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Phylogenetic relationships of the North American chorus frogs (*Pseudacris*: Hylidae)

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Abstract

We examined phylogenetic relationships of the North American chorus frogs (*Pseudacris*: Hylidae) from 38 populations using 2.4 kb 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 US populations of *feriarum* and *nigrita*, and (3) northeastern US 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*.

Keywords: *Pseudacris*; Chorus frogs; Molecular phylogeny; Mitochondrial DNA; Phylogeography

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; Gerhardt and Huber, 2002; Ryan, 2001). 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 (Cocroft, 1994; Da Silva, 1997; Hedges, 1986).

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*, *Hyla cadaverina*, *Hyla 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,

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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 1960–1970s, 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 (Conant and Collins, 1998; Kramer, 1973, 1974; Stebbins, 1985). 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 (Chantell, 1968a; Mittleman and List, 1953; Neill, 1949; Platz and Forester, 1988; Platz, 1989; Schwartz, 1957). 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* (Collins and Taggart, 2002; Conant and Collins, 1998; Da Silva, 1997; Duellman, 2001; Harper, 1939a; Jameson et al., 1966; Platz and Forester, 1988; Platz, 1989; Schwartz, 1957; Smith, 1951). *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 (Burbrink, 2000; Burbrink, 2002; Shaffer and McKnight, 1996). (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.

2. Materials and methods

2.1. Taxa sampled

We sampled 38 populations of *Pseudacris* in the United States and Canada (Appendix A), 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 °C. Based on information from previous phylogenetic analyses (Cocroft, 1994; Hedges, 1986), *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 (Appendix A).

2.2. DNA extraction, amplification, purification, and sequencing

DNA was extracted from liver and muscle tissue using the Qiagen DNeasy kit. Eight primers were used to amplify a 2.4-kb 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 GCRCTGAARATGCTGA

GATGARCCC (#30) and tRNAval-H GGTGTAAG CGARAGGCTTTKGTTAAG (#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).

2.3. Sequence alignment and phylogenetic analyses

Contiguous sequences from eight overlapping fragments were constructed in Sequencher 4.1 (GeneCodes). 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 1000 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 Nos. 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 Γ distributed substitution rates and invariable sites; Lanave et al., 1984; Hasegawa et al., 1985; Rodríguez, 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 (100,000 generations) were discarded as the burn-in. Bipartition posterior probabilities (bpp) were estimated using a consensus of 19,000 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). These datasets were 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.

2.4. 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 (Goldman et al., 2000; Shimodaira and Hasegawa, 1999), 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. 1A), Hypothesis B: Hedges' (1986) distance Wagner topology (Fig. 1B), Hypothesis C: the parsimony topology from our re-analysis of the Hedges dataset (Fig. 1C), and Hypothesis D: Cocrofts' (1994) parsimony topology (Fig. 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.

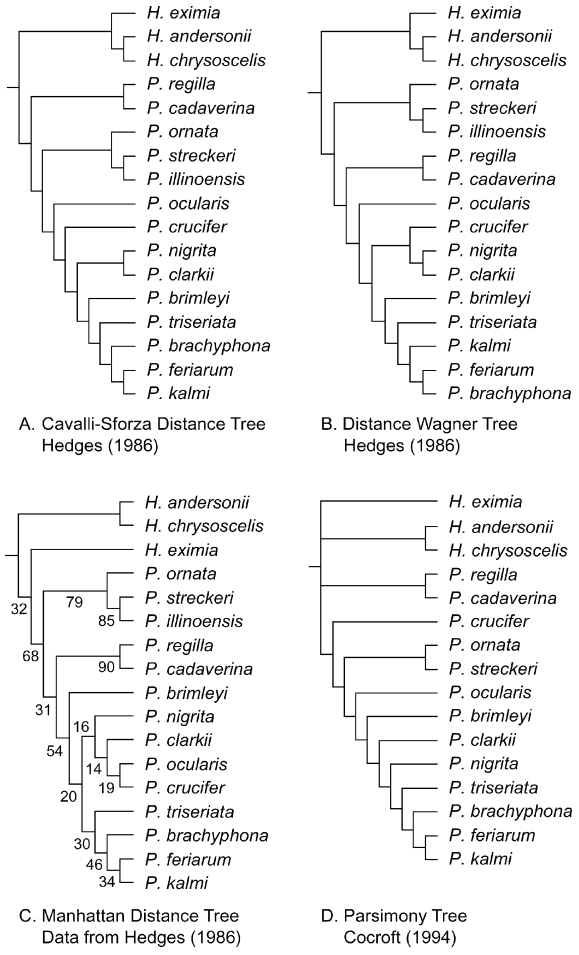

Fig. 1. Previous hypotheses of *Pseudacris* phylogenetic relationships tested in this study. (A) Hedges (1986) Cavalli-Sforza distance topology (allozyme data). (B) Hedges (1986) distance Wagner topology (allozyme data). (C) Topology based on parsimony re-analysis of Hedges (1986) dataset (allozyme data). (D) Cocroft (1994) parsimony topology (allozyme and morphological data).

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; and (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.

3. Results

3.1. 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*, *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. 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 ln $L = -10264.31767$ (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. 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 in the likelihood tree in Fig. 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 (Smith and Smith, 1952; Wright and Wright, 1949). 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. 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 US populations and Canadian populations both east and northwest of the Great Lakes (Fig. 3).

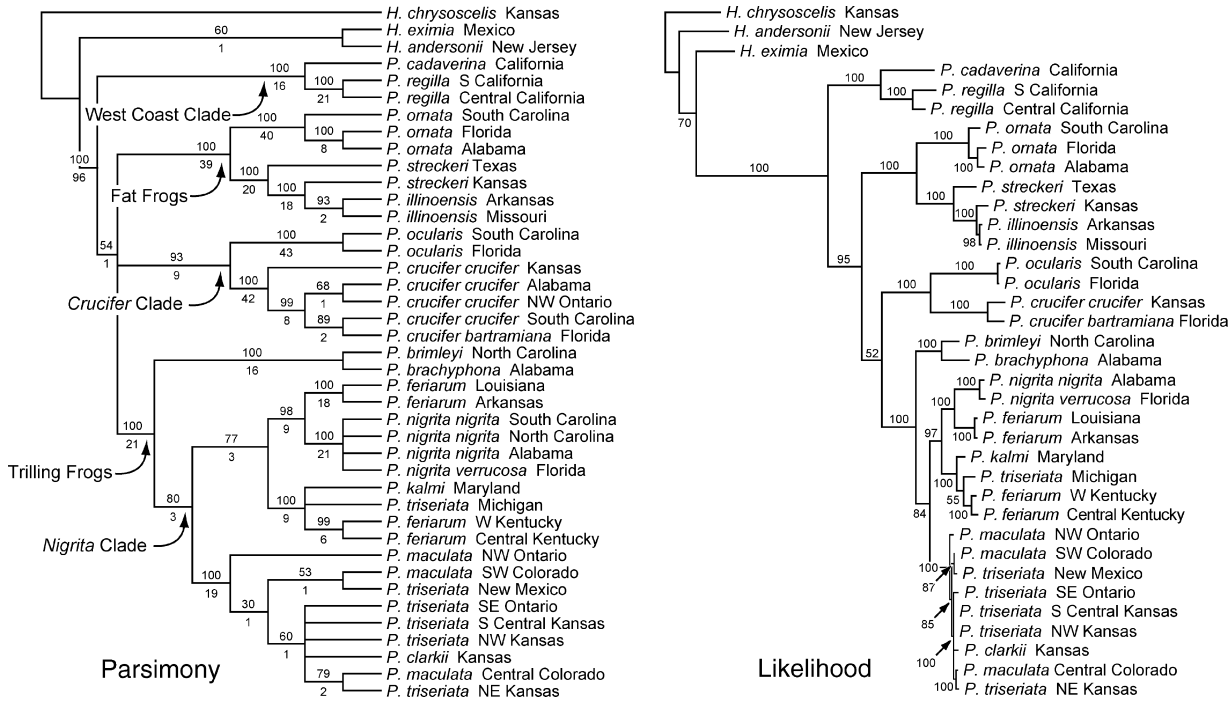

Fig. 2. Maximum parsimony tree rooted with *H. chrysoscelis* (left). Tree shown is a strict consensus of 504 equally parsimonious trees (CI = 0.55, RI = 0.83). Numbers above branches indicate nonparametric bootstrap values greater than 50% based on 1000 pseudoreplicates. Decay indices are listed below branches. Maximum likelihood tree under the GTR + Γ + I model rooted with *H. chrysoscelis* (right). Bayesian bpp values are shown above each branch. Populations of several species were excluded from the likelihood analysis.

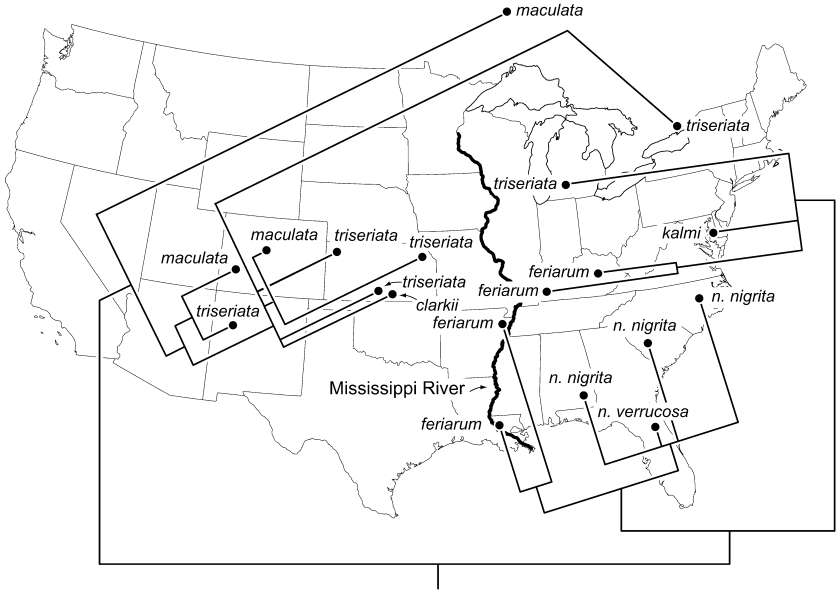

Fig. 3. Geographic distributions of the *Nigrita* Clade. Map shows geographic division of this group into eastern and western clades by the Mississippi River (thick line). The eastern clade is further subdivided into a northeastern lineage and a southeastern lineage. Current taxonomy does not reflect the phylogenetic relationships among these populations. Branch lengths are not proportional to distance.

3.2. Re-analysis of allozyme data

Our re-analysis of the Hedges (1986) allozyme dataset resulted in >50% bootstrap support for two of the four basal clade relationships for *Pseudacris* described above (the Fat Frog and Trilling Frog Clades; Fig. 1C). Although this analysis places *ocularis* and *crucifer* as sister taxa, the bootstrap proportion for

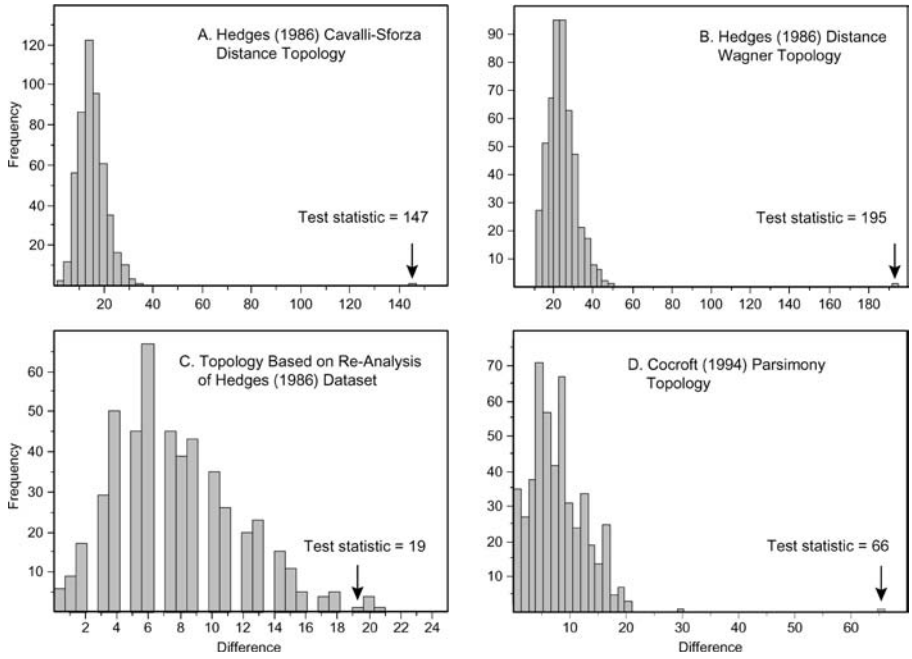

Fig. 4. Null distributions for the parametric bootstrap tests. These tests examine the validity of previous phylogenetic hypotheses for *Pseudacris*. Values of the test statistic that fall outside the 95% limits of the distribution are significant.

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.

3.3. Comparisons to alternative hypotheses

All four null hypotheses outlined in Fig. 1 were rejected. The two topologies of Hedges (1986, Figs. 1A and B) and the topology of Cocroft (1994, Fig. 1D) are rejected at $p < 0.002$. The tree from the Manhattan distance analysis of the Hedges (1986, Fig. 1C) dataset is rejected at $p < 0.014$ (Fig. 4). Overall, the reexamined phylogenetic hypotheses based on allozymes, or allozymes combined with morphological and behavioral data, are largely incongruent with the mtDNA dataset.

4. Discussion

4.1. 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 (Anderson, 1991; Chantell, 1968a; Cocroft, 1994; Da Silva, 1997; Delahoussaye, 1966; Gaudin, 1974; Hardy and Borroughs, 1986; Harper, 1939b; Hedges, 1986; Mittleman and List, 1953). 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. 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. 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, 1936; Brandt and Walker, 1933; Highton and Hedges, 1995; Hoffman, 1983; E. Moriarty, unpublished 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 Platz and Forester, 1988; Schwartz, 1957) ranges from Florida to northwestern Canada. Members of the *triseriata* complex form a subset of the *Nigrita* Clade (Fig. 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 (Joshua Rest, unpublished data; Moriarty and Berendzen, unpublished data; Platz and Forester, 1988; Platz, 1989; Schmidt, 1938; Smith and Smith, 1952; Smith, 1956). 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 (Chantell, 1968b; Harper, 1955; 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 (Platz and Forester, 1988; Platz, 1989; Smith and Smith, 1952; Smith, 1956). 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 (Cocroft, 1994; Da Silva, 1997; Hedges, 1986). 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 (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. *P. 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 (Lindsay, 1958; Lord and Davis, 1956; 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 (Conant and Collins, 1998; Platz and Forester, 1988; Platz, 1989; Smith and Smith, 1952; Smith, 1956). 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 US/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.

4.2. Phylogeographic considerations

The Mississippi River has contributed to genetic divergence in many vertebrate groups (Austin et al., 2002; Burbrink, 2000; 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. 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 US 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 *P. 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.

4.3. Status of subspecies

Collins and Taggart (2002) elevated *P. 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 (Conant and Collins, 1998; Smith, 1951). 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 *P. 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 et al. (2002), we do not find the maintenance of subspecies for *P. crucifer* and *P. nigrita* necessary or informative.

4.4. 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, 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 *H. crucifer*, *H. regilla*, *H. cadaverina*, and *L. 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*, *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*.

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Appendix A.

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 K2 tissue samples do not have voucher specimens.

Species	Sample/Voucher number	GenBank Accession No.	Collection locality
Pseudacris triseriata	KU224560	AY291090	Douglas:Kansas (NE)
P. triseriata	KU224558	AY291092	Cheyenne:Kansas (NW)
P. triseriata	KU289219	AY291091	Berrien:Michigan
P. triseriata	ECM K2	AY291088	Kingman:Kansas (S central)
P. triseriata	KU224630	AY291089	McKinley:New Mexico
P. triseriata	TNHC62324	AY291081	Frontenac, Ontario: Canada (SE)
P. ornata	KU288911	AY291104	Liberty:Florida
P. ornata	TNHC62183	AY291105	Aiken:South Carolina
P. ornata	TNHC62178	AY291106	Barbour:Alabama
P. crucifer crucifer	KU288677	AY291102	Linn:Kansas
P. crucifer crucifer	KU290341	AY291101	Lac Seul, Ontario: Canada (NW)
P. crucifer crucifer	TNHC62210	AY291099	Barbour:Alabama
P. crucifer crucifer	TNHC62216	AY291100	Barnwell:South Carolina
P. crucifer bartramiana	TNHC62369	AY291103	Ocala:Florida
P. streckeri	KU289036	AY291107	Harper:Kansas
P. streckeri	TNHC62317	AY291108	Travis:Texas
P. illinoensis	TNHC62346	AY291110	Scott:Missouri
P. illinoensis	TNHC62351	AY291109	Clay:Arkansas
P. clarkii	KU289035	AY291093	Chautauqua:Kansas
P. feriarum	KU289227	AY291084	Calloway:Kentucky (W)
P. feriarum	R. Highton 71747	AY291096	Lincoln:Kentucky (central)
P. feriarum	TNHC62265	AY291085	East Baton Rouge: Louisiana
P. feriarum	TNHC62255	AY291086	Craighead:Arkansas
P. kalmi	KU289235	AY291087	Kent:Maryland
P. maculata	KU290342	AY291082	Lac Seul, Ontario: Canada (NW)
P. maculata	KU224624	AY291080	Gunnison:Colorado (central)
P. maculata	KU224625	AY291083	Archuleta:Colorado (SW)
P. nigrita nigrita	MVZ11452	AY291077	Scotland:North Carolina
P. nigrita nigrita	TNHC62201	AY291078	Barbour:Alabama
P. nigrita nigrita	TNHC62208	AY291076	Barnwell:South Carolina
P. nigrita verrucosa	TNHC62364	AY291079	Brevard:Florida
P. brimleyi	TNHC62337	AY291094	Pitt:North Carolina
P. brachyphona	TNHC62303	AY291095	Tallapoosa:Alabama
P. ocularis	TNHC62234	AY291097	Barnwell:South Carolina
P. ocularis	TNHC62241	AY291098	Gulf:Florida
H. chrysoscelis	KU289034	AY291116	Douglas:Kansas
H. eximia	UTA A-13225	AY291113	Morelia, Michoacán:Mexico
P. cadaverina	KU207382	AY291114	San Diego:California
P. regilla	KU207396	AY291111	San Diego:California (S)
P. regilla	TNHC62409	AY291112	Berkeley:California (S central)
H. andersonii	KU207335	AY291115	Burlington:New Jersey

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