Red River barrier and Pleistocene climatic fluctuations shaped the genetic structure of Microhyla fissipes complex (Anura: Microhylidae) in southern China and Indochina

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Abstract

South China and Indochina host striking species diversity and endemism. Complex tectonic and climatic evolutions appear to be the main drivers of the biogeographic patterns. In this study, based on the geologic history of this region, we test 2 hypotheses using the evolutionary history of Microhyla fissipes species complex. Using DNA sequence data from both mitochondrial and nuclear genes, we first test the hypothesis that the Red River is a barrier to gene flow and dispersal. Second, we test the hypothesis that Pleistocene climatic cycling affected the genetic structure and population history of these frogs. We detect 2 major genetic splits that associate with the Red River. Time estimation suggests that late Miocene tectonic movement associated with the Red River drove their diversification. Species distribution modeling (SDM) resolves significant ecological differences between sides of the Red River. Thus, ecological divergence also probably promoted and maintained the diversification. Genogeography, historical demography, and SDM associate patterns in southern China with climate changes of the last glacial maximum (LGM), but not Indochina. Differences in geography and climate between the 2 areas best explain the discovery. Responses to the Pleistocene glacial-interglacial cycling vary among species and regions.

Key words: demographic histories, ecological divergence, genogeography, LGM, pigmy narrow-mouth frog.
Southern China and Indochina harbor extraordinary high levels of species diversity and share a zoogeographic fauna in the Oriental Region (Myers et al. 2000; Holt et al. 2013). The region includes China, Vietnam, Laos, Cambodia, Thailand, and Myanmar. This biodiversity has attracted much attention (e.g., Stuart et al. 2006; Matsui et al. 2010; McLeod 2010). High species richness in these areas has been attributed in large part to its complex geological and climatic history (e.g., Pleistocene climatic oscillations) and resulting changes in topography and environment (Woodruff 2010; de Bruyn et al. 2014). Advances in speciation and diversification, conservation, biogeography, and geology depend on understanding how these historic processes shaped the spatial patterns of genetic diversity, i.e., the distributions of species.

Tectonic movement and orogenesis can drive habitat fragmentation and create barriers to gene flow. Such events can drive genetic diversification and speciation (Che et al. 2010). Probably, the most famous geological formation of this area is the Red River shear zone, a major geological strike-slip fault zone that demarks the boundary between South China and Indochina tectonic plates (Leloup et al. 1995; Hall 1998). This shear zone runs southward from the southeastern corner of Tibet to the Gulf of Tonkin along the Red River (Figure 1). The Red River shear zone has a complex geological history (Hall 1998; Replumaz et al. 2001). For example, left-lateral Red River shearing occurred in the Oligocene and early Miocene (30–15 Ma; Tapponnier et al. 1990; Searle 2006). This was followed several times by right-lateral strike-slip events during late Miocene (e.g., 12.17–9.19, 8.14–6.18, Zhang et al. 2009). These events caused habitat changes along the Red River, which could act as potential geographic barriers for species dispersal and, thus, promote speciation. Many taxa, including both plants and animals, occur in 1 side of the Red River only (reviewed in Li and Li 1992, 1997; Bain and Hurley 2011; Fan et al. 2013; and references therein), or show genetic differences between populations on either side of the Red River (Zhang et al. 2010a, 2010b).

Climatic cycling, especially during the Late Pleistocene, shaped the current distributions, genetic diversification, and demographic dynamics of many species (Hewitt 2000, 2004). Although southern China and Indochina were not glaciated, they experienced cooler and possibly drier climates during the Pleistocene (Williams et al. 1998; Li et al. 2004). In pace with the cyclical cooling-warming climate, many species experienced periodic habitat expansions and contractions to meet their ecological requirements. For example, during glacial times, species retracted into small refugia and during postglacial periods their ranges expanded. In southern China, both some plants (Qi et al. 2012; Tian et al. 2015) and animals (Zhang et al. 2008; Zhong et al. 2008; Blair et al. 2012) exhibit this pattern, as do several Indochinese forest-dwelling animals (e.g., Luo et al. 2004; Fuchs et al. 2008; Patow et al. 2010; Morgan et al. 2011). By comparison, several recent studies on southern China montane species have detected unusual expansions during the glacial periods, with demographic contractions and genetic divergence occurring after the glacial periods. For example, this occurs in tits (Dai et al. 2011; Wang et al. 2012), Chinese bamboo partridge (Huang et al. 2010), and Elliot’s laughing thrush (Qu et al. 2011). Several studies have questioned if the glacial climate adversely affected population sizes and genetic structures. For example, several forest-dwelling taxa from southern China (e.g., Song et al. 2009; Lei et al. 2012; Yan et al. 2012; Yan et al. 2013; Wu et al. 2013) and Indochina (Latine et al. 2015) presented long-term stable demographic histories and deep genetic diversification that predated the Pleistocene. Most of these controversies likely owe to studying different parts of these areas and being based on species with different ecological requirements (e.g., lowland vs. montane species). Thus, studies involving additional widely distributed taxa can contribute to understanding how Pleistocene climate fluctuations affected the distributions and genetic patterns among species and regions.

Pigmy narrow-mouth frogs Microhyla fissipes complex (Microhylidae) constitute an ideal model group for testing hypotheses on how tectonic and climatic processes shape the patterns of organisms in southern China and Indochina. Similar to most other amphibians, they have low vagility and low physiological tolerance to extremely cold temperatures and dry environmental conditions. These characteristics can lead to isolation, which, in turn, makes them ideal taxa for carrying footprints of historical processes (Zeisset and Beebee 2008). These frogs occur in open lowlands (lowland scrub forest, grassland, agricultural land, pastureland, and some urban areas), and they range from western Myanmar eastward through Indochina and northward into southern China including Hainan and Taiwan (AmphibiaWeb 2016; Frost 2016). Because the populations show subtle differences in morphological characters and occur in similar habitats, the group was long considered to constitute the single species M. fissipes only (Poyarkov et al. 2014).

Recently, Hasan et al. (2014) combined mitochondrial DNA (mtDNA) analyses and morphological comparisons to reveal 2 species within this complex. Populations from Bangladesh were assigned to *M. mukhlesuri*, and populations from China (1 locality from Anhui, 2 localities from Taiwan) were considered “true” *M. fissipes* (Hasan et al. 2014). However, the absence of extensive sampling leaves the Hasan et al. (2014) hypothesis with 2 possible scenarios of Indochinese species: “*M. fissipes*” from Thailand and Laos might be same as *M. mukhlesuri*, or they correspond to an undescribed and/or cryptic species. Presently, their ranges are unclear. The widespread distribution of this complex not only makes it as good model for testing the hypothesis that the Red River is a geographic barrier to dispersal, but also the hypothesis that the Pleistocene climate changes impacted both species, which occur in similar habitats, equally.

Herein, we use both mitochondrial DNA and nuclear DNA (nuDNA) markers to dissect the impact of geologic events and Pleistocene climatic cycling on the current geographical patterns of the distribution and genetic diversity of *M. fissipes* complex. More specifically, we test 2 following hypotheses. First, we test the null hypothesis (H0) that the Red River is not a barrier to dispersal and gene flow for the *M. fissipes* complex. Rejection of the H0 will require investigations into the potential mechanisms of isolation that promote diversification. Second, considering ice sheets did not cover both southern China and Indochina during the Pleistocene, we hypothesize that Pleistocene climatic changes had little effect on populations. This predicts that populations will have persisted in situ, exhibit a high level of divergence, and keep stable population demography throughout the Pleistocene. Alternatively, Pleistocene climatic cycling affected regions differently because of different landscapes. Signals of population shrinkages or expansions associated with cyclical Pleistocene glaciations should occur in regions where the climate experiences extreme changes.

### Materials and Methods

#### Sampling, sequencing, and alignment

For the ingroup, we obtained 324 individuals from the *M. fissipes* complex representing 76 localities from southern China and Indochina (Supplementary Table S1; Figure 1). For the outgroups, 1

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individual each of *M. heymonsi*, *M. mixtura*, *M. okinavensis* and *M. mymensinghensis* was included based on the studies of Matsui et al. (2005) and Hasan et al. (2014). Totally, new sequences from 319 individuals were analyzed, along with 9 individuals from GenBank (Supplementary Table S1). The Animal Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences approved the protocols for collection of specimens of this study. Toe samples were collected mostly from adults and preserved in 95% ethanol; several adult individuals from each population were euthanized using benzocaine and preserved as voucher specimens (Supplementary Table S1).

Total genomic DNA was extracted using standard phenol-chloroform extraction protocol (Sambrook et al. 1989). A partial fragment of the mitochondrial gene encoding cytochrome b (CYTB) was amplified for 319 individuals. Partial sequences of the nuclear gene encoding recombinase activating 2 protein (RAG2) were amplified for 201 individuals representing each matriline and most localities (Supplementary Table S1). Primers were summarized in Supplementary Table S2. Standard polymerase chain reactions (PCR) were conducted in a 25 μL volume reaction using the following cycling conditions: an initial denaturing step at 95°C for 5 min; 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C (Supplementary Table S2) for 1 min, and extension at 72°C for 1 min; and a final extension step of 72°C for 10 min. PCR products were purified with Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai, China) or a modification of the Exo-SAP method (Werle et al. 1994). Cleaned products were sequenced directly with the corresponding PCR primers using the BigDye Terminator Cycle Sequencing Kit (v.2.0, Applied Biosystems) and an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Sequences from each gene were aligned using MEGA 5.05 (Tamura et al. 2011). For CYTB, identical haplotypes were collapsed using DNAsp 5.10 (Librado and Rozas 2009), and the overall value of nucleotide diversity (π) and haplotype diversity (H) were also estimated using the same program.

**Phylogenetic analyses**

Matrilineal relationships among mitochondrial haplotypes of CYTB and a nuclear gene tree based on RAG2 gene were independently constructed using Bayesian inference (BI) and maximum likelihood (ML) methods. Best-fit nucleotide substitution models were selected for the 3 codon position partitions using Akaike information criterion (AIC) in jmodeltest 2.1.6 (Guindon and Gascue 2003; Darriga...
Divergence time estimation

Time to the most recent common ancestor (TMRCA) of matrilineal complex was estimated using a Bayesian approach implemented in BEAST 1.7.5 (Drummond and Rambaut 2007). We employed a likelihood ratio test (LRT) to assess if our CYTB data followed a constant rate of molecular evolution (Huelsbeek and Crandall 1997). The test was based on unconstrained and clock-enforced matrilineal genealogies estimated using PHYLIP 3.6.9 (Felsenstein 2004). Due to the absence of a fossil record, we assumed a range of substitution rate of 0.65–1.00% (mean = 0.8%) per Ma for CYTB based on evolutionary rates commonly proposed for anurans (Macey et al. 1998, 2001; Monsen and Blouin 2003; Yan et al. 2013) and generally for mtDNA (Brown et al. 1979). Five tree-priors (Bayesian skyline, constant size, expansion growth, exponential growth, and logistic growth) were performed with a different model of nucleotide substitution for each of the 3 codon positions. The best prior was determined using a Bayes factor test with TRACER 1.4 (Rambaut and Drummond 2007). For each process, Markov chains were run for 30 million generations. Trees were sampled every 1000 generations. Burn-in and convergence of the chains were determined with TRACER 1.4 (Rambaut and Drummond 2007). Further, ESSs were required to have values greater than 200.

Historical demography

Two analyses of the CYTB data served to assess historical changes in effective population size. Considering that population subdivision could have influenced the effect of expansion (Grant et al. 2012; Heller et al. 2013), we analyzed well-supported (BPP >0.95) matrilineal N1, N2, S1, S2, S3, and S5, which harbored more than 10 samples and contained 3 haplotypes. First, we used non-genealogical lines N1, N2, S1, S2, S3, and S5, which harbored more than 10 samples as a data partition as BI analyses. Support values were estimated from 1000 non-parametric bootstrap replicates. Uncorrected pair-wise genetic distances (p distances) between major lineages and sublineages were calculated for CYTB using MEGA 5.0 (Tamura et al. 2011). Support values were estimated from 1000 non-parametric bootstrap replicates. Uncorrected pair-wise genetic distances (p distances) between major lineages and sublineages were calculated for CYTB using MEGA 5.0 (Tamura et al. 2011).

Point-based analysis of environmental variables

A point-based method of analysis was performed to compare ecological niche differences between the 2 species. The values for each of the 19 present bioclimatic variables were extracted for all sampling sites using DIVA-GIS (Hijmans et al. 2005). Principal component analysis (PCA) was conducted in sps 16.0 to convert the 19 variables to a set of values that accounted for most of the variance. Principal components with eigenvalues >1 that explained >10% of the variation were retained. We used independent sample T test for the 19 bioclimatic variables to determine significant differences in environmental conditions between the populations from each side of the Red River.

Results

Sequence characteristics

For CYTB, we obtained sequences for 324 individuals of the M. fissipes complex, and the 4 outgroup samples (Supplementary Table S1). For this dataset, we sequenced 319 individuals de novo and retrieved 9 from the previous studies (Matsui et al. 2005; Zhang et al. 2005; Hasan et al. 2014). After trimming the ends, 588 base pairs (bp) were retained for downstream analyses. All nucleotide sequences successfully translated to amino acids without premature stop codons and heterozygotes. A total of 136 potentially parsimony-informative sites resulted in 89 haplotypes for the ingroup. The overall nucleotide diversity (π) was 0.0534; haplotype diversity (H) was 0.948. The best-fit models for the first, second, and third codon positions of CYTB were K80 + G, HKY + I, and GTR + G, respectively.
One nuclear gene fragment, RAG2, was obtained from a subset of our samples representing matrilines of *M. fissipes* complex and 1 individual of *M. mixtura* (Supplementary Table S1). The data included 201 sequences. After trimming the ends, a 587-bp fragment was resolved, of which 42 sites were potentially parsimony informative. The best-fit models for the first, second, and third codon positions were all GTR + G. All newly obtained sequences were deposited in GenBank (Supplementary Table S1).

Matrilineal genealogy and nuclear gene tree

The BI and ML analyses produced extremely similar matrilineal based on the mtDNA data. The BI tree was presented in Figure 2B. Distinctive matrilineal N and S corresponded to *M. fissipes* and *M. mukhlesuri*, respectively. *Microhyla fissipes* (Lineage N) was broadly distributed mainly north of the Red River where it covered most regions of southern China including Taiwan and Hainan (circles, Figure 1; Figure 2). It contained 6 sublineages (N1–N6) whose relationships were unresolved. Only N1, N2, N4, and N5 were highly supported. Sublineage N1 occurred in coastal southern China, Hainan, and Taiwan (type locality of *M. fissipes*). Sublineage N2 mainly occurred from west of N1 from eastern Sichuan to Jiangxi. Sublineage N3 occurred at localities 19–21 (Figure 1). Sublineage N4 occurred at localities 11 and 36–38. Sublineage N5 occurred at localities 17, 18, and 26. Finally, sublineage N6 occurred at localities 17, 18, 22, 25, 27–34. Haplotypes 9 (localities 1–13, 31) and 10 (localities 9, 10, 22, 25, 27–34) from sublineage N1 and N2, respectively, were broadly distributed (Supplementary Table S1).

*Microhyla mukhlesuri* (Lineage S) hosted 5 matriline (Figure 2, S1–S5). The species occurred mainly in Indochina and extreme southwestern China south of Red River (triangles, Figure 1). Sublineage S1, the sister group of S2, consisted of individuals from central Vietnam to eastern Thailand. Sublineage S2 occurred at locality 76 in northern Vietnam only. Sublineage S3 was distributed in northwestern Thailand and 1 site in Yunnan (locality 53). Two haplotypes from southern Yunnan and 1 haplotype from Bangladesh (the type locality of *M. mukhlesuri*) formed sublineage
S4 but without high support. Sublineages S3 and S4 clustered together with strong support. Sublineage S5 occurred mainly in southern Vietnam. Locality 53 contained sympatric sublineages S3 and S4, and locality 65 hosted both S1 and S3. No single haplotype occurred across the distribution of the species (Figure 1, Supplementary Table S1). The p-distances for CYTB between matrielines N and S were 9.1%; distances between the 6 sublineages of matriiline N ranged from 1.0% to 2.4% and the 5 sublineages of matriiline S ranged from 3.4% to 6.8% (Supplementary Table S3).

BI and ML analyses of the RAG2 data (Figure 2A) also resolved well-supported matrilines N and S (M. fissipes and M. mukhlesuri, respectively). Owing to a limited number of potentially parsimony-informative sites, no strong historical patterning was recovered within either group. Within each species, the nuclear gene did not form geographic units, but were mixed together.

**Estimated divergence times**

The null hypothesis of clock-like evolution for CYTB was not rejected by the LRT ($\chi^2 = 37.22, df = 63, P > 0.05$). Therefore, a strict molecular clock was used. The Bayes factor test suggested Bayesian skyline as being the best tree prior process (Supplementary Table S4).

Historical demography

Based on the CYTB data, ESSs values were generally high (> 200) for all parameters in all BSPs analyses, indicating good MCMC mixing in the combined chains. Sublineages N1 and N2 from north of the Red River had similar demographic histories. Significant negative values of Tajima’s $D$ and Fu’s $F_s$ indicated population expansions (Table 1). The most prominent feature of the BSPs was a slight decline followed by a sharp increase in effective population size. As shown in Figure 4A and Figure 4B, the population sizes of sublineages N1 and N2 were relatively stable until approximately 25,000 years near the LGM. The decline spanned from 25,000 to 5,000 years ago with expansion starting about 5,000 years ago.

According to the coalescent-based BSPs analyses, the effective population sizes of sublineages S1–S3 and S5 were stable during LGM (Figure 4C–F). Significant negative values ($P < 0.05$) of Tajima’s $D$ and Fu’s $F_s$ were also revealed for sublineages S1 and S5 (Table 1). However, compared with BSP, which directly quantified demography from gene genealogies (Drummond et al. 2005), these summary statistics did not take into account tree structure and they were not based on all available information (Pybus et al. 2000).
Historical dynamics of distributions

The ENMs had excellent predictive power for occurrences under conditions of both today and the LGM. The AUC had values higher than 0.85 in all analyses, which implied that the results greatly differed from random prediction (AUC = 0.5). For *M. fissipes*, suitable habitats occurred presently north of the Red River in southern China and coastal northern Vietnam (Figure 5A). In contrast, the LGM MAXENT projection predicted a more narrow distribution and shift to 4 major areas including coastal southeastern China, Taiwan, Zhejiang and northern Fujian, and eastern Sichuan, Chongqing and Guizhou (Figure 5B). For *M. mukhlesuri*, suitable habitats occurred presently south of the Red River including Hainan (Figure 5C). The resulting LGM MAXENT projection predicted a similar continuous distribution compared with the current range, although some subtle differences existed for the most suitable areas (Figure 5D).

Point-based analysis

Four principal components (PC1 = 49.86%, PC2 = 17.34%, PC3 = 15.13%, PC4 = 7.80%) explained 90.13% of the total variation for the present ecological niche differences (Table 2). Plots from PC1 and PC3 (Figure 6A), PC1 and PC4 (Figure 6B) indicated that *M. fissipes* and *M. mukhlesuri* were well differentiated across the Red River. The independent sample *T* test indicated that the habitats of these 2 species differed significantly (*P* < 0.05) between the Red River for 12 of the 19 bioclimatic variables (Table 3).

Discussion

Species boundary

Hasan et al. (2014) hypothesized the existence of 2 or more species within *M. fissipes* complex based on morphology and mtDNA
sequences, though with limited sampling. Analyses of our nuDNA sequences also resolve 2 major clades (N and S) and these correspond with the matrilines. This discovery supports the recognition of both *M. fissipes* and *M. mukhlesuri*. Further, our extensive sampling clearly defines the distributions of both species. *Microhyla fissipes* occurs north of the Red River throughout southern China, including Taiwan (type locality) and Hainan Island (Figure 1). Samples of *M. mukhlesuri* are from Bangladesh (type locality), southern Yunnan, Thailand, Laos, and Vietnam south of the Red River. Although *M. mukhlesuri* has 5 divergent matrilines, the nuclear gene tree shows no sign of further division. Despite the absence samples from Myanmar and Cambodia, the widespread occurrence of *M. mukhlesuri* from Bangladesh to Thailand, Laos, and Vietnam implies that it also occurs in these areas. Further studies in these regions can fine-tune the distribution of *M. mukhlesuri*.

**Red River barrier**

Our study supports the allopatric occurrence of *M. fissipes* and *M. mukhlesuri* along the Red River shear zone (Figure 1). No locality examined hosts both species. This finding implies the absence of gene flow across the Red River. Two other species of frogs exhibit the same pattern: the spiny frog *Nanorana yunnanensis* (Zhang et al. 2010a) and the megophryid frog *Leptobrachium ailaonicum* (Zhang et al. 2010b). Both of these species occur around the Red River in Yunnan, which suggests that the river serves as an effective geographical barrier to contemporary dispersal. The Red River forms a major geological strike-slip fault zone that marks the boundary between South China and Indochina tectonic plates (Leloup et al. 1995; Hall 1998). The complex geology of the Red River was suggested to divide once continuous populations (Hall 1998; Zhang et al. 2010a, 2010b). The divergence of *M. fissipes* and *M. mukhlesuri* dates to 7.89 Ma, which broadly coincides with 1 period of the extrusion of the Indochina block shearing along the Red River fault in the Late Miocene (Hall 1998; Xiang et al. 2004; Zhang 2009). These geological events coincided with extremely dry, hot climatic conditions along the Red River basin and were likely responsible for the initial fragmentation of habitats.

Differing environmental conditions between north and south of the Red River likely promoted and maintained the divergence of

*Figure 5. ENMs for lineages N (A, B) and S (C, D). SDMs at present (A, C) and LGM (~21 ka; B, D) were presented. Color scale refers to probability of occurrence (habitat suitability) from MAXENT.*
The Red River largely demarks the location of change between subtropical and tropical climates (Peel et al. 2007; Chen and Chen 2013) where temperature and precipitation can differ greatly. Differing climate and landscape features can promote local adaptations (Sobel et al. 2010) especially for frogs whose reproductive periods are strongly influenced by environmental variables. Both the ENMs and T tests indicate significant ecological differences between *M. fissipes* and *M. mukhlesuri* both at present and during the LGM (Figure 5). The PCAs based on the present environment data are consistent with these findings. Bioclimatic variables for the populations on either side of Red River generally form 2 clusters (Figure 6). Twelve of the 19 bioclimatic variables involved in temperature and precipitation differ significantly (*T* test, *P* < 0.05) across the Red River (Table 3). Ecological differences between populations could generate localized adaptations (Zhou et al. 2012). This process probably likely plays the key role in speciation within the *M. fissipes* complex.

Previous studies were limited to the narrow confines of geographic regions (e.g., Yunnan; Zhang et al. 2010a, 2010b) or involved either side of the Red River (e.g., reviewed in Li and Li 1992; Bain and Hurley 2011; Fan et al. 2013). Thus, they focused on the Red River as being a geographical barrier to dispersal alone;
**Effect of the LGM**

The effects of Pleistocene climatic cycling varied with regions, despite the *M. fissipes* complex having similar habitat requirements (lowland scrub forest and grassland, agricultural land and pastureland). Our results demonstrate that late Pleistocene climatic had little influence on the distribution of *M. mukhlesuri* in Indochina. By contrast, it greatly affected the current genetic structure and population demography of *M. fissipes* in southern China.

LGM climatic change does not appear to have affected the population size and genetic structuring of *M. mukhlesuri* from Indochina. Demographic reconstructions do not detect any bottlenecks for any sublineage but rather predict stable demographic histories (Figure 4C–F). Further, the absence of widely distributed haplotypes rejects a hypothesis of recent population expansion. Divergence estimates for the 5 sublineages predate Pleistocene climatic changes, which implies that population structuring does not associate with climatic cycling. However, analyses of the nuDNA data do not obtain the same pattern. This is not surprising given the slower divergence rates of the nuDNA markers and usually higher effective population sizes, which could result in incomplete lineage sorting (Zhang and Hewitt 2003; Brito and Edwards 2009).

Large areas of suitable habitat during the LGM explain the stable demographic history of *M. mukhlesuri*. ENMs analyses reveal widespread suitable climatic conditions for *M. mukhlesuri* during the LGM (Figure 5C, D). In tropical Indochina, temperatures lowered during the LGM, but the consequences of this on humidity and types of vegetation are a hot topic of debate. Some palaeoclimatic studies suggested that most of the tropical rain forest was eliminated and replaced by pine grassland savannah during the LGM (Hope et al. 2004; Bird et al. 2005; Wurster et al. 2010). Consistent with this, several studies of forest-dwelling species in these regions identified population shrinkages during the LGM followed by drastic expansions, such as for Asian colobine monkeys (Brandon-Jones 1996), stone oaks (Cannon and Manos 2003), mosquito *Anopheles dirus* (O’Loughlin et al. 2008; Morgan et al. 2010), and the black fly *Simulium tani* (Framual et al. 2005). In contrast, records of pollen and phyoliths indicated the existence of a large and continuous tract of tropical lowland rain forest during the LGM (Cannon et al. 2009; Wang et al. 2009b). Latimee et al. (2015) compared the suitable habitats for 3 Southeast Asian forest-dwelling murine rodents (*Leopoldamys*) in the present and LGM. They reported that these species did not experience significant range contractions during the LGM. However, little is known about how lowland species responded to these climate changes, especially in mainland Indochina. Our study documents a stable demographic history for 1 species of lowland frog.

*Microbryla fissipes* from southern China underwent demographic and range contractions in response to climate changes during the late Pleistocene, especially sublineages N1 and N2. Our coalescent-based BSP analyses suggest both population sizes of the sublineages N1 and N2 experienced declines during the LGM followed by recent, rapid expansions (Figure 4A, B). Further, widely distributed haplotypes (Hap 9, 10) occur across large areas of southern China, which possibly indicates recent population expansions. These 2 sublineages cover most of the distribution of *M. fissipes*, which suggests the climatic change possibly affected most of the populations of *M. fissipes*. The occurrence of several recently divergent sublineages within southern China reflects a pattern of range fragmentation into multiple refugia during the LGM. Moreover, the 9 or more pairs of sympatric haplotypes (Figure 1) may indicate secondary contact owing to recent population expansions from different refugia.

Drastic historic climatic change in southern China possibly contributed to the genetic pattern and population history of *M. fissipes*. Although no glacier covered these regions, the temperature of southern China was reduced by 6–7°C and precipitation decreased by 400–600 mm/year during the LGM (Zhou et al. 1991). Under this climate, drastic population size changes are likely to have happened, especially for the lowland frogs that are sensitive to the environment oscillations. Consistent with this scenario, our ENMs analyses suggest that the predicted range of *M. fissipes* from southern China shrink extensively during the LGM compared with today (Figure 5A, B).

Considering previous studies in southern China, we suggest that species with different ecological requirements vary in their responses to the same climatic changes even in similar regions. For example, the lowland and montane species in southern China show different responses to Late Pleistocene climatic changes. Similar to *M. fissipes*, population contractions and postglacial range expansions were also found in the lowland dwelling cricket frogs of southern China (*Fejervarya multifilis*; Zhong et al. 2008). In contrast, montane spiny frogs (*Quasipaa boulengeri*; Yan et al. 2013) and stream newts (*Pachytriton*; Wu et al. 2013) in southern China appear to have deep population divergences and stable demographic histories. The opposing patterns also correspond with differing types of vegetation in these regions, such as plants growing in thickets at forest margins (e.g., Qi et al. 2012; Tian et al. 2015) vs. those limited to montane forests of mixed evergreen and deciduous broadleaf trees (e.g., Wang and Ge 2006; Wang et al. 2009a). Montane species can shift their habitats along elevational gradients and survive environmental perturbations intact, but not lowland species (Hewitt 2000, 2004; Wu et al. 2013). These studies indicate that it is necessary to consider the ecological requirements of species when investigating how historic changes in climate shape genetic patterns.

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**Table 3. Results of T test for the 19 biological environmental variables, and 4 principal components derived from the PCA**

| Bi-variable | F    | P value |
|-------------|------|---------|
| BI01 = Annual Mean Temperature | 0.383 | 0.537   |
| BI02 = Mean Diurnal Range* | 69.433 | 0.000   |
| BI03 = Isothermality | 1.963 | 0.163   |
| BI04 = Temperature Seasonality* | 12.092 | 0.001   |
| BI05 = Max Temperature of Warmest Month* | 6.935 | 0.009   |
| BI06 = Min Temperature of Coldest Month | 0.091 | 0.764   |
| BI07 = Temperature Annual Range | 3.122 | 0.079   |
| BI08 = Mean Temperature of Wettest Quarter* | 7.692 | 0.006   |
| BI09 = Mean Temperature of Driest Quarter | 1.637 | 0.203   |
| BI10 = Mean Temperature of Warmest Quarter* | 4.357 | 0.039   |
| BI11 = Mean Temperature of Coldest Quarter* | 4.217 | 0.035   |
| BI12 = Annual Precipitation* | 15.186 | 0.000   |
| BI13 = Precipitation of Wettest Month* | 8.284 | 0.005   |
| BI14 = Precipitation of Driest Month* | 11.975 | 0.001   |
| BI15 = Precipitation Seasonality | 0.974 | 0.325   |
| BI16 = Precipitation of Wettest Quarter* | 10.311 | 0.002   |
| BI17 = Precipitation of Driest Quarter* | 8.746 | 0.004   |
| BI18 = Precipitation of Warmest Quarter* | 18.085 | 0.000   |
| BI19 = Precipitation of Coldest Quarter | 0.841 | 0.361   |

**Note:** * indicates significant differences (P < 0.05) between the habitats of *M. fissipes* and *M. mukhlesuri* at the Red River.
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Supplementary material

Supplementary material can be found at http://www.cz.oxfordjournals.org/.

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