

# GEOLOGICAL AND CLIMATIC FORCES DRIVING SPECIATION IN THE CONTINENTALLY DISTRIBUTED TRILLING CHORUS FROGS (*PSEUDACRIS*)

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Tertiary geological events and Quaternary climatic fluctuations have been proposed as important factors of speciation in the North American flora and fauna. Few studies, however, have rigorously tested hypotheses regarding the specific factors driving divergence of taxa. Here, we test explicit speciation hypotheses by correlating geologic events with divergence times among species in the continentally distributed trilling chorus frogs (*Pseudacris*). In particular, we ask whether marine inundation of the Mississippi Embayment, uplift of the Appalachian Mountains, or modification of the ancient Teays-Mahomet River system contributed to speciation. To examine the plausibility of ancient rivers causing divergence, we tested whether modern river systems inhibit gene flow. Additionally, we compared the effects of Quaternary climatic factors (glaciation and aridification) on levels of genetic variation. Divergence time estimates using penalized likelihood and coalescent approaches indicate that the major lineages of chorus frogs diversified during the Tertiary, and also exclude Quaternary climate change as a factor in speciation of chorus frogs. We show the first evidence that inundation of the Mississippi Embayment contributed to speciation. We reject the hypotheses that Cenozoic uplift of the Appalachians and that diversion of the Teays-Mahomet River contributed to speciation in this clade. We find that by reducing gene flow, rivers have the potential to cause divergence of lineages. Finally, we demonstrate that populations in areas affected by Quaternary glaciation and aridification have reduced levels of genetic variation compared to those from more equable regions, suggesting recent colonization.

**KEY WORDS:** Appalachian uplift, aridification, glaciation, Mississippi Embayment, *Pseudacris*, speciation, Teays River.

Two of the most important factors thought to drive speciation are formation of geological barriers and climatic fluctuations (Mayr 1942; Hewitt 2000). Geological changes such as uplift of mountain systems and the development of river systems may form barriers to gene flow between populations, resulting in diversification along these boundaries (Nielson et al. 2001; Brant and Ortí 2003; Carstens et al. 2004; Funk et al. 2005; Steele et al. 2005; Howes et al. 2006; Kozak et al. 2006). Additionally, rapid climate change during Pleistocene glaciation events may isolate populations in multiple refugia, leading to genetic divergence, and potentially to

speciation (Sewell et al. 1996; Hewitt 2000; Good and Sullivan 2001; Knowles 2001; Tzedakis et al. 2002; Church et al. 2003; Zamudio and Savage 2003; Carstens et al. 2005a). Although a number of recent studies have examined the effects of a particular process in a limited geographic region, few have elucidated the relative importance of climate change and barrier formation on a continental scale.

Many phylogeographic studies have addressed questions of speciation by employing a retrospective, interpretive approach rather than a predictive hypothesis-testing approach. The former

method attempts post hoc to identify historical processes that might have produced observed patterns of genetic variation. This can lead to overinterpretation of data because there is no well-defined null hypothesis to set bounds on the expected pattern. Additionally, this approach frequently does not consider whether the timing of proposed events coincides with the speciation event. The more powerful approach employed here formulates temporally and geographically explicit hypotheses prior to data collection and analysis, and thus uses information from independently derived datasets to test factors proposed to drive speciation.

The late Tertiary period (2.6–34 million years ago [mya]; Gradstein et al. 2004) is characterized by several major geological changes in the eastern United States. First, sea levels fluctuated dramatically, leaving a series of scarps along the Coastal Plain (Haq et al. 1987; Dowsett and Cronin 1990). These marine transgressions, which may have been indirectly related to climate change, filled the Mississippi Embayment, a geologic trough formed during the Cretaceous through faulting in the Mississippi River Valley (Cox and Van Arsdale 2002; Fig. 1A). Marine depositional sediments indicate that sea transgressions from the Gulf of Mexico extended as far north as southern Missouri during the Paleocene and as far north as Jackson, Mississippi, during the Miocene (Reed et al. 2005). These marine inundations likely presented a formidable barrier to salt-intolerant species such as amphibians, although this hypothesis has not yet been tested (Fig. 1A).

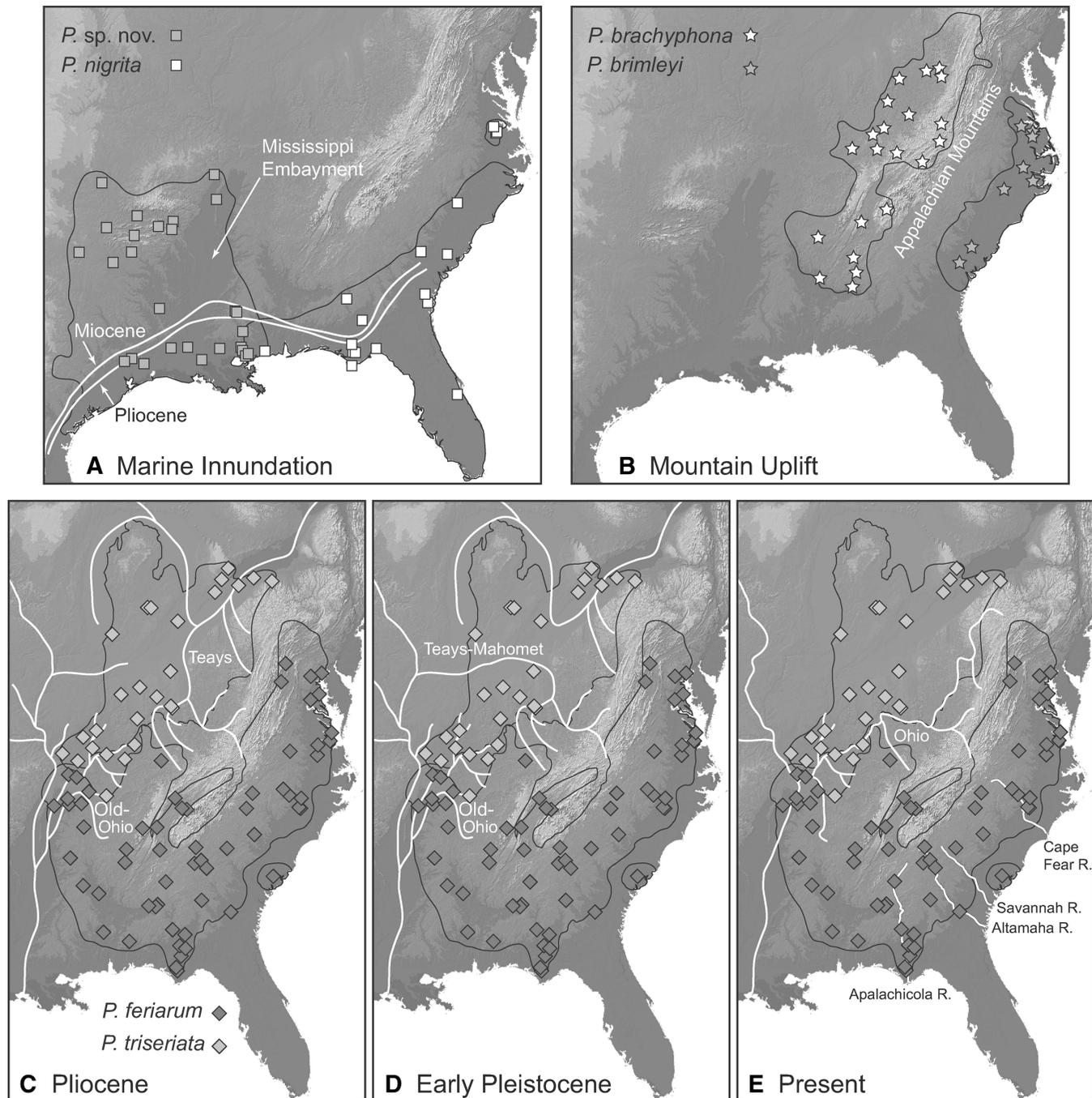
A second major geological change during the Tertiary was renewed uplift of the Appalachian Mountains (Fig. 1B). The Paleozoic Appalachian Mountains had been largely eroded to a plain (Dunbar and Waage 1969; Cleaves 1989) by the end of the Mesozoic. Data from sedimentation rates and fault ages indicate that another major uplift occurred during the late Oligocene to Miocene (Prowell and O'Connor 1978; Hack 1982; Reinhardt et al. 1984; Poag and Sevon 1989; Prowell 1989; Prowell and Christopher 2000, 2006; Dennison 2001). The uplift may have created both an elevational barrier and an ecological barrier through the development of a rain shadow on the eastern slope. In this case, the western slope of the Appalachians was probably wet although the eastern side was arid, similar to the Sierra Nevada Range today (Stanley 1989). We might expect, therefore, that this geologic feature contributed to the divergence of taxa inhabiting the region (Fig. 1B).

A third major geological change involves development of North American river systems. During the late Pliocene and early Pleistocene, the ancient Teays-Mahomet River and its tributaries flowed northward from the western side of the Appalachian Mountains into Ohio, and west through central Indiana and Illinois before joining with the Mississippi River system (Fig. 1C–E; Ver Steeg 1946; Hocutt et al. 1986; Gray 1991; Melhorn and Kempton

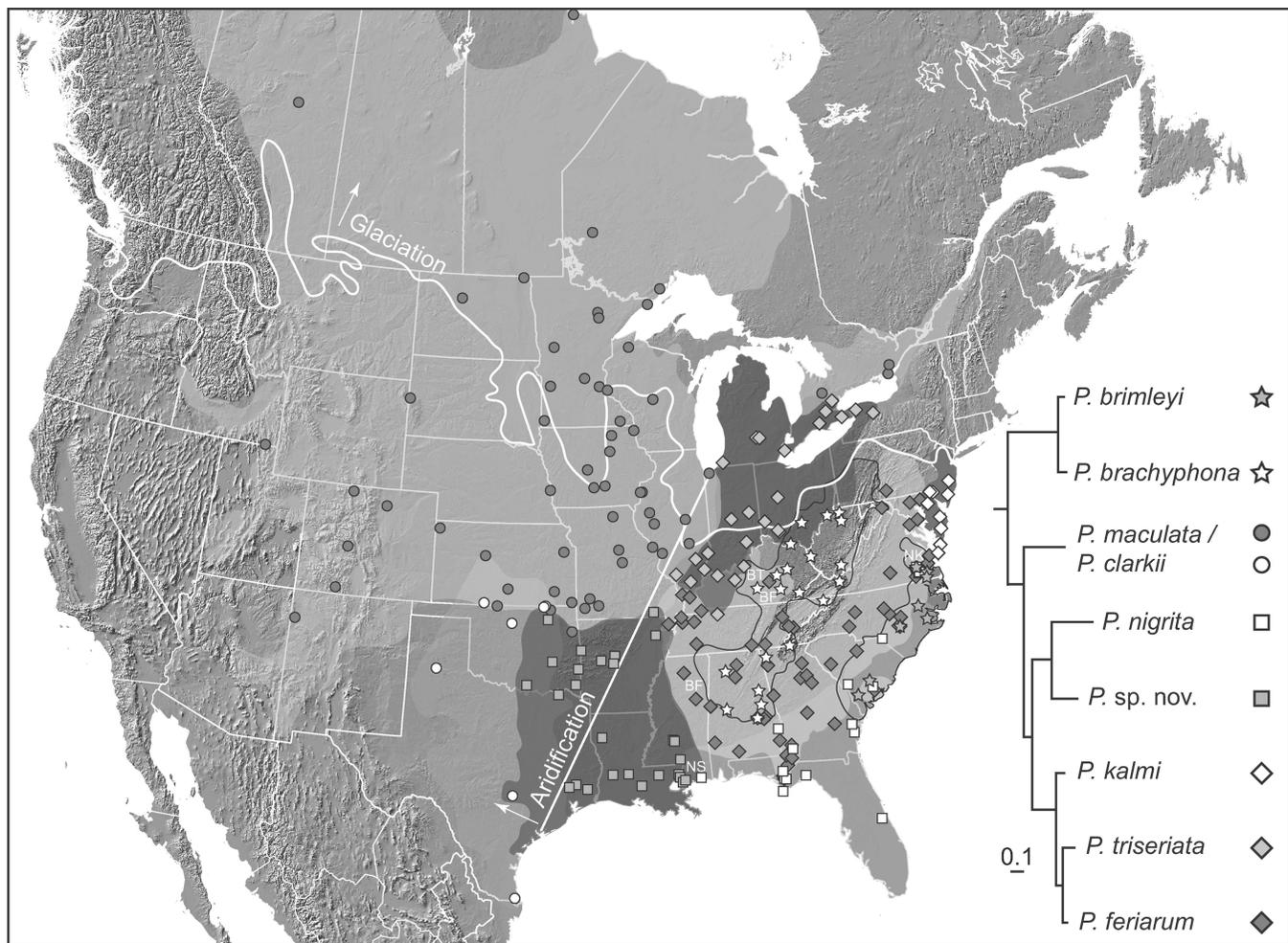
1991). Glacial advances in the Pleistocene (about 0.8 mya) dammed the Teays-Mahomet River in southern Ohio, forming a vast lake that lasted several thousand years before overflow resulted in the formation of new river channels. These channels cut a path westward to the Old-Ohio drainage system, forming the basis of the modern Ohio River (Fig. 1D–E; Gray 1991). When the Teays-Mahomet River joined the Old-Ohio, a land passageway between Kentucky and Indiana was cut off, potentially blocking gene flow between populations of terrestrial organisms on either side of the newly formed Ohio River (Fig. 1D–E). Development of these river systems has been implicated as a cause of speciation in a number of fish taxa (Hocutt et al. 1978; Mayden 1988; Strange and Burr 1997; Berendzen et al. 2003).

The onset of the Quaternary (present–2.6 mya; Gibbard and Van Kolfschoten 2004) marked the beginning of a period of rapid climate fluctuations, with advances of massive ice sheets across much of North America alternating with warmer interglacial periods (Brown and Lomolino 1998). The most recent Wisconsin glaciation extended as far south as southern Illinois (Denton and Hughes 1981; Fig. 2). According to paleoclimatic models, as the ice sheet receded (12–110 thousand years ago [kya]; glacial maximum 14 kya; Denton and Hughes 1981; Gibbard and Kolfschoten 2004), a period of extreme aridification (desertification) ensued throughout much of the western United States, due to ice sheet-induced displacement of the jet stream. The eastern boundary of this arid region stretched from approximately northern Illinois to east Texas, and lasted until around 11 kya (Fig. 2; Bartlein et al. 1998; Brown and Lomolino 1998). The rapid onset of this xeric period is proposed to have caused local extinction of wetland-restricted species, which later re-colonized these regions as more favorable climatic conditions returned (Starkey et al. 2003). Given these drastic changes, we expect to find reduced genetic variation in organisms from recently glaciated or aridified areas. We also predict that these climatic factors caused isolation of populations in refugia, potentially contributing to divergence of taxa during the Pleistocene.

Hylid treefrogs have undergone two independent radiations into North America from Central America and Mexico. One radiation includes members of the genus *Hyla*, the other the genera *Acris* and *Pseudacris* (Smith et al. 2005). Both of the latter genera are endemic to North America and are thought to have diverged at least 33 mya (Smith et al. 2005). The trilling chorus frogs are a continentally distributed clade within *Pseudacris* (Moriarty and Cannatella 2004). Members of this group range from northern Mexico to Canada and throughout the eastern two-thirds of the United States (Conant and Collins 1998). We determined the species diversity and range limits of the nine species in this group based on 2.4 kb of 12S/16S mitochondrial DNA data from 237 populations in combination with published morphological and



**Figure 1.** Hypotheses for geological factors contributing to speciation. Panel A illustrates the hypothesis that marine inundation of the Mississippi Embayment led to speciation between *P. nigrita* (white boxes) and *P. sp. nov.* (gray boxes). White lines indicate the maximum extent of inundation during the Miocene and Pliocene. Note that each of these inundations bisects the current distributions of these taxa (indicated by black lines). Panel B describes the hypothesis that uplift of the Appalachian Mountains caused divergence of *P. brachyphona* (white stars) and *P. brimleyi* (gray stars). Current high elevation areas are shown in light gray. Panels C–E show development of the Teays–Mahomet–Ohio River systems from the Pliocene (C), to early Pleistocene (D), to present (E) positions. Panels D and E illustrate the hypothesis that glacially induced diversion of the Teays–Mahomet River caused speciation between *P. feriarum* (dark gray diamonds) and *P. triseriata* (light gray diamonds). Panel E also shows four modern river systems (Apalachicola, Altamaha, Savannah, and Cape Fear) that bisect the range of *P. feriarum* and that contribute to intraspecific divergence.



**Figure 2.** Distributions of North American trilling chorus frogs. Species boundaries are based on the phylogeny (Fig. 3; Lemmon et al., 2007) and county-level taxon records from Lannoo (2005). Markers indicate populations sampled and correspond to species in the phylogeny on the right. Ranges of *P. brachyphona* and *P. brimleyi* are outlined in black for visual clarity. Capital letters indicate the following hybrid DNA identity of the individual: NS—*P. nigrita*–*P. sp. nov.*, BF—*P. brachyphona*–*P. feriarum*, BT—*P. brachyphona*–*P. triseriata*, and NK—*P. nigrita*–*P. kalmi*. Degree of overlap between species is indicated where known; if no overlap is shown between parapatric taxa, the degree of overlap has not been determined. White lines point to boundaries of Pleistocene climatic events. The glaciated region is north of the line labeled Glaciation; the aridified region is west of the line labeled Aridification. The scale of the branch lengths on the phylogeny is substitutions per site. This figure is available in color in the online version of Lemmon et al. (2007).

behavioral data (Lemmon et al., 2007; Fig. 2). This article provided a foundation for testing specific hypotheses about factors driving speciation on the North American continent.

Due to their broad geographic distribution, we expect the patterns of diversity within the trilling *Pseudacris* to be potentially influenced by a spectrum of historical processes, both geological and climatic. Here, we ask the general question: Are speciation events correlated primarily with geological events of the Tertiary or with climatic fluctuations of the Quaternary? To address this question, we first test specific hypotheses that barriers promoting speciation formed through inundation of the Mississippi Embayment, uplift of the Appalachian Mountains, and diversion of the ancient

Teays-Mahomet River. Second, to investigate the plausibility that ancient rivers caused divergence, we test the prediction that current river systems reduce gene flow. Third, we test the prediction that Quaternary climate change caused speciation. Finally, we test the hypothesis that these climatic factors reduced patterns of genetic diversity within species. We employ the Lemmon et al. (2007) chorus frog dataset to test these hypotheses by correlating fossil-based and coalescent-based divergence times among species to the timing of geological events, by examining levels of genetic variation across riverine barriers, and by comparing levels of genetic variation throughout areas affected by Quaternary climate change. This multi-tiered approach integrates phylogenetics and population

genetics as well as new statistics for phylogeographic applications to rigorously test hypotheses for the factors driving speciation in North America.

## Methods

### TESTS OF SPECIATION HYPOTHESES

#### *Geological changes driving speciation*

To test the historical effects of geological barriers on species diversification, we asked whether the formation of particular barriers (Mississippi Embayment inundation, Appalachian Mountains, Teays-Mahomet-Ohio River) occurred within the confidence limits of estimated divergence times for three species pairs. Divergence times were estimated using two different approaches. The first approach uses a coalescent model to estimate the rate of migration between, and time of divergence for two populations specified a priori (Nielsen and Wakeley 2001). This method assumes a panmictic ancestral population splits into two populations, which then may or may not exchange migrants (asymmetric migration is allowed). Populations may have different effective population sizes, but these are assumed to be constant in time. The method also assumes that the genetic loci are selectively neutral and that there is no additional population subdivision. Divergence times were estimated for *Pseudacris nigrita*–*P. sp. nov.*, *P. brimleyi*–*P. brachyphona*, and *P. triseriata*–*P. feriarum* (Figs. 1–3). Population boundaries for these three pairs are delineated by the Pearl River, Appalachian Mountains, and the Ohio River, respectively.

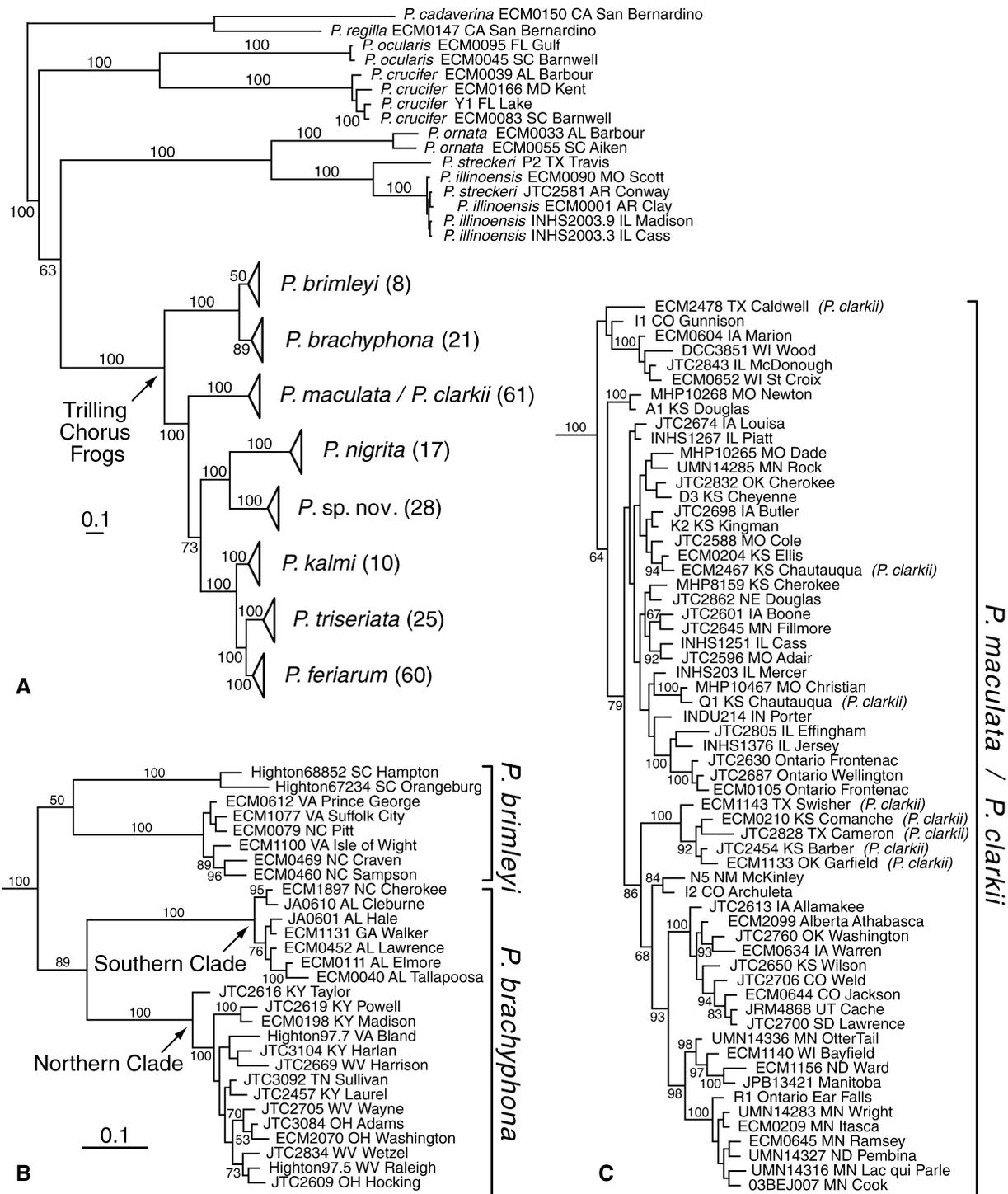
Analyses were performed using MDIV (<http://ser-loop.tc.cornell.edu/cbsu/mdiv.htm>) following Carstens et al. (2005b). For each of the three species pairs, we performed preliminary analyses using the default settings to determine an appropriate prior for the scaled divergence time,  $T$ , the scaled migration rate,  $M$ , and the measure of genetic diversity,  $\theta$ . Analyses were performed using the HKY model of substitution (Hasegawa et al. 1985). All prior distributions were assumed to be uniform with a lower bound equal to zero. Based on preliminary analyses, the upper bounds for  $M$  were assumed to be 3.0, 1.0, and 3.0 for *Pseudacris nigrita*–*P. sp. nov.*, *P. brimleyi*–*P. brachyphona*, *P. triseriata*–*P. feriarum*, respectively. Likewise, the upper bounds for  $T$  were assumed to be 1.0, 4.0, and 2.7, respectively. Finally, the upper bounds for  $\theta$  were assumed to be 120.0, 62.1, and 70.0, respectively. After discarding 500,000 cycles as burnin, the posterior probability distribution was estimated using 1.5 million cycles of the Markov chain. Estimates of  $T$  and  $\theta$  were used to solve for divergence time ( $T_{\text{div}}$ ) using the following equations:  $T = T_{\text{div}}/2Ne$  and  $\theta = 4Ne\mu$ , where the units of  $\mu$  are substitutions per sequence per generation. To calculate divergence time in years, a mutation rate of 0.00249 substitutions per site per million years was assumed, as estimated for the same mitochondrial region (12S/16S) in the frog family

Pipidae by Evans et al. (2004). This rate was converted to the units used in MDIV by assuming a generation time in *Pseudacris* of one year (Green 1964; Caldwell 1987; Smith 1987).

The second approach to quantifying divergence times, based on penalized likelihood, uses fossil calibrations and branch lengths to estimate absolute dates (Sanderson 2002). This method assumes that the species identity and date of fossils are accurate and that the gene tree used represents the species tree. With this method, divergences were estimated across the entire *Pseudacris* phylogeny. Because there are no pre-Pleistocene *Pseudacris* fossils with known species identity, external calibration points from the hylid phylogeny were used. Following Smith et al. (2005), minimum ages of clades were constrained in the genus *Hyla* and at the base of the *Acris/Pseudacris* split. To calculate divergence times, a dataset of the same 12S/16S region was used with 35 hylid frog sequences (D. Cannatella and A. Holloway, unpubl. data; see Supplementary Appendix online) in combination with a single representative of each *Pseudacris* species from our dataset. This hylid dataset has taxon sampling comparable to Smith et al. (2005). A Bayesian analysis was performed on the combined dataset using the methods described in Lemmon et al. (2007) except with four runs, a sample frequency of 100, and 40,000 total samples.

Our phylogeny was generally congruent with the tree of Smith et al. (2005) with respect to the nodes to which fossils were assigned. The one exception is the position of *Hyla gratiosa*, which we found to be the sister taxon of *H. cinerea*, rather than affiliated with *H. versicolor* and *H. avivoca*. The position of this taxon in our tree is more reasonable because the former two species are more similar morphologically and acoustically, and they hybridize in nature, suggesting their close relationship (Oldham and Gerhardt 1975; Gerhardt et al. 1980; Höbel and Gerhardt 2003). Four of the five fossil calibrations (phylogenetic position and age) employed by Smith et al. (2005) were used. The *H. avivoca/H. gratiosa/H. versicolor* calibration found by Smith et al. (2005) could not be used because we did not recover those taxa as a monophyletic group. Instead, the minimum age of the *H. versicolor/H. chrysoscelis/H. avivoca* clade was constrained to at least 14 mya and the *H. gratiosa/H. cinerea* clade to at least 15 mya (Holman 2003; Smith et al. 2005). In sum, we employed six fossil calibration points. The “root” of the tree was constrained to 42 mya, following Smith et al. (2005).

Analyses were performed using r8s 1.70 (Sanderson 2003). An appropriate smoothing parameter was chosen following eight preliminary analyses using a range of smoothing parameters (smoothing parameter =  $10^{1+0.3n}$ , where  $n = \{0,1,2,3,4,5,6,7\}$ ). The parameter producing the smallest cross-validation score was used in the final analysis (Sanderson 2002). To assess uncertainty in the divergence time estimates, we repeated the analysis using 1000 trees randomly sampled from the posterior distribution



**Figure 3.** Phylogeny of *Pseudacris*. Tree A shows the phylogenetic relationships of the genus. Numbers of populations sampled from each trilling chorus frog species are indicated in parentheses. Trees B–E illustrate the population level relationships of the trilling chorus frogs on the fully resolved tree. Each tip on the phylogeny is described by a field number, state/province, and county/region of origin. Bayesian posterior probabilities above 50% are located near corresponding branches. Species names in parentheses indicate species allocation based on morphology and behavior, if this conflicted with the mitochondrial clade identity. The scale of the branch lengths on the phylogeny is substitutions per site. Note that the branch length scale for tree A is 25% of the scale for trees B–E.

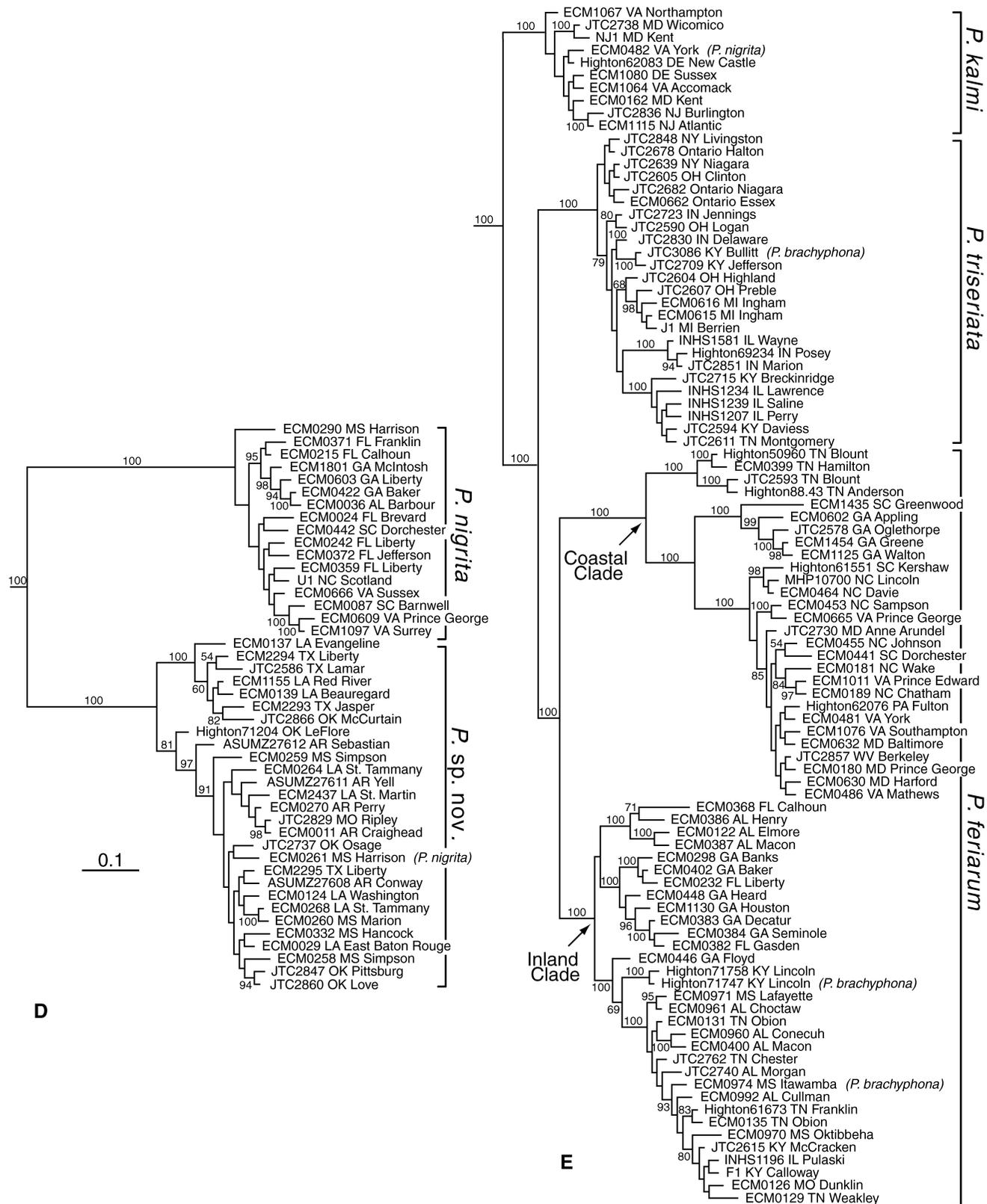


Figure 3. Continued.

(see Bayesian phylogenetic analysis above). Because preliminary analyses suggested that the optimal smoothing parameter did not vary substantially across the 1000 replicates, we used the value 79, chosen in the above analysis, for all replicate analyses. Confidence intervals (95%) were calculated as the range in divergence times estimated for each node after removing the lower 2.5% and upper 2.5% of the distribution of times estimated for that node. This procedure is analogous to the bootstrapping approach used by Evans et al. (2004). For the complete hybrid chronogram, see Supplementary Material available online.

#### *Uncertainty in timing of geological processes*

The timing of Mississippi Embayment inundation is based on relative sea-level estimates that have been measured on a fine temporal scale (Haq et al. 1987). Timing of recent Appalachian uplift is somewhat uncertain. Data based on sedimentation rates and fault ages bracket uplift from late Oligocene to Miocene, suggesting that orogenic activity slowed substantially by the late Miocene, and the mountains then rapidly eroded (Hack 1982; Pazzaglia and Brandon 1996; Prowell and Christopher 2000, 2006). More extensive geological data are needed to refine this estimate. The estimate for Teays-Mahomet River divergence is based on paleomagnetic and stratigraphic data, indicating that this event occurred between 0.79 and 0.88 mya (Bigham et al. 1991; Bonnett et al. 1991; Goldthwait 1991). In this article, we consider a geological event to be consistent with a speciation event if the confidence intervals of the two events overlap.

#### *Rivers as barriers to gene flow*

Modern river systems have been suggested as important barriers to gene flow (Kozak et al. 2006; Liu et al. 2006; Pauly et al. 2007). To determine whether this is the case for *Pseudacris*, patterns of genetic variation were examined in one exemplar species (*P. feriarum*) that spans several major rivers. We expect that if a particular river inhibits gene flow among populations, then genetic distances between populations spanning the river should be greater than genetic distances between populations on the same side of the river. Partial Mantel tests (Mantel 1967; Smouse et al. 1986) were employed to test hypotheses that the following rivers are barriers to gene flow in *P. feriarum*: Apalachicola/Chattahoochee (within inland clade only), Altamaha/Oconee (all *P. feriarum*), Savannah (coastal clade only), and Cape Fear/Haw (coastal clade only; Fig. 1E, 3E). This type of test permits integration of geospatial data into population genetic analyses (Kidd and Ritchie 2006). Within-clade tests were performed to maximize the independence of each test (to reduce the effects of other rivers). The partial Mantel test calculates partial correlations between a response variable and multiple independent variables (Smouse et al. 1986). In this way, we can test for a correlation between two variables (genetic distance, position relative to barrier) while controlling for a third

variable (geographic distance). Pairwise genetic distances were measured in terms of patristic distance, calculated as the sum of branch lengths between a pair of populations on the majority-rule Bayesian topology (Fig. 3E). Redundant haplotypes were included in these analyses by inserting them with zero length branches next to their identical haplotype. Geographic distance was measured as the great-circle distance between populations (Sinnott 1984). Geographic distances were not log-transformed because the relationship between genetic and geographic distance was approximately linear. Position of two populations relative to the river was coded as either same or opposite sides. To perform the test, we calculated (1) a matrix of pairwise patristic distances, (2) a matrix of pairwise geographic distances, and (3) a matrix of binary variables indicating whether a pair of populations spans the barrier or not. All tests were performed in FSTAT 2.9.3 (Goudet 1995) with 10,000 randomizations. A significant result suggests that the river inhibits gene flow.

As a corollary to the barrier tests, we asked whether coastal *P. feriarum* shows evidence of northward expansion east of the Appalachian Mountains in response to recent climatic change along the eastern Piedmont (Williams et al. 2000, 2004). Specifically, we hypothesize that if northward expansion has occurred, populations should exhibit lower pairwise genetic distances on the north side of the Savannah River than on the south side. To test this, we performed a randomization test, hereafter referred to as the *Range Expansion Test*: (1) all pairwise patristic and geographic distances among populations were calculated, (2) population pairs from one side of the barrier were placed in one category, and pairs from the other side of the barrier were placed in a second category, (3) to remove the effect of geographic distance, pairwise patristic distances were divided by great-circle distances between populations, (4) this standardized patristic distance ( $\bar{v}$ ) was averaged for all population pairs north of the barrier ( $\bar{v}_N$ ) and for those south of the barrier ( $\bar{v}_S$ ), and the difference between these values was used as the test statistic ( $\Delta\bar{v}_{test}$ ), (5) the categories assigned to the population pairs (north or south) were randomized, and  $\Delta\bar{v}_{rand}$  was calculated; this step was performed 100,000 times to generate a null distribution, and (6) the distribution of  $\Delta\bar{v}_{rand}$  was compared to the test statistic  $\Delta\bar{v}_{test}$ . A significant result suggests that the species has undergone a recent expansion.

#### *Climatic changes affecting genetic diversity*

We examined the effects of climatic fluctuations on genetic diversity in seven trilling *Pseudacris* clades, three of which occupy formerly glaciated or aridified areas (*P. brimleyi* was not included due to small sample size). If these clades have expanded their ranges into climatically disturbed areas, we expect to observe both a pattern of recent population growth and reduced genetic variation relative to geographic area.

To test for population growth, the coalescent model of population growth developed by Kuhner et al. (1998) was employed. Populations experiencing growth are expected to have many coalescent events near the tips of the tree, whereas stable populations are expected to have a relatively larger proportion of deeper coalescent events (Kuhner et al. 1998). Growth rate ( $g$ ) was estimated for each of the seven clades following Carstens et al. (2004). First,  $\theta$  was estimated for each clade using the coalescent model implemented in MIGRATE 2.1.2 (Beerli and Felsenstein 1999). Using these estimates of diversity as the starting parameters,  $g$  was estimated for each clade using FLUCTUATE 1.4 (Kuhner et al. 1998). To avoid potential bias in estimating confidence in  $g$  (Abdo et al. 2004), we tested for significance of each value of  $g$  by generating a null distribution for each clade. The null distributions were obtained by first simulating 100 datasets using TREEEVOLVE 1.3.2 (Grassly et al. 1999) and the values of  $\theta$  estimated above, assuming constant population size ( $g = 0$ ). Then  $g$  was estimated for each of those datasets using FLUCTUATE (with the same settings as above). Finally, the values of  $g$  estimated with the empirical datasets were compared to the null distributions of  $g$  to test for significance. Substitution model parameters required for the analyses were estimated in PAUP\* version 4.0b10 (Swofford 1998).

If taxa have expanded their ranges into climatically disturbed areas, we predict that: (1) clades in climatically disturbed areas will have lower genetic variation than clades in undisturbed areas and (2) in clades that span the boundaries of these regions, populations within the affected region will show lower genetic variation ( $\bar{v}$ ) than populations outside the region. To test the first prediction, the amount of standardized genetic variation ( $\bar{v}$ ) within each clade was quantified and compared across clades. The values were compared by first sorting the species-level clades by  $\bar{v}$ , and then by testing for significance using a randomization test that is analogous to a Tukey test (Zar 1998). The test statistic is the difference between  $\bar{v}$  for two adjacent clades in the sorted list. The null distribution was simulated by randomizing  $v$ s between two adjacent clades and calculating  $\Delta v$  (with 100,000 randomizations).

To test the second prediction, we considered only clades with part of their range in climatically disturbed areas. In the trilling *Pseudacris*, one clade is found in glaciated areas (*P. triseriata*), one inhabits aridified areas (*P. sp. nov.*), and one is in both (*P. maculata*/*P. clarkii*; Fig. 2). The glacial boundary was designated using the maximum extent of the most recent glaciation (Wisconsin), based on Denton and Hughes (1981). The aridification boundary was approximated using data from Bartlein et al. (1998). To assess whether populations in climatically disturbed areas have significantly lower genetic variation, we performed the range expansion test described above.

## Results

### TIMING OF SPECIATION

All speciation events in *Pseudacris* occurred in the Tertiary rather than the Quaternary. This result is supported by both approaches for estimating divergence times, which give remarkably congruent estimates (Fig. 4). The youngest split is between *P. maculata* and *P. clarkii*, which occurred near the end of the Pliocene. These results demonstrate that Pleistocene climatic factors did not cause the major species-level divergences in this group.

Inundation of the Mississippi Embayment is consistent with timing of speciation between *P. nigrita* and *P. sp. nov.* During the late Miocene and early Pliocene, sea levels fluctuated and rose above current levels (Haq et al. 1987; Figs. 1, 4). The peak sea level of this period, which was sufficient to geographically isolate the current ranges of these species (Fig. 2), occurred at the same time as the speciation event, approximately 4.8 mya (approximate age based on average of  $T_{div1A}$  and  $T_{div2}$  from Table 1; Fig. 4). Prior to this peak, sea levels dropped to at or below current levels potentially allowing passage of the ancestor of these taxa.

The most recent uplift of the Appalachian Mountains occurred well before the divergence of *P. brachyphona* and *P. brimleyi*. Although orogenic activity took place in the Oligocene and Miocene (5.3–33.9 mya), speciation between these taxa is estimated to be during the Pliocene, approximately 4.6 mya (Table 1; Fig. 4). Although the Appalachians may currently restrict gene flow, the actual uplift of the mountains did not cause divergence of these species.

The Teays-Mahomet River shifted to form the Ohio River after speciation between *P. feriarum* and *P. triseriata* (Fig. 1D–E). Damming of the Teays-Mahomet River occurred from 0.79 to 0.88 mya, whereas divergence of these taxa is estimated at approximately 2.6 mya (Table 1; Fig. 4). Therefore, this particular channel shift does not appear to be a factor in speciation.

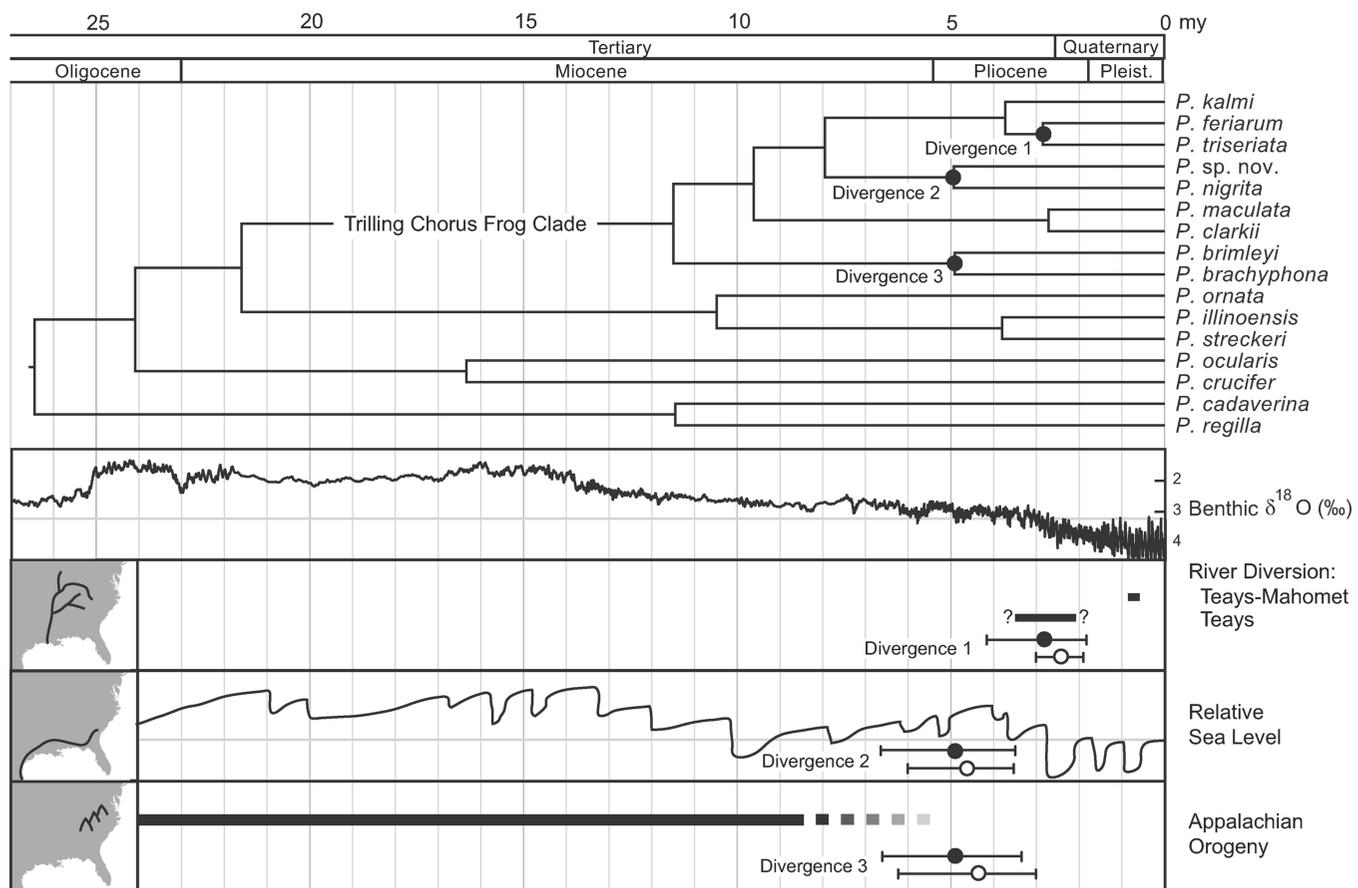
The average 12S/16S mutation rate that we estimated for hylid frogs from r8s (0.00277 substitutions per site per million years) is highly consistent with estimates from distantly related pipid frogs (0.00249; Evans et al. 2004). For illustration purposes, we show the results of the MDIV estimates of divergence times using this new mutation rate, although we favor the Evans et al. (2004) estimate for the MDIV calculations because it is from an independent source (Table 1).

### RIVERS AS BARRIERS TO GENE FLOW

The results from the partial Mantel tests suggest that each of the four rivers restricts gene flow in *P. feriarum*. A significantly different genetic distance exists between populations on the same side of the river compared to the populations on different sides of the river, even after controlling for geographic distance (Table 2).

**Table 1.** Estimates of divergence times in three sister species pairs using coalescent (MDIV) and penalized likelihood (r8s) approaches. The columns indicate: the species pair that diverged, a measure of genetic diversity ( $\theta$ ), mutation rate ( $\mu$ ) in units of substitutions per site per million years, divergence time in millions of years (bold) using the  $\mu$  from Evans et al. (2004;  $T_{div1A}$ ) and  $\mu$  estimated from analysis of the hylid dataset ( $T_{div1B}$ ), 95% lower and upper confidence limits on  $T_{div1}$ , divergence time in millions of years derived from the r8s analysis ( $T_{div2}$ ), and 95% lower and upper confidence limits on  $T_{div2}$ .

Species Pair	MDIV results								r8s results	
	Evans et al. $\mu$				Estimated $\mu$				$T_{div2}$	CI
	$\theta$	$\mu$	$T_{div1A}$	CI	$\theta$	$\mu$	$T_{div1B}$	CI		
<i>P. nigrita</i> /sp. nov.	38.08	0.00249	<b>4.63</b>	3.56–6.08	38.08	0.00277	<b>4.16</b>	3.20–4.46	<b>4.97</b>	3.50–6.72
<i>P. brachyphona</i> / <i>P. brimleyi</i>	24.59	0.00249	<b>4.17</b>	3.03–6.23	24.59	0.00277	<b>3.75</b>	2.72–5.6	<b>4.95</b>	3.37–6.68
<i>P. feriarum</i> / <i>P. triseriata</i>	68.88	0.00249	<b>2.40</b>	1.93–3.03	68.88	0.00277	<b>2.16</b>	1.73–2.72	<b>2.86</b>	1.86–4.12



**Figure 4.** Chronogram of species divergence in *Pseudacris* and chronology of geological events from the late Oligocene to present. Divergences between sister species (top half, labeled with black dots) correspond to hypothesized geological events (bottom half). Estimated divergence times with 95% error bars are shown for the penalized likelihood (black dots) and coalescent (white dots) analyses. Horizontal gray lines indicate present-day levels of benthic  $\delta^{18}\text{O}$  and sea level. Because benthic  $\delta^{18}\text{O}$  levels are negatively correlated with temperature, this trace is shown as a proxy for temperature fluctuations through time (Zachos et al. 2001; Liseicki and Raymo 2005). Units for benthic  $\delta^{18}\text{O}$  are described in Zachos et al. (2001, ref. 19). River diversion estimates are derived from Gray (1991), Melhorn and Kempton (1991), and Strange and Burr (1997). The sea level curve is taken from Haq et al. (1987) and illustrates changes in sea level compared to present levels. The Appalachian orogeny bars represent periods of uplift (solid bar) and erosion (broken bar) based on Prowell and O'Connor (1978), Hack (1982), Reinhardt et al. (1984), Poag and Sevon (1989), Prowell (1989), Prowell and Christopher (2000, 2006), and Dennison (2001).

These data suggest that river systems may form important barriers to gene flow in chorus frogs.

The randomization test for expansion in coastal *P. feriarum* points to significantly greater genetic distances among populations south of the Savannah River than populations on the north side ( $P < 0.0001$ ). In addition, the phylogeny shows that the earliest-branching populations in this clade are from Tennessee and Georgia, and the deeply embedded populations are from the Carolinas and northward (Fig. 3E). These results are consistent with a pattern of northward expansion.

#### EFFECTS OF CLIMATE ON GENETIC VARIATION

Estimates of the growth parameter ( $g$ ) show evidence of recent population growth in all clades examined except *P. brachyphona* (Table 3). Although estimates of  $g$  are not directly comparable among clades due to their different genealogical histories and population sizes, the  $P$ -values for expansion in the *P. maculata/clarkii* (glaciated/aridified range) and *P. sp. nov.* (partially aridified range) clades are highly significant.

Comparisons of the standardized genetic variation among clades also suggest that the two clades existing in previously glaciated areas have undergone recent geographic expansion into these areas (Table 3). The *P. maculata/clarkii* clade has significantly lower genetic variation than all other clades, and *P. triseriata* has the next lowest value (although this is not significantly less than *P. nigrita*). This result is supported by the Range Expansion Tests: both *P. maculata/clarkii* and *P. triseriata* show significantly lower variation in glaciated parts of their ranges. If we omit populations from glaciated areas and repeat the Range Expansion Tests, *P. maculata/clarkii* still has significantly lower genetic variation than any other clade ( $\bar{v} = 0.000419$ ;  $P = 0.019$ ), suggesting that aridification may have reduced variation in this clade. Interestingly, after northern populations are removed from the *P. triseriata* sample, this species has the highest amount of genetic variation of all clades ( $\bar{v} = 0.001110$ ). These results are

**Table 2.** Role of modern river systems in restricting gene flow. Partial Mantel tests were conducted for four river systems. A significant result suggests that a river inhibits gene flow between populations. The Mantel test statistic ( $r$ ) is analogous to the Pearson product-moment correlation coefficient. This statistic tests the partial correlation between genetic distance and rivers after controlling for geographic distance. Significant  $P$ -values are indicated by an asterisk.

River	$P$	$r$
Apalachicola	<0.0001*	0.285
Altamaha	<0.0001*	0.362
Savannah	<0.0001*	0.569
Cape Fear	<0.0001*	0.181

consistent with the idea that glaciation and aridification have acted in concert to reduce genetic diversity in *P. maculata/clarkii*. We found equivocal support for range expansion of *P. sp. nov.* into formerly aridified areas (the population growth test was significant whereas the Range Expansion Test was not significant; Table 3).

#### Discussion

Divergence times estimated using two independent approaches indicate that the major lineages of trilling chorus frogs diversified during the Tertiary (late Miocene), and therefore Quaternary climatic change was not an important factor driving speciation. Instead, we show evidence that at least one geological event, the inundation of the Mississippi embayment during the Pliocene, resulted in speciation. To our knowledge, this is the first study to demonstrate that this event contributed to speciation in any group. We also show that modern river systems reduce gene flow in *Pseudacris* and thus potentially promote diversification. Lastly, *Pseudacris* populations inhabiting areas affected by Quaternary climatic change have reduced levels of genetic variation compared to populations from more equable regions, suggesting that these areas have been recently colonized.

#### GEOLOGICAL PROCESSES AND SPECIES DIVERSIFICATION

Identifying the primary factors that have caused speciation is notoriously difficult and frequently speculative. Here, we employed a predictive hypothesis-testing approach to test for correlations between timing of species divergence and timing of geological events that are relevant to the species distribution. Divergence times estimated using the two approaches were remarkably concordant, despite the different assumptions of these methods. The geological events examined include inundation of the Mississippi Embayment, uplift of the Appalachian Mountains, and diversion of the Teays-Mahomet River. Although the latter two geological events were ruled out as the causes of species divergence, the timing of the first event is consistent with timing of speciation between *P. nigrita* and *P. sp. nov.*

#### Inundation of the Mississippi Embayment

Marine inundation of the Mississippi Embayment is correlated with at least one speciation event in *Pseudacris*. Sea levels in the Embayment reached the maximum inland extent during the early Cenozoic, then sea levels fluctuated, and gradually receded through the late Cenozoic (Fig. 1A; Reed et al. 2005). A peak level of the late Pliocene seas corresponds to the divergence time of *P. nigrita*–*P. sp. nov.* Immediately prior to this high sea stand was a drop to present-day levels, which potentially allowed passage of the ancestor of *P. nigrita*–*P. sp. nov.* across the Embayment before

**Table 3.** Measures of genetic variation ( $\bar{v}$ ), range expansion ( $\Delta v$ ), and growth ( $g$ ) in seven trilling *Pseudacris* clades. Taxa are identified as from glaciated (G), aridified (A), or unaffected (–) regions. Clades are listed in order from lowest to highest standardized genetic variation ( $\bar{v}$ ). Each  $P$ -value corresponds to a test between the  $\bar{v}$  in that row and the  $\bar{v}$  immediately below (see text for details). For the range expansion test, the statistic  $\Delta v$  quantifies the within-clade difference between  $\bar{v}$  of populations in a climatically disturbed area and  $\bar{v}$  of populations outside the area. An asterisk next to the  $P$ -value indicates evidence for population expansion into a climatically disturbed region. The *Pseudacris maculata/clarkii* clade was only tested for expansion into glaciated regions and not for expansion into aridified regions because no populations are located outside aridified regions. Growth parameters ( $g$ ) were estimated using FLUCTUATE and significance of these values was determined through simulation of null distributions. A significant value, denoted by an asterisk, indicates that the population has undergone recent population growth.

Clade	Region	Genetic Variation		Range Expansion		Growth	
		$\bar{v}$	$P$	$\Delta v$	$P$	$g$	$P$
<i>P. maculata/clarkii</i>	G A	0.000330	< 0.01*	0.000161	< 0.01*	1035	<0.01*
<i>P. triseriata</i>	G	0.000558	0.25	0.000701	< 0.01*	1191	0.01*
<i>P. nigrita</i>	–	0.000645	0.37	–	–	494	0.01*
<i>P. kalmi</i>	–	0.000677	0.36	–	–	4055	0.02*
<i>P. sp. nov.</i>	A	0.000805	0.48	–0.000059	0.56	1252	<0.01*
<i>P. brachyphona</i>	–	0.000806	0.06	–	–	61	0.45
<i>P. feriarum</i>	–	0.000906	–	–	–	192	<0.01*

speciation (Fig. 4; Haq et al. 1987). Currently the two species form a narrow contact zone along the eastern side of the Mississippi Embayment (Fig. 1A; Gartside 1980). These data are consistent with the interpretation that inundation of the Embayment contributed to this speciation event.

This study is the first to find a correlation between timing of speciation and inundation of the Mississippi Embayment. Although the Mississippi River has been implicated as a barrier to gene flow and the potential cause of speciation in numerous taxa (Moncrief 1993; Burbrink et al. 2000; Austin et al. 2002, 2004; Burbrink 2002; Leaché and Reeder 2002; Brant and Ortí 2003; Zamudio and Savage 2003; Hoffman and Blouin 2004; Howes et al. 2006; Ray et al. 2006), only a handful of studies have attempted to estimate timing of divergence between populations currently divided by the river. Although all of these divergence estimates may be compromised by methodological problems (strict molecular clock; Hillis et al. 1996), two studies suggest that divergences occurred in the Pleistocene (Brant and Ortí 2003; Howes et al. 2006), one suggests the late Pliocene (Hoffman and Blouin 2004), and one supports both Pleistocene and Pliocene divergences (Austin et al. 2004). Pleistocene divergences are more likely due to geographic isolation caused by ice sheets or glacial outwash in the Mississippi River Valley rather than marine inundation because seas did not extend much further into the Embayment than present during this period (Fig. 4; Reed et al. 2005). The Pliocene divergence of northern leopard frogs (Hoffman and Blouin 2004) is not related to marine inundation because the frogs are distributed north of the Mississippi Embayment. No geological or climatic factor has been suggested as the cause of the Pliocene divergence between *P. crucifer* clades (Austin et al. 2004).

#### Appalachian uplift

The most recent uplift of the Appalachian Mountains is not correlated with the divergence of *P. brachyphona*–*P. brimleyi*, which occurred well after the uplift (Fig. 4). Although the eroded mountains may still have played a role in divergence if the ancestor dispersed across the mountains, the uplift itself did not cause speciation. An alternative hypothesis for the cause of divergence between these species is bisection of their ancestral range through competition from another chorus frog, the ancestor of *P. feriarum*–*P. kalmi*–*P. triseriata*. Evidence that this ancestor existed along the corridor between the modern distributions of *P. brachyphona* and *P. brimleyi* comes from the fact that *P. kalmi* was left behind in the northeastern United States after speciation occurred during the middle Pliocene. Although this idea is speculative, *P. brimleyi* and *P. feriarum* are rarely found sympatrically, suggesting their distributions may be restricted by interspecific competition (E. M. Lemmon, unpubl. data). Therefore, we hypothesize that historical competition among lineages caused allopatric divergence.

The Appalachians have been proposed to be an important geographic feature causing divergence in other taxa (Burbrink et al. 2000; Austin et al. 2002; Leaché and Reeder 2002; Church et al. 2003; Zamudio and Savage 2003; Austin et al. 2004; Runck and Cook 2005). Divergence times between clades spanning the mountains have only been estimated in two of these studies (Church et al. 2003; Austin et al. 2004); in both, divergences are thought to have occurred during the Pleistocene, long after the uplift and erosion of the Appalachians had occurred. Both of the latter studies attributed the divergences to the effects of glaciation rather than to Cenozoic uplift of this mountain system.

### Teays River development

Although our results suggest that modern river systems can be important barriers to gene flow, the data indicate that diversion of the ancient Teays-Mahomet River did not cause speciation in the trilling *Pseudacris*. Rather, this event occurred well after the divergence of *P. feriarum*–*P. triseriata* (Fig. 4). Prior to the formation of the westward flowing Teays-Mahomet River, however, the Teays flowed northward, emptying into the Lake Erie basin (Fig. 1C; Gray 1991; Melhorn and Kempton 1991). At some point during the late Pliocene, the northward path of the Teays was diverted west to form the Teays-Mahomet, thereby cutting off the land connection between northern and southern Indiana, Ohio, and Illinois (Gray 1991; Melhorn and Kempton 1991; Strange and Burr 1997). Because this event has not been well studied in the geological literature, there are no precise estimates for the timing of this channel shift. The general time frame (late Pliocene), however, is consistent with the divergence estimates for *P. feriarum*–*P. triseriata*, and may therefore be involved in this speciation event (Fig. 4). Additional geological data are needed to test this hypothesis.

Although not important for speciation in chorus frogs, which are terrestrial, modification of the Teays-Mahomet River may have caused divergence in some aquatic taxa (Hocutt et al. 1978; Mayden 1988; Strange and Burr 1997; Berendzen et al. 2003; Kozak et al. 2006). Although many phylogeographic patterns are consistent with this event, evidence from divergence time estimates is tenuous. In the only two studies that estimated timing of genetic divergences (Strange and Burr 1997; Kozak et al. 2006), estimates (based on a molecular clock) pre-dated the Quaternary, thereby ruling out glacial blockage of the Teays-Mahomet as a cause of divergence. Future research should not only identify genetic patterns consistent with positions of ancient river drainages but also attempt to correlate timing of speciation events with timing of drainage modification.

### CLIMATIC FACTORS AND REDUCED GENETIC DIVERSITY

Quaternary climatic factors did not cause speciation, but rather have reduced genetic variation in *Pseudacris*. In particular, clades from glaciated areas have the lowest genetic variation. Furthermore, within clades that span the glacier boundary, populations in glaciated regions have significantly lower genetic diversity than those from unglaciated regions. These clades also show evidence for significant recent population growth, supporting the idea that glaciated regions have been recently colonized. These results are consistent with a myriad of other studies that have found similar colonization patterns following glacial recession (see Hewitt 1999, 2000, 2004 for reviews).

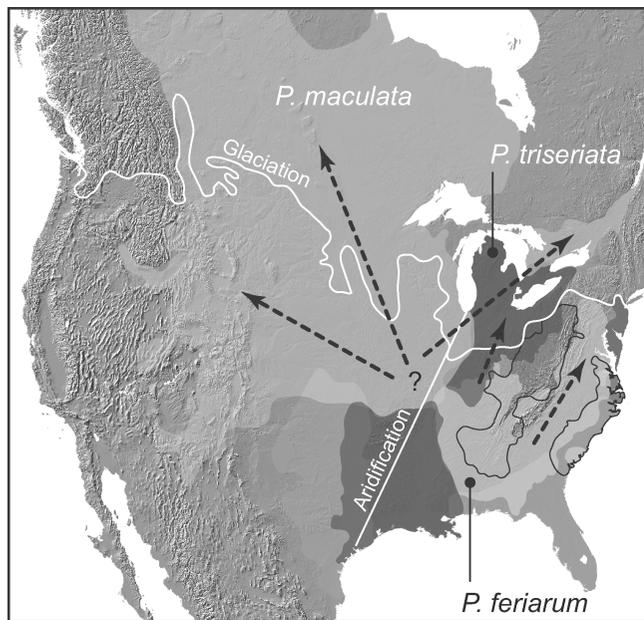
The hypothesis that aridification of the western United States affected the demographic history of the *P. maculata/clarkii* lin-

age is supported by the strikingly low level of genetic variation throughout the clade. Despite the age (about 9.4 mya) and the broad distribution of this lineage, populations show little geographic structuring, and subclades have only shallow divergences. Even when only populations in unglaciated areas are examined, genetic variation is lower than in any other trilling *Pseudacris* clade. This supports the idea that aridification caused local extinction and suggests that aridified areas have only recently been colonized by this clade. A different pattern, however, was found in *P. sp. nov.*, in which genetic variation is slightly higher in the aridified region. One possible explanation is that the Ozark Mountains may have formed a refugium for *P. sp. nov.*, allowing populations to survive during the drought. This idea is supported by studies of freshwater fishes, which not only persisted but even diversified during this period (Mayden 1988). To test the Ozark refugium hypothesis, however, more intense sampling of *P. sp. nov.* should be conducted in the area.

Whereas recent expansion into glaciated areas is supported by many taxa, expansion into aridified regions has not been as well studied. An alternative explanation for reduced genetic variation is that a selective sweep (Hartl and Clark 1997) erased mitochondrial variation in aridified areas. Although comparison of data from nuclear markers with mitochondrial data would be the optimal approach for testing this hypothesis, a selective sweep in *Pseudacris* is unlikely because a similar pattern of low genetic variation exists in several other western wetland-restricted taxa, including tiger salamanders (Shaffer and McKnight 1996), painted turtles (Starkey et al. 2003), and leopard frogs (Hoffman and Blouin 2004). The improbability that selective sweeps occurred in multiple taxa in the same region suggests, rather, that the same climatic processes affected contraction and expansion of these species' geographic distributions.

### ROUTES OF GEOGRAPHIC EXPANSION AND CONTACT ZONES

Following Quaternary climatic changes due to glaciation and aridification, chorus frogs expanded geographically to colonize previously uninhabitable areas of North America (Fig. 5). Signatures of these expansions can be detected in several taxa. The genetic patterns in *P. triseriata* suggest that the species expanded northward from unglaciated areas in southern Illinois, Indiana, and Ohio. This expansion is congruent with the routes of other taxa (*P. crucifer* clade D: Austin et al. 2002; *Ambystoma maculatum* interior clade: Zamudio and Savage 2003). The close phylogenetic relationship of *P. maculata* haplotypes in southeast Ontario and in southern Illinois suggests that the species expanded across Illinois, Indiana, and Michigan before entering southeast Ontario between Lake Erie and Lake Huron. This scenario suggests that *P. maculata* (a freeze-tolerant species; Storey and Storey 1987; Jenkins and Swanson 2005) expanded into formerly glaciated



**Figure 5.** Proposed expansion routes in several trilling chorus frog lineages following Quaternary glaciation and aridification. Hypothesized expansion patterns, indicated by dashed arrows, are based on phylogenetic structure and levels of intraspecific genetic variation. Glaciated and aridified regions correspond to areas indicated by arrows in Figure 2. Distributions of unlabeled taxa correspond to species in Figure 2.

areas prior to *P. triseriata*, which later bisected the distribution of *P. maculata* (Fig. 5). Alternative entry routes for *P. maculata* into southeast Ontario (e.g., via upstate Michigan) are less likely because these would require frogs to traverse large areas of currently (and presumably, historically) unsuitable habitat (Fig. 2; Bleakney 1959; Cook 1964; Lannoo 2005). *Pseudacris maculata* also expanded across the western United States and Canada into formerly glaciated and aridified areas.

Species that currently inhabit the Coastal Plain of the eastern and southern United States likely underwent frequent range expansion and contraction as sea levels fluctuated throughout the Pliocene and Pleistocene (Haq et al. 1987). One such fluctuation (Hobbs 2004) allowed gene flow between populations on the Delmarva Peninsula (Delaware/Maryland/Virginia; *P. kalmi*) and populations of eastern mainland Virginia (*P. nigrita*), indicated by a putative hybrid between the two species (Figs. 2, 3; Lemmon et al., 2007). The finding that Coastal Plain species (*P. kalmi*, *P. nigrita*, *P. sp. nov.*) have relatively lower genetic variation (Table 3) than inland species (*P. brachyphona* and *P. feriarum*) is consistent with the prediction that sea level fluctuations had a demographic effect similar to glaciation. This hypothesis can be tested by examining genetic variation in other organisms with similar distributions.

The strongest evidence for expansion is found in the coastal *P. feriarum* clade: northern populations have significantly lower

genetic variation (Table 3) and southern populations are phylogenetically basal (Fig. 3). Although northern *P. feriarum* populations did not experience glaciation, they were indirectly affected through southward expansion of boreal forests (Davis 1983; Williams 2000, 2004). Thus, it is probable that coastal *P. feriarum* contracted its range southward during the last glacial maximum and later expanded northward (Fig. 5), a hypothesis testable by combining ecological niche and paleoclimate models (e.g., Carstens and Richards 2007). *Pseudacris crucifer* shows a similar expansion route (clades A+B: Austin et al. 2002).

An extremely interesting finding is the strong congruence between proposed suture zones or contact zone hot spots (Remington 1968; Swenson and Howard 2004, 2005) and areas of contact among mitochondrial lineages of the trilling chorus frogs (Fig. 2). In particular, the southeastern Ontario hybrid zone hot spot (No. 5, Swenson and Howard 2005) corresponds closely to the contact between *P. maculata* and *P. triseriata*. The central-southeastern Alabama tree contact zone hot spot (No. 3, Swenson and Howard 2005) matches with an area of contact between *P. brachyphona*, *P. feriarum*, *P. nigrita*, and *P. sp. nov.* The central Texas suture zone (No. 3, Remington 1968) lines up with the contact between *P. clarkii* and *P. sp. nov.* Perhaps most significantly, the southern Indiana/Illinois/Missouri–northern Kentucky/Arkansas hot spot (suture zone No. VIIG, Remington 1968; tree contact zone No. 4, Swenson and Howard 2005) corresponds closely to the contact between two species pairs: *P. feriarum* and *P. triseriata* in the eastern region, and *P. maculata* and *P. sp. nov.* in the west. Although Swenson and Howard (2005) did not include data from amphibians, our study suggests that suture zones apply to this group as well. Future studies attempting to uncover biodiversity should be careful to sample these North American hot spots.

## Conclusion

In this study, we have highlighted the importance of developing testable a priori hypotheses with respect to phylogeographic questions and evaluating these hypotheses within a statistical framework. We provide a novel combination of approaches: correlation of the timing of barrier development and species divergence times, and examination of the effects of climatic fluctuations on genetic variation. This strategy allowed us to test several geological events thought to promote diversification in North America. Our study offers insight into general patterns of speciation and provides a guide for future phylogeographic studies attempting to identify the specific features driving divergence.

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### *Supplementary Material*

The following supplementary material is available for this article:

**Appendix S1.** List of taxa included in a Bayesian phylogenetic analysis of hylid frogs.  
Figure S1.

This material is available as part of the online article from:

<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1558-5646.2007.00181.x>

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