to detect historical population-size change. Assuming neutral alleles, a significantly negative value indicates recent demographic growth, whereas a significantly positive value indicates a very recent bottleneck event. Summary statistics were calculated in DNAsp v5 (Librado and Rozas, 2009); associated confidence intervals were estimated through 1000 coalescent simulations.

2.6. Tempo of diversification

If Pliocene warming had a detrimental impact on *Pachytriton*, we expect to find a slowdown of lineage accumulation after initial speciation in the genus. We tested a deviation from the null model of constant diversification rate using the constant-rate (CR) test and the gamma statistic (γ) by Pybus and Harvey (2000). Negative values of γ imply a deceleration of diversification rate on a given chronogram. The null model can be rejected at the 95% significance level when $\gamma < -1.645$ (Pybus and Harvey, 2000). Because we included 6 of 8 described species in the genus, incomplete sampling at the species level should have little effect on γ estimation (Pybus and Harvey, 2000; Cusimano and Renner, 2010). However, to account for new species yet to be discovered, we performed a Monte Carlo constant-rate (MCCR) test by simulating 100 phylogenies under the constant-rate model (pure-birth process) with a full sampling of total 10 and 20 species assumed in *Pachytriton*, respectively. Phylogenies were randomly pruned to retain six species to reflect the actual sampling. The observed γ was compared to the distribution of gamma values estimated from those pruned trees. If the observed value is statistically different from the null distribution, the constant-rate model will be rejected given incomplete taxon sampling (Pybus and Harvey, 2000). Both CR test and MCCR test were conducted using the LASER package in R.

3. Results

3.1. Gene-tree estimation

No premature stop codon or insertion-deletion is detected in the protein-coding regions of *ND2*, *cytb* or *RAG-1*. A single insertion-deletion is present in the *NCRT* fragment, which exhibits moderate interspecific variation in salamandrids comparable to the level observed in *RAG-1*. Individual gene trees reveal different relationships among species of *Pachytriton* (Fig. 2). Tree topologies are almost identical from Bayesian and the ML analyses. The mtDNA and *NCRT* gene trees are congruent with each other and with the result from Wu et al. (2010, 2012b) in recovering *P. inexpectatus* as the sister taxon to a clade comprising the rest of the sampled species. In contrast, the *RAG-1* data recognize a sister-taxon relationship between *P. inexpectatus* and *P. archospotus*. All sampled species of *Pachytriton* are monophyletic with strong support in the mitochondrial gene tree, but populations of *P. brevipes*, *P. feii*, *P. granulosus* and *P. xanthospilos* are admixed in the two independent nuclear gene trees. The latter three species were once considered conspecific populations of *P. brevipes* (Fei et al., 2006), and were recently elevated to full species based on mtDNA data (Nishikawa et al., 2011; Wu et al., 2012b). Notably, the mtDNA gene tree estimates that the most recent common ancestors (MRCAs) of sampled populations of *P. archospotus* and *P. granulosus* date only to the end of the Pliocene, although both species likely originated before the Pliocene. Because gene trees overestimate divergence times (Edwards and Beerli, 2000), diversification within each of these species likely is even more recent.

3.2. Species-tree estimation

Consistent with mtDNA and *NCRT* gene trees, the multi-locus species tree resolves the basal split between *P. inexpectatus* and other sampled species (Fig. 3). *Pachytriton archospotus* forms the sister taxon to a clade comprising *P. brevipes*, *P. feii*, *P. granulosus* and *P. xanthospilos*. This topology is favored by 90.9% of the 75,000 post-burn-in MCMC trees. However, relationships among the latter four species cannot be resolved with high node support (Supplementary Fig. S1). Given their admixture pattern in nuclear gene trees, the four species are most appropriately treated as comprising a species complex (*P. brevipes* complex), which requires further studies of species delimitation. Only 6.4% of the MCMC trees suggest that *P. inexpectatus* and *P. archospotus* are sister species, and the remaining 2.7% of the trees group *P. inexpectatus* with the *P. brevipes* complex. The first speciation event in *Pachytriton* occurred in the late Miocene, 9.4 Ma (95% CI: 6.1–12.9 Ma). Subsequent divergence between *P. archospotus* and the *P. brevipes* complex dates to 6.8 Ma (95% CI: 4.5–9.1 Ma). Both speciation events largely overlap the period when the East Asian summer monsoon underwent a significant intensification at 7–10 Ma. Star BEAST also estimates a mitochondrial substitution rate of 7.7×10^{-9} per site per year (95% Highest Posterior Density, HPD: 5.9×10^{-9} -9.9×10^{-9}), which corresponds to a sequence divergence of 1.54% between taxa per million years of separation.

3.3. Impact of Pliocene warming

Temperature buffer zones were derived from temperature variables for each of 24 populations. Considerable variation in the size of buffer zones occurs among species and localities (Fig. 4). Because the only available Pliocene temperature data are estimated using mean annual temperatures (Novacek, 1999; Ravelo et al., 2004; Salzmann et al., 2011), we focus on temperature buffer zones derived from AMT. The other six temperature variables yield a very similar trend of variation (Supplementary Fig. S2). When collecting localities are treated as the lowest elevational limits, buffer zones of eight populations are below the 2-degree threshold, which is likely the minimal global temperature increase in the Pliocene. These populations were physiologically stressed and likely declined during the warming period. When temperature buffer zones are calculated more liberally by inferring the lowest elevational limits from absence localities, two thirds of the populations still have buffer zones narrower than 5 °C. Buffer zones derived from temperature variables measuring winter conditions (the coldest and driest times) all infer a more severe and widespread decline compared to estimates from AMT, whereas those derived from measurements of summer conditions (the warmest and wettest times) show less stress. Regardless of which temperature variable is used, conservative estimates, which are closer to true values than liberal estimates, reveal that none of the mountains sampled from the known distribution of *P. archospotus* and *P. granulosus* provides adequate temperature buffer zones. Similarly, *P. xanthospilos* from the eastern Nanling Mountain Range and *P. brevipes* from the

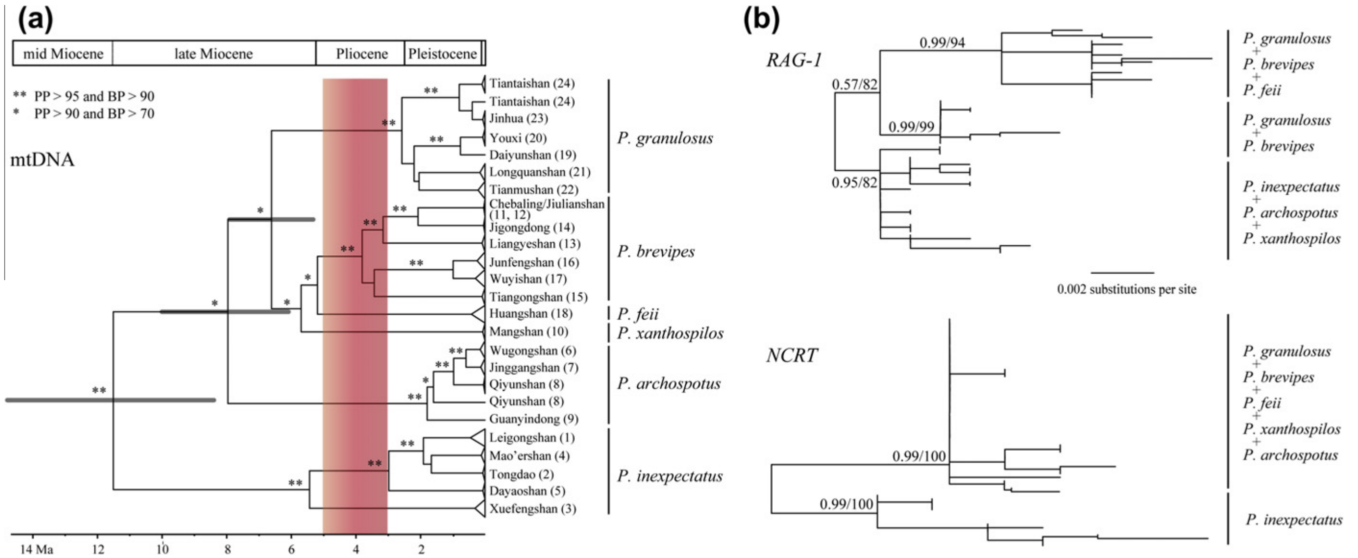

Fig. 2. (a) Bayesian mtDNA gene tree and (b) ML nuclear gene trees. The mtDNA gene tree is scaled with geological times; nuclear gene trees are scaled with substitution units. Numbers at the tips of the mtDNA tree correspond to sampling localities. Grey bars represent 95% HPD of the node age. The red vertical column indicates the warm Pliocene. Numbers and asterisks on branches are Bayesian posterior probabilities and ML bootstrap values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

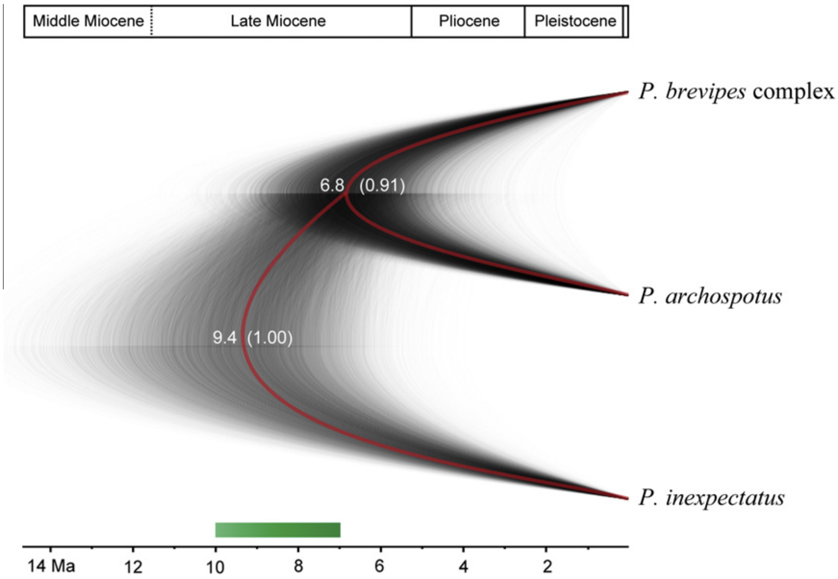

Fig. 3. Species tree of *Pachytriton* inferred from two nuclear and two mitochondrial loci. This topology is supported by 90.9% of the 75,000 total post-burn-in MCMC trees, each of which is displayed by a thin black line. The consensus tree is shown in red. Nodes are marked by divergence times with Bayesian posterior probabilities of branch support in parentheses. The green horizontal bar indicates the start of substantial Asian summer monsoon intensification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

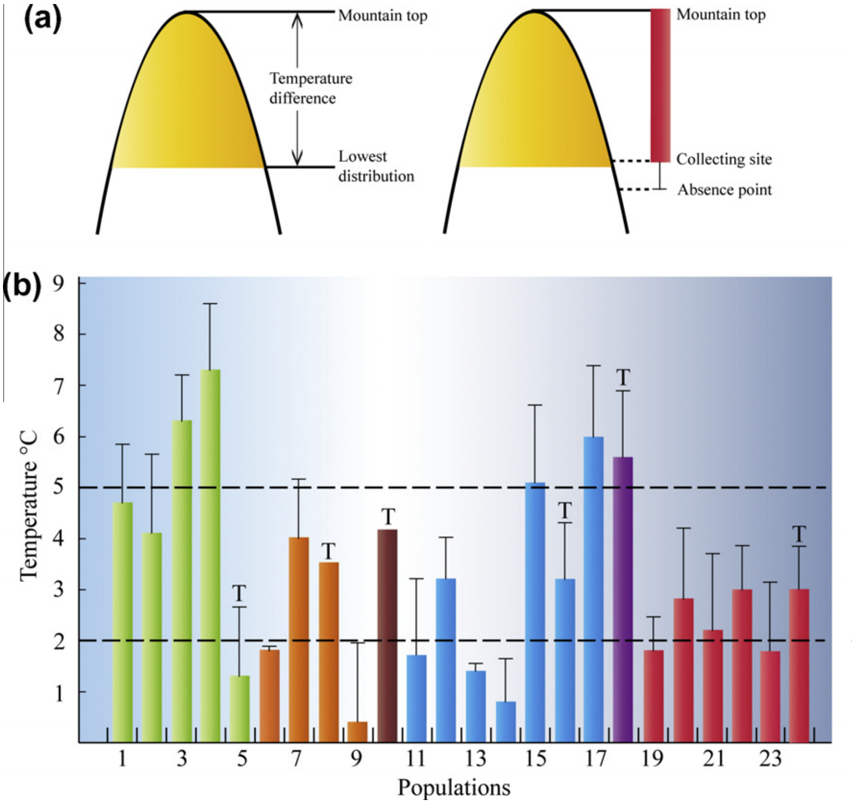

Fig. 4. (a) Left: schematic depiction of the temperature buffer zone on a mountain, with a salamander species' distribution shaded orange. Right: conservative (red bar) and more liberal (red bar plus the whisker) estimates of the temperature buffer zone inferred from known collecting locality and absence points. (b) Temperature buffer zones estimated for 24 populations of *Pachytriton*. The horizontal axis corresponds to sampling sites (populations): *P. inexpectatus* light green, *P. archospotus* orange, *P. xanthospilos* brown, *P. brevipes* blue, *P. feii* purple, and *P. granulosus* red. Lowest distributions are known for sites 8 and 10 (Xu et al., 2002; Shen et al., 2008), so their bars represent the actual buffer zones. T, type locality. Dashed lines represent the 2–5 °C increment of global temperature in the Pliocene. If the buffer zone does not or only slightly exceeds this increment, *Pachytriton* at this local area could have declined or even been extirpated during the Pliocene warming period.

middle-to-southern portion of the Wuyi Mountain Range are at high risk. Except for *P. feii*, all species from their respective type localities could be threatened by global warming. On the other hand, we identify several high mountains that are capable of buffer-

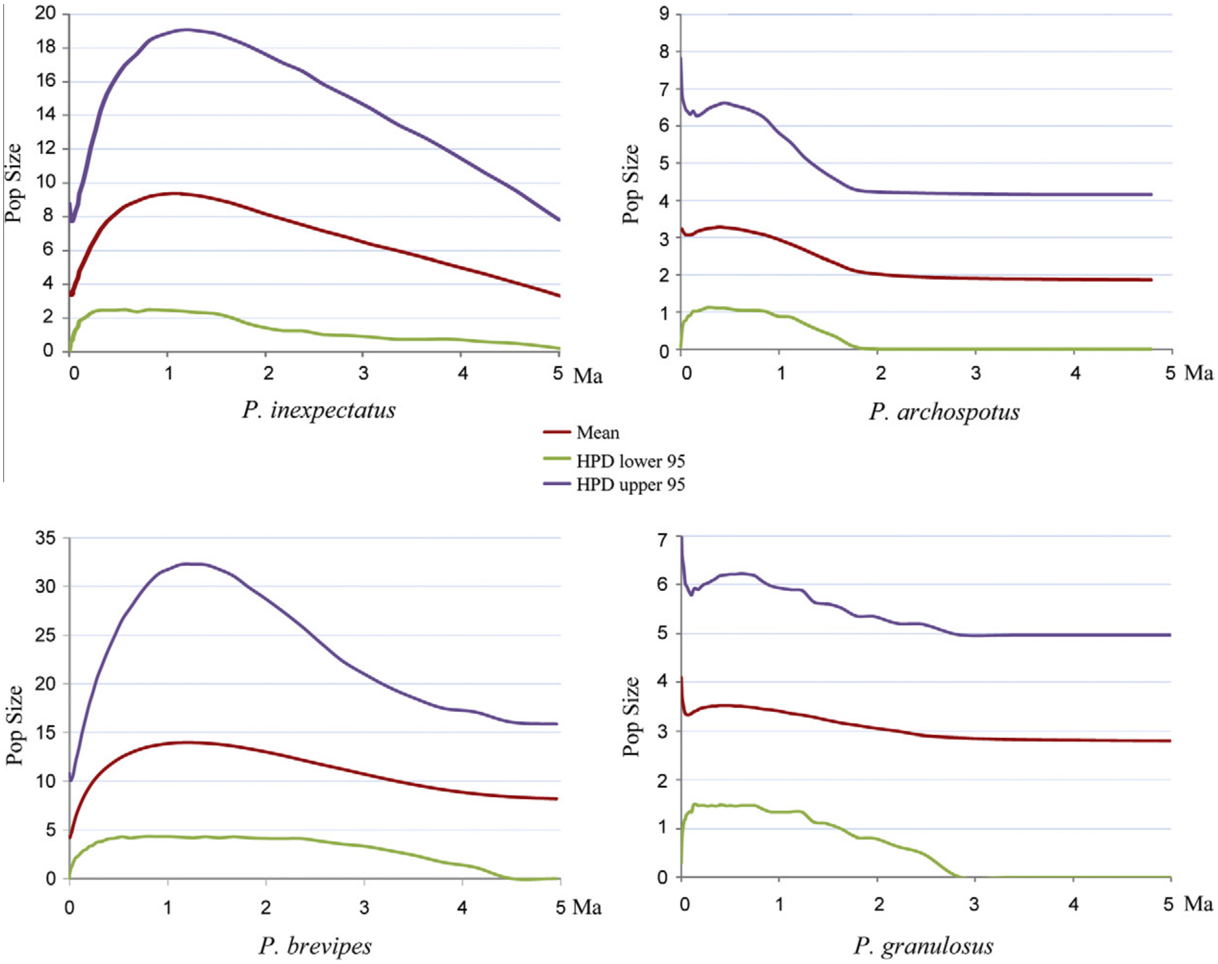

Fig. 5. Extended Bayesian Skyline Plots (EBSPs) showing demographic dynamics through geologic time. The central red line measures the mean value of the product of effective population size and generation time in millions of years (Ne × τ); the upper blue line and lower green line represent the 95% HPD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ing large temperature changes. These include Xuefengshan[1] (site 3) and Mao'ershan (site 4) for *P. inexpectatus*, Tiangongshan (site 15) and Wuyishan (site 17, at the northern end of the Wuyi Mountain Range) for *P. brevipes*, and Huangshan (site 18) for *P. feii*.

3.4. Historical demography

The coalescent-based EBSPs reveal distinct demographic history among species (Fig. 5). *Pachytriton inexpectatus* and *P. brevipes* share a gradual demographic growth from 5 Ma until 1 Ma, when population size started to decrease rapidly. In contrast, population sizes of *P. archospotus* and *P. granulosus* remained low from 5 to 3 Ma. Weak growth occurred in *P. archospotus* after 2 Ma and in *P. granulosus* after 3 Ma. Non-genealogical summary statistics, including Tajima's D and Fu and Li's D*, fail to reject the null model of a stable population demography for all four species (Supplementary Table S2). However, compared to EBSPs, which directly quantify demography from gene genealogies (Ho and Shapiro, 2011), these summary statistics do not take tree structure into account and thus are not based on all available information (Pybus et al., 2000).

3.5. Tempo of diversification

Because the species tree did not resolve relationships within the *P. brevipes* complex, we performed the CR test on the fully resolved mtDNA chronogram, which is co-estimated with the species tree and pruned to leave one individual per species. The gamma statistic ($\gamma = -1.662807$) significantly rejects the null model of a constant diversification rate in favor of a deceleration in the accumulation of lineages in *Pachytriton*. When assuming a total of 10 or 20 extant species in the genus (i.e., 4 or 14 species more than we included; the latter of which is unlikely given our extensive sampling), the observed γ value is still significantly smaller than the null distribution generated by Monte Carlo simulations assuming a constant rate ($P = 0.039$ and $P = 0.049$, respectively). This confirms that the slowdown of the rate of lineage accumulation is not an artifact.

4. Discussion

Analyses of conspecific populations may reveal significant geographic structure and variation within a species, whereas interspecific comparisons advance understanding of historical processes that generate regional biodiversity (Bermingham and Moritz, 1998). Our study provides a unique perspective from which to analyze the significance of pre-Quaternary climate change for East Asian organisms by incorporating both population-level and species-level components. The results suggest a potentially critical role of monsoon activity and global warming in shaping the genetic diversity and distributional pattern in *Pachytriton*.

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[1] We use -shan (Chinese pinyin for mountain) to refer to a mountain, and the capitalized plural Mountains to refer to a more inclusive mountain range.

4.1. Summer monsoon and initial speciation in *Pachytriton*

Onset and intensification of Asian monsoons has been one of the most important factors in global climatic change since the middle Miocene (Clemens et al., 1996; Ravelo et al., 2004). However, the likely impact on organismal diversity has rarely been assessed in a phylogenetic context. The multi-locus coalescent model indicates that all three major lineages of *Pachytriton* originated well before the Quaternary glacial period, and initial speciation occurred around a critical period in the development of the East Asian monsoon system. These divergence times are reasonable because the mitochondrial substitution rate, which is co-estimated with the species tree, is very similar to rates of *ND2* or *cytb* in other salamanders and frogs (Weisrock et al., 2001; Crawford, 2003; Mueller, 2006). When summer monsoons underwent substantial intensification, precipitation increased due to strengthened circulation (An et al., 2006). This climatic shift is regarded as an environmental response to the rapid uplift of much of the Tibetan Plateau as it approached its present elevation during the same time period (Harrison et al., 1992; An et al., 2001). In southern China, the summer monsoon begins in May, peaks in June and extends to late August (Qiang and Yang, 2008). The monsoon season corresponds to the breeding season of *Pachytriton* (Fei et al., 2006). Rainfall increases dramatically compared to the non-monsoon period and accounts for most of the annual precipitation (Qiang and Yang, 2008). Downpours are especially prevalent in mountainous regions, flooding streams that *Pachytriton* inhabit. Overflows form temporary waterways that connect neighboring stream systems, thereby providing opportunities for aquatic organisms such as *Pachytriton* to disperse across otherwise separated habitats that are hundreds of meters apart. After summer monsoonal rains, we have found salamanders in temporary waterways caused by stream flooding as well as in small catchments fed by overflows (unpublished personal observations). This after-rain dispersal mechanism has been proposed for North American aquatic salamanders after lab experiments, which demonstrate much higher dispersal ability when salamanders are submerged in water compared to when they are exposed on dry land (Schalk and Luhring, 2010).

Given that contemporary species distributions are generally non-overlapping in *Pachytriton*, speciation most likely occurred in allopatry, which is the predominant mode of animal diversification (Mayr, 1963; Barraclough and Vogler, 2000). Recent studies reveal that range expansion associated with subsequent vicariance may accelerate speciation rate (Moyle et al., 2009; Van Bocxlaer et al., 2010). Yet, direct factors that reduce gene flow within formerly continuous populations vary among regions and taxa. It is possible that weakening of the summer monsoon led to vicariant isolation in *Pachytriton*. The strength of summer monsoonal circulation exhibits considerable variability and cyclicity over both short and long terms, from tens to thousands of years (An et al., 2001; Cosford et al., 2008). When the summer monsoon enters its weakened stage, connectivity among streams is lost due to the lack of stream flooding. Without sufficient gene flow to maintain a common gene pool, populations undergo differentiation and eventually speciation.

On the other hand, colonization of unoccupied streams could promote local adaptation within new habitats, which may facilitate phenotypic diversification and ultimately speciation. One potential example in *Pachytriton* is evolution of the unusual hyobranchial skeleton in *P. archospotus*. Ceratobranchial bones in this species are greatly enlarged, which causes a reconfiguration of the posterior hyobranchial skeleton (Wu et al., 2012a). The epibranchial bones are straight and rod-like in *P. archospotus* but strongly bowed and dorsolaterally flared in other species (Shen et al., 2008). The medial epibranchial flange, a feature unique to *Pachytriton* among salamandrids (Özeti and Wake, 1969), also is absent from *P. archospotus* (Wu et al., 2012a). Given the inferred species tree, these characters of *P. archospotus* are likely autapomorphies. The hyobranchial skeleton of *Pachytriton* is specialized for underwater suction feeding (Özeti and Wake, 1969). Reconfiguration of the skeleton in *P. archospotus* implies a modified feeding habit in this species, which in turn may reflect novel prey availability (Bouton et al., 1999).

4.2. Global warming and temperature buffer zones

Modeling *Pachytriton*'s response to a warming climate predicts that many extant populations are at high risk for future decline if environmental temperature should again rise to levels seen in the Pliocene. Populations that occur in mountains of low elevations and are not heat-tolerant are the most likely to be stressed. Furthermore, the model assumes that over time salamander populations retreat to the very top of the mountain to escape environmental warming. In reality, stringent habitat requirements often preclude *Pachytriton* from occupying mountain tops where suitable streams and broadleaf-forest are absent. The actual sizes of buffer zones thus are equal to or smaller than our predictions, indicating an even more severe impact. The eastern Nanling Mountain Range and the middle-to-southern portion of the Wuyi Mountain Range, which generally do not exceed 1300 m in elevation, may not provide sufficiently cool habitat for *Pachytriton* as global temperatures rise. Even high mountains may not offer refuge. If salamanders that occur on high mountains are found only at high elevations, the resulting temperature buffer zone is still narrow. This phenomenon occurs in those populations for which a small conservative estimate is associated with a large liberal estimate (e.g., Fig. 4, sites 9 and 21). For *P. archospotus* and *P. granulosus*, the highest mountains within their geographic range reach 2000 m above sea level, but corresponding collecting sites are over 1000 m. Because all high mountains have been sampled for these two species, it is unlikely that any large temperature buffer zone remains to be discovered. *Pachytriton xanthospilos* also lacks a large buffer zone at its type locality, but the western Nanling Mountains remain to be surveyed adequately.

We identify several mountains capable of buffering large temperature increases for populations of *P. inexpectatus*, *P. brevipes* and *P. feii* that occur there. Among those mountains, Huangshan (site 18), the type locality of *P. feii*, is located at the lower reaches of the Yangtze River, a region that has been proposed as a Pleistocene refugium for eastern Asian conifers, frogs and non-migratory birds based on patterns of intraspecific genetic variation (Gao et al., 2007; Zhang et al., 2008a; Li et al., 2009). Huangshan is characterized by a heterogeneous topography with numerous high peaks interspersed among low valleys, where refugia may have been abundant and supported habitats with relatively stable microclimates when the global climate underwent major changes (Qian and Ricklefs, 2000; Li et al., 2009; Song et al., 2009). Moreover, nucleotide diversity is highest in the Huangshan population of the Chinese giant salamander and the sharp-snouted pitviper (Murphy et al., 2000; Huang et al., 2007), which further suggests preservation of old gene lineages in this region. Huangshan may have served as a refugium for eastern Asian species during both warming and cooling periods over geologic time.

The novel buffer-zone modeling approach provides a unique opportunity to understand the temperature constraints of *Pachytriton* populations and their possible response to climatic change in the absence of physiological data. This approach is particularly useful when there is very limited occurrence data (even as few as one observation per locality). By combining knowledge of geographic distribution with the global climate database, we establish a mountain-based model that can be projected back to the Pliocene warming period. This model intuitively identifies locations that are potential climatic refugia. Most importantly, our approach can incorporate future climatic models to reveal the association between global warming and worldwide amphibian declines. Particularly, precipitation-related variables should be incorporated along with temperature to derive more accurate projections. According to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, global temperature is likely to rise 1.1–6.4 °C by the year 2100 relative to 1980 levels if greenhouse gas emissions are not reduced (IPCC, 2007). *Pachytriton*, as well as other cold-adapted montane amphibians, may be forced to higher and higher elevations and ultimately vanish in some locations. Species with elevational ranges but essentially no latitudinal or longitudinal ranges are at highest risk of extinction (Wake, 2012).

Our model can be made more precise by incorporation of finer-scale climatic and elevational data. The resolution of data currently available (30 arcsec, $\sim$1 km^2) is still coarse for analyses that involve temperature gradients on mountains. We also assume species-climate equilibrium by treating absence localities as unsuitable habitats and predictors of maximum temperature tolerance (Nogués-Bravo, 2009). Instead of using a correlative model, implementation of mechanistic niche models may differentiate the relative roles of physiological tolerance and biotic constraints (interspecific competition, predation and anthropogenic activity) in determining a population's lowest distribution (Gifford and Kozak, 2012). Using actual water temperature instead of air temperature may also improve model precision. However, a large number of samples (24 populations in our analysis) helps to reduce noise and to reveal shared trends among populations and species.

4.3. Molecular evidence for Pliocene warming

Phylogenetic analyses based on DNA sequences are consistent with the buffer-zone model regarding a detrimental impact of Pliocene warming on *Pachytriton*. The CR and MCCR tests significantly reject a model of constant diversification rate over time and suggest a slowdown of lineage accumulation in the genus. Our conclusion is not affected by incomplete taxon sampling at the species level. Furthermore, although the mtDNA chronogram indicates that both *P. archospotus* and *P. granulosus* may extend back as early as the late Miocene, lineage accumulation within these two species occurred only after the end of the warming period. In contrast, we find much earlier intraspecific divergences in *P. brevipes*, which is younger than both *P. archospotus* and *P. granulosus*. The contrasting coalescent patterns are congruent with the hypothesis that extinction terminated early branches and that all surviving populations have a recent origin (Harvey and Rambaut, 1998). Consistently, large-temperature buffer zones are identified for *P. brevipes* but not for *P. archospotus* or *P. granulosus*. This result is not an artifact of including only slightly genetically differentiated populations from the latter two species. Instead, all localities were chosen without *a priori* knowledge of their genetic affinity and are evenly distributed across the corresponding geographic range. Due to limited availability of samples of *P. feii* and *P. xanthospilos* (one population each), it is unclear whether the pattern of early origin contrasting with recent divergence occurs in either species. The EBSPs indicate that demographic growth occurred in *P. archospotus* and *P. granulosus* only after the warming period, whereas *P. brevipes* and P. *inexpectatus* continuously increased in population size from 5 Ma until 1 Ma. Multiple climatic refugia identified for *P. brevipes* and *P. inexpectatus* (e.g., Mao'ershan, Xuefengshan, Wuyishan, and Tiangongshan) may account for the overall population growth, even though some local populations with narrow buffer zones may have declined.

5. Conclusions

Molecular phylogenetic analyses that incorporate two nuclear and two mitochondrial DNA sequences, combined with climatic and geographic data, highlight the potential significance of historical monsoonal activities in promoting speciation in aquatic salamanders through range expansion followed by vicariance. Comparative phylogeographic analyses can test whether the same processes have affected other East Asian montane organisms with stringent water requirements. Evaluation of the impact of Pliocene warming by estimating temperature buffer zones represents an effective approach that can be applied to other studies of how environmental changes, including those that involve temperature or precipitation, affect montane species, both in the past and in the future. Such studies serve a pivotal conservation purpose in light of the threat posed by future global temperature change to a majority of the world's species (Hansen et al., 2006), and especially montane amphibians (Stuart et al., 2004; Wake and Vredenburg, 2008; Rovito et al., 2009; Wake, 2009, 2012).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.10.011.

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