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# Genealogy and Demographic History of a Widespread Amphibian throughout Indochina

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## Abstract

Relatively little is known about spatial patterns of cryptic diversity in tropical species and the processes that generate them. Few studies examine the geographic distribution of genetic lineages in Southeast Asia, an area hypothesized to harbor substantial cryptic diversity. We investigated the evolutionary history of Asian tree frogs of the *Polypedates leucomystax* complex ($n$ = 172) based on 1800 bp of the mtDNA genes *ND1* and cytochrome b and tested hypotheses pertaining to climate, geology, and dispersal patterns. Analyses revealed substantial genetic diversity and lineage divergence throughout the region with evidence for widespread sympatric lineages and a general north versus south clustering. Relaxed molecular clock analysis and tests for demographic expansion identified an initial cladogenesis during the Miocene with subsequent Plio–Pleistocene diversification, with the former corresponding to periods of increased aridity and the onset of monsoonal weather systems. Rates of diversification were relatively constant until the Early Pleistocene when rates increased exponentially. We found equivocal evidence for isolation-by-distance and a potential role of some landscape features as partial barriers to dispersal. Finally, our analyses showed that divergence between insular and mainland populations occurred before *Homo sapiens* colonized Southeast Asia, suggesting that historical human-mediated dispersal did not drive insular diversification. Our results suggested that demographic expansion in the Late Pleistocene resulted in widespread sympatric lineages in the *P. leucomystax* complex throughout southern China and Indochina and further clarified the evolutionary history of lineages within *P. leucomystax*.

**Key words:** *climatic shift, cryptic species, demographic expansion, phylogeography,* Polypedates leucomystax, *Red River, Rhacophoridae, Tibetan Plateau, Vietnam, whipping frogs*

The relative role climatic oscillation plays in shaping modern patterns of genetic structure is debated (e.g., Hewitt 2000; Bennett 2004). Proponents of the glacial refugia hypothesis suggest that the ranges of species in tropical areas became fragmented during the mid-to-late Pleistocene due to increased aridity and/or decreased temperature (Haffer 1969). This hypothesis states that glacial retreat and its concomitant climatic conditions resulted in reuniting of previously allopatric populations (secondary contact) through range expansion. However, studies of Nearctic fauna suggest that divergence in some vertebrate groups occurred well before Pleistocene climatic shifts (e.g., Zink

et al. 2004) and that the usefulness of the refugia model may be restricted to taxa that have limited tolerance of climatic perturbations, including dryness (Murphy and Aguirre-León 2002).

Several recent studies elucidate the patterns and timing of lineage divergences in both temperate and tropical organisms. Whereas some Neotropical groups show rapid divergence throughout the Pleistocene (Noonan and Gaucher 2005; Bryson et al. 2011), others appear to have a much older date of divergence (e.g., Becerra 2005). Phylogenetic studies on palaeofauna reach a wide range of conclusions. For example, cladogenesis and demographic expansion of some East and Southeast Asian vertebrates are correlated with Late Pleistocene climatic oscillations (Huang et al. 2007; Zhang et al. 2008; Li et al. 2009a, 2009b; Ding et al. 2011), but others are associated with geological and palaeogeographic events (Song et al. 2009; Zhang et al. 2010). Some initial cladogenesis may also date back to the mid-late Miocene, a period of tectonic and climatic instability in Asia (Song et al. 2009; Che et al. 2010).

Southeast Asia is topologically complex due to its dynamic geologic and climatic history (Voris 2000; Huang et al. 2005; Che et al. 2010). Uplift of the Himalayan Mountains and the Qinghai-Tibetan Plateau starting approximately 50 million years ago (Ma) has profoundly impacted both continental climatic patterns and biotic evolution throughout central and southern Asia (Zhisheng et al. 2001; Che et al. 2010). Further, landscape features such as the Red River in southern China (i.e., Yuan River) and northern Vietnam (i.e., Sông Cái) act as barriers to dispersal for some taxa (e.g., Zhang et al. 2010). Thus, this region likely contains undocumented genetic diversity (Stuart et al. 2006). In species complexes where cryptic variation is suspected but not confirmed, molecular data and dating may serve to clarify patterns of variation and identify the probable drivers of these patterns.

The common Asian tree frog or whipping frog, *Polypedates leucomystax* Gravenhorst 1829 (Rhacophoridae), ranges from India and Nepal eastward through Indochina and the Sunda Shelf and northwards into mainland China (Frost 2011). The taxon exhibits substantial morphological and call variation across its range and is therefore recognized as a complex of cryptic species (Matsui et al. 1986; Narins et al. 1998; Trépanier et al. 1999; Orlov et al. 2001; Brown et al. 2010; Diesmos et al. 2004; Sheridan et al. 2010). The complex occurs in a variety of habitats, including the edges of secondary growth and even major metropolitan areas including Hanoi and Ho Chi Minh City in Vietnam and Haikou on Hainan Island in China (Pope 1931; Liu 1950; Manthey and Grossmann 1997). Because of its apparent ubiquity, the complex is listed as Least Concern by the International Union for the Conservation of Nature (Diesmos et al. 2004).

Subsequent to Narins et al. (1998) and Trépanier et al. (1999), Brown et al. (2010) reported evidence of substantial cryptic genetic diversity based on 16S mitochondrial sequences and showed signs of recent population expansion in *P. leucomystax* throughout insular Southeast Asia. Based on their matrilineal genealogy, they suggest that humans mediated recent dispersal and range expansion of *P. leucomystax* among islands. Their results also showed signs of highly divergent sympatric lineages on the mainland. However, their mainland sampling was limited, and thus they could not clarify matrilineal genealogical relationships, diversity, and biogeographic patterns throughout a broader range of the species complex.

We combine broad geographic sampling throughout Vietnam and southern China with limited sampling in adjacent areas of Indochina to clarify the matrilineal history and genetic structure of *P. leucomystax*. Using phylogenetic and population genetic tools along with molecular clocks, we investigate two main questions. 1) How has historical climatic cycling influenced cladogenesis and historical demography of *P. leucomystax*? By combining molecular dating estimates and changes in demographic parameters through time, along with palaeoclimatic data from the literature, we assess concordance between genetic patterns and climatic cycling throughout Southeast Asia and 2) how are patterns of genetic diversity spatially structured? For example, do highly divergent lineages correspond with geography? We test a null hypothesis of isolation-by-distance (IBD), which predicts a strong correlation between Euclidean distance and genetic differentiation. Where IBD does not explain the observed patterns, we test two further hypotheses. First, we test the hypothesis that the Red River acts as a barrier to dispersal, and second, we test the hypothesis that humans are responsible for establishing the initial insular populations of *P. leucomystax*. Failure to refute the null hypothesis—that humans did not mediate divergence in this species complex—requires that insular lineages diverged before the earliest estimated arrival of *Homo sapiens* in Southeast Asia (60 000 years ago; Shi et al. 2008).

## Materials and Methods

### Specimens Examined

We sampled 172 individuals from the *P. leucomystax* complex throughout Southeast Asia. Sampling included 23 localities throughout Vietnam and southern China as well as specimens from 12 localities in total from Laos, Cambodia, Thailand, Myanmar, Sabah/Borneo, and the Philippines (see Supplementary Materials online). Heart, skeletal muscle, or liver tissues were used for DNA analysis and were either flash frozen or ethanol-preserved.

### DNA Extraction, Amplification, and Sequencing

We sequenced segments of NADH subunit 1 (*ND1*) and cytochrome b (*cyt b*). Genomic DNA from liver or muscle tissue was first digested with proteinase K for 7–12 h and subsequently extracted using three phenol-chloroform isoamyl alcohol and one chloroform isoamyl alcohol extractions. We amplified DNA fragments using the polymerase chain reaction (PCR; 92 °C for 30 s, 47–53 °C for 40 s, and 72 °C for 1.0 min) performed in 25-µl reactions for 37 cycles. Each reaction contained the following mix: 18.79-µl $ddH_20$, 1-µl 10 mM of each primer, 2.5-µl PCR buffer containing 1.5 mM $MgCl_2$, 0.56-µl 10 mM dNTPs, 0.15-µl 5 U *Taq* DNA polymerase, and 1-µl template DNA. Different combinations of primers were used to obtain the longest reliable reads (see Supplementary Materials online).

Annealing temperatures ranged from 47 to 53 °C, and we obtained optimal temperatures for each primer combination by running temperature gradients. PCR products were cleaned using QiaColumns (Qiagen), eluted in Qiagen EB buffer, and sequenced using 8μl BigDye® (Applied Biosystems International) with 25 cycles under the following conditions: 92 °C for 30 s, 47–53 °C for 30 s, and 72 °C for 1.5 min. We then ran out resuspended DNA on either an ABI 377 automated sequencer using long plates or an ABI 3100 automated sequencer (Applied Biosystems).

### DNA Sequence Alignment and Genealogical Reconstructions

Sequences were edited and assembled in BioEdit v. 7.0.5 (Hall 1999), and the identities of the specimens were confirmed using a BLASTn search. We checked for premature stop codons, which would have indicated the amplification of nuclear pseudogenes. For outgroup sequences, we downloaded orthologs from *Polypedates cruciger* (Blyth 1852; GenBank Accession No. AF249089.1 and AY708131.1), *Rhacophorus malabaricus* (Jerdon 1870; 14718832 and AY708130.1), and *R. schlegelii* (Günther 1858; AB202078.1). These taxa lay outside the ingroup (Li et al. 2009a). The sequence from a putative representative of *P. megacephalus* (AY458598; locality undefined) was also included because the evolutionary distinctiveness and identification of this specimen remains questionable (Sheridan et al. 2010). Alignment was performed using ClustalW v. 1. (Thompson et al. 1994) and subsequently confirmed by eye.

We used Bayesian inference (BI; Huelsenbeck and Ronquist 2001) to hypothesize the matrilineal genealogy of *P. leucomystax*. Using the Akaike information criterion (AIC) in MrModeltest v. 2.2 (Nylander 2004), we selected the GTR+I+Γ model of nucleotide substitution. Because mtDNA is inherited as a single, linked molecule, we concatenated the data into a single matrix. Phylogenetic inference was implemented using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). The analysis started with random trees, used six Markov chains (five heated chains and one cold), and ran for $4.5 \times 10^6$ generations, sampling trees every 100 generations. We ran two independent analyses with different starting trees to avoid being trapped on local optima. Fluctuating likelihood values and standard deviation of split frequencies were monitored to determine when stationarity and convergence was achieved (Huelsenbeck and Ronquist 2001). We monitored effective sample size (ESS) values with Tracer v. 1.4.1 (Rambaut and Drummond 2007). After discarding 25% of the sampled trees as burn-in, we used the remaining trees to generate a majority rule consensus tree. Nodal support values (Bayesian posterior probabilities; BPP) were mapped on the resulting topology.

We also assessed genealogical relationships under a maximum likelihood (ML) framework using RAxML v. 7.0.4 (Stamatakis 2006) implemented through the raxmlGUI interface (Silvestro and Michalak 2011). The GTRGAMMA model was specified for the concatenated mtDNA data, and we implemented a ML + rapid bootstrap search (Stamatakis et al. 2008) with the number of repetitions determined based on the *autoMR* option. We accepted bootstrap support values greater than 70 as strong support for a given node.

### Population Genetic and Historical Demographic Analyses

DnaSP v. 5 (Librado and Rozas 2009) was used to estimate diversity via the number of haplotypes ($H$), haplotype diversity ($h$), average number of nucleotide differences assuming no recombination ($k$), number of segregating positions ($S$), and nucleotide diversity ($\pi$). Sequence divergence (uncorrected $p$-distance and Tamura-Nei distance) was calculated using Mega5 (Tamura et al. 2011). We quantified historical demography using mismatch distributions (Rogers and Harpending 1992) calculated with DnaSP; these values evaluated the frequencies of pairwise nucleotide differences within predefined groups (in our case, major mtDNA lineages). A unimodal population curve representing a large frequency of small pairwise differences signified a recent population expansion. We used the Raggedness Index (Harpending 1994), calculated using DnaSP, to statistically compare the observed empirical curve to that expected under an equilibrium or expansion model.

To date approximate times of population expansion in lineages showing a unimodal distribution, we estimated three parameters from the data: $\theta_0 = 2\mu N_0$, $\theta_1 = 2\mu N_1$, and $\tau = 2ut$, where $u = \mu k$, $t$ was time in years or generations, and $N$ represented effective population size (Rogers and Harpending 1992). Calculations of $u$ assumed a divergence estimate of $\mu = 0.0069$ substitutions per site per million years ($6.9 \times 10^{-9}$ substitutions per site per year) for anuran mitochondrial genes (Hoffman and Blouin 2004 and references therein) multiplied by our total sequence length ($k$) and assuming a generation time of 1 year.

Because the Raggedness Index has low statistical power (Librado and Rozas 2009), we searched for the presence of nonequilibrium conditions within lineages using the Ramos-Onsins and Rozas' (2002) $R_2$ statistic, Tajima's (1989) $D$, and Fu's (1997) $F$s. Fu's $F$s test excels when testing for past demographic changes in large populations, whereas $R_2$ is sensitive in smaller populations (Ramos-Onsins and Rozas 2002). Although significantly positive or negative values of these tests may indicate either demographic changes or selection, comparisons of these results and to those of a mismatch distribution are assumed to yield more robust interpretations of demographic history. Critical values of $D$ followed Tajima (1989). Significance of $F$s and $R_2$ was determined from 1000 coalescent simulations assuming no recombination. All tests for demographic expansion were conducted in DnaSP.

We used Bayesian skyline plots to visualize recent changes in the effective population sizes of the major lineages using Beast v. 1.6.1 (Drummond et al. (2005); Drummond and Rambaut 2007). Input files were created with Beauti v. 1.6.1 (Drummond and Rambaut 2007), selecting an extended Bayesian skyline coalescent prior using a linear model of demographic change. We selected the GTR+I+Γ model and ran the analysis for 10 000 000 generations, sampling every

1000 generations and specifying a burn-in value of 10%. Run diagnostics were monitored in TRACER to visualize parameter estimates and ESS values.

We also assessed genetic differentiation between sample sites using spatial analysis of molecular variance (SAMOVA). SAMOVA used a simulated annealing approach to cluster geographically homogeneous populations together to minimize differentiation within groups and maximize differentiation between groups (Dupanloup et al. 2002). We first used ARLEQUIN v. 3.0 (Excoffier et al. 2005) to create an *.arp* file containing the sequence data sorted by sample site. Next we created a *.geo* file containing the geographic coordinates for each sample site. We then used SAMOVA v. 1.0 to determine the degree of genetic structure between our sites. Separate analyses were run for the two major lineages recovered from our phylogenetic analysis (lineages A and B in "Results"). The number of initial conditions was set to 100, and the molecular distance was set to pairwise difference. To select the most appropriate number of groups, *K*, we ran a number of different analyses for both lineages, specifying a range of different *K*-values. We then generated plots illustrating the relationship between *K* and differentiation between groups as measured by the $F_{CT}$ statistic. The most-likely number of groups was determined when the rate of change between successive *K*-values began to decline.

### Isolation-by-Distance

IBDWS v. 3.2.3 (Jensen et al. 2005) was used to test for correlations between Euclidean and genetic distance between populations (Wright 1943). We employed Rousset's measure of differentiation based on sequence data (PhiST/[1–PhiST]) to estimate genetic distance (Rousset 1997). Significance was assessed by Mantel tests (Mantel 1967) using 10 000 randomizations. To determine if the Red River served as a barrier to dispersal, we employed partial Mantel tests (Smouse et al. 1986). A significant riverine effect was tested both on the entire data set and within the major lineages A and B (below). Simple binary coding was used for the effect matrix. Population pairs on the same side of the Red River were assigned a value of 0 and pairs on opposite sides a value of 1. Partial correlations controlled for the influence of Euclidean distance while testing for a significant barrier effect. Significance of partial correlations was assessed with 10 000 randomizations. We excluded the few Philippine and Bornean samples from the barrier analysis because these islands could not be considered as being north or south of the Red River.

### Molecular Dating

Molecular dating assumed a rate of substitution identical to that used to date population expansions. This was cautiously pursued because even closely related groups may differ significantly in evolutionary rates (Welch and Bromham 2005). Ideally, we would use multiple fossil or geological calibration points (Benton and Ayala 2003; Reisz and Müller 2004), but such data were not available.

We dated the BI tree using BEAST v. 1.6.1. Substitution rates, gamma shape parameters, and proportion of invariable positions were obtained from MRMODELTEST (GTR+I+Γ). The analysis was constrained to the topology of the BI tree by specifying monophyly of the lineages. Because several studies have provided evidence of cryptic species in this complex (Matsui et al. 1986; Narins et al. 1998; Trépanier et al. 1999; Orlov et al. 2001; Diesmos et al. 2004; Brown et al. 2010; Sheridan et al. 2010), we considered the Yule process tree prior and used an uncorrelated relaxed lognormal molecular clock model in BEAST (Drummond et al. 2006). Two Markov chain Monte Carlo (MCMC) runs were made, each with $10^8$ generations, sampling every 10 000 generations for a final sample of 10 000 states.

We assessed convergence of the two BEAST runs by examining output files for each run in TRACER. Next, we combined the results of the runs using LOGCOMBINER v. 1.6.1 (Drummond and Rambaut 2007) while sampling the combined runs every 200 generations with a burn-in of 25%. We considered ESS values > 200 to indicate good mixing and a valid estimate of continuous parameters and likelihoods, given the specified priors (Drummond and Rambaut 2007). The maximum lineage credibility tree was computed for the combined runs using TREEANNOTATOR v. 1.6.1 (Drummond and Rambaut 2007).

Based on data from Y-chromosome markers and 5000 males, Shi et al. (2008) estimated that *Homo sapiens* first reached Asia 60 000 years ago (see Stanyon et al. 2009 for thorough review). Thus, we required insular lineages to diverge more recently than 60 000 years ago in order to consider human relocations of frogs as a possible cause of lineage divergence.

### Diversification Rates

If Pleistocene climatic cycling had a substantial influence on rates of cladogenesis in *P. leucomystax*, we expected this to be reflected in increases in diversification rates concordant with these processes. To test this prediction, we used the R package LASER (Rabosky 2006a, 2006b) to test for significant departures from diversification-rate constancy over the evolutionary history of these frogs. LASER improves over previous methods in its ability to test for temporal *increases* in diversification rate versus decreases as measured by the gamma statistic ($\gamma$) based on a constant rate test (Pybus and Harvey 2000). We calculated the test statistic $\Delta AIC_{RC}$ and compared the observed value to a null distribution of rate constancy by simulating 5000 genealogies with the same number of terminals as our empirical phylogeny. All rate-constant models and rate-variable models were compared simultaneously. A significantly positive value of this statistic signified that a rate-variable model was a better fit to the data than a rate-constant model. To visualize trends in lineage accumulation through time, we generated lineage through time plots in LASER. We scaled branching times for the plot by the basal divergence time estimated in the BEAST analysis. Finally, we calculated $\gamma$ to test for a decrease in diversification through time as would be expected if the number of available

niches was being depleted. All LASER analyses were based on the dated BEAST topology.

## Results

### Sequence Characteristics

We sequenced 172 *P. leucomystax* for *ND1* and *cyt b* and added sequences from GenBank. All new sequences were deposited in GenBank (JX393322-JX393651). No premature stop codons were found. Thus, the data were concatenated, and this resulted in 1832 positions.

### Genealogy

The BI genealogy was composed of two highly divergent lineages, each with several sublineages (Figure 1). Lineage A comprised four major sublineages. Support values for sublineage relationships were high (BPP ≥ 0.95), except for the relationship between sublineage A1 and the other sublineages in A (BPP = 0.54). Lineage B consisted of seven sublineages. Sublineage relationships were highly supported (BPP ≥ 0.95), except for the relationship between sublineages B2 and B3–B7 (BPP = 0.84). The putative sample of *P. megacephalus* in GenBank was placed in the middle of the genealogy in lineage B3. The ML genealogy was similar to the BI topology except that ML placed sublineage A1 as sister to lineage B. However, support for this relationship was weak (bootstrap = 31). The remaining relationships between lineages received high support (Figure 1).

### Patterns of Divergence

Most sampling locations were represented in both lineages A and B. Lineages B1, B2, and B6 were especially widespread geographically. The general pattern of divergence within the lineages suggested a northern origin and southward dispersal (Figures 1 and 2). The Mantel test failed to detect significant IBD when considering the entire dataset ($r = -0.006$, $P = 0.535$). Mantel tests obtained significant IBD within lineage A ($r = 0.313$, $P = 0.016$), but not lineage B ($r = 0.088$, $P = 0.235$). Lineage B had limited sample sizes from several areas, including Laos, Myanmar, Cambodia, Borneo, and the Philippines (sublineages B2 and B6). Although these samples were informative with regard to relationships, they did not accurately reflect diversity at these sites. These sites were removed for the Mantel test, after which a significant pattern of IBD was obtained ($r = 0.234$; $P = 0.0120$). Based on partial Mantel tests, the Red River was resolved as a significant barrier to dispersal within lineage B (with the exclusion of insular samples; $r = 0.136$; $P = 0.049$), but not for lineage A ($r = -0.054$; $P = 0.651$) or for the data set as a whole ($r = -0.023$; $P = 0.671$).

### Sympatric Lineages

Widespread, genetically distinct sympatric lineage pairs occurred at 16 of 24 sites in southern China, Vietnam, and southern Laos (Figure 2), of which 10 occurred between lineages A and B, and six within B only. We also detected sympatry in the Philippines and Borneo despite extremely limited sampling as well as sympatry on Hainan Island when considering the island as a whole; the two lineages on Hainan were sampled at different locations. We detected no more than two sympatric sublineages at any one locality.

### Population Genetics and Demographics

In general, diversity was high within each lineage as revealed by the number of haplotypes and haplotype and nucleotide diversities (Table 1). Diversity in sublineage B2 (Laos, Myanmar, and Yunnan, China) was exceptionally high ($k = 58.194$; $\pi = 0.05025$). Sequence divergence values based on both uncorrected *p*-distances and Tamura-Nei distances suggested high levels of divergence (up to 21%) between some lineages (Table 2). Significant deviations from neutral expectations were present in several lineages, suggesting recent demographic expansions. However, results differed depending on the statistical test performed (Table 1). For example, Tajima's *D* detected significant deviations for lineages B3 and B7 only. Conversely, Fu's *Fs* found significant deviation in lineages A1, A2, A3, B1, B3, and B7. All four tests suggested demographic expansion for sublineages A1, A2, and B7 (Table 1). The estimated times of expansion within the lineages occurred during the Late Pleistocene, approximately 170 000–13 400 years before present. Extended Bayesian skyline plots also supported a hypothesis of recent demographic expansion in each recovered lineage (results not shown).

The SAMOVA results generally agreed with the results from the phylogenetic analyses. For lineage A, the rate of change in $F_{CT}$-values started to decline at four groups, which largely corresponded to the four sublineages recovered in the mtDNA gene tree ($F_{CT} = 0.944$; $F_{ST} = 0.973$; $F_{SC} = 0.511$). Lineage B showed a slower decline in the rate of change in $F_{CT}$ with increasing values of *K*. However, one decline was observed at five groups ($F_{CT} = 0.695$; $F_{ST} = 0.824$; $F_{SC} = 0.423$) and another at 10 groups ($F_{CT} = 0.801$; $F_{ST} = 0.817$; $F_{SC} = 0.081$). The partitions suggested by SAMOVA were shown in Figure 3. All $F_{CT}$-values were statistically significant based on 1023 permutations ($P < 0.0001$). Overall, SAMOVA corroborated the phylogenetic results and suggested a general north-south clustering.

### Temporal Diversification

All ESS values for the two BEAST runs were high, indicating good mixing and sampling. Neither the standard deviation of the uncorrelated lognormal relaxed clock model nor its coefficient of variation approached zero in either run, indicating that a strict clock model was inappropriate.

Estimated divergence dates for the major lineages were mapped onto the BI tree (Figure 1), and estimated dates and the upper and lower bounds of the 95% highest posterior density (HPD) for each were summarized in Table 3. The oldest divergence in our tree, *Rhacophorus* and *Polypedates*, was estimated at approximately 9.74 Ma. These genera occurred

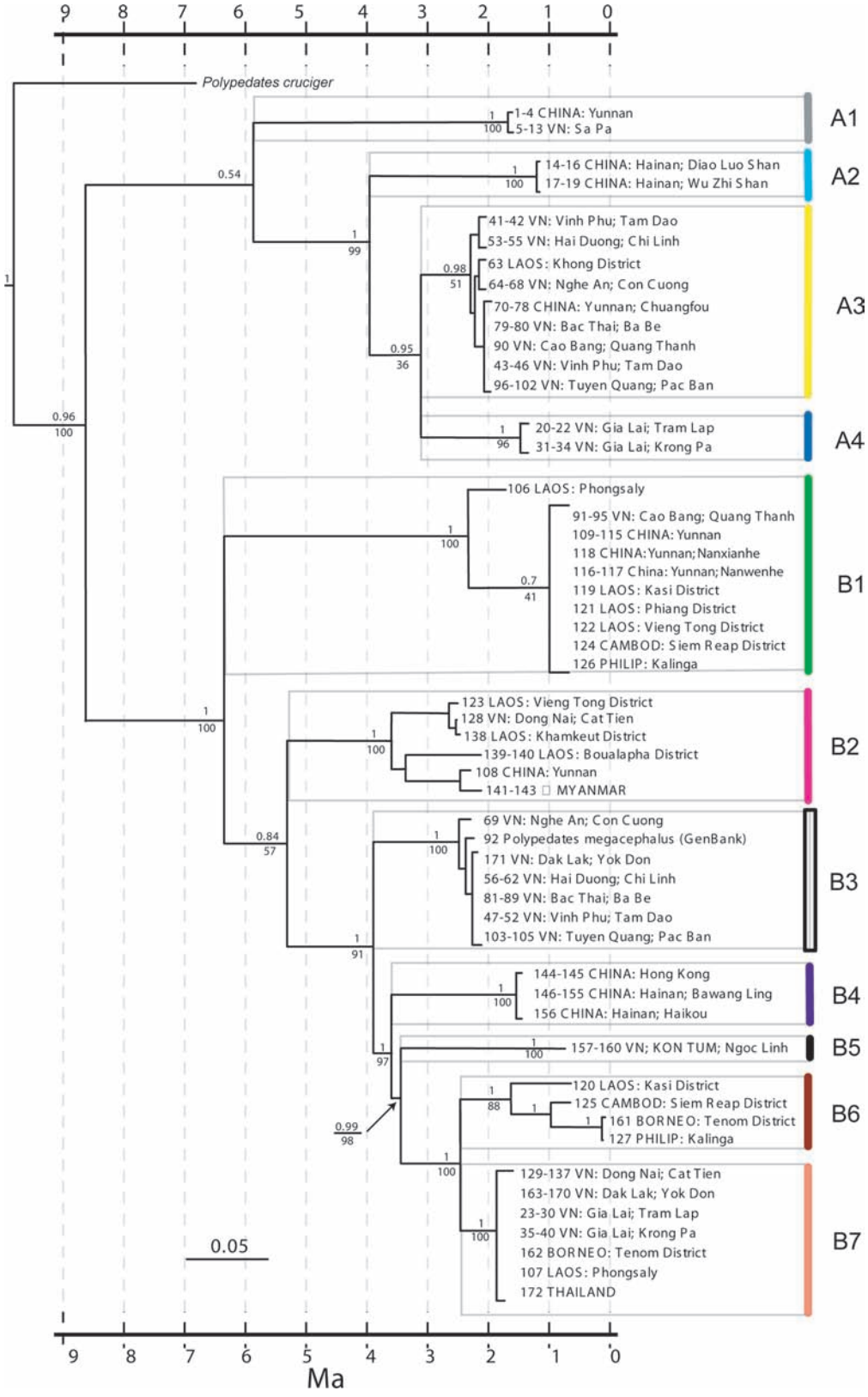


**Figure 1.** Bayesian majority rule phylogram depicting matrilineal relationships of the *P. leucomystax* species complex based on partial sequences from *ND1* and *cyt b*. Numbers above nodes represent Bayesian posterior probability values. Numbers below nodes represent maximum likelihood bootstrap proportions. Identical haplotypes and polytomies were collapsed for clarity. ID numbers refer to those shown in Appendix I (Supplementary Materials online). Color version of Figure 1 available at http://jhered.oxfordjournals.org.

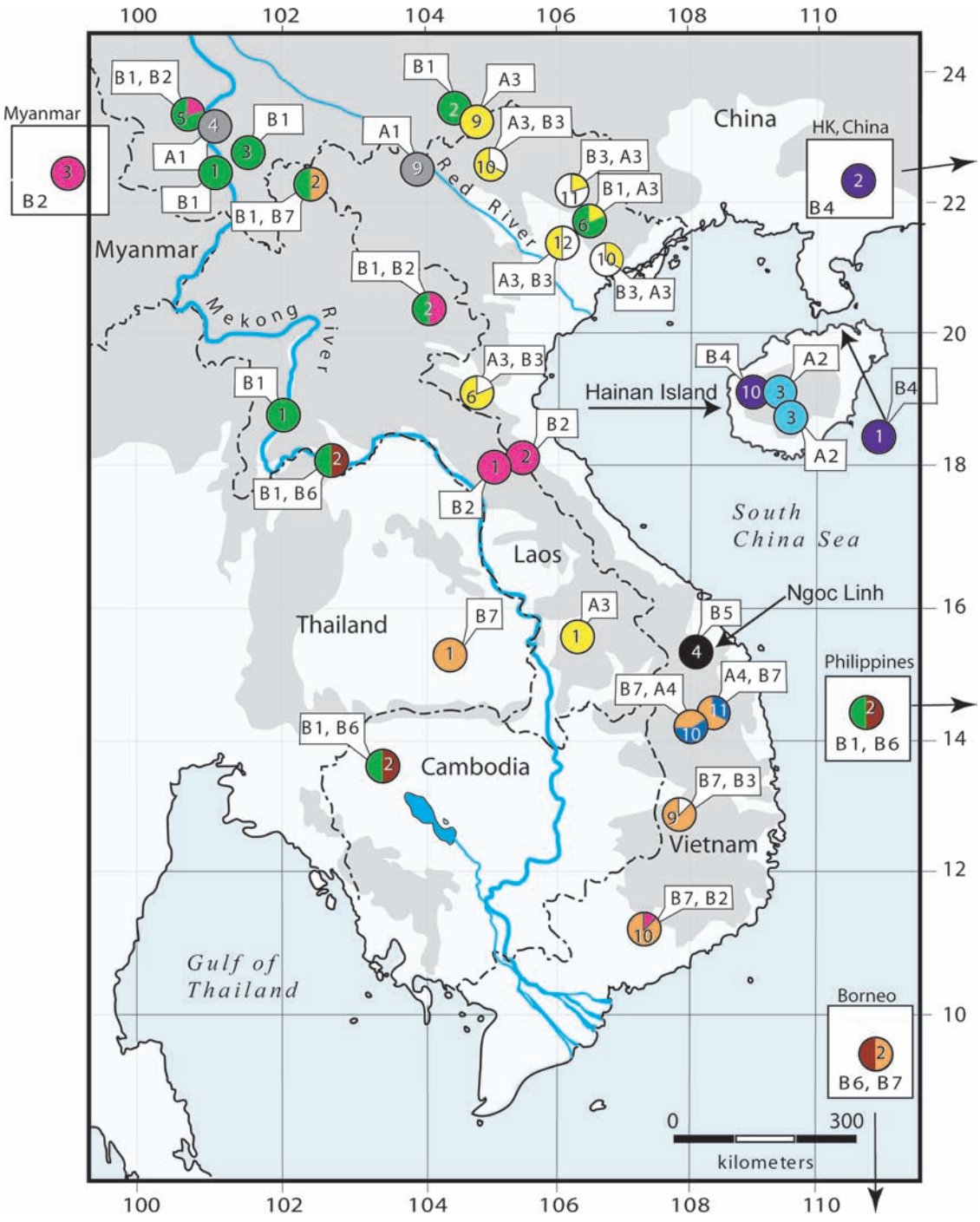


**Figure 2.** Geographic distribution of major mitochondrial lineages of the *P. leucomystax* species complex throughout southern China and Indochina. Black circle, sublineage B5, indicates the locality of Ngoc Linh; light blue (A2) and purple (B4) circles are on Hainan Island. Colored pie diagrams show the lineages recovered in the genealogical analysis (Figure 1). Pies with more than one color represent sites containing sympatric lineages. Numbers within pies indicate sample size. Shading represents high-elevation regions. Color version of Figure 2 available at http://jhered.oxfordjournals.org.

within Group 3 of Li et al. (2009a) where *Rhacophorus* is the sister group to *Polypedates*, *Feihyla,* and *Chiromantis* (the divergence between *Polypedates* and *Rhacophorus* is not shown in Figure 1 due to long branch-lengths).

Divergence of lineages A and B within the *P. leucomystax* complex was estimated at approximately 8.74 Ma (95% HPD = 11.54–6.11 Ma). Cladogenesis within each lineage began during the Plio–Pleistocene boundary in all sublineages

**Table 1** Nucleotide diversity statistics and tests for demographic expansion for select sublineages

| Lineage | *n* | *H* | *h* | *S* | *k* | $\pi$ | Tajima's *D* | Fu's *Fs* | $R_2$ | *r* |
|---|---|---|---|---|---|---|---|---|---|---|
| A1 | 11 | 8 | 0.945 ± 0.00287 | 9 | 2.255 ± 1.798 | 0.00204 | −1.119 | **−4.012** | **0.118** | **0.072** |
| A2 | 6 | 5 | 0.933 ± 0.01481 | 5 | 1.867 ± 1.524 | 0.00127 | −0.825 | **−2.263** | **0.158** | **0.111** |
| A3 | 32 | 25 | 0.984 ± 0.00014 | 54 | 9.163 ± 18.728 | 0.00635 | −1.308 | **−10.45** | 0.072 | 0.012 |
| A4 | 7 | 6 | 0.952 ± 0.00912 | 18 | 5.667 ± 9.559 | 0.00339 | −1.508 | −0.884 | 0.205 | 0.038 |
| B1 | 21 | 13 | 0.933 ± 0.00146 | 26 | 4.276 ± 4.869 | 0.00384 | −1.567 | **−4.249** | 0.086 | 0.018 |
| B2 | 9 | 9 | 1.000 ± 0.00274 | 127 | 58.194 ± 774.123 | 0.05025 | 1.073 | 0.217 | 0.217 | **0.047** |
| B3 | 25 | 16 | 0.897 ± 0.00286 | 44 | 4.583 ± 5.424 | 0.00296 | **−2.392** | **−6.387** | **0.049** | 0.017 |
| B4 | 13 | 6 | 0.859 ± 0.00400 | 12 | 3.154 ± 3.045 | 0.00205 | −0.752 | 0.041 | 0.126 | 0.030 |
| B5 | 4 | 4 | 1.000 ± 0.03125 | 7 | 3.500 ± 5.023 | 0.00200 | −0.817 | −1.012 | 0.211 | 0.055 |
| B6 | 4 | 4 | 1.000 ± 0.03125 | 47 | 24.333 ± 186.345 | 0.01550 | −0.528 | 1.326 | 0.237 | **0.222** |
| B7 | 33 | 25 | 0.977 ± 0.00021 | 58 | 6.663 ± 10.406 | 0.00466 | **−2.002** | **−13.585** | **0.054** | **0.018** |

*n* = number of individuals; *H* = number of haplotypes; *h* = haplotype diversity ± variance; *k* = average number of nucleotide differences ± variance; *S* = segregating positions; $\pi$ = nucleotide diversity; $R_2$ = Ramos-Onsins and Rozas statistic; *r* = raggedness index. Bold values represent significance at $P < 0.05$.

**Table 2** Mitochondrial DNA divergence between major lineages of the *Polypedates leucomystax* species complex

| Lineage | A1 | A2 | A4 | A3 | B1 | B2 | B3 | B4 | B5 | B6 | B7 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | | 0.167 | 0.162 | 0.167 | 0.195 | 0.196 | 0.182 | 0.189 | 0.190 | 0.196 | 0.193 |
| A2 | 0.145 | | 0.043 | 0.041 | 0.201 | 0.207 | 0.202 | 0.200 | 0.203 | 0.208 | 0.207 |
| A4 | 0.142 | 0.041 | | 0.031 | 0.194 | 0.201 | 0.192 | 0.200 | 0.192 | 0.200 | 0.198 |
| A3 | 0.145 | 0.039 | 0.030 | | 0.200 | 0.199 | 0.198 | 0.200 | 0.193 | 0.203 | 0.200 |
| B1 | 0.166 | 0.171 | 0.166 | 0.169 | | 0.103 | 0.101 | 0.100 | 0.098 | 0.100 | 0.103 |
| B2 | 0.167 | 0.175 | 0.171 | 0.169 | 0.094 | | 0.092 | 0.088 | 0.091 | 0.091 | 0.093 |
| B3 | 0.157 | 0.172 | 0.165 | 0.169 | 0.092 | 0.085 | | 0.060 | 0.063 | 0.063 | 0.064 |
| B4 | 0.162 | 0.170 | 0.170 | 0.170 | 0.091 | 0.082 | 0.057 | | 0.043 | 0.039 | 0.045 |
| B5 | 0.162 | 0.172 | 0.164 | 0.165 | 0.090 | 0.083 | 0.059 | 0.042 | | 0.039 | 0.041 |
| B6 | 0.166 | 0.175 | 0.170 | 0.172 | 0.091 | 0.084 | 0.060 | 0.038 | 0.038 | | 0.029 |
| B7 | 0.164 | 0.175 | 0.168 | 0.170 | 0.094 | 0.086 | 0.060 | 0.043 | 0.039 | 0.029 | |

Values below diagonal represent uncorrected *p*-distances. Values above diagonal represent Tamura-Nei corrected distances.

except B2, which was composed of two sublineages that began diverging approximately 3.7 Ma. These were combined into a single, larger lineage for analysis due to limited sampling from these sites, although they should be considered as two separate groups.

All estimated dates of divergence for island lineages were older than 60 000 years ago (Table 3). One Philippine lineage diverged from the mainland 1.04 Ma (95% HPD = 2.2–0.1 Ma). The other Philippine lineage, the sister to a sample from Tenom (Borneo), diverged from its most recent mainland ancestor approximately 1.05 Ma (95% HPD = 1.92–0.31 Ma). Divergence of sublineage A2 from Hainan was estimated at 1.2 Ma (95% HPD = 2.82–0.3 Ma), whereas the samples within sublineage B4 diverged from the nearest mainland lineage (Hong Kong) about 1.6 Ma (95% HPD = 2.82–0.68 Ma).

### Diversification Rates

Maximum likelihood analyses in LASER detected no decrease in diversification rate through time over the evolutionary history of these frogs ($\gamma$ = 2.3507; $P$ = 0.9906). The simultaneous comparison of multiple rate-constant and rate-variable models favored the rate-variable yule2rate model (AIC = −2564.951) over a constant birth death model (AIC = −2545.441), indicating that rates of diversification increased through time ($\Delta AIC_{RC}$ = 19.5095). Significance of the calculated $\Delta AIC_{RC}$ statistic based on 5 000 simulated trees under a Yule process indicated a significant difference from a pure birth model ($P < 0.001$). Diversification through time progressed gradually until the Pleistocene reached approximately 1.8 Ma. Lineage accumulation then followed a more exponential distribution versus linear and never reached an asymptote (Figure 4).

## Discussion

Our results are consistent with the hypothesis that palaeoclimatic changes in the Miocene may have contributed to the isolation and subsequent divergence of the *P. leucomystax* complex. Cladogenesis within the complex began about 8.74 Ma, and diversification within the two major lineages began at the Plio–Pleistocene boundary (about 2 Ma; with the exception of sublineage B2). Our results resolve multiple widespread, sympatric lineages on the mainland, and these generally agree with those of other studies of this complex. Genetic diversity within and between many mainland populations is extremely

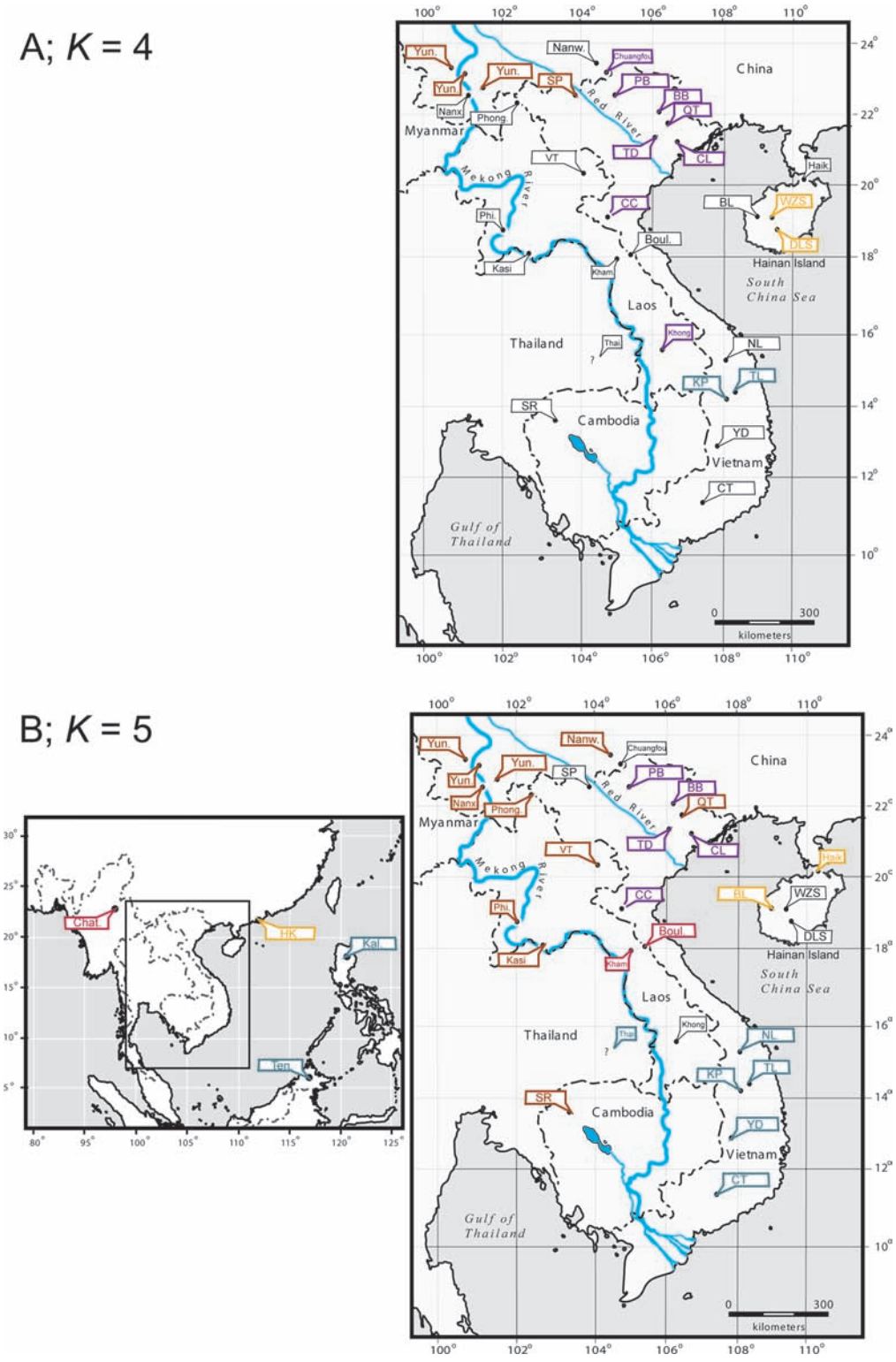


**Figure 3.** SAMOVA results illustrating the spatial clustering of haplotypes for lineage A (A) and lineage B (B). Number of clusters *K* is based on a decrease in the rate of change of $F_{CT}$ with increasing *K*. Site abbreviations are as follows: Yun. = Yunnan; Nanx. = Nanxianhe; Phong. = Phongsaly; SP = Sa Pa; Nanw. = Nanwenhe; PB = Pac Ban; BB = Ba Be; QT = Quang Thanh; CL = Chi Linh; TD = Tam Dao; CC = Con Cuong; Boul. = Boulapha; NL = Ngoc Linh; TL = Tram Lap; KP = Krong Pa; YD = Yok Don; CT = Cat Tien; SR = Siem Reap; Thai. = Thailand; Kham. = Khamkeut; Phi. = Phiang; VT = Vieng Tong; Haik. = Haikou; BL = Bawang Ling; DLS = Diao Luo Shan WZS = Wu Zhi Shan; HK = Hong Kong; Ten. = Tenom; Kal. = Kalinga; Chat. = Chatthin. Color version of Figure 3 available at http://jhered.oxfordjournals.org.

**Table 3** Mean estimated divergence times for the sampled lineages and sublineages of *Polypedates leucomystax* and upper and lower bounds of the highest posterior density (HPD) for each estimate

| Divergence: | Mean (Ma) | HPD 95% upper (Ma) | HPD 95% lower (Ma) |
|---|---|---|---|
| Lineage A from Lineage B | 8.74 | 11.54 | 6.11 |
| Lineage A: | | | |
| A1 from A2–A4 | 6.00 | – | – |
| A2 (Wu Zhi Shan, Hainan) from A3–A4 | 4.10 | 2.39 | 5.89 |
| A3 from A4 | 3.30 | 4.70 | 2.00 |
| Lineage B: | | | |
| B1 from B2–B7 | 6.40 | 8.66 | 4.38 |
| B2 from B3–B7 | 5.54 | 7.51 | 3.85 |
| B3 from B4–B7 | 4.56 | 6.15 | 3.14 |
| B4 (Bawang Ling, Hainan) from B5–B7 | 3.75 | 5.16 | 2.53 |
| B5 (Ngoc Linh) from B6–B7 | 3.24 | 4.44 | 2.12 |
| B6 from B7 | 2.64 | 3.72 | 1.69 |
| Divergence within sublineages: | | | |
| Within A1 (= Pu'er from Sa Pa) | 1.80 | 3.23 | 0.71 |
| Within A2 | 1.20 | 2.82 | 0.30 |
| Within A3 | 2.46 | 3.57 | 1.44 |
| Within A4 | 1.73 | 2.95 | 0.72 |
| Within B1 | 2.58 | 4.13 | 1.31 |
| Within B2 | 3.73 | 5.44 | 2.02 |
| Within B3 | 2.64 | 4.04 | 1.36 |
| Within B4 (= Hainan from Hong Kong) | 1.68 | 2.82 | 0.68 |
| Within B5 | 0.88 | 1.93 | 0.15 |
| Within B6 | 1.79 | 2.81 | 0.78 |
| Within B7 (= southern Vietnam from Laos/ Borneo/Thailand | 1.99 | 2.86 | 1.19 |
| Within B1 (= Philippines sample 126 from mainland B1 samples) | 1.04 | 2.20 | 0.10 |
| Within B6 (= Borneo/ Philippines samples 161/127) from mainland B6 samples) | 1.05 | 1.92 | 0.31 |

Estimates are shown in millions of years ago (Ma) and correspond to the most recent common ancestors of the lineages shown in Figure 1. Divergence times were estimated in BEAST using the priors described in Materials and Methods.

high (up to 21% uncorrected *p*-distances), and our analyses identify recent demographic expansions. With regard to patterns of dispersal, we cannot refute the null hypothesis of IBD within the major lineages, both of which show a general pattern of north-to-south dispersal (Figures 1 and 2). However, IBD does not explain the overall pattern of diversification, and we also find that the Red River has formed a partial barrier to dispersal in lineage B. With regard to human-mediated dispersal of *P. leucomystax,* we cannot refute the null hypothesis that *P. leucomystax* dispersed to the Sunda Shelf and the Philippines without human mediation.

## Climatic Oscillations and Genealogical Divergence

The initial divergence between lineages A and B (between 11.54 and 6.11 Ma) may have been caused by increased aridity

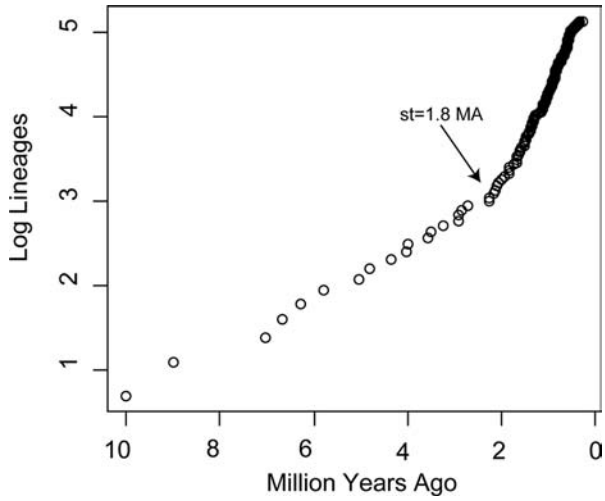


**Figure 4.** Lineage through time plot illustrating the accumulation of mitochondrial lineages as a function of time for *Polypedates leucomystax* throughout southern China and Indochina. Divergence times were scaled to the basal divergence of *Polypedates* and *Rhacophorus* estimated from the BEAST analysis. Arrow indicates approximate time at which rate increased significantly.

on the mainland about 10 Ma when sea levels were low and the Sunda Shelf was connected to the mainland (Hall 1998). The exposed Sunda Shelf affected wind and rainfall and caused conditions on the present-day mainland to be much drier than today (Verstappen 1997). The increased aridity corresponds to a reduction in forest habitat (Heaney 1991), and amphibian populations may have been imprisoned in remnant patches of suitable habitat.

Divergences within both major lineages of *P. leucomystax* correspond to a period of increased humidity on the mainland. Rising sea levels from 10 to 5 Ma gradually reduced the land area of Indochina, separating the mainland from the islands and completely submerging Hainan (Hall 1998). Stratigraphic and paleoecological analyses suggest that Asia experienced drastic climatic change about 9–8 Ma due to further uplift of the Qinghai–Tibetan Plateau (Zhisheng et al. 2001). Central Asia experienced increased aridity, whereas the monsoon-dominated weather patterns that persist today started in Southeast Asia, which became warmer, more humid, and more densely covered by tropical forest (Verstappen 1997). Previously isolated amphibian populations on the mainland were likely able to expand their range, diversifying and coming into secondary contact with divergent lineages, while rising sea levels isolated insular populations.

The Late Miocene divergence of the *P. leucomystax* complex corresponds to that of Asian dicroglossid frogs (Che et al. 2010; Zhang et al. 2010) and to periods of rapid divergence in Southeast Asian birds (Outlaw and Voelker 2008; Song et al. 2009). Several widespread species of *Ficedula* appear to have diversified about 6.5 Ma, around the same time as divergences began within lineages A and B. The close correspondence between these divergence dates suggests

that climatic conditions associated with the formation of the Qinghai-Tibetan Plateau are the driver of these widespread divergences.

Explosive radiations occur within each of the sublineages of the *P. leucomystax* complex between 2.64 and 0.88 Ma, likely due to a combination of climatic cycling and resulting changes in the landscape. Climatic change during the Pliocene leading up to the repeated glacial/interglacial cycles of the Pleistocene contributed to global changes in both temperature and aridity (Williams 1975; Zachos et al. 2001; Abramowski et al. 2006). Repeated climatic cycling altered the structure of tropical ecosystems, fragmenting populations into isolated pockets of suitable habitat until an interglacial period when expansion of these habitats occurred as temperature and rainfall increased. Pleistocene climatic cycles also affected sea level, thus mediating dispersal of some species to islands (Song et al. 2009). Thus, repeated cases of habitat cycling driven by climatic shifts are a likely explanation for the initial and subsequent diversifications of the many lineages present in the *P. leucomystax* species complex. The Plio–Pleistocene divergence events revealed in our analyses are also similar to those in the region's other vertebrates (Zhang et al. 2008; Song et al. 2009; Zhang et al. 2010).

Hainan Island (Figure 2), a Pleistocene land-bridge island (Voris 2000), appears to have been colonized independently at least twice (Figure 1). The estimated dates of isolation are consistent with the hypothesis that *P. leucomystax* first colonized Hainan Island before it was isolated from the mainland by the formation of the Qiongzhou Strait about 2 Ma. Hainan Island was reconnected to mainland China four times during the Pleistocene (600 000–10 000 years ago; Zhao et al. 2007; Shi et al. 2008). Our results do not indicate recolonization of the mainland from Hainan Island during any of these land-bridge events. Interestingly, our estimates for isolation of *P. leucomystax* on Hainan are similar to those for the rhesus macaque (1.6 Ma, *Macaca mulatta*; Zhang and Shi 1993). Similarly, Mao et al. (2010) found the Early Pleistocene to be important in the separation of Hainanese horseshoe bats (*Rhinolophus hainanensis*) from their mainland ancestor.

The relatively recent divergence of the two Bornean lineages from mainland lineages (1.05 and 1.38 Ma, respectively) is consistent with colonization of Borneo while the Sunda Shelf was connected to the mainland; a time when sea levels were low during the Pliocene, approximately 5 Ma (Hall 1998). Subsequent periods of higher sea levels likely resulted in fragmentation leading to cladogenesis of these insular populations. The isolation and reconnection of the Sunda Shelf to the mainland also occurred several times throughout the Quaternary, most recently during the last glaciation.

### Landscape Effects on Genealogical Divergence

Landscape features such as the Red River may play an important role in shaping the distribution of haplotypes in this species complex. Partial Mantel tests suggest that the Red River forms a partial barrier to dispersal for some lineages. However, this hypothesis does not explain the overall pattern in the genealogy (Figures 1 and 2). For example, sublineages A3, B1, and B3 occur both north and south of the Red River. Further, five of seven lineages within sublineage B occur both north and south of Ngoc Linh, yet B5 occurs at Ngoc Linh and B7 occurs south and west of Ngoc Linh only. Thus, our genealogy and SAMOVA analyses provided some evidence for historical north-to-south dispersal for both lineages A and B, with the Red River acting as a partial barrier in some cases.

Finally, the Annamite Range (Truong Son Mountains) that separates southern Vietnam from Laos and Cambodia may have isolated populations in southern coastal Vietnam from those to the west, especially because *Polypedates* is generally a lowland species. Additional sampling from sites west of the Annamite Range is required to test this prediction.

### Sympatric Lineages

Several incidences of sympatric lineages occur throughout the mainland, on Borneo and in the Philippines (Figure 2). Our results suggest that this is most likely a result of demographic expansion during the Pleistocene. Brown et al. (2010) report sympatry on Hainan Island at Bawangling National Nature Reserve where our more limited sampling detected only one haplotype. Despite widespread overall sympatry, no sympatric sublineages occur from lineage A, and no site contains representatives from more than two distinctive lineages. Hong Kong, Laos (Phongsaly and Vientiane), and the Philippines contain two sublineages from lineage B only (the specimen of *P. megacephalus* from GenBank clustered within lineage B3, and Sheridan et al. 2010 suggest that this specimen may have been misidentified).

Brown et al. (2010) summarize the extreme taxonomic uncertainty surrounding the *P. leucomystax* complex; even the distinction between *P. leucomystax* and *P. megacephalus* remains unclear (Figure 1). Our study does not have a taxonomic focus, but the widespread sympatry, high genetic diversity, and sequence divergence values combined with a failure to detect significant IBD for the whole data set provide further support for the presence of undocumented cryptic species. This is consistent with ecological, morphological, and behavioral evidence (Inger and Tan 1996; Narins et al. 1998; Trépanier et al. 1999; Riyanto et al. 2011). Additional data from other sources (e.g., ecology, acoustic analyses, nuclear genes) are required to clarify the taxonomy of this complex. Until the taxonomic status of this species complex is resolved, it will be difficult to effectively test further hypotheses regarding dispersal and potential geographic, ecological, and behavioral barriers and mechanisms of isolation in this system.

### Widespread Anuran Species and Human-Mediated Dispersal

Stuart et al. (2006) question whether widespread amphibian species exist in Southeast Asia or not. Many widespread species of anurans are now known to be complexes of geographically restricted cryptic species (Wynn and Heyer 2001; Bain et al. 2003; Stuart et al. 2006). As discussed above, the taxonomy of this complex is unresolved. It is difficult to delimit species in large, morphologically conservative species complexes. Nevertheless, existence of widespread lineages indicates that

even if each lineage or sublineage were considered as a unique taxon, widespread species with extremely broad distributions exist and these involve sublineages B1, B2, and B6.

Our results show that humans did not mediate the initial dispersal of *P. leucomystax* from the mainland to the islands, but some of the widespread sympatry on the mainland may have been caused by human-mediated transport. Sample 171 from Yok Don National Park, a southern site, falls into sublineage B3, otherwise composed only of northern samples. Sample 128 from Cat Tien National Park also falls into an otherwise north-central sublineage (B2) on the opposite side of the Truong Son Mountains. We cannot reject the hypothesis of human-mediated relocations as an explanation for the presence of these sympatric lineages, especially as we are aware that confiscated animals are often released into national parks, and this may have some relevance. However, further evidence is required to address this possibility.

Although the initial divergence between insular and mainland *P. leucomystax* was not human-mediated, recent dispersal among islands is likely to have increased with increased recent human dispersal, as hypothesized by Brown et al. (2010). Evidence certainly exists for occasional accidental transport of these frogs. For example, humans introduced *P. leucomystax* to Japan and West Papua New Guinea (Maeda and Matsui 1999; Kuraishi et al. 2009), and a specimen was found hitchhiking on an airplane to Guam (Wiles 2000; Christy et al. 2007). Kuraishi et al. (2009) identify the Philippines as the most likely source of *P. leucomystax* introduced to the Ryukyu archipelago of Japan.

## Conclusions

Our study adds to a growing body of literature investigating the historical forces shaping the contemporary spatial distribution of genetic lineages throughout East and Southeast Asia (e.g., Huang et al. 2007; Zhang et al. 2008; Li et al. 2009a, 2009b; Song et al. 2009; Zhang et al. 2010; Ding et al. 2011). Overall, our population genetic analyses demonstrate that demographic expansion is at least partially responsible for the widespread sympatry in the sampled lineages. The apparently similar habitat requirements yet strikingly different ranges of the two highly divergent lineages all beg the question: given the existence of widespread lineages, why are some lineages widespread whereas others are not? Future research will identify the factors that allow one lineage to disperse freely and become widespread while its sister lineage remains geographically restricted.

## Supplementary Material

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

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