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Patterns and Processes of Speciation in North American Chorus Frogs (*Pseudacris*)

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Patterns and Processes of Speciation in North American Chorus Frogs (*Pseudacris*)

by

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Patterns and Processes of Speciation in North American Chorus Frogs (*Pseudacris*)

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During speciation, populations become spatially separated from each other by biotic or abiotic factors, and this leads to genetic divergence and reproductive isolation. Here, I study the process of speciation and the patterns resulting from this process in the chorus frogs (*Pseudacris*). I first lay the foundation for this work by constructing phylogenies based on molecular data. I then address broad-scale questions regarding the abiotic factors thought to drive speciation. I examine evolution of reproductive signals within a phylogenetic context, and finally, I address fine-scale questions regarding the completion of reproductive isolation in contact zones between recently-evolved species.

In chapter 1, I estimate the phylogenetic relationships across the genus *Pseudacris*. I find that several species of unclear status (*regilla*, *cadaverina*, *crucifer*, *ocularis*) belong to this genus, and that *P. ocularis* is the sister species of *P. crucifer*. In chapter 2, I

examine the phylogeography of a clade within *Pseudacris*, the trilling chorus frogs. I find support for at least nine species and delineate their geographic distributions. In chapter 3, I test geological and climatic hypotheses proposed to drive speciation in North American flora and fauna. By estimating divergence times in the trilling chorus frogs and correlating these divergences with timing of geologic events, I find that marine inundation of the Mississippi Embayment may have caused speciation in this group. Additionally, I find that climatic events led to reduced genetic variation rather than divergence within species. In chapter 4, I study the evolution of acoustic signals of all species of *Pseudacris*. Using a comparative method approach, I find that physiology-based call variables are more evolutionarily labile than morphology-based call variables. In addition, I find that sympatric signals are more different than allopatric signals, suggesting that these frogs have partitioned the acoustic niche. In chapter 5, I examine evolution of reproductive isolation between two chorus frog species in sympatry. I find that male signals show a repeated pattern of divergence in sympatry, and that different axes of the signal diverge in different populations, suggesting that heterospecific overlap may lead to reproductive isolation among conspecific populations. I also find that female preferences have evolved in sympatry, suggesting that divergence in the contact zone is due to reinforcement.

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Introduction

Chorus frogs (genus *Pseudacris*) are a clade of winter-breeding hylid treefrogs that are widely distributed across North America. This group was the subject of pioneering research on speciation, particularly during the mid-1900's. A large proportion of this work was done by students and collaborators in the laboratory of W. Frank Blair, at the University of Texas, Austin. Here, I continue this tradition with new research on chorus frogs. To introduce *Pseudacris* as a model system for studying speciation, I first briefly review some highlights of previous research.

In one of the first applications of the oscillograph to frog calls, Thompson and Martof (1957) demonstrated that several chorus frog species differ from each other with respect to one or more call variables, suggesting that frog species, in general, have unique calls. Through laboratory observation of chorus frog reproductive behaviors, Martof and Thompson (1958) tested the function of the male call. They found that gravid females not only respond to male calls when they sexually receptive, but that they can locate the male based on acoustic cues alone. Littlejohn and Michaud (1959) demonstrated the importance of acoustic signals in premating isolation between chorus frog species. In one of the first female discrimination experiments, they showed that females distinguish between conspecific and heterospecific signals and strongly prefer conspecific calls.

Crenshaw and Blair (1959) provided evidence that multiple reproductive isolating mechanisms prevent hybridization between species. They found that in chorus frogs, acoustic signals are critical, but spatial separation (by habitat and calling site) is also important. In a further investigation of isolating mechanisms, Blair and Littlejohn (1960) found that male signals and female preferences for these signals prevent hybridization. They suggested that call divergence between species is a byproduct of ecological selection on body size.

Work by Littlejohn (1960) demonstrated that frogs distinguish between conspecific calls and those of a non-sister species, but not between conspecific calls and those of the sister species. Martof (1961) showed experimental evidence that acoustic signals prevent hybridization. Through a series of female preference tests, he found that species differ in their ability to identify conspecific signals when paired with different heterospecific stimuli: some taxa totally ignore signals of other species while others confuse signals and potentially hybridize. Michaud (1962) found that hybrids can have intermediate calls between the parental species and females cannot distinguish the hybrid from the parental call in choice tests. To identify the characteristics of the signal that are salient to females, Martof and Thompson (1964) dissected the male call and performed a series of female preference tests, varying one character at a time. Their study suggests that frequency, duration, intensity of the call as well as call rate are important to females.

To understand the genetic mechanisms that prevent fusion of species when hybridization occurs, Mecham (1965) created 13 hybrid crosses of *Pseudacris* species

and estimated hybrid viability. He found that postzygotic isolation is low for most hybrid combinations, at least through early larval development. He was unable, however, to study hybrid fitness later in the life cycle.

After the idea of reinforcement was introduced by Dobzhansky (1940) and popularized by Blair (1955, 1958), researchers looked for this phenomenon in *Pseudacris* by testing for reproductive character displacement, a pattern that sometimes results from reinforcement (Lemmon et al. 2004). Michaud (1964) found no evidence for character displacement in acoustic signals of two Texas species. Working on a different species pair, however, Fouquette (1975) found strong evidence for acoustic character displacement (and no hybridization) in a contact zone through the Florida panhandle. Interestingly, in a contact zone further west (Louisiana and Mississippi), a genetic study by Gartside (1980) demonstrated a high frequency of hybridization and low call differentiation between the same two species. Gartside (1980) suggested that a possible reason for this geographic disparity is that ecological conditions are more favorable to hybrids in the western zone compared to the eastern zone. No further work was done at this contact zone.

Despite the fascinating early work on chorus frogs, for some mysterious reason, speciation research on this system basically ceased after 1980, though behavioral research continued on the spring peeper (*Pseudacris crucifer*; e.g., Wilczynski et al. 1984; Schwartz 1987; Schwartz and Gerhardt 1998) and pacific chorus frog (*P. regilla*; e.g., Brenowitz and Rose 1994; Alder and Rose 1998; Rose and Brenowitz 2002) and some

ecological research continued (e.g., Caldwell 1987; Hensley 1993; Smith and Van Buskirk 1995; Wente and Phillips 2003). One possible explanation for this may be that taxonomic work stalled before this date, due to the difficulties of delineating species based on morphology alone. As molecular techniques were developed, genetic work by Hedges (1986) and a combined genetic/morphology study by Cocroft (1994) helped to clarify some of the coarse-scale phylogenetic problems (e.g., by placing "*Hyla crucifer*" into *Pseudacris*) but left extensive ambiguity with respect to the morphologically-similar "*Pseudacris nigrita* group", which includes most species that were the subject of these early speciation studies. Of course, without clear understanding of phylogenetic relationships, it is not possible to determine whether the organism under study is a single species or multiple species. This is a major obstacle for speciation researchers, who may have chosen other systems to avoid the taxonomic mess.

With the hope of again generating excitement for evolutionary research on *Pseudacris*, below I present coarse-scale phylogenetic relationships among chorus frogs (Chapter 1) and detailed phylogeographic data concerning positions of contact zones among taxa (Chapters 2 and 3). I then build on this phylogenetic foundation through speciation studies of broad-scale patterns of acoustic signal evolution across the genus (Chapter 4) and fine-scale investigation of the evolution of premating isolation in a specific contact zone (Chapter 5). This work answers some long-standing questions, but raises far more new ones, thus opening the doors to a whole new generation of evolutionary research on chorus frogs.

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Chapter 1

Phylogenetic Relationships of the North American Chorus Frogs (*Pseudacris*: Hylidae)*

Abstract: We examined phylogenetic relationships of the North American chorus frogs (*Pseudacris*: Hylidae) from 38 populations using 2.4kb of 12S and 16S mtDNA to elucidate species relationships and examine congruence of previous phylogenetic hypotheses. Parsimony, maximum likelihood, and Bayesian phylogenies are consistent and reveal four strongly supported clades within *Pseudacris*: 1) A West Coast Clade containing *regilla* and *cadaverina*, 2) a Fat Frog Clade including *ornata*, *streckeri*, and *illinoensis*, 3) a *Crucifer* Clade consisting of *crucifer* and *ocularis*, and 4) a Trilling Frog Clade containing all other *Pseudacris*. Explicit hypothesis testing using parametric bootstrapping indicates that previous phylogenetic hypotheses are rejected by our sequence dataset. Within the Trilling Frog Clade, *brimleyi* and *brachyphona* form the sister group to the *Nigrita* Clade: *nigrita*, *feriarum*, *triseriata*, *kalmi*, *clarkii*, and *maculata*. The *Nigrita* Clade shows geographic division into three clades: 1) populations of *maculata* and *triseriata* west of the Mississippi River and Canadian populations, 2) southeastern U.S. populations of *feriarum* and *nigrita*, and 3) northeastern U.S. populations of *feriarum*, *kalmi*, and *triseriata*. We find that subspecific epithets for *crucifer* (*crucifer* and *bartramiana*) and *nigrita* (*nigrita* and *verrucosa*) are uninformative,

therefore we discourage recognition of these subspecies. *Pseudacris regilla*, *cadaverina*, *ocularis*, and *crucifer* are maintained in *Pseudacris*.

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1.1 INTRODUCTION

A substantial component of our knowledge of animal behavior, ecology, and evolution is derived from studies of North American treefrogs (family Hylidae) (e. g., Andersson, 1994; Ryan, 2001; Gerhardt and Huber, 2002). Insight into the origin of behaviors and evolution of traits requires a phylogenetic framework. However, our understanding of the relationships among North American hylid frogs remains ambiguous despite the availability of some morphological, molecular, and behavioral data for phylogeny estimation (Hedges, 1986; Cocroft, 1994; Da Silva, 1997).

Although most hylids are tropical, there is a significant Holarctic radiation (the extra-tropical North American and Eurasian regions). The Nearctic (extra-tropical North American) component of this radiation includes *Hyla* (tree frogs; 10 species), and two endemic genera *Acris* (cricket frogs; 2 species) and *Pseudacris* (chorus frogs; 15 species). Prior to 1975, overall similarity of morphology or advertisement calls was used to justify taxonomic groupings of *Pseudacris* and other Holarctic hylids. Maxson and Wilson

(1975) first incorporated a phylogenetic perspective into Holarctic hylid systematics in their use of microcomplement fixation data from albumins. Hedges (1986) transferred *Hyla crucifer*, *H. cadaverina*, *H. regilla*, and *Limnaeodus ocularis* to *Pseudacris*, based primarily on an allozyme phylogeny. Later, Cocroft (1994) combined Hedges (1986) allozyme data with a suite of morphological characters in a total evidence analysis of *Pseudacris*. He concluded that the transferral of *crucifer*, *cadaverina*, and *regilla* to *Pseudacris* was unnecessary, and returned these species to *Hyla*. Most recently, after including two additional morphological characters to the Cocroft (1994) dataset, Da Silva (1997) returned these species to *Pseudacris*, noting that their phylogenetic position was consistent with placement in either genus.

As defined in the 1960–70s, chorus frogs (*Pseudacris* sensu stricto) are broadly distributed from the southern tip of Florida to northern Canada and from the east to west coasts of North America (Conant and Collins, 1998). *Pseudacris* occur in a variety of habitats from hardwood forests, to plains, to mountainous regions. These frogs congregate to breed in late winter and early spring, primarily in temporary bodies of water and disperse to woodlands and prairies for the remainder of the year (Kramer, 1973; Kramer, 1974; Stebbins, 1985; Conant and Collins, 1998). One characteristic of chorus frogs is their preference for cold weather breeding. Choruses may form shortly after the ice thaws from breeding pools (Whitaker, 1971). The mating season tapers off as nighttime temperatures rise and the breeding of other hylids commences (Conant and Collins, 1998).

Because chorus frogs are morphologically conservative, taxonomic confusion at the subspecific level has been common (Neill, 1949; Mittleman and List, 1953; Schwartz, 1957; Chantell, 1968a; Platz and Forester, 1988; Platz, 1989). Until recently the subspecies of *triseriata* (*t. feriarum, t. kalmi, t. maculata,* and *t. triseriata*; fide Schwartz, 1957) were treated as part of a wide-ranging polytypic species. Platz and Forester (1988) and Platz (1989) elevated the four subspecies to specific level based on differences in advertisement calls. These taxonomic changes have been controversial, in part because it is unclear whether call variation is clinal or differences in calls are used as prezygotic isolating mechanisms for species recognition.

Presently, *Pseudacris* includes 15 species: *brachyphona*, *brimleyi*, *cadaverina*, *clarkii*, *crucifer* (two subspecies, *c. crucifer* and *c. bartramiana*), *feriarum*, *illinoensis*, *kalmi*, *maculata*, *nigrita* (two subspecies, *n. nigrita* and *n*. *verrucosa*), *ocularis*, *ornata*, *regilla* (seven subspecies, *r. cascadae, r. curta, r. hypochondriaca, r. pacifica, r. palouse, r. regilla, r. sierra*), *streckeri*, and *triseriata* (Harper, 1939a; Smith, 1951; Schwartz, 1957; Jameson *et al.*, 1966; Platz and Forester, 1988; Platz, 1989; Da Silva, 1997; Collins and Taggart, 2002; Conant and Collins, 1998; Duellman, 2001). *Pseudacris illinoensis* was recognized as a full species by Collins and Taggart (2002) without discussion, and *kalmi* is recognized by some workers as a subspecies of *feriarum* (Crother, 2001). Although we arbitrarily treat these units as species, our use of this taxonomy should not be taken as agreement with this action (see Discussion).

The goals of this study are multifold. 1) We resolve persistent ambiguities in *Pseudacris* phylogenetic relationships using 2.4 kb of mitochondrial DNA sequence data. We utilize rapidly evolving mitochondrial genes because these markers have been shown to facilitate resolution of phylogenetic relationships among closely related taxa (Shaffer and McKnight, 1996; Burbrink et al., 2000; Burbrink, 2002). 2) We reanalyze the Hedges (1986) allozyme dataset using allele frequency information and test the congruence of this and several other previous phylogenetic hypotheses with our sequence data. 3) We incorporate multiple exemplars of species spanning broad geographic areas and include all currently recognized or disputed species of *Pseudacris* (sensu lato). Inclusion of multiple populations of *Pseudacris* species is extremely important because the monophyly of many currently recognized taxa in this genus has not been established. Our study represents the first to include multiple populations of *Pseudacris* species in a genus-level phylogenetic analysis. Our results provide a phylogenetic context for ongoing studies of signal evolution and speciation in these frogs.

1.2 MATERIALS AND METHODS

Taxa. We sampled 38 populations of *Pseudacris* in the United States and Canada (Supplemental Data 1.1), which encompassed all 23 species and subspecies of *Pseudacris* (except 5 of the *P. regilla* subspecies *sensu* Jameson et al., 1966). Widespread taxa were sampled from multiple populations; collection permits were obtained from all relevant states. Tissue samples were frozen in liquid nitrogen or immersed in tissue buffer, then

stored at –80^{o} C. Based on information from previous phylogenetic analyses (Hedges, 1986; Cocroft, 1994), *Hyla chrysoscelis*, *H. andersoni*, and *H. eximia* were chosen as outgroups. Most specimens are deposited in the Museum of Natural History, University of Kansas and the Texas Memorial Museum, University of Texas, Austin (Supplemental Data 1.1).

DNA amplification and sequencing. DNA was extracted from liver and muscle tissue using the Qiagen DNeasy™ kit. Eight primers were used to amplify a 2.4kb region spanning the 12S, tRNAval, and 16S rRNA mitochondrial genes via polymerase chain reaction: 5' to 3' 12Sm GGCAAGTCGTAACATGGTAAG (designed in our lab) and 16Sa ATGTTTTTGGTAAACAGGCG (modified from #87 in Goebel et al., 1999); 16Sc GTRGGCCTAAAAGCAGCCAC (designed in our lab) and 16Sd CTCCGGTCTGAACTCAGATCACGTAG (modified from #95); 16Sh GCTAGACCATKATGCAAAAGGTA (#76) and 12L1 AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT (#46); tRNAphe-L GCRCTGAARATGCTGAGATGARCCC (#30) and tRNAval-H GGTGTAAGCGARAGGCTTTKGTTAAG (#73). Samples were purified under the QIAquick Gel Extraction protocol. Sequencing reactions were done with the same primers listed above, using the ABI Big Dye terminator ready-mix. Sequencing was performed on an ABI 3100 PRISM™ sequencer (Applied Biosystems Inc.).

Sequence Alignment and Phylogenetic Analyses. Contiguous sequences from eight overlapping fragments were constructed in Sequencher 4.1 (GeneCodes Corp.). All

regions were sequenced in both directions with three exceptions: 1) a 300 bp region between the 16Sc and 16Sh primers (16 samples), 2) a 100-250 bp region between tRNAval and 12Sm primers (4 samples), 3) a 100 bp region on the 3' side of 16Sa primer (2 samples). Except for *P. clarkii* and *P. brimleyi*, at least one sample for each species had complete double-stranded sequence. DNA sequences were aligned using Clustal X 1.8 (Thompson et al., 1997). Alignments were manually adjusted to minimize informative sites and ambiguously aligned regions were defined as character sets for possible exclusion using MacClade 4.0 (Maddison and Maddison, 2000).

Phylogenetic analyses were performed using PAUP* 4.0b8 (Swofford, 2000) unless otherwise noted. Heuristic searches were executed under maximum parsimony (MP; Camin and Sokal, 1965) with TBR branch swapping, random addition sequence of taxa, and 100 replicates per search. Characters were unordered and equally weighted for parsimony analyses. Clade support was evaluated using nonparametric bootstrapping (Felsenstein, 1985) with heuristic searches of 1,000 replicates, and by decay indices (Bremer support; Bremer, 1994) using PAUP* 4.0b8. Exclusion of all ambiguously aligned regions yielded no difference in tree topology and minimal change in bootstrap values for parsimony searches. Thus, these regions were excluded from all further analyses. Sequences are deposited with Genbank accession numbers AY291076–AY291116.

For maximum likelihood (ML; Felsenstein, 1981) analyses, we employed successive likelihood ratio tests of six nested models to determine an appropriate model

of evolution (Huelsenbeck and Crandall, 1997). The likelihood ratio test indicated that GTR+Γ+I (general time reversible model with gamma distributed substitution rates and invariable sites; Lanave et al., 1984; Hasegawa et al., 1985; Rodriguez et al., 1990; Yang, 1993) is the best-fitting model for these data. For the ML analysis, we used only 36 of the 41 sequences used in the MP analysis above (we excluded TNHC62210, TNHC62216, KU290341, MVZ11452, and TNHC62208) to reduce computation time because these sequences were nearly identical to other sequences included in the analysis. Intra-clade genetic distances were calculated using a GTR+Γ+I correction implemented in PAUP* 4.0b8 (Swofford, 2000).

Two identical Bayesian analyses were conducted using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) assuming the GTR+Γ+I model. The four Markov chains employed were sampled every 100 generations. Analyses were run for two million generations and the first 1000 sampled trees (100000 generations) were discarded as the burn-in. Bipartition posterior probabilities (bpp) were estimated using a consensus of 19000 sampled trees. We compared these posterior probabilities from the two analyses using a correlation analysis to assure that the estimates were reliable.

The allozyme dataset of Hedges (1986) was re-coded using frequency information to calculate Manhattan distances between taxa. Each locus (a character) was assigned a user-defined step matrix in PAUP*, and each taxon was assigned a unique state for this character. The cost of change from one state to another was set equal to the Manhattan

distance between the species (Berlocher and Swofford, 1997). This dataset was analyzed under parsimony as described above.

Our comparison of the morphological datasets of Cocroft (1994) and Da Silva (1997) with skeletal material indicated discrepancies, and thus we are hesitant to use these data without comprehensive verification of character states. The integration of morphological data is relegated to a future project.

Hypothesis Testing. In order to test alternative hypotheses against our ML topology, we performed parametric bootstrap tests. We chose to employ parametric bootstrapping instead of nonparametric tests, such as the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999; Goldman et al., 2000), because of the increased power compared to nonparametric methods (Huelsenbeck and Hillis, 1996; but see Buckley, 2002). We tested four *a priori* (=null) hypotheses against our phylogeny: Hypothesis A: Hedges' (1986) UPGMA Cavalli-Sforza tree (Fig. 1.1A), Hypothesis B: Hedges' (1986) distance Wagner topology (Fig. 1.1B), Hypothesis C: the parsimony topology from our re-analysis of the Hedges dataset (Fig. 1.1C), and Hypothesis D: Cocroft's (1994) parsimony topology (Fig. 1.1D). We constrained the complete topology of Hypotheses A and B for the tests. For Hypotheses C and D, however, the points of conflict with our MP tree were narrowed to two (C) and three (D) nodes with >50% bootstrap support. Only these nodes were constrained for estimation of the best tree under the null hypothesis. By minimizing the number of constrained nodes, rejection of the null hypothesis was made more difficult.

The parametric bootstrap generates a null distribution against which one can test a statistic of interest. The test is performed as follows: 1) simulate N datasets under the null hypothesis using ML parameter estimates derived from observed sequence data; 2) for each *simulated* dataset, find the shortest tree under the null hypothesis and the overall shortest tree; 3) calculate the differences in tree lengths of the topologies in 2); 4) calculate the test statistic from the difference in tree length under the null hypothesis and overall shortest tree for the *observed* dataset; 5) if the test statistic falls outside the 95% limits of the distribution of tree length differences, the null hypothesis is rejected (Goldman et al., 2000). Our methods followed those described for the parametric bootstrap (SOWH-test) in Goldman et al. (2000), except that we analyzed the datasets under parsimony rather than likelihood as implemented by Hillis et al. (1996) and Sullivan et al. (2000). We used the program MacSimum written by Mark Holder to simulate sequence data.

1.3 RESULTS

Phylogenetic Relationships. After exclusion of ambiguous nucleotide regions, 2333 characters were included in the phylogenetic analyses; 625 of these sites were variable and 519 were parsimony informative. Parsimony analysis resulted in 504 equally parsimonious trees of length 1407 (CI=0.55, excluding uninformative characters and RI=0.83). The large number of trees is due to very short branch lengths of *P. nigrita*, *feriarum*, *kalmi*, and *clarkii*, *maculata*, and *triseriata*. Four major clades of *Pseudacris*

are identified: 1) the West Coast Clade, *regilla* and *cadaverina*; 2) the Fat Frog Clade, *ornata*, *streckeri*, and *illinoensis*; 3) the *Crucifer* Clade, *ocularis* and *crucifer*; and 4) all other *Pseudacris* (Fig. 1.2). Species in the last group produce trilled calls only and thus will be referred to as the Trilling Frog Clade.

Maximum likelihood analysis under the GTR+Γ+I model resulted in a topology with lnL = -10264.31767 (Γ-shape parameter with four discrete rate categories = 0.672558; proportion of invariable sites = 0.505709; nucleotide frequencies: A = 0.352462, C = 0.214354, G = 0.179525, and T = 0.253660). The ML topology, which is consistent with the parsimony tree, offers better resolution. The *Crucifer* Clade is sister to the Trilling Frogs. The Fat Frogs form the sister group of the *Crucifer* + Trilling Frog Clades. The West Coast Clade is the sister-group to remaining ingroup species (Fig. 1.2). A majority-rule consensus of 19,000 trees from the Bayesian analysis revealed the same topology as the maximum likelihood search. Bipartition posterior probability values (bpp) are shown on the likelihood tree in Fig. 1.2. Comparison of these values from the parallel Bayesian runs using a correlation analysis indicated that these estimates of branch support were reliable (r^2=0.99).

Within the Trilling Frogs, the clade of *brimleyi* + *brachyphona* is the sister group to a clade containing *clarkii*, *nigrita*, *triseriata*, *maculata*, *feriarum*, and *kalmi*. We refer to the latter group as the *Nigrita* Clade (Wright and Wright, 1949; Smith and Smith, 1952). The wide-ranging *Nigrita* Clade shows geographic division into two lineages divided by the Mississippi River. The eastern *Nigrita* Clade includes *nigrita* nested

within populations of *feriarum*, *kalmi*, and *triseriata* (as their distributions are currently delineated), such that *feriarum* is paraphyletic with respect to *nigrita* (Fig. 1.2) The intra-clade genetic distance for the eastern *Nigrita* Clade is 0.09–4.00%. In the western *Nigrita* Clade, *clarkii* is nested within populations of *triseriata* and *maculata*. The intra-clade genetic distance for the western *Nigrita* Clade is 0.04–0.54%. This western clade includes U.S. populations and Canadian populations both east and northwest of the Great Lakes (Fig. 1.3).

Re-analysis of Allozyme Data. Our re-analysis of the Hedges (1986) allozyme dataset resulted in >50% bootstrap support for 2 of the 4 basal clade relationships for *Pseudacris* described above (the Fat Frog and Trilling Frog Clades; Fig. 1.1C). Although this analysis places *ocularis* and *crucifer* as sister taxa, the bootstrap proportion for this clade is only 19. In addition, *ocularis* and *crucifer* fall within the Trilling Frog Clade. The Fat Frog group forms the sister to all other *Pseudacris*, though this relationship is poorly supported. This topology does not show *brimleyi* and *brachyphona* as sister taxa.

Comparisons to Alternative Hypotheses. All four null hypotheses outlined in Fig. 1.1 were rejected. The two topologies of Hedges (1986, Fig. 1.1A and 1.1B) and the topology of Cocroft (1994, Fig. 1.1D) are rejected at $p<0.002$. The tree from the Manhattan distance analysis of the Hedges (1986, Fig. 1.1C) dataset is rejected at $p<0.014$ (Fig. 1.4). Overall, the reexamined phylogenetic hypotheses based on allozymes, or allozymes combined with morphological and behavioral data, are largely incongruent with the mtDNA dataset.

1.4 DISCUSSION

Species Relationships. The sister–group relationship of *P. crucifer* and *ocularis* is a novel finding. Because of certain advertisement call and morphological features, the relationship of these species to other hylid frogs has long been debated, even at the generic level (Harper, 1939b; Mittleman and List, 1953; Delahoussaye, 1966; Chantell, 1968a; Gaudin, 1974; Hardy and Borroughs, 1986; Hedges, 1986; Anderson, 1991; Cocroft, 1994; Da Silva, 1997). Although the two species have differentiated with respect to morphology (*ocularis* has undergone miniaturization relative to other *Pseudacris*) and advertisement calls (*crucifer* produces a frequency sweep whereas *ocularis* produces a complex call consisting of a sweep followed by a trill), this study strongly supports (bootstrap value 93%, bpp 100) the inclusion of both taxa in *Pseudacris* sensu stricto (Fig. 1.2). The existence of the other three major clades (the Fat Frogs, the West Coast Clade, and the Trilling Frogs) was suggested by earlier workers (Cocroft, 1994; Da Silva, 1997; Fig. 1.1D).

Within the Trilling Frogs, the sister-group relationship of *P. brimleyi* and *brachyphona* is rather unexpected given that the two are morphologically dissimilar. The eastern coastal plain species *brimleyi* phenotypically resembles the narrowly sympatric *feriarum* more than it does the Appalachian *brachyphona*. However, advertisement calls of *brimleyi* and *brachyphona* are very similar; both have very rapid trills compared to other members of the Trilling Frog group (Brandt and Walker, 1933; Brandt, 1936; Hoffman, 1983; Highton and Hedges, 1995; E. Moriarty unpubl. data). Based on Hedges

(1986) phylogenetic hypothesis, Highton and Hedges (1995) speculated that similarity in calls of the two species was due to convergence. Thus they rejected an alternative hypothesis that *brimleyi* and *brachyphona* possess an ancestral call type relative to other members of the Trilling Frog Clade. In our phylogeny, the basal position which *brimleyi* and *brachyphona* occupy with reference to other Trilling Frogs supports, rather, their "ancestral call type" hypothesis.

The paraphyletic "*Pseudacris triseriata* species complex", consisting of *feriarum*, *triseriata*, *kalmi*, and *maculata* (sensu Schwartz, 1957; Platz and Forester, 1988) ranges from Florida to northwestern Canada. Members of the *triseriata* complex form a subset of the *Nigrita* Clade (Fig. 1.2) and have traditionally been grouped together because they resemble each other morphologically more than they resemble other members of the *Nigrita* Clade. This complex exhibits morphological variation (relative tibia to body length ratios are large in the southeast and small in the northwest), behavioral shifts (jumping vs. "scooting" escape strategy in long-legged vs. short-legged frogs), and variation in advertisement calls across its range (Schmidt, 1938; Smith and Smith, 1952; Smith, 1956; Platz and Forester, 1988; Platz, 1989; Joshua Rest, unpubl. data; Moriarty and Berendzen, unpubl. data). Historically, taxa in the *triseriata* complex have been distinguished mainly by tibia/body length ratios (Smith and Smith, 1952; Smith, 1956), but also by several other morphological and advertisement call characters (Harper, 1955; Chantell, 1968b; Platz and Forester, 1988; Platz, 1989). Geographic boundaries between species are poorly defined due to the apparent broad sympatry and lack of clearly diagnostic characters (Smith and Smith, 1952; Smith 1956; Platz and Forester, 1988;

Platz, 1989). Although there is substantial genetic, behavioral, and morphological variation across the range of the *triseriata* complex, three things are unclear: 1) How many lineages the complex contains, 2) how extensive reproductive isolation among lineages is, and 3) where the boundaries of these lineages lie.

Previous phylogenetic studies of *Pseudacris* did not sample western populations of the *triseriata* complex (Hedges, 1986; Cocroft, 1994; Da Silva, 1997). Our broader population sampling allowed us to detect at least two major lineages within the complex, which are apparently separated by the Mississippi River. The exception is the *P. feriarum* population from the west side of the river in Jonesboro, Arkansas (Craighead Co.), which is part of the eastern lineage. Prior to the Wisconsin stage of the Pleistocene, the Mississippi flowed west of Crowley's Ridge, upon which this population is situated. During the early Wisconsin the channel shifted to its current position on the eastern side of the ridge (Blum et al., 2000). Although the Jonesboro population was recently separated from eastern *Pseudacris*, it retains affinities with the eastern lineage. This pattern is also found in rat snakes (*Elaphe obsoleta* group) from Craighead Co, Arkansas. Although this population is situated on the west side of the Mississippi River, it is a member of the eastern clade (Burbrink, 2000). More extensive sampling in the region is needed to assess the effect of this alluvial system on the phylogeography of other vertebrates.

Pseudacris nigrita and *clarkii* are nested within the eastern and western *triseriata* complex lineages, respectively. These members of the *Nigrita* Clade border the

geographic range of the *triseriata* complex. *P. nigrita* lies to the south and *clarkii* to the west (Conant and Collins, 1998). The presence of these species in the *Nigrita* Clade makes the *triseriata* complex paraphyletic.

With regard to interactions among eastern *Nigrita* Clade members, Fouquette (1975) found evidence for character displacement in advertisement calls between *P. nigrita* and *feriarum* populations in a zone of sympatry (Alabama, Florida, and Georgia). Therefore gene flow between species may be restricted due to evolution of premating isolating mechanisms. However, at the far western extent of the sympatric zone between *nigrita* and *feriarum* (Louisiana and Mississippi), Gartside and Dessauer (1980) examined allozyme allele frequencies and discovered substantial hybridization between species. These studies suggest that *Pseudacris* species may develop disparate reproductive interactions at different areas of contact.

The western lineage of the *Nigrita* Clade contains all populations of *P. maculata*, *clarkii*, and most populations of *triseriata*. The position of the *clarkii* sequence (bootstrap value 100%) may be explained by either incomplete lineage sorting or by gene introgression via hybridization. *Pseudacris clarkii* and *feriarum* have been reported to call syntopically in breeding pools (Texas), and although hybrids have been produced in the laboratory, they are found very rarely in nature (Lord and Davis, 1956; Lindsay, 1958; Michaud, 1962; Michaud, 1964). Some evidence from female preference studies indicates that female *clarkii* and *feriarum* prefer conspecific male calls when presented with a choice between *clarkii* and *feriarum*, suggesting that advertisement calls have

diverged sufficiently to create a premating isolating mechanism between species (Michaud, 1962; Michaud, 1964; *feriarum* discussed as *nigrita*). Thus, although extensive hybridization between *clarkii* and *feriarum* seems improbable, broader population and gene sampling will be necessary to discriminate between this and the hypothesis of incomplete lineage sorting following divergence of these species.

Our results do not concur with the range limits of several *Nigrita* Clade members (*triseriata* complex: *feriarum*, *kalmi*, *maculata*, and *triseriata*) as currently delineated (Smith and Smith, 1952; Smith, 1956; Platz and Forester, 1988; Platz, 1989; Conant and Collins, 1998). There do appear to be several mitochondrial lineages within the contiguous distributions of these taxa, specifically, a northeastern group (Maryland, Michigan, and Kentucky), a southeastern group (Louisiana, Arkansas), and a western U.S./Canadian group (Colorado, Kansas, New Mexico, and Ontario, Canada). At this time our geographic sampling is not broad enough to delimit the borders of these lineages. Therefore, taxonomic recommendations resulting from a finer-scale phylogeographic analysis of the Trilling Frog Clade will be discussed elsewhere.

Phylogeographic Considerations. The Mississippi River has contributed to genetic divergence in many vertebrate groups (Burbrink et al. 2000; Austin et al. 2002; Burbrink, 2002; Leache and Reeder, 2002). Geographic division observed within the *Nigrita* Clade is consistent with these studies. Burbrink (2000) found that morphological variation in color pattern was not useful for distinguishing mitochondrial lineages of rat snakes (*Elaphe obsoleta* group). Rather, these characters may have evolved multiple times

within the clade during adaptation to local ecological conditions on both sides of the Mississippi River. Similarly, morphological variation (particularly of tibia/body length ratios) within chorus frogs from the southeastern to the northwestern part of their range may be the product of local selective pressures. Thus, previous attempts to delineate ranges of species or subspecies of the *Nigrita* Clade may have been confounded rather than helped by use of these characters. Molecular evidence suggests instead that major breaks among chorus frog lineages occur along river drainages and other geographic barriers.

A surprisingly low amount of genetic variation is found in western *Nigrita* Clade populations (0.04–0.54%) relative to eastern populations (0.09–4.00%; Fig. 1.2, compare likelihood tree branch lengths). A similar pattern has been observed for painted turtles (*Chrysemys picta*), tiger salamanders (*Ambystoma tigrinum*), and snapping turtles (*Chelydra serpentina*) (Shaffer and McKnight, 1996; Starkey et al., 2003; H.B. Shaffer, pers. comm.). Based on a paleoclimatology model of Bartlein et al. (1998), Starkey et al. (2003) postulated that after recession of the most recent glaciers, a period of extreme aridification (approx. 14,000 years ago) in the western and north central parts of the U.S. may have eliminated aquatic turtle species and amphibians from these regions. Following the aridification event, aquatic taxa were able to recolonize these areas rapidly, and the low genetic variation in western and central regions reflects this recent expansion. Our data from the *Nigrita* Clade support this proposition. However, this hypothesis must be tested more rigorously using a greater number of populations and individuals.

Austin et al. (2002) described three major mitochondrial lineages within *Pseudacris crucifer* (eastern, central, and western lineages). Following glaciation, the eastern lineage (east of the Appalachian Mountains) expanded northward into Canada and west around the north side of the Great Lakes into SW Ontario (between Lakes Erie/Ontario and Lake Huron), into northwestern Ontario, and into Minnesota and Wisconsin. Along the corridor of SW Ontario, the eastern *crucifer* lineage contacted a deeply diverged central *crucifer* lineage. The eastern lineage also contacted a western *crucifer* lineage on the west side of Lake Michigan. In contrast, within the *Nigrita* Clade, it appears that the *western* clade expanded its range northward into Canada. Our limited data suggest that the western and eastern *Nigrita* Clades may connect through the same corridor as *P. crucifer* lineages in SW Ontario and/or E Ontario. The contact zones of *Nigrita* Clade lineages on the south side of the Great Lakes cannot be determined from our data; however, we are currently conducting a broader phylogeographic study to identify these contacts.

Status of Subspecies. Collins and Taggart (2002) elevated *Pseudacris streckeri illinoensis* to species status without discussion. Populations of *P. illinoensis* are restricted to a narrow region in the sandhill prairies of northeastern Arkansas, southeastern Missouri and southern Illinois (Smith, 1951; Conant and Collins, 1998). These populations are separated from the much broader range of *P. streckeri* by approximately 150 miles. Our trees indicate that *P. streckeri* is paraphyletic with respect to *P. illinoensis*. Smith (1951) suggested that *P. illinoensis* represents relict populations of the broader-ranging ancestor of these two taxa. He postulated that this ancestor and other

prairie species once occupied a wider and more easterly range during expansion of the prairie peninsula approximately 4000 years ago; with the recession of the peninsula and growth of forests, many prairie species such as *P. streckeri* (now considered *P. illinoensis*) survived only in small pockets of suitable habitat (Smith, 1957). Under this scenario, the position of the *illinoensis* sequences, nested within *streckeri* sequences, is not surprising. Although our population samples are limited, the character evidence for paraphyly is strong. At least eight synapomorphies have a CI of 1.0, and a deletion with a CI of 1.0, unite the sequence of *streckeri* from Kansas with the two sequences of *illinoensis*.

However, a tree based on mitochondrial genes alone is not sufficient to address the complex issues surrounding the recognition of taxonomic species. The question of whether *streckeri* and *illinoensis* have differentiated sufficiently in allopatry to merit status as different species deserves further study. Female choice experiments are useful for addressing the question. If female *illinoensis* and *streckeri* consistently discriminate calls of their own species from those of heterospecific males, this would suggest that calls may have diverged enough between populations to serve as premating isolating mechanisms between species. This information would provide support for the action of Collins and Taggart (2002) in designating *illinoensis* and *streckeri* separate species. Data from nuclear genes would also be desirable.

In contrast to the *illinoensis*/*streckeri* example, the subspecies ranges of *Pseudacris crucifer* (*bartramiana* and *crucifer*) and *P. nigrita* (*verrucosa* and *nigrita*) are

contiguous (Brady and Harper, 1935; Harper, 1939a). Recognition of the subspecies *P. crucifer bartramiana* and *P. nigrita verrucosa* renders the nominate subspecies of each species paraphyletic. Our intraspecific samples are not extensive; however, given the data at hand and in agreement with Austin (2002), we do not find the maintenance of subspecies for *P. crucifer* and *P. nigrita* necessary or informative.

Taxonomy. Of any single *Pseudacris* species, the taxonomic position of *P. ocularis* has perhaps been most puzzling to systematists (for detailed 19th century taxonomic history, see Harper, 1939b). The species was transferred from *Pseudacris* to *Hyla* by Harper (1939b) based on external morphology and behavioral characters. Mittleman and List (1953) erected a new monotypic genus, *Limnaeodus*, for *ocularis* because of substantial osteological differences between *ocularis* and other hylids. However, they maintained that *ocularis* either shares an immediate common ancestor with *Pseudacris* or is a direct offshoot of the group. Lynch (1963), Chantell (1968a), and Gaudin (1974) found additional skeletal characters to support recognition of *Limnaeodus*. The latter two studies suggested, instead, that *ocularis* is more closely related to *Acris* than *Pseudacris*. Anderson (1991) recommended placement of *ocularis* in *Hyla* based on karyological evidence. This arrangement was not well accepted.

The sister species of *ocularis*, *Pseudacris crucifer*, has also been shuffled among genera. Using evidence from a review of morphological, and molecular, and behavioral studies, Hardy and Borroughs (1986) named a new genus for *crucifer*, *Parapseudacris*, and transferred the species from *Hyla*. This action was widely ignored. Based on an

allozyme phylogeny, Hedges (1986) moved *Hyla crucifer*, *H. regilla*, *H. cadaverina* and *Limnaoedus ocularis* to *Pseudacris*. He justified his action in part by pointing out that these species share features such as a cold-weather breeding season, a round or ovoid testis, and a black pigment covering on the testis. In contrast, other hylids in North America are warm-weather breeders, and have a white (unpigmented) and elongate testis.

The phylogenetic analyses of Cocroft (1994) and Da Silva (1997) are consistent with Hedges (1986) transferral of *crucifer*, *regilla*, *cadaverina*, and *ocularis* to a monophyletic *Pseudacris*. Our tree provides strong support for the monophyly of the genus including these taxa. Given that most checklists and field guides (Stebbins, 1985, is an exception) follow Hedges' taxonomy, we continue it here.

In summary, our study supports the taxonomic arrangement of Hedges (1986), in recognizing *cadaverina*, *crucifer*, and *ocularis*, and *regilla* as members of a monophyletic *Pseudacris*. We find the taxonomic status of *illinoensis* to be ambiguous, and recommend further study of its relationship to *streckeri*. Finally, we suggest use of specific names only for populations of *nigrita* and *crucifer*.

Supplemental Data 1.1. Specimens used in molecular analyses. Museum collections are abbreviated as follows: TNHC = Texas Natural History Collection, University of Texas, Austin; MVZ = Museum of Vertebrate Zoology, University of California, Berkeley; UTA = University of Texas, Arlington; KU = University of Kansas. The R. Highton and ECM tissue samples do not have voucher specimens.

Species	Sample/ Voucher Number	GenBank Accession	Collection Locality
Pseudacris triseriata	KU224560	AY291090	Douglas:Kansas (NE)
Pseudacris triseriata	KU224558	AY291092	Cheyenne:Kansas (NW)
Pseudacris triseriata	KU289219	AY291091	Berrien:Michigan
Pseudacris triseriata	ECM K2	AY291088	Kingman:Kansas (S central)
Pseudacris triseriata	KU224630	AY291089	McKinley:New Mexico
Pseudacris triseriata	TNHC62324	AY291081	Frontenac, Ontario: Canada (SE)
Pseudacris ornata	KU288911	AY291104	Liberty:Florida
Pseudacris ornata	TNHC62183	AY291105	Aiken:South Carolina
Pseudacris ornata	TNHC62178	AY291106	Barbour:Alabama
Pseudacris crucifer crucifer	KU288677	AY291102	Linn:Kansas
Pseudacris crucifer crucifer	KU 290341	AY291101	Lac Seul, Ontario:Canada (NW)
Pseudacris crucifer crucifer	TNHC62210	AY291099	Barbour:Alabama
Pseudacris crucifer crucifer	TNHC62216	AY291100	Barnwell:South Carolina
Pseudacris crucifer bartramiana	TNHC62369	AY291103	Ocala:Florida
Pseudacris streckeri	KU289036	AY291107	Harper:Kansas
Pseudacris streckeri	TNHC62317	AY291108	Travis:Texas
Pseudacris illinoensis	TNHC62346	AY291110	Scott:Missouri
Pseudacris illinoensis	TNHC62351	AY291109	Clay:Arkansas
Pseudacris clarkii	KU289035	AY291093	Chautauqua:Kansas

Pseudacris feriarum	KU289227	AY291084	Calloway:Kentucky (W)
Pseudacris feriarum	R. Highton 71747	AY291096	Lincoln:Kentucky (central)
Pseudacris feriarum	TNHC62265	AY291085	East Baton Rouge:Louisiana
Pseudacris feriarum	TNHC62255	AY291086	Craighead:Arkansas
Pseudacris kalmi	KU289235	AY291087	Kent:Maryland
Pseudacris maculata	KU290342	AY291082	Lac Seul, Ontario:Canada (NW)
Pseudacris maculata	KU224624	AY291080	Gunnison:Colorado (central)
Pseudacris maculata	KU224625	AY291083	Archuleta:Colorado (SW)
Pseudacris nigrita nigrita	MVZ11452	AY291077	Scotland:North Carolina
Pseudacris nigrita nigrita	TNHC62201	AY291078	Barbour:Alabama
Pseudacris nigrita nigrita	TNHC62208	AY291076	Barnwell:South Carolina
Pseudacris nigrita verrucosa	TNHC62364	AY291079	Brevard:Florida
Pseudacris brimleyi	TNHC62337	AY291094	Pitt:North Carolina
Pseudacris brachyphona	TNHC62303	AY291095	Tallapoosa:Alabama
Pseudacris ocularis	TNHC62234	AY291097	Barnwell:South Carolina
Pseudacris ocularis	TNHC62241	AY291098	Gulf:Florida
Hyla chrysoscelis	KU289034	AY291116	Douglas:Kansas
Hyla eximia	UTA A-13225	AY291113	Morelia, Michoacán:Mexico
Pseudacris cadaverina	KU207382	AY291114	San Diego:California
Pseudacris regilla	KU207396	AY291111	San Diego:California (S)
Pseudacris regilla	TNHC62409	AY291112	Berkeley:California (S central)
Hyla andersonii	KU207335	AY291115	Burlington:New Jersey

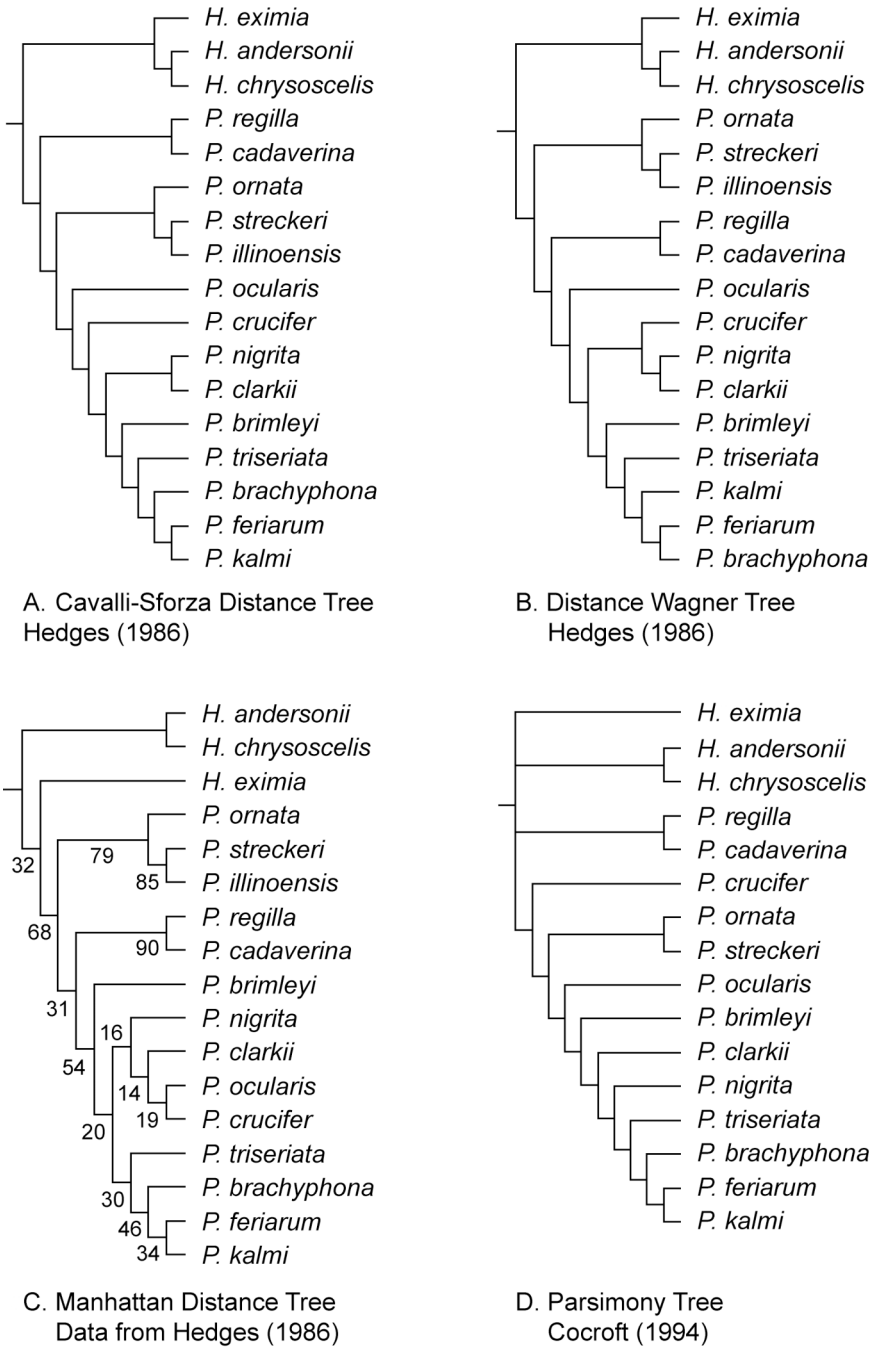

Figure 1.1. Previous hypotheses of *Pseudacris* phylogenetic relationships tested in this study**.** A. Hedges (1986) Cavalli-Sforza distance topology (allozyme data). B. Hedges (1986) distance Wagner topology (allozyme data). C. Topology based on parsimony re-analysis of Hedges (1986) dataset (allozyme data). Cocroft (1994) parsimony topology (allozyme and morphological data).

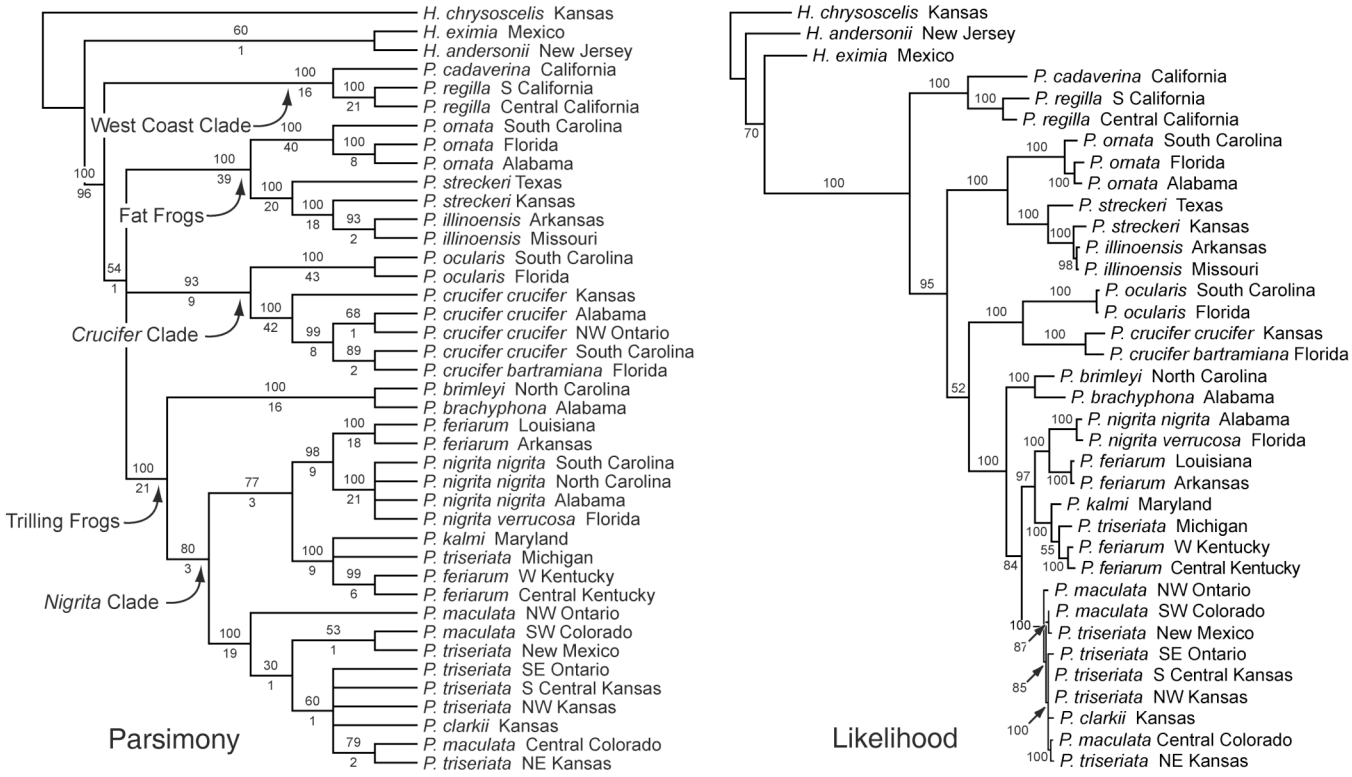

Figure 1.2. Maximum parsimony tree rooted with *Hyla chrysoscelis* (left). Tree shown is a strict consensus of 504 equally parsimonious trees (CI=0.55, RI=0.83). Numbers above branches indicate nonparametric bootstrap values greater than 50% based on 1000 pseudoreplicates. Decay indices are listed below branches. Maximum likelihood tree under the GTR+Γ+I model rooted with *Hyla chrysoscelis* (right). Bayesian bpp values are shown above each branch. Populations of several species were excluded from the likelihood analysis.

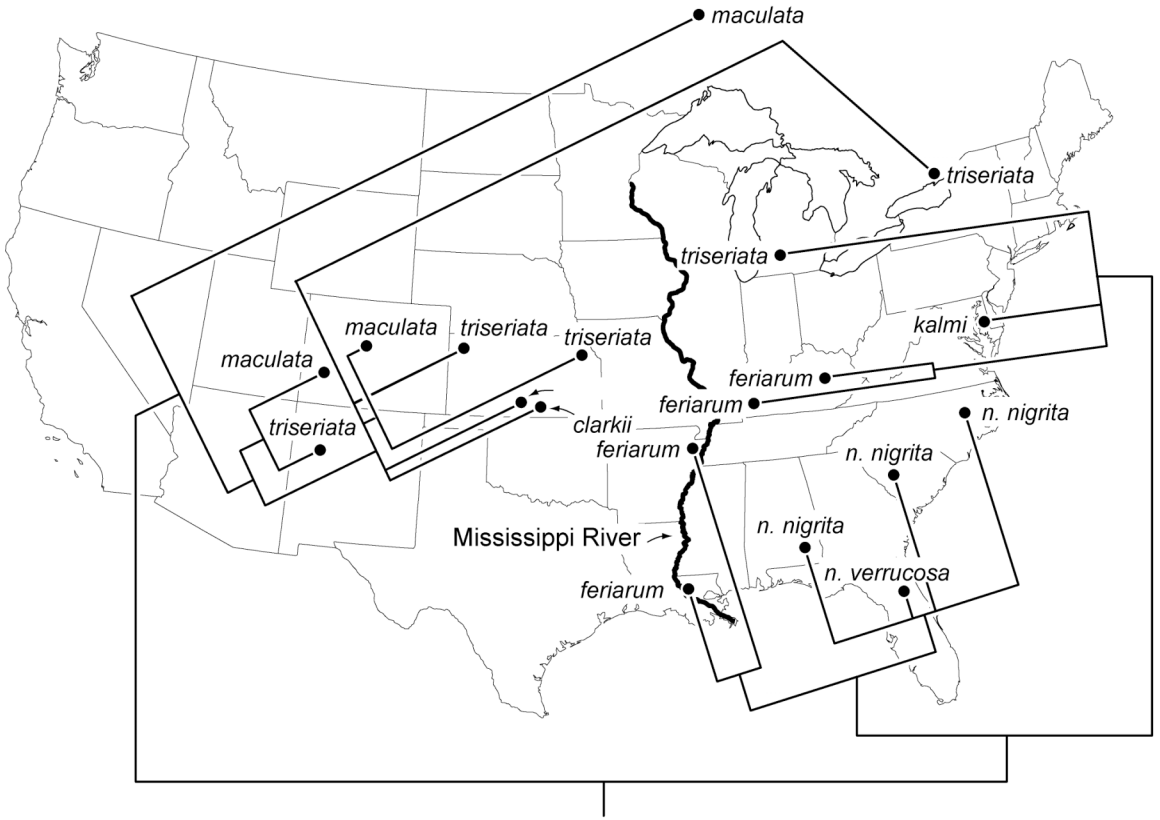

Figure 1.3. Geographic distributions of the *Nigrita* Clade. Map shows geographic division of this group into eastern and western clades by the Mississippi River (thick line). The eastern clade is further subdivided into a northeastern lineage and a southeastern lineage. Current taxonomy does not reflect the phylogenetic relationships among these populations. Branch lengths are not proportional to distance.

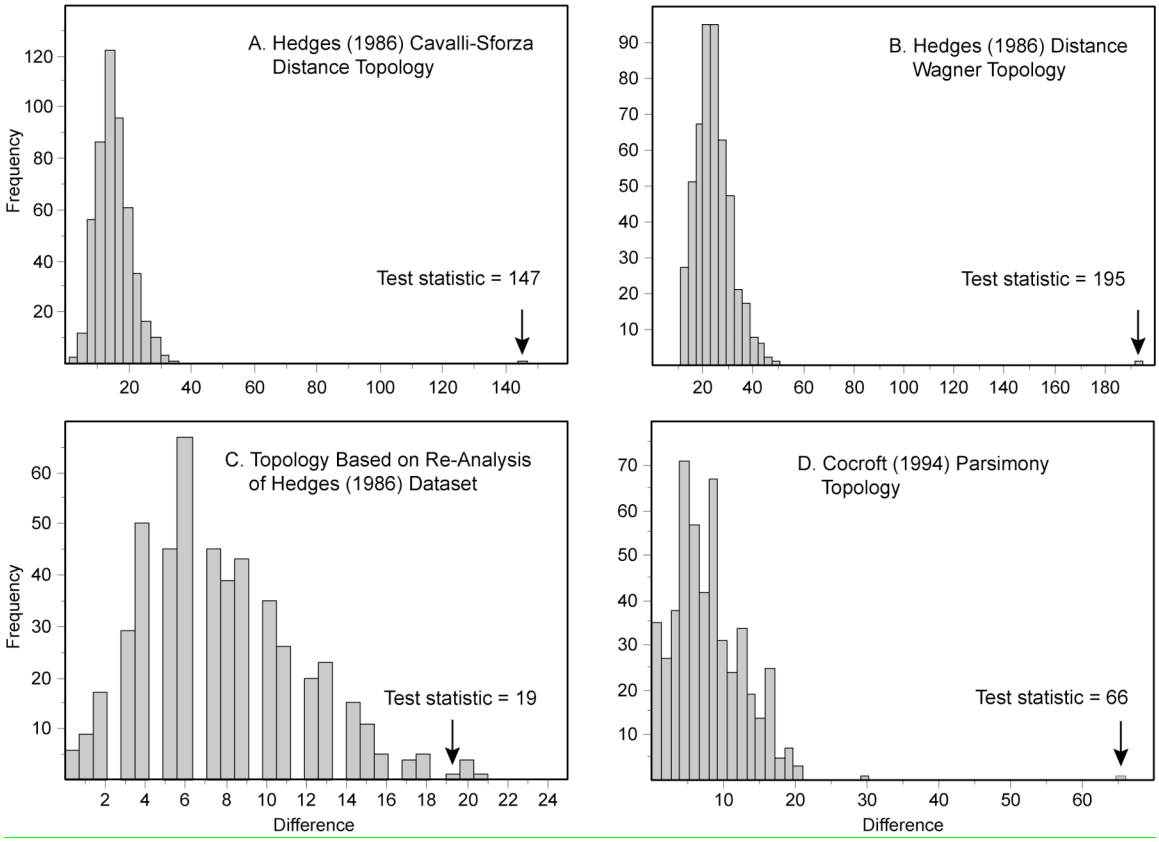

Figure 1.4. Null distributions for the parametric bootstrap tests. These tests examine the validity of previous phylogenetic hypotheses for *Pseudacris*. Values of the test statistic that fall outside the 95% limits of the distribution are significant.

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Chapter 2

Phylogeny-based Delimitation of Species Boundaries and Contact Zones in the Trilling Chorus Frogs (*Pseudacris*)*

Abstract: Although the trilling chorus frogs (subclade within *Pseudacris*: Hylidae) have been important in studies of speciation, continental patterns of genetic diversity within and among species have not been elucidated. As a result, this North American clade has been the subject of substantial taxonomic debate. In this study, we examined the phylogenetic relationships among the trilling *Pseudacris* and tested previously hypothesized scenarios for speciation using 2.4 kb of mitochondrial 12S and 16S rRNA from 253 populations. Bayesian phylogenetic analyses, in combination with published morphological and behavioral data, support recognition of at least nine species, including an undescribed species from the south-central United States. Evidence is presented for substantial geographic subdivision within *P. brachyphona* (northern and southern clades) and *P. feriarum* (coastal and inland clades). Discordance between morphology/behavior and molecular data in several individuals suggests occasional hybridization between sympatric species. These results require major revision of range limits for several taxa, in particular, *P. maculata*, *P. triseriata*, and *P. feriarum*. Hypothesis tests using parametric bootstrapping strongly reject previously proposed scenarios for speciation in the group.

The tests also support recognition of the geographically restricted taxon *P. kalmi* as a distinct species. Results of this study provide both a firm phylogenetic basis for future studies of speciation in the trilling *Pseudacris* and a taxonomic framework for conservation efforts.

*Significant portions of this chapter are currently in press as Lemmon, Lemmon, Lee-Yaw, Collins, and Cannatella 2007. Molecular Phylogenetics and Evolution.

2.1 INTRODUCTION

Deciphering the phylogenetic relationships among taxa and determining how the patterns observed relate to known historical events are important to our understanding of speciation. Although the biogeographic origins and higher-order relationships among hylid tree frogs of North America have been elucidated (Middle American Clade: Smith et al. 2005), additional work is required to understand evolutionary relationships within each of the three main North American genera (*Hyla*, *Acris*, and *Pseudacris*). The trilling chorus frogs form a clade within *Pseudacris* (Moriarty and Cannatella 2004). The eight putative species (*P. brachyphona*, *P. brimleyi*, *P. clarkii*, *P. feriarum*, *P. kalmi*, *P. maculata*, *P. nigrita*, and *P. triseriata*) generally have parapatric distributions across the continent from northern Mexico to northern Canada and from the East Coast to the Rocky Mountains (Conant and Collins, 1998; Moriarty and Cannatella, 2004). The taxonomic

status of half of these species is supported by morphological and behavioral data. The other four taxa (*P. feriarum*, *P. kalmi*, *P. maculata*, *P. triseriata*) were elevated from subspecies to species primarily on the basis of acoustic data, and there is ambiguity with respect to geographic boundaries based on these data (Platz, 1989; Platz and Forester, 1988). To gain insight into the process of speciation in this group, the phylogenetic relationships and range boundaries of these taxa must first be resolved using genetic data.

Although some trilling chorus-frog species are distinguishable based on color pattern or advertisement call structure (Brandt and Walker, 1933; Neill, 1949; Smith, 1934; Walker, 1932), a number of putative lineages are more cryptic. Smith and Smith (1952) and Smith (1956) defined the distributions of several trilling *Pseudacris* taxa based on geographic patterns of tibia length to body length ratios. By plotting population means of ratios on a map, they identified morphometric clines, which were then used to define the boundaries between taxa. These authors found a general trend of relatively shorter leg lengths in populations from the northwestern U.S. and Canada and relatively longer legs in populations in the southeastern U.S. These morphological differences translate into behavioral differences as well: short-legged frogs tend to walk rather than hop (Smith and Smith, 1952). Taxonomic designations based on these morphological studies have been generally accepted, (Fig. 2.1; Conant and Collins, 1998) but recent genetic work cast doubt on the accuracy of these designations (Moriarty and Cannatella 2004). Molecular data point to a need for wider sampling to identify cryptic lineages and to delineate geographic distributions of species.

The trilling chorus frogs have been the subject of important studies of speciation. Fouquette (1975) and Gartside (1980) independently studied the contact zone between *Pseudacris feriarum* and *P. nigrita* and found disparate outcomes of secondary contact in different areas of sympatry. In the Apalachicola River drainage (Florida), the two species show reproductive character displacement of their acoustic signals (Fouquette 1975). In the Pearl River drainage (Louisiana/Mississippi), they hybridize freely and lack differentiation of calls (Gartside 1980). Although these apparent differences are extremely interesting from an evolutionary standpoint, what remains unclear is whether these authors examined the same species pair across the contact zone or whether a third species was involved. If a single species pair was studied across the zone, each species, as currently defined, should be monophyletic.

Two scenarios have been put forward to explain the origin of trilling *Pseudacris* in eastern North America. Smith (1957) proposed that following the Wisconsin glaciation (12–110 ka; Denton and Hughes 1981; Gibbard and Kolfschoten 2004), *P. kalmi* diverged from *P. triseriata* after an eastward expansion of the latter species left relict populations in New Jersey and the Delmarva Peninsula (Fig. 2.2). These relict populations became what is now called *P. kalmi*. Therefore, *P. kalmi* and *P. triseriata* are predicted to be sister species. Smith (1957) also proposed that when *P. triseriata* expanded eastward, it bisected the range of the widespread *P. feriarum*, leaving populations of *P. feriarum* in the eastern Great Lakes region that were isolated from the main distribution of the species (Fig. 2.2). Thus, populations in the Great Lakes region are predicted to form a monophyletic group with other *P. feriarum* populations. These

scenarios of migration and divergence were based on morphometric data for chorus frogs throughout North America (Smith, 1956; Smith and Smith, 1952), but these hypotheses have not been tested within a genetic framework.

The goals of this study are twofold. First, we elucidate the phylogenetic relationships and establish geographic ranges of the trilling *Pseudacris*, using 2.4 kb of mitochondrial DNA from a dense taxon sample. Second, we test three scenarios for speciation and the associated taxonomic hypotheses, based on assumptions or predictions of previous authors. Detailed tests of timing of speciation and geographic expansion are presented elsewhere (Lemmon et al., in press). The results of this study advance our understanding of the patterns and processes of speciation in this group. In addition, identification of more precise species distributions facilitates efforts to conserve these frogs.

2.2 MATERIALS AND METHODS

Sampling. We sampled chorus frogs from 253 populations (258 total individuals) across North America (Fig. 2.3; Supplemental Data 2.1). Approximately 30% of the populations were collected by ECM; 10% were borrowed from museum or personal tissue collections; the remaining 60% were collected for this project by herpetologists across the continent (Supplemental Data 2.1; see Acknowledgments). Appropriate scientific permits were obtained for collection of specimens. The sample includes 16 basal *Pseudacris*

populations (outgroups: *Pseudacris regilla*, *P. cadaverina*, *P. crucifer*, *P. ocularis*, *P. ornata*, *P. streckeri*, and *P. illinoensis*) and 237 trilling *Pseudacris* populations (ingroup: *P. brachyphona*, *P. brimleyi*, *P. maculata*, *P. clarkii*, *P. nigrita*, *P.* sp. nov., *P. kalmi*, *P. feriarum*, *P. triseriata*; Moriarty and Cannatella, 2004). Our sample encompasses all currently described chorus-frog species, with the exception of two recently resurrected members of the *P. regilla* species group (Recuero et al., 2006). We focused our sampling efforts on potential contact zones among taxa, particularly along major river drainages and mountain systems, as well as on the edges of species' distributions. Tissues were either frozen in liquid nitrogen or placed in tissue buffer or 95% ethanol and then stored at -80°C. Specimens were deposited into museums listed in Supplemental Data 2.1.

DNA Sequencing and Data Alignment. Following the methods described in Moriarty and Cannatella (2004), we sequenced eight DNA fragments from a ~2.4-kb region spanning the 12S, $tRNA^{Val}$, and 16S mitochondrial rRNA genes. Contiguous sequences were constructed using Sequencher 4.5 (GeneCodes). Sequences were aligned in Clustal X 1.8 (Thompson et al. 1997) and the alignment was manually checked in MacClade (4.08; Maddison and Maddison 2005). Uneven leading and trailing sequence as well as ambiguously aligned regions were identified in MacClade and excluded from further analysis. The 12S, $tRNA^{Val}$, and 16S genes were used as character partitions described below. To maximize the genetic diversity and geographic area sampled, we sequenced 1–2 individuals from many populations rather than several individuals from fewer populations. This sampling minimized the number of redundant haplotypes in the dataset, which were omitted from phylogenetic analyses. All sequences were deposited in

Genbank (Supplemental Data 2.1) and the dataset was deposited in TreeBase (www.treebase.org; SN3302).

Phylogenetic Methods. We used a Bayesian approach to estimate phylogenetic relationships. To determine the appropriate model of evolution for each of the three partitions (12S: GTR+I+G, $tRNA^{Val}$: TRN+I, 16S: GTR+I+G), we employed the Akaike information criterion (Akaike 1974) as implemented in MODELTEST 3.06 (Posada and Crandall 1998). Since the TRN+I model is not available in MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003), we used the more general GTR+I model because overparameterization is less likely to cause bias than underparameterization (Lemmon and Moriarty 2004). We performed six separate partitioned Bayesian analyses (with four heated chains per analysis) using MrBayes with default prior (prset) and proposal (prop) settings. All parameters were unlinked across partitions except branch lengths, which were not unlinked for two reasons. First, we desired branch lengths that represented the average number of substitutions per site across the entire region sequenced. Second, because recombination among mitochondrial partitions is unlikely, all partitions share a common gene tree.

The posterior probability distribution was estimated using the last 75% of the Markov chain samples. Convergence of the Markov chains on the posterior distribution was assessed by comparing bipartition posterior probability estimates across the six runs. We sampled from the chains every 1000 generations until the *maximum* standard deviation of bipartition posterior probability estimates across runs was less than 0.0625.

Running the chains until this level of agreement among the independent runs was reached assured that the runs converged on the posterior distribution and that enough samples were taken to estimate the phylogeny accurately. We also compared distributions for the model parameters across the six runs, which reached stationarity at 3000 samples. A total of 92,682 samples was used to estimate the posterior distribution. A fully resolved tree was obtained by constructing a majority-rule consensus tree from the posterior distribution. Branch lengths were estimated as the average across the 92,682 samples.

Phylogenetic Hypothesis Testing. We tested three previously proposed taxonomic hypotheses concerning the phylogenetic origin of *Pseudacris* lineages. Hypothesis 1 posits that *P. kalmi* populations from New Jersey and the Delmarva Peninsula are relict populations of *P. triseriata* from an eastward expansion of the latter species. Therefore, *P. kalmi* is predicted to be most closely related to *P. triseriata* (Smith, 1957; Fig. 2.2). Hypothesis 2 predicts that chorus frogs in southeastern Ontario and New York are relict *P. feriarum* from a northward expansion of the species (Smith, 1957; Fig. 2.2). Hypothesis 3 states that chorus frogs in Louisiana, Arkansas, and westward are *P. feriarum* (Fouquette, 1975; Gartside, 1980; Smith and Smith, 1952). Following Moriarty and Cannatella (2004), we used a parametric bootstrapping approach to test these hypotheses. The null hypotheses are: 1) *P. kalmi* and *P. triseriata* populations form a monophyletic group, 2) *P. feriarum*, southeastern Ontario, and New York populations form a monophyletic group, and 3) eastern and western *P. feriarum* populations form a monophyletic group. For computational efficiency, we used subsets of the full dataset for each test; each subset included up to 10 geographically dispersed populations of the focal

species, 3 populations of each of the other trilling *Pseudacris* species, and 3 representative outgroups. This arrangement produced datasets of 47, 58, and 51 terminals, respectively (see Supplemental Data 2.1 for populations included).

Designating Species. Geographic populations corresponding to haplotype clades were assigned species names based on the inclusion of the type locality within the range of the haplotype clade. Each major haplotype clade contained no more than one type locality, and therefore species designations were straightforward. Type localities for the trilling chorus frogs are listed by Frost (2006).

2.3 RESULTS

Phylogenetic relationships. A dataset of 2401 characters was used for the phylogenetic analyses, after exclusion of 164 edge or ambiguously aligned sites; 685 sites were variable and 567 were parsimony-informative. Twelve individuals with redundant haplotypes were identified from the 258 sequences (Supplemental Data 2.1). The phylogeny shows strong support for the monophyly of most currently recognized trilling *Pseudacris* species (Fig. 2.4A).

Pseudacris feriarum is the sister taxon of *P. triseriata*. There is geographic separation of *P. feriarum* into a coastal lineage and an inland lineage, separated roughly by the Altamaha River in eastern Georgia (Fig. 2.4E; following the nomenclature of Wright and Wright 1949). The distribution of *P. triseriata* is more restricted than

previously described (Smith 1956; Smith 1957; Smith and Smith 1952; Figs. 1, 3). The New Jersey/Delmarva Peninsula native, *P. kalmi*, is the sister taxon of the *P. feriarum* + *P. triseriata* clade (Fig. 2.4E). *Pseudacris nigrita* is the sister lineage to an undescribed species (*P.* sp. nov.; Fig. 2.4D), previously thought to be a western extension of *P. feriarum*. *Pseudacris nigrita* + *P.* sp. nov. form the sister clade of *P. triseriata* + *P. feriarum* + *P. kalmi* (Fig. 2.4A). The *P. maculata*/*P. clarkii* clade (Fig. 2.4C) is the sister-group of these five species (Fig. 2.4A). Although *P. maculata* and *P. clarkii* are readily distinguishable in sympatry by morphology and behavior, their mitochondrial gene trees are not reciprocally monophyletic, suggesting recent mitochondrial introgression or incomplete lineage sorting. In concordance with Moriarty and Cannatella (2004), *P. brachyphona* and *P.brimleyi* are sister species (Fig. 2.4B); this clade is the sister taxon of the remaining trilling *Pseudacris* (Fig. 2.4A). There is not clear support for reciprocal monophyly of the gene trees of these two species, despite their allopatry and morphological differentiation, suggesting incomplete lineage sorting. Within *P. brachyphona* there is geographic division into a northern lineage and a southern lineage (Fig. 2.4B).

Phylogenetic hypothesis tests. The parametric bootstrapping results do not support previous hypotheses regarding the biogeographic origin of trilling chorus frogs. In all three tests, the null hypothesis was rejected. Test 1 indicates that populations of *Pseudacris kalmi* in New Jersey and the Delmarva Peninsula are not simply relictual *P. triseriata* ($P = 0.028$). In fact, the phylogeny indicates that *P. kalmi* separated from an ancestral lineage of *P. triseriata* and *P. feriarum* prior to the divergence of the latter two

species (Fig. 2.4). Based on this phylogenetic evidence and also previous work on advertisement calls (Platz and Forester, 1988), we advocate continued recognition of *P. kalmi* as a distinct species. Test 2 shows that southeastern Ontario and New York populations are not derivatives of *P. feriarum* ($P < 0.002$). Rather, these populations encompass a contact zone between *P. maculata* and *P. triseriata* (Figs. 3, 4). Test 3 indicates that the distribution of *P. feriarum* does not extend from Pennsylvania to Texas ($P < 0.002$). Instead, populations in Louisana, Arkansas, and westward represent a new species of chorus frog, which is the sister taxon of *P. nigrita* (Figs. 3 and 4). This previously unidentified species is currently being described elsewhere (Lemmon et al., unpub. ms).

Evidence for hybridization. The phylogeny points to several cases where the mitochondrial clade to which an individual belongs does not correspond to its morphological and behavioral identity. In particular, we found evidence for hybridization between: *Pseudacris kalmi*-*P. nigrita* (1 individual), *P. feriarum*-*P. brachyphona* (2), *P. triseriata*-*P. brachyphona* (1), and *P.* sp. nov.-*P. nigrita* (1; Figs. 3 and 4; Supplemental Data 2.1). In each of these cases, the mitochondrial clade to which an individual belongs is listed first and the identity of the individual based on morphological and acoustic data is listed second. The data do not allow us to distinguish between recent hybrids and advanced-generation hybrids. These results support the idea that occasional introgression occurs between trilling *Pseudacris* lineages.

2.4 DISCUSSION

Species diversity within the trilling chorus frogs. Our phylogenetic analysis of mitochondrial lineages and tests of speciation hypotheses support the recognition of at least nine species within the trilling *Pseudacris* clade: *P. brachyphona*, *P. brimleyi*, *P. clarkii*, *P. feriarum*, *P. kalmi*, *P. maculata*, *P. nigrita*, *P.* sp. nov., and *P. triseriata*. These analyses are corroborated by previously published behavioral and morphological data. Revised range distributions of these taxa are shown in Fig. 2.3. We also found evidence for substantial genetic and geographic structure within *P. brachyphona* (northern and southern clades) and *P. feriarum* (coastal and inland clades), but we refrain from decisions about splitting these taxa until morphological, ecological, or behavioral differentiation between lineages of these species has been demonstrated.

An interesting finding is that *Pseudacris maculata* and *P. clarkii* are not genetically differentiated with respect to their mitochondrial DNA. If only the mitochondrial gene trees were considered, *P. clarkii* would be synonymized under *P. maculata*. However, substantial differences in morphology and behavior exist (Smith, 1934; Lemmon et al. unpub. ms), suggesting that the mitochondrial pattern results from hybridization and/or incomplete lineage sorting. Evidence suggests that both processes may be occurring in this species pair. Fieldwork in the contact zone has yielded individuals with intermediate advertisement calls and morphology between the two species, supporting the hypothesis of hybridization (E. M. Lemmon, unpub. data). On the other hand, the fact that allopatric *P. clarkii* populations far from the contact zone (west

and south Texas) do not form a separate clade from *P. maculata*, is more suggestive of incomplete lineage sorting (Figs. 3 and 4). In this scenario, the two species have undergone rapid morphological and behavioral differentiation relative to molecular divergence, resulting in taxa with distinct phenotypes and acoustic signals that are not, however, reciprocally monophyletic. Data from nuclear markers are needed, however, to determine whether hybridization or incomplete lineage sorting contributed more to the patterns of genetic variation observed. Given the degree of morphological and behavioral divergence between the taxa, we maintain *P. maculata* and *P. clarkii* and as separate species, until further data suggest otherwise.

This study provides genetic identification for several marginal populations of uncertain origin including *Pseudacris maculata* from Arizona and New Mexico (formerly *P. triseriata*; Smith, 1952; Platz 1989), disjunct *P. feriarum* from Berkeley, Charleston, and Dorchester Counties, South Carolina (*P. feriarum*; Schwartz, 1957), disjunct *P. nigrita* from eastern Virginia (new state record; Hobson and Moriarty, 2003), and *P. maculata* and *P. triseriata* from southeastern Ontario (formerly *P. triseriata* only Bleakney, 1959; Cook, 1964; Figs. 3 and 4). In addition, this study provides strong support for recognition of the geographically restricted taxon, *P. kalmi* (formerly *P. feriarum kalmi*, Hedges, 1986) as a distinct species. Furthermore, we have found evidence for a new cryptic species of chorus frog, previously undetected within *P. feriarum* (Smith, 1952). In fact, this new species is the sister species of *P. nigrita*, with which it forms a narrow hybrid zone in the Pearl River drainage along the boundary between southern Louisiana and Mississippi (Gartside, 1980; Figs. 3 and 4D).

Earlier studies of the contact zone between *Pseudacris nigrita* and presumed "*P. feriarum"* in the Apalachicola River drainage of Florida (Fouquette, 1975) and the Pearl River drainage of Louisiana and Mississippi (Gartside, 1980) found disparate outcomes of secondary contact between these taxa. Fouquette (1975) observed a strong pattern of reproductive character displacement between taxa and found no evidence for hybridization, although an allozyme study of Gartside (1980) described a high frequency of hybridization between taxa (center of zone has 60% hybrids). We purposely sampled the majority of the *P. feriarum* populations examined in the Fouquette (1975) and Gartside (1980) studies to ascertain their taxonomic identity and found that all Fouquette (1975) localities are true *P. feriarum* (inland clade) and all Gartside (1980) localities are a third, cryptic species, *P.* sp. nov. (Figs. 3 and 4). This clarifies why there is a higher incidence of hybridization along the Pearl River (sister taxa) compared to the apparently low incidence along the Apalachicola River (non-sister taxa). Our results indicate that reproductive character displacement occurs between non-sister species in this group (*P. nigrita and P. feriarum*; Figs. 3 and 4; Fouquette, 1975).

Results of the parametric bootstrapping tests have important implications for both conservation and speciation in chorus frogs. The tests do not support the biogeographic scenarios proposed by Smith (1957) for speciation in the trilling *Pseudacris* (Figs. 2 and 3). Intriguingly, however, one of the morphological clines identified by Smith and Smith (1952) corresponds very closely to boundaries between mitochondrial lineages. These authors found a steep cline in relative leg length that runs perpendicular to a line stretching from southern Indiana and Illinois (Ohio River drainage), across the boundary

between Missouri and Arkansas, and into eastern Oklahoma (Fig. 2.1). They interpreted this line as the boundary between *P. feriarum* and *P. triseriata*. Our data show that, in fact, four lineages come into contact along this line: *P. feriarum* and *P. triseriata* in the east and *P. maculata*/*clarkii* and *P.* sp. nov. in the west (Fig. 2.3). Although Smith and Smith (1952) did not find east-west morphological differentiation at species boundaries, they were able to identify the border between the two north-south species pairs. The congruence between molecular and morphological data provides further support for delineation of these species boundaries.

Evidence for hybridization among species. An interesting pattern that emerges is that most trilling *Pseudacris* lineages hybridize with nearby relatives. Prior to this study, natural hybridization was known only between *P. nigrita* and *P.* sp. nov. (Gartside, 1980) and between *P. clarkii* and *P.* sp. nov. (Michaud, 1964), although laboratory experiments had demonstrated viability of several other hybrid crosses (Mecham, 1965). We show evidence for sporadic mitochondrial introgression in nature between three additional species pairs: *P. kalmi*-*P. nigrita*, *P. feriarum*-*P. brachyphona*, and *P. triseriata*-*P. brachyphona* as well as further evidence for hybridization between *P.* sp. nov-*P. nigrita*. These data suggest that despite large differences in reproductive behaviors (measured by acoustic signals, Lemmon et al., unpub. ms), frogs occasionally fail to avoid heterospecific mates. The potential for hybridization can lead to reinforcement (Howard, 1993) and, in some cases, result in differentiation of reproductive signals in sympatry (Fouquette, 1975). This pattern of hybridization underlines the importance of using multiple lines of evidence to delimit species (e.g., morphology, genetics, behavior). It

also illustrates the usefulness of mitochondrial genes in identifying areas of genetic admixture. Future studies should incorporate nuclear markers to establish the utility of mitochondrial DNA in defining species boundaries.

Implications for conservation of *Pseudacris*. Declining amphibian species have been reported from many regions of North America where *Pseudacris* are found (Gray et al., 2005; Reeder et al., 2005; Rorabaugh, 2005). Whereas several other frog taxa (in particular, *Rana* and *Acris*) have experienced declines in parts of the United States, *Pseudacris* populations appear less affected or stable in some areas (Corn et al., 1989; Fisher and Shaffer, 1996). This disparity may be due, in part, to the different natural histories of these taxa. Although *Acris* and *Rana* spend much of their life cycle near their natal ponds, *Pseudacris* disappear from breeding ponds after metamorphosis, dispersing to nearby fields and woods, and returning only for the next year's brief breeding season (Kramer, 1973; Kramer, 1974). Because a number of emerging amphibian diseases are transmitted via water (Daszak et al., 1999; Jancovich et al., 2001; Lips et al., 2006), *Pseudacris* may have an advantage over more aquatic frogs by avoiding bodies of water for the majority of their life cycle.

There are some notable exceptions, however, to the overall pattern of stability in chorus-frog populations. Recent field surveys have suggested that several species are declining in parts of the northeastern U.S. and southeastern Canada (Gibbs et al., 2005; Picard and Desroches, 2004; Pollio and Kilpatrick, 2002; Sias, 2006; Weeber and Vallianatos, 2000; J. Andrews and M. Ferguson, unpub. data; C. Pollio, unpub. data). In

addition, several species have been listed by state wildlife agencies as species of conservation concern (*Pseudacris feriarum*: Pennsylvania, West Virginia; *P. triseriata*: Pennsylvania; *P. brachyphona*: Pennsylvania; *P. maculata*: Michigan), state threatened (*P. brachyphona*: Maryland), or state endangered (*P. kalmi*: Pennsylvania). In southeastern Ontario and New York, surveys have found that eastern populations have declined but the western populations appear stable (Gibbs et al., 2005; Picard and Desroches, 2004; F. Schueler, unpub. data). Our data indicate that declining eastern populations are *P. maculata* whereas stable western populations are *P. triseriata* (Fig. 2.3). The apparent declines have been attributed to several factors, including habitat loss, agricultural runoff, and industrial pollution (Gibbs et al., 2005; Pollio and Kilpatrick, 2002; Sias, 2006). Clearly, more focused research is needed to track the causes of these declines. Our study contributes to conservation efforts by defining the taxonomic status and range limits of these taxa. Currently, the trilling *Pseudacris* species that presents the most urgent conservation challenge is the New Jersey Chorus Frog, *P. kalmi*. In this study we have demonstrated that *P. kalmi* is a distinct species. Due to its restricted range, which is located in one of the most densely populated areas of the U.S., *P. kalmi* faces extinction particularly through habitat loss. Conservation measures must be undertaken immediately to preserve the remaining populations of this species.

Supplemental Data 2.1. List of *Pseudacris* specimens included in this study. The list uses updated taxonomy for each population. Field number, museum voucher number, and Genbank accession numbers are listed. Specimens with the same superscript letter following the field number have identical haplotypes. Putative hybrids are denoted by the following symbols after the field number: α (*P. brachyphona* with *P. feriarum* mtDNA), β (*P. brachyphona* with *P. triseriata* mtDNA), ε (*P. nigrita* with *P.* sp. nov. mtDNA), and ϕ (*P. nigrita* with *P. kalmi* mtDNA). A "n/a" under the museum number header means no voucher specimen is available for that specimen. Vouchers that have not been cataloged are listed as such for respective collections. Superscript numbers following the museum numbers refer to footnotes at the end of the list. An asterisk next to a Genbank number indicates previously published sequences from Moriarty and Cannatella (2004). The test column denotes which taxa were used in each of the three parametric bootstrapping tests (e.g. ECM0041 was included in all three tests). State or province of origin and county, township, or region information is listed for each specimen in addition to GPS coordinates. Museum collection codes are as follows: Arkansas State Museum Herpetology Collection, Jonesboro (ASUMZ), Bell Museum of Natural History, Minneapolis (JFBM), Canadian Museum of Nature, Ottawa (CMN), Cincinnati Museum Center, Museum of Natural History and Science (CMC), Illinois Natural History Survey (INHS), Museum of Vertebrate Zoology, Berkeley (MVZ), North Carolina State Museum of Natural History, Raleigh (NCSM), Royal Ontario Museum, Toronto (ROM), Smithsonian National Museum of Natural History, Washington, D.C. (USNM), Sternberg Museum of Natural History, Fort Hays State University (MHP), Texas Natural History Collection, University of Texas, Austin (TNHC), University of Alabama Herpetology Collection, Tuscaloosa (UAHC), and University of Kansas Museum of Natural History, Lawrence (KU).

Species	Field No.	Museum No.	Genbank No.	Test	State/Prov.	County/Twshp.	Latitude	Longitude
P. brachyphona	ECM0040	TNHC62303	**AY291095***		AL	Tallapoosa	33.0064	-85.7603
P. brachyphona	ECM0041[A]	TNHC62304	**EF472011**	1 2 3	AL	Tallapoosa	33.0064	-85.7603
P. brachyphona	ECM0111	TNHC62305	**EF472014**		AL	Elmore	32.5175	-86.0071
P. brachyphona	ECM0198	TNHC62315	**EF472022**		KY	Madison	37.6503	-84.2417
P. brachyphona	ECM0452	TNHC63121	**EF472012**		AL	Lawrence	34.3344	-87.3503
P. brachyphona	ECM0974α	TNHC63443	**EF472190**		MS	Itawamba	34.1679	-88.3754
P. brachyphona	ECM1131	n/a	**EF472013**	1 2 3	GA	Walker	34.7048	-85.2819
P. brachyphona	ECM1897[A]	NCSM71330	**EF472017**		NC	Cherokee	35.0414	-84.0520
P. brachyphona	ECM2070	n/a	**EF472028**		OH	Washington	39.5478	-81.2141
P. brachyphona	JA-06-01	UAHC15645	**EF472016**		AL	Hale	32.9222	-87.4403
P. brachyphona	JA-06-10	UAHC15646	**EF472015**		AL	Cleburne	33.5136	-85.8284
P. brachyphona	JTC2457	TNHC62402	**EF472019**		KY	Laurel	37.1333	-84.1333

P. brachyphona	JTC2609	TNHC63535	**EF472020**	1 2 3	OH	Hocking	39.4161	-82.6018
P. brachyphona	JTC2616	TNHC63389	**EF472021**		KY	Taylor	37.2469	-85.3197
P. brachyphona	JTC2619	TNHC63387	**EF472023**		KY	Powell	37.8169	-83.6811
P. brachyphona	JTC2669	TNHC66044	**EF472018**		WV	Harrison	39.2715	-80.5192
P. brachyphona	JTC2705	TNHC66046	**EF472026**		WV	Wayne	38.1679	-82.3779
P. brachyphona	JTC2834	TNHC66047	**EF472027**		WV	Wetzel	39.5597	-80.5567
P. brachyphona	JTC3084	CMC10360	**EF472031**		OH	Adams	38.7156	-83.3233
P. brachyphona	JTC3086β	MHP12900	**EF472183**		KY	Bullitt	37.8636	-85.6356
P. brachyphona	JTC3092	MHP12896	**EF472030**		TN	Sullivan	36.4866	-82.0717
P. brachyphona	JTC3104	MHP12898	**EF472029**		KY	Harlan	36.9279	-83.2154
P. brachyphona	R.Highton71747α	R. Highton uncat.[1]	**AY291096***		KY	Lincoln	37.4358	-84.6878
P. brachyphona	R.Highton97-5	n/a	**EF472024**		WV	Raleigh	37.7489	-80.9236
P. brachyphona	R.Highton97-7	n/a	**EF472025**		VA	Bland	37.0372	-81.1094
P. brimleyi	ECM0079	TNHC62337	**AY291094***	1 2 3	NC	Pitt	35.7006	-77.4094
P. brimleyi	ECM0460	TNHC63571	**EF472033**		NC	Sampson	35.0992	-78.4772
P. brimleyi	ECM0469	TNHC63573	**EF472036**		NC	Craven	35.1892	-77.0814
P. brimleyi	ECM0612	TNHC63667	**EF472032**		VA	Prince George	37.1229	-77.1094
P. brimleyi	ECM1077	TNHC63669	**EF472035**	1 2 3	VA	Suffolk City	36.6930	-76.6953
P. brimleyi	ECM1100	TNHC63670	**EF472034**		VA	Isle of Wight	36.8666	-76.6194
P. brimleyi	R.Highton67234	R. Highton uncat.	**EF472037**	1 2 3	SC	Orangeburg	33.3227	-80.4137
P. brimleyi	R.Highton68852	R. Highton uncat.	**EF472038**		SC	Hampton	32.5594	-81.2844
P. cadaverina	ECM0150	TNHC62247	**EF472006**	2	CA	San Bernardino	34.1132	-117.1422
P. clarkii	ECM0210	TNHC63497	**EF472105**		KS	Comanche	37.1247	-99.3258
P. clarkii	ECM1133	TNHC63548	**EF472102**	1 2 3	OK	Garfield	36.3956	-97.8784
P. clarkii	ECM1143	TNHC63159	**EF472103**	1 2 3	TX	Swisher	34.6464	-101.5722
P. clarkii	ECM2467	TNHC65044	**EF472107**		KS	Chautauqua	37.0401	-96.1815
P. clarkii	ECM2478	TNHC65763	**EF472106**		TX	Caldwell	30.0205	-97.6946
P. clarkii	JTC2454[E]	TNHC63533	**EF472109**		KS	Barber	37.0139	-98.6492
P. clarkii	JTC2455 [E]	TNHC63534	**EF472108**		KS	Barber	37.0139	-98.6492
P. clarkii	JTC2828	TNHC63138	**EF472104**	1 2 3	TX	Cameron	26.1809	-97.5198
P. clarkii	Q-1	KU289035	**AY291093***		KS	Chautauqua	37.0044	-96.2764

P. crucifer	ECM0039	TNHC62210	**AY291099***		AL	Barbour	32.0369	-85.0889
P. crucifer	ECM0083	TNHC62216	**AY291100***	1 2 3	SC	Barnwell	33.3177	-81.4840
P. crucifer	ECM0166	TNHC62221	**EF472007**		MD	Kent	39.3122	-75.8485
P. crucifer	Y-1	TNHC62369[2]	**AY291103***		FL	Lake	29.0833	-81.5833
P. feriarum	ECM0122	TNHC62268	**EF472173**		AL	Elmore	32.5175	-86.0071
P. feriarum	ECM0126	TNHC62380	**EF472189**	2 3	MO	Dunklin	36.2435	-89.9622
P. feriarum	ECM0129	TNHC62271	**EF472169**		TN	Weakley	36.2579	-88.6676
P. feriarum	ECM0131	TNHC62273	**EF472170**		TN	Obion	36.2579	-89.2597
P. feriarum	ECM0135	TNHC62276	**EF472176**		TN	Obion	36.4529	-89.3035
P. feriarum	ECM0180	TNHC62280	**EF472202**		MD	Prince George	38.6909	-77.0137
P. feriarum	ECM0181	TNHC62385	**EF472206**		NC	Wake	35.6238	-78.8999
P. feriarum	ECM0189	TNHC62287	**EF472205**		NC	Chatham	35.8530	-79.1271
P. feriarum	ECM0232	TNHC63303	**EF472167**	1 2 3	FL	Liberty	30.1626	-85.0666
P. feriarum	ECM0298	TNHC63326	**EF472196**		GA	Banks	34.3322	-83.5654
P. feriarum	ECM0368	TNHC63322	**EF472175**		FL	Calhoun	30.2847	-85.1073
P. feriarum	ECM0382	TNHC63323	**EF472177**		FL	Gasden	30.6591	-84.8323
P. feriarum	ECM0383	TNHC63358	**EF472172**		GA	Decatur	30.9081	-84.5979
P. feriarum	ECM0384	TNHC63359	**EF472178**		GA	Seminole	31.0223	-84.8292
P. feriarum	ECM0386	TNHC63122	**EF472174**		AL	Henry	31.6083	-85.0710
P. feriarum	ECM0387	TNHC63123	**EF472168**		AL	Macon	32.5290	-85.6016
P. feriarum	ECM0399	TNHC63685	**EF472163**		TN	Hamilton	35.1915	-85.2459
P. feriarum	ECM0400	TNHC63133	**EF472161**		AL	Macon	32.4703	-85.6908
P. feriarum	ECM0402	TNHC63333	**EF472179**		GA	Baker	31.3835	-84.5430
P. feriarum	ECM0441	TNHC63537	**EF472197**	2 3	SC	Dorchester	32.9552	-80.2613
P. feriarum	ECM0446	TNHC63361	**EF472171**	1 2 3	GA	Floyd	34.4076	-85.2216
P. feriarum	ECM0448	TNHC63362	**EF472180**		GA	Heard	33.2765	-85.1211
P. feriarum	ECM0453	TNHC63562	**EF472208**		NC	Sampson	35.1418	-78.5562
P. feriarum	ECM0455	TNHC63564	**EF472201**		NC	Johnson	35.4392	-78.3706
P. feriarum	ECM0464	TNHC63567	**EF472212**	1 2 3	NC	Davie	35.8982	-80.5764
P. feriarum	ECM0481	TNHC63627	**EF472200**		VA	York	37.1779	-76.5007
P. feriarum	ECM0486[I]	TNHC63642	**EF472209**		VA	Mathews	37.4451	-76.3424

P. feriarum	ECM0601[I]	TNHC63643	**EF472204**		VA	Mathews	37.4451	-76.3424
P. feriarum	ECM0602	TNHC63364	**EF472203**	2 3	GA	Appling	31.9522	-82.3848
P. feriarum	ECM0630	TNHC63520	**EF472217**	1 2 3	MD	Harford	39.5078	-76.2195
P. feriarum	ECM0632	TNHC63522	**EF472198**		MD	Baltimore	39.4964	-76.7617
P. feriarum	ECM0665	TNHC63644	**EF472207**		VA	Prince George	37.1229	-77.1094
P. feriarum	ECM0960	TNHC63465	**EF472162**		AL	Conecuh	31.3546	-87.0267
P. feriarum	ECM0961[H]	TNHC63466	**EF472188**		AL	Choctaw	31.7484	-88.1277
P. feriarum	ECM0969[H]	TNHC63467	**EF472191**		AL	Pickens	33.0979	-88.2033
P. feriarum	ECM0970	TNHC63439	**EF472192**	1 2 3	MS	Oktibbeha	33.4282	-88.8768
P. feriarum	ECM0971	TNHC63440	**EF472193**		MS	Lafayette	34.4114	-89.3729
P. feriarum	ECM0992	TNHC63468	**EF472186**	2 3	AL	Cullman	34.0928	-86.8825
P. feriarum	ECM1011	TNHC63645	**EF472214**		VA	Prince Edward	37.0973	-78.4770
P. feriarum	ECM1076	TNHC63652	**EF472216**		VA	Southampton	36.7804	-77.2316
P. feriarum	ECM1125	n/a	**EF472218**		GA	Walton	33.7948	-83.7132
P. feriarum	ECM1130	n/a	**EF472187**		GA	Houston	32.4960	-83.6077
P. feriarum	ECM1435	TNHC65775	**EF472221**		SC	Greenwood	34.1505	-82.1591
P. feriarum	ECM1454	TNHC65747	**EF472220**		GA	Greene	33.5745	-83.2012
P. feriarum	F-1	KU289227	**AY291084***		KY	Calloway	36.6333	-88.2667
P. feriarum	INHS1196	INHS18810	**EF472181**		IL	Pulaski	37.2769	-89.1833
P. feriarum	JTC2578	TNHC63355	**EF472199**		GA	Oglethorpe	33.8628	-83.4089
P. feriarum	JTC2593	TNHC63686	**EF472165**	1 2 3	TN	Blount	35.7564	-83.9706
P. feriarum	JTC2615	TNHC63393	**EF472182**		KY	McCracken	37.1597	-88.7972
P. feriarum	JTC2730	TNHC63524	**EF472210**		MD	Anne Arundel	38.8283	-76.5389
P. feriarum	JTC2740	TNHC63134	**EF472194**		AL	Morgan	34.5448	-86.7639
P. feriarum	JTC2762	TNHC63689	**EF472195**		TN	Chester	35.4392	-88.6414
P. feriarum	JTC2857	TNHC66049	**EF472211**		WV	Berkeley	39.4927	-78.2772
P. feriarum	MHP10700	MHP10700	**EF472219**		NC	Lincoln	35.4135	-80.9719
P. feriarum	R.Highton50960	n/a	**EF472166**		TN	Blount	35.6911	-83.7989
P. feriarum	R.Highton61551	USNM uncat.	**EF472215**		SC	Kershaw	34.4782	-80.8017
P. feriarum	R.Highton61673	USNM uncat.	**EF472184**		TN	Franklin	35.2031	-85.9211
P. feriarum	R.Highton62076	USNM uncat.	**EF472213**		PA	Fulton	40.0708	-77.8839

P. feriarum	R.Highton71758	R. Highton uncat.	**EF472185**		KY	Lincoln	37.4019	-84.8092
P. feriarum	R.Highton88-43	n/a	**EF472164**		TN	Anderson	36.1408	-84.1047
P. illinoensis	ECM0001	TNHC62351	**AY291109***		AR	Clay	36.3308	-90.1090
P. illinoensis	ECM0090	TNHC62346	**AY291110***		MO	Scott	37.0667	-89.5667
P. illinoensis	INHS2003.3	n/a	**EF472008**		IL	Cass	40.0175	-90.4242
P. illinoensis	INHS2003.9	n/a	**EF472010**		IL	Madison	38.7969	-90.0389
P. kalmi	ECM0162	TNHC62354	**EF472224**	1 2 3	MD	Kent	39.3122	-75.8485
P. kalmi	ECM1064	TNHC63671	**EF472225**	1 2 3	VA	Accomack	37.7501	-75.6663
P. kalmi	ECM1067	TNHC63674	**EF472223**	1	VA	Northampton	37.4749	-75.8583
P. kalmi	ECM1080	TNHC63135	**EF472230**	1	DE	Sussex	38.7459	-75.3809
P. kalmi	ECM1115[J]	TNHC63544	**EF472226**	1 2 3	NJ	Atlantic	39.4765	-74.7106
P. kalmi	JTC2738	TNHC63403	**EF472227**	1	MD	Wicomico	38.3215	-75.4499
P. kalmi	JTC2836	TNHC63546	**EF472228**	1	NJ	Burlington	39.9593	-74.5093
P. kalmi	NJ-1	KU289235	**AY291087***	1	MD	Kent	39.3122	-75.8485
P. kalmi	R.Highton62067[J]	USNM uncat.	**EF472229**	1	NJ	Salem	39.6834	-75.4905
P. kalmi	R.Highton62083	USNM uncat.	**EF472222**	1	DE	New Castle	39.7153	-75.6259
P. maculata	03BEJ007	TNHC63622	**EF472090**		MN	Cook	47.9105	-90.0075
P. maculata	03EKH001[D]	TNHC63621	**EF472101**		MN	St. Louis	47.6988	-93.0484
P. maculata	A-1	KU224560	**AY291090***		KS	Douglas	39.0068	-95.2233
P. maculata	D-3	KU224558	**AY291092***		KS	Cheyenne	39.7722	-101.7994
P. maculata	DCC3851	n/a	**EF472080**		WI	Wood	44.4500	-90.0500
P. maculata	ECM0105	TNHC62324	**AY291081***		Ontario	Frontenac	44.2333	-76.5000
P. maculata	ECM0204	TNHC62296	**EF472123**		KS	Ellis	38.8352	-99.3363
P. maculata	ECM0209	TNHC62389	**EF472078**		MN	Itasca	47.5000	-93.0000
P. maculata	ECM0604	TNHC63365	**EF472088**		IA	Marion	41.3875	-92.9526
P. maculata	ECM0634	TNHC63370	**EF472092**		IA	Warren	41.3375	-93.5570
P. maculata	ECM0644	TNHC65773	**EF472091**	2	CO	Jackson	40.8348	-106.5705
P. maculata	ECM0645	n/a	**EF472089**		MN	Ramsey	45.0051	-93.1011
P. maculata	ECM0652	TNHC65814	**EF472093**		WI	St. Croix	44.8614	-92.6236
P. maculata	ECM1140[B]	TNHC65824	**EF472100**		WI	Bayfield	46.3941	-91.2938
P. maculata	ECM1156	TNHC63139	**EF472094**		ND	Ward	48.1817	-101.2924

P. maculata	ECM2099	n/a	**EF472132**		Alberta	Athabasca	54.6155	-113.3466
P. maculata	I-1	KU224624	**AY291080***		CO	Gunnison	38.8221	-106.5744
P. maculata	I-2	KU224625	**AY291083***		CO	Archuleta	37.2898	-106.9754
P. maculata	INDU214	n/a	**EF472121**		IN	Porter	41.6100	-87.2353
P. maculata	INHS1251	INHS18890	**EF472127**		IL	Cass	39.9242	-90.3904
P. maculata	INHS1267	INHS13035	**EF472115**		IL	Piatt	40.0114	-88.7261
P. maculata	INHS1372[F]	INHS13057	**EF472124**		IL	Madison	38.8294	-90.0628
P. maculata	INHS1376	INHS13062	**EF472122**		IL	Jersey	39.0778	-90.5555
P. maculata	INHS203	INHS16769	**EF472113**		IL	Mercer	41.1031	-90.9339
P. maculata	JPB13421	CMN32633	**EF472084**	2	Manitoba	Churchill	58.7667	-94.1667
P. maculata	JPB22607[B]	ROM uncat.	**EF472081**	2	Ontario	Fraleigh	48.4500	-89.2000
P. maculata	JRM4868	TNHC62405	**EF472083**		UT	Cache	42.0778	-111.7222
P. maculata	JTC2588	TNHC63697	**EF472117**		MO	Cole	38.5767	-92.1733
P. maculata	JTC2596	TNHC63699	**EF472126**		MO	Adair	40.2540	-92.5821
P. maculata	JTC2600[F]	TNHC63702	**EF472111**	1 2 3	MO	Boone	39.0333	-92.3333
P. maculata	JTC2601	TNHC63425	**EF472118**		IA	Boone	41.9900	-93.8841
P. maculata	JTC2613	TNHC63423	**EF472099**		IA	Allamakee	43.3621	-91.2264
P. maculata	JTC2630	TNHC63704	**EF472116**	2	Ontario	Frontenac	44.5500	-76.3333
P. maculata	JTC2645	TNHC63612	**EF472112**		MN	Fillmore	43.7208	-91.9767
P. maculata	JTC2650	TNHC63504	**EF472134**		KS	Wilson	37.5667	-95.7333
P. maculata	JTC2674	TNHC63431	**EF472120**		IA	Louisa	41.0997	-91.0444
P. maculata	JTC2687	TNHC63717	**EF472129**	2	Ontario	Wellington	43.9822	-80.4039
P. maculata	JTC2698	TNHC63428	**EF472125**		IA	Butler	42.6382	-92.6233
P. maculata	JTC2700	TNHC65819	**EF472079**	1 2 3	SD	Lawrence	44.4060	-103.9573
P. maculata	JTC2706	TNHC63146	**EF472086**		CO	Weld	40.4233	-104.7086
P. maculata	JTC2708[C]	TNHC63430	**EF472098**		IA	Howard	43.2130	-92.4899
P. maculata	JTC2760	TNHC63552	**EF472095**		OK	Washington	36.8836	-95.9259
P. maculata	JTC2805	TNHC63752	**EF472128**		IL	Effingham	39.1235	-88.6194
P. maculata	JTC2832	TNHC63554	**EF472114**		OK	Cherokee	36.0895	-94.8505
P. maculata	JTC2843	TNHC63733	**EF472097**		IL	McDonough	40.3325	-90.6046
P. maculata	JTC2862	TNHC63543	**EF472119**		NE	Douglas	41.2586	-95.9378

P. maculata	K-2[F]	n/a	**AY291088***		KS	Kingman	37.6458	-98.1133
P. maculata	MHP 8159	MHP8159	**EF472130**		KS	Cherokee	37.1692	-94.8441
P. maculata	MHP10265	MHP10265	**EF472133**		MO	Dade	37.3897	-93.9138
P. maculata	MHP10268	MHP10268	**EF472131**		MO	Newton	36.9416	-94.1717
P. maculata	MHP10467	MHP10467	**EF472135**		MO	Christian	37.0265	-93.4604
P. maculata	N-5	KU224630	**AY291089***		NM	McKinley	36.0023	-108.8162
P. maculata	R-1	KU290342	**AY291082***	2	Ontario	Lac Seul	50.6333	-93.2167
P. maculata	UMN14283[C]	JFBM14283	**EF472082**		MN	Wright	45.3194	-93.9417
P. maculata	UMN14285	JFBM14285	**EF472110**		MN	Rock	43.7917	-96.2667
P. maculata	UMN14316	JFBM14316	**EF472096**		MN	Lac qui Parle	45.0417	-95.9167
P. maculata	UMN14327	JFBM14327	**EF472087**		ND	Pembina	48.9861	-97.5544
P. maculata	UMN14336[D]	JFBM14336	**EF472085**	1 2 3	MN	Otter Tail	46.4583	-95.7056
P. nigrita	ECM0024	TNHC62364	**AY291079***	3	FL	Brevard	28.2006	-80.8678
P. nigrita	ECM0036	TNHC62201	**AY291078***	3	AL	Barbour	32.0369	-85.0889
P. nigrita	ECM0087	TNHC62208	**AY291076***	1 2 3	SC	Barnwell	33.3177	-81.4840
P. nigrita	ECM0215	TNHC63210	**EF472039**		FL	Calhoun	30.4477	-85.0922
P. nigrita	ECM0242	TNHC63187	**EF472045**	1 2 3	FL	Liberty	30.1626	-85.0666
P. nigrita	ECM0261ε	TNHC63585	**EF472052**		MS	Harrison	30.5010	-88.9084
P. nigrita	ECM0290	TNHC63593	**EF472040**	3	MS	Harrison	30.5010	-88.9084
P. nigrita	ECM0359	TNHC63191	**EF472050**		FL	Liberty	30.1437	-84.9766
P. nigrita	ECM0371	TNHC63200	**EF472049**		FL	Franklin	29.7035	-85.1901
P. nigrita	ECM0372	TNHC63201	**EF472042**	3	FL	Jefferson	30.1981	-84.0500
P. nigrita	ECM0422	TNHC63345	**EF472043**		GA	Baker	31.2380	-84.5017
P. nigrita	ECM0442	TNHC63538	**EF472041**	3	SC	Dorchester	33.0956	-80.3156
P. nigrita	ECM0482ϕ	TNHC62399	**EF472231**		VA	York	37.1779	-76.5007
P. nigrita	ECM0603	TNHC63354	**EF472046**		GA	Liberty	31.8470	-81.5960
P. nigrita	ECM0609	TNHC63658	**EF472044**	1 2 3	VA	Prince George	37.1229	-77.1094
P. nigrita	ECM0666	TNHC63662	**EF472048**		VA	Sussex	36.8921	-77.0628
P. nigrita	ECM1097	TNHC63664	**EF472047**		VA	Surrey	36.9277	-77.0406
P. nigrita	ECM1801	TNHC65785	**EF472051**	3	GA	McIntosh	31.5343	-81.5376
P. nigrita	FC11452	MVZ145452[3]	**AY291077***	3	NC	Scotland	34.7739	-79.4631

P. ocularis	ECM0045	TNHC62234	**AY291097***		SC	Barnwell	33.1606	-81.6908
P. ocularis	ECM0095	TNHC62241	**AY291098***		FL	Gulf	29.6801	-85.3287
P. ornata	ECM0033	TNHC62178	**AY291106***		AL	Barbour	32.0369	-85.0889
P. ornata	ECM0055	TNHC62183	**AY291105***	1 2 3	SC	Aiken	33.2167	-81.7500
P. regilla	ECM0147	TNHC62195	**EF472005**	1 2 3	CA	San Bernardino	34.1132	-117.1422
P. sp. nov.	ASUMZ27608	ASUMZ27608	**EF472058**	3	AR	Conway	35.1508	-92.7439
P. sp. nov.	ASUMZ27611	ASUMZ27611	**EF472057**		AR	Yell	35.0003	-93.4167
P. sp. nov..	ASUMZ27612	ASUMZ27612	**EF472056**		AR	Sebastian	35.3858	-94.3983
P. sp. nov.	ECM0011	TNHC62255	**AY291086***	1 2 3	AR	Craighead	35.8546	-90.6626
P. sp. nov.	ECM0029	TNHC62265	**AY291085***	1 2 3	LA	EastBatonRouge	30.6889	-90.8894
P. sp. nov.	ECM0124	TNHC62269	**EF472066**		LA	Washington	30.6787	-89.9480
P. sp. nov.	ECM0137	TNHC62277	**EF472064**		LA	Evangeline	30.7801	-92.2819
P. sp. nov.	ECM0139	TNHC62384	**EF472060**	3	LA	Beauregard	30.7821	-93.0143
P. sp. nov.	ECM0258	TNHC63598	**EF472054**	3	MS	Simpson	31.9682	-90.1125
P. sp. nov.	ECM0259	TNHC63599	**EF472069**		MS	Simpson	31.9274	-90.0544
P. sp. nov.	ECM0260	TNHC63600	**EF472053**		MS	Marion	31.2358	-89.8228
P. sp. nov.	ECM0264	TNHC63480	**EF472068**		LA	St.Tammany	30.5655	-89.8715
P. sp. nov.	ECM0268	TNHC63483	**EF472059**		LA	St.Tammany	30.3840	-89.7554
P. sp. nov.	ECM0270	TNHC63380	**EF472055**		AR	Perry	34.8916	-92.8044
P. sp. nov.	ECM0332	TNHC63609	**EF472067**		MS	Hancock	30.4399	-89.6576
P. sp. nov.	ECM1155	TNHC63496	**EF472061**	3	LA	Red River	32.1649	-93.4799
P. sp. nov.	ECM2293	TNHC65744	**EF472075**		TX	Jasper	30.2577	-94.2141
P. sp. nov.	ECM2294	TNHC65745	**EF472076**		TX	Liberty	30.4451	-94.7405
P. sp. nov.	ECM2295	TNHC65746	**EF472077**	3	TX	Liberty	30.3517	-95.0632
P. sp. nov.	ECM2437	TNHC65022	**EF472074**		LA	St. Martin	30.3309	-91.6964
P. sp. nov.	JTC2586	TNHC63583	**EF472062**	1 2 3	TX	Lamar	33.7803	-95.5353
P. sp. nov.	JTC2737	TNHC63551	**EF472072**	3	OK	Osage	36.5356	-96.0507
P. sp. nov.	JTC2829	TNHC63703	**EF472071**		MO	Ripley	36.7069	-90.6938
P. sp. nov.	JTC2847	TNHC63556	**EF472070**	3	OK	Pittsburg	34.9927	-95.8385
P. sp. nov.	JTC2860	TNHC63557	**EF472065**		OK	Love	34.1330	-97.1062
P. sp. nov.	JTC2866	TNHC63559	**EF472063**		OK	McCurtain	34.1405	-94.6958

P. sp. nov.	R.Highton71204	R. Highton uncat.	**EF472073**		OK	LeFlore	34.7107	-94.5497
P. streckeri	JTC2581	TNHC63382	**EF472009**		AR	Conway	35.2503	-92.6833
P. streckeri	P-2	TNHC62317	**AY291108***		TX	Travis	30.3218	-97.8034
P. triseriata	ECM0615	TNHC63682	**EF472146**	2	MI	Ingham	42.7222	-84.4275
P. triseriata	ECM0616	TNHC63683	**EF472155**	1	MI	Ingham	42.6890	-84.2830
P. triseriata	ECM0662	n/a	**EF472142**	2	Ontario	Essex	42.1216	-82.9715
P. triseriata	INHS1207	INHS18840	**EF472138**		IL	Perry	38.0188	-89.4181
P. triseriata	INHS1234	INHS18853	**EF472136**	2	IL	Lawrence	38.7128	-87.6768
P. triseriata	INHS1239	INHS18857	**EF472153**	1	IL	Saline	37.7358	-88.6941
P. triseriata	INHS1581	INHS19242	**EF472159**	2	IL	Wayne	38.5257	-88.3456
P. triseriata	J-1	KU289219	**AY291091***	1 2	MI	Berrien	41.9500	-86.4167
P. triseriata	JTC2590	TNHC63412	**EF472151**	1 2	OH	Logan	40.4614	-83.6700
P. triseriata	JTC2594	TNHC63392	**EF472139**		KY	Daviess	37.8661	-87.2855
P. triseriata	JTC2604	TNHC63405	**EF472144**		OH	Highland	39.2124	-83.8362
P. triseriata	JTC2605	TNHC63408	**EF472149**	2	OH	Clinton	39.2599	-83.8828
P. triseriata	JTC2607	TNHC63410	**EF472148**	2	OH	Preble	39.6478	-84.5272
P. triseriata	JTC2611	TNHC63687	**EF472137**	1 3	TN	Montgomery	36.4501	-87.4767
P. triseriata	JTC2639	TNHC63691	**EF472156**	1 2	NY	Niagara	43.1706	-78.6906
P. triseriata	JTC2678^{G}	TNHC63708	**EF472141**	2	Ontario	Halton	43.6500	-79.9167
P. triseriata	JTC2679^{G}	TNHC63709	**EF472140**	2	Ontario	Oxford	42.9089	-80.8341
P. triseriata	JTC2682	TNHC63712	**EF472145**	1 2 3	Ontario	Niagara R.M.	43.0085	-79.5393
P. triseriata	JTC2690^{G}	TNHC63720	**EF472143**	1 2	Ontario	Waterloo R.M.	43.2984	-80.3735
P. triseriata	JTC2709	TNHC63394	**EF472147**	2	KY	Jefferson	38.1111	-85.8703
P. triseriata	JTC2715	TNHC65812	**EF472157**		KY	Breckinridge	37.6495	-86.4241
P. triseriata	JTC2723	TNHC63510	**EF472154**		IN	Jennings	38.9848	-85.6094
P. triseriata	JTC2830	TNHC63517	**EF472150**	2	IN	Delaware	40.0400	-85.3000
P. triseriata	JTC2848	TNHC63694	**EF472152**	2	NY	Livingston	42.9377	-77.7739
P. triseriata	JTC2851	TNHC63518	**EF472158**	1	IN	Marion	39.8641	-86.2904
P. triseriata	R.Highton69234	R. Highton uncat.	**EF472160**	1 2 3	IN	Posey	38.1298	-87.9350

[1] R.Highton71747 was labeled as *Pseudacris feriarum* in Moriarty and Cannatella (2004); according to R. Highton (pers. comm.), who collected the specimen, this specimen is morphologically and acoustically a *P. brachyphona.* However, its mitochondrial DNA suggests that this individual is a hybrid between the two species.
[2] TNHC62369 is from Lake Co. not Ocala Co, as reported in Moriarty and Cannatella (2004).
[3] MVZ145452 was mislabeled in Moriarty and Cannatella (2004) as MVZ11452.

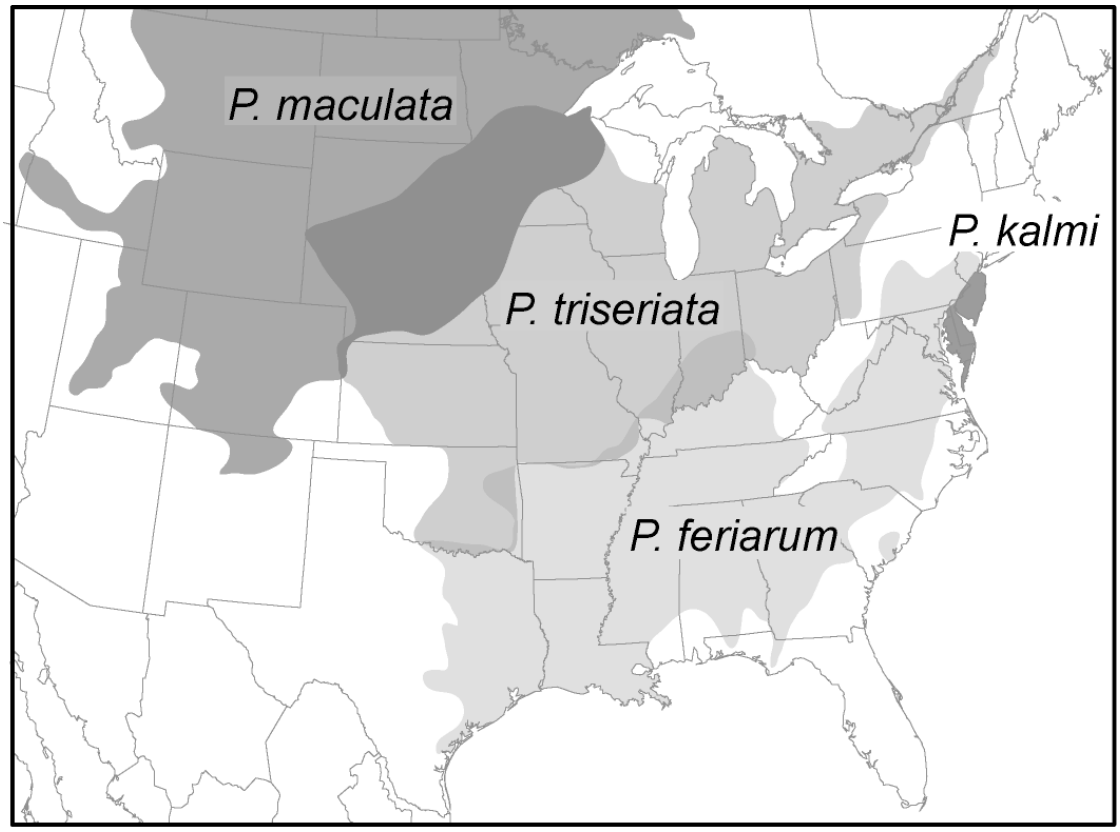


Figure 2.1. Distributions of four trilling chorus-frog taxa based on earlier non-genetic studies. This figure was modified from Conant and Collins (1998), which was largely based on morphometric data of Smith (1956), Smith (1957), and Smith and Smith (1952). Distributions of *Pseudacris brachyphona*, *P. brimleyi*, *P. clarkii*, *P. nigrita*, and *P.* sp. nov. are not shown because ranges of these species have not changed substantially with the addition of genetic data.

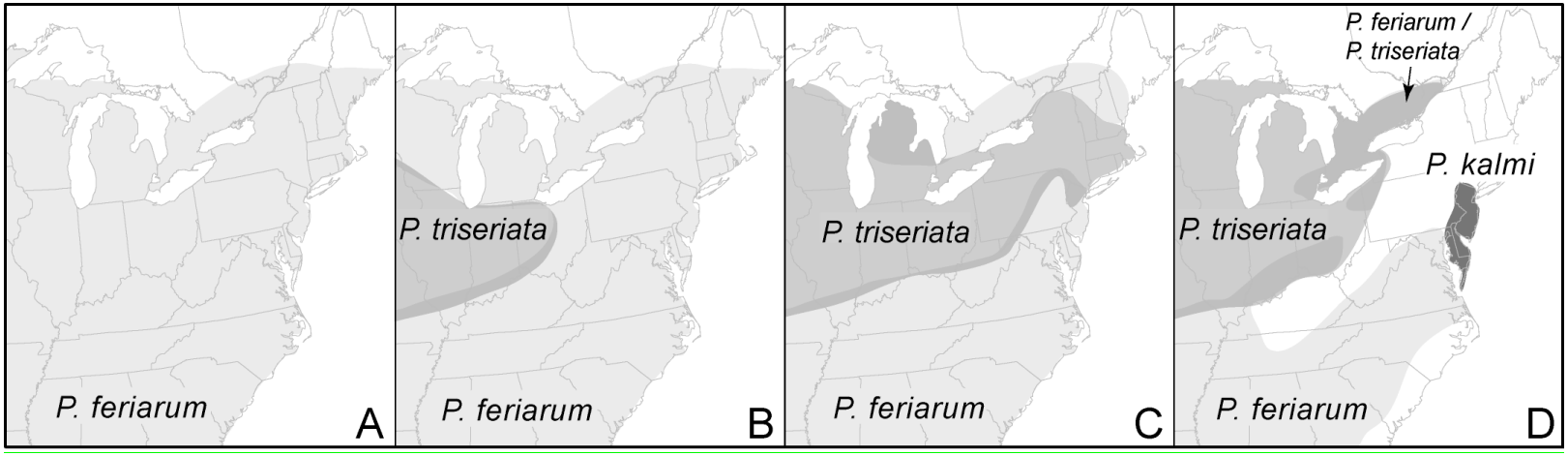


Figure 2.2. Scenarios for migration and speciation in the trilling *Pseudacris* proposed by Smith (1957). According to this scenario, the distribution of the wide-ranging *P. feriarum* (A) was bisected by eastward expansion of *P. triseriata* (B), leading to geographic isolation of northern *P. feriarum* populations (C). *Pseudacris triseriata* reached the East Coast where it left relict populations when its range contracted (D). These relict populations are now known as *P. kalmi.*

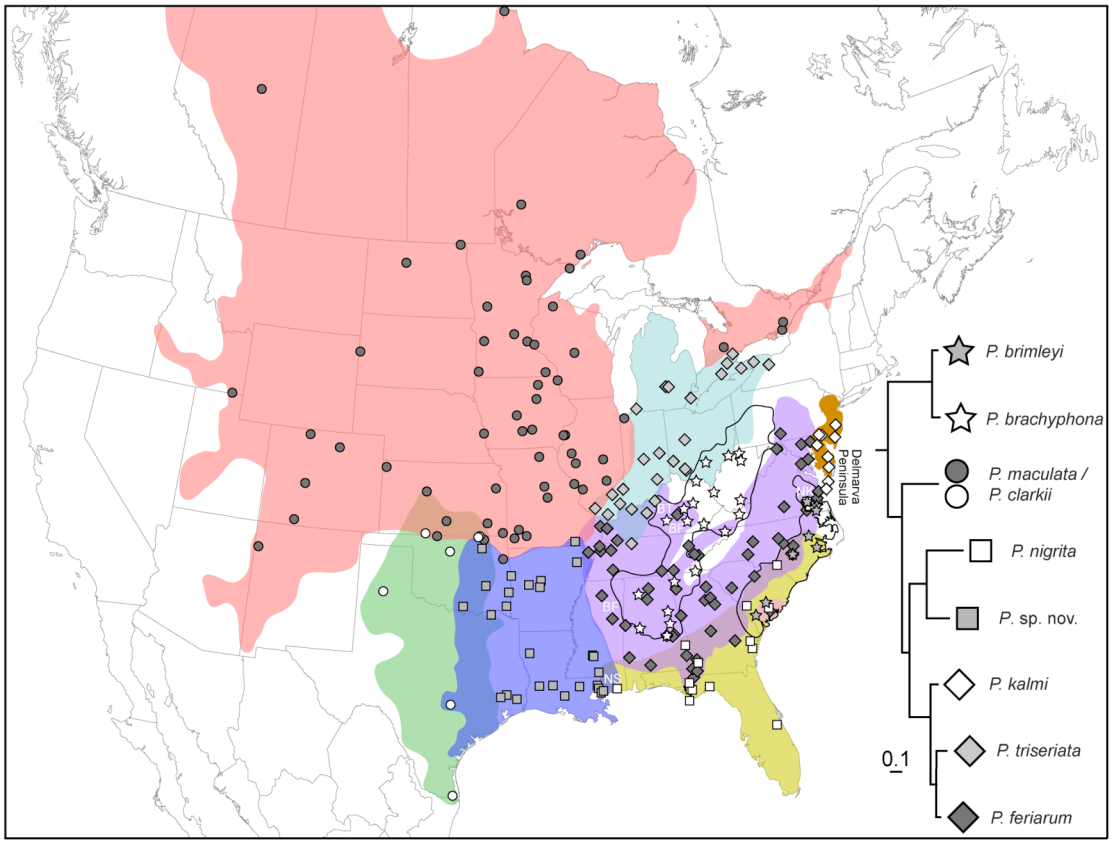
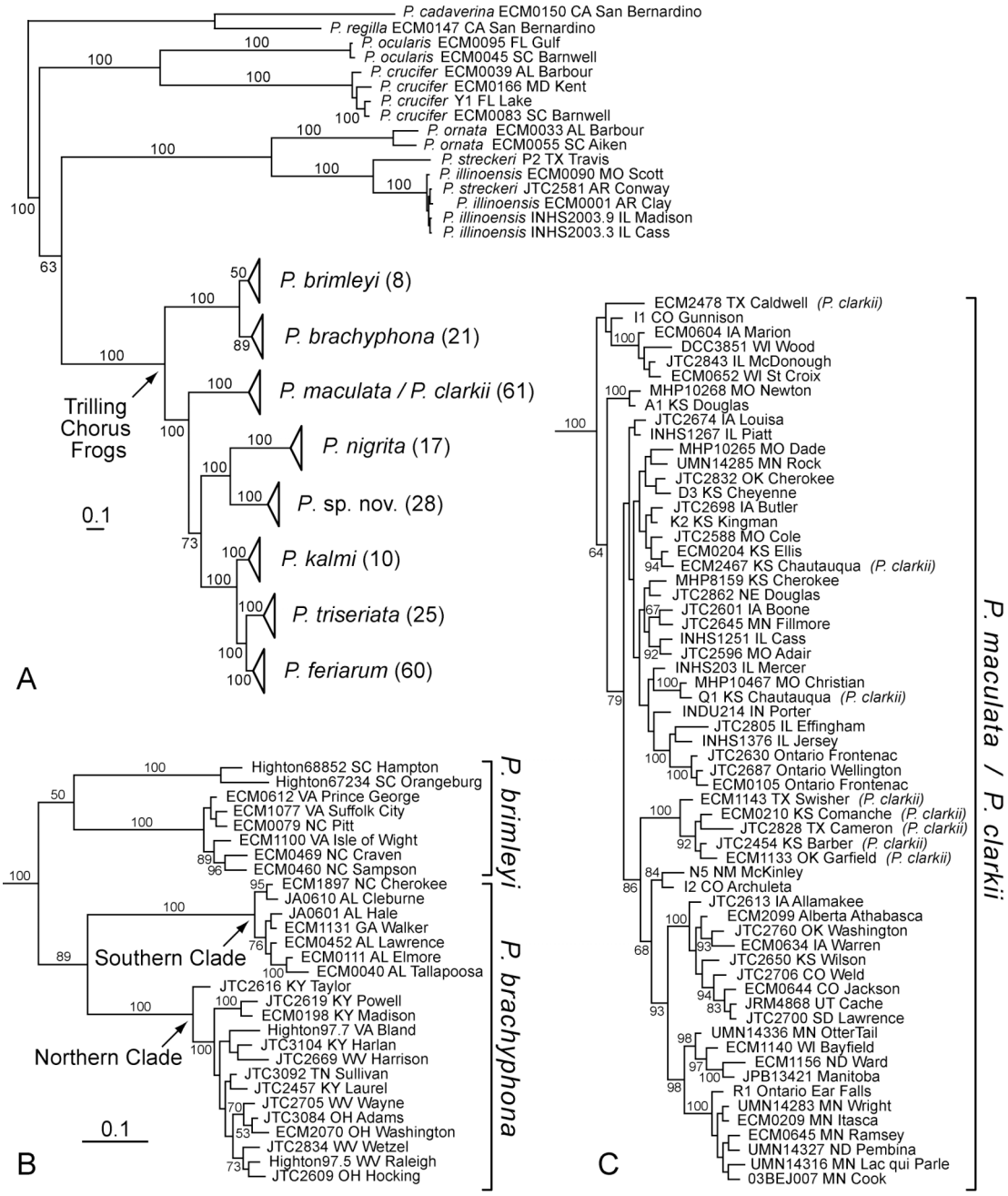
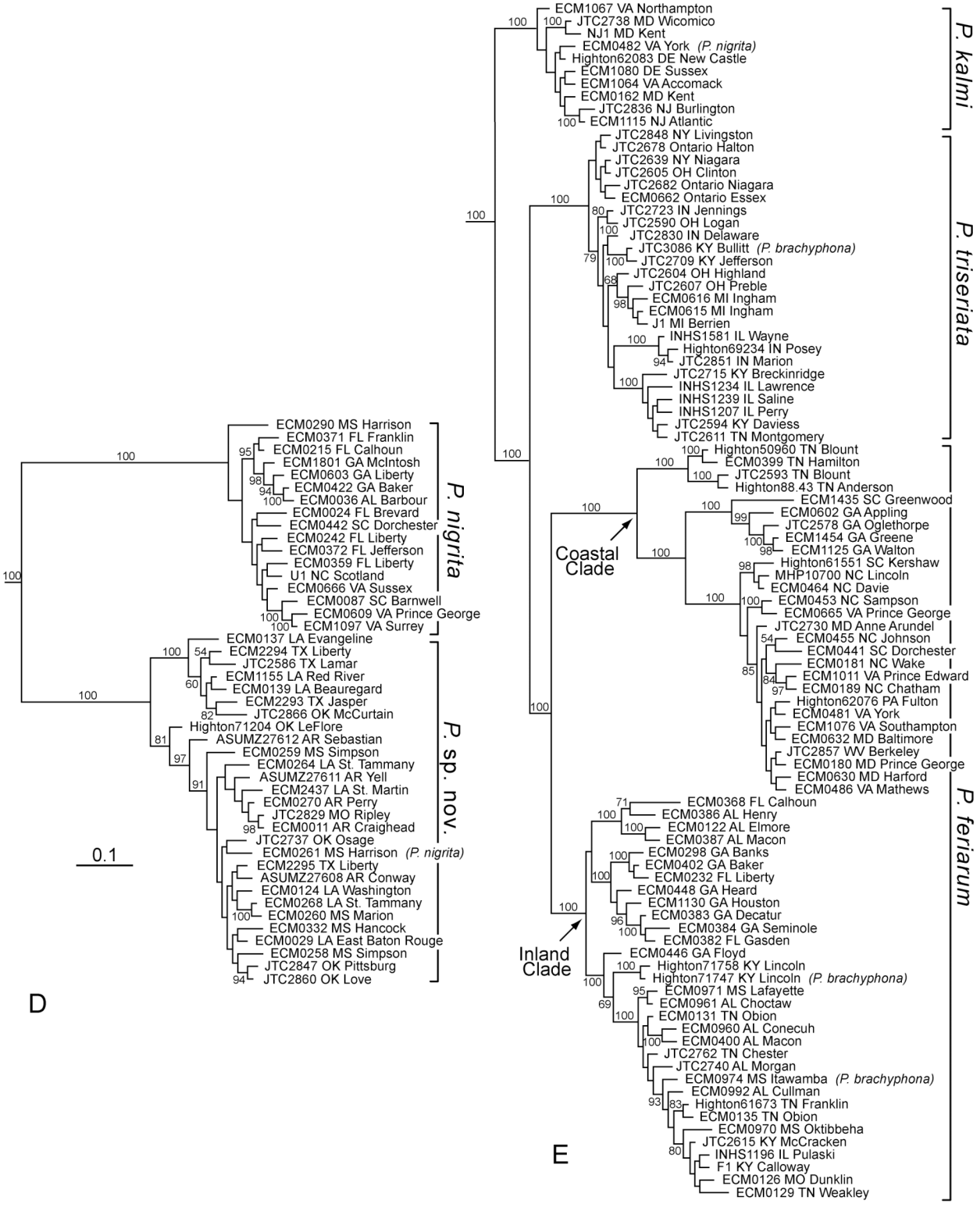


Figure 2.3. Updated distributions of all North American Trilling Frogs. Species boundaries are based on the phylogeny (Fig. 2.4) and county-level taxon records from Lannoo (2005). Markers indicate populations sampled that correspond to species in the phylogeny to the right. Ranges of *Pseudacris brachyphona* and *P. brimleyi* are outlined in black for visual simplicity. Capital letters indicate hybrids and represent the following hybrid combinations: NS–*P. nigrita*-*P.* sp. nov., BF–*P. brachyphona*-*P. feriarum*, BT–*P. brachyphona*-*P. triseriata*, and NK–*P. nigrita*-*P. kalmi*, where the first species listed refers to the behavioral/morphological identity and the second to the mitochondrial DNA identity of the individual. Degree of geographic overlap between species is indicated on map if known; if no overlap is shown between parapatric taxa, then the amount of overlap is unknown.

Figure 2.4. Bayesian phylogeny of *Pseudacris*. Tree A shows the phylogenetic relationships of the entire genus. Numbers of populations sampled from each species are indicated in parentheses. Trees B-E illustrate the population-level relationships of each subclade. 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 the morphological/behavioral identity of individuals when this conflicted with the mitochondrial clade identity. Note that the branch length scale for phylogeny A is 25% of the scale for phylogenies B-E


P. cadaverina ECM0150 CA San Bernardino
P. regilla ECM0147 CA San Bernardino
P. ocularis ECM0095 FL Gulf
P. ocularis ECM0045 SC Barnwell
P. crucifer ECM0039 AL Barbour
P. crucifer ECM0166 MD Kent
P. crucifer Y1 FL Lake
P. crucifer ECM0083 SC Barnwell
P. ornata ECM0033 AL Barbour
P. ornata ECM0055 SC Aiken
P. streckeri P2 TX Travis
P. illinoensis ECM0090 MO Scott
P. streckeri JTC2581 AR Conway
P. illinoensis ECM0001 AR Clay
P. illinoensis INHS2003.9 IL Madison
P. illinoensis INHS2003.3 IL Cass
P. brimleyi (8)
P. brachyphona (21)
P. maculata / P. clarkii (61)
P. nigrita (17)
P. sp. nov. (28)
P. kalmi (10)
P. triseriata (25)
P. feriarum (60)
Trilling Chorus Frogs
0.1
A
Highton68852 SC Hampton
Highton67234 SC Orangeburg
ECM0612 VA Prince George
ECM1077 VA Suffolk City
ECM0079 NC Pitt
ECM1100 VA Isle of Wight
ECM0469 NC Craven
ECM0460 NC Sampson
ECM1897 NC Cherokee
JA0610 AL Cleburne
JA0601 AL Hale
ECM1131 GA Walker
ECM0452 AL Lawrence
ECM0111 AL Elmore
ECM0040 AL Tallapoosa
Southern Clade
JTC2616 KY Taylor
JTC2619 KY Powell
ECM0198 KY Madison
Highton97.7 VA Bland
JTC3104 KY Harlan
JTC2669 WV Harrison
JTC3092 TN Sullivan
JTC2457 KY Laurel
JTC2705 WV Wayne
JTC3084 OH Adams
ECM2070 OH Washington
JTC2834 WV Wetzel
Highton97.5 WV Raleigh
JTC2609 OH Hocking
Northern Clade
0.1
B
P. brimleyi
P. brachyphona
ECM2478 TX Caldwell (P. clarkii)
I1 CO Gunnison
ECM0604 IA Marion
DCC3851 WI Wood
JTC2843 IL McDonough
ECM0652 WI St Croix
MHP10268 MO Newton
A1 KS Douglas
JTC2674 IA Louisa
INHS1267 IL Piatt
MHP10265 MO Dade
UMN14285 MN Rock
JTC2832 OK Cherokee
D3 KS Cheyenne
JTC2698 IA Butler
K2 KS Kingman
JTC2588 MO Cole
ECM0204 KS Ellis
ECM2467 KS Chautauqua (P. clarkii)
MHP8159 KS Cherokee
JTC2862 NE Douglas
JTC2601 IA Boone
JTC2645 MN Fillmore
INHS1251 IL Cass
JTC2596 MO Adair
INHS203 IL Mercer
MHP10467 MO Christian
Q1 KS Chautauqua (P. clarkii)
INDU214 IN Porter
JTC2805 IL Effingham
INHS1376 IL Jersey
JTC2630 Ontario Frontenac
JTC2687 Ontario Wellington
ECM0105 Ontario Frontenac
ECM1143 TX Swisher (P. clarkii)
ECM0210 KS Comanche (P. clarkii)
JTC2828 TX Cameron (P. clarkii)
JTC2454 KS Barber (P. clarkii)
ECM1133 OK Garfield (P. clarkii)
N5 NM McKinley
I2 CO Archuleta
JTC2613 IA Allamakee
ECM2099 Alberta Athabasca
JTC2760 OK Washington
ECM0634 IA Warren
JTC2650 KS Wilson
JTC2706 CO Weld
ECM0644 CO Jackson
JRM4868 UT Cache
JTC2700 SD Lawrence
UMN14336 MN OtterTail
ECM1140 WI Bayfield
ECM1156 ND Ward
JPB13421 Manitoba
R1 Ontario Ear Falls
UMN14283 MN Wright
ECM0209 MN Itasca
ECM0645 MN Ramsey
UMN14327 ND Pembina
UMN14316 MN Lac qui Parle
03BEJ007 MN Cook
C
P. maculata / P. clarkii


ECM0290 MS Harrison
ECM0371 FL Franklin
ECM0215 FL Calhoun
ECM1801 GA McIntosh
ECM0603 GA Liberty
ECM0422 GA Baker
ECM0036 AL Barbour
ECM0024 FL Brevard
ECM0442 SC Dorchester
ECM0242 FL Liberty
ECM0372 FL Jefferson
ECM0359 FL Liberty
U1 NC Scotland
ECM0666 VA Sussex
ECM0087 SC Barnwell
ECM0609 VA Prince George
ECM1097 VA Surrey
P. nigrita
ECM0137 LA Evangeline
ECM2294 TX Liberty
JTC2586 TX Lamar
ECM1155 LA Red River
ECM0139 LA Beauregard
ECM2293 TX Jasper
JTC2866 OK McCurtain
Highton71204 OK LeFlore
ASUMZ27612 AR Sebastian
ECM0259 MS Simpson
ECM0264 LA St. Tammany
ASUMZ27611 AR Yell
ECM2437 LA St. Martin
ECM0270 AR Perry
JTC2829 MO Ripley
ECM0011 AR Craighead
JTC2737 OK Osage
ECM0261 MS Harrison (P. nigrita)
ECM2295 TX Liberty
ASUMZ27608 AR Conway
ECM0124 LA Washington
ECM0268 LA St. Tammany
ECM0260 MS Marion
ECM0332 MS Hancock
ECM0029 LA East Baton Rouge
ECM0258 MS Simpson
JTC2847 OK Pittsburg
JTC2860 OK Love
P. sp. nov.
0.1
D
ECM1067 VA Northampton
JTC2738 MD Wicomico
NJ1 MD Kent
ECM0482 VA York (P. nigrita)
Highton62083 DE New Castle
ECM1080 DE Sussex
ECM1064 VA Accomack
ECM0162 MD Kent
JTC2836 NJ Burlington
ECM1115 NJ Atlantic
P. kalmi
JTC2848 NY Livingston
JTC2678 Ontario Halton
JTC2639 NY Niagara
JTC2605 OH Clinton
JTC2682 Ontario Niagara
ECM0662 Ontario Essex
JTC2723 IN Jennings
JTC2590 OH Logan
JTC2830 IN Delaware
JTC3086 KY Bullitt (P. brachyphona)
JTC2709 KY Jefferson
JTC2604 OH Highland
JTC2607 OH Preble
ECM0616 MI Ingham
ECM0615 MI Ingham
J1 MI Berrien
INHS1581 IL Wayne
Highton69234 IN Posey
JTC2851 IN Marion
JTC2715 KY Breckinridge
INHS1234 IL Lawrence
INHS1239 IL Saline
INHS1207 IL Perry
JTC2594 KY Daviess
JTC2611 TN Montgomery
P. triseriata
Highton50960 TN Blount
ECM0399 TN Hamilton
JTC2593 TN Blount
Highton88.43 TN Anderson
ECM1435 SC Greenwood
ECM0602 GA Appling
JTC2578 GA Oglethorpe
ECM1454 GA Greene
ECM1125 GA Walton
Coastal Clade
Highton61551 SC Kershaw
MHP10700 NC Lincoln
ECM0464 NC Davie
ECM0453 NC Sampson
ECM0665 VA Prince George
JTC2730 MD Anne Arundel
ECM0455 NC Johnson
ECM0441 SC Dorchester
ECM0181 NC Wake
ECM1011 VA Prince Edward
ECM0189 NC Chatham
Highton62076 PA Fulton
ECM0481 VA York
ECM1076 VA Southampton
ECM0632 MD Baltimore
JTC2857 WV Berkeley
ECM0180 MD Prince George
ECM0630 MD Harford
ECM0486 VA Mathews
ECM0368 FL Calhoun
ECM0386 AL Henry
ECM0122 AL Elmore
ECM0387 AL Macon
ECM0298 GA Banks
ECM0402 GA Baker
ECM0232 FL Liberty
ECM0448 GA Heard
ECM1130 GA Houston
ECM0383 GA Decatur
ECM0384 GA Seminole
ECM0382 FL Gasden
Inland Clade
ECM0446 GA Floyd
Highton71758 KY Lincoln
Highton71747 KY Lincoln (P. brachyphona)
ECM0971 MS Lafayette
ECM0961 AL Choctaw
ECM0131 TN Obion
ECM0960 AL Conecuh
ECM0400 AL Macon
JTC2762 TN Chester
JTC2740 AL Morgan
ECM0974 MS Itawamba (P. brachyphona)
ECM0992 AL Cullman
Highton61673 TN Franklin
ECM0135 TN Obion
ECM0970 MS Oktibbeha
JTC2615 KY McCracken
INHS1196 IL Pulaski
F1 KY Calloway
ECM0126 MO Dunklin
ECM0129 TN Weakley
P. feriarum
E

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Chapter 3

Geological and Climatic Forces Driving Speciation in the Continentally Distributed Trilling Chorus Frogs (*Pseudacris*)*

Abstract: 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.

*Significant portions of this chapter have been previously published as Lemmon, Lemmon, and Cannatella 2006. Evolution, in press.

3.1 INTRODUCTION

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 over-interpretation 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 [ma]; 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. 3.1A). 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. 3.1A).

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 development of a rain shadow on the eastern slope. In this case, the western slope of the Appalachians was probably wet while the eastern side was arid, similar to the Sierra Nevada Range today (Stanley 1989). We might expect, therefore, that this geologic feature contributed to divergence of taxa inhabiting the region (Fig. 3.1B).

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. 3.1C–E; Ver Steeg 1946; Hocutt et al. 1986; Gray 1991; Melhorn and Kempton 1991). Glacial advances in the Pleistocene (~0.8 ma) dammed the Teays-Mahomet River in southern Ohio, forming a vast lake that lasted

several thousand years before overflow resulted in 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. 3.1D-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. 3.1D-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 ma; 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. 3.2). According to paleoclimatic models, as the ice sheet receded (12–110 thousand years ago [ka]; glacial maximum 14 ka; 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 ka (Fig. 3.2; 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 ma (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 behavioral data (Lemmon et al. in press; Fig. 3.2). This paper 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 cause 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. (in press) 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.

3.2 MATERIALS AND 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, θ. Analyses were performed using the HKY model of substitution. 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 θ 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 θ were used to solve for divergence time (T_{div}) using the following equations: $T = T_{div} / 2Ne$ and $\theta = 4Ne\mu$, where the units of μ 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 (Cannatella and Holloway, unpub. data; see Supplemental Data 3.1) 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. (in press) except with 4 runs, a sample frequency of 100, and 40000 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 ma and the *H. gratiosa*/*H. cinerea* clade to at least 15 ma (Holman 2003; Smith et al. 2005). In sum, we employed six fossil calibration points. The "root" of the tree was constrained to 42 ma, following Smith et al. (2005).

Analyses were performed using r8s 1.70 (Sanderson 2003). An appropriate smoothing parameter was chosen following seven 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 (see Bayesian phylogenetic analysis above). Since 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 hylid chronogram see Supplemental Figure 3.1.

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 and suggest that orogenic activity slowed substantially by the late Miocene and the mountains 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 this event occurred between 0.79 and 0.88 ma (Bigham et al 1991; Bonnett et al. 1991; Goldthwait 1991). In this paper, 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). In order 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. 3.1E, 3.3E). 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. 3.3E). 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 *Pseudacris 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 (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.

In order 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, θ 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). In order 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 TREEVOLVE 1.3.2 (Grassly et al. 1999) and the values of θ 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* v4.0b10 (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 Δv (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 (*Pseudacris triseriata*), one inhabits aridified areas (*P*. sp. nov.) and one is in both (*P*. *maculata*/*P. clarkii*; Fig. 3.2). 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.

3.3 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. 3.4). 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 *Pseudacris nigrita* and *P.* sp. nov. During the late Miocene and early Pliocene, sea levels fluctutated 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. 3.2), occurred at the same time as the speciation event, approximately 4.8 ma (Fig. 3.4). 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 *Pseudacris brachyphona* and *P. brimleyi*. Although orogenic activity took place in the Oligocene and Miocene (5.3–33.9 ma), speciation between these taxa is estimated to be during the Pliocene, approximately 4.6 ma (Table 3.1; Fig. 3.4). 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 *Pseudacris feriarum* and *P. triseriata* (Fig. 3.1D-E). Damming of the Teays-Mahomet River occurred from 0.79–0.88 ma, whereas divergence of these taxa is estimated at approximately 2.6 ma (Table 3.1; Fig. 3.4). 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 3.1).

Rivers as Barriers to Gene Flow

The results from the partial Mantel tests suggest that each of the four rivers restrict gene flow in *Pseudacris feriarum*. A significantly different genetic distance exists between populations on the same side of the river compared to populations on different sides of the river, even after controlling for geographic distance (Table 3.2). These data suggest that river systems may form important barriers to gene flow in chorus frogs.

The randomization test for expansion in coastal *Pseudacris 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. 3.3E). 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 *Pseudacris brachyphona* (Table 3.3). 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 3.3). The *Pseudacris 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 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 3.3).

3.3 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. 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 causes of species divergence, the timing of the first event is consistent with timing of speciation between *Pseudacris 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. 3.1A; 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 *Pseudacris nigrita*-*P*. sp. nov. across the Embayment before speciation (Fig. 3.4; Haq et al. 1987). Currently the two species form a narrow contact zone along the eastern side of the Mississippi Embayment (Fig. 3.1A; 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 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. Though all of these divergence estimates may be compromised by methodological problems (strict molecular clock; Hillis et al. 1996), two studies suggest 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. 3.4; 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 *Pseudacris crucifer* clades (Austin et al. 2004).

Austin et al. (2004), who studied *Pseudacris crucifer*, did not suggest a factor of divergence for the Pliocene split.

Appalachian uplift. The most recent uplift of the Appalachian Mountains is not correlated with the divergence of *Pseudacris brachyphona-P. brimleyi*, which occurred

well after the uplift (Fig. 3.4). Though 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 U.S after speciation occurred during the middle Pliocene. Though 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, unpub. 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 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. 3.4). Prior to formation of the westward flowing Teays-Mahomet River, however, the Teays flowed northward, emptying into the Lake Erie basin (Fig. 3.1C; 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. 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). Though 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 U.S. affected the demographic history of the *P. maculata*/*clarkii* lineage is supported by the strikingly low level of genetic variation throughout the clade. Despite the age (~9.4 ma) 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, supporting the idea that aridification caused local extinction. This suggests that aridified areas were recently 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. 3.5). Signatures of these expansions can be detected in several taxa. The genetic patterns in *Pseudacris triseriata* suggest the species expanded northward from unglaciated areas in southern Illinois, Indiana, and Ohio. This expansion is congruent

with the routes of other taxa (*Pseudacris crucifer* clade D: Austin et al. 2002; *Ambystoma maculatum* interior clade: Zamudio and Savage 2003). The close phylogenetic relationship of *P. maculata* haplotypes in SE Ontario and in southern Illinois suggest that the species expanded across Illinois, Indiana, and Michigan before entering SE 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 areas prior to *P. triseriata*, which later bisected the distribution of *P. maculata* (Fig. 3.5). Alternative entry routes for *P. maculata* into SE 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. 3.2; Bleakney 1959; Cook 1964; Lannoo 2005). *Pseudacris maculata* also expanded across the western U.S. and Canada into formerly glaciated and aridified areas.

Species that currently inhabit the Coastal Plain of the eastern and southern U.S. 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; *Pseudacris kalmi*) and populations of eastern mainland Virginia (*P. nigrita*), indicated by a putative hybrid between the two species (Figs. 2, 3; Lemmon et al. in press). The finding that Coastal Plain species (*P. kalmi*, *P. nigrita*, *P.* sp. nov.) have relatively lower genetic variation (Table 3.3) 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 *Pseudacris feriarum* clade: northern populations have significantly lower genetic variation (Table 3.3) and southern populations are phylogenetically basal (Fig. 3.3). Although the northern *P. feriarum* distribution did not experience glaciation, it was 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. 3.2). In particular, the southeastern Ontario hybrid zone hot spot (No. 5, Swenson and Howard 2005) corresponds closely to the contact between *Pseudacris 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.

3.5 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.

Table 3.1. 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 (θ), mutation rate (μ) in units of substitutions per site per million years, divergence time in millions of years (bold) using the μ from Evans et al. (2004; T_{div1A}) and μ 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}.

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

Table 3.2. 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 and r^2 is the coefficient of determination. 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	r^2
Apalachicola	< 0.0001*	0.285	0.375
Altamaha	< 0.0001*	0.362	0.569
Savannah	< 0.0001*	0.569	0.646
Cape Fear	< 0.0001*	0.181	0.355

Table 3.3. Measures of genetic variation ($\bar{v}$), range expansion (Δv), 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 smallest to largest standardized genetic variation ($\bar{v}$). Each *P*-value corresponds to a test between the neighboring $\bar{v}$ and the $\bar{v}$ immediately below (see text for details). For the range expansion test, the statistic Δv 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.

		Genetic Variation		Range Expansion		Growth	
Clade	Region	$\bar{v}$	*P*	Δv	*P*	g	*P*
P. maculata/*clarkii*	G A	0.000330	0.00*	0.000161	0.00*	1035	0.00*
P. triseriata	G	0.000558	0.25	0.000701	0.00*	1191	0.01*
P. nigrita	–	0.000645	0.37	-	-	494	0.01*
P. kalmi	–	0.000677	0.36	-	-	4055	0.02*
P. sp. nov.	A	0.000805	0.48	-0.000059	0.56	1252	0.00*
P. brachyphona	–	0.000806	0.06	-	-	61	0.45
P. feriarum	–	0.000906	-	-	-	192	0.00*

Supplemental Data 3.1. List of taxa included in a Bayesian phylogenetic analysis of hylid frogs. All taxa except the outgroups *Agalychnis callidryas*, *Hyla microcephala*, *Phrynohyas venulosa*, and *Trachycephalus jordani* were included in the penalized likelihood analysis to estimate divergence times. The *Acris*, *Hyla*, and *Smilisca* sequences are taken from Cannatella and Holloway (unpub. data) and the *Pseudacris* are either published in Moriarty and Cannatella (2004) or Moriarty et al. (in press). The combined dataset includes complete 12S, tRNAval and 16S mitochondrial sequence data for these taxa.

Species	Field Number	Genbank Number
Acris crepitans	DCC3535	EF566969
Acris crepitans	MGP01060103	EF566970
Acris gryllus	ECM0052	EF566971
Agalychnis callidryas	DCC2134	EF566944
Hyla andersonii	HAFL04	EF566956
Hyla andersonii	HASCB01	EF566955
Hyla andersonii	WED54451	AY291115
Hyla arenicolor	DCC3043	EF566960
Hyla arenicolor	DCC3897	EF566958
Hyla arenicolor	HCG2	EF566959
Hyla avivoca	H146	EF566947
Hyla avivoca	HCG28	EF566946
Hyla chrysoscelis	DCC3095	EF566948
Hyla chrysoscelis	DCC3829	EF566949
Hyla cinerea	DCC3511	AY680271
Hyla euphorbiacea	JAC8895	EF566961
Hyla eximia	JAC8527	EF566957
Hyla eximia	JAC8160	AY291113
Hyla femoralis	DCC3858	EF566964
Hyla gratiosa	H15929	EF566966
Hyla japonica	WED2	EF566952
Hyla meridionalis	KU207371	EF566953
Hyla microcephala	MVZ203881	EF566945
Hyla pentheter	JAC7808	EF566972
Hyla plicata	JAC8599	EF566962
Hyla savignyi	KU207344	EF566954
Hyla squirella	H487	EF566965
Hyla versicolor	DCC3512	EF566951
Hyla versicolor	DCC3800	EF566950
Hyla walkeri	JAC7861	EF566963
Hyla zeteki	MVZ203913	EF566968
Phrynohyas venulosa	DCC3069	AY326048
Pseudacris brachyphona	ECM0040	AY291095
Pseudacris brimleyi	ECM0079	AY291094

Pseudacris cadaverina	ECM0150	EF472006
Pseudacris clarkii	JTC2828	EF472104
Pseudacris crucifer	ECM0039	AY291099
Pseudacris feriarum	ECM0382	EF472177
Pseudacris illinoensis	ECM0001	AY291109
Pseudacris kalmi	JTC2836	EF472228
Pseudacris maculata	DCC3851	EF472080
Pseudacris nigrita	ECM0372	EF472042
Pseudacris ocularis	ECM0045	AY291097
Pseudacris ornata	ECM0033	AY291106
Pseudacris regilla	ECM0147	EF472005
Pseudacris sp. nov.	ECM0029	AY291085
Pseudacris streckeri	P-2	AY291108
Pseudacris triseriata	J-1	AY291091
Smilisca baudinii	DMH86_269	EF566967
Smilisca phaeota	DMH86_115	AY326040
Trachycephalus jordani	DCC2917	AY326042

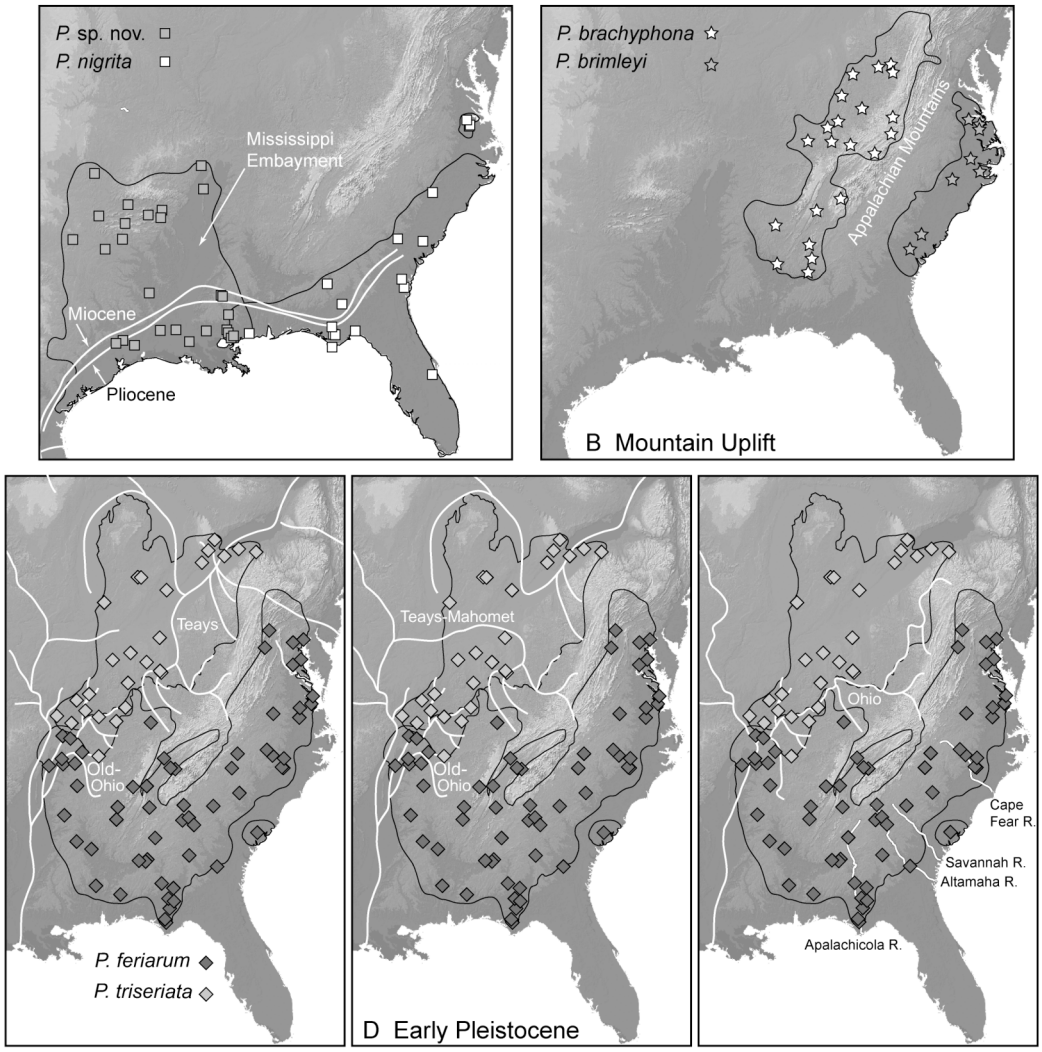


Figure 3.1. Hypotheses for geological factors contributing to speciation. Panel A illustrates the hypothesis that marine inundation of the Mississippi Embayment led to speciation between *P. nigrita* (white boxes) and *P.* sp. nov. (gray boxes). White lines indicate the maximum extent of inundation during 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–E illustrate the hypothesis that glacially induced diversion of the Teays-Mahomet River caused speciation between *Pseudacris 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.

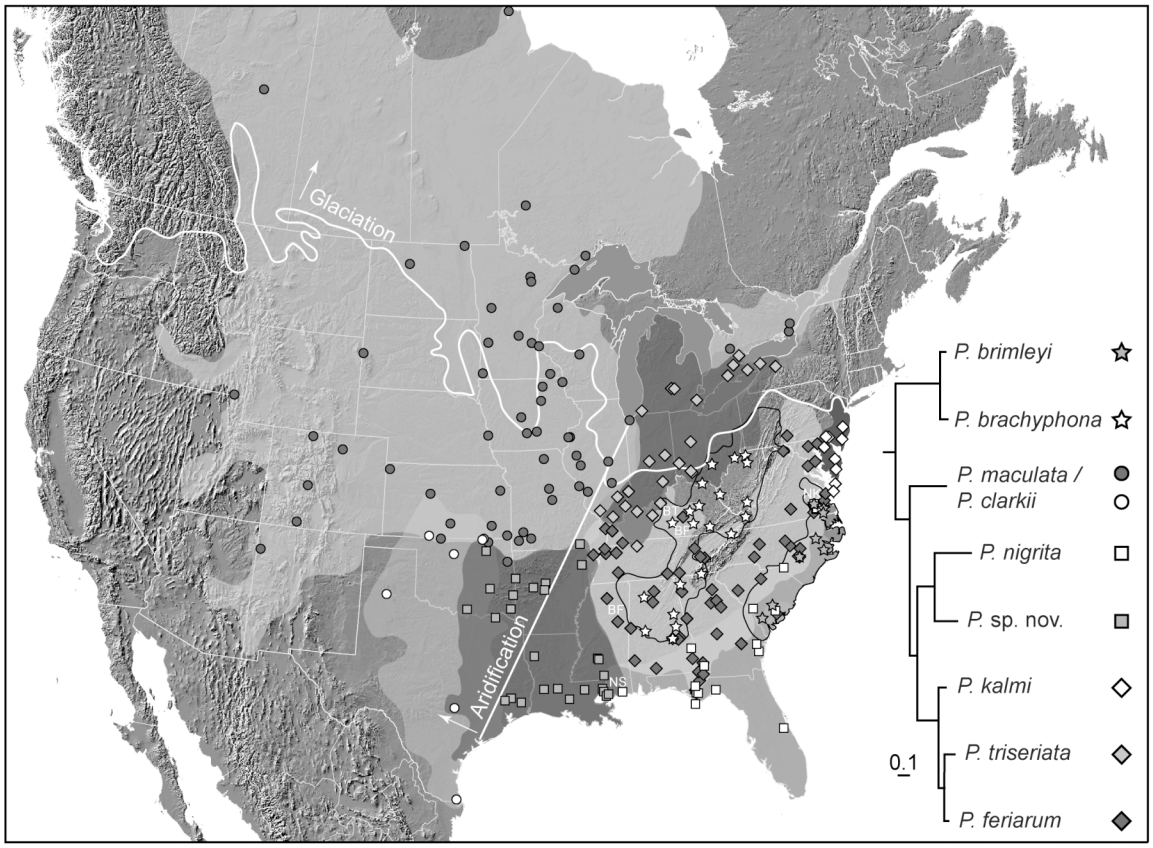
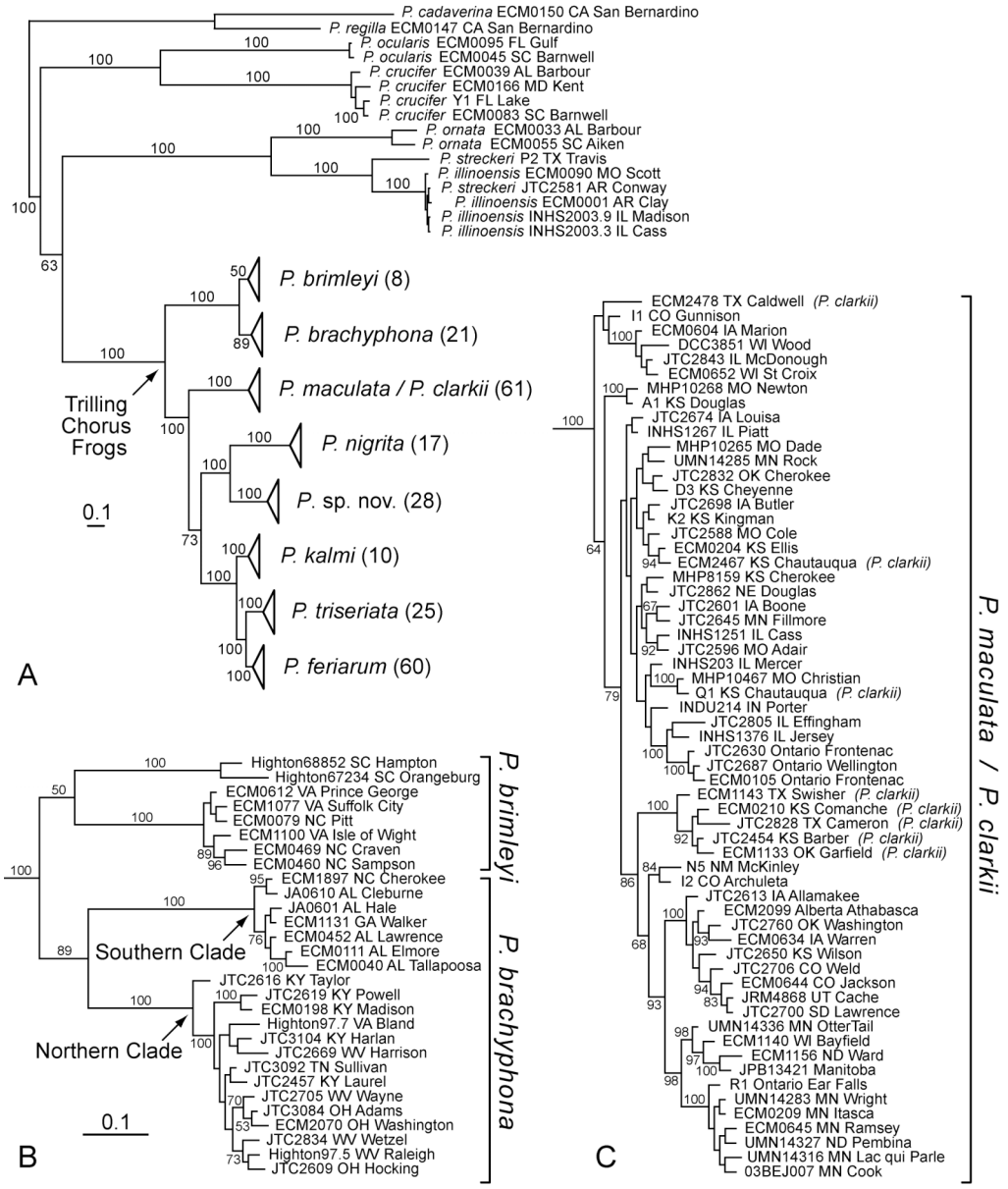
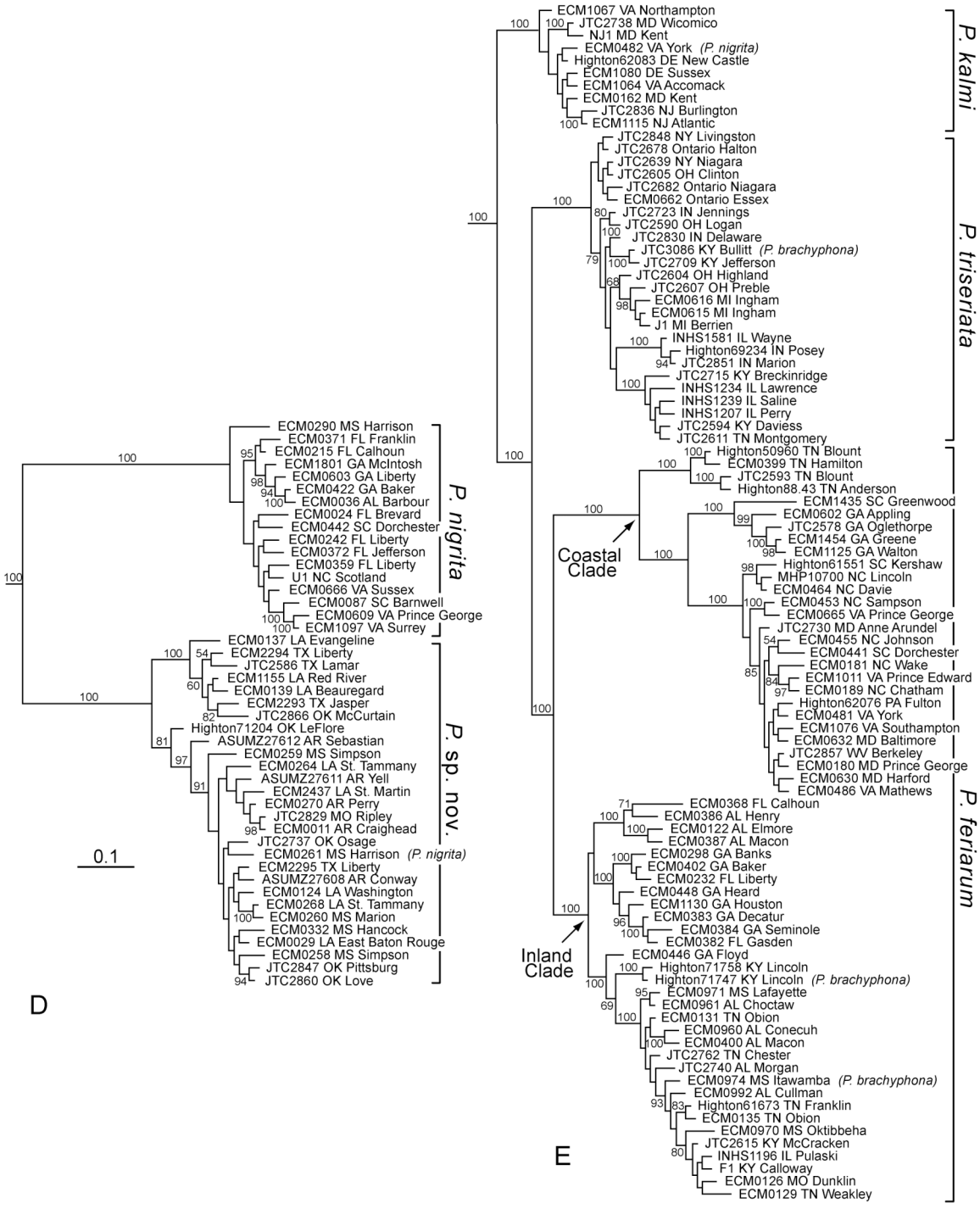


Figure 3.2. Distributions of North American Trilling Chorus Frogs. Species boundaries are based on the phylogeny (Fig. 3.3; Lemmon et al. in press) and county-level taxon records from Lannoo (2005). Markers indicate populations sampled and correspond to species in the phylogeny on the right. Ranges of *P. brachyphona* and *P. brimleyi* are outlined in black for visual clarity. Capital letters indicate the following hybrid combinations, in which the first species listed refers to the behavioral/morphological identity and the second to the mitochondrial 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. (in press).

Figure 3.3. 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.


P. cadaverina ECM0150 CA San Bernardino
P. regilla ECM0147 CA San Bernardino
P. ocularis ECM0095 FL Gulf
P. ocularis ECM0045 SC Barnwell
P. crucifer ECM0039 AL Barbour
P. crucifer ECM0166 MD Kent
P. crucifer Y1 FL Lake
P. crucifer ECM0083 SC Barnwell
P. ornata ECM0033 AL Barbour
P. ornata ECM0055 SC Aiken
P. streckeri P2 TX Travis
P. illinoensis ECM0090 MO Scott
P. streckeri JTC2581 AR Conway
P. illinoensis ECM0001 AR Clay
P. illinoensis INHS2003.9 IL Madison
P. illinoensis INHS2003.3 IL Cass
P. brimleyi (8)
P. brachyphona (21)
P. maculata / P. clarkii (61)
P. nigrita (17)
P. sp. nov. (28)
P. kalmi (10)
P. triseriata (25)
P. feriarum (60)
Trilling Chorus Frogs
0.1
A
Highton68852 SC Hampton
Highton67234 SC Orangeburg
ECM0612 VA Prince George
ECM1077 VA Suffolk City
ECM0079 NC Pitt
ECM1100 VA Isle of Wight
ECM0469 NC Craven
ECM0460 NC Sampson
ECM1897 NC Cherokee
JA0610 AL Cleburne
JA0601 AL Hale
ECM1131 GA Walker
ECM0452 AL Lawrence
ECM0111 AL Elmore
ECM0040 AL Tallapoosa
Southern Clade
JTC2616 KY Taylor
JTC2619 KY Powell
ECM0198 KY Madison
Highton97.7 VA Bland
JTC3104 KY Harlan
JTC2669 WV Harrison
JTC3092 TN Sullivan
JTC2457 KY Laurel
JTC2705 WV Wayne
JTC3084 OH Adams
ECM2070 OH Washington
JTC2834 WV Wetzel
Highton97.5 WV Raleigh
JTC2609 OH Hocking
Northern Clade
0.1
B
P. brimleyi
P. brachyphona
ECM2478 TX Caldwell (P. clarkii)
I1 CO Gunnison
ECM0604 IA Marion
DCC3851 WI Wood
JTC2843 IL McDonough
ECM0652 WI St Croix
MHP10268 MO Newton
A1 KS Douglas
JTC2674 IA Louisa
INHS1267 IL Piatt
MHP10265 MO Dade
UMN14285 MN Rock
JTC2832 OK Cherokee
D3 KS Cheyenne
JTC2698 IA Butler
K2 KS Kingman
JTC2588 MO Cole
ECM0204 KS Ellis
ECM2467 KS Chautauqua (P. clarkii)
MHP8159 KS Cherokee
JTC2862 NE Douglas
JTC2601 IA Boone
JTC2645 MN Fillmore
INHS1251 IL Cass
JTC2596 MO Adair
INHS203 IL Mercer
MHP10467 MO Christian
Q1 KS Chautauqua (P. clarkii)
INDU214 IN Porter
JTC2805 IL Effingham
INHS1376 IL Jersey
JTC2630 Ontario Frontenac
JTC2687 Ontario Wellington
ECM0105 Ontario Frontenac
ECM1143 TX Swisher (P. clarkii)
ECM0210 KS Comanche (P. clarkii)
JTC2828 TX Cameron (P. clarkii)
JTC2454 KS Barber (P. clarkii)
ECM1133 OK Garfield (P. clarkii)
N5 NM McKinley
I2 CO Archuleta
JTC2613 IA Allamakee
ECM2099 Alberta Athabasca
JTC2760 OK Washington
ECM0634 IA Warren
JTC2650 KS Wilson
JTC2706 CO Weld
ECM0644 CO Jackson
JRM4868 UT Cache
JTC2700 SD Lawrence
UMN14336 MN OtterTail
ECM1140 WI Bayfield
ECM1156 ND Ward
JPB13421 Manitoba
R1 Ontario Ear Falls
UMN14283 MN Wright
ECM0209 MN Itasca
ECM0645 MN Ramsey
UMN14327 ND Pembina
UMN14316 MN Lac qui Parle
03BEJ007 MN Cook
C
P. maculata / P. clarkii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ECM0024 FL Brevard
ECM0442 SC Dorchester
ECM0242 FL Liberty
ECM0372 FL Jefferson
ECM0359 FL Liberty
U1 NC Scotland
ECM0666 VA Sussex
ECM0087 SC Barnwell
ECM0609 VA Prince George
ECM1097 VA Surrey
P. nigrita
ECM0137 LA Evangeline
ECM2294 TX Liberty
JTC2586 TX Lamar
ECM1155 LA Red River
ECM0139 LA Beauregard
ECM2293 TX Jasper
JTC2866 OK McCurtain
Highton71204 OK LeFlore
ASUMZ27612 AR Sebastian
ECM0259 MS Simpson
ECM0264 LA St. Tammany
ASUMZ27611 AR Yell
ECM2437 LA St. Martin
ECM0270 AR Perry
JTC2829 MO Ripley
ECM0011 AR Craighead
JTC2737 OK Osage
ECM0261 MS Harrison (P. nigrita)
ECM2295 TX Liberty
ASUMZ27608 AR Conway
ECM0124 LA Washington
ECM0268 LA St. Tammany
ECM0260 MS Marion
ECM0332 MS Hancock
ECM0029 LA East Baton Rouge
ECM0258 MS Simpson
JTC2847 OK Pittsburg
JTC2860 OK Love
P. sp. nov.
0.1
D
ECM1067 VA Northampton
JTC2738 MD Wicomico
NJ1 MD Kent
ECM0482 VA York (P. nigrita)
Highton62083 DE New Castle
ECM1080 DE Sussex
ECM1064 VA Accomack
ECM0162 MD Kent
JTC2836 NJ Burlington
ECM1115 NJ Atlantic
P. kalmi
JTC2848 NY Livingston
JTC2678 Ontario Halton
JTC2639 NY Niagara
JTC2605 OH Clinton
JTC2682 Ontario Niagara
ECM0662 Ontario Essex
JTC2723 IN Jennings
JTC2590 OH Logan
JTC2830 IN Delaware
JTC3086 KY Bullitt (P. brachyphona)
JTC2709 KY Jefferson
JTC2604 OH Highland
JTC2607 OH Preble
ECM0616 MI Ingham
ECM0615 MI Ingham
J1 MI Berrien
INHS1581 IL Wayne
Highton69234 IN Posey
JTC2851 IN Marion
JTC2715 KY Breckinridge
INHS1234 IL Lawrence
INHS1239 IL Saline
INHS1207 IL Perry
JTC2594 KY Daviess
JTC2611 TN Montgomery
P. triseriata
Highton50960 TN Blount
ECM0399 TN Hamilton
JTC2593 TN Blount
Highton88.43 TN Anderson
Coastal Clade
ECM1435 SC Greenwood
ECM0602 GA Appling
JTC2578 GA Oglethorpe
ECM1454 GA Greene
ECM1125 GA Walton
Highton61551 SC Kershaw
MHP10700 NC Lincoln
ECM0464 NC Davie
ECM0453 NC Sampson
ECM0665 VA Prince George
JTC2730 MD Anne Arundel
ECM0455 NC Johnson
ECM0441 SC Dorchester
ECM0181 NC Wake
ECM1011 VA Prince Edward
ECM0189 NC Chatham
Highton62076 PA Fulton
ECM0481 VA York
ECM1076 VA Southampton
ECM0632 MD Baltimore
JTC2857 WV Berkeley
ECM0180 MD Prince George
ECM0630 MD Harford
ECM0486 VA Mathews
ECM0368 FL Calhoun
ECM0386 AL Henry
ECM0122 AL Elmore
ECM0387 AL Macon
ECM0298 GA Banks
ECM0402 GA Baker
ECM0232 FL Liberty
ECM0448 GA Heard
ECM1130 GA Houston
ECM0383 GA Decatur
ECM0384 GA Seminole
ECM0382 FL Gasden
Inland Clade
ECM0446 GA Floyd
Highton71758 KY Lincoln
Highton71747 KY Lincoln (P. brachyphona)
ECM0971 MS Lafayette
ECM0961 AL Choctaw
ECM0131 TN Obion
ECM0960 AL Conecuh
ECM0400 AL Macon
JTC2762 TN Chester
JTC2740 AL Morgan
ECM0974 MS Itawamba (P. brachyphona)
ECM0992 AL Cullman
Highton61673 TN Franklin
ECM0135 TN Obion
ECM0970 MS Oktibbeha
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INHS1196 IL Pulaski
F1 KY Calloway
ECM0126 MO Dunklin
ECM0129 TN Weakley
P. feriarum
E

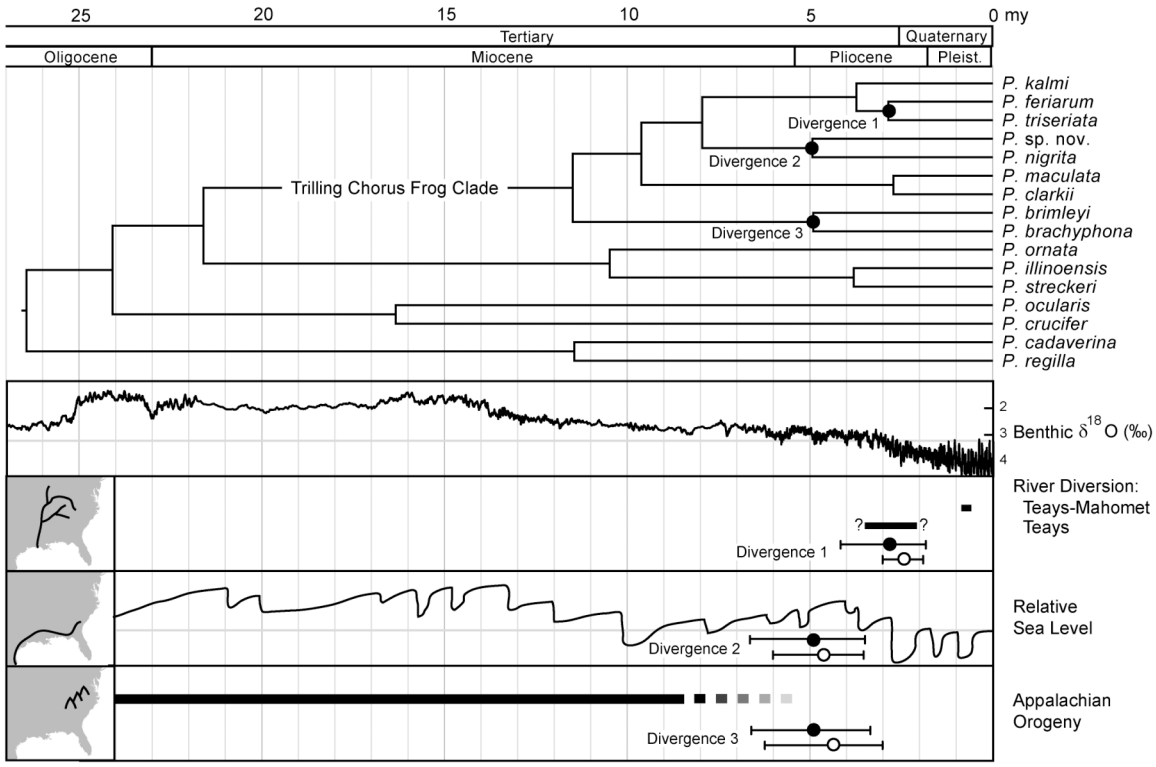


Figure 3.4. Chronogram of species divergence in *Pseudacris* and chronology of geological events from the late Oligocene to present. Divergences between sister species (top half, labeled with black dots) correspond to hypothesized geological events (bottom half). Estimated divergence times with 95% error bars are shown for the penalized likelihood (black dots) and coalescent (white dots) analyses. Horizontal gray lines indicate present day levels of benthic $\delta^{18}O$ and sea level. Because benthic $\delta^{18}O$ levels are negatively correlated with temperature, this trace is shown as a proxy for temperature fluctuations through time (Zachos et al. 2001; Lisiecki and Raymo 2005). Units for benthic $\delta^{18}O$ 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).

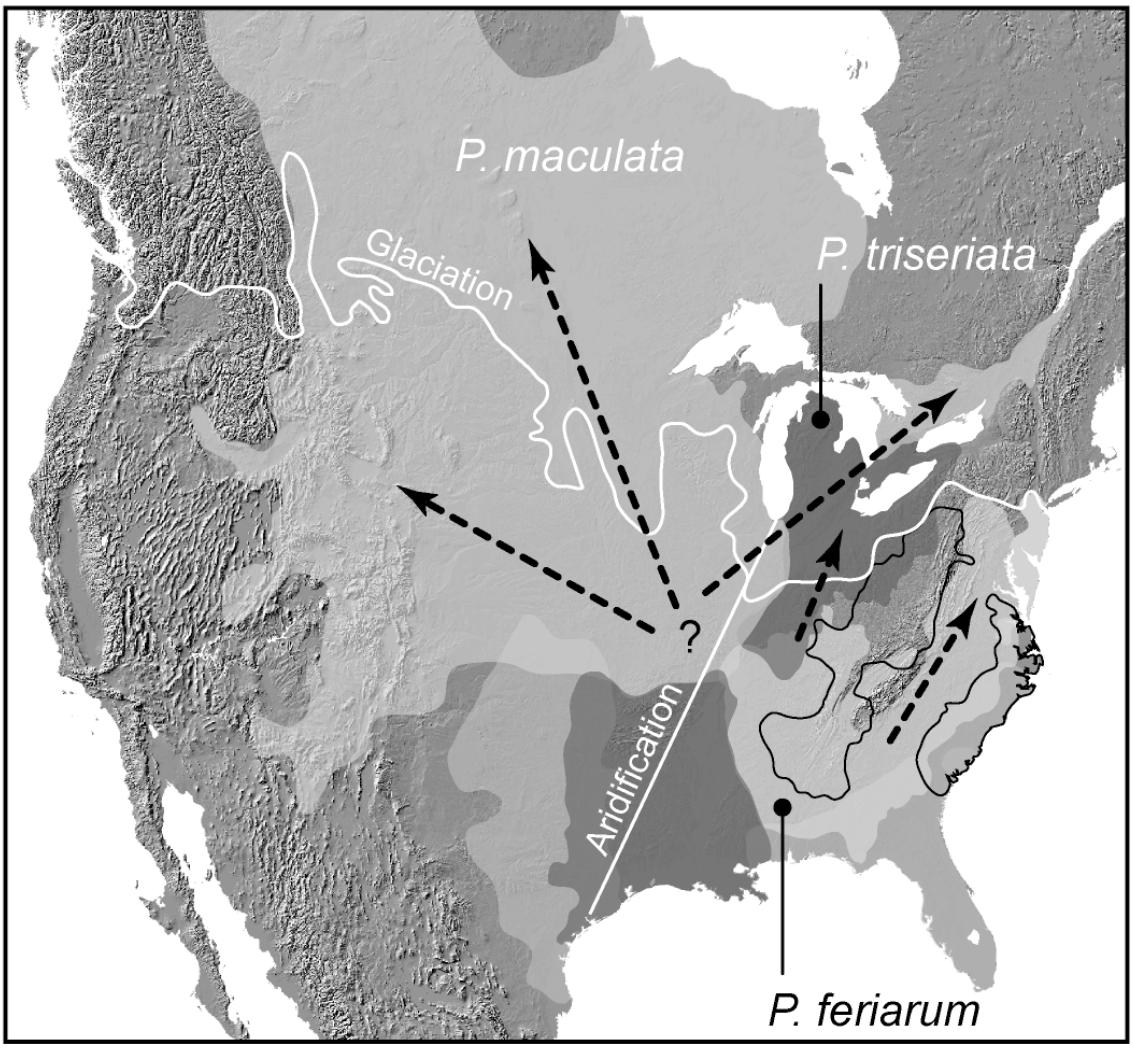
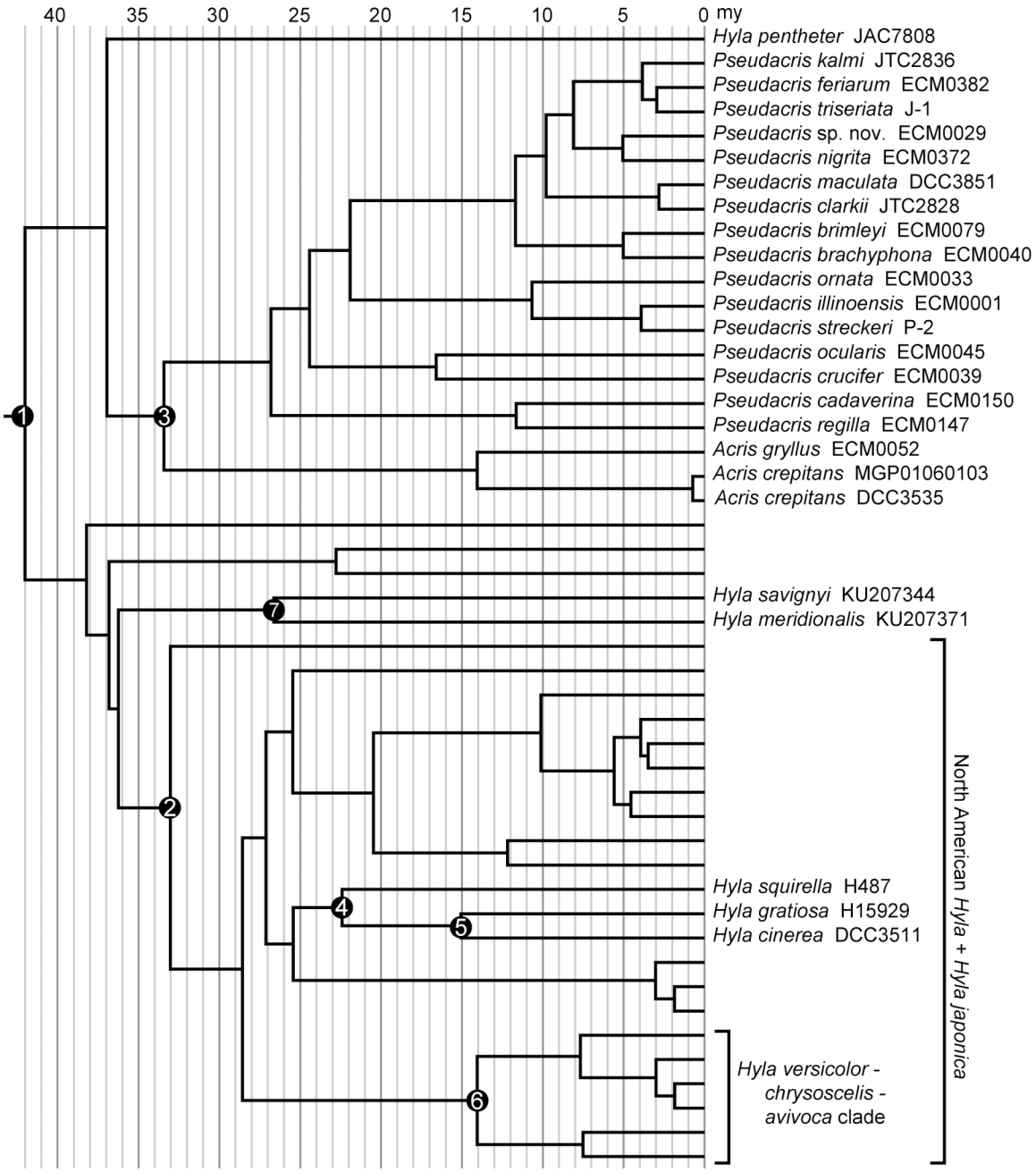


Figure 3.5. Proposed expansion routes in several trilling chorus frog lineages following Quaternary glaciation and aridification. Hypothesized expansion patterns, indicated by dashed arrows, are based on phylogenetic structure and levels of intraspecific genetic variation. Glaciated and aridified regions correspond to areas indicated by arrows in Fig. 3.2. Distributions of unlabeled taxa correspond to species in Figure 3.2.



Supplemental Figure 3.1. Full chronogram from a penalized likelihood analysis of divergence times across North American hylid frogs based on a 12S/16S mitochondrial dataset. Scale is in millions of years (my). Calibration points and root constraint follow Smith et al. (2005) except as explained in the methods: 1) root constraint, 42 my, 2) North American *Hyla*, 33 my, 3) *Acris–Pseudacris*, 15 my, 4) *Hyla squirrella–H. gratiosa–H. cinerea*, 15 my, 5) *Hyla gratiosa–H. cinerea*, 15 my, 6) *Hyla chrysoscelis*–H. *versicolor*, 14 my, and 7) European *Hyla*, 10 my. The four outgroups used in the Bayesian analysis are not shown (full phylogeny can be obtained from TreeBase).

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Chapter 4

Acoustic Niche Partitioning and Signal Divergence in Chorus Frogs (*Pseudacris*)

Abstract. Acoustic interference of reproductive signals in breeding aggregations can reduce the ability of individuals to locate conspecific mates. When this reproductive cost is high, species may be under selection to avoid acoustically similar taxa or to evolve signals that occupy new acoustic space. Because different components of acoustic signals are subject to different selection pressures and constraints, we expect some components (physiology-controlled characters) to be more evolutionarily labile than other components (morphology-controlled characters). Signal components that are under less evolutionary constraint or stronger divergent selection are predicted to form the more important axis of differentiation for acoustically similar sympatric species. We test these predictions by 1) examining the degree of signal divergence between sympatric and allopatric species pairs, and by 2) estimating evolutionary rates of signal components, using an acoustically diverse clade, the North American chorus frogs (*Pseudacris*). We find that signal divergence is greater among sympatric species pairs than allopatric species pairs, providing evidence that acoustic niche partitioning occurs in chorus frogs. Acoustic divergence is significant along the axis of physiology-controlled characters but not along the axis of morphology-controlled characters. The former axis is not correlated

with body size, a variable that is indicative of the species' ecological niche within the community, supporting the idea that acoustic divergence is not merely a by-product of ecological differentiation. Maximum likelihood estimates of the degree of phylogenetic effect indicate that physiology-controlled characters are more evolutionarily labile, supporting the prediction that these characters are more likely to evolve during acoustic niche partitioning among sympatric species.

4.1 INTRODUCTION

The raucous playlist of songs emanating from a frog pond on a warm summer night begs the question: In choruses of multiple species, how do acoustically signaling organisms avoid interference? What are the ecological and evolutionary consequences of this interference?

Assuming that species compete for acoustic space—the multidimensional acoustic representation of signal structure (Nelson and Marler 1990)—several scenarios are possible: species with no acoustic competitors experience no competitive exclusion and co-exist without difficulty, while species with very similar calls cannot co-exist in the same region or calling site. Alternatively, interference from sympatric species may lead to divergent evolution of behaviors. For example, species may diverge with respect to acoustic signals, breeding time, or breeding habitat (Duellman 1967; Hödl 1977; Drewry and Rand 1983; Donnelly and Guyer 1994; Lüddecke et al 2000; Gottsberger and Gruber

2004). This behavioral axis of divergence may be regulated by the available ecological space. If optimal breeding time and habitat are limited, however, species may be under pressure to partition the acoustic niche—meaning the axes of call variables such as pitch (frequency) or temporal components—to minimize signal interference. If species diverge acoustically in response to heterospecific interactions, do components of the signal diverge uniformly, or are some components more evolutionarily labile?

Among frogs, acoustic signals are the primary method of communication. Males announce their presence to potential mates through an unlearned advertisement call (B. Dawson, unpub. data), and females then make mating decisions based on information conveyed through the acoustic structure of the signal (Ryan 2001; Gerhardt and Huber 2002). Frogs frequently call in aggregations of multiple species, each with a species-specific signal. The anuran advertisement call is subject to both natural and sexual selection (Ryan 1980, 1985, 1991; Gerhardt and Huber 2002). The direction and magnitude of call evolution may be affected, however, by biotic and abiotic constraints that can have unequal effects on different components of the call. These constraining factors include acoustically-oriented predators (Ryan 1985), heterospecific signalers (Hödl 1977; Drewry and Rand 1983; Duellman and Pyles 1983), environmental acoustics of the calling sites (Ryan and Wilczynski 1991), vocal morphology and size-related traits (Ryan 1988), and energetic costs (Wells 2001). Given the diverse selection pressures and constraints affecting signals, we might generally predict that individual call variables (e.g., call rate, dominant frequency) evolve at different rates. Only Ryan (1988) and Cocroft and Ryan (1995) have examined this prediction in a phylogenetic context, testing

the prediction that physiology-controlled characters are more evolutionarily labile than morphology-controlled characters (see description below).

If acoustic space is limited, sympatric species pairs are expected to show greater signal divergence than allopatric species pairs, a prediction that can be tested by measuring call parameters of replicate sympatric and allopatric species pairs. In addition, those call characters that are crucial for partitioning acoustic space are predicted to be the least constrained, i.e., the most evolutionary labile in general. This prediction can be tested by determining which acoustic characters most closely track the phylogeny; i.e., retain phylogenetic signal. Finally, if acoustic trait divergence in sympatry results from competition for acoustic rather than ecological space, we expect these traits to be uncorrelated with variables related to the ecological niche. This prediction can be tested by examining correlations between each call variable and body size (an important axis along which frogs partition ecological space; Parmalee 1999).

The North American tree frog genus *Pseudacris* (chorus frogs) is an excellent system for testing these predictions. Advertisement calls of *Pseudacris* include both single-note and pulsed calls that vary across a range of frequencies (Straughan 1975; Platz and Forester 1988; Owen and Tucker 2006). Chorus frogs (including spring peepers) generally breed syntopically and synchronically following winter and early spring rains, before most other species have emerged into the breeding pools (E. C. Moriarty, unpub. data), and then disappear after their short breeding season. Any occasional other species breeding at this time (e.g., true frogs [*Rana*] or cricket frogs [*Acris*]) is acoustically very

different than *Pseudacris* in several temporal or frequency parameters of the call. Thus, other *Pseudacris* species are the primary competitors for acoustic space during the early spring breeding season, and represent the entire acoustic community at most sites.

An advantage of this system is that consistent mechanisms of sound production among chorus frog species allow comparison of homologous call characters. Additionally, community structure and behavioral interactions among members of this group are well studied (Brandt 1936; Harper 1937, 1939; Schwartz 1957; Gosner and Black 1958; Michaud 1964; Gerhardt 1973; Caldwell, 1987). Geographic ranges and overlap among taxa have been well documented using museum records and molecular data (Lannoo 2005; Moriarty et al., in press). Finally, a well-supported phylogeny is available (Moriarty and Cannatella, 2004; see additional data below).

This study has four goals, the first three of which relate to the hypothesis that acoustic space is partitioned in these small acoustic communities. First, we ask whether sympatric species pairs are more acoustically divergent than allopatric species pairs. Second, we ask which characters are associated with partitioning of acoustic space. Third, we examine correlations between call variables and body size to determine which characters are influenced by size-related ecology. Fourth, we re-examine the general hypothesis (Ryan, 1988; Cocroft and Ryan 1995) that physiologically-controlled call characters are more evolutionarily labile than morphologically-controlled characters. We test these hypotheses using a phylogeny from new mitochondrial and nuclear DNA sequences and a dataset of 15 call characters measured across the genus *Pseudacris*.

4.2 MATERIALS AND METHODS

Sampling. We recorded and collected 15 species of *Pseudacris* from 21 populations across North America. For five species, multiple populations were recorded to reflect intraspecific call variation. At the time call sampling was done, we sampled all recognized species. However, recent work has clarified species boundaries. The former distribution of *triseriata* is now known to include some populations of *P. maculata*, a new undescribed species under description (Moriarty et al. in press), as well as true *P. triseriata*. Thus, our original recordings were not made in the proper location to capture true *P. triseriata*. Also, only data from the southern species (*P. hypochondriaca*) of the three species formerly included in the Pacific chorus frog (recently divided into *P. regilla*, *P. sierra*, and *P. hypochondriaca*; Recuero et al. 2006a, 2006b) were examined here. Most species were recorded in allopatric locations with respect to *Pseudacris* species with similar calls. Areas where reproductive character displacement is known to occur (Fouquette 1975) were avoided. This sampling strategy minimizes the effect of call divergence between sympatric species.

Ten or more individuals were recorded from all species except *Pseudacris kalmi* and *P. streckeri*, each represented by eight individuals. Ten or more calls were recorded from most individuals, resulting in a total dataset of 2611 calls (Supplemental Data 4.1). Only advertisement calls were analyzed because other call types, such as aggressive or courtship calls, are structurally different (Owen and Tucker 2006).

Acoustic analysis. A Sennheiser ME67 directional microphone was used to record calls onto TDK MA90 metal bias tape cassettes with a portable Sony stereo cassette recorder (WM-D6C). The microphone was held at approximately one meter from the calling frog during recording. The frog was then captured and the location and temperature of its position were noted. Frogs were euthanized, tissue samples were removed, and specimens were preserved and deposited into the Texas Memorial Museum (Austin, Texas) under IACUC protocol 06022701. Snout-urostyle length of preserved specimens was measured with a precision of 0.01 mm using digital calipers.

Recordings were digitized using SoundEdit16 version 2 (Macromedia) at a sampling rate of 44100 Hz with sample size of 16 bits. Calls were analyzed using SoundRuler version 0.941 (http://soundruler.sourceforge.net/; reviewed by Bee 2004). This program was designed to accommodate the variety of calls in *Pseudacris*. Frequency measurements were taken from spectrograms generated with FFT length of 1024 samples and 900 samples of overlap among subsequent FFTs. All call variables were taken directly or calculated from SoundRuler's raw data output. Definitions of call characters are in Supplemental Data 4.2.

Because some call variables change with the frog's temperature, we corrected the appropriate variables to a common temperature of 14°C using linear regression analyses (Sokal and Rohlf 1995). All fifteen call variables were regressed against temperature for all species using an $\alpha = 0.01$ to assess whether a variable was significantly correlated with temperature. If a variable's correlation with temperature was significant in three or

more species, that variable was corrected in all species; otherwise, the variable was not corrected. Calls were corrected using either a species-specific slope (when significant) or the significant slope of the species with the most structurally similar call. Structural similarity was established by conducting a discriminant function analysis of the raw call variables by species and using the Mahalanobis distance between species centroids to identify the most similar species. This approach was taken because we observed that irrespective of phylogenetic relationships, species with comparable calls are affected similarly by temperature. Four call variables were strongly correlated with temperature and were thus corrected to the common temperature: call length, call rise time, call rate, and call period. These results are consistent with those from intraspecific studies of various anurans (Harper 1937; Bellis 1957; Zweifel 1959; Michaud 1964; Zweifel 1967, 1970; Platz 1989; Gerhardt and Huber 2002; Forester et al. 2003).

Selection and classification of call characters. We selected call characters known to be salient for species recognition in frogs (Klump and Gerhardt 1987; Gerhardt 1991; Gerhardt 1994; Gerhardt 1996; Murphy and Gerhardt 2000; Gerhardt and Huber 2002). Characters not known to be important in mate recognition but that showed low intraspecific and high interspecific variation were also included. To ensure structural homology of call components, we define a call as the sound produced by a single exhalation during vocalization. We define a pulse as a section of the call delimited by amplitude decreases below 50% of the maximum call amplitude.

Comparative and experimental studies of call production have suggested that call characters fall into two natural categories (Drewry et al. 1982; Martin 1972). The frog's physiology primarily controls temporal properties of the call, including call duration, call shape, call rhythm, and pulse number. These characters are produced by active contractions of the body wall and laryngeal muscles as the frog forces air from the lungs (Schmidt 1965; Martin 1972; Martin and Gans 1972). Morphology, including the shape and size of the frog's larynx and associated arytenoid cartilages (Martin 1971, 1972; reviewed in Schneider 1988), controls mostly spectral properties of calls, including dominant frequency and relative energy. A dataset of 15 call variables was constructed to examine call evolution across the genus *Pseudacris*. Within this dataset, six characters have been surmised to be under morphological control and eight under physiological control, and one has not been studied in detail (Table 4.1).

We examined the distribution for each call character for each species. Normality was tested by calculating skewness and kurtosis for each distribution and identifying significant deviations from expected values (Sokal and Rohlf 1995). Within a species, most variables were normally distributed. Because transformation did not improve the overall number of species with normal distributions, all analyses were performed on the untransformed data.

DNA sequencing. Sequence data for a ~2.4kb region spanning the 12S rRNA, tRNA-val, and 16S rRNA mitochondrial genes were collected following Moriarty and Cannatella (2004). Seven new sequences from this region were added to the Moriarty and

Cannatella (2004) dataset. Data were also collected for the first exon of the nuclear gene rhodopsin. Two primers were used to amplify 292 bp: 5' to 3' Rhod1U AACGGAACAGAAGGCCCAAACTT (modified from Hoegg et al. 2004) and Rhod1L GCCAAAGCCATGATCCAGGTGA (designed in our lab). PCR conditions were as follows: 1) 2 min 92°C, 2) 30 sec 92°C, 3) 45 sec 48-55°C, 4) 1 min 72°C, 5) 7 min 72°C, and 35 cycles of steps 2-4. Annealing temperature (step 3) was adjusted within the range above to optimize amplification.

Samples were purified using the Viogene Gel-M™ gel extraction protocol. Sequencing reactions were done with the primers listed above, using the ABI BigDye version 3.1 terminator ready-mix. Several internal primers were developed to obtain complete sequence data from problematic samples. These primers are: 5' to 3' RhodE1U GAAGGCTTCTTTGCTACYCTTGGTG, RhodE2U GCTTCTTTGCTACYCTTGGT, RhodE3U GATATTCACACCCCATGCTAAGCAA, RhodI4U AGGTGGMAGATAGTTTAGTT, RhodI5U GTGGMAGATAGTTTAGTTGGGAATG, RhodE1L GGACCAAAGGGCAATTTCACCTGTC, RhodE2L ACCAGGGACCAAAGGGCAATTTCAC, RhodI4L TCMTGATACWTCACAGYCTG, and RhodI5L CTGCMATGTAAAATGGCATATAC (designed in our lab). An ABI 3100 PRISM™ sequencer was used to sequence samples (Applied Biosystems Inc.).

Phylogenetic analyses. Contiguous sequences were constructed from 8 overlapping mitochondrial fragments and 2-11 nuclear fragments using Sequencher 4.5 (GeneCodes Corp.). Sequence data were aligned using Clustal X 1.8 (Thompson et al., 1997).

Alignments were examined and adjusted manually to minimize informative sites; regions of ambiguous alignment were defined as character sets and excluded from further analyses. To confirm the alignment, nuclear data were translated to codons using the universal genetic code in MacClade 4.08 for OSX (Maddison and Maddison, 2005). Data partitions and character sets were also defined using MacClade.

The mitochondrial and nuclear datasets were concatenated and phylogenetic analyses were conducted simultaneously on six data partitions: 12S, tRNA-val, 16S (mitochondrial genes), and first, second, and third codon positions of rhodopsin (nuclear gene). Prior to phylogenetic analysis, the appropriate model of evolution for each partition was chosen using the Akaike information criterion (AIC) in ModelTest 3.06 (Posada and Crandall 1998). We performed four independent Bayesian analyses using MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003) with four heated chains for 300,000 generations (sampling every 100 generations). Convergence was determined by comparing bipartition posterior probabilities across the four runs, and the first 166,000 samples were discarded as burnin from each analysis. The remaining samples were then combined using a majority-rule consensus tree to calculate the posterior probabilities. Branch lengths were obtained by averaging branch lengths across all samples that included the bipartition (posterior probability of the branch length).

Acoustic niche partitioning test. We categorized each of the 105 pairwise combinations of taxa as sympatric or allopatric. Geographic overlap between species was calculated from range maps constructed from county-level museum records compiled by Lannoo

(2005). Additional information was obtained from a detailed molecular phylogeographic study of *Pseudacris* (Moriarty et al. in press). To estimate degree of overlap, the area of sympatry between two species was divided by total area of the species with the smaller range (Chesser and Zink 1994). Each species pair was then placed into one of two categories, allopatric (<10% overlap) or sympatric (>10% overlap). This degree of overlap was chosen because it represented a natural break in the percent overlap data. Of the 105 pairs, 29 were sympatric and 76 were allopatric. Geographic overlap values between 5% and 20% were also tested and produced consistent results.

We quantified call distances between each species pair using a multivariate approach. First, principal component analyses (PCA; from correlation matrices, using JMP 5.1 SAS Institute Inc.) were performed on species means of the physiology-controlled characters. Second, pairwise call distances between species were calculated using scores on PC1. The mean pairwise call distance was then calculated for each category, allopatric pairs and sympatric pairs. The difference in means between categories was calculated and compared to a null distribution created by randomizing species pairs between the two categories for 1000 replicates. This test was repeated for the morphology-controlled characters. Because we predicted that sympatric pairs would be more divergent than allopatric pairs, a one-tailed test was used.

Phylogenetic effect on call evolution. We used a generalized least squares (GLS) approach to assess the degree of phylogenetic effect on character evolution, using a maximum likelihood approach implemented in the program Continuous (Pagel 1994,

1997, 1999). The estimated parameter λ equals 1.0 if character evolution is explained perfectly by the phylogeny and λ equals 0 if phylogenetic history has no effect on character evolution. Characters that have evolved under the Brownian motion model are expected to exhibit a strong phylogenetic effect (λ = 1.0; Pagel 1999; Freckleton et al. 2002). Likelihood-ratio tests are used to test if λ is significantly different than predicted by the null hypothesis (λ = 1). If the null hypothesis is rejected, the interpretation is that character evolution cannot be explained by a neutral model of evolution, suggesting that the character may be under selection (Darst et al. 2005).

We also used the software Continuous to assess changes in the rate of trait evolution by estimating the parameter δ (Pagel 1999). If most trait evolution has occurred early in the radiation of *Pseudacris* then $\delta < 1.0$. This scenario corresponds to early divergence of a call trait variable deep in phylogenetic history, such that these ancestral species might easily share geographic space because their acoustic niches are separated. If trait evolution has largely occurred late in the phylogeny, near the tips of the tree, then $\delta > 1.0$. This situation would suggest that call variables diverged recently, providing support for recent divergence of acoustic characters.

Correlations between traits were tested within a phylogenetic context by estimating λ under the null and alternative hypotheses, where covariance between two traits is constrained to be 0 under the null hypothesis (Pagel 1994, 1997, 199). Correlations between call variables (or their multivariate counterparts) and body size

were tested to determine whether signal divergence is correlated with ecological divergence.

Character evolution along the phylogeny was assessed using mean population character values (Fig. 4.1). To obtain population means, each call variable was averaged across calls within an individual, and then averaged across individuals within a population (21 populations; Supplemental Data 4.1). Parameters were estimated for each call variable and likelihood-ratio tests were performed to test for significance.

Call variables were reduced to principal components using JMP 5.1 (SAS Institute Inc.). These analyses were performed to remove some of the correlations among variables and to identify differences in degree of phylogenetic effect among characters and character suites. Principal component analyses were performed on population means for each variable.

We performed the first set of principal component analyses on character "suites." Here, we combined related measures of a particular call feature. The suites examined here include call dominant frequency (composed of dominant frequency begin, end, and peak), call relative energy (call relative energy begin and end), call duration (call length, call rise time, call fall time), and call rhythm (call rate, call period, and call duty cycle).

The second set of PC analyses was performed as described under "acoustic niche partitioning test." PC analyses were performed separately on the physiological and morphological categories; λ was estimated using population PC scores as trait values. To

test the hypothesis that physiology-controlled characters are more labile than morphology-controlled characters, we used a randomization approach to test for a significant difference in λ scores for the two categories. Call variables were randomized between categories, while maintaining the original number of variables in each group. PC analyses were then performed on the randomized set of characters and λ scores were estimated. This procedure was repeated 500 times and the differences in scores between the two categories were used to create a null distribution against which the observed difference was tested.

It is common, almost mandatory, in analyses of tables of statistics to "correct" the experiment-wise alpha (probability of a type I error) by application of the Bonferroni inequality (see for example, Rice 1989). This correction, however, is problematic in that it increases the probability of committing a Type II error (Perneger 1998). In addition, there is currently broad controversy regarding the proper application of this correction (Perneger 1998; Moran 2003; Nakagawa 2004). For these reasons, we did not apply the Bonferroni correction here. Although we conducted multiple tests on univarate characters and present these results for visual purposes, we rely on the single randomization test described above to evaluate the character lability hypothesis.

4.3 RESULTS

Phylogenetic relationships of *Pseudacris*. The Bayesian analysis of the combined mitochondrial and nuclear data supports a majority-rule tree consistent with the results of Moriarty and Cannatella (2004). The addition of nuclear data provided greater support for the basal position of *P. crucifer*/*P. ocularis* relative to the clade containing *P. ornata*, *P. streckeri*, and *P. illinoensis*, which was an unresolved node in the previous study (Fig. 4.1). Only four branches had Bayesian posterior probabilities less than 1.00; these are listed in Figure 4.1. Branch lengths are provided in Supplemental Data 4.3.

Acoustic niche partitioning. The randomization test supported the hypothesis that sympatric species are more acoustically divergent than allopatric species. The difference between allopatric and sympatric pairs was significant for the physiology PC1 scores ($p = 0.041$, one-tailed test) but not for the morphology PC1 scores ($p = 0.186$, one-tailed test). These axes explain 68% and 69% of the variation, respectively (Table 4.2).

Correlations between call variables and body size. No significant correlations were found between body size and any physiology-controlled character. In contrast, body size is strongly correlated with multiple morphology-controlled characters (Tables 4.1–4.3).

Phylogenetic effect on call evolution. For univariate characters, the null hypothesis of perfect covariance with phylogeny ($\lambda = 1$) was rejected for 5 of 8 physiology-controlled characters and 1 of 6 morphology-controlled characters (Table 4.1). These results should be regarded cautiously, however, because several of these characters are highly correlated.

When call variables were combined into character suites and analyzed with PCA, the null hypothesis was rejected for 2 of 2 physiology-controlled suites and for 0 of 2 morphology-controlled suites on PC1 (Table 4.3). The maximum likelihood estimate of λ for PC1 was significantly different than 1 for the physiology-controlled category (λ = 0.89, p = 0.03; likelihood-ratio test) but not for the morphology-controlled category (λ = 1.00, p = 1.00; likelihood-ratio test; Table 4.2). More directly, the randomization test rejected the null hypothesis that the difference in λ values for the physiological and morphological categories was due to chance (p = 0.04, one-tailed test). These results are consistent with the predictions of Cocroft and Ryan (1995) and Ryan (1988) that physiology-controlled characters should be more evolutionarily labile than morphology-controlled characters in the genus *Pseudacris*.

Rate of signal evolution. The GLS analysis found the δ scaling parameter (Pagel 1999) to be >1.0 for 10 of 15 call variables and significantly >1.0 for 4 of 15 variables (Table 4.1). This suggests that most change in call variables occurs near the tips of the tree. Values of δ are significantly >1.0 for PC2 of both physiology-controlled character suites (call duration: high call rise time and call fall time loadings and call rhythm: high call duty cycle loading), but not for PC1 or any axes of morphology-controlled characters (Table 4.3). The null hypothesis of gradual evolution was not rejected for PC axes representing combinations of physiology-controlled or morphology-controlled characters (Table 4.2), although δ>1.0 for all axes except PC1 of the morphology-controlled characters.

4.4 DISCUSSION

Our data demonstrate that the calls of sympatric species pairs are on average more divergent than the calls of allopatric species pairs, providing evidence that acoustic niche partitioning occurs in frog communities. Interestingly, the results suggest that for *Pseudacris* the divergence of physiology-controlled characters is more important for partitioning acoustic space than the divergence of morphology-controlled characters. This result is supported by Cocroft and Ryan (1995) and by the other major finding of this study, that physiology-controlled characters are more evolutionarily labile than morphology-controlled characters in chorus frogs. No correlations were found between physiology-controlled characters and body size, suggesting that acoustic differences in sympatry are not merely the result of ecological differentiation.

Comparison with previous work. Whereas there is some support for acoustic niche partitioning in other taxa (e.g., bats: Siemers and Schnitzler 2004; birds: Nelson and Marler 1990; cicadas: Sueur 2002), there is only equivocal support for this phenomenon from previous studies of anuran communities. In a study of three tropical tree frog communities, Duellman and Pyles (1983) predicted that species occupying the same acoustic space should be allopatric. Based on a cluster analysis of species using call variables, they identified 15 species pairs with similar calls. Six pairs were allopatric and nine were sympatric. Although they interpreted these results as evidence for acoustic niche partitioning, this proportion is not significantly greater than random expectation (one-tailed binomial test). In an examination of several frog communities, Chek et al.

(2003) predicted that species in a community should have calls that are evenly distributed across acoustic space. They tested this hypothesis using simulations of random call variables and found some support for their prediction in only for 3 of 11 communities.

Neither of the studies above directly tests the critical prediction of the acoustic niche partitioning hypothesis: that sympatric species pairs are more acoustically divergent than allopatric species pairs. Our study specifically tests this prediction, by comparing levels of call divergence for sympatric species pairs to the null expectation determined from allopatric species pairs. Additionally, earlier studies compared species from deeply diverged lineages with diverse mechanisms for signal production. Our study, in contrast, focuses on a single clade of closely related species. This is advantageous for two reasons. First, we can appropriately compare homologous characters of the signals. Second, we can identify characters important for differentiating recently diverged taxa. Therefore, given the contrasting phylogenetic scales and our results on character lability, it is not surprising that we find partitioning by physiology-controlled characters whereas previous studies claim some support for partitioning by morphology-controlled characters, especially those related to size (dominant frequency; Duellman and Pyles 1983; Hödl 1977; Drewry and Rand 1983; Chek et al. 2003).

Our results with respect to character lability may not apply all anurans, however. We might expect species with simple spectral structure and complex temporal structure (e.g., *Pseudacris*, *Bufo*) to have more labile temporal components. In contrast, we might predict that species with complex spectral structure and simple temporal structure (e.g.,

Leptodactylus) have more labile spectral components. Preliminary evidence from túngara frogs (*Engystomops*), which fall into the latter category, supports this prediction (S. Ron, ms), suggesting that some taxa diversify with respect to morphology-controlled (spectral) characters.

Processes driving acoustic niche partitioning. At least two processes can lead to the observed pattern of acoustic niche partitioning in *Pseudacris*. In the first, co-existence only occurs when signals of species are already sufficiently different to avoid acoustic interference (Passmore 1981). We refer to this as signal assortment, a co-option and modification of "size assortment," used by Losos (1990). This process has not been studied in detail. In the second, signals diverge in sympatry, thus reducing interference, and the divergent signal(s) may then spread into allopatric populations.

Can we discriminate between these two processes? If signal divergence has occurred, we expect to find a pattern of reproductive character displacement between taxa. If signal assortment is the basis for acoustic niche partitioning, we expect to find equivalent levels of signal differentiation among sympatric and allopatric populations of these species. These predictions are complicated, however, by the situation where signals diverge in sympatry and then spread into allopatry, thereby obliterating the pattern expected from signal divergence. For this reason, it may only be possible to determine the process driving acoustic niche partitioning if a clear pattern of reproductive character displacement is present.

Estimates of δ from the GLS analyses lend some support for the signal divergence hypothesis. Two-thirds of the characters show a trend of accelerated evolution near the tips of the tree (although the trend is significant for only four characters), suggesting more recent interspecific divergence. The pattern for call variables summarized by principal components (suites and categories) is consistent with this trend, except for PC axes with high loadings of dominant frequency variables. Interestingly, dominant frequency shows δ estimates consistently less than 1.00, whether individual variables, suites, or principal component axes are examined. Although these estimates are not significantly different from the expectation of gradual evolution, the trend suggests diversification of these variables early rather than late in the phylogeny.

Selection pressures driving signal divergence. Several types of selection pressures can lead to acoustic reproductive character displacement. First, interaction of non-hybridizing taxa possessing similar signals and the resulting loss of mating efficiency lead to divergence (facilitated reproductive character displacement, Howard 1993; Noor 1999). The strength of this selection is predicted to increase when predation pressures are high or when breeding resources (space or time) are in short supply. Second, limited ecological resources can lead to ecological character displacement (including divergence in body size) to reduce competition among species (Brown and Wilson 1956; Schluter 2000, 2001). If acoustic signals are correlated with ecological characters under selection, pleiotropic divergence of signals can occur. Third, maladaptive hybridization between acoustically-similar species can lead to reinforcement of male signals and female

preferences, resulting in reduction of signal confusion and decrease in frequency of hybridization (Dobzhansky 1940; Blair 1955, 1958; Howard 1993).

How can we distinguish among these selection pressures? Table 4.4 presents predictions for each type of selection. Using these, we can design critical tests for the primary selection pressure contributing to the observed pattern. To provide evidence for facilitated reproductive character displacement, no hybrids should be found in sympatry and females should require more time to choose conspecific signals in the presence of heterospecific signals (Howard, 1993; Noor 1999; Amézquita et al. 2006). To support ecological character displacement, a correlation between acoustic characters and an ecology-related character (e.g., body size or signal transmission environment) must be shown (Schluter 2000). To demonstrate reinforcement with reproductive character displacement, five conditions are needed (Howard 1993): 1) hybridization occurs in nature, 2) selection acts against hybridization, 3) female preferences have evolved in sympatry, 4) the displaced reproductive character is not correlated with ecological characteristics, and 5) signals are heritable.

Processes of acoustic niche partitioning in *Pseudacris*. Evidence to date provides support for the hypothesis of signal divergence (reproductive character displacement) in *Pseudacris*. For example, in the contact zone between *Pseudacris feriarum* and *P. nigrita*, the former species has undergone reproductive character displacement with respect to physiology-controlled call characters (pulse rate and pulse number; Fouquette 1975). Preliminary genetic and behavioral data suggest that this pattern may be due to

reinforcement (E. Moriarty unpub. data). Because hybridization is known between several other species pairs (*P. clarkii*/*maculata*, *P. kalmi*/*nigrita*, *P. brachyphona*/*feriarum*, *P. nigrita*/sp. nov.: Moriarty et al. in press; *P. clarkii*/sp. nov.: Michaud, 1964), signal divergence by reinforcement may be more common than previously thought. Future work will elucidate whether this selective force is primarily responsible for acoustic niche partitioning in chorus frogs. Several call variables (dominant frequency, relative energy) are highly correlated with body size, a character that is related to the ecological niche occupied by a species (Parmelee 1999). Our analyses indicate, however, that characters involved in acoustic niche partitioning (physiology-controlled variables) are not correlated with body size, thus providing evidence that acoustic characters have not simply diverged in sympatry as the result of ecological character displacement.

Our study found a significant difference in call variables in sympatric species, but not allopatric species. We interpret this pattern as acoustic niche partitioning. To identify the processes responsible for acoustic niche partitioning, future work should examine signals of a species in populations both allopatric and sympatric with another species. This fine-scale comparison will be necessary to test for reproductive character displacement. If not present, signal assortment rather than signal divergence is implicated (given the caveat described above). If signal displacement is detected, additional ecological, behavioral, and genetic data (Table 4.3) should be collected to differentiate among the selective pressures driving signal divergence.

4.5 CONCLUSION

In summary, divergence in advertisement calls is greater among sympatric species than allopatric species, indicating acoustic niche partitioning occurs in chorus frogs. Call components that are constrained by the frog's morphology show a stronger phylogenetic effect than components that are constrained by physiology, indicating that the latter characters are more evolutionarily labile in this clade. Chorus frogs partition acoustic space using labile physiology-controlled characters and not by morphology-controlled characters.

Table 4.1. Estimates of phylogenetic effect, mode of trait evolution, and correlation with body size for univariate call variables. Two categories of call characters were examined: physiology-controlled characters (P) and morphology-controlled characters (M). Phylogenetic effect is measured by λ, and p is the probability of the null hypothesis, $\lambda = 1$. Mode of trait evolution is measured by δ and p indicates the probability of the null hypothesis of gradual trait evolution (H_o: $\delta = 1$). With accelerated evolution near the tips of the phylogeny, $\delta > 1.0$ and with rapid evolution early in the phylogeny, $\delta < 1.0$. Correlations between body size and call variables are shown (ρ), and p indicates whether the null hypothesis of no correlation ($\rho = 0$) is rejected.

Variable	Category	λ	p($\lambda = 1$)	δ	p($\delta = 1$)	ρ	p($\rho = 0$)
Call length	P	0.86	0.01*	1.81	0.42	–	0.46
Call rise time	P	0.77	0.00*	2.31	0.24	–	0.84
Call fall time	P	0.54	0.00*	3.00	0.04*	–	0.42
Call rate	P	0.88	0.08	3.00	0.01*	–	0.09
Call period	P	0.87	0.01*	2.09	0.33	–	0.18
Call duty cycle	P	0.82	0.01*	0.57	0.49	–	0.73
Call shape onset	P	0.99	0.83	2.34	0.22	–	0.64
Pulse number	P	1.00	0.77	0.97	0.97	–	0.63
Dominant frequency begin	M	1.00	1.00	0.59	0.51	-0.73	0.00*
Dominant frequency end	M	1.00	1.00	0.70	0.66	-0.72	0.00*
Dominant frequency peak	M	1.00	1.00	0.69	0.65	-0.66	0.00*
Call relative energy begin	M	0.98	0.33	1.72	0.50	-0.69	0.00*
Call relative energy end	M	1.00	1.00	3.00	0.02*	–	0.27
Tuning of call	M	0.77	0.00*	2.54	0.22	–	0.47
Crest factor	?	0.00	0.01*	3.00	0.00*	–	0.20

Table 4.2. Estimates of phylogenetic effect, mode of evolution, and body size correlations for multivariate call variables and loadings of univariate characters on principal component axes. Physiology and morphology headers denote principal component analyses of physiology-controlled and morphology-controlled characters, respectively. Tests and symbols are described in Table 4.1. Proportion of variance explained by the PC axes is indicated. The lower portion of the table is presented to facilitate comparison of the the loadings of call variables on PC1 and PC2 with the comparative analyses. Two categories of call characters were analyzed: physiology-controlled characters (P) and morphology-controlled characters (M).

	Physiology		Morphology	
	PC1	PC2	PC1	PC2
λ	0.89	0.99	1.00	0.94
$p(\lambda = 1)$	0.03*	0.81	1.00	0.11
δ	1.75	1.86	0.80	1.59
$p(\delta = 1)$	0.45	0.39	0.79	0.51
ρ	–	–	-0.70	–
$p(\rho = 0)$	0.33	0.72	0.00*	0.34
Proportion of variance explained	0.68	0.16	0.69	0.24
Eigenvalue	5.47	1.31	4.16	1.44

Call Variables	Category	Loadings on PC Axes			
Call length	P	0.42	-0.09	–	–
Call rise time	P	0.40	-0.02	–	–
Call fall time	P	0.38	-0.21	–	–
Call rate	P	-0.36	-0.20	–	–
Call period	P	0.40	-0.13	–	–
Call duty cycle	P	0.38	0.27	–	–
Call shape onset	P	-0.22	0.58	–	–
Pulse number	P	0.19	0.69	–	–
Dominant frequency begin	M	–	–	0.47	0.24
Dominant frequency end	M	–	–	0.48	0.12
Dominant frequency peak	M	–	–	0.48	0.15
Call relative energy begin	M	–	–	0.47	0.10
Call relative energy end	M	–	–	-0.25	0.64
Tuning of call	M	–	–	0.19	-0.70

Table 4.3. Estimates of phylogenetic effect, mode of evolution, and body size correlation for multivariate character suites. Suites are composed of physiology-controlled (P) or morphology-controlled (M) characters (see text). Tests and symbols are described in Table 4.1. Proportion of variance explained by the PC axes is indicated. Loadings of univariate characters on principal component axes are not shown. λ, δ, and ρ were not estimated for call dominant frequency PC2 (n/a) because this axis explains less than 1% of the variation.

	Call Duration (P)		Call Rhythm (P)		Call Dom. Freq (M)		Call Rel. Ener. (M)	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
λ	0.85	0.00	0.84	0.90	1.00	n/a	0.99	0.96
p (λ = 1)	0.01*	0.00*	0.02*	0.04*	1.00	n/a	0.58	0.25
δ	2.00	3.00	2.12	3.00	0.63	n/a	3.00	1.92
p (δ = 1)	0.33	0.02*	0.27	0.04*	0.56	n/a	0.11	0.31
ρ	–	–	–	–	-0.72	n/a	0.56	-0.45
p (ρ = 0)	0.40	0.66	0.15	0.18	0.00*	n/a	0.01*	0.03*
Prop. Var. Expl.	0.91	0.09	0.83	0.11	0.99	n/a	0.70	0.30
Eigenvalue	2.73	0.27	2.50	0.34	3.00	n/a	1.41	0.60

Table 4.4. Predictions for processes driving acoustic niche partitioning. Critical predictions supporting each process are in bold. The question mark means a particular process may or may not produce the phenomenon listed in the first column.

RCD	Differential Community Formation	Facilitated RCD	Ecological CD	Reinforcement +
Hybridization	No	**No**	Yes/No	**Yes**
Delayed mate choice in sympatry	No	**Yes**	?	?
Correlation with ecological character	?	No	**Yes**	No
Female preference evolution in sympatry	No	?	?	Yes
Male signal evolution in sympatry	No	Yes	Yes	Yes

Supplemental Data 4.1. List of specimens recorded and sequenced. Population numbers correspond to tips of the phylogeny on Fig. 4.1. Number of calls analyzed from each individual are is listed under No. Calls. Museum numbers refer to the Texas Natural History Collection, Texas Memorial Museum, University of Texas. Individuals not collected were not assigned a museum number. Genbank numbers are listed for the sequenced representative of each population. Collection locality is the county and state of origin.

Species	Pop.	No. Calls	Field No.	Museum No.	Genbank Mt	Genbank Nu	Collection Locality
Pseudacris brachyphona	9	14	ECM111	62305	AY291xxx	AY291xxx	Elmore:AL
Pseudacris brachyphona	9	13	ECM112	62306			Elmore:AL
Pseudacris brachyphona	9	14	ECM114	62307			Elmore:AL
Pseudacris brachyphona	9	19	ECM115	62308			Elmore:AL
Pseudacris brachyphona	9	14	ECM116	62309			Elmore:AL
Pseudacris brachyphona	9	13	ECM117	62310			Elmore:AL
Pseudacris brachyphona	9	12	ECM119	62312			Elmore:AL
Pseudacris brachyphona	9	11	ECM120	62313			Elmore:AL
Pseudacris brachyphona	9	11	ECM002365	na			Elmore:AL
Pseudacris brachyphona	9	19	ECM040	62303			Tallapoosa:AL
Pseudacris brachyphona	9	11	ECM041	62304			Tallapoosa:AL
Pseudacris brimleyi	10	12	ECM071	62329			Pitt:NC
Pseudacris brimleyi	10	10	ECM072	62330			Pitt:NC
Pseudacris brimleyi	10	12	ECM073	62331			Pitt:NC
Pseudacris brimleyi	10	12	ECM074	62332			Pitt:NC
Pseudacris brimleyi	10	11	ECM075	62333			Pitt:NC
Pseudacris brimleyi	10	11	ECM076	62334			Pitt:NC
Pseudacris brimleyi	10	14	ECM077	62335			Pitt:NC
Pseudacris brimleyi	10	13	ECM077a	na			Pitt:NC
Pseudacris brimleyi	10	12	ECM077b	na			Pitt:NC
Pseudacris brimleyi	10	13	ECM077c	na			Pitt:NC
Pseudacris brimleyi	10	12	ECM078	62336			Pitt:NC
Pseudacris brimleyi	10	12	ECM080	62338			Pitt:NC
Pseudacris brimleyi	11	12	ECM469	63573			Craven:NC

Pseudacris brimleyi	11	11	ECM470	63574	Craven:NC
Pseudacris brimleyi	11	13	ECM471	na	Craven:NC
Pseudacris brimleyi	11	8	ECM472	63575	Craven:NC
Pseudacris brimleyi	11	13	ECM473	63576	Craven:NC
Pseudacris brimleyi	11	12	ECM474	63577	Craven:NC
Pseudacris brimleyi	11	12	ECM475	63578	Craven:NC
Pseudacris brimleyi	11	13	ECM476	63579	Craven:NC
Pseudacris brimleyi	11	11	ECM477	63580	Craven:NC
Pseudacris brimleyi	11	14	ECM478	63581	Craven:NC
Pseudacris cadaverina	2	12	ECM143	62245	San Bernardino:CA
Pseudacris cadaverina	2	10	ECM143a	na	San Bernardino:CA
Pseudacris cadaverina	2	11	ECM145	62246	San Bernardino:CA
Pseudacris cadaverina	2	3	ECM150	62247	San Bernardino:CA
Pseudacris cadaverina	2	13	ECM150a	na	San Bernardino:CA
Pseudacris cadaverina	2	5	ECM151	62248	San Bernardino:CA
Pseudacris cadaverina	2	3	ECM153a	na	San Bernardino:CA
Pseudacris cadaverina	2	12	ECM155	62249	San Bernardino:CA
Pseudacris cadaverina	2	11	ECM155a	na	San Bernardino:CA
Pseudacris cadaverina	2	11	ECM156	62250	San Bernardino:CA
Pseudacris cadaverina	2	10	ECM156a	na	San Bernardino:CA
Pseudacris cadaverina	2	11	ECM156b	na	San Bernardino:CA
Pseudacris clarkii	12	13	ECM102	62253	Chautauqua:KS
Pseudacris clarkii	12	14	ECM102a	na	Chautauqua:KS
Pseudacris clarkii	12	14	ECM103	62254	Chautauqua:KS
Pseudacris clarkii	12	9	ECM103a	na	Chautauqua:KS
Pseudacris clarkii	12	14	ECM104	na	Chautauqua:KS
Pseudacris clarkii	12	16	ECM2464	na	Chautauqua:KS
Pseudacris clarkii	12	12	ECM2465	65043	Chautauqua:KS
Pseudacris clarkii	12	13	ECM2466	na	Chautauqua:KS
Pseudacris clarkii	12	15	ECM2468	na	Chautauqua:KS
Pseudacris clarkii	12	12	ECM2469	65045	Chautauqua:KS

Pseudacris clarkii	12	13	ECM2470	na	Chautauqua:KS
Pseudacris crucifer	4	14	Y1	62369	Lake:FL
Pseudacris crucifer	4	15	Y2	62370	Lake:FL
Pseudacris crucifer	4	15	Y3	62371	Lake:FL
Pseudacris crucifer	4	12	ECM2336	na	Calhoun:FL
Pseudacris crucifer	4	14	ECM2338	na	Calhoun:FL
Pseudacris crucifer	4	16	ECM2339	na	Calhoun:FL
Pseudacris crucifer	4	14	ECM2341	na	Calhoun:FL
Pseudacris crucifer	4	18	ECM2344	na	Calhoun:FL
Pseudacris crucifer	4	10	ECM2345	na	Calhoun:FL
Pseudacris crucifer	4	18	ECM2367	na	Calhoun:FL
Pseudacris crucifer	4	17	ECM2432	na	Calhoun:FL
Pseudacris crucifer	4	17	ECM2433	na	Calhoun:FL
Pseudacris crucifer	4	6	ECM2434	na	Calhoun:FL
Pseudacris crucifer	4	17	ECM2435	na	Calhoun:FL
Pseudacris crucifer	4	16	ECM2436	na	Calhoun:FL
Pseudacris feriarum	15	11	ECM387	63123	Macon:AL
Pseudacris feriarum	15	14	ECM 388	63124	Macon:AL
Pseudacris feriarum	15	7	ECM 389	63125	Macon:AL
Pseudacris feriarum	15	12	ECM 390	63126	Macon:AL
Pseudacris feriarum	15	12	ECM 391	63127	Macon:AL
Pseudacris feriarum	15	8	ECM 392	63128	Macon:AL
Pseudacris feriarum	15	7	ECM 393	63129	Macon:AL
Pseudacris feriarum	15	5	ECM 394	na	Macon:AL
Pseudacris feriarum	15	4	ECM 396	na	Macon:AL
Pseudacris feriarum	15	13	ECM 397	63131	Macon:AL
Pseudacris feriarum	15	6	ECM 398	63132	Macon:AL
Pseudacris feriarum	15	18	ECM 400	63133	Macon:AL
Pseudacris feriarum	16	12	ECM129	62271	Weakley:TN
Pseudacris feriarum	16	10	ECM130	62272	Weakley:TN
Pseudacris feriarum	16	11	ECM131	62273	Obion:TN

Pseudacris feriarum	16	11	ECM132	62274	Obion:TN
Pseudacris feriarum	16	13	ECM135	62276	Obion:TN
Pseudacris feriarum	16	14	ECM136	62383	Obion:TN
Pseudacris illinoensis	6	14	ECM001	62351	Clay:AR
Pseudacris illinoensis	6	16	ECM002	62352	Clay:AR
Pseudacris illinoensis	6	11	ECM003	62353	Clay:AR
Pseudacris illinoensis	6	11	ECM004	62339	Clay:AR
Pseudacris illinoensis	6	15	ECM005	62340	Clay:AR
Pseudacris illinoensis	6	15	ECM006	62341	Clay:AR
Pseudacris illinoensis	6	15	ECM007	62342	Clay:AR
Pseudacris illinoensis	6	13	ECM008	62343	Clay:AR
Pseudacris illinoensis	6	13	ECM009	62344	Clay:AR
Pseudacris illinoensis	6	13	ECM010	62345	Clay:AR
Pseudacris kalmi	14	13	ECM162	62354	Kent:MD
Pseudacris kalmi	14	13	ECM162a	na	Kent:MD
Pseudacris kalmi	14	10	ECM163	62355	Kent:MD
Pseudacris kalmi	14	10	ECM164	62356	Kent:MD
Pseudacris kalmi	14	12	ECM170	62358	Kent:MD
Pseudacris kalmi	14	11	ECM171	62359	Kent:MD
Pseudacris kalmi	14	11	ECM172	62360	Kent:MD
Pseudacris kalmi	14	11	ECM172a	na	Kent:MD
Pseudacris maculata	13	11	ECM099	62377	Douglas:KS
Pseudacris maculata	13	11	ECM099a	na	Douglas:KS
Pseudacris maculata	13	12	ECM100	na	Douglas:KS
Pseudacris maculata	13	11	ECM101	62378	Douglas:KS
Pseudacris maculata	13	12	ECM2448	65031	Douglas:KS
Pseudacris maculata	13	7	ECM2450	65033	Douglas:KS
Pseudacris maculata	13	13	ECM2452	65035	Douglas:KS
Pseudacris maculata	13	16	ECM2454	65036	Douglas:KS
Pseudacris maculata	13	12	ECM2456	65038	Douglas:KS
Pseudacris maculata	13	12	ECM2457	65039	Douglas:KS

Pseudacris maculata	13	13	ECM2458	65040	Douglas:KS
Pseudacris maculata	13	12	ECM2459	65041	Douglas:KS
Pseudacris maculata	13	13	ECM2460	65042	Douglas:KS
Pseudacris maculata	13	14	ECM2462	na	Douglas:KS
Pseudacris maculata	13	10	ECM2463	na	Douglas:KS
Pseudacris nigrita	19	10	ECM024	62364	Brevard:FL
Pseudacris nigrita	19	10	ECM025	62365	Brevard:FL
Pseudacris nigrita	19	12	ECM026	62366	Brevard:FL
Pseudacris nigrita	19	11	ECM027	62367	Brevard:FL
Pseudacris nigrita	19	12	ECM028	62368	Brevard:FL
Pseudacris nigrita	21	14	ECM372	63201	Jefferson:FL
Pseudacris nigrita	21	13	ECM373	63202	Jefferson:FL
Pseudacris nigrita	21	14	ECM374	63203	Jefferson:FL
Pseudacris nigrita	21	12	ECM375	63204	Jefferson:FL
Pseudacris nigrita	21	13	ECM376	63205	Jefferson:FL
Pseudacris nigrita	21	12	ECM377	63206	Jefferson:FL
Pseudacris nigrita	21	11	ECM378	63207	Jefferson:FL
Pseudacris nigrita	21	10	ECM379	63208	Jefferson:FL
Pseudacris nigrita	21	14	ECM381	na	Jefferson:FL
Pseudacris nigrita	20	11	ECM062a	na	Barnwell:SC
Pseudacris nigrita	20	12	ECM065	62203	Barnwell:SC
Pseudacris nigrita	20	8	ECM066a	na	Barnwell:SC
Pseudacris nigrita	20	11	ECM067	62204	Barnwell:SC
Pseudacris nigrita	20	10	ECM069	62206	Barnwell:SC
Pseudacris ocularis	3	20	ECM042	62231	Barnwell:SC
Pseudacris ocularis	3	12	ECM043	62232	Barnwell:SC
Pseudacris ocularis	3	12	ECM044	62233	Barnwell:SC
Pseudacris ocularis	3	11	ECM044a	na	Barnwell:SC
Pseudacris ocularis	3	15	ECM044b	na	Barnwell:SC
Pseudacris ocularis	3	15	ECM045	62234	Barnwell:SC
Pseudacris ocularis	3	13	ECM046	62235	Barnwell:SC

Pseudacris ocularis	3	15	ECM047	62236	Barnwell:SC
Pseudacris ocularis	3	15	ECM048	62237	Barnwell:SC
Pseudacris ocularis	3	5	ECM050	62239	Barnwell:SC
Pseudacris ornata	8	15	ECM033	62178	Barbour:AL
Pseudacris ornata	8	19	ECM034	62179	Barbour:AL
Pseudacris ornata	8	16	ECM035	62180	Barbour:AL
Pseudacris ornata	8	17	ECM037	62181	Barbour:AL
Pseudacris ornata	8	14	ECM038	62182	Barbour:AL
Pseudacris ornata	7	11	ECM057	62185	Barnwell:SC
Pseudacris ornata	7	12	ECM059	na	Barnwell:SC
Pseudacris ornata	7	12	ECM061	62186	Barnwell:SC
Pseudacris ornata	7	11	ECM062	62187	Barnwell:SC
Pseudacris ornata	7	13	ECM063	62188	Barnwell:SC
Pseudacris ornata	7	14	ECM063a	na	Barnwell:SC
Pseudacris ornata	7	10	ECM066	62189	Barnwell:SC
Pseudacris regilla	1	15	ECM140	62190	San Bernardino:CA
Pseudacris regilla	1	12	ECM140a	na	San Bernardino:CA
Pseudacris regilla	1	12	ECM140b	na	San Bernardino:CA
Pseudacris regilla	1	16	ECM141	62191	San Bernardino:CA
Pseudacris regilla	1	13	ECM142	62192	San Bernardino:CA
Pseudacris regilla	1	13	ECM144	62193	San Bernardino:CA
Pseudacris regilla	1	11	ECM147	62195	San Bernardino:CA
Pseudacris regilla	1	14	ECM147a	na	San Bernardino:CA
Pseudacris regilla	1	20	ECM148	62196	San Bernardino:CA
Pseudacris regilla	1	14	ECM149	62197	San Bernardino:CA
Pseudacris regilla	1	12	ECM152	62198	San Bernardino:CA
Pseudacris regilla	1	14	ECM153	62199	San Bernardino:CA
Pseudacris sp. nov.	18	16	ECM011	62255	Craighead:AR
Pseudacris sp. nov.	18	11	ECM012	62256	Craighead:AR
Pseudacris sp. nov.	18	13	ECM013	62257	Craighead:AR
Pseudacris sp. nov.	18	13	ECM014	62258	Craighead:AR

Pseudacris sp. nov.	18	11	ECM015	62259	Craighead:AR
Pseudacris sp. nov.	18	16	ECM016	62260	Craighead:AR
Pseudacris sp. nov.	18	14	ECM017	62261	Craighead:AR
Pseudacris sp. nov.	18	13	ECM018	62262	Craighead:AR
Pseudacris sp. nov.	18	15	ECM019	62263	Craighead:AR
Pseudacris sp. nov.	18	14	ECM020	62264	Craighead:AR
Pseudacris sp. nov.	17	10	ECM124	62269	Washington:LA
Pseudacris sp. nov.	17	10	ECM125	62379	Washington:LA
Pseudacris sp. nov.	17	14	ECM137	62277	Evangeline:LA
Pseudacris sp. nov.	17	14	ECM138	62278	Evangeline:LA
Pseudacris sp. nov.	17	10	ECM029	62265	East Baton Rouge:LA
Pseudacris sp. nov.	17	14	ECM030	62266	East Baton Rouge:LA
Pseudacris sp. nov.	17	14	ECM031	62267	East Baton Rouge:LA
Pseudacris sp. nov.	17	12	ECM304	63471	East Baton Rouge:LA
Pseudacris sp. nov.	17	15	ECM305	63472	East Baton Rouge:LA
Pseudacris sp. nov.	17	11	ECM306	63473	East Baton Rouge:LA
Pseudacris sp. nov.	17	12	ECM307	63474	East Baton Rouge:LA
Pseudacris sp. nov.	17	11	ECM308	63475	East Baton Rouge:LA
Pseudacris sp. nov.	17	14	ECM309	63476	East Baton Rouge:LA
Pseudacris sp. nov.	17	10	ECM310	63477	East Baton Rouge:LA
Pseudacris sp. nov.	17	13	ECM311	63478	East Baton Rouge:LA
Pseudacris sp. nov.	17	15	ECM313	na	East Baton Rouge:LA
Pseudacris streckeri	5	11	P3	62318	Travis:TX
Pseudacris streckeri	5	12	P4	62319	Travis:TX
Pseudacris streckeri	5	12	P5	62320	Travis:TX
Pseudacris streckeri	5	13	P6	62321	Travis:TX
Pseudacris streckeri	5	11	P7	62322	Travis:TX
Pseudacris streckeri	5	10	P8	62323	Travis:TX
Pseudacris streckeri	5	17	ECM021	62301	Travis:TX
Pseudacris streckeri	5	10	ECM023	62302	Travis:TX
Acris gryllus	-	-	ECM052	62372	Barnwell:SC

Hyla andersonii	-	-	54451	KU 207335	Burlington:NJ
Hyla chrysoscelis	-	-	T-1	n/a	Douglas:KS

Supplemental Data 4.2. Definitions of call characters examined in *Pseudacris*.

Call length	Duration of call from 10% maximum amplitude (call onset) to 10% maximum amplitude (offset)
Call rise time	Duration of call from 10% maximum amplitude (onset) to maximum amplitude
Call fall time	Duration of call from maximum amplitude to 10% of maximum amplitude (offset)
Call shape onset	Time from 10% to 50% of maximum amplitude (onset)/time from 10% to 90% (onset)
Call rate	1/time from 10% maximum amplitude (onset) to 10% maximum amplitude (onset) for next call
Call period	Time from 10% maximum amplitude (onset) to 10% maximum amplitude (onset) for next call
Call duty cycle	Call length/Call period
Pulse number	Number of pulses
Call dominant frequency begin	Call dominant frequency at 10% maximum amplitude (onset)
Call dominant frequency end	Call dominant frequency at 10% maximum amplitude (offset)
Call dominant frequency peak	Call dominant frequency at the call maximum amplitude
Call relative energy begin	Call energy from 10% to 50% maximum amplitude call onset/Call energy from 90% to 50% maximum amplitude call offset
Call relative energy end	Call energy from 90% to 50% maximum amplitude call offset/Call energy from 90% to 50% maximum amplitude call offset
Tuning of call (Q3)	Width of dominant frequency at half the height of spectrum peak/dominant frequency, in an amplitude spectrum centered on the point of maximum amplitude
Crest factor	Maximum amplitude/root mean square of amplitude

Supplemental Data 4.3. Branch lengths in substitutions per site from Figure 4.1.

Branch Number	Branch Length
1	0.02779
2	0.04393
3	0.04492
4	0.05267
5	0.01543
6	0.01550
7	0.00649
8	0.00743
9	0.01429
10	0.00128
11	0.00290
12	0.00385
13	0.00205
14	0.00600
15	0.00476
16	0.00291
17	0.00167
18	0.00133
19	0.00226
20	0.00205
21	0.00166
22	0.00081
23	0.01711
24	0.01118
25	0.00730
26	0.00542
27	0.00994
28	0.00288
29	0.01647
30	0.00508
31	0.00956
32	0.01935
33	0.02745
34	0.03680
35	0.02374
36	0.05484
37	0.01015
38	0.03333
39	0.01225
40	0.04529
41	0.02906

42	0.12924
43	0.03424
44	0.03144
45	0.11606

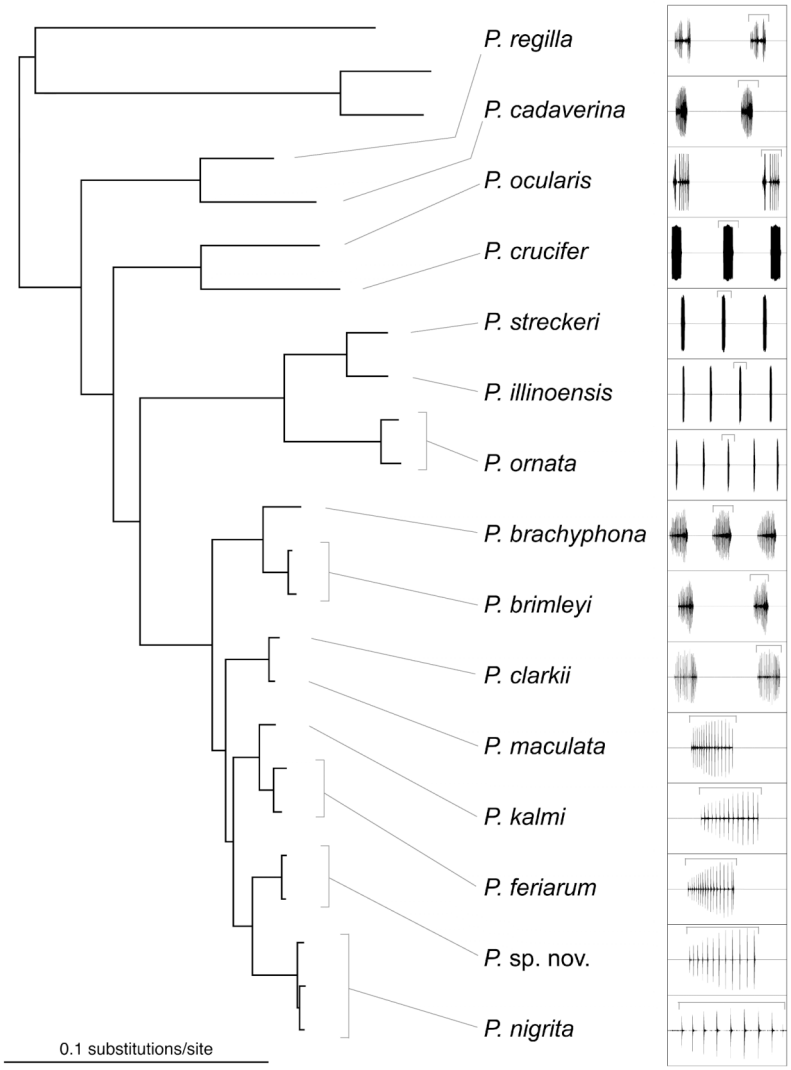

Figure 4.1. Phylogram and oscillograms of *Pseudacris* species. Branches 42-44 are outgroup taxa (*Acris gryllus*, *Hyla andersonii*, and *H. chrysoscelis*, respectively). Branch lengths are listed in Table 4.2. Only four branches have Bayesian posterior probability values less than 1.00. These are: branch 22 (0.66), branch 28 (0.70), branch 30 (0.84), branch 39 (0.96). Oscillograms are from natural calls that were recorded at temperatures varying from 12.4°C to 17.4°C. Time is indicated on the x-axis and amplitude on the y-axis. A gray bracket on each oscillogram indicates a full call. Warmer temperatures result in greater horizontal spacing of pulses and calls. Call temperatures are listed from top to bottom: *P. regilla* (12.4), *P. cadaverina* (12.4), *P. ocularis* (17.3), *P. crucifer* (14), *P. streckeri* (15), *P. illinoensis* (14), *P. ornata* (15.2), *P. brachyphona* (16), *P. brimleyi* (15), *P. clarkii* (12.7), *P. maculata* (17.4), *P. kalmi* (13), *P. feriarum* (13.8), *P.* sp. nov. (11.8), and *P.nigrita* (12.6°C).

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Chapter 5

Heterospecific Overlap Generates Divergent Reproductive Character Displacement in Chorus Frogs

Abstract. Recent neural network models have suggested that geographic overlap between two or more species can promote divergence of mate recognition systems among conspecific sympatric populations. This can occur when reinforcement leads to reproductive character displacement along different signal axes in different populations. Here, I test this hypothesis by assessing patterns of acoustic signal divergence in a contact zone between two frog species, *Pseudacris feriarum* and *P. nigrita* in the southeastern United States. In addition, I test one criterion for reinforcement, by examining the evolution of female preferences in the contact zone. Patterns of signal evolution based on analysis of 16 populations indicate: 1) the magnitude of reproductive character displacement in sympatry varies geographically, 2) only *P. feriarum* has diverged in sympatry, and 3) populations of *P. feriarum* have displaced along different axes of the signal. Preference experiments on females from an allopatric and a sympatric *P. feriarum* population indicate that in sympatry the propensity of females to hybridize has been reduced by ~60%. Data also suggest that divergence of the female preference rather than the male call has led to greater reproductive isolation between taxa. Sympatric females strongly prefer sympatric over allopatric *P. feriarum*, providing evidence that reinforcement is driving displacement in this system. Geographic variation in male

signals and published phylogeographic data support the hypothesis that these taxa are in an early stage of speciation in the northern part of their contact zone and later stage in the southern region. Signal and preference data suggest that overlap with heterospecific taxa can promote divergent reproductive character displacement, potentially leading to reproductive isolation among conspecific populations.

5.1 INTRODUCTION

Effective communication between conspecific individuals is essential for maintenance of species (Dobzhansky 1940; Blair 1958; Littlejohn and Michaud 1959; Blair and Littlejohn 1960). A signaler must convey information about species identity to a receiver to procure a conspecific mate. Failure to do so may lead to hybridization, which tends to erode the boundaries delineating species (Sanderson et al. 1992; Howard et al. 2004). Though the importance of communication in the maintenance of species is clear, we have an inadequate understanding of how communication systems diverge in nature and how the evolution of such systems contributes to the process of speciation (Howard 1993; Noor 1999).

Interference of reproductive signals by heterospecific individuals can decrease the efficiency of signal propagation and hinder the ability of a receiver to decode information (Gerhardt and Huber, 2002). Individuals may waste time, energy, and gametes attracting, approaching, or mating with heterospecifics. As a result of this interference, species may evolve greater differences in signals and signal preferences in sympatry relative to

allopatry. This pattern is called reproductive character displacement (RCD; Brown and Wilson 1956).

Divergence of reproductive characters can result from several evolutionary processes (Howard, 1993; Noor, 1999). Selection against signal interference in two non-hybridizing species may cause divergence (facilitated RCD or noisy neighbors hypothesis: Littlejohn, 1965; Howard, 1993; Noor 1999; Amezquita 2006). Selection against ecological interference in two non-hybridizing species may indirectly cause divergence of reproductive characters if they are correlated with non-reproductive characters, such as body size, during ecological adaptation in sympatry (ecological RCD hypothesis: Brown and Wilson 1956; Schluter, 2001). Selection against hybridization can also drive divergence, causing evolution of greater prezygotic isolation in sympatry (reinforcement hypothesis: Dobzhansky 1940; Blair 1955, 1958). The amount of data implicating each of these factors varies. Because the processes are not mutually exclusive, most studies attempt to identify the primary selective force driving the pattern of RCD.

Substantial effort has been invested in documenting patterns of RCD in sympatry. Patterns observed include symmetric signal divergence (both species; Littlejohn, 1965; Gerhardt and Huber, 2002), asymmetric signal divergence (one species; Fouquette, 1975; Butler, 1988; Loftus-Hills and Littlejohn, 1992), displacement of the receiver preference but not displacement of the signal (Marquez and Bosch, 1997; Ratcliffe and Grant, 1983; Gerhardt, 1994), and displacement of both the preference and the signal (Littlejohn and Loftus-Hills, 1968; Sætre et al., 1997; Marshall and Cooley, 2000; Höbel and Gerhardt

2003). Evidence supporting a role for reinforcement (Waage, 1975, 1979; Gerhardt, 1994; Noor, 1995; Sætre et al., 1997; Rundle and Schluter, 1998; Hoskin et al. 2005; Kronforst et al. 2007) or ecological factors (Nagel and Schluter, 1998; Podos, 2001) in driving these patterns is accumulating rapidly. Only a few studies, however, have tested whether levels of RCD vary among sympatric populations (Fouquette, 1975; Waage, 1975, 1979; Loftus-Hills and Littlejohn, 1992; Gabor and Ryan, 2001). Additionally, it is unclear whether the same components of the signal always diverge in different populations or whether different signal components may evolve (Pfennig and Ryan 2006).

Here, I examine geographic variation in patterns of RCD in the acoustic signals of chorus frogs (*Pseudacris*). I focus on interactions between *Pseudacris feriarum* and *P. nigrita*, which form a narrow contact zone in the southeastern United States. In addition, I test one criterion for reinforcement (Howard 1993) by examining the evolution of female *P. feriarum* preferences in sympatry. Previous research in this contact zone demonstrated a strong pattern of RCD in sympatric populations of *P. feriarum* in a localized area of southern Alabama/Georgia and the Florida Panhandle (Fouquette 1975). I extend this work by examining sympatric and allopatric populations of both species across a broader geographic area, from Florida to Virginia.

I address three questions regarding evolution of reproductive signals: 1) Is there heterogeneity in the amount of sympatric divergence among localities? 2) Does RCD occur in both species? 3) Have the same signal components diverged across the contact zone? With respect to female preference evolution, I ask: 4) Do females prefer

conspecific males? 5) Has the female preference evolved in sympatry? 6) Has the propensity to hybridize been reduced? 7) Is the displacement perceptible to the opposite sex? I address these questions by measuring levels signal divergence across multiple transects spanning the contact zone and by testing female preferences in one of these transects.

5.2 MATERIALS AND METHODS

Male Signal Analyses

Sampling. To examine geographic variation in acoustic signals, male *Pseudacris feriarum* and *P. nigrita* were recorded and collected from eight populations in allopatry (four per species) and eight populations from sympatry (four per species) in the southeastern United States (Fig. 5.1). These populations correspond to four rough transects spanning the contact zone; each transect includes an allopatric *P. feriarum* population, an allopatric *P. nigrita* population, and sympatric *P. feriarum* and *P. nigrita* from the same locality. The transects span the following geographic regions: 1) Alabama/Florida (transect studied by Fouquette [1975]), 2) Georgia/Florida, 3) South Carolina/Georgia, and 4) Virginia (Fig. 5.1). Hereafter, the first state listed will be used as the transect name. Because *P. nigrita* do not exist in allopatry in Virginia and because loss of suitable habitat in eastern North Carolina hindered my ability to locate *P. nigrita* at historic localities, this population was lacking from the Virginia transect. Instead, I increased the number of allopatric *P. nigrita* populations by including one from southern

Mississippi. None of the statistical analyses described below depend on the geographic layout of the transects, which are presented here for visual purposes only. Representative calls of each species are presented in Figure 5.2.

During the course of this study, our genetic work revealed that populations of putative *P. feriarum* in Louisiana and Mississippi are actually an undescribed, cryptic species (Moriarty et al. in press) that hybridizes with *P. nigrita* in a narrow contact zone (Gartside 1980). Genetic analysis of all other putative *P. feriarum* and *P. nigrita* populations included here along with a broader population and species sample (250+ populations) revealed that each species forms a monophyletic group (Moriarty et al. in press). Therefore, in this study, I am examining interactions between only two species.

Areas of sympatry between the two species were located based on published studies, museum databases, personal communications, and my field surveys. In the contact zone, *P. feriarum* and *P. nigrita* can be found calling in close proximity, often alternating calls with each other (Crenshaw and Blair 1959; E. Moriarty, unpub. data). The species show some ecological separation in sympatry: *P. feriarum* prefers cypress/gum swamps, whereas *P. nigrita* prefers pine flatwoods ponds (Carr 1940; Crenshaw and Blair 1959). Interaction between the species most often occurs at the interface of these habitats or in artificial habitats, such as roadside ditches (E. Moriarty, unpub. data). At several of these sites (in Florida and Virginia), putative hybrids have been found that are morphologically and acoustically intermediate between the parental species (E. Moriarty, unpub. data). Where possible, individuals calling syntopically were

sampled; to obtain a large enough sample, however, frogs were collected from some ponds that were dominated by one of the species.

A total of 318 individuals were recorded from the 16 populations. Each population included 5 to 43 individuals (mean=18 frogs). Between 2 and 17 calls were sampled per individual (mean=10 calls), depending on the quality of the recording and activity level of the frog (Supplemental Data 5.1). Calls were recorded onto TDK MA90 metal bias tape cassettes with a Sony stereo cassette-recorder (WM-D6C) using a Sennheiser ME67 directional microphone. The microphone was held roughly one meter from the calling individual during recording. When possible, the frog was then captured. In all cases, the temperature of the frog's calling location (aquatic or terrestrial) was measured. Tissue samples were taken from euthanized frogs (following IACUC protocol 06022701) and voucher specimens were deposited into the Texas Memorial Museum (Austin, Texas).

Acoustic Analyses. Recordings were digitized using SoundEdit16 version 2 (Macromedia) with a sample size of 16 bits at a sampling rate of 44100 Hz. Calls were analyzed using SoundRuler version 0.941 (http://soundruler.sourceforge.net/; reviewed by Bee 2004). Frequency measurements were taken from spectrograms generated with FFT length of 1024 and 900 samples of overlap among subsequent FFTs. All call variables were taken directly or calculated from SoundRuler's raw data output.

Each individual call file was analyzed twice: the first time, only the highest quality calls were analyzed (though calls were not necessarily sequential) to collect data

related to the spectral (e.g., dominant frequency) and temporal structure (e.g., pulse rate) within calls. The second time, the longest series of sequential calls was measured, to collect information related to the temporal spacing between calls (e.g., call rate). In some cases, the quality of a recording was high enough that both categories of data could be measured at once. The two datasets, containing 3046 and 2751 calls, respectively, were merged after averaging call characters within individuals (Supplemental Data 5.1).

A total of 16 call variables were examined to explore patterns of evolution in acoustic signals. These variables were chosen for two reasons: 1) they show high interspecific and low intra-population variation and/or 2) they are known to be important for species recognition in other frogs (Loftus-Hills and Littlejohn 1971; Klump and Gerhardt 1987; Gerhardt 1991; Gerhardt 1994; Gerhardt 1996; Murphy and Gerhardt 2000; Gerhardt and Huber 2002). The call variables, described in Supplemental Data 5.2, include both spectral characters and temporal characters.

Several components of the frog's acoustic signal are influenced by changes in temperature (Gerhardt and Huber 2002). To control for this effect, I tested for correlations between temperature and each call variable. If the effect of temperature was strong ($p<0.01$), I adjusted the variable to a common temperature of 14ºC, using species-specific slopes. Regression slopes used in the corrections are shown in Supplemental Data 5.3.

Statistical Analyses. Randomization tests were performed to address two questions: 1) Is there variation in the amount of divergence between sympatric *Pseudacris feriarum* and

P. nigrita among localities? and 2) In each sympatric locality, does reproductive character displacement occur in *P. feriarum* and/or *P. nigrita*?

Normality of signal variables was first assessed using a Shapiro-Wilk's test in R Version 1.16 (R Foundation for Statistical Computing), then a principal components analysis (PCA) was conducted for all individuals using JMP 5.1 (SAS Institute Inc.).

To address the first question, the standard deviation in level of divergence between sympatric populations was calculated and compared to a null distribution. For each sympatric locality, the level of divergence was quantified as follows: a random *P. feriarum* and a random *P. nigrita* were selected and the distance between the pair along PC1 was calculated (*d*). This was repeated 10,000 times (with replacement) and the mean *d* was calculated ($\bar{d}$). The variation in the level of divergence among localities was then calculated as the standard deviation of $\bar{d}$, denoted $\sigma_{\bar{d}}$, which was used as the test statistic. A point from the null distribution was generated by computing $\sigma_{\bar{d}}$ after randomizing individuals within species across the four localities (sample sizes were maintained). A total of 100,000 points were generated from the null distribution for this and all randomization tests that follow. The test statistic was considered to be significant for this and all subsequent randomization tests if it fell outside the 95% limits of this distribution. These analyses were repeated for PC2 and PC3.

To address the second question, the difference between the allopatric calls of the two species was compared to the difference between the allopatric call of one species and the sympatric call of the other species. This quantifies how much the signal of a species

has changed (displaced) since secondary contact relative to the pre-contact state. Allopatric individuals were pooled by species for this test. The test was performed for each of the four sympatric populations, for each of the two species, and along the first three PC axes for a total of 24 tests. An example of one of these tests follows. First, an allopatric *P. feriarum* and an allopatric *P. nigrita* were randomly drawn from the pooled sample and the distance between the pair along PC1 was calculated. This was repeated 10,000 times and the mean *d* was calculated ($\overline{d}_A$). Second, a random sympatric *P. feriarum* (e.g., from Florida) and a random allopatric *P. nigrita* (pooled sample) were selected and the distance between the pair along PC1 was calculated (*d*). This was repeated 10,000 times with replacement and the mean *d* was calculated ($\overline{d}_S$). After taking the absolute value of $\overline{d}_S$ and $\overline{d}_A$, the difference between the values was calculated (Δd). This number was used as the test statistic. A point from the null distribution was generated by computing Δd after randomizing individuals between the sympatric and allopatric *P. feriarum* groups (sample sizes were maintained). A sequential Bonferroni correction was applied to correct for multiple tests (Rice 1989).

A discriminant function analysis (DFA) of the 16 call variables was performed to test the null hypothesis that the sympatric populations are diverging with respect to the same components of the acoustic signal. The analysis was performed on the four sympatric *P. feriarum* populations using JMP 5.1, where call variables were stepped into the model until the next variable had a p-value > 0.05. This resulted in five call variables that were included in the analysis (pulse rate, pulse number, call rise time, call fall time,

and dominant frequency peak). Scores on the first two canonical axes (CVs) were saved for further analysis.

To determine which sympatric populations differ significantly from each other, Tukey-type randomization tests were conducted. To reduce the number of tests, populations were ranked by mean canonical score and only rank neighbors were compared. For each pairwise comparison, an individual was drawn randomly from each population and the distance between them along CV1 was calculated (*d*). This was repeated 10,000 times with replacement and mean *d* was used as the test statistic ($\bar{d}$). A point from the null distribution was generated by computing $\bar{d}$ after randomizing individuals between populations. This test was also conducted on CV2.

To calculate the standardized coefficients from the discriminant analysis, which indicate how important a call variable is in discriminating among groups, the canonical vector coefficients were multiplied by the pooled standard deviation within groups for each variable (equivalent to root mean square error). These coefficients were calculated for CV1 and CV2.

Female Preference Tests

To determine whether female preference evolution is driving male signal divergence, phonotaxis experiments were conducted on *Pseudacris feriarum* females from allopatric and sympatric populations (Macon/Lee Cos., Alabama and Liberty Co., Florida, respectively; Fig. 5.1). Following the general methodology of Ryan and Rand

(1999), I performed three binary mate-choice experiments on *P. feriarum* females. The females were given a choice between two acoustic stimuli in each experiment as follows: A) sympatric *P. feriarum* vs. *P. nigrita*, B) allopatric *P. feriarum* vs. *P. nigrita*, and C) sympatric *P. feriarum* vs. allopatric *P. feriarum*. Sympatric *P. nigrita* calls were used because preliminary analyses indicated that sympatric calls did not differ from allopatric calls in Florida. Tests were presented in random order for each female. Natural calls from these populations are shown in Figure 5.2.

Construction of Acoustic Stimuli. The three synthetic acoustic stimuli were constructed based on natural male calls from the local populations of females as follows: 1) pulse number from multiple calls per individual was extracted from the raw SoundRuler output, 2) the mode number of pulses was determined for the population, and calls containing the mode pulse number were extracted (other calls were not used further), 3) homologous pulses were aligned (e.g., pulse 1 from individual A was aligned to pulse 1 from individual B) and character data (e.g., pulse dominant frequency) were averaged across individuals, 4) the average call character values were used to synthesize acoustic stimuli for the phonotaxis experiments. Individuals used for constructing the stimuli include: 1) allopatric *P. feriarum*, 101 calls from 13 indivs., mode number pulses = 17, 2) sympatric *P. feriarum*, 178 calls from 18 indivs., mode number pulses = 29, 3) *P. nigrita*, 205 calls from 23 indivs., mode number pulses = 10 (Supplemental Data 5.4).

Individual pulses were synthesized using the program JOSHSYN (SYN16bt.exe) written by Joshua Schwartz that uses the following information: pulse rise time, pulse fall

time, rise time to half maximum amplitude, fall time to half maximum amplitude, pulse duration, fundamental frequency, dominant frequency, third harmonic frequency, relative amplitude of fundamental frequency, relative amplitude of third harmonic, maximum amplitude, and relative amplitude of each pulse (obtained from SoundRuler). After synthesis, pulses were assembled into calls using appropriate interpulse intervals (spacing between pulses) as described below.

Examination of the relationship between temperature and the call variables revealed that call duration is strongly correlated with temperature whereas pulse duration is not. This indicates that only the interpulse intervals change with temperature. Therefore, to construct stimuli with the proper temporal structure for experimental conditions, the following steps were performed: 1) interpulse intervals (IPIs) between successive pairs of pulses within the call were calculated, 2) proportion of total IPI time was calculated for each IPI, 3) homologous IPI proportions were averaged across individual frogs within the population, 4) population-specific regressions were calculated for temperature versus call duration, 5) call duration was corrected to the testing temperatures (18ºC and 20ºC, respectively) and total pulse duration within the call was subtracted to give the corrected total IPI, 6) mean IPI proportions were converted to absolute IPIs using the corrected total IPI, 7) mean absolute IPIs were inserted between synthesized pulses to construct the full acoustic stimuli.

Experimental Conditions. Experiments involving allopatric and sympatric females were conducted at Auburn University (Auburn, Alabama) and the Florida Department of

Environmental Protection research dormitory (Eastpoint, Florida), respectively, in 2004 and 2005. Each of these testing locations was within an hour drive of the frog collection localities. Females were collected from breeding ponds and tested within 48 hours of capture. Most females were collected in amplexus but gravid single females were also used for the experiments. Upon return to the research station, each female was separated from its male, and placed into a 6 x 6 x 2-inch plastic container filled with leaves and water and allowed to acclimate to room temperature in a darkened closet for at least two hours. A mixed *P. feriarum*/*P. nigrita* chorus was played to females during the acclimation period.

Phonotaxis tests were conducted in a 56-inch diameter wading pool containing approximately 2 inches of water. The pool was first lined with white duct-tape to permit easier visualization of frogs. A 3-inch wide ring of black plastic was taped around the top edge of the pool to prevent escape. Two facing Mineroff SME-AFS speakers were set on 2-inch tall cinder blocks, just above the surface of the water on opposite sides of the pool. A Sony CCD-TRV67 infrared video camera with a wide-angle lens and a Sony HVL-IRM satellite light attached was placed above the pool to allow viewing of female responses on a monitor in the next room. Several floating sticks were placed in the pool in a symmetric fashion to allow females to perch and listen to stimuli while swimming around the pool. This situation mimics the natural habitat of the frogs. Preliminary tests indicated that without these perches, females are unresponsive. All tests were performed in a completely darkened room (black plastic was taped over all windows and crevices

emitting light). All sources of noise (refrigerators, air conditioning, etc.) in the building were disconnected prior to testing.

Test Protocol. Prior to testing, the water temperature in the pool was recorded and the appropriate stimuli (corrected to 18ºC or 20ºC) were selected. For each test, a container with an acclimated female was placed in the center of the pool and the lid was carefully removed. After the researcher left the room, the stimuli were played through the speakers. Females that did not make a choice within 20 min. were scored as unresponsive. Females that did not climb out of the container within 15 min were also scored as unresponsive. A choice was scored if the female exhibited clear phonotactic behavior (head scanning, swimming in loops near the speaker, etc.) followed by swimming into physical contact with the front of the speaker. If a female simply followed the edge of the pool and speaker base to the front of the speaker, a choice was not scored. After each choice, the female was gently placed back into the container for the next test. Each female was tested until she either failed to respond or completed all three tests.

Stimuli were played at 75 db, one second apart, on different tracks (right vs. left) antiphonally from opposing speakers. Sounds were played from a Macintosh computer outside the test chamber using SoundEdit15. Stimulus amplitude of the two speakers was standardized using a Radio Shack digital sound pressure level meter. In each experiment, a continuous chorus of *P. feriarum* was played in the background at 9 db less than the stimuli. The chorus was played from both speakers, thereby mimicking the natural sound environment during the breeding season, where males call in very large choruses.

Preliminary tests showed that without the background chorus, females are unresponsive. All tests were conducted blind. Female choices were scored in real time. The three experiments were presented in random order to each female and the leads to the speakers (right or left) were randomized between tests.

Statistical Analyses. Four questions were addressed with statistical tests, 1) Do females prefer conspecific males? 2) Has the female preference diverged in sympatry? 3) Has the propensity to hybridize been reduced? 4) Is the displacement in male calls perceptible to the females? This last question is derived from a criterion of Howard (1993) for demonstrating that reproductive character displacement is due to reinforcement.

To answer the first question, one-tailed exact binomial tests were conducted on the results of experiments A and B under the null hypothesis of no preference (proportion = 0.5). One-tailed tests were chosen because the *a priori* expectation was that females would choose the conspecific stimulus. To answer the second question, Fisher's exact test was used to compare the proportion of allopatric females and sympatric females that chose the sympatric *P. feriarum* stimulus in test A. This test was also performed for experiment B. To address the third question, Fisher's exact test was performed to compare the proportion of sympatric females that chose the conspecific stimulus in experiment A to the proportion of allopatric females that chose the conspecific signal in experiment B. To address the last question, a two-tailed exact binomial test was conducted on the results of experiment C. A sequential Bonferroni correction was applied to correct for multiple (nine) tests (Rice 1989).

5.3 RESULTS

Male signal variation. The first randomization test indicates that there is significant heterogeneity in the magnitude of reproductive character displacement among sympatric localities of *P. feriarum* and *P. nigrita* along the first three principal component axes: PC1, $p<0.00001$; PC2, $p=0.00234$; PC3, $p<0.00001$. PC loadings are given in Table 5.1. The second test indicates that signal divergence has occurred along PC1 in sympatric *P. feriarum* from Florida ($p<0.00001$), Georgia, ($p<0.00001$), and South Carolina ($p<0.00024$) but not Virginia ($p=1.0000$). The South Carolina population has also diverged significantly along PC2 ($p<0.00001$) and PC3 ($p<0.00001$) and Georgia has diverged along PC3 ($p<0.00001$). There was no evidence for displacement of *P. nigrita* at any locality, although sympatric Virginia *P. nigrita* approached significance (Table 5.2). General patterns of divergence across the four transects are shown in Figure 5.3.

The discriminant analysis shows that the four sympatric *P. feriarum* populations separate along both the first and second canonical axes, which explain 98.82% of the variation (Fig. 5.4; Table 5.3). The standardized coefficients indicate that pulse rate contributes substantially to the first axis but little to the second axis whereas pulse number loads heavily on the second axis, but contributes little to the first (Table 5.4).

The multiple comparison randomization test indicates that the Georgia population is significantly higher ($p<0.00001$) along the pulse rate-dominated axis (CV1), whereas the Virginia population is significantly lower ($p<0.00001$) than the other two populations. In contrast, the South Carolina and Florida populations are significantly different from

each other ($p<0.00001$) and higher than the other two populations ($p<0.00001$) along the pulse number-dominated axis (CV2; Table 5.5). Raw temperature-corrected pulse rate and pulse number data are presented in Table 5.6.

Female preference tests. Results of the phonotaxis experiments indicate that allopatric and sympatric *Pseudacris feriarum* females prefer conspecific signals over heterospecific signals in both experiments A and B (A, allopatric females, $p=0.00469962$; A, sympatric females, $p=1.63\text{x}10^{-9}$; B, allopatric females, $p=0.02443695$; B, sympatric females, $p=1.55\text{x}10^{-8}$). Female preferences have evolved in sympatry such that sympatric females choose the conspecific stimulus significantly more often than allopatric females (experiment A, 0.87 vs. 0.67, $p=0.01502$; experiment B, 0.88 vs. 0.63, $p=0.003949$). The propensity of females to hybridize has been substantially reduced from 37% in allopatric females (experiment B) to 13% in sympatric females (experiment A; $p= 0.00367$). Finally, displacement of the signal in sympatry is perceptible to females: sympatric females have a strong preference for the sympatric signal ($p=1.35 \text{ x } 10^{-7}$), whereas allopatric females exhibit a weak preference ($p=0.046$). The results are summarized in Fig. 5.5.

5.4 DISCUSSION

I have shown that sympatric *Pseudacris feriarum* exhibits a pattern of acoustic reproductive character displacement in three of four localities where the species is

sympatric with *P. nigrita*. The displaced populations not only vary in the magnitude of character displacement but also in the direction of divergence. The Georgia population has diverged significantly in pulse rate, the South Carolina population has diverged substantially in pulse number, and the Florida population has diverged to a lesser degree along both axes of the signal. This indicates that heterospecific overlap can generate divergent selection on populations of the same species, resulting in displacement along different signal axes.

The female preference tests support a role for reinforcement driving the pattern of reproductive character displacement. I found that preferences have diverged in sympatry to such a degree that females choose the heterospecific signal ~60% less than allopatric females. When given a choice between an allopatric or sympatric conspecific call, sympatric females show a strong preference for the divergent sympatric signal, suggesting they exert strong directional selection on the male signal. Females make the same number of mating mistakes whether they are presented with an allopatric or sympatric conspecific signal. This implies that evolution of the female preference rather than evolution of the male signal has led to reduced hybridization. Additional research is needed to determine the cost of hybridizing to females and quantify the frequency of hybridization in the field.

Why does the degree of sympatric signal displacement vary geographically?

Our results show geographic variation in the degree of RCD among populations. In particular, the northernmost population (Virginia) shows no divergence in sympatry,

whereas the other three populations show strong divergence. There are several possible reasons for this pattern. First, hybrid fitness may vary geographically (Parris 2001; Sweigart et al. 2007). In this situation, we would expect high fitness of hybrids in non-divergent populations and low fitness in divergent populations. Second, depth of the population within the contact zone (distance from allopatry) may be related to the degree of divergence (Littlejohn 1965). Gene flow from allopatry may swamp divergent alleles in shallow sympatry but not in deep sympatry, thus causing variation in the degree of displacement, depending on where populations were sampled (e.g. Hoskin et al. 2005). Third, relative abundance of each species can affect divergence. In areas where a species is relatively rare, it may be under stronger selection to diverge from conspecific signals than in areas where it is common (Howard 1993; Noor 1995). Selection may be counterbalanced, however, by gene flow from heterospecific populations (when heterospecific individuals are more abundant), such that the greatest divergence occurs when relative abundances are nearly equal (Nosil 2003). Fourth, ecological overlap between species may vary geographically (Gow et al. 2006; Taylor et al. 2006). For example, extrinsic factors, such as preferred breeding habitat or timing of optimal breeding conditions for two species, may be discrete in space or time one region and continuous in another, thereby affecting the probability of interaction between species and the strength of selection promoting divergence. Finally, the two species may have come into secondary contact at different times in different parts of the contact zone, resulting in a greater degree of divergence in the older contact area because populations have had more time to evolve differences (Borge et al. 2005).

The first hypothesis could be tested by performing controlled hybridization experiments in different parts of the contact zone and testing for variation in hybrid fitness (e.g., Parris 2001). The second hypothesis could be examined by testing for a positive correlation between distance to allopatry versus degree of displacement across multiple populations. Similarly, the third hypothesis could be tested, by looking for a negative correlation between density of conspecific individuals and displacement in that species. The expected pattern may be confounded, however, by interaction of these two factors (distance to allopatry and density) because density should decline near the edges of species ranges (Kirkpatrick and Barton 1997). The fourth hypothesis could be tested by estimating the ecological niches of sympatric species in different parts of the contact zone and by determining the degree of spatial and temporal overlap (and probability of interaction) during the breeding season. The last hypothesis could be evaluated using multi-gene phylogeographic data to test for population stability or recent expansion in different parts of the zone.

Available data lend support for several hypotheses (2, 3, and 5) in the *Pseudacris feriarum*/*P. nigrita* contact zone, which are not mutually exclusive. Within the Florida transect, Fouquette (1975) found increasing displacement with distance from allopatry in *P. feriarum*. This pattern may also occur in other parts of the contact zone. Published distributions (Crenshaw and Blair 1959; Lannoo 2005), phylogeographic data (Moriarty et al. in press), and my field surveys indicate that *P. feriarum* only penetrate the Coastal Plain distribution of *P. nigrita* along river corridors. Although population densities are of *P. feriarum* are high within the river floodplain, the species is surrounded by *P. nigrita*

outside of this area, suggesting in that *P. feriarum* is the relatively rarer species. The Virginia locality is an exception to this rule. In this region, *P. nigrita* populations are isolated from their main distribution and form a sympatric island, surrounded by *P. feriarum*. For this reason, *P. nigrita* is predicted to be the rarer species in Virginia and may therefore be under stronger selection to diverge. Data supporting the timing of secondary contact hypothesis are the strongest. Phylogeographic and population genetic data indicate that *P. feriarum* underwent a recent expansion into the northern part of its range, probably in response to climate change since the last glacial maximum (Moriarty et al. in press). Therefore, the two species came into contact only recently in Virginia, whereas they have been interacting for a longer period in the southern part of the range. This suggests that speciation-in-action may be observed in this system, where populations are in an early stage of divergence in northern areas and in a later stage in the southern region.

Why is reproductive character displacement asymmetric?

I have shown evidence for asymmetric RCD in sympatry, where only *Pseudacris feriarum* has diverged substantially in the contact zone. This pattern is found frequently in taxa that have undergone RCD (Fouquette, 1975; Butler, 1988; Loftus-Hills and Littlejohn, 1992). Several hypotheses have been proposed to explain this pattern, some of which invoke maladaptive hybridization as the selective force driving divergence, whereas others require only reduced mating efficiency due to interference of signals in sympatry. First, taxa may experience asymmetric postzygotic isolation. In this case, the

species experiencing the greater cost to hybridization may be under stronger selection to evolve more effective communication mechanisms (Gabor and Ryan, 2001). Second, species may face asymmetric prezygotic isolation (but equal postzygotic isolation). In this situation, the species with poor species recognition ability is predicted to diverge in sympatry. Third, as described above, when relative abundances are unequal, the rarer species may undergo RCD. If one species is consistently less abundant in sympatry, that taxon may diverge in all populations. Fourth, there may be a greater cost to evolving in one species (fitness tradeoff), so selection acts more efficiently to cause displacement in the other species. Finally, divergent characters may spread from sympatry to allopatry in one species, either because they are preferred by allopatric females or because demographic factors (such as extinction in allopatry) lead to colonization of allopatry by sympatric individuals.

Because these factors are not mutually exclusive, identifying a single process that caused asymmetric RCD can be difficult. To determine the primary forces driving the pattern, each hypothesis can be tested individually. To test the first hypothesis, symmetry of fitness effects can be assessed through reciprocal hybridization experiments. The second hypothesis can be tested through species recognition tests on allopatric populations of each species. The third hypothesis can be assessed by quantifying levels of reproductive isolation across populations that vary in relative abundance (e.g., Peterson et al. 2005). The fourth hypothesis is perhaps the most difficult to test. One approach would be to examine the energetic costs of different signals (Wells 2001). If the extreme calls (most different from the heterospecific) of the putatively constrained species are more

costly to produce, this would suggest that the signal of this species is physiologically constrained. For testing the fifth hypothesis, phylogeographic data from multiple genes could be used to determine directions of gene flow, into or out of the contact zone.

Future behavioral, ecological, and genetic work will test each of these hypotheses in turn, however, some data are available regarding the emigration the displaced signal from sympatry to allopatry (hypothesis 5). Ancestors of *Pseudacris feriarum* and *P. nigrita* speciated in the late Miocene, ~8 million years ago (Moriarty et al. in press). Since this time, multiple sea level fluctuations have covered much of the current distribution of *P. nigrita* throughout the Coastal Plain (Dowsett and Cronin 1990), likely causing local extinction in all but more inland areas. Genetic data support this prediction: *P. nigrita* has extremely low genetic variation per unit geographic area compared to other *Pseudacris* species, suggesting population contraction and re-expansion (Moriarty et al. in press; Lemmon and Moriarty, manuscript). Given that marine inundation of large areas of allopatry would have forced *P. nigrita* inland toward *P. feriarum*, it is probable that after sea level recession, sympatric *P. nigrita* recolonized coastal regions, spreading the diverged signal through allopatry.

Why does the axis of signal divergence vary?

A novel finding of this study is that different acoustic signal components may diverge in different sympatric populations. This pattern may be the result of stochastic processes or geographic variation in the fitness landscape. In the first scenario, there may be multiple solutions to the problem of signal interference, and the component of the

signal that diverges depends on the level of genetic variation at loci controlling these components. Therefore, selection may be more efficient at one locus in some populations and at a different locus in other populations simply due to the level of variation at these loci at the time of secondary contact. Alternatively, different components may displace due to geographic variation in the fitness of signal types. For instance, if habitat varies geographically, some types of signals may transmit more efficiently in some habitats than others, leading to environmental selection on signal components (Ryan and Wilczynski 1991). Additionally, signal-oriented predators may vary spatially, such that individuals with one signal phenotype are disfavored in one area but not another (Jiggins et al. 2001). Another important factor is the presence of multiple heterospecific taxa in the breeding habitat. If a species interacts with more than one other taxon in some parts of its range, this third species could drive evolution of the signal in a new direction (Pfennig and Ryan 2006).

These hypotheses can be assessed through several experimental approaches. To test for stochastic divergence in nature, experiments with replicate captive populations could be conducted, where unidirectional and bidirectional strengths of selection are imposed on each population. This would elucidate whether populations always evolve along the same signal paths. To test the environmental selection hypothesis, signal transmission experiments could be conducted in the different sympatric habitats to determine whether signals degrade more rapidly with distance in non-native habitats and which values of signal components transmit most effectively at each site. To test the acoustic-predation hypothesis, acoustic playback tests of the sympatric signals could be

performed to determine the proportion of predators attracted by each signal type. Testing the multiple heterospecific overlap hypothesis requires several steps. First, candidate species with acoustically similar signals should be identified. Second, populations with two and three species should be examined to determine whether signals in three-species contacts diverge consistently in a different direction from two-species contacts. Third, female preference tests should be performed to identify which signal components are salient to females in different sympatric regions.

Although the proposed hypotheses still need to be thoroughly tested in the chorus frog system, some evidence supports the multiple heterospecific overlap hypothesis. Sympatric *Pseudacris feriarum* and *P. nigrita* overlap with a third species of chorus frog, *P. brimleyi*, from South Carolina and northward. *Pseudacris feriarum* has a higher pulse rate signal than *P. nigrita*, but a lower pulse rate than *P. brimleyi*. This suggests that divergence from *P. nigrita* along the pulse rate axis may cause *P. feriarum* to experience acoustic interference from *P. brimleyi*, and therefore, selection may favor a different signal component to evolve. In the sympatric South Carolina population, this is indeed the case: *P. feriarum* has displaced in pulse number rather than pulse rate (Fig. 5.3). Further tests should be done to ascertain whether *P. brimleyi* has influenced this direction of signal evolution. At least one other empirical study, however, has found evidence that overlap with multiple heterospecific taxa causes divergent selection on signals. In *Heliconius* butterflies, sister species *H. melpomene* and *H. cydno* have diverged in visual signals to mimic different sympatric model species. This divergence has led to assortative mating based on theses signals and speciation in their ancestor (Jiggins et al. 2001;

Jiggins et al. 2004). Additionally, neural network simulations by Pfennig and Ryan (2006) found that signals of conspecific populations diverged along different axes in response to overlap with different heterospecifics. These data suggest that variation in the type of species interactions can can promote the evolution of reproductive isolation among conspecific populations and potentially lead to speciation.

Patterns of female preference evolution

The mate choice experiments indicate that the preferences of female *Pseudacris feriarum* have undergone reproductive character displacement, such that sympatric females make significantly fewer mating mistakes than allopatric females. The propensity to hybridize has been reduced through evolution of the female preference in sympatry and not through evolution of the male trait itself. This can be seen by the fact that whether females are given allopatric or sympatric conspecific calls paired with the heterospecific call, they make the same number of mistakes. This result begs the question, "Why then did the male signal evolve?" The answer can be found in experiment C, where sympatric females strongly prefer the sympatric over the allopatric signal. This suggests that sympatric females exert directional selection on the male call, causing it to diverge from that of *P. nigrita*.

Physiological data from a close relative to *Pseudacris*, *Hyla versicolor*, indicate that increasing the number of pulses within calls is metabolically costly (Taigen and Wells 1985; Wells and Taigen 1986), suggesting that males are energetically constrained in the degree of divergence along the pulse number axis. The *Pseudacris* preference data

are consistent with this finding: allopatric females have a weak preference for the sympatric call. This provides evidence for stabilizing selection on the male signal—female preference drives the signal in one direction and metabolic limitations constrain the signal in the other. Because female preferences have diverged in sympatry, the male signal has evolved to a new optimum in this tradeoff. Additional work will be done to elucidate the energetic costs of allopatric versus sympatric calls and to identify the costs of diverging along the pulse rate versus the pulse number axis.

Through a series of female preference tests on Georgia frogs, Martof and Thompson (1964) found that pulse number and pulse rate are not essential for species recognition by allopatric *Pseudacris feriarum*. Instead, frequency, call rate, call duration, and intensity were implicated as important to females. In contrast, our results based on male call divergence suggest that in sympatric regions, pulse number and pulse rate may be critical to females for identifying conspecifics. Further experiments will be conducted to identify the specific call characters used by females. Because the critical components may vary geographically (Pfennig and Ryan 2007), experiments will be performed in multiple sympatric populations to determine the direction of the female preference in different regions.

Why has the female preference evolved?

The pattern of reproductive character displacement in both the male signal and the female preference may be caused by selection against signal interference (facilitated RCD: Howard 1993), selection against ecological interference (ecological RCD: Brown

and Wilson 1956), or a consequence of reinforcement (Dobzhansky 1940). Evidence to date supports that reinforcement is driving divergence in chorus frogs. First, putative hybrids, which are morphologically and acoustically intermediate (Fig. 5.3), have been found in nature, thereby ruling out the facilitated RCD hypothesis. Second, comparative analyses of acoustic signal evolution across the entire genus *Pseudacris* indicate that pulse rate and pulse number are uncorrelated with body size, which is related to the ecological niche in frogs (Parmelee 1999). This suggests that signal displacement is not merely a byproduct of ecological character divergence. Third, preliminary data from ongoing laboratory experiments suggest that hybrids have lower viability (developmental problems) and decreased fertility (sperm disfunction; E. Moriarty Lemmon, unpub. data), supporting the idea that reinforcement is driving the evolution of premating isolation in this system. Further research on the degree of natural and sexual selection against hybridization and on the frequency of hybridization in the field is currently underway.

5.5 CONCLUSION

This study demonstrates disparate directions and levels of acoustic reproductive character displacement among populations of sympatric *Pseudacris feriarum* in different parts of the contact zone with *P. nigrita*. The data shows that female *P. feriarum* preferences have diverged in sympatry, resulting in reduced probability of hybridization and greater discrimination among conspecific signals. Additionally, results indicate that divergence of the female preference and not the male signal has reduced the frequency of

hybridization between species. Together, the signal and preference data suggest that female preference evolution drives divergence of the male signal, and this has resulted in displacement of the signal along different, uncorrelated axes, potentially leading to reproductive isolation among conspecific populations.

Table 5.1. Loadings for the first five principal components from the multivariate analysis of 16 call variables.

	I	II	III	IV	V
Call Duration	-0.11778	0.17442	0.57065	0.15823	0.20002
Call Duty Cycle	0.30068	0.27963	0.10249	0.09426	0.24682
Call Fall Time	-0.22475	-0.17671	0.02628	0.23356	0.34591
Call Rate	0.35266	0.13813	-0.29511	0.00594	-0.03008
Call Rise Time	0.06556	0.31677	0.53826	-0.02834	-0.03261
Dom Freq Beg	-0.29563	0.16204	-0.203	0.28288	0.07181
Dom Freq End	-0.24575	0.24131	-0.27826	0.34668	0.16185
Dom Freq Peak	-0.23838	0.3689	-0.22711	0.27619	0.01863
Pulse Duration	0.22076	-0.34827	0.09348	0.45338	0.11451
Pulse Duty Cycle	0.3889	0.07678	-0.17279	0.12058	0.01289
Pulse Fall Time	0.22376	-0.27481	0.06351	0.37387	0.33738
Pulse Number	0.33678	0.2813	0.07313	0.01472	0.16151
Pulse Rate	0.36717	0.18309	-0.20303	0.01799	-0.01896
Pulse Rise Time	0.1144	-0.32407	0.08376	0.28781	-0.41079
Pulse Shape Off	0.02034	0.31446	0.11115	0.29121	-0.35979
Pulse Shape On	-0.01177	0.02988	0.08699	0.32581	-0.54222
Eigenvalue	5.5984	2.5013	2.0343	1.578	1.2217
Percent of Variance	34.99	15.63	12.71	9.86	7.64
Cumulative Percent	34.99	50.62	63.34	73.20	80.84

Table 5.2. Results of randomization test for detecting reproductive character displacement in different sympatric populations. Populations that were significant after sequential Bonferroni correction are denoted with asterisks. A * indicates significance at $p<0.0001$ and ** means significance $p<0.00001$.

		P. feriarum	***P. nigrita***
Axis	**Population**	**P-value**	**P-value**
PC1	Florida	0.00001**	0.55342
PC1	Georgia	0.00001**	0.11231
PC1	South Carolina	0.00024*	0.79796
PC1	Virginia	1	0.00507
PC2	Florida	0.89705	0.44288
PC2	Georgia	0.10654	0.5018
PC2	South Carolina	0.00001**	0.87185
PC2	Virginia	0.53837	0.97422
PC3	Florida	0.84688	0.13446
PC3	Georgia	0.00001**	0.90916
PC3	South Carolina	0.00001**	0.28278
PC3	Virginia	0.06276	0.86451

Table 5.3. Loadings for canonical variates analyses. These are unstandardized eigenvectors.

	I	II	III
Call Fall Time	0.76843	-3.23757	4.96293
Call Rise Time	2.53892	-0.16805	7.57221
Dom Freq Peak	0.00037	-0.00357	0.00222
Pulse Number	-0.02908	0.32090	-0.24022
Pulse Rate	0.43479	-0.11495	0.07638
Eigenvalue	17.95393	4.10132	0.26353
Percent of Variance	80.44	18.38	1.18
Cumulative Percent	80.44	98.82	100.00
Canonical Correlation	0.97326	0.89664	0.45669

Table 5.4. Standardized coefficients for the three canonical axes from the discriminant analysis. Variables that are more important for distinguishing groups have higher values (positive or negative, in bold). The percent of variation explained by each axis is listed below the call variables.

Call Variable	**Std. Coeff. 1**	**Std. Coeff. 2**	**Std. Coeff. 3**
Pulse Number	-0.095	**1.050**	-0.786
Dom Freq Peak	0.058	-0.561	0.349
Call Fall Time	0.096	-0.405	0.621
Call Rise Time	0.452	-0.030	1.347
Pulse Rate	**1.091**	-0.288	0.192
Percent Var.	80.44%	18.37%	1.18%

Table 5.5. Pairwise comparisons of populations along canonical axes 1 and 2 with randomization tests. For each canonical axis, populations were compared in rank order from low mean canonical scores to high mean scores. Significant comparisons at $p<0.00001$ are indicated with an asterisk.

Axis	Comparison	P-value
CV1	Virginia vs. South Carolina	0.00001*
CV1	South Carolina vs. Florida	0.20925
CV1	Florida vs. Georgia	0.00001*
CV2	Georgia vs. Virginia	0.15049
CV2	Virginia vs. Florida	0.00001*
CV2	Florida vs. South Carolina	0.00001*

Table 5.6. Temperature-corrected raw data for two call variables. The mean ± standard deviation and range (on line below) of pulse number and pulse rate are shown for each of the 16 populations examined. Transect numbers correspond to those listed in the Methods and position refers to allopatry (A) or sympatry (S). State refers to the location of the population and *N* indicates the number of individuals recorded from the population.

Species	**Transect**	**Position**	**State**	***N***	**Pulse Number**	**Pulse Rate**
P. feriarum	1	A	FL	13	17.40±1.97	21.83±1.78
					14.75–22.00	16.96–23.65
P. feriarum	1	S	FL	20	24.33±3.60	30.68±2.46
					19.13–31.00	26.92–35.84
P. nigrita	1	A	FL	9	10.22±1.28	10.34±0.79
					8.37–12.25	8.74–11.23
P. nigrita	1	S	FL	20	9.65±1.47	8.54±1.10
					7.24–11.68	6.47–10.62
Hybrid ECM2326	1	S	FL	1	15.54	18.97
					—	—
Hybrid ECM2327	1	S	FL	1	14.16	14.91
					—	—
P. feriarum	2	A	GA	14	21.97±2.16	25.43±1.41
					18.73–25.50	23.38–27.93
P. feriarum	2	S	GA	14	24.68±3.17	50.69±3.04
					20.90–30.60	44.01–54.94
P. nigrita	2	A	FL	5	9.33±1.51	10.46±1.50
					8.12–11.91	8.99–12.95
P. nigrita	2	S	GA	17	8.24±0.59	8.51±1.24
					7.10–9.19	4.84–9.84
P. feriarum	3	A	SC	20	20.17±2.32	25.65±1.28
					15.20–25.33	23.99–27.66
P. feriarum	3	S	SC	17	32.79±3.94	27.73±2.13
					25.25–40.80	23.54–30.54
P. nigrita	3	A	GA	20	9.97±0.78	8.26±0.53
					8.94–11.65	6.95–9.17
P. nigrita	3	S	SC	21	8.77±0.90	8.86±1.69
					7.08–10.10	5.61–11.32
P. feriarum	4	A	VA	20	18.58±2.44	24.79±1.69
					12.67–23.33	21.58–27.46
P. feriarum	4	S	VA	44	17.50±2.85	21.79±2.48
					13.67–32.14	16.67–26.77
P. nigrita	4	S	VA	41	8.06±1.03	7.58±1.05
					6.10–11.19	5.08–9.90
Hybrid ECM1984	4	S	VA	1	13.00	13.08
					—	—
Hybrid ECM2003	4	S	VA	1	15.53	15.59

P. nigrita	n/a	A	MS	19	— 10.36±1.04 8.81–12.32	— 9.39±1.20 6.69–11.86

Supplemental Data 5.1. List of male *Pseudacris feriarum*, *P. nigrita*, and putative hybrids examined for signal variation. Specimens are organized by field number. Position indicates whether the individual was found in allopatry (A) or sympatry (S). Transect refers to the geographic transects listed in Fig. 5.3 and described in the Methods. Recording temperature (Temp.) is listed in degrees Celcius. Signal indicates whether the individual was included in the call variation portion of the study. Preference tests (Pref. Tests) indicates whether the individual's call was used to synthesize stimuli for the female phonotaxis tests. Dataset 1 and 2 list the number of calls extracted from a frog's calling sequence for quantifying within-call variation (1) and between call-variation (2) within individuals. The distinction between these datasets is explained further in the methods section.

Field No.	Species	Position	Transect	State	County	Temp.	Signal	Pref. Tests	Dataset 1	Dataset 2
ECM0024	*P. nigrita*	A	3	FL	Brevard	12.6	Y	N	9	11
ECM0025	*P. nigrita*	A	3	FL	Brevard	12.8	Y	N	9	12
ECM0026	*P. nigrita*	A	3	FL	Brevard	12.6	Y	N	11	14
ECM0027	*P. nigrita*	A	3	FL	Brevard	13.2	Y	N	10	13
ECM0028	*P. nigrita*	A	3	FL	Brevard	19	Y	N	11	11
ECM0218	*P. feriarum*	S	2	FL	Calhoun	11.8	N	Y	10	n/a
ECM0221	*P. feriarum*	S	2	FL	Calhoun	11.8	N	Y	10	n/a
ECM0222	*P. feriarum*	S	2	FL	Calhoun	11.8	N	Y	10	n/a
ECM0224	*P. nigrita*	S	2	FL	Liberty	12.4	N	Y	2	n/a
ECM0225	*P. nigrita*	S	2	FL	Liberty	12.4	Y	Y	6	6
ECM0226	*P. nigrita*	S	2	FL	Liberty	12.4	N	Y	6	n/a
ECM0227	*P. nigrita*	S	2	FL	Liberty	12.4	Y	Y	4	4
ECM0228	*P. nigrita*	S	2	FL	Liberty	12.4	Y	Y	5	5
ECM0229	*P. nigrita*	S	2	FL	Liberty	12.4	Y	Y	10	5
ECM0230	*P. nigrita*	S	2	FL	Liberty	12.4	Y	Y	11	11
ECM0231	*P. nigrita*	S	2	FL	Franklin	19	N	Y	10	n/a
ECM0232	*P. feriarum*	S	2	FL	Liberty	12.4	Y	Y	15	8
ECM0233	*P. feriarum*	S	2	FL	Liberty	12.4	Y	Y	12	11
ECM0234	*P. feriarum*	S	2	FL	Liberty	12.4	Y	Y	8	12
ECM0235	*P. feriarum*	S	2	FL	Liberty	12.4	Y	Y	5	10
ECM0236	*P. nigrita*	S	2	FL	Liberty	15.8	Y	Y	7	3

ECM0237	*P. nigrita*	S	2	FL	Liberty	15.8	Y	Y	12	13
ECM0238	*P. nigrita*	S	2	FL	Liberty	15.8	Y	Y	9	13
ECM0239	*P. nigrita*	S	2	FL	Liberty	15.8	Y	Y	10	7
ECM0240	*P. nigrita*	S	2	FL	Liberty	15.8	Y	Y	7	11
ECM0241	*P. nigrita*	S	2	FL	Liberty	15.8	Y	Y	9	11
ECM0244	*P. feriarum*	S	2	FL	Liberty	12.2	Y	N	5	5
ECM0245	*P. feriarum*	S	2	FL	Liberty	12.2	Y	Y	6	10
ECM0248	*P. feriarum*	S	2	FL	Liberty	12.2	Y	Y	6	6
ECM0262	*P. nigrita*	A	1	MS	Harrison	20	Y	N	11	11
ECM0280	*P. nigrita*	A	1	MS	Harrison	15.4	Y	N	11	11
ECM0281	*P. nigrita*	A	1	MS	Harrison	12.4	Y	N	11	11
ECM0282	*P. nigrita*	A	1	MS	Harrison	12.4	Y	N	4	3
ECM0283	*P. nigrita*	A	1	MS	Harrison	13.8	Y	N	11	11
ECM0284	*P. nigrita*	A	1	MS	Harrison	13	Y	N	11	7
ECM0285	*P. nigrita*	A	1	MS	Harrison	13	Y	N	12	12
ECM0286	*P. nigrita*	A	1	MS	Harrison	13	Y	N	11	7
ECM0287	*P. nigrita*	A	1	MS	Harrison	12.2	Y	N	11	7
ECM0288	*P. nigrita*	A	1	MS	Harrison	12.2	Y	N	7	5
ECM0289	*P. nigrita*	A	1	MS	Harrison	12.2	Y	N	10	10
ECM0290	*P. nigrita*	A	1	MS	Harrison	19.2	Y	N	11	11
ECM0291	*P. nigrita*	A	1	MS	Harrison	19.2	Y	N	12	12
ECM0292	*P. nigrita*	A	1	MS	Harrison	19.8	Y	N	11	11
ECM0293	*P. nigrita*	A	1	MS	Harrison	19.8	Y	N	12	12
ECM0294	*P. nigrita*	A	1	MS	Harrison	19.8	Y	N	11	11
ECM0295	*P. nigrita*	A	1	MS	Harrison	19.8	Y	N	11	8
ECM0296	*P. nigrita*	A	1	MS	Harrison	19.8	Y	N	11	11
ECM0297	*P. nigrita*	A	1	MS	Harrison	19.8	Y	N	11	11
ECM0336	*P. feriarum*	S	2	FL	Liberty	14.4	Y	Y	8	11
ECM0337	*P. feriarum*	S	2	FL	Liberty	14.4	Y	Y	10	11
ECM0338	*P. feriarum*	S	2	FL	Liberty	17.8	Y	Y	11	11
ECM0340	*P. nigrita*	S	2	FL	Liberty	17.8	Y	Y	12	11

ECM0343	*P. feriarum*	S	2	FL	Liberty	17.8	Y	Y	11	11
ECM0344	*P. feriarum*	S	2	FL	Liberty	17.8	Y	Y	10	12
ECM0345	*P. feriarum*	S	2	FL	Liberty	17.8	Y	Y	10	11
ECM0346	*P. feriarum*	S	2	FL	Liberty	17.8	Y	Y	7	11
ECM0347	*P. feriarum*	S	2	FL	Liberty	17.8	Y	N	11	11
ECM0348	*P. feriarum*	S	2	FL	Liberty	17.8	Y	Y	8	6
ECM0359	*P. nigrita*	S	2	FL	Liberty	16	Y	Y	8	11
ECM0360	*P. nigrita*	S	2	FL	Liberty	16	Y	Y	8	11
ECM0361	*P. nigrita*	S	2	FL	Liberty	16	Y	Y	2	3
ECM0362	*P. nigrita*	S	2	FL	Liberty	16	Y	Y	11	11
ECM0363	*P. nigrita*	S	2	FL	Liberty	16	Y	Y	11	11
ECM0364	*P. nigrita*	S	2	FL	Liberty	16	Y	Y	9	8
ECM0365	*P. nigrita*	S	2	FL	Liberty	16	Y	Y	9	11
ECM0366	*P. nigrita*	S	2	FL	Liberty	16	Y	Y	9	11
ECM0367	*P. feriarum*	S	2	FL	Liberty	16	Y	Y	6	11
ECM0372	*P. nigrita*	A	2	FL	Jefferson	17	Y	N	12	13
ECM0373	*P. nigrita*	A	2	FL	Jefferson	17	Y	N	10	9
ECM0374	*P. nigrita*	A	2	FL	Jefferson	17	Y	N	11	12
ECM0375	*P. nigrita*	A	2	FL	Jefferson	17	Y	N	9	13
ECM0376	*P. nigrita*	A	2	FL	Jefferson	17	Y	N	10	14
ECM0377	*P. nigrita*	A	2	FL	Jefferson	17	Y	N	10	13
ECM0378	*P. nigrita*	A	2	FL	Jefferson	16	Y	N	9	13
ECM0379	*P. nigrita*	A	2	FL	Jefferson	16	Y	N	9	14
ECM0381	*P. nigrita*	A	2	FL	Jefferson	16	Y	N	11	12
ECM0387	*P. feriarum*	A	2	AL	Macon	14	Y	Y	12	12
ECM0388	*P. feriarum*	A	2	AL	Macon	14	Y	Y	12	14
ECM0389	*P. feriarum*	A	2	AL	Macon	14	Y	Y	6	4
ECM0390	*P. feriarum*	A	2	AL	Macon	14	Y	Y	9	12
ECM0391	*P. feriarum*	A	2	AL	Macon	14	Y	Y	10	12
ECM0392	*P. feriarum*	A	2	AL	Macon	14	Y	Y	7	5
ECM0393	*P. feriarum*	A	2	AL	Macon	14	Y	Y	5	13

ECM0394	*P. feriarum*	A	2	AL	Macon	14	Y	Y	4	10
ECM0395	*P. feriarum*	A	2	AL	Macon	14	Y	Y	10	9
ECM0396	*P. feriarum*	A	2	AL	Macon	14	Y	Y	2	8
ECM0397	*P. feriarum*	A	2	AL	Macon	14	Y	Y	6	12
ECM0398	*P. feriarum*	A	2	AL	Macon	14	Y	Y	4	7
ECM0400	*P. feriarum*	A	2	AL	Macon	18	Y	Y	9	14
ECM0401	*P. feriarum*	S	3	GA	Baker	18	Y	N	11	11
ECM0402	*P. feriarum*	S	3	GA	Baker	18	Y	N	12	11
ECM0403	*P. feriarum*	S	3	GA	Baker	18	Y	N	12	12
ECM0404	*P. feriarum*	S	3	GA	Baker	18	Y	N	11	11
ECM0405	*P. feriarum*	S	3	GA	Baker	18	Y	N	11	5
ECM0406	*P. feriarum*	S	3	GA	Baker	18	Y	N	10	4
ECM0407	*P. feriarum*	S	3	GA	Baker	18	Y	N	12	8
ECM0408	*P. feriarum*	S	3	GA	Baker	18	Y	N	10	4
ECM0409	*P. feriarum*	S	3	GA	Baker	18	Y	N	11	6
ECM0410	*P. feriarum*	S	3	GA	Baker	18	Y	N	12	12
ECM0411	*P. feriarum*	S	3	GA	Baker	18	Y	N	12	12
ECM0412	*P. feriarum*	S	3	GA	Baker	18	Y	N	11	11
ECM0413	*P. feriarum*	S	3	GA	Baker	18	Y	N	11	11
ECM0414	*P. feriarum*	S	3	GA	Baker	18	Y	N	10	10
ECM0422	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	12	12
ECM0423	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	13	13
ECM0424	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	12	12
ECM0425	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	12	12
ECM0426	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	11	11
ECM0427	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	7	7
ECM0428	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	12	12
ECM0429	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	12	8
ECM0430	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	11	11
ECM0431	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	11	11
ECM0432	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	12	12

ECM0433	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	10	10
ECM0434	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	10	10
ECM0435	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	11	11
ECM0436	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	12	12
ECM0437	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	11	11
ECM0438	*P. nigrita*	S	3	GA	Baker	18.6	Y	N	11	11
ECM0441	*P. feriarum*	S	4	SC	Dorchester	15	Y	N	8	4
ECM0442	*P. nigrita*	S	4	SC	Dorchester	14.8	Y	N	6	6
ECM0443	*P. nigrita*	S	4	SC	Dorchester	14.8	Y	N	6	6
ECM0444	*P. nigrita*	S	4	SC	Dorchester	14.8	Y	N	9	7
ECM0481	*P. feriarum*	S	5	VA	York	18.6	Y	N	11	7
ECM0482	*P. nigrita*	S	5	VA	York	18.6	Y	N	11	11
ECM0483	*P. nigrita*	S	5	VA	York	18.6	Y	N	11	9
ECM0487	*P. nigrita*	S	5	VA	York	15.8	Y	N	11	11
ECM0488	*P. nigrita*	S	5	VA	York	15.8	Y	N	11	11
ECM0489	*P. feriarum*	S	5	VA	York	15.8	Y	N	10	7
ECM0490	*P. feriarum*	S	5	VA	York	16	Y	N	9	8
ECM0491	*P. feriarum*	S	5	VA	York	16	Y	N	11	11
ECM0492	*P. feriarum*	S	5	VA	York	16	Y	N	9	9
ECM0493	*P. feriarum*	S	5	VA	York	16	Y	N	7	7
ECM0494	*P. feriarum*	S	5	VA	York	15.2	Y	N	11	10
ECM0495	*P. feriarum*	S	5	VA	York	15.2	Y	N	10	10
ECM0496	*P. feriarum*	S	5	VA	York	15.2	Y	N	17	10
ECM0497	*P. feriarum*	S	5	VA	York	16	Y	N	11	11
ECM0498	*P. nigrita*	S	5	VA	York	16.6	Y	N	10	6
ECM0996	*P. feriarum*	A	5	VA	Prince Edward	7.2	Y	N	10	4
ECM0997	*P. feriarum*	A	5	VA	Prince Edward	7.2	Y	N	3	4
ECM0998	*P. feriarum*	A	5	VA	Prince Edward	7.2	Y	N	11	11
ECM0999	*P. feriarum*	A	5	VA	Prince Edward	7.2	Y	N	10	11
ECM1000	*P. feriarum*	A	5	VA	Prince Edward	7.2	Y	N	8	11
ECM1002	*P. feriarum*	A	5	VA	Prince Edward	7.2	Y	N	14	6

ECM1003	*P. feriarum*	A	5	VA	Prince Edward	8.2	Y	N	3	2
ECM1004	*P. feriarum*	A	5	VA	Prince Edward	8.2	Y	N	5	2
ECM1005	*P. feriarum*	A	5	VA	Prince Edward	8.2	Y	N	10	13
ECM1006	*P. feriarum*	A	5	VA	Prince Edward	8.2	Y	N	6	5
ECM1007	*P. feriarum*	A	5	VA	Prince Edward	8.2	Y	N	2	4
ECM1008	*P. feriarum*	A	5	VA	Prince Edward	8.2	Y	N	8	11
ECM1009	*P. feriarum*	A	5	VA	Prince Edward	8.2	Y	N	3	10
ECM1010	*P. feriarum*	A	5	VA	Prince Edward	8.2	Y	N	10	11
ECM1013	*P. feriarum*	A	5	VA	Prince Edward	10.6	Y	N	10	11
ECM1014	*P. feriarum*	A	5	VA	Prince Edward	10.6	Y	N	11	4
ECM1015	*P. feriarum*	A	5	VA	Prince Edward	10.6	Y	N	10	4
ECM1016	*P. feriarum*	A	5	VA	Prince Edward	10.6	Y	N	7	4
ECM1017	*P. feriarum*	A	5	VA	Prince Edward	10.6	Y	N	4	9
ECM1018	*P. feriarum*	A	5	VA	Prince Edward	10.6	Y	N	3	8
ECM1022	*P. feriarum*	S	5	VA	York	9.2	Y	N	10	14
ECM1023	*P. feriarum*	S	5	VA	York	9.2	Y	N	10	8
ECM1024	*P. feriarum*	S	5	VA	York	9.8	Y	N	10	6
ECM1026	*P. feriarum*	S	5	VA	York	8.8	Y	N	11	8
ECM1027	*P. feriarum*	S	5	VA	York	8.8	Y	N	3	8
ECM1028	*P. feriarum*	S	5	VA	York	8.8	Y	N	8	11
ECM1029	*P. feriarum*	S	5	VA	York	8.8	Y	N	14	12
ECM1030	*P. feriarum*	S	5	VA	York	9	Y	N	10	10
ECM1031	*P. feriarum*	S	5	VA	York	9	Y	N	10	11
ECM1389	*P. feriarum*	S	4	SC	Dorchester	10	Y	N	5	5
ECM1390	*P. feriarum*	S	4	SC	Dorchester	10	Y	N	6	6
ECM1392	*P. feriarum*	S	4	SC	Dorchester	10	Y	N	7	7
ECM1393	*P. feriarum*	S	4	SC	Dorchester	10	Y	N	6	6
ECM1396	*P. feriarum*	S	4	SC	Dorchester	10	Y	N	11	11
ECM1397	*P. feriarum*	S	4	SC	Dorchester	10	Y	N	7	3
ECM1399	*P. feriarum*	S	4	SC	Dorchester	10	Y	N	11	11
ECM1401	*P. feriarum*	S	4	SC	Dorchester	10	Y	N	10	10

ECM1405	*P. feriarum*	S	4	SC	Dorchester	8	Y	N	7	3
ECM1407	*P. feriarum*	S	4	SC	Dorchester	8	Y	N	12	12
ECM1410	*P. feriarum*	S	4	SC	Dorchester	8.6	Y	N	8	3
ECM1411	*P. feriarum*	S	4	SC	Dorchester	8	Y	N	12	12
ECM1412	*P. feriarum*	S	4	SC	Dorchester	8	Y	N	11	11
ECM1422	*P. nigrita*	S	4	SC	Dorchester	7.8	Y	N	11	5
ECM1423	*P. nigrita*	S	4	SC	Dorchester	7.8	Y	N	8	2
ECM1424	*P. nigrita*	S	4	SC	Dorchester	7.8	Y	N	7	3
ECM1425	*P. nigrita*	S	4	SC	Dorchester	7.8	Y	N	2	2
ECM1426	*P. nigrita*	S	4	SC	Dorchester	7.8	Y	N	7	7
ECM1427	*P. nigrita*	S	4	SC	Dorchester	7.8	Y	N	11	5
ECM1428	*P. nigrita*	S	4	SC	Dorchester	7.8	Y	N	11	3
ECM1429	*P. nigrita*	S	4	SC	Dorchester	7.8	Y	N	11	4
ECM1430	*P. feriarum*	A	4	SC	Greenwood	6.8	Y	N	3	2
ECM1431	*P. feriarum*	A	4	SC	Greenwood	6.8	Y	N	6	3
ECM1432	*P. feriarum*	A	4	SC	Greenwood	7	Y	N	4	4
ECM1433	*P. feriarum*	A	4	SC	Greenwood	7	Y	N	2	2
ECM1434	*P. feriarum*	A	4	SC	Greenwood	8	Y	N	11	7
ECM1435	*P. feriarum*	A	4	SC	Greenwood	8	Y	N	14	8
ECM1436	*P. feriarum*	A	4	SC	Greenwood	8	Y	N	10	8
ECM1437	*P. feriarum*	A	4	SC	Greenwood	8	Y	N	9	9
ECM1438	*P. feriarum*	A	4	SC	Greenwood	8	Y	N	11	7
ECM1439	*P. feriarum*	A	4	SC	Greenwood	8	Y	N	11	4
ECM1440	*P. feriarum*	A	4	SC	Greenwood	8	Y	N	8	8
ECM1441	*P. feriarum*	A	4	SC	Greenwood	8	Y	N	12	12
ECM1442	*P. feriarum*	A	4	SC	Greenwood	8	Y	N	4	4
ECM1443	*P. feriarum*	A	4	SC	Greenwood	9.2	Y	N	8	8
ECM1444	*P. feriarum*	A	4	SC	Greenwood	9.2	Y	N	9	9
ECM1445	*P. feriarum*	A	4	SC	Greenwood	9.2	Y	N	10	6
ECM1446	*P. feriarum*	A	4	SC	Greenwood	9.2	Y	N	11	11
ECM1447	*P. feriarum*	A	4	SC	Greenwood	9.2	Y	N	10	10

ECM1448	*P. feriarum*	A	4	SC	Greenwood	9.2	Y	N	11	5
ECM1449	*P. feriarum*	A	4	SC	Greenwood	9.2	Y	N	10	5
ECM1454	*P. feriarum*	A	3	GA	Greene	11	Y	N	11	7
ECM1455	*P. feriarum*	A	3	GA	Greene	11	Y	N	8	4
ECM1456	*P. feriarum*	A	3	GA	Greene	11	Y	N	5	5
ECM1457	*P. feriarum*	A	3	GA	Greene	11	Y	N	6	4
ECM1458	*P. feriarum*	A	3	GA	Greene	11	Y	N	10	10
ECM1459	*P. feriarum*	A	3	GA	Greene	11	Y	N	6	3
ECM1460	*P. feriarum*	A	3	GA	Greene	10.8	Y	N	13	6
ECM1461	*P. feriarum*	A	3	GA	Greene	10.8	Y	N	10	10
ECM1462	*P. feriarum*	A	3	GA	Greene	10.8	Y	N	10	10
ECM1463	*P. feriarum*	A	3	GA	Greene	10.8	Y	N	11	11
ECM1464	*P. feriarum*	A	3	GA	Greene	11	Y	N	11	11
ECM1465	*P. feriarum*	A	3	GA	Greene	11	Y	N	8	4
ECM1466	*P. feriarum*	A	3	GA	Greene	11	Y	N	10	4
ECM1467	*P. feriarum*	A	3	GA	Greene	11	Y	N	10	5
ECM1468	*P. nigrita*	S	4	SC	Dorchester	12	Y	N	13	13
ECM1469	*P. nigrita*	S	4	SC	Dorchester	12	Y	N	11	11
ECM1470	*P. nigrita*	S	4	SC	Dorchester	13	Y	N	8	6
ECM1471	*P. nigrita*	S	4	SC	Dorchester	13	Y	N	9	6
ECM1472	*P. nigrita*	S	4	SC	Dorchester	13	Y	N	11	11
ECM1477	*P. nigrita*	S	4	SC	Dorchester	14	Y	N	9	9
ECM1478	*P. nigrita*	S	4	SC	Dorchester	14	Y	N	13	13
ECM1479	*P. nigrita*	S	4	SC	Dorchester	14	Y	N	11	11
ECM1480	*P. nigrita*	S	4	SC	Dorchester	14	Y	N	8	4
ECM1481	*P. nigrita*	S	4	SC	Dorchester	14	Y	N	12	12
ECM1482	*P. feriarum*	S	4	SC	Colleton	15	Y	N	5	3
ECM1483	*P. feriarum*	S	4	SC	Colleton	15	Y	N	11	11
ECM1484	*P. feriarum*	S	4	SC	Colleton	15	Y	N	5	5
ECM1491	*P. nigrita*	A	4	GA	McIntosh	12.2	Y	N	11	11
ECM1492	*P. nigrita*	A	4	GA	McIntosh	12.2	Y	N	3	3

ECM1493	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	11	4
ECM1494	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	8	3
ECM1495	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	11	11
ECM1496	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	12	12
ECM1497	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	11	11
ECM1498	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	11	11
ECM1800	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	5	5
ECM1801	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	9	6
ECM1802	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	11	11
ECM1803	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	10	10
ECM1804	*P. nigrita*	A	4	GA	McIntosh	12.8	Y	N	10	10
ECM1805	*P. nigrita*	A	4	GA	McIntosh	12.2	Y	N	11	9
ECM1806	*P. nigrita*	A	4	GA	McIntosh	12.2	Y	N	12	12
ECM1807	*P. nigrita*	A	4	GA	McIntosh	12.2	Y	N	9	9
ECM1808	*P. nigrita*	A	4	GA	McIntosh	12.2	Y	N	10	10
ECM1809	*P. nigrita*	A	4	GA	McIntosh	12.2	Y	N	10	10
ECM1811	*P. nigrita*	A	4	GA	McIntosh	12.2	Y	N	9	6
ECM1812	*P. nigrita*	A	4	GA	McIntosh	12.2	Y	N	11	11
ECM1982	*P. feriarum*	S	5	VA	Sussex	11.4	Y	N	11	9
ECM1983a	*P. nigrita*	S	5	VA	Sussex	11.4	Y	N	11	11
ECM1983b	*P. nigrita*	S	5	VA	Sussex	11.4	Y	N	11	4
ECM1984	*P.* hybrid	S	5	VA	Sussex	11.4	Y	N	13	7
ECM1985	*P. feriarum*	S	5	VA	Sussex	15.8	Y	N	11	11
ECM1986	*P. feriarum*	S	5	VA	Sussex	15.8	Y	N	11	11
ECM1988	*P. feriarum*	S	5	VA	Sussex	11	Y	N	11	11
ECM1989	*P. feriarum*	S	5	VA	Sussex	11	Y	N	12	8
ECM1990	*P. feriarum*	S	5	VA	Sussex	10.4	Y	N	11	3
ECM1991	*P. feriarum*	S	5	VA	Sussex	10.2	Y	N	11	5
ECM1992	*P. feriarum*	S	5	VA	Sussex	10.2	Y	N	12	12
ECM1993	*P. feriarum*	S	5	VA	Sussex	10.2	Y	N	12	6
ECM1994	*P. feriarum*	S	5	VA	Sussex	10.2	Y	N	11	5

ECM1995	*P. feriarum*	S	5	VA	Sussex	10.2	Y	N	13	13
ECM1996	*P. feriarum*	S	5	VA	Sussex	10.4	Y	N	11	8
ECM1997	*P. feriarum*	S	5	VA	Sussex	10.4	Y	N	10	4
ECM1998	*P. feriarum*	S	5	VA	Sussex	10.4	Y	N	12	5
ECM1999	*P. feriarum*	S	5	VA	Sussex	10.4	Y	N	11	11
ECM2003	*P.* hybrid	S	5	VA	Sussex	8.2	Y	N	12	4
ECM2004	*P. nigrita*	S	5	VA	Sussex	8.2	Y	N	8	2
ECM2005	*P. feriarum*	S	5	VA	Sussex	8.2	Y	N	11	4
ECM2007	*P. feriarum*	S	5	VA	Sussex	10	Y	N	11	11
ECM2008	*P. feriarum*	S	5	VA	Sussex	10.4	Y	N	11	11
ECM2009	*P. feriarum*	S	5	VA	Sussex	10	Y	N	5	5
ECM2010	*P. feriarum*	S	5	VA	Sussex	10	Y	N	3	2
ECM2012	*P. feriarum*	S	5	VA	Sussex	10	Y	N	4	4
ECM2013	*P. feriarum*	S	5	VA	Sussex	10.6	Y	N	10	4
ECM2031	*P. feriarum*	S	5	VA	Sussex	15.8	Y	N	10	6
ECM2032	*P. nigrita*	S	5	VA	York	11.4	Y	N	11	11
ECM2033	*P. nigrita*	S	5	VA	York	11.4	Y	N	10	10
ECM2034	*P. nigrita*	S	5	VA	York	11.4	Y	N	11	11
ECM2035	*P. nigrita*	S	5	VA	York	11.4	Y	N	9	7
ECM2036	*P. nigrita*	S	5	VA	York	12	Y	N	10	10
ECM2037	*P. nigrita*	S	5	VA	York	12	Y	N	12	12
ECM2038	*P. nigrita*	S	5	VA	York	12	Y	N	11	11
ECM2039	*P. nigrita*	S	5	VA	York	12	Y	N	13	13
ECM2040	*P. nigrita*	S	5	VA	York	12	Y	N	4	4
ECM2041	*P. nigrita*	S	5	VA	York	12.8	Y	N	11	11
ECM2044	*P. nigrita*	S	5	VA	York	12.8	Y	N	11	4
ECM2045	*P. nigrita*	S	5	VA	York	12.8	Y	N	9	9
ECM2046	*P. nigrita*	S	5	VA	York	12.8	Y	N	9	9
ECM2047	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	11
ECM2048	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	11
ECM2049	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	11

ECM2050	*P. feriarum*	S	5	VA	Sussex	12.6	Y	N	11	11
ECM2051	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	4
ECM2052	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	10	10
ECM2053	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	10	10
ECM2054	*P. feriarum*	S	5	VA	Sussex	12.6	Y	N	11	11
ECM2055	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	7
ECM2056	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	11
ECM2057	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	5
ECM2058	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	11
ECM2059	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	12	6
ECM2060	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	10	5
ECM2061	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	15	15
ECM2062	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	10	7
ECM2063	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	11
ECM2064	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	12	12
ECM2065	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	11	11
ECM2066	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	12	6
ECM2067	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	7	7
ECM2068	*P. nigrita*	S	5	VA	Sussex	12.6	Y	N	12	7
ECM2326	*P.* hybrid	S	2	FL	Liberty	17.2	Y	N	9	9
ECM2327	*P.* hybrid	S	2	FL	Liberty	16.2	Y	N	11	4
ECM2388	*P. feriarum*	S	2	FL	Liberty	17	Y	N	11	11
ECM2389	*P. feriarum*	S	2	FL	Liberty	17	Y	N	12	12
ECM2390	*P. feriarum*	S	2	FL	Liberty	17	Y	N	11	8

Supplemental Data 5.2. Definitions of call characters examined across *Pseudacris*. All pulse-related variables except pulse number were averaged across pulses within the call.

Call Length	Duration of call from 10% maximum amplitude (call onset) to 10% maximum amplitude (offset)
Call Duty Cycle	Call length / time from 10% maximum amplitude (call onset) to 10% maximum amplitude (onset) of next call
Call Fall Time	Duration of call from maximum amplitude to 10% maximum amplitude (call offset)
Call Rate	1 / time from 10% maximum amplitude (call onset) to 10% maximum amplitude (onset) for next call
Call Rise Time	Duration of call from 10% maximum amplitude (call onset) to maximum amplitude
Call dominant frequency begin	Call dominant frequency at 10% maximum amplitude (call onset)
Call dominant frequency end	Call dominant frequency at 10% maximum amplitude (call offset)
Call dominant frequency peak	Call dominant frequency at the call maximum amplitude
Pulse Duration	Duration of pulse from 10% maximum amplitude (pulse onset) to 10% maximum amplitude (offset)
Pulse Duty Cycle	Pulse length / time from 10% maximum amplitude (pulse onset) to 10% maximum amplitude (onset) of next pulse
Pulse Fall Time	Duration of pulse from maximum amplitude to 10% of maximum amplitude (pulse offset)
Pulse number	Number of pulses in the call
Pulse Rate	1 / time from 10% maximum amplitude (pulse onset) to 10% maximum amplitude (onset) of next pulse
Pulse Rise Time	Duration of pulse from 10% maximum amplitude (onset) to maximum amplitude
Pulse Shape Offset	Duration of pulse from 50% to 10% maximum amplitude (offset) / duration from 90% to 10% (offset)
Pulse Shape Onset	Duration of pulse from 10% to 50% maximum amplitude (onset) / duration from 10% to 90% (onset)

Supplemental Data 5.3. Correlations between temperature and call variables. A * indicates significance at p<0.001. Call variables are described in detail in Supplemental Data 5.2.

	P. feriarum			*P. nigrita*		
	Slope	P-value	r2	Slope	P-value	r2
Call Length	-0.099	0.001*	0.641	-0.068	0.001*	0.572
Call Duty Cycle	0.001	0.445	0.004	0.008	0.001*	0.172
Call Fall Time	-0.011	0.011	0.044	-0.007	0.098	0.014
Call Rate	0.040	0.001*	0.842	0.030	0.001*	0.716
Call Rise Time	-0.087	0.001*	0.504	-0.061	0.001*	0.582
Dom Freq Beg	22.851	0.001*	0.167	25.427	0.001*	0.115
Dom Freq End	25.139	0.001*	0.152	30.194	0.001*	0.158
Dom Freq Peak	24.611	0.001*	0.127	20.938	0.001*	0.080
Pulse Duration	0.000	0.007*	0.049	0.000	0.001*	0.058
Pulse Duty Cycle	0.014	0.001*	0.520	0.005	0.001*	0.414
Pulse Fall Time	0.000	0.552	0.002	0.000	0.691	0.001
Pulse Number	0.009	0.951	0.000	0.177	0.001*	0.064
Pulse Rate	2.065	0.001*	0.758	0.838	0.001*	0.610
Pulse Rise Time	0.000	0.001*	0.125	0.000	0.001*	0.140
Pulse Shape Offset	-0.001	0.662	0.001	-0.006	0.001*	0.070
Pulse Shape Onset	-0.003	0.140	0.015	-0.009	0.001*	0.063

Supplemental Data 5.4. Female allopatric and sympatric *Pseudacris feriarum* tested in the preference experiments.

Field Number	State	County
ECM0672	FL	Liberty
ECM0674	FL	Liberty
ECM0742	FL	Liberty
ECM0750	FL	Liberty
ECM0750	FL	Liberty
ECM0758	FL	Liberty
ECM0759	FL	Liberty
ECM0761	FL	Liberty
ECM0762	FL	Liberty
ECM0766	FL	Liberty
ECM0768	FL	Liberty
ECM0770	FL	Liberty
ECM0774	FL	Liberty
ECM0776	FL	Liberty
ECM0776	FL	Liberty
ECM0778	FL	Liberty
ECM0780	FL	Liberty
ECM0782	FL	Liberty
ECM0782	FL	Liberty
ECM0807	FL	Liberty
ECM0814	FL	Liberty
ECM0821	FL	Liberty
ECM0824	FL	Liberty
ECM0825	FL	Liberty
ECM0827	FL	Liberty
ECM0828	FL	Liberty
ECM0830	FL	Liberty
ECM0832	FL	Liberty
ECM0834	FL	Liberty
ECM0835	FL	Liberty
ECM0840	FL	Liberty
ECM0841	FL	Liberty
ECM0842	FL	Liberty
ECM0843	FL	Liberty
ECM0844	FL	Liberty
ECM0846	FL	Liberty
ECM0847	FL	Liberty
ECM0848	FL	Liberty
ECM0849	FL	Liberty
ECM0850	FL	Liberty

ECM0851	FL	Liberty
ECM0852	FL	Liberty
ECM0853	FL	Liberty
ECM0854	FL	Liberty
ECM0855	FL	Liberty
ECM0856	FL	Liberty
ECM0879	FL	Liberty
ECM0881	FL	Liberty
ECM0889	FL	Liberty
ECM0890	FL	Liberty
ECM0891	FL	Liberty
ECM0892	FL	Liberty
ECM0893	FL	Liberty
ECM0894	FL	Liberty
ECM0896	FL	Liberty
ECM0897	FL	Liberty
ECM0899	FL	Liberty
ECM0900	FL	Liberty
ECM0901	FL	Liberty
ECM0902	FL	Liberty
ECM0903	FL	Liberty
ECM0904	FL	Liberty
ECM0905	FL	Liberty
ECM0906	FL	Liberty
ECM0908	FL	Liberty
ECM0909	FL	Liberty
ECM0910	FL	Liberty
ECM0936	AL	Macon
ECM0940	AL	Macon
ECM0951	AL	Lee
ECM0952	AL	Lee
ECM0953	AL	Lee
ECM0978	AL	Macon
ECM0980	AL	Macon
ECM0982	AL	Macon
ECM0986	AL	Lee
ECM0987	AL	Lee
ECM0988	AL	Lee
ECM0993	AL	Lee
ECM0994	AL	Lee
ECM1247	AL	Macon
ECM1248	AL	Macon
ECM1258	AL	Macon
ECM1262	AL	Macon

ECM1264	AL	Macon
ECM1265	AL	Macon
ECM1266	AL	Macon
ECM1267	AL	Macon
ECM1268	AL	Macon
ECM1272	AL	Macon
ECM1278	AL	Macon
ECM1280	AL	Macon
ECM1282	AL	Macon
ECM1284	AL	Macon
ECM1285	AL	Macon
ECM1298	AL	Macon
ECM1304	AL	Macon
ECM1306	AL	Macon
ECM1308	AL	Macon
ECM1312	AL	Macon
ECM1314	AL	Macon
ECM1322	AL	Macon
ECM1326	AL	Macon
ECM1330	AL	Macon
ECM1332	AL	Macon
ECM1333	AL	Macon
ECM1334	AL	Macon
ECM1335	AL	Macon
ECM1336	AL	Macon
ECM1338	AL	Macon
ECM1340	AL	Macon
ECM1348	AL	Macon
ECM1350	AL	Macon
ECM1352	AL	Macon
ECM1354	AL	Macon
ECM1362	AL	Macon
ECM1364	AL	Macon
ECM1366	AL	Macon
ECM1372	AL	Macon
ECM1374	AL	Macon
ECM1383	AL	Macon
ECM1386	AL	Macon

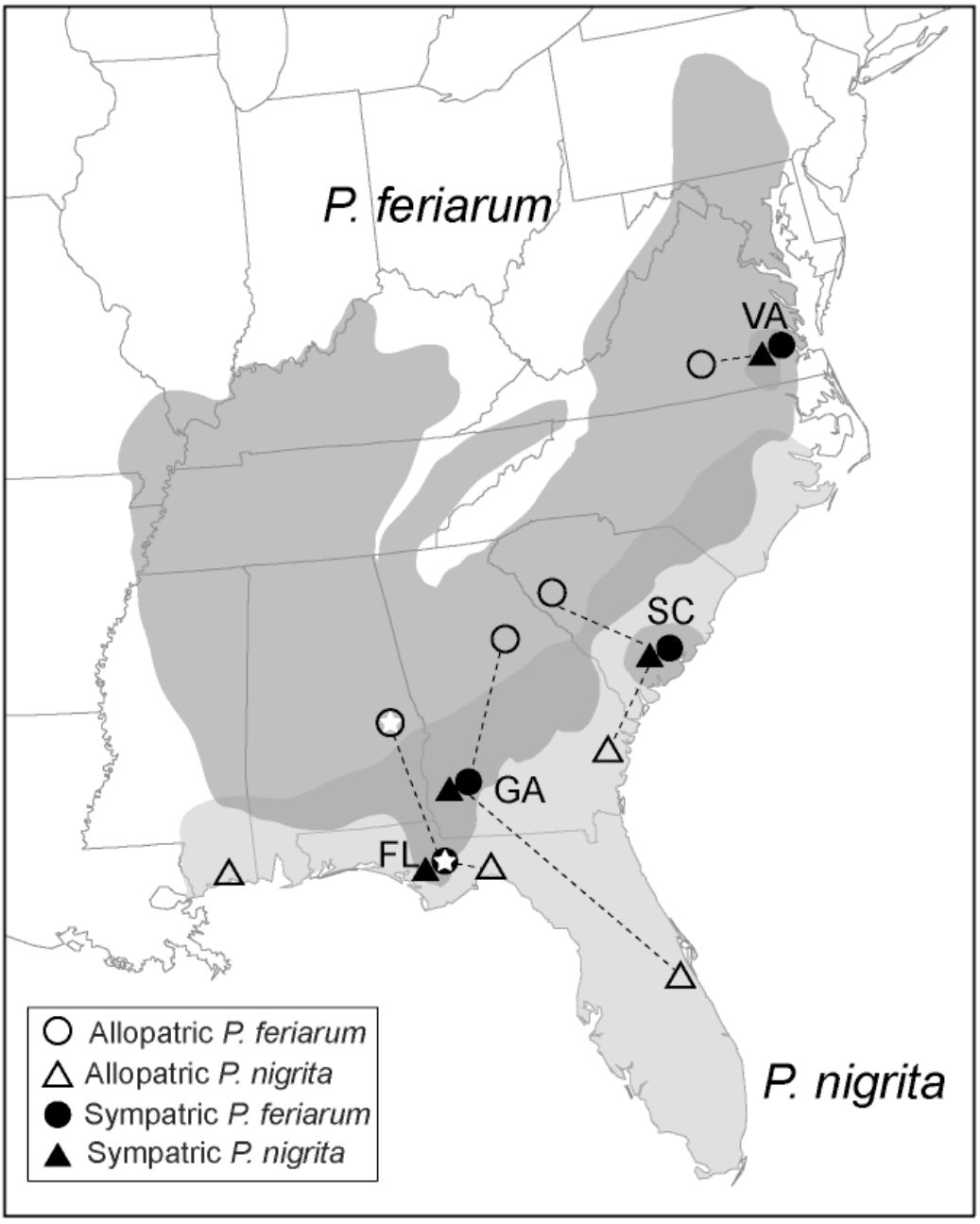

Figure 5.1. Distributions of *Pseudacris feriarum* and *P. nigrita* in the southeastern United States and populations sampled in this study. Call transects are indicated by dashed lines and state abbreviations. Female preference experiments were conducted in the two populations marked with a white star.

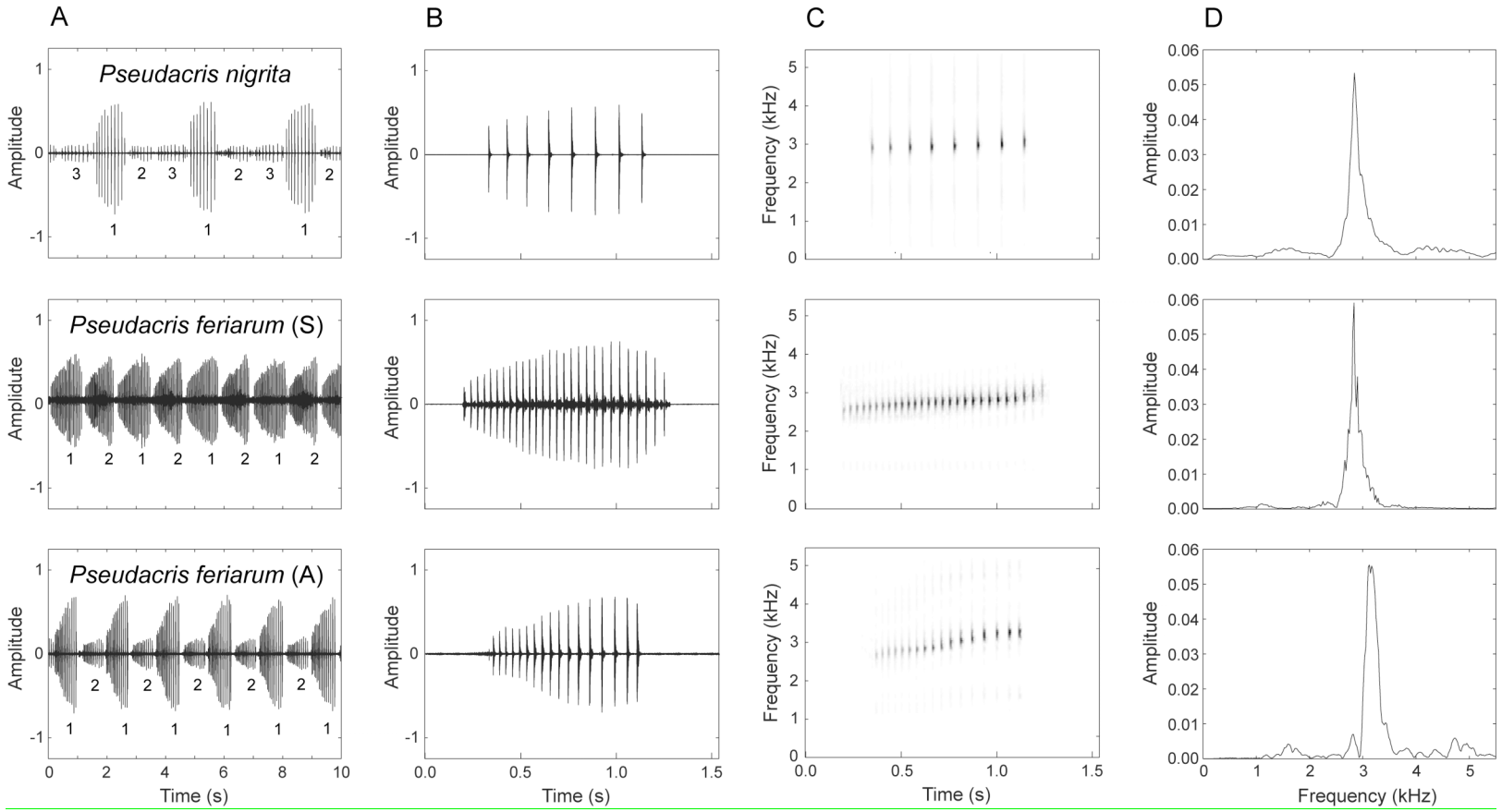

Figure 5.2. Male acoustic signal structure, represented by oscillograms (column A–10 sec sequence, column B–1.5 sec sequence), spectrograms (column C), and power spectra (column D) for allopatric *Pseudacris nigrita* (Florida), sympatric *P. feriarum* (Florida), and allopatric *P. feriarum* (Tennessee; by row). Oscillograms in column A show multiple individuals call in sequence; the different individuals are indicated by a number below each call. Calls were recorded between 12.4 and 13.8°C, therefore, temporal differences are not due to temperature variation.

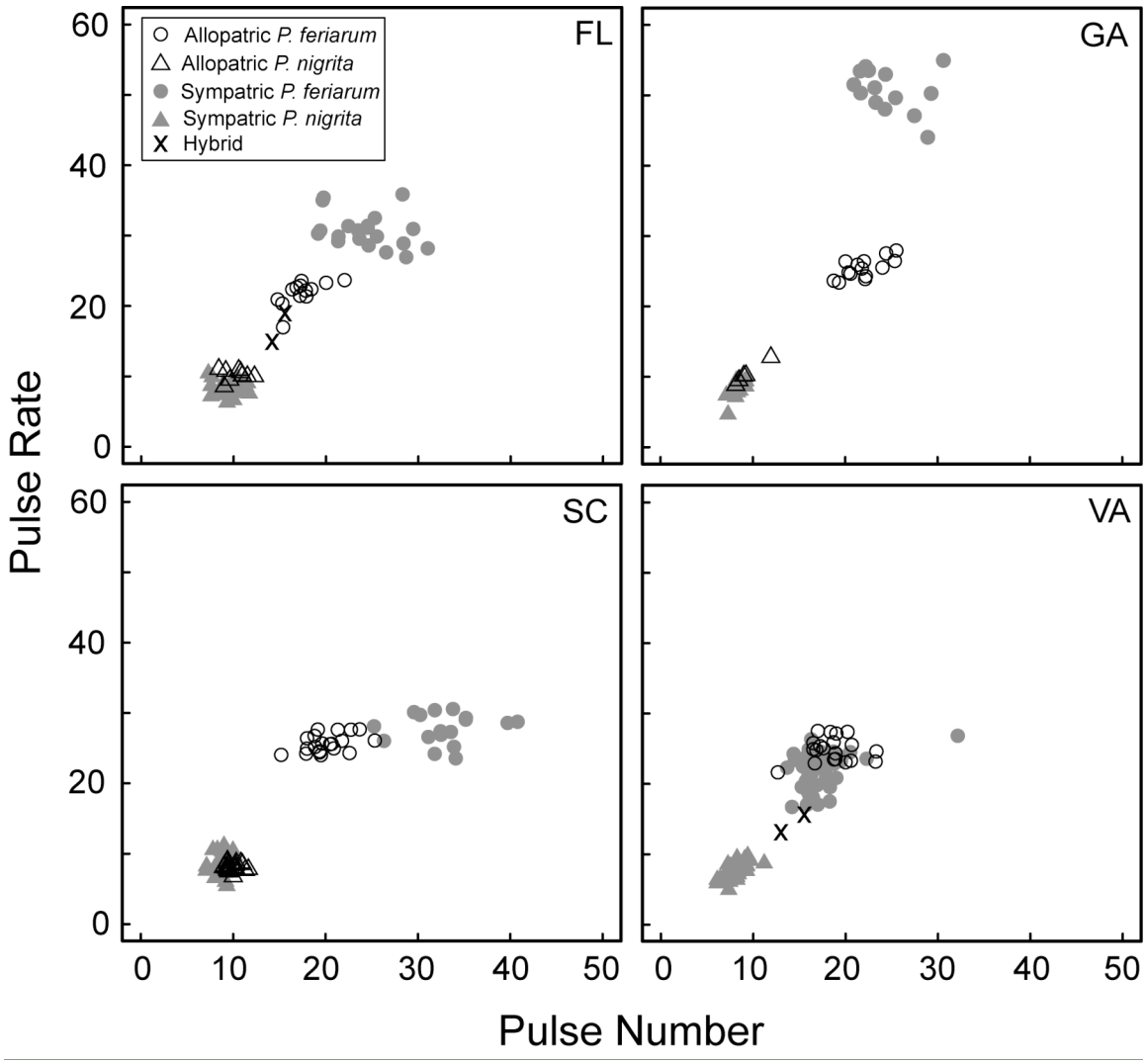

Figure 5.3. Signal divergence in the four call transects: Florida (FL), Georgia (GA), South Carolina (SC), and Virginia (VA). Putative hybrids collected in sympatry are denoted with a black "X".

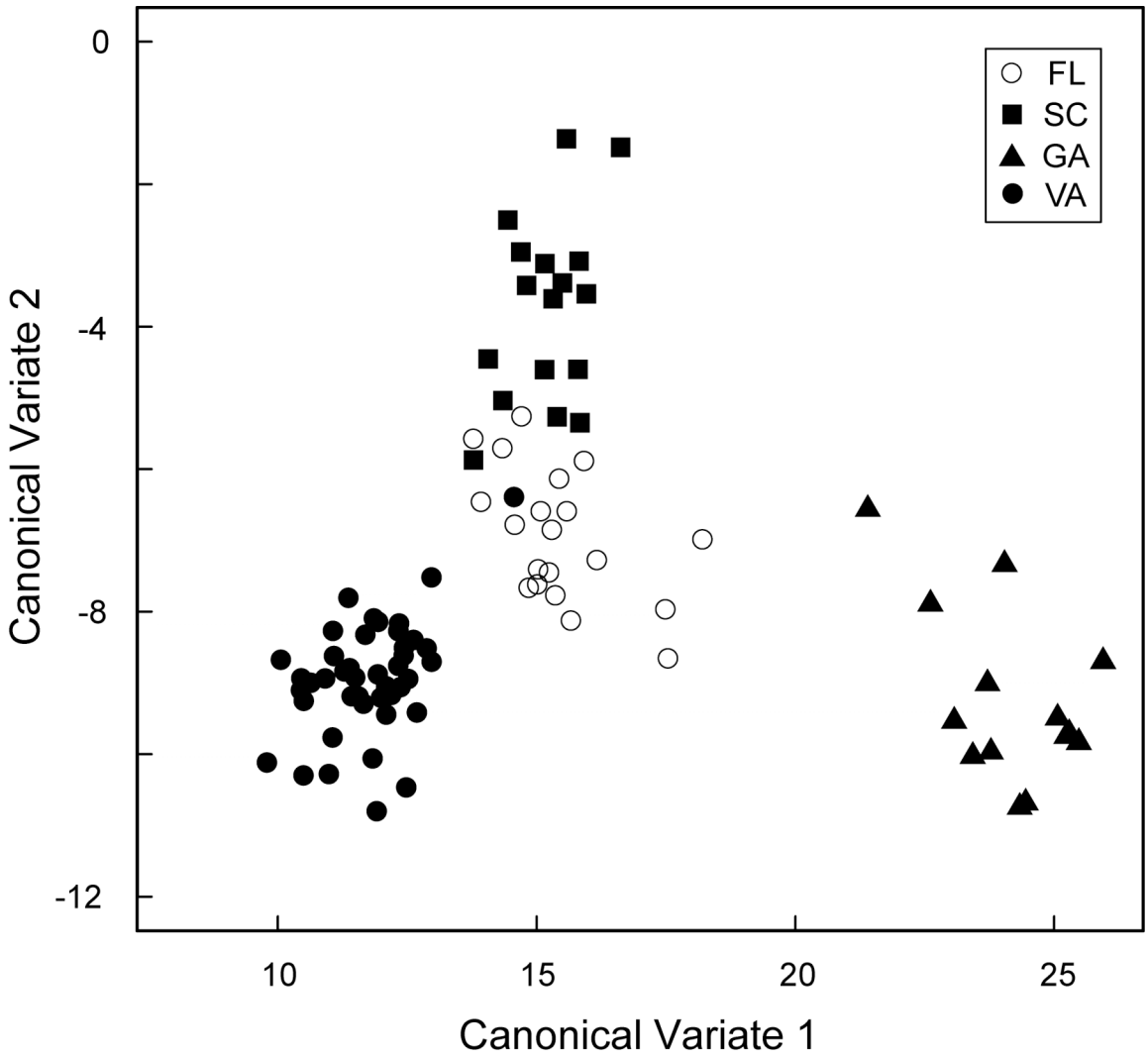

Figure 5.4. Divergence of the four sympatric *Pseudacris feriarum* populations along CV1 (pulse rate-dominated axis) and CV2 (pulse number-dominated axis). State abbreviations are the same as in Fig. 5.3.

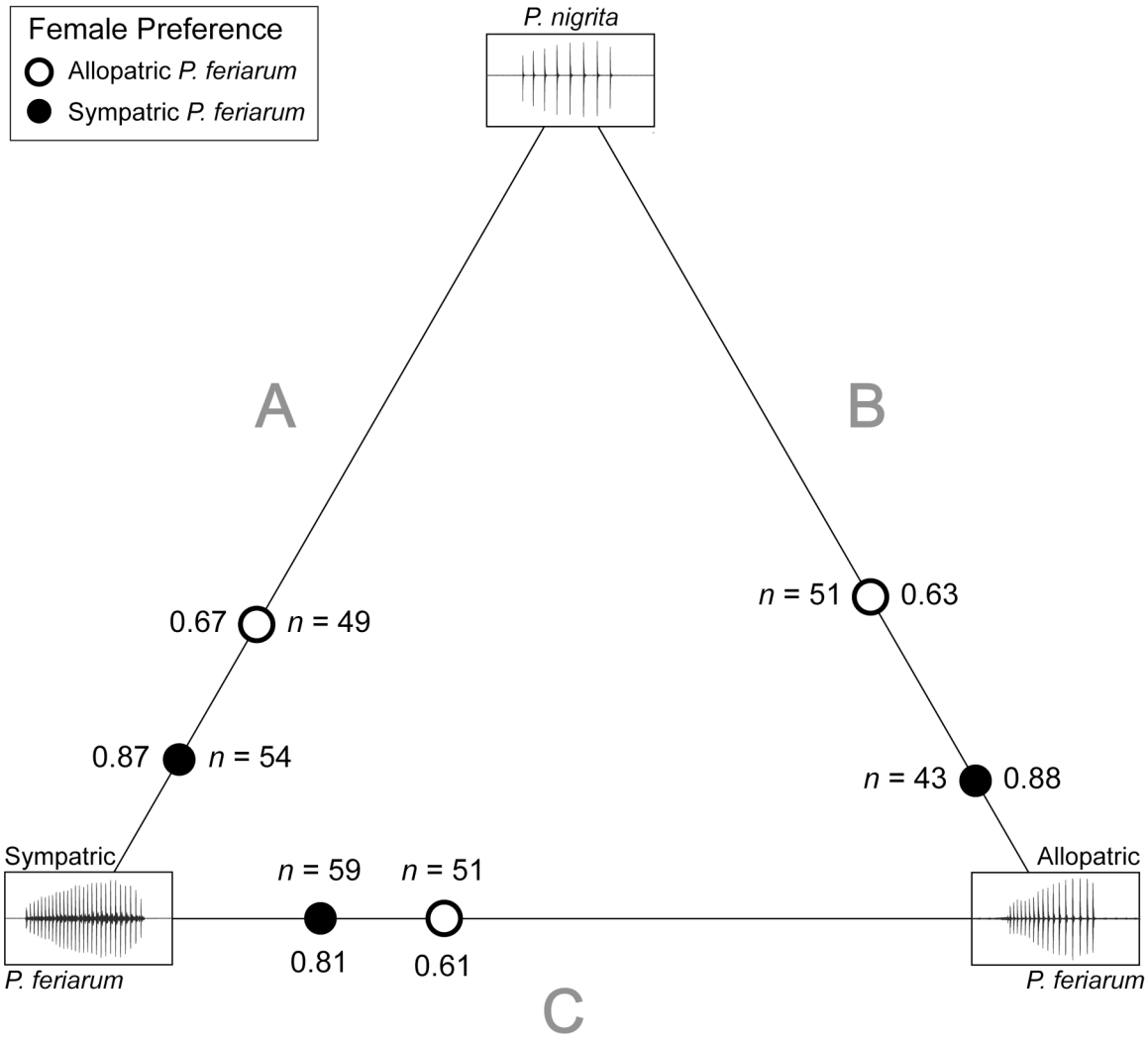

Figure 5.5. Summary of female preference results from the three experiments (A, B, and C). An oscillogram of a natural call from the respective populations is shown in the box at each vertex of the triangle. The proportion of females that chose the more popular stimulus is shown outside the triangle and the sample size is indicated inside the triangle next to each dot.

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Vita

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