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Cryptic lineages in a small frog: the post-glacial history of the spring peeper, *Pseudacris crucifer* (Anura: Hylidae)

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Abstract

The spring peeper (*Pseudacris crucifer*) is believed to have been a primary herpetological invader of eastern North America following the most recent period of glacial retreat. We examined the phylogeographic pattern and population structure of *P. crucifer* to determine whether the distribution of haplotypic variants reflect post-Pleistocene recolonization dynamics. A number of geographically isolated evolutionary lineages were supported by both maximum parsimony and neighbor-joining analyses, and by coalescence approaches applied to mtDNA. South-western Ontario represents a high level of genotypic diversity (π) due to the presence of two divergent lineages. The geographic distribution of these lineages are interpreted as reflecting post-glacial recolonization dynamics from separate, isolated refugia during the late Pleistocene that have come into secondary contact in SW Ontario. The phylogenetic placement of haplotypes from the range of *P. crucifer bartramiana* (Florida and South Carolina) does not allow for monophyly of *P. crucifer crucifer*, and therefore the *bartramiana* subspecies designation does not reflect a separate evolutionary lineage.

Keywords: *Pseudacris*; Phylogeography; Gene flow; North America; Pleistocene; Secondary contact; Xerothermic

1. Introduction

Climatic events of the Pleistocene in North America are well documented (Anderson and Lewis, 1992; Webb et al., 1993) and have had a large effect on the historical distribution of a number of North American taxa including amphibians (e.g., Green et al., 1996). As such, the origin and distribution of contemporary populations in temperate regions can only be fully understood with reference to the historical events of the Pleistocene (Blair, 1965). Genetic methods for studying intraspecific relationships (Avise et al., 1987) have demonstrated the linkage between gene genealogy and post-glacial history in a wide array of taxa. It is still the case that most studies on the phylogeographic impact of Pleistocene events have been conducted on southern continental USA species, particularly vertebrates (but see Bernatchez and Dodson, 1991; Billington et al., 1992; Green et al., 1996), with few broad scale phylogeographic studies on taxa that have recolonized previously glaciated regions of North America (e.g., Omland et al., 2000; Wilson and Hebert, 1998). Such studies could be key to understanding the impact of large-scale recolonization and refugial dynamics. Range adjustments in the Great Lakes region were dramatic because of the extension of the Pleistocene ice sheet deep into the midwest United States approximately 20,000 years before present (ybp), and the subsequent rapid deglaciation over the present-day Great Lakes beginning approximately 15,000 ybp (Holman, 1995).

Amphibians are excellent model species for the study of gene genealogy in relation to post-glacial history due to their limited vagility (relative to birds and large mammals), the tendency to be insufficiently motile to cross moderate barriers of unfavorable habitat, and the

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fact that some temperate species are widely distributed, permitting comparison of genetic structure between previously glaciated and unglaciated regions.

Amphibian species experienced numerous range adjustments during the late Wisconsonian as the ice sheets waxed and waned along with concomitantly altered climate patterns. The timing and speed of movement of amphibians into previously glaciated habitat would have depended on the habitat requirements and dispersal ability of the species in question. Reinvasion of major plant species is believed to have occurred quickly and from different major refugia (e.g., southern Appalachian Highlands, Mississippi Embayment, or Coastal Plain; Stuckey, 1993), during the time period lasting roughly 12,000–9000 ybp (Bennett, 1985, 1986), and some herpetological species would have closely followed the northward expanding habitat at the end of the Wisconsinian (Holman, 1995). If this is the case, it may have allowed for a 'patchwork' of reinvasion (i.e., regions being invaded from multiple refugia) as areas deglaciated in a latitudinally non-uniform manner. Paleoclimatic events such as melt water-induced climate change would have repeatedly altered the microclimate (and habitat) of the Great Lakes region (Anderson and Lewis, 1992) possibly having important affects on recolonization in areas where glacial runoff was great, creating possible barriers to gene flow.

A hypothetical pattern of colonization in which primary amphibian and reptilian invaders closely followed the non-uniform retreat of the ice sheet into southern Michigan and then SW Ontario, has been proposed based on geological data, paleobotanical and paleovertebrate assemblages, and ecological tolerances of modern amphibian fauna (Holman, 1992). Species entered southern Michigan first through what is now Indiana and Ohio, and would have gained access to southern Ontario between what is now Lakes Huron and Erie, and between Lakes Erie and Ontario. Later invasions into eastern Ontario and Quebec would have occurred across the St. Lawrence lowlands once the Chaplain Sea subsided (<10,000 ybp), and from the west around Lake Superior. Due to its probable history as a primary invader of deglaciated regions of eastern North America (Holman, 1992), *Pseudacris crucifer* is an excellent model for the study of phylogeography and genetic effects of post-glacial range expansion.

Pseudacris crucifer is a small hylid frog ubiquitous throughout eastern North America. Although widespread, it is restricted to woodland areas, where it breeds early in the season (April–May in northern populations, January–March in the south), in temporary or semipermanent ponds (Conant and Collins, 1998). The species current range extends as far north as James Bay (approximately 52°N), in part due to its freeze tolerance (Churchill and Storey, 1996). Subspecies designation (*P. crucifer bartramiana*) was afforded to southern

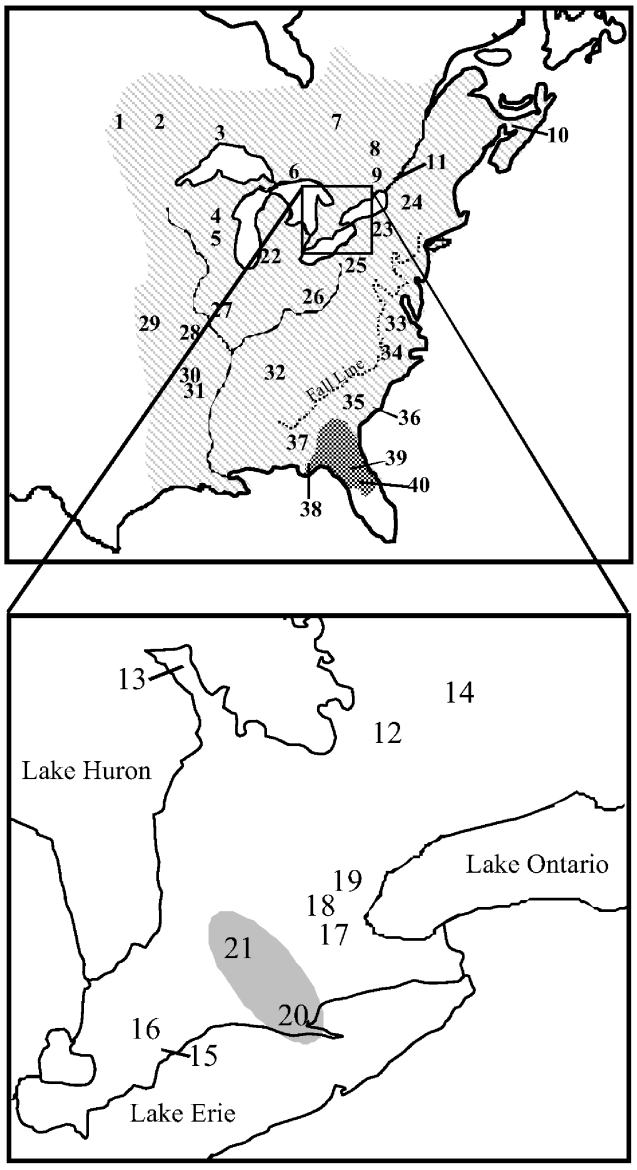

Fig. 1. Distribution of 40 sampled spring peeper populations in eastern North America (see Appendix A for locations and sample sizes). The distribution of *Pseudacris crucifer crucifer* (light shade) and *P. c. bartramiana* (dark shade) are indicated (after Conant and Collins, 1998). Inset: SW Ontario with genetic secondary contact zone indicating populations (circled in gray) with diverse mtDNA haplotypes from the central and northern clades. The fall line represents the erosional scarp separating the Appalachian Piedmont from the Coastal Plain.

populations (S. Georgia and N. Florida; see Fig. 1) based primarily on color pattern variation (Harper, 1939). However, the genetic basis of this designation has not been extensively studied (but see Chippindale, 1989). Our objective was to determine whether the genealogical structure of *P. crucifer* reflects historical factors, such as the patterns of glacial retreat. Specifically we ask the following: did post-glacial expansion follow single or multiple routes? If so, did these stem from multiple refugia? Is the pattern of haplotypic variation in

the Great Lakes region consistent with historical (i.e., pattern of glacial retreat) geography? And to what degree are northern and southern populations genetically distinct?

2. Materials and methods

2.1. Taxon sampling

Mitochondrial haplotypic variation was examined in 151 individuals of *P. crucifer* from 40 locations (Fig. 1, Appendix A). Sampling was concentrated in the Great Lakes region to examine fine scale patterns of haplotypic variation, with additional sites examined from across much of the range to resolve larger geographic patterns of haplotype radiation following the Wisconsin glaciation, and to examine the genealogical relationship between northern and southern populations. Samples were obtained from numerous sources, either as ethanol preserved or frozen muscle tissue, toe clips from live specimens subsequently released at the point of capture, or previously used elecrotrophoretic samples (see Appendix A for sources). We chose two closely related species as outgroups, *Pseudacris ocularis* and *Pseudacris triseriata* (Cocroft, 1994). Recent phylogenetic work on North American chorus frogs suggests that *P. ocularis* is the sister species to *P. crucifer* (based on complete sequences of the 12S and 16S genes, E. Moriarty, unpublished data). Template DNA was extracted using QIAamp DNA Mini Kit (Qiagen) following manufacturers instructions and stored at −20 °C.

2.2. mtDNA surveys

Polymerase chain reaction (PCR) amplifications of double-stranded product were preformed on a Perkin–Elmer 9600 thermocycler. Amplification of a 692 bp segment of cytochrome *b* (cyt-*b*) was done using primers MVZ15-L and MVZ18-H (Moritz et al., 1992). A 616 bp segment of the 16S gene was amplified using primers 16Sar-L and 16Sbr-H (Palumbi et al., 1991). The PCR products correspond to positions 16243–16934 (cyt-*b*) and 3956–4571 (16S) of the total mtDNA sequence of *Xenopus laevis* (Roe et al., 1985). A negative control was included for all PCR reactions. Twenty-five μl reactions were used, containing 25–50 ng of template, 1 μM of each primer, 2 mM $MgCl_2$, 1× Gibco BRL reaction buffer, 10 mM dNTPs, 0.4 U of Gibco BRL Taq polymerase. Amplification conditions for both genes involved a denaturation stage at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 10 s, primer annealing at 50 °C for 20 s and elongation at 72 °C for 20 s. A final elongation of 5 min at 72 °C was followed by cooling to 4 °C. Product was run out on 2% agarose gels (Boehringer–Mannheim) in 1× TBE buffer, with subsequent staining in ethidium bromide and visualization under UV light. Bands were cut out of low melt agarose and cleaned using Agarase (Boehringer–Mannheim) following manufacturer's instructions. PCR product was cycle-sequenced (Amersham—Thermo-Sequenase) using MVZ15-L and 16Sbr-H with the following profile: 35 cycles of 94, 55, and 72 °C for 30, 30, and 70 s respectively, followed by a final extension of 70 °C for 7 min. Product was run out on 6% PAA gels for 2–6 h, blotted, vacuum-dried and placed on film (Kodak—Biomax) for 24–48 h. Autoradiograms were read by eye and sequences were aligned using GeneWorks (IntelliGenetics, Mountain View, CA, USA) with subsequent verification by eye.

2.3. Analysis of mitochondrial DNA data

Nucleotide diversity (π, or the mean of pairwise sequence differences) and its standard error were estimated as in Nei (1987) using ARLEQUIN vers 2.0 (Schneider et al., 1999). We calculated π for: (1) the entire ingroup sample, (2) clades identified by both a tree building algorithm and a parsimony network (see below), and (3) for each population sample, where $n > 3$. Tajima's test for selective neutrality (Tajima, 1989a,b) was used to compare two estimates of the parameter θ among clades; one being derived from π the other (θ_P) being estimated from the number of segregating sites. Under the infinite-sites model Tajima's test can be used to assess whether impact of selection or population change (i.e., expansion) can be detected. Significance was assessed by comparing calculated D values with confidence intervals listed in Tajima's (1989a, Table 2). To further assess demographic changes we constructed mismatch distributions using Arlequin (ver. 2.0, Schneider et al., 1999), and compared them to Poisson distributions (Slatkin and Hudson, 1991), randomly generated using Data Desk (vers. 6.0.2, Data Description, Ithaca, NY, USA).

Permutation tail probability (PTP) tests (Faith and Cranston, 1991, as implemented in PAUP* vers. 4.0; Swofford, 1999) were used to evaluate phylogenetic signal within each data partition. Prior to tree building using the total evidence approach (Huelsenbeck et al., 1996), we tested for conflicts between the data partitions (cyt-*b*—16S) for ingroup taxa using the partition homogeneity test (Farris et al., 1995) as implemented in PAUP* using 100 rounds of data swapping and tree estimation with TBR branch swapping. We tested for saturation of our sequence data by plotting sequence divergence (raw distance) verses Kimura 2-parameter (Kimura, 1980) distance for cyt-*b* (first, second, and third base pair separately) and 16S (Berbee et al., 1995; Burns, 1997).

Phylogenetic relationships among mtDNA haplotypes representing 151 *P. crucifer*, one *P. ocularis* and one *P. triseriata* were estimated using maximum

parsimony (MP) and Neighbor-joining (NJ) criteria implemented in PAUP* 4.0 (Swofford, 1999). Maximum parsimony analyses consisting of 100 heuristic searches using random stepwise addition, followed by tree-bisection-reconstruction (TBR) branch swapping. Support for the clades was estimated with non-parametric bootstrapping (Felsenstein, 1985) using 100 pseudoreplicates. Pseudoreplicates were executed as heuristic searches with 10 additions per replicate. NJ analysis was based on Kimura 2-parameters distances (Kimura, 1980) that corrects for multiple substitutions per site. Support for the NJ tree was estimated with 1000 nonparameteric bootstraps.

Traditional methods of phylogenetic reconstruction were created for interspecific studies, and these methods harbor assumptions that are violated by intraspecific studies (Crandall and Templeton, 1996). For example, in higher level studies ancestral taxa are assumed to be extinct, whereas coalescent theory predicts that, at the intraspecific level, ancestral haplotypes are not only still present, but are the most common haplotypes in a population (Donnelly and Tavarée, 1986; Watterson and Guess, 1977). At the intraspecific level, phylogenetic relationships among haplotypes can best be estimated using the algorithm of Templeton et al. (1992). Moreover, when parsimony-informative characters are few, the algorithm of Templeton et al. (1992) reconstructs phylogenetic relationships more accurately than maximum parsimony (Crandall, 1994). The network obtained gives a finer-grained pattern of phylogenetic relationships with more than 95% probability that the observed steps are parsimonious. Parsimony probabilities for the haplotype pairs differing at one or more sites (P_j) were calculated with the ParsProb program (D. Posada, available at http://bioag.byu/zoology/crandall_lab/programs.htm). We then constructed a network according to Templeton et al. (1992).

Population structure was also assessed by performing analysis of molecular variance (AMOVA). Φ statistics (analogous to the F-statistics of Wright, 1965), were calculated using ARLEQUIN (ver. 2.0, Escoffier et al., 1992; Schneider et al., 1999). Analyses were preformed with clades A, C, D, and subclades a and b of clade B (see below) designated as the highest (i.e., regional) level of genetic apportionment. The regional apportionment of variation with respect to all haplotypes is described by Φ_{CT}, Φ_{SC} describes the apportionment within populations of the defined regions, and Φ_{ST} refers to the variation in a single population relative to all haplotypes. Unfortunately small sample sizes precluded the use of some locations; however, we combined samples based on their close geographic proximity. Omitted were populations 1, 2, 22, 26, 29, and 34. We combined populations 30 with 31, 35 with 36, 37 with 38, and 39 with 40 (see Appendix A). The AMOVA assumes that groupings represent populations and that the populations are in drift-migration equilibrium, conditions that may be unrealistic for the regional and combined population groupings. Levels of significance of the Φ statistics were determined through 1000 permutation replicates.

3. Results

3.1. Mitochondrial DNA data

There were two possible insertions or deletions observed in the 16S partition in the ingroup and another four insertions in the outgroup sequence (see Appendix B for accession numbers). These were treated as missing for phylogenetic analyses resulting in 585 bp of mtDNA (291 bp of cyt-*b* and 294 bp of 16S), for which there were a total of 60 variable characters. For cyt-*b* there were 45 variable positions including 28 potentially phylogenetically informative sites. For 16S there were 15 variable characters for which 12 were parsimony-informative. The absence of stop codons and indels in the cyt-*b* sequence, as well as strand bias against guanine on the light strand, a notable 3rd codon bias, and an overall conservation of codons among the 151 samples suggests that our cyt-*b* sequences are not nuclear homologs. Overall there was a thymine bias within the ingroup (mean cyt-*b*—$A = 0.2442$, $C = 0.2436$, $G = 0.1507$, $T = 0.3614$; mean 16S—$T = 0.2786$, $G = 0.1710$, $C = 0.2189$, $A = 0.3314$; mean combined—$A = 0.2868$, $C = 0.2319$, $G = 0.1614$, $T = 0.3198$). Mean pairwise ti/tv ratios for cyt-*b* and 16S were 5.51 and 3.84, respectively, combined mean = 5.07 (excluding pairwise comparisons where transversions were not detected). Mean pairwise sequence divergence varied from 0.3% to 3.5% for 16S among ingroup haplotypes, 8% to 11% between *P. crucifer* and *P. triseriata*, and 10% to 12% between *P. crucifer* and *P. ocularis*. Mean pairwise distances for cyt-*b* ranged from 0.3% to 6% among ingroup haplotypes, 16% to 20% between the ingroup and *P. triseriata*, and 14% to 17% between *P. crucifer* and *P. ocularis*. Combined data partitions produced distances ranging from 0.2% to 3.8% among ingroup haplotypes, 12.1% to 14.6% and 12.4% to 14.1% among ingroup and *P. triseriata* and *P. ocularis*, respectively. The nucleotide diversity (π) across all ingroup samples averaged 0.0177 (SE = 0.0090).

3.2. Genetic structure and phylogeography

Partition homogeneity test indicated that the two data sets were not significantly heterogeneous ($P > 0.05$). PTP results on the combined data partitions suggest phylogenetic signal significantly different from random ($P < 0.001$). For these reasons, as well as the fact that mtDNA genes are geneologically linked, we chose to examine the data set as one combined rather

than as separate partitions. Plots of Kimura 2-parameter by *p*-distance suggested a negligible level of saturation at 16S and the first and second base pair of cyt-*b*, with possible saturation evident at the third base pair position (plots not shown).

A total of 52 unique mtDNA sequences were identified from *P. crucifer*. MP analyses resulted in 2784 equally parsimonious trees with a score of 230. There was congruence among NJ and MP trees where bootstrap support exceeded 50%; however, resolution was lower for the MP algorithm. Bootstrap support for the NJ analysis robustly supported a number of clades including: (i) A western clade (99%) consisting of haplotypes found in the Arkansas, Missouri, Kansas, and SW Illinois samples (clade C, Fig. 2). (ii) A central clade (81%) consisting of populations extending from Tennessee north into SW Ontario (clade D, Fig. 2). This clade represents all the haplotypes from Tennessee, Ohio, Michigan, and populations 15 and 16 in the extreme SW portion of Ontario (see Appendix A). (iii) A SE clade (60%) consisted of haplotypes from Florida, Alabama, South Carolina, North Carolina, and Virginia (clade B). Within this clade, two sub-clades consisting of (a) haplotypes from North Carolina and Virginia (94%) and the haplotypes from Florida, South Carolina, and Alabama (94%). Clades A and B were sister clades (64%) while clades C and D were basally positioned. Bootstrap support for MP analyses provided strong support for the sub-clades within clade B and high support for clade D (Fig. 2). Three geographically divergent haplotypes (U—Ottawa, V—Virginia, WW—Long Point) were grouped together by the NJ analyses although bootstrap support was greater that 50% for only haplotypes U and V. The remaining haplotypes, representing the northern populations of the US and Canada formed a large polytomy reflecting the close phylogenetic relationship among most haplotypes as revealed by the parsimony network (see below). Two populations in central SW Ontario (Long Point and Wildwood; Nos. 20 and 21, Fig. 1) have representative divergent haplotypes from the central clade and northern clade (see Fig. 3). The geographic position as well as admixture of haplotypes suggests secondary contact.

The maximum number of differences for parsimonious connections among haplotypes at the 95% level was 10. The resulting parsimony network reveals relationships among haplotypes that could not be resolved through MP or NJ analyses. For most of the northern populations (north of the last glacial maximum), three haplotypes dominate (A, K, and O), with a number of unique haplotypes, restricted to single individuals with 1 or 2 bp differences derived from these three haplotypes (see Fig. 3). This typical starburst pattern is suggestive of a widespread species having originated from a small number of founding individuals (Avise, 2000). The large number of haplotypes differing by only 1 or 2 bp may be the result of artificial substitution due to high *Taq* error rate during PCR (Cline et al., 1996; Kobayashi et al., 1999; Lundberg et al., 1991). This would not effect our analysis since our major findings represent divergences that could not be explained by PCR error. However, the mitochodrial haplotype diversity seen here is not atypical, and in fact is relatively low for intraspecific studies of anuran amphibians (e.g., *Litoria fallax*, James and Moritz, 2000; *Hyla arenicolor*, Barber, 1999; *Epipedobates femoralis*, Lougheed et al., 1999). The number of mutational differences between the western haplotypes (clade C) and the rest of the network exceeded the number required for 95% probability (Fig. 3).

Mismatch distributions for all examined clades were significantly different from Poisson expectation (χ^2, all $P > 0.05$) with the exception of clade D (Fig. 4). However, clade C was weakly non-significant ($0.1 > P > 0.05$). Clades A and B had broad, multimodal shapes suggestive of a stationary rather than expanding population (Slatkin and Hudson, 1991). This pattern was also observed for sub-clade a, populations 35–40 that are located in Florida, Alabama, and South Carolina. Clade D has a unimodal pattern that can be associated with either a pattern of population expansion (Harpending, 1994; Slatkin and Hudson, 1991), or, may also be due to variation in substitution rate among sites (Aris-Brozou and Excoffier, 1996). The latter case may have influenced the observed pattern since the third base pair position in cyt-*b* is known to accumulate mutations at a faster rate than first or second base pair positions. However, our saturation plots suggest minimal levels of saturation (not shown) and one would expect the unimodal pattern to be observed across more than just clade D if rate variation were an important factor.

Nucleotide diversity is highest in clade B, but not significantly so (Fig. 5). Comparisons of nucleotide diversity among populations shows the highest amount of diversity in areas of secondary contact (populations 20 and 21) and areas that may have been close to, if not in, refugial areas (population 32). Unfortunately, small sample sizes from the Southeast precluded comparisons from there. There was no significant difference between nucleotide diversity (θ_π) and θ_P in the total sample (Tajima's $D = -0.327$, $n = 151$, $P = 0.39$), nor for the central (clade D) or SE (clade B) haplotype groups analysed separately (central: Tajima's $D = -0.887$, $n = 18$, $P = 0.21$; SE: Tajima's $D = 0.069$, $n = 12$, $P = 0.58$). Tajima's D remained non-significant when sub-clades a and b were examined separately (clade a: Tajima's $D = -0.278$, $n = 9$, $P = 0.42$; clade b: Tajima's $D = 0.000$, $n = 3$, ns). The western group had a significant D value (Tajima's $D = -1.955$, $n = 19$, $P = 0.01$), while the northern group was nearly significant (Tajima's $D = -1.328$, $n = 96$, $P = 0.08$). Overall, the

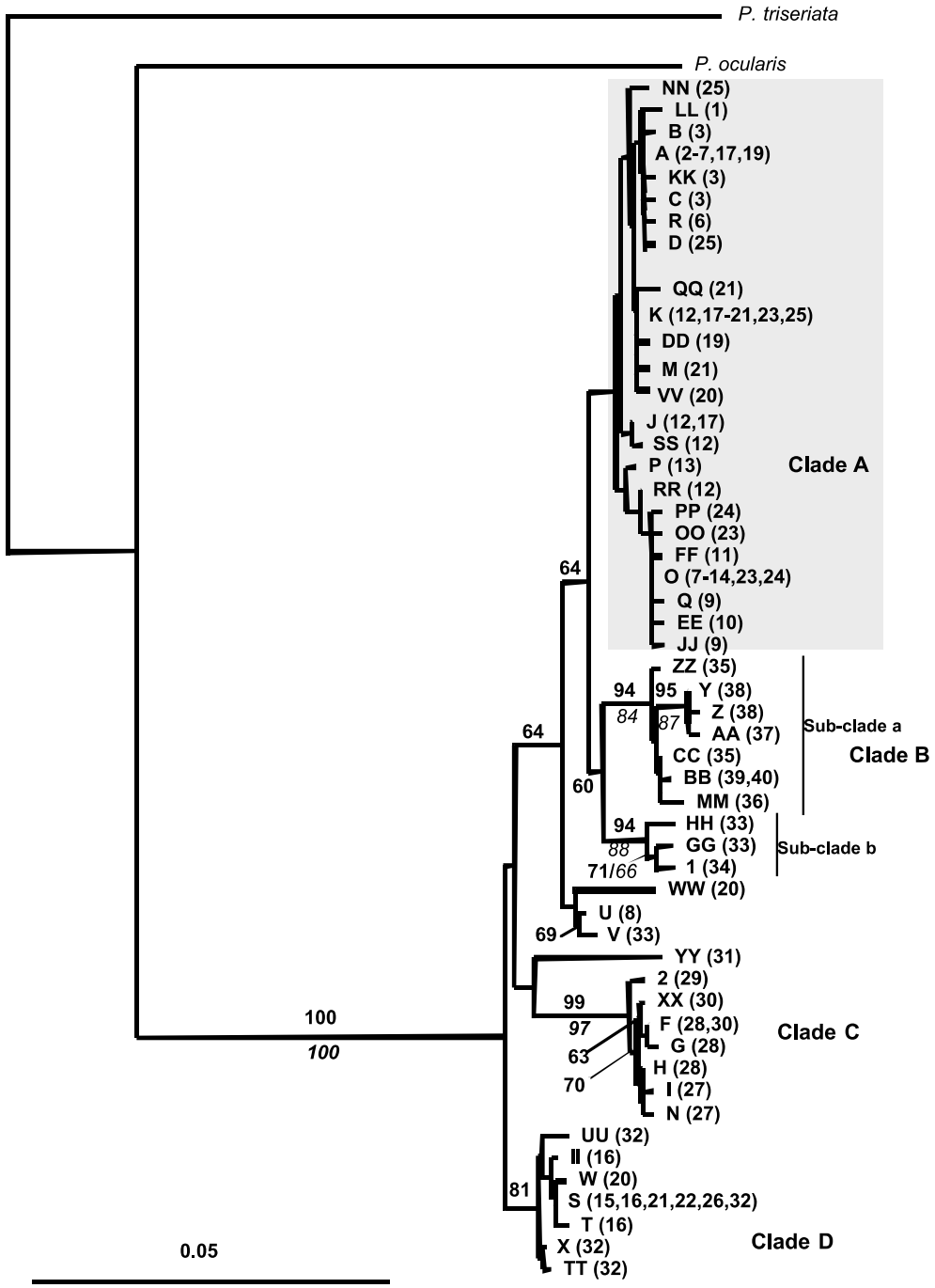

Fig. 2. Phylogeny of the spring peeper mtDNA sequences. The tree was derived using Neighbor-joining (NJ) criteria (Kimura 2-parameter). Bootstrap support for nodes derived for NJ (1000 replicates) are indicated above the branch, with maximum-parsimony bootstrap support (100 replicates) are italicized below branches. Low resolution of the MP consensus tree (see Section 3) precluded displaying it separately. Haplotype designations match those from Appendix B. Next to haplotypes are population numbers from which the haplotypes were found. Note that most haplotypes were restricted to single populations with a few wide-spread haplotypes in some clades.

non-significant negative D values reflect the bimodal or flat mismatch distributions within clades, suggesting modest population expansion confounded by rate heterogenity (Aris-Brozou and Excoffier, 1996).

For the AMOVA analysis, we grouped populations by clade represented in Fig. 2 (western, central, SE subclades, and northern haplotypes). The AMOVA revealed significant genetic structuring across all hierarchical levels (all $P < 0.0001$). Over all, 75.2% of the variation was a result of differences between clades ($\Phi_{CT} = 0.752$). Only 14.5% of the total variation resulted from differences among populations of these

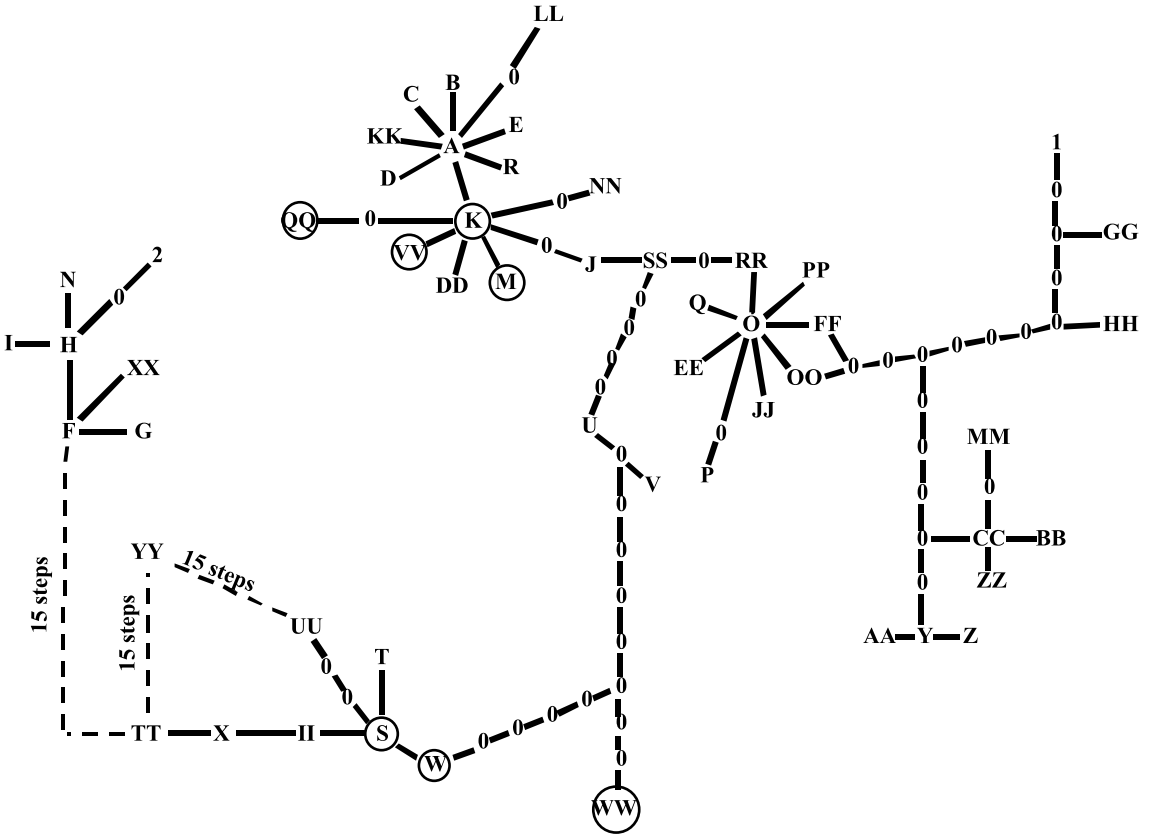

Fig. 3. Parsimony network of the 54 spring peepers mtDNA haplotypes. Cladogram estimation follows Templeton et al. (1992). This method gives a finer-grained pattern of phylogenetic relationships than maximum parsimony when mutations are few (Crandall, 1994). Parsimony probabilities ($\geqslant$95%) for the haplotype pairs were calculated using ParsProb program (http://bioag.byu.edu/zoology/crandall_lab/programs.htm). Haplotype connections up to 10 differences were parsimonious at the 95% level. Haplotypes correspond to those in Appendix B. Zeros represent unsampled or ancestral haplotypes. Haplotypes found in populations from the zone of secondary contact in SW Ontario (populations #20 and 21) have been circled. Dashed lines represent non-parsimonious connections at the 95% level.

groups ($\Phi_{SC} = 0.586$), while the remaining 10.3% was due to within population variation ($\Phi_{ST} = 0.897$).

4. Discussion

Our analyses of *P. crucifer* presents a number of important patterns: first, the presence of divergent haplotypes in two central SW Ontario populations, representing highly divergent lineages suggests that this portion of SW Ontario may represent a zone of secondary contact. Second, phylogenetic and AMOVA results support our contention that there are a number of evolutionary significant units (ESUs, Ryder, 1986) across the species range. Finally, the phylogenetic trees do not support the recognition of the putative subspecies *P. c. bartramiana*.

4.1. Divergence among northern lineages

The level of divergence among the haplotypes of clades A–D combined with strong geographic structure of haplotypes implies that there have been historical barriers to gene flow in *P. crucifer*. This interpretation is supported by findings in another amphibian species. Phillips (1994) found that a highly divergent lineage (genetic group I; Fig. 4 of Phillips, 1994) of *Ambystoma maculatum* exists allopatrically within the range of a second widespread lineage (Group II) ranging from Missouri to the Atlantic coast. The genetic group I of *A. maculatum* ranges from Michigan south to Alabama, and is concordant with the geographic position of the divergent central lineage of *P. crucifer* (clade D). The geographic location of six of the seven *P. crucifer* populations (populations 15, 16, 20, 21, 22, and 26, Fig. 1) where clade D is detected are all located in formally glaciated locations, and it is likely that such divergence is due to the presence of separate glacial refugia and recolonization dynamics. This clade extends south into Tennessee (population 32) which has a relatively high π-value (Fig. 5) as would be expected from a refugial area. *A. maculatum* and *P. crucifer* overlap in range, and also share similar ecologies (e.g., both woodland vernal pool breeders), and therefore

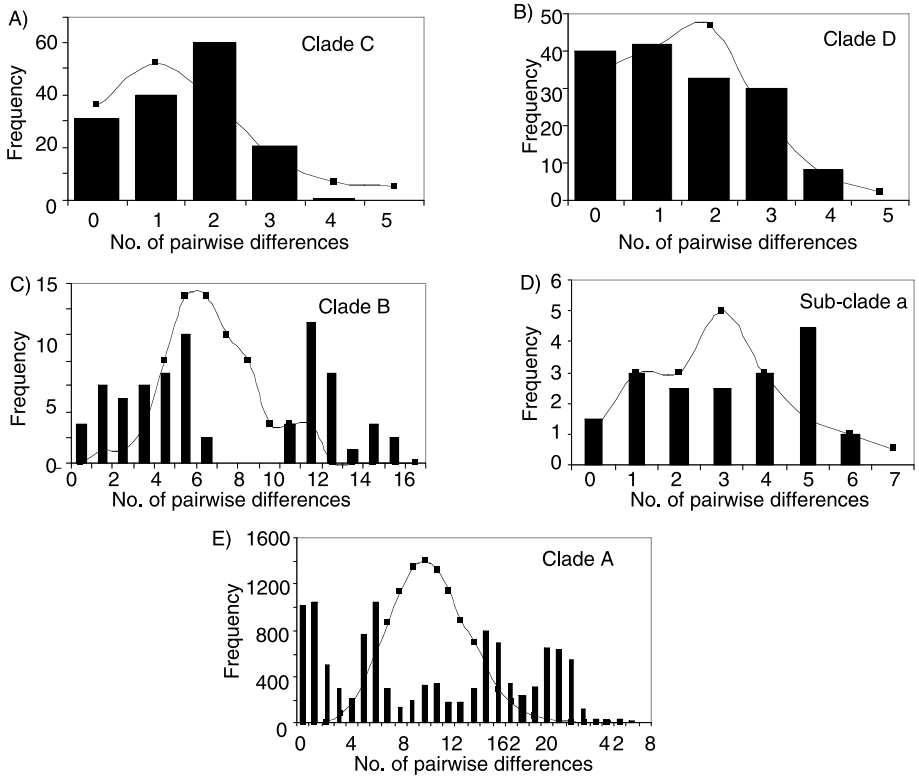

Fig. 4. Mismatch distributions or histograms of pairwise differences for mtDNA sequences. (A) Western populations (clade C); (B) Central populations (clade D); Southeast US (clade B); (D) sub-clade a of clade B, representing populations from Florida, Alabama, and South Carolina. (E) Northern populations (clade A). The solid line in each graph is the randomly generated Poisson distribution calculated based on the respective mismatch mean values. The expected (Poisson) is significantly different from the observed values in all but (B).

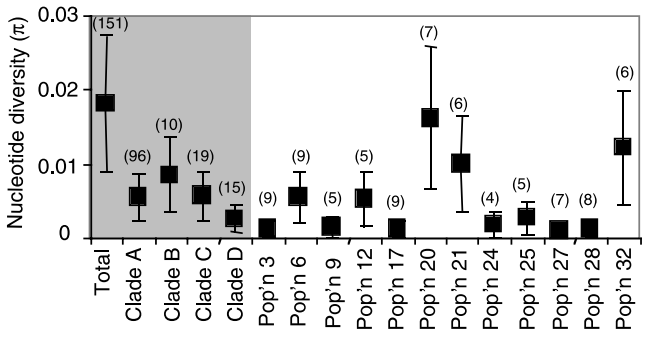

Fig. 5. Plot of mean nucleotide diversity (π) of mtDNA sequences within the total sample and each of the identified clades (shaded area). Populations where sample sizes are greater than 3 and where $\pi > 0$ are also shown. Means and SE are calculated following Nei (1987).

may have had similar recolonization dynamics. Phillips (1994) argues that the most likely scenario for the presence of the two lineage groups is the presence of a eastern highland refugium for the group I clade, while the widespread group II clade likely originated from one or more southern refugia from which *A. maculatum* could have dispersed north along the Atlantic Coastal Plain and across the Mississippi Valley. This pattern of population divergence in *A. maculatum* is also supported by mtDNA sequence data (K. Zamudio, personal communication). In his continental scale allozyme study of *P. crucifer*, Chippindale (1989) found that the most obvious differences in allozyme allele frequencies were between SW Ontario (locations 15 and 16, Fig. 1) and other northern populations, supporting the idea that this region may be a zone of secondary contact. Southern Michigan and SW Ontario were deglaciated and open to colonization well before the Champlain Sea would have subsided, opening eastern Ontario to colonizing amphibians (Holman, 1992).

There appears to be numerous haplotypes that are common within various regions, such as haplotype A in the NW, haplotype O in the NE and haplotype K in the SE regions of the Great Lakes. Haplotypes A and K are not highly divergent (Fig. 3) and their relative occurrence diminishes as they move east to west, north of the Great Lakes. This pattern, as well as the lack of phylogenetic resolution, suggests that the majority of the northern haplotypes are closely related and probably originated in one or more closely related refugia with subsequent saltatory colonization north into eastern Ontario and then west. The large genetic distance

between the western clade C and clade A relative to the geographic distance where both occur in the western portion of the range suggests that barriers existed to prevent western haplotypes from colonizing into western Ontario and Manitoba prior to the arrival of eastern haplotypes.

Pleistocene climatic oscillations involving both temperature and moisture may account for much of the pattern of diversification found in western *P. crucifer*. Two highly divergent clades (A and C) meet west of the Great Lakes, and it seems unlikely that clade A originated from the western portion of the range due to: (i) the large genetic distance between the two clades (see Fig. 3), and (ii) the close phylogenetic relationship between clades A and B (the later being restricted to the east coast). It may be that part of the recolonization dynamics of the western region was strongly impacted by the extension of the 'Prairie Peninsula' (Transeau, 1935) east into the Great Lakes States and SW Ontario during the Xerothermic period some 6000–4000 years ago (see Fig. 6). As a woodland species, *P. crucifer* would have been restricted to isolated woodland refugia or displaced in either a northern or southern direction. As a barrier to gene flow the prairie peninsula may have been the driving force behind such high levels of divergence in this region. Random lineage sorting following colonization from a single refugia is an unlikely explanation for the observed divergence between clades A and C due to the necessarily high level of refugial diversity (>3.5% sequence divergence), as well as the geographic structuring of haplotype distributions.

Hybridization between *P. crucifer* and other hylids is a possible cause of the divergent lineages of mtDNA. There are few potential candidates for hybridization in much of the range. The most likely candidate, based on the timing of the breeding season and phylogenetic relationship is *P. triseriata* (Cocroft, 1994). However, sequence divergence between *P. triseriata* and *P. crucifer* is quite high, relative to the maximum sequence divergence within *P. crucifer* (15% vs. 4%). Further, although genetic compatibility has

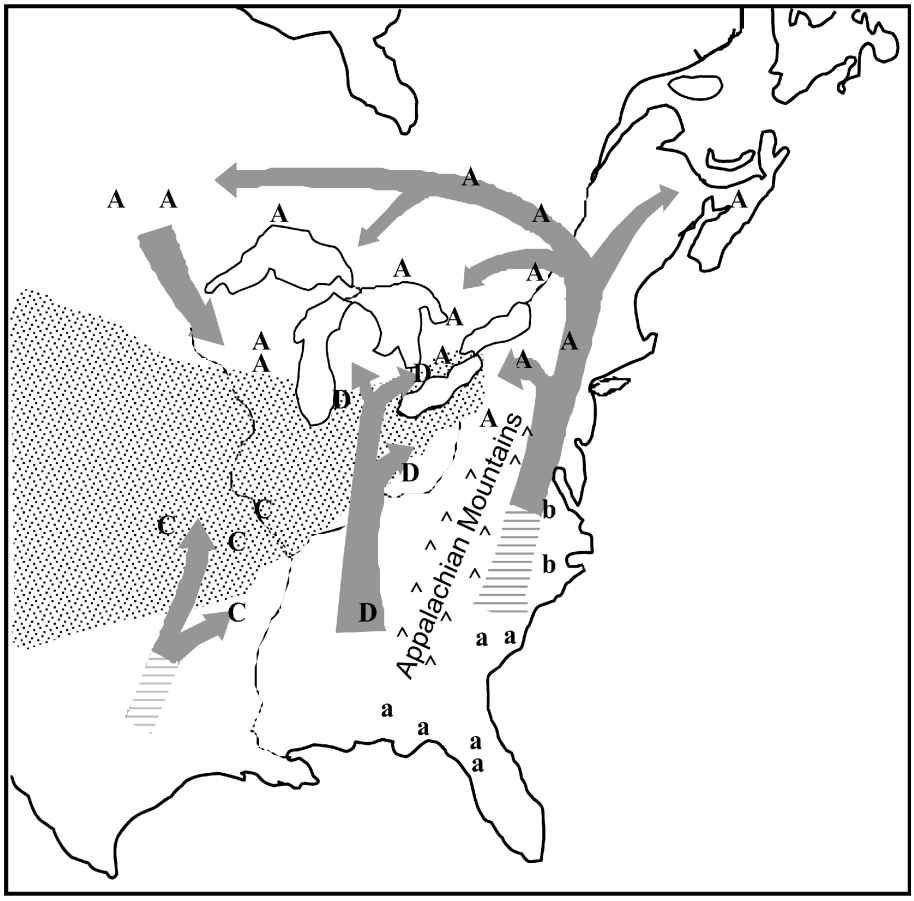

Fig. 6. Map showing the current distribution and proposed dispersal patterns (arrows) of mtDNA clades from southern refugia following glacial retreat. Letters represent the geographic locations of the identified mitochondrial clades (Figs. 2 and 3; A = Northern clade; b, c = subclades of clade B; C = Western clade; D = Central clade). Stippled area represents the proximate maximal extent of the prairie peninsula (after Transeau, 1935).

been examined in the laboratory between *P. crucifer* and other species of hylids (Mecham, 1965; Ralin, 1970), the viability of offspring is extremely low (all hatching eggs fail to transform in crosses between *P. crucifer* and *P. triseriata*, and surviving offspring of crosses with *Hyla versicolor* tend to be pentaploids (J. Bogart, personal communication). In summary, the introgression of mtDNA, although possible, is not likely to be the source of the high variability observed here, in part due to the limited evidence of natural hybridization.

Perhaps more problematic in terms of interpretation is the presence of divergent haplotypes (U, V, and WW), occurring in three populations (8, 20, and 33). The three haplotypes are highly differentiated from sympatric haplotypes. Given their small body size, and the presumed high site fidelity associated with most anurans (e.g., Berven and Grudzen, 1990; Kusano et al., 1999), one would predict that in *P. crucifer* gene flow should be low, particularly over such a large geographic scale. Low gene flow would mean that the distribution of haplotypes of relatively rapidly evolving genes should reflect population relationships rather than random sorting events. As such, the presence of divergent haplotypes in Ottawa, Virginia, and Long Point may reflect recent invasions from multiple refugia with subsequent incomplete sorting. As previously alluded to, if the divergent haplotypes in Ottawa and Virginia did arise from one refugium it would have to have harbored a large amount of genetic diversity through the Pleistocene. Alternatively, gene flow may be underestimated in small anurans. Widespread, divergent haplotypes are not documented in populations of organisms with low gene flow (Avise et al., 1987), and given what is understood about their ecology, and the glacial history of the region, the scenario of high levels of gene flow seems unlikely.

The geographic incidence of mitochondrial haplotypes provides us with clues to the post-glacial history of *P. crucifer*. Significant phylogenetic structuring and non-random distribution of haplotypes across much of the range emphasize the role of refugia and dynamic post-glacial colonization processes in shaping present day genetic structure.

4.2. Cryptic species and subspecies designation

At higher taxonomic levels frogs are, in general, conservative in their morphological evolution (Chek et al., 2001; Cherry et al., 1978; Hass et al., 1995; Maxson, 1984; Richards and Moore, 1996). Thus, the potential for identifying morphologically cryptic species becomes an important aspect of recognizing biological diversity (Hanken, 1999). In *P. crucifer*, subspecies designation has been dictated by morphological characters (Harper, 1939), and is not supported by our mitochondrial data. Although Florida haplotypes differ from other haplotypes by up to 3.5%, still greater genetic pairwise divergence is found among haplotypes within *P. crucifer crucifer* (up to 4%). Although 4% intraspecific sequence divergence is high relative to some vertebrate groups (e.g., birds, Klicka and Zink, 1997), it is low relative to many anuran amphibians where intraspecific genetic distances are as high as 7–14% for mitochondrial DNA (e.g., *Litoria* spp. James and Moritz, 2000; McGuigan et al., 1998; *H. arenicolor* Barber, 1999; *E. femoralis* Lougheed et al., 1999). Allozyme markers show a lack of genetic distinction between *P. c. bartramiana* and other populations, with genetic distances that are intermediate among population comparisons and low incidence of fixed alleles in populations of *P. c. bartramiana* (Chippindale, 1989). The *bartramiana* lineage is typically distinguished morphologically from the putative 'northern' subspecies by various characteristics such as larger size, broken stripe on upper lip (i.e., spots), broader dorsal strips and more pronounced spotting on the ventral surface (Harper, 1939). However, color patterns and size differences may not be unique to *P. c. bartramiana* (Chippindale, 1989), and it is likely that the differences are either clinal or the result of some selective forces on morphology. The lability of these and other morphological characters needs to be examined in detail. Furthermore, the extent of contemporary intermixing of historically isolated lineages would benefit from further sampling. A recent study (Burbrink et al., 2000) demonstrated the problematic nature of recognition of evolutionary lineages based on highly labile characters and emphasizes the need for more work to be done to verify whether subspecies reflect historical divisions.

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Appendix A. Sample locations and mtDNA haplotype distributions for 40 populations of *P. crucifer*

Population numbers correspond to Fig. 1.

	Location	Approx. coordinates		*n*	mtDNA haplotype (frequency)	Source (museum/field No.)[a]
		N	W			
1	Saint Rita, Manitoba	49°53	096°15	1	LL	JPB
2	Lac Seul, Ontario	50°15	092°10	2	A(2)	TMM (M2, M3)
3	Thunder Bay, Ontario	48°40	089°00	9	A(6), B, C, KK	JPB
4	Springfield Township, Wisconsin	43°47	089°20	4	A(4)	USP
5	Sherry Township, Wisconsin	44°15	090°05	5	A(5)	USP
6	Espanola, Ontario	46°17	081°48	9	O(5), A(2), R(2)	JPB
7	Hanna Township, Ontario	49°00	081°58	3	O, A(2)	ROM (5569–5571)
8	Ottawa-Carleton, Ontario	45°19	075°48	3	U, O(2)	ROM (5575–5577)
9	Lake Opinicon, Ontario	44°33	076°20	5	Q, JJ, O(3)	Toe clips
10	Oxford, Nova Scotia	45°44	063°53	3	EE, O(2)	ROM (5578–5580)
11	Oro, Ontario	44°44	078°56	3	O(2), FF	ROM (5581–5583)
12	Innisfil, Ontario	44°22	079°36	5	J, O, SS, RR, K	Toe clips
13	St. Edmunds Township, Ontario	45°10	081°31	3	P, O(2)	ROM (5563–5565)
14	Landon's Bay, Ontario	44°21	076°04	7	O(7)	Toe clips
15	Rodney, Ontario	42°34	081°40	3	S(3)	ROM (5590–5592)
16	Skunk's Misery, Ontario	42°41	081°45	3	S, T, II	ROM (5587–5589)
17	Dundas, Ontario	43°14	080°01	9	A, J, K(7)	ROM (5566–5568), JPB
18	Aberfoyle, Ontario	43°29	080°09	4	K (4)	JPB
19	Acton, Ontario	43°33	079°58	3	***K***, ***DD***, ***A***	ROM (5572–5574)
20	Long Point, Ontario	42°37	080°28	7	W(2), K(3), WW, VV	Toe clips
21	Wildwood Lake, Ontario	43°15	081°00	6	K(3), M, S, QQ	Toe clips
22	Duck Lake, Michigan	42°24	085°23	2	S(2)	Toe clips
23	Ithaca, New York	42°27	076°27	3	K, O, OO	Toe clips
24	Burden Lake, New York	42°36	073°34	4	PP, O(3)	TH
25	Slippery Rock Cr., Pennsylvania	41°05	079°60	5	D, K,(3), NN	RL
26	Stroud's Run State Park, Ohio	39°21	082°02	1	S	PL
27	Madison, Illinois	38°15	090°00	7	I, N(6)	CP
28	Baskett Research Area, Missouri	38°45	092°12	8	F(5), G, H(2)	RD
29	Linn, Kansas	39°40	094°04	1	2	TMM (M1)
30	Stone, Arkansas	35°50	092°00	2	F, XX	MM
31	White, Arkansas	35°20	091°50	1	YY	MM
32	Murfreesburo, Tennessee	35°50	086°24	6	UU, T, S, X(3)	BM
33	Wakefield, Virginia	38°02	077°90	3	V, HH, GG	ROM (5584–5586)
34	Pitt, North Carolina	35°35	077°23	1	1	TMM (ecm 81)
35	Barnwell, South Carolina	33°18	081°15	2	ZZ, CC	TMM (ecm 58, ecm 60)
36	Berkeley, South Carolina	33°10	080°00	1	MM	PM
37	Barbour, Alabama	31°50	085°27	1	AA	TMM (ecm 39)
38	Liberty, Florida	30°15	084°45	2	Y, Z	PM
39	Alachula, Florida	29°50	082°30	1	BB	PM
40	Ocala, Florida	29°10	082°20	2	BB(2)	TMM (Y1, Y2)

[a] JPB: J. Bogart, Guelph University; UWSP: T.E. Wild, University of Wisconsin-Stevens Point; ROM: Royal Ontario Museum, Toronto; RL: R. Layne, Slippery Rock University, PA; P. Larson, Ohio University; TH: T. Hunsinger, NY State Museum, Albany; PM: P. Moler, Florida Game and Fresh Water Fish Commission, Gainsville; TMM: D. Cannatella, Texas Memorial Museum, Austin; RD: R. Daniel, University of Missouri, Columbus; CP: C. Phillips, University of Illinois Museum of Natural History, Champlain; BM: B. Miller, Middle Tennessee St. University, Murfreesburo; MM: M. McCallum, Arkansas St. University, Jonesburo.

Appendix B. Mitochondrial DNA fragments from cytochrome *b* and 16S isolated in this study

Each gene fragment is provided with the respective GenBank accession number and the combined mtDNA haplotype for which it is a part of (corresponding to figures and Appendix A). 16S haplotype number 18 is not assigned to a haplotype because is was unique from haplotype number 4 only by an observed insertion; it was therefore excluded from analysis (see Section 2)

Cytochrome *b* segment			16S segment		
Cytochrome *b* haplotypes	GenBank Accession No.	Found in mtDNA haplotypes:	16S haplotype	GenBank Accession No.	Found in mtDNA haplotypes:
1	AF488308	A	1	AF487352	F, H, N, XX
2	AF488309	F, G	2	AF487353	II, S, TT, W, X
3	AF488310	H, I	3	AF487354	EE, FF, J, JJ, O, OO, PP, RR, SS, U, Y
4	AF488311	J, K, M			
5	AF488312	N	4	AF487355	A, DD, K, KK, NN, P, QQ, R, VV, WW
6	AF488313	O, P, Q			
7	AF488314	R	5	AF487356	BB
8	AF488315	S, T, X	6	AF487357	CC, ZZ
9	AF488316	U, V	7	AF487358	1, GG, V
10	AF488317	W	8	AF487359	HH
11	AF488318	X	9	AF487360	T
12	AF488319	AA, Y	10	AF487361	Q
13	AF488320	BB, CC	11	AF487362	B
14	AF488321	DD	12	AF487363	C
15	AF488322	EE	13	AF487364	LL
16	AF488323	FF	14	AF487365	Z
17	AF488324	GG	15	AF487366	MM
18	AF488325	HH	16	AF487367	D
19	AF488326	II	17	AF487368	M
20	AF488327	JJ	18	AF487369	—
21	AF488328	KK	19	AF487370	UU
22	AF488329	LL	20	AF487371	G
23	AF488330	MM	21	AF487372	YY
24	AF488331	NN	22	AF487373	AA
25	AF488332	OO	23	AF487374	1
26	AF488333	PP	24	AF487375	2
27	AF488334	QQ	*P. triseriata*	AF487376	
28	AF488335	RR	*P. ocularis*	AF487377	
29	AF488336	SS			
30	AF488337	TT			
31	AF488338	UU			
32	AF488339	VV			
33	AF488340	WW			
34	AF488341	XX			
35	AF488342	YY			
36	AF488343	ZZ			
37	AF488344	1			
38	AF488345	2			
P. triseriata	AF488806				
P. ocularis	AF488805				

References

Anderson, T.W., Lewis, C.F.M., 1992. Climatic influences of deglacial drainage changes in southern Canada at 10–8 ka suggested by pollen evidence. Géogr. Physiq. Quatern. 46, 255–272.

Aris-Brozou, S., Excoffier, L., 1996. The impact of population expansion and mutation rate heterozygosity on DNA sequence polymorphism. Mol. Biol. Evol. 13, 494–504.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., Saunders, N.C., 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Ann. Rev. Ecol. Syst. 18, 489–522.

Avise, J.C., 2000. Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge.

Barber, P.H., 1999. Phylogeography of the canyon treefrog, *Hyla arenicolor* (Cope) based on mitochondrial DNA sequence data. Mol. Ecol. 8, 547–562.

Bennett, K.D., 1985. The spread of *Fagus grandifolia* across eastern North America during the last 18,000 years. J. Biogeogr. 12, 146–166.

Bennett, K.D., 1986. The rate of spread and population increase of forest trees during the postglacial. Phil. Trans. R. Soc., Lond. B 314, 523–531.

Berbee, M.L., Yoskimura, A., Sugiyama, J., Taylor, J.W., 1995. Is Penicillium monophyletic? An evaluation of phylogeny in the family Trichocomaceae from 18S, 5.8S, and ITS ribosomal DNA sequence data. Mycologia 87, 210–222.

Bernatchez, L., Dodson, J.J., 1991. Phylogeographic structure in mitochondrial DNA of the lake whitefish (*Coregonus clupeaformis*) and its relation to Pleistocene glaciations. Evolution 45, 1016–1035.

Berven, K.A., Grudzen, T.A., 1990. Dispersal in the wood frog (*Rana sylvatica*): implications for genetic population structure. Evolution 44, 2047–2056.

Billington, N., Barrette, R.J., Hebert, P.D.N., 1992. Management implications of mitochondrial DNA variation in walleye stocks. N. Am. J. Fish. Manage. 12, 276–284.

Blair, W.F., 1965. Amphibian speciation. In: Wright, H.E., Frey, D.G. (Eds.), The Quaternary of the United States. Princeton University Press, Princeton, NJ, pp. 543–555.

Burbrink, F.T., Lawson, R., Slowinski, J.B., 2000. Mitochondrial DNA phylogeography of the polytypic Norht American rat snake (*Elaphe obsoleta*): a critique of the subspecies concept. Evolution 54, 2107–2118.

Burns, K.J., 1997. Molecular systematics of tanagers (Thraupinae): evolution and biogeography of a diverse radiation of Neotropical birds. Mol. Phylogenet. Evol. 8, 334–348.

Chek, A.A., Lougheed, S.C., Bogart, J.P., Boag, P.T., 2001. Perception and history: molecular phylogeny of a diverse group of Neotropical frogs, the 30-chromosome *Hyla* (Anura: Hylidae). Mol. Phylogenet. Evol. 18, 370–385.

Cherry, M.L., Case, S.M., Wilson, A.C., 1978. Frog perspective on the morphological difference between humans and chimpanzees. Science 200, 209–211.

Chippindale, P.T., 1989. Intra- and interpopulation genetic variation in the spring peeper, *Hyla crucifer*. Unpublished M.Sc. thesis, University of Toronto, 133 p.

Churchill, T.A., Storey, K.B., 1996. Organ metabolism and cryoprotectant synthesis in spring peepers, *Pseudacris crucifer*. Copeia 1996, 517–525.

Cline, J., Braman, J.C., Hogrefe, H.H., 1996. PCR fidelity of *Pfu* polymerase and other thermostable DNA polymerases. Nucleic Acids Res. 24, 3546–3551.

Cocroft, R.B., 1994. A cladistic analysis of chorus frog phylogeny (Hylidae: *Pseudacris*). Herpetologica 50, 420–437.

Conant, R., Collins, J.T., 1998. A Field Guide to Reptiles and Amphibians Eastern and Central North America, third ed. Houghton Mifflin, Boston.

Crandall, K.A., 1994. Intraspecific cladogram estimation: accuracy at higher levels of divergence. Syst. Biol. 43, 222–235.

Crandall, K.A., Templeton, A.R., 1996. Applications of intraspecific phylogenetics. In: Harvey, P.H., Leigh Brown, A.J., Maynard Smith, J., Sean, Nee (Eds.), New Uses fort New Phylogenies. Oxford University Press, Oxford, pp. 81–99.

Donnelly, P., Tavaré, S., 1986. The ages of alleles and a coalescent. Adv. Appl. Prob. 18, 1–19.

Escoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.

Faith, D.P., Cranston, P.S., 1991. Could a cladogram this short have arisen by chance alone?: on permutation tests for cladistic structure. Cladistics 7, 1–28.

Farris, J.S., Kallersjo, M., Kluge, A.G., Bult, C., 1995. Testing significance of incongruence. Cladistics 10, 315–319.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Green, D.M., Sharbel, T.F., Kearsley, J., Kaiser, H., 1996. Postglacial range fluctuations, genetic subdivision and speciation in the western North American spotted frog complex, *Rana pretiosa*. Evolution 50, 374–390.

Hanken, J., 1999. Why are there so many new amphibian species when amphibians are declining? Trends Ecol. Evol. 14, 7.

Harpending, H.C., 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. Hum. Biol. 66, 591–600.

Harper, F., 1939. A southern subspecies of the spring peeper (*Hyla crucifer*). Notulae Naturae 27, 1–4.

Hass, C.A., Dunski, J.F., Maxson, L.R., 1995. Divergent lineages within the Bufo margaritifera complex (Amphibia: Anura; Bufonidae) revealed by albumin immunology. Biotropica 27, 238–249.

Holman, J.A., 1992. Late quaternary herpetofauna of the central Great Lakes region, USA: zoogeographical and paleoecological implications. Quart. Sci. Rev. 11, 345–351.

Holman, J., 1995. Pleistocene Amphibians and Reptiles in North America. Oxford University Press, Oxford.

Huelsenbeck, J.P., Bull, J.J., Cunningham, C.W., 1996. Combining data in phylogenetic analyses. Trends Ecol. Evol. 11, 152–158.

James, C.H., Moritz, C., 2000. Intraspecific phylogeography in the sedge frog *Litoria fallax* (Hylidae) indicates pre-Pleistocene vicariance of an open forest species from eastern Australia. Mol. Ecol. 9, 349–358.

Kimura, M., 1980. A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. J. Mol. Evol. 16, 111–120.

Klicka, J., Zink, R.M., 1997. The importance of recent ice ages in speciation: a failed paradigm. Science 227, 1666–1669.

Kobayashi, N., Tamura, K., Aotsuka, T., 1999. PCR error and molecular population genetics. Biochem. Genet. 37, 317–321.

Kusano, T., Maruyama, K., Kanenko, S., 1999. Breeding site fidelity in the Japonese toad, *Bufo japonicus formosus*. Herpetol. J. 9, 9–13.

Lougheed, S.C., Gascon, C., Jones, D.A., Bogart, J.P., Boag, P.T., 1999. Ridges and rivers: a test of competing hypotheses of Amazonian diversification using a dart-poison frog (*Epipedobates femoralis*). Proc. R. Soc. Lond. B 266, 1–7.

Lundberg, K.S., Shoemaker, D.D., Adams, M.W.W., Short, J.M., Sorge, J.A., Marthur, E.J., 1991. High fidelity amplification using thermostable DNA polymerase isolated from *Pyrococcus furiosius*. Gene 108, 1–6.

Maxson, L.R., 1984. Molecular probes of phylogeny and biogeography in toads of the widespread genus. Bufo. Mol. Biol. Evol. 4, 345–356.

McGuigan, K., Donald, K., Parris, K., Moritz, C., 1998. Mitochondrial DNA diversity and historical biogeography of a wet

forest-restricted frog from mid-east Australia (*Litoria pearsoniana*). Mol. Ecol. 7, 175–186.

Mecham, J.S., 1965. Genetic relationships and reproductive isolation in southeastern frogs of the genera *Pseudacris* and *Hyla*. Am. Midl. Nat. 74, 269–308.

Moritz, C., Schneider, C.J., Wake, D.B., 1992. Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. Syst. Biol. 41, 273–291.

Nei, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Omland, K.E., Tarr, C.L., Boarman, W.I., Marzluff, J.M., Fleischer, R.C., 2000. Cryptic genetic variation and paraphyly in ravens. Proc. R. Soc. Lond. B 267, 2475–2482.

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. The Simple Fools Guide to PCR Vers. 2.0. University of Hawaii.

Phillips, C.A., 1994. Geographic distribution of mitochondrial DNA variants and the historical biogeography of the spotted salamander, *Ambystoma maculatum*. Evolution 48, 597–607.

Ralin, D.B., 1970. Genetic compatibility and a phylogeny of the temperate North American hylid fauna. PhD dissertation, University of Texas at Austin, p. 117.

Richards, C.M., Moore, W.S., 1996. A phylogeny for the African treefrog family Hyperoliidae based on mitochondrial DNA. Mol. Phylogenet. Evol. 5, 522–532.

Roe, B.A., Ma, D.-P., Wilson, R.K., Wong, J.F.-H., 1985. The complete nucleotide sequence of *Xenopus laevis* mitochondrial genome. J. Biol. Chem. 260, 9759–9774.

Ryder, O.A., 1986. Species conservation and the dilemma of subspecies. Trends Ecol. Evol. 1, 9–10.

Schneider, S., Roessli, D., Excoffier, L., 1999. Arlequin: A Software for Population Genetics Data Analysis. Ver. 2.000. University of Geneva, Geneva, Switzerland.

Slatkin, M., Hudson, R.R., 1991. Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. Genetics 129, 555–562.

Stuckey, R.L., 1993. Phytogeographical outline of aquatic and wetland angiosperms in continental eastern North America. Aquat. Bot. 44, 259–301.

Swofford, D.L., 1999. PAUP*: Phylogenetic Analysis using Parsimony (*and other Methods): Ver. 4.0. Sinauer Associates, Sunderland, MA.

Tajima, F., 1989a. Statistical methods for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

Tajima, F., 1989b. The effect of change in population size on DNA polymorphism. Genetics 123, 597–601.

Templeton, A.R., Crandall, K.A., Sing, C.F., 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonucleases mapping and DNA sequence data. III. Cladogram estimation. Genetics 132, 619–633.

Transeau, E.N., 1935. The prairie peninsula. Ecology 16, 423–437.

Watterson, G.A., Guess, H.A., 1977. Is the most frequent allele the oldest? Theor. Pop. Biol. 11, 141–160.

Webb III, T., Bartlein, P.J., Harrison, S.P., Anderson, K.H., 1993. Vegitation, lake levels and climate in eastern North America for the past 18,000 years. In: Wrightm, H.E., Kutzbach, J.E., Webb III, T., Ruddiman, W.F., Street-Perrott, F.A., Bartlein, P.J. (Eds.), Global Climates Since the Last Glacial Maximum. University of Minnesota Press, Minneapolis, pp. 415–467.

Wilson, C.C., Hebert, P.D.N., 1998. Phylogeography and postglacial dispersal of lake trout (*Salvelinus namaycush*) in North America. Can. J. Fish. Aquat. Sci. 55, 1010–1024.

Wright, S., 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 19, 395–420.