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Discordant temporal and geographic patterns in maternal lineages of eastern north American frogs, *Rana catesbeiana* (Ranidae) and *Pseudacris crucifer* (Hylidae)

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Abstract

Whether the Pleistocene has had a disproportionate impact on the recent diversification of temperate species, or played a lesser role in a more protracted process, has been a prominent evolutionary debate for the past decade. We used cytochrome *b* sequences to reconstruct the evolutionary histories of two widely co-distributed, and ecologically divergent frogs (*Rana catesbeiana* and *Pseudacris crucifer*) to examine the role of the Pleistocene in structuring these species. Results for *R. catesbeiana* reflect a pattern of allopatric fragmentation, likely in Coastal Plain refugia on either side of the Mississippi River dating to the mid to early Pleistocene. In contrast, *P. crucifer* contains numerous divergent lineages, including one west of the Mississippi River in the Interior Highlands, and in the east, multiple lineages that likely expanded from a number of southern Appalachian refugia with lineage sundering originating in the late Pliocene. Large-scale phylogeographic comparisons between these and other eastern North American species reflect both congruent and independent patterns of diversification, possibly reflecting the relative importance of dispersal ability and habitat associations. Although intra-lineage diversification has been structured by repeated Pleistocene glaciations, lineage sundering likely dates at least to the Pliocene in most (but not all) northern temperate amphibian and reptile species studied to date.

1. Introduction

A major evolutionary debate of the past decade has centered on whether recent glacial events had a disproportionate impact on the process of temperate speciation, or whether such events played a lesser role as part of more protracted processes of diversification (Arbogast and Slowinski, 1998; Avise and Walker, 1998; Avise et al., 1998; Klicka and Zink, 1997, 1999; Veith et al., 2003; Zink and Slowinski, 1995). For example, Zink and colleagues have argued that temperate passerine bird species (for which arguably the largest amount of phylogeographic data exist) have older (>250,000 years) origins and thus do not conform to the late Pleistocene origin model (Hubbard, 1973; Mengel, 1970), and that indeed, many passerines may trace their origins to pre-Pleistocene events (Klicka and Zink, 1997). In contrast, Avise and colleagues contend that, while many species may trace their origins to earlier Pliocene events, the Pleistocene had a major impact on the completion of the *process* of speciation in birds and other vertebrates. The importance of the Pleistocene on species diversification (and lineage extinction) remains an important area of investigation. For example, examining the comparative phylogeography of co-distributed species can test predictions of the generality of Pleistocene forces acting to diversify regional biota, and enhances our understanding of the development and maintenance of genetic diversity across regional and continental scales.

The dominant biogeographic paradigm relating to Pleistocene events holds that species have responded independently to the correlated events of past climatic fluctuations (Brown and Lomolino, 1998; Sullivan et al.,

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2000; Valentine and Jablonski, 1993). However, in many instances regional biota are relatively understudied, and thus the generality and scale of congruent versus independent responses of taxa, and the relative impact of Pleistocene glaciation on diversification are unclear, despite the recognized importance of genetic variation as a currency of biodiversity (Ehrlich and Wilson, 1991; Humphries et al., 1995; Moritz and Faith, 1998). Only through coordinated study of an array of co-distributed species will we become better informed of the role of the Pleistocene in lineage diversification.

The North American bullfrog (Ranidae: *Rana catesbeiana*) and the spring peeper (Hylidae: *Pseudacris crucifer*) inhabit both previously glaciated and unglaciated regions of eastern North America from the Gulf of Mexico, north as far as 47°N (Conant and Collins, 1998) and 54°N (Bider and Matte, 1996), respectively. Both species' ranges extend from the Atlantic to west of the Mississippi River (Figs. 1 and 2). The western range limit of *R. catesbeiana* has been blurred due to introductions and habitat alteration; however, fossil records (Holman, 1995; Fig. 1) suggest that this species occurred naturally well west of the Mississippi River over the past two million years. Here we have assembled cytochrome *b* sequence data from populations spanning the geographic ranges of *R. catesbeiana* and *P. crucifer* to elucidate historical patterns within each species and to determine the concordance in geographic patterns and tempo of genetic diversification. Specifically, we ask: (1) Can we confidently differentiate among alternative refugial hypotheses for *P. crucifer* and *R. catesbeiana*? (2) What do the phylogenetic patterns suggest about the role of the Pleistocene in intra-specific diversification? (3) Based on our results and those from other comprehensive surveys of widespread eastern North American taxa, what hypotheses can be generated to explain congruence of phylogeographic patterns, should they exist?

2. Materials and methods

2.1. Specimen information

We obtained tissue samples (toe clips or blood) from 215 *R. catesbeiana* from 42 sites, and 238 *P. crucifer* from 60 sites ($n = 1$–10 per site) across their respective ranges in eastern North America. Listed below are sample locations (number as in Figs. 1 and 2), haplotype

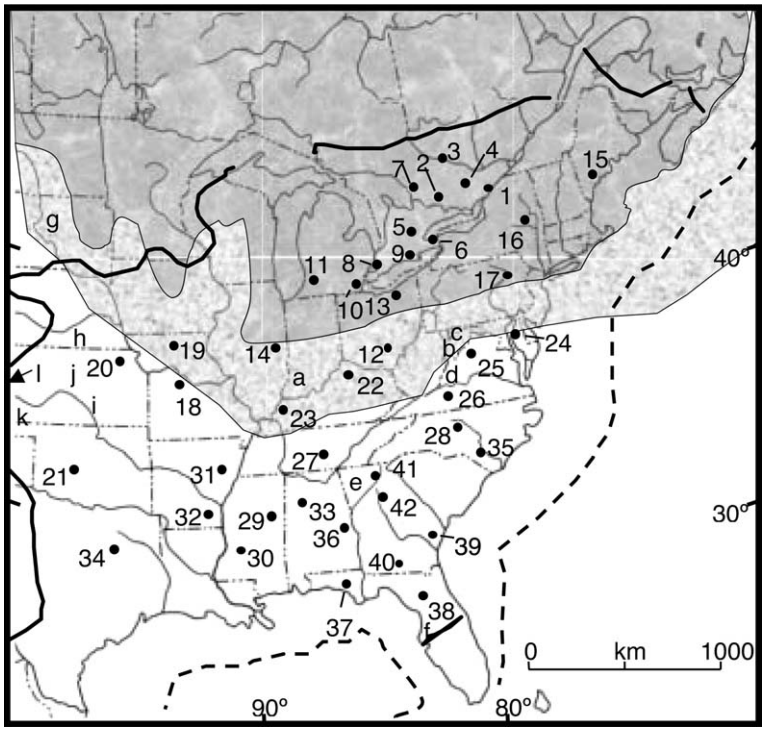

Fig. 1. Geographic distribution (bounded by the dark line) and locations (●) of *R. catesbeiana* sample sites. Dark marble pattern outlines approximate southern limit of Wisconsinian glacial stage (~20,000 ybp), and light marble represents maximal glaciation resulting from earlier glacial periods. The approximate extent of exposed continental shelf is indicated with dashed lines. Lowercase letters indicate approximate locations of selected fossil records for *R. catesbeiana*. Estimated age in brackets, all data from Holman (1995) and references therein: (a) Prairie Creek, IN (15,000–14,000 ybp); (b) Hamilton Cave, WV (900,000–400,000 ybp); (c) Clark's Cave VA (11,000 ybp); (d) Straight Canyon Fissure, VA (~30,000 ybp); (e) Kingston Saltpeter Cave, GA (10,300 ybp); (f) Inglis IA Site, FL (1,900,000–1,400,000); (g) Java Local Fauna, SD (1,900,000–1,400,000); (h) Albert Ahrens Local Fauna, NE (1,900,000–400,000); (i) Williams Local Fauna, KS (Illinoian Glacial Stage); (j) Courland Canal, KS (1,900,000–1,500,000); (k) Jinglebob Local Fauna, KS (Wisconsinian Glacial Age); (l) Hansen Bluff Fauna, CO (1,500,000–1,900,000).

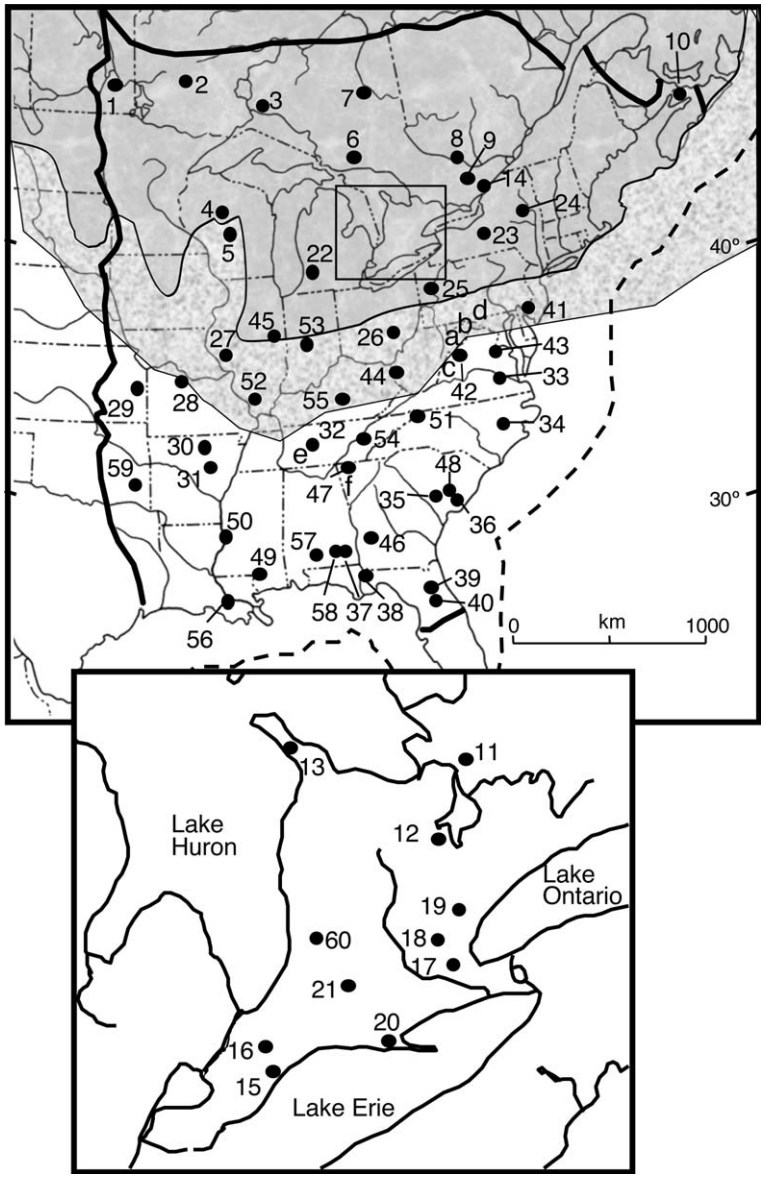

Fig. 2. Geographic distribution (bounded by the dark line) and locations (●) of *P. crucifer* sample sites. Inset: Southwest Ontario populations. Dark marble pattern outlines approximate southern limit of Wisconsinian glacial stage (~20,000 ybp), and light marble represents maximal glaciation resulting from earlier glacial periods. The approximate extent of exposed continental shelf is indicated with dashed lines. Lowercase letters indicate approximate locations of fossil records for *P. crucifer*. Estimated age in brackets (ybp, years before present), all data from Holman (1995) and references therein: (a) Hamilton Cave (900,000–400,000 ybp), New Trout Cave, WV (~17,060 ybp); (b) Cumberland Cave, MY (900,000–400,000 ybp); (c) Clark's Cave VA (11,000 ybp); (d) New Paris 4 Site (~12,000 ybp), Frankstown Cave, PA (10,000–400,000); (e) Cheek Bend Cave, TN (12,000–16,000 ybp); (f) Kingston Saltpeter Cave (10,300 ybp), and Ladds Quarry Site, GA (10,000–11,000 ybp).

number (as in Figs. 3 and 4), GenBank accession numbers at haplotype first occurrence and population haplotype frequency (in parentheses). *R. catesbeiana*: [1] St. Lawrence Isl. Natl. Park, Grenadier Island, ON 44°25′N 075°52′W, 9—AY210375 (4); [2] Pigeon River, Omemee, ON 44°16′N 078°35′W, 9 (4), 37—AY210397 (2); [3] Muskrat River, ON 45°44′N 077°02′W, 9 (5), 33—AY210393 (1); [4] Bug Gull Lake, Clarendon and Miller Township, ON 44°50′N 076°56′W, 9 (9), 24—AY210366 (1); [5] Aberfoyle Mill Pond, Hwy 6, Aberfoyle, ON 43°28′N 080°09′W, 8—AY083288 (10); [6] Mud Lake, Welland, ON 42°56′N 079°15′W, 9 (5), 40—AY210373 (1); [7] Little Lake Joseph, RR 362, 7 km N. Minett, ON 45°12′N 079°40′W, 8 (5), 30—AY210390 (1); [8] St. Clair Natl. Wildlife Area, ON 42°23′N 082°24′W, 2—AY210370 (1), 7—AY083292 (1), 8 (1), 11—AY210365 (4); [9] Big Creek Natl. Wildlife Area, Port Rowan, ON 42°35′N 080°27′W, 8 (2), 9 (2), 16—AY210376 (4); [10] Sterling State Park, Monroe, MI 41°55′N 083°20′W, 8 (1), 11 (2); [11] Duck Lake, Allegan, MI 42°24′N 085°23′W, 10—AY210378 (1), 11 (5); [12] Lake Alma State Park, OH 39°09′N 082°31′W, 1—AY083293 (1), 8

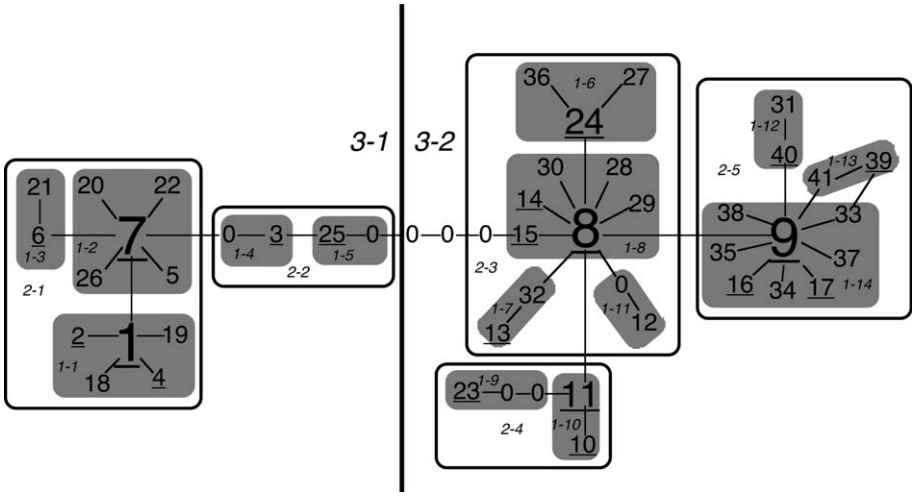

Fig. 3. *Rana catesbeiana* cytochrome *b* haplotype parsimony network. Each line represents a single mutational change, irrespective of length, zeros represent unsampled or extinct haplotypes. Size of font reflects haplotype frequency (see Section 2 for numbers and distributions). Italicized numbers identify haplotype clade-groups and level. Underlined haplotypes were those chosen for additional sequencing (see Fig. 5).

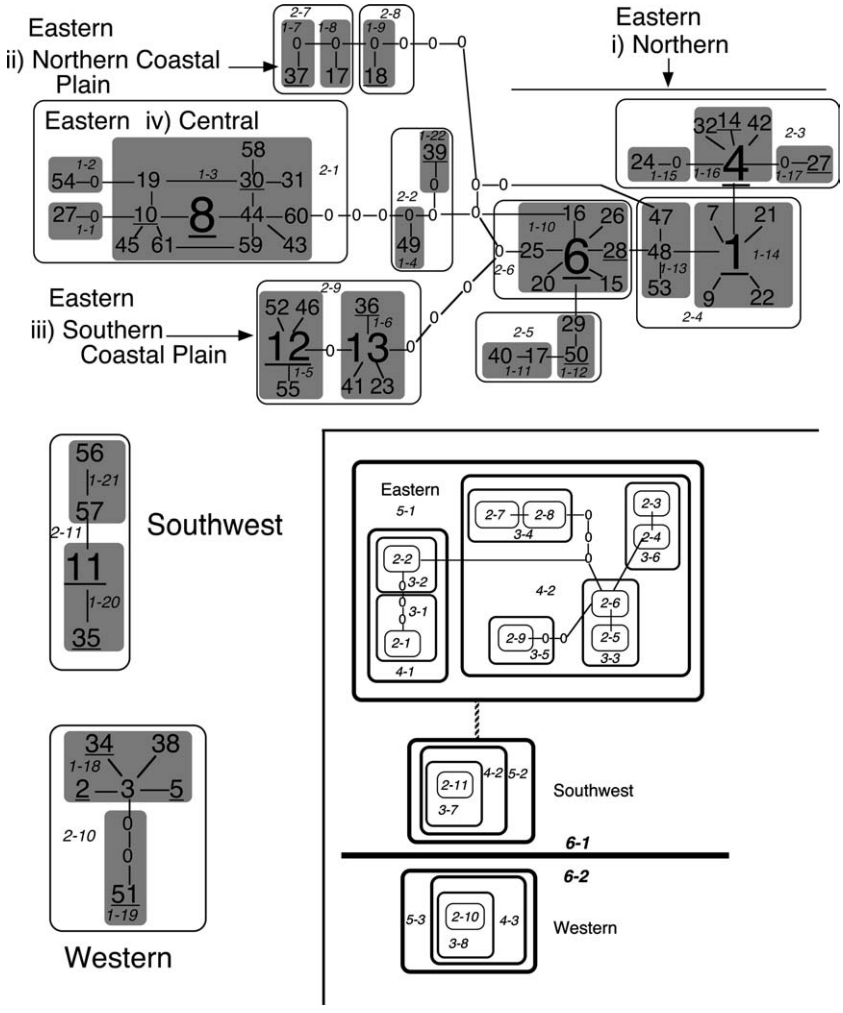

Fig. 4. *Pseudacris crucifer* cytochrome *b* haplotype parsimony network. Each line represents a single mutational change, irrespective of length, zeros represent unsampled or extinct haplotypes. Size of font reflects haplotype frequency. Italicized numbers identify haplotype clade-groups and level. Underlined haplotypes were those chosen for additional sequencing (see Fig. 6). Main figure illustrates haplotype (i.e., *0*-step) through *2*-step clade-groups, inset, *3*-step through *6*-step clade-groups. Regional labels correspond to those in text and Fig. 6.

(3); [13] Holden Arboretum, Lake, OH 41°37′N 081°18′W, 1 (2), 8 (2), 38—AY210398 (1); [14] Middle Fork State Fish and Wildlife Area, Road 2350 N. of Route 74, Vermillion, IL 40°11′N 087°44′W, 2 (1), 11 (4); (15) Long Lake, Cumberland, Maine, 9 (1); [16] Saratoga Lake, Saratoga, NY 43°01′N 073°45′W, 9 (3), 31—AY210391 (1), 40 (2); [17] Lake Independence, Luzerne, PA 41°09′N 076°01′W, 9 (3); [18] E. Shelton Lane, Baskett Research Area, Boone MO 38°45′N 092°12′W, 1 (2), 18—AY210381 (1), 23—AY210362 (1); [19] Lake Paho, Mercer, MO, Route 136, 40°24′N 093°39′W, 1 (3), 2 (2), 4—AY210369 (1); [20] NESA, Jefferson, KS 39°03′N 095°11′W, 5—AY210379 (3), 8 (1), 19—AY210382 (1); [21] Norman, Cleveland, OK 35°12′N 097°26′W, 5 (5); [22] Route 127 at Indian Gap Road, Franklin, KY 38°19′N 084°51′W, 1 (1), 7 (3), 30 (1); [23] Sauerherber Unit, Sloughs Wildlife Management Area, Henderson, KY 37°51′N 087°47′W, 8 (2), 28—AY210308 (4), 29—AY210389 (1); [24] St. Mary's University Campus, MD 38°11′N 076°26′W, 9 (3), 35—AY210395 (1); [25] Augusta, VA 37°59′N 078°60′W, 8 (5), 13—AY210367 (1); [26] Otter Lake, Blue Ridge Parkway, VA 37°33′N 079°21′W, 8 (1), 17—AY083291 (1); [27] Arnold Air Force Base, Coffee, TN 35°25′N 086°03′W, 1 (1), 8 (3), 11 (1); [28] Mason Farm, Chapel Hill, Orange, NC 35°54′N 079°00′W, 8 (1), 17 (5); [29] Starkville, Oktibbeha, MS 33°25′N 088°47′W, 7 (2), 12—AY210380 (1), 32—AY210392 (1); [30] Ross Barnett Reservoir, Lake Harbor Road, Rankin, MS 32°32′N 089°54′W, 7 (2), 12 (1), 22 (1), 26—AY210386 (1); [31] L′Anguille, CR 221 and CR 220, Cross, AR 35°40′N 090°93′W, 1(1), 6—AY210372 (2), 7 (2), 21—AY210384 (1); [32] Dumas, Route 165 between Oakwood Bayou and Canal #43, Desha, AR 33°53′N 090°90′W, 3—AY210371 (1), 7 (4), 20—AY210383 (1); [33] Walker Mines, Haul Road, Walker, AL 33°19′N 087°20′W, 1 (1), 7 (1), 12 (1), 24 (2); [34] Tyler, TX 33°47′N 095°34′W, 3 (5), 25—AY210368 (1); [35] Waccamaw River at Lake Waccaamaw, Columbus, NC, 34°16′N 078°31′W, 8 (1); [36] Tsinia Wildlife Viewing Area, Tuskasgee National Forest, Macon, AL 32°27′N 085°39′W, 13 (1), 24 (1), 27—AY210387 (1), 32 (1), 36—AY210396 (1); [37] Leon , FL 30°25′N 084°00′W, 8 (1), 39—AY210374 (1), 41—AY210399 (1); [38] NW 71st St., Gainsville, Alachua, FL 29°44′N 082°25′W, 8 (7), 34—AY210394 (1); [39] Savannah-Ogeechee Canal, Chatham, GA, 32°01′N 081°16′W, 8 (2); [40] Reed Bingham State Park, Colquitt, GA 31°10′N 083°33′W, 8 (2), 14—AY210363 (1); [41] Peidmont College, Demorest, Habersham, GA 34°34′N 083°33′W, 24 (4); [42] Thompson Mills Forest, Athens, Clarke, GA 33°35′N 083°22′W, 8 (1), 9 (1), 24 (5). *P. crucifer* : [1] St. Rita, MB 49°53′N 096°15W, 22—AF488329 (1); [2] LacSeul, ON 50°15′N 092°10′W, 1—AF488308 (2); [3] Thunder Bay, ON 48°40′N 089°00′W, 1 (8), 21—AF488328 (1); [4] Springfield Township, WI 43°47′N 089°20′W, 1 (4); [5] Sherry Township, WI 44°15′N 090°05′W, 1 (5); [6] Espanola, Sudbury, ON 46°17′N 081°48′W, 1 (2), 6—AF488313 (5), 7—AF488314 (2); [7] Hanna Township, Cochrane, ON 49°00′N 081°58′W, 1 (2), 6 (1); [8] Ottawa, ON 45°19′N 075°48′W, 6 (3); [9] Lake Opinicon, Frontenac, ON 44°33′N 076°20′W, 6 (4), 20—AF488327 (1); [10] Oxford, Cumberland, NS 45°44N 063°53′W, 6 (2), 15—AF488322 (1); [11] Oro, Victoria, ON 44°44′N 078°56′W, 6 (2), 16—AF488323 (1); [12] Innisfil, Simcoe, ON 44°22′N 079°36′W, 4—AF488311 (2), 6 (1), 28—AF448335 (1); [13] St. Edmund's, Bruce, ON 45°10′N 081°31′W, 6 (3); [14] Landon's Bay, Leeds, and Grenville, ON 44°21′N 076°04′W, 6 (8); [15] Rodney, Elgin, ON 42°34′N 081°40′W, 8—AF488315 (3); [16] Skunk's Misery, Middlesex, ON 42°41′N 081°45′W, 8 (2), 19—AF488326 (1); [17] Dundas, Hamilton—Wentworth, ON 43°14′N 080°01′W, 4 (8), 1 (1); [18] Aberfoyle, Wellington, ON 43°29′N 080°09′W, 4 (4); [19] Acton, Halton, ON 43°33′N 079°58′W, 4 (1), 9—AF488316 (1), 14—AF488321 (1); [20] Long Point, Haldimand-Norfolk, ON 42°37′N 080°28′W, 4 (3), 10—AF488317 (2), 32—AF488339 (1), 33—AF488340 (1); [21] Wildwood Lake, Oxford, ON 43°15′N 081°00′W, 4 (4), 6 (1), 8 (1), 27—AF488334 (1); [22] Duck Lake, Kalamazoo, MI 42°24′N 085°23′W, 8 (2); [23] Ithaca, Tompkins, NY 42°27′N 076°27′W, 4 (1), 6 (1), 25—AF488332 (2); [24] Burden lake, Rensselaer, NY 42°36′N 073°34′W, 6 (3), 26—AF488333 (1); [25] Slippery Rock Cr., Butler, PA 41°05′N 079°60′W, 1 (1), 4 (3), 24—AF488331 (1); [26] Stroud's Run SP, Athens, OH 39°21′N 082°02′W, 8 (1); [27] Edwardsville, Madison, IL 38°15′N 090°00′W, 3—AF488310 (1), 5 — AF488312 (5), 51—AY210878; [28] Baskett RA, Boone, MO 38°45′N 092°12′W, 2—AF488309 (6), 3 (2); [29] Linn, KS, 39°40′N 094°04′W, 38—AF488345(1); [30] Stone, AR 35°50′N 092°00′W, 2 (1), 54—AF488341 (1); [31] White, AR 35°20′N 091°50′W, 35—AY210877 (1); [32] Murfreesburo, Rutherford, TN 35°50′N 086°24′W, 8 (2), 30—AF488337 (1), 31—AF488338 (1), 43—AY210852 (1), 58—AY210854 (1); [33] Wakefield, Sussex, VA 38°02′N 077°90′W, 17—AF488324 (2), 18—AF488325 (1); [34] Pitt, NC 35°35′N 077°23′W, 37—AF488344 (1); [35] Barnwell, SC 33°18′N 081°15′W, 13—AF488320 (1), 36—AF488343 (1); [36] Berkeley, SC 33°10′N 080°00′W, 23—AF488330 (1); [37] Barbour, Houston, AL 31°50′N 085°27′W, 12—AF488319 (1); [38] Liberty, FL 30°15′N 084°45′W, 12 (2); [39] Alachula, FL 29°50′N 082°30′W, 13 (1); [40] Ocala, FL 29°10′N 082°20′W, 13 (2); [41] Deepwater, Salem, NJ 39°32′N 075°30′W, 17 (3); [42] Augusta, VA 38°22′N 079°10′W 4 (1), 29—AY210872 (3), 40—AY210869 (2); [43] Dawn, Caroline, VA 37°39′N 077°17′W 39—AY210857 (1), 50—AY210870 (1); [44] Wayne, WV 38°18′N 082°19′W 4 (1), 29 (2), 42—AY210872 (1); [45] Danville, Virmillion, IL 40°11′N 087°44′W, 8 (6), 44 — AY210868 (1), 45—AY210867 (1); [46] Herod, Terrill, GA 31°40′N 084°27′W, 12 (2), 46—

AY210863 (1); [47] Lafayette, Walker, GA 34°42′N 085°20′W, 11 — AF488318 (1), 13 (3), 59—AY210853 (1); [48] Holly Hill, Orangeburg , SC 33°15′N 080°17′W, 13 (2), 41—AY210859 (1); [49] Salem, Pearl River, MS, 30°29′N 089°36′W, 11 (4); [50] Vicksberg, Warren, MS 32°16′N 090°51′W, 11 (3); [51] Boone, Watauga, NC 36°13′N 081°40′W, 47—AY210858 (1), 48—AY210874 (2), 49—AY210855 (1); [52] Carbondale, Jackson, IL 37°44′N 089°20′W, 30 (2), 44 (2), 51 (1); [53] Danville, Hendrick′s, IN 39°45′N 086°31′W, 8 (2), 41 (1); [54] Alcoa, Blount, TN 35°47′N 83°60′W, 1 (1), 53—AY210882 (1), 54—AY210860 (1), 59 (1), 60—AY210862 (2); [55] Mt. Salem, Lincoln, KY 37°25′N 084°47′W, 59 (3), 61—AY210864 (1); [56] St. Martin Pa., LA 30°21′N 091°43′W, 11—AY210856 (4); [57] Salt Pond, Covington, AL 31°10′N 086°32′W, 12 (5), 55—AY210865 (1); [58] Ft. Rucker, Dale, AL 31°22′N 085°44′W, 12 (4), 52—AY210866 (1), 55 (1); [59] Beaver′s Bend SP, McCurtain, OK 34°00′N 094°45′W, 51 (1), 56—AY210875 (1), 57—AY210876 (1); [60] Clinton, Huron, ON 43°38′N 081°27′W, 1 (1), 8 (2).

2.2. Sequencing protocol

Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen) following manufacturer's instructions. We amplified a segment of the cytochrome *b* mitochondrial gene using the MVZ 15L primer (Moritz et al., 1992) in combination with MVZ arH (Goebel et al., 1999) for *R. catesbeiana*, or MVZ 18H (Moritz et al., 1992) for *P. crucifer* (PCR conditions as in Austin et al., 2002). PCR products were cleaned with QIAquick PCR purification kits (Qiagen) according to the manufacturer's specifications and cycle sequenced (Amersham—Thermo-Sequenase) using MVZ 15L. Products were run on 6% PAA gels for 2–6 h, and upon completion, 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 using `MacClade` 4.0 (Maddison and Maddison, 2002). A subset of representative divergent haplotypes (see Section 3) was further sequenced to help resolve basal phylogenetic structure. These subsequent PCR products were cycle sequenced using MVZ arH (*R. catesbeiana*) or MVZ 18H (*P. crucifer*).

2.3. Phylogenetic analyses

Statistical parsimony (Templeton et al., 1992) cladogram networks were constructed using `TCS` version 1.13 (Clement et al., 2000). A complimentary approach involving maximum likelihood (ML, Felsenstein, 1981) was performed on long-sequenced exemplar haplotypes to help determine the basal relationships among clades. We first evaluated 24 models of molecular evolution using `Mr. Modeltest` version 1.1 (Nylander, 2002). A ML heuristic search with 'as-is' random additions was conducted using `Paup*` 4.10 (Swofford, 1998) and support was estimated using 500 nonparameteric bootstraps. Bayesian analysis, as implemented in `MR. BAYES` 3.0b4 (Huelsenbeck and Ronquist, 2001), was also conducted to compare posterior probabilities with ML boostrap values. Bayesian analyses began with random starting trees and run for 2.0×10^6 generations, implementing Metropolis-coupled MCMC using four incrementally heated Markov chains per generation, and Markov chains sampled every 500 generations. Stationarity of the Markov chain was determined by plotting the sampled $\ln L$ scores against generation time, and the points generated prior to stationarity were discarded and subsequent generations used to form the posterior probability distribution. The analysis was conducted twice to ensure convergence on the same $\ln L$ values. We combined remaining trees from both analyses to create a majority rule consensus tree.

For the *R. catesbeiana* data set, *R. virgatipes* and *R. septentrionalis* were included as outgroup taxa, as they represent the basal lineages within the *R. catesbeiana* species group (Austin et al., 2003). *Rana heckscheri*, *R. clamitans*, and *R. okaloosae* were also included but not designated as outgroup taxa. Recent work on chorus frogs strongly supports the sister-group relationship between *Pseudacris ocularis* and *P. crucifer* (Moriarty and Cannatella, 2004). *P. ocularis* and two other taxa representing separate lineages within *Pseudacris*, *P. triseriata*, and *P. streckeri* were therefore included as outgroups for the *P. crucifer* data set. For *P. crucifer* we repeated ML analysis using midpoint rooting without outgroups to compare ingroup topologies between methods.

We determined whether the application of a molecular clock was valid for our data by conducting a ML analysis with a molecular clock enforced for each ingroup and compared this with a ML estimate without the clock enforced using a likelihood ratio test (Huelsenbeck and Crandall, 1997). Because intra-specific gene divergence may predate population isolation (Nei, 1987; Nichols, 2001), we calculated point estimates of divergence times (p) among clades represented by the long-sequenced haplotypes using the moment method of Nei and Li (1979), which corrects for the ancestral portion of the within-phylogroup diversity ($p_{\text{net}} = p_{\text{AB}} - 0.5[p_{\text{A}} + p_{\text{B}}]$). We were unable to find an anuran cytochrome *b* calibrated molecular clock, although previous work on the *R. catesbeiana* species group suggests a similar rate of lineage divergence between cytochrome *b* and ND2 (Austin et al., 2003). The latter has been 'calibrated' to geological events in a number of poikilothermic vertebrates and these relatively similar rates of lineage diversification argue against heterogeneity due to life history characteristics (Weisrock et al., 2001 and references therein). Given the necessity for caution when using a molecular clock

calibrated for another poikilothermic species, we conservatively bounded estimates of divergence using rate of change of between 0.5 and 1% per million years.

2.4. Nested clade analyses and inferences of demographic histories

The 95% plausible sets of haplotype cladograms (see above) were nested into evolutionary hierarchies using the algorithm of Templeton et al. (1987), and nesting rules for resolving cladogram ambiguity (Templeton and Sing, 1993). Tip-interior status (the temporal component) was assigned to each clade-group, and geographic information was 'overlayed' on the nested set of haplotypes (using `GeoDis` version 2.0; Posada et al., 2000), Significance ($\alpha = 0.05$) of clade-group distances (D_c, D_n, and I–T) measures were determined by comparison with a null distribution from 10,000 permutation replicates which assume no geographic association within a particular clade-group given haplotype frequencies and sample sizes per location (Templeton, 1998). Inferences were made following the most recent published key at: http://inbio.byu.edu/Faculty/kac/crandall_lab/geodis.htm(Downloaded January 2004, see Templeton, 2004).

Finally, we examined haplotype variation using the approach of Grant and Bowen (1998) where patterns of haplotype (h) and nucleotide (π) diversity were used to infer historical demographic events. h measures the relative frequencies of haplotypes in a population, without consideration of their relationships, and π is the weighted mean of pairwise divergence among haplotypes. Results were categorized as reflecting: (1) prolonged bottleneck [low h (> 0.5) and low π ($< 0.5\%$)], (2) rapid population growth from ancestral population with low $N_{e(f)}$ (high h and low π), (3) a brief bottleneck where some haplotypes are lost but with limited effect on π (low h and high π), and (4) stable populations with large historical $N_{e(f)}$ or admixed populations (high h and high π) (Avise, 2000; Grant and Bowen, 1998). We treated major groups identified through statistical parsimony as gene populations for calculating h and π using `Arlequin` version 2.2 (Schneider et al., 2000).

3. Results

3.1. Cytochrome b variation

We found 41 haplotypes among the 215 short (408 bp) *R. catesbeiana* sequences varying from 0.03 to 2.76% HKY sequence divergence (Table 1). Of the 408 characters, 42 were variable and 24 parsimony-informative. Third base codon positions accounted for 30 of the total variable sites, five for second codon positions, and seven for first codon positions. This, together with a bias against guanine on the light strand (mean G = 15.8%, A = 23.7%, C = 28.2%, and T = 32.3%), and a lack of stop codons, strongly suggests the target is mtDNA cyt *b* sequence, and not a nuclear homologue (Zhang and Hewitt, 1996). The guanine bias increased slightly in the 925 bp data set discussed below (mean G = 13.9%, A = 23.7%, C = 31.3%, and T = 31.1%).

This portion of the cyt *b* gene has been previously characterized for *P. crucifer* (Austin et al., 2002). The addition of 87 individuals, primarily from the southern portion of the species' range, did not affect the bp composition (but see below). We found 62 *P. crucifer* haplotypes from the 305 bp sequenced. Pairwise sequence divergence ranged from 0.3 to as high as 6.62% (Table 1). The 622 bp data set (discussed below) did have an increased bias against guanine (mean G = 14.7%, A = 25.4%, C = 24.7%, and T = 35.2%). Of the 66

Table 1
Range of cytochrome *b* HKY genetic divergences among major intra- and inter-lineage comparisons for *R. catesbeiana* and *P. crucifer* identified in Figs. 3 and 4

Rana catesbeiana					
Lineage	West	East			
West	0–1.24%	0–1.74%			
East	1.24–2.76%				
p_{net}	1.14%				
P. crucifer					
Lineage	SW	West	Central	Southeast	North
SW	0–0.99%				
West	3.75–5.16%	0–1.33%			
p_{net}	5.06%				
Central	2.70–5.51%	4.11–6.62%	0–2.00%		
p_{net}	3.37%	5.44%			
Southeast	3.38–5.16%	4.43–6.62%	3.38–5.86%	0–1.33%	
p_{net}	3.75%	3.09%	3.14%		
North	4.11–6.62%	4.10–6.27%	3.03–6.58%	2.34–4.78%	0–3.39%
p_{net}	4.42%	4.69%	2.91%	1.87%	

p_{net} is the point estimate of percentage HKY divergence correcting for ancestral lineage diversity (see text) calculated from sequences used for ML analyses.

variable positions (57—3rd, 3rd—2nd, and six—1st codon positions), 51 were parsimony-informative.

3.2. Cytochrome b phylogenetics

3.2.1. Statistical Parsimony

With 408 bp of cyt *b*, *R. catesbeiana* haplotypes connected by up to eight mutational steps lie within the 95% limits of parsimony. All 41 haplotypes were connected within a single network (Fig. 3). There was a larger than average number of mutational steps (minimum of five) between two main groups of haplotypes corresponding to eastern and western clades. Most other haplotypes differed by a single mutational step.

With 305 bp, *P. crucifer* haplotypes separated by up to seven mutational steps were within the limits of parsimony. High pairwise sequence differentiation within this data set resulted in three independent networks (Fig. 4). Individual networks were highly regionalized, with a western, southwestern, and a large eastern group that consisted of widespread: (i) northern, (ii) a northern Coastal Plain, (iii) a southern Coastal Plain, and (iv) central groups. Connections among haplotype groups that could not be determined using the parsimony network were inferred from the ML topology (see Fig. 5).

3.2.2. Maximum likelihood and Bayesian methods

The simplest model of evolution not rejected by the χ^2 goodness of fit tests for both *R. catesbeiana* and *P. crucifer* was the HKY + Γ model. Likelihood parameters for *R. catesbeiana* and *P. crucifer*, respectively were: ln $L = -1708.886$; transition/transversion ratio = 2.485; shape parameter = 0.017, and ln $L = -1602.464$; transition/transversion ratio = 5.673; shape parameter = 0.124.

Results from the ML analysis reflect low levels of sequence divergence even within 925 bp of cyt *b* for *R. catesbeiana*. Topology was shallow, with limited bootstrap and Bayesian posterior support (Fig. 5). There was moderate bootstrap (69%) and significant Bayesian posterior probability (99%) support for the monophyly of the western group, which is nested within a paraphyletic grouping of eastern haplotypes. This pattern reflects the relative ages of the eastern and western groups, a conclusion supported by biogeographic analysis of the entire *R. catesbeiana* species group in which the southeast Coastal Plain Region was identified as the likely biogeographic origin (Austin et al., 2003).

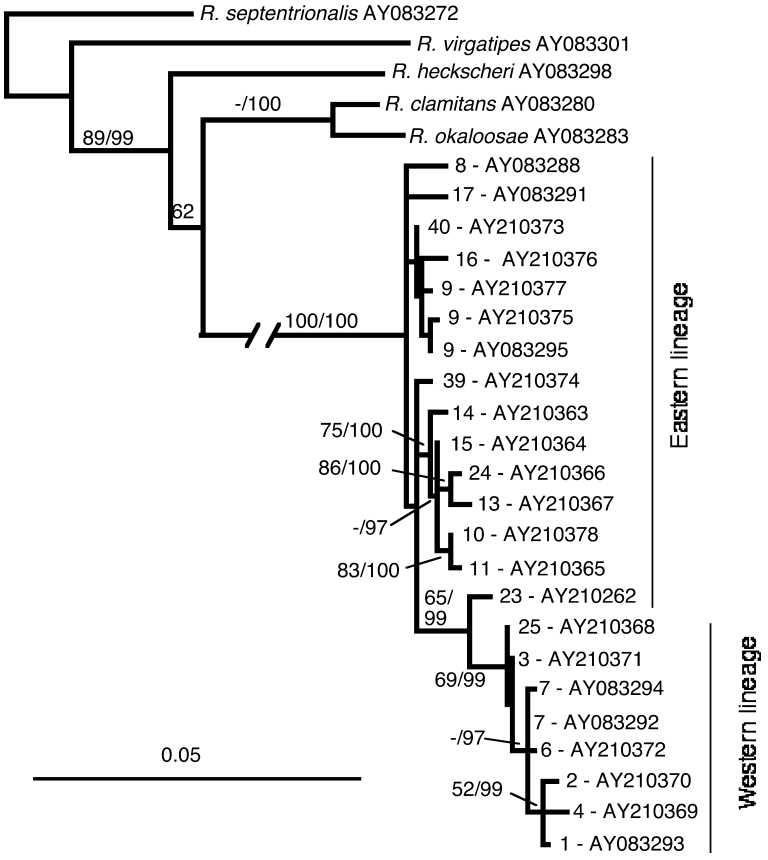

Fig. 5. Maximum likelihood phylogram for 23 exemplar *R. catesbeiana* haplotypes based on 925 bp of cytochrome *b*. Bootstrap support greater than 60% of 500 replicates are indicated first, followed by Bayesian posterior probabilities $\geqslant 95\%$. Identifiers are haplotype and Genbank accession number. Haplotypes were identified as eastern or western based on results from parsimony network (Fig. 3).

Although interspecific biogeographic and intraspecific phylogeographic approaches (this study) may reflect different historical time scales, *R. catesbeiana* haplotypes in both studies coalesce to eastern North America. We therefore use the relative coalescence times of western haplotypes versus all haplotypes to root the statistical parsimony network in the NCA (see below).

Maximum-likelihood results from 622 bp of cyt *b* for 23 *P. crucifer* exemplars correspond to the groupings determined from the parsimony network (Fig. 6). Haplotypes from the west group with good bootstrap and posterior support (87 and 100%, respectively) and form the sister group to a clade containing all other haplotypes. Haplotypes from both the southwest and central lineages group with good support (99%/100% and 85%/100%, respectively for bootstrap/posterior probabilities, Fig. 6). The remaining eastern haplotypes [representing the: (i) northern, (ii) northern Coastal Plain, and (iii) southern Coastal Plain regional lineages] group with moderate bootstrap support (66%) and high posterior probability (99%), with varying degrees of support for their regional placement (Fig. 6). The ML topology is the same using mid-point rooting with the exclusion of outgroups (not shown).

We are unable to reject the null hypothesis of clock-like rate of evolution within either species (*R. catesbeiana* $\chi^2_{32.67,21} = 32.04$; $P > 0.05$; *P. crucifer* $\chi^2_{32.67,21} = 27.10$; $P > 0.05$). Under the assumption of a slow poikilothermic mtDNA evolutionary rate of 0.5–1% divergence per million years, Pliocene divergence accounts for most inter-lineage diversification seen in *P. crucifer*, while the eastern and western *R. catesbeiana* lineages appear to have diverged early in the Pleistocene (Fig. 7). The presence of fossils in Kansas, Colorado, and Florida dating to the early Pleistocene supports our contention that *R. catesbeiana* was present in both geographic regions at this time (cf. Holman, 1995; Fig. 1).

3.3. Nested-clade phylogeographic analysis

The hierarchical nesting procedure results in three nesting levels for *R. catesbeiana* and six levels for *P. crucifer* (Figs. 3 and 4). The NCA results in nine *R. catesbeiana* and 13 *P. crucifer* groups with geographic distributions that are significantly different from random (Tables 2 and 3). Past fragmentation, contiguous range expansion, and isolation-by-distance are, at various levels, inferred for both species.

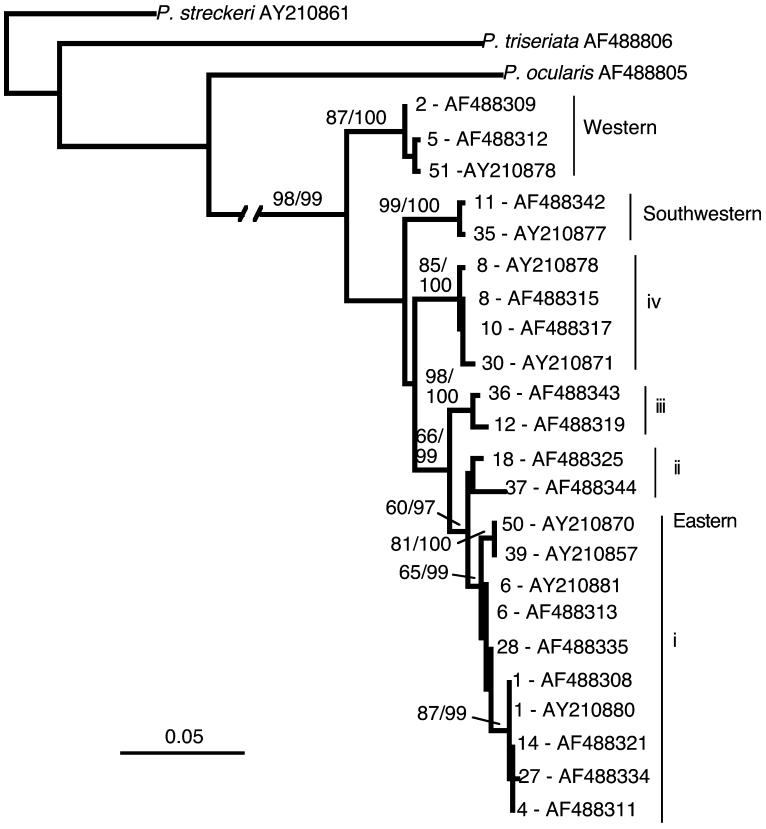

Fig. 6. Maximum likelihood phylogram for 23 exemplar *P. crucifer* haplotypes based on 622 bp of cytochrome *b*. Bootstrap support greater than 60% of 500 replicates are indicated first, followed by Bayesian posterior probabilities $\geqslant 95\%$. Identifiers are haplotype and Genbank accession number. Geographic groups correspond to those of Fig. 4 and in text: Western, Southwestern, and Eastern haplotypes. The latter is grouped into a: (i) Northern, (ii) Northern Coastal Plain, (iii) Southern Coastal Plain, and a (iv) Central lineages.

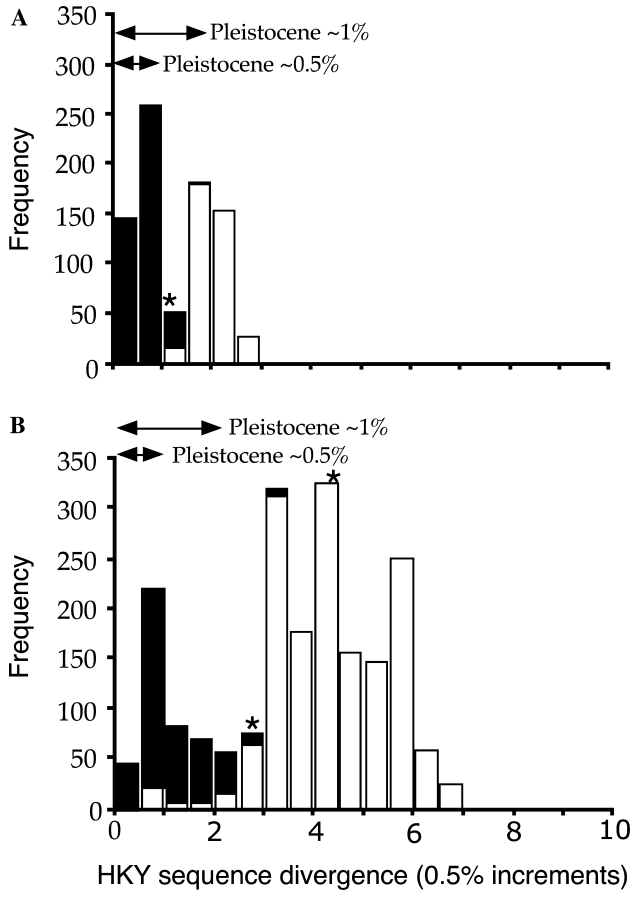

Fig. 7. Histogram of: (A) 820 *R. catesbeiana* and (B) 2016 *P. crucifer* HKY pair-wise sequence comparisons based on 408 and 305 bp of cytochrome *b*, respectively. Black columns are within-lineage comparisons, white columns are comparisons among lineages (see text). Point estimates of major lineage divergence (*pnet*) based on long sequences (i.e., 925 and 622 bp, respectively) are indicated with *. Western versus eastern *R. catesbeiana* lineages $p_{net} = 0.0114$. Due to the shallow topology among most of the *P. crucifer* lineages (Fig. 5), we illustrated the average p_{net} comparison between the western and all other lineages (mean $p_{net} = 0.0435$, SD = 0.0102) and the average among other comparisons excluding the western lineage (mean $p_{net} = 0.0247$, SD = 0.0139). See Table 2 for values.

At the total cladogram level (i.e., deepest coalescent time), past fragmentation is inferred for *R. catesbeiana*. The greater than average number of mutational differences between the western and eastern haplotypes supports this interpretation, while the geographic location of the ancestral groups (*2–2* and *2–3*) within each of the highest nesting clade-groups (Fig. 8) implies past isolation on either side of the Mississippi River. Contiguous range expansion from the southwest occurred west of the Mississippi, followed by restricted gene flow across much of the range (group *2–1*). In the east, isolation-by-distance is a repeated pattern, found at both the *2*-step and *3*-step clade-group levels (Fig. 8 and Table 2). Contiguous range expansion from the southern Appalachians into Canada is also implied (group *2–5*), likely reflecting post-glacial colonization dynamics.

The patterns at the deep coalescent periods (groups *5–1* and *6–1*) in *P. crucifer* are inconclusive due to the lack of polarity (i.e., no tip-interior differentiation) within each clade (Table 3). Past fragmentation is interpreted within group *4–1* (Figs. 4 and 9). The distribution of clade-group *4–2* suggests range expansion, although the geographic gap between some of the groups does not allow us to distinguish between contiguous range expansion or long-distance migration. Two widespread northern groups that have expanded throughout the previously glaciated region (*3–3* and *3–6*, Fig. 9) display different geographic patterns. Range expansion was inferred in the northeast (group *3–3*), although again contiguous expansion versus long-distance dispersal could not be differentiated due to the large unsampled gap separating groups *2–5* and *2–6* (Fig. 9). In contrast, isolation-by-distance was identified within the group that extends from the central Appalachians, north and west into Canada and Wisconsin (group *3–6* ; Fig. 9). Furthermore, contiguous range expansion occurred along the Gulf Coast (group *2–11*) and Ozark (*2–10*) regions. Isolation-by-distance or past fragmentation may be the cause of the geographic disjunction between the two southeastern groups (*1–5* and *1–6*). Haplotypes of group *1–5* are located on either side of the Apalachicola River, an important biogeographic barrier for many taxa (Avise, 1992; Blainey, 1971; Church et al., 2003; Swift et al., 1985). This distribution and the large unsampled geographic area between the two nested-groups (Fig. 9) precludes comment on the biogeographic effect of the river on *P. crucifer*.

We estimated h and π from the eastern ($h = 0.824$, $\pi = 0.359$) and western ($h = 0.851$, $\pi = 0.413$) haplotype groups of *R. catesbeiana* and for the central ($h = 0.787$, $\pi = 0.849$), northern ($h = 0.835$, $\pi = 1.081$), southern Coastal Plain ($h = 0.720$, $\pi = 0.489$), southwest ($h = 0.371$, $\pi = 0.017$) and western ($h = 0.781$, $\pi = 0.044$) groups of *P. crucifer*. Following the interpretation of Grant and Bowen (1998), the pattern for both *R. catesbeiana* groups represents large values of h and low π (i.e., $h > 0.5$, $\pi < 0.5\%$), implying population bottlenecks followed by rapid growth and accumulation of new mutations. The same interpretation applies to the western and southern Coastal Plain groups of *P. crucifer*. However, the large northern and central groups have large h and π (π is twice that of 'low' values), signifying either large stable populations or secondary contact between differentiated lineages. Population stability is unlikely given the recent glacial history of these northern regions. Rather, this pattern may reflect the presence of numerous common haplotypes (star-like phylogenies) in the north and central regions, indicative of population expansion from different Appalachian refugia. Finally, the southwest group had low h and π, suggestive of a population bottleneck or founder event, as is evident from the presence of few haplotypes in the

Table 2
Results of nested-clade analysis for *R. catesbeiana*

	Position	D_c	*P*	D_n	*P*
Clade *1–2*					
5	Tip	220.94	−0.012	540.70	+0.025
7	Interior	347.83	ns	374.88	ns
20	Tip	0.00	ns	165.80	ns
22	Tip	0.00	ns	344.37	ns
26	Tip	0.00	ns	344.38	ns
I–T		187.15	ns	−96.04	ns
1–2–3–5–15–16–18: Inconclusive					
Clade *1–7*					
8	Interior	574.75	ns	578.32	ns
14	Tip	0.00	ns	700.02	ns
15	Interior	0.00	ns	306.22	ns
28	Tip	0.00	−0.001	475.27	ns
29	Tip	0.00	ns	475.82	ns
30	Tip	435.08	ns	535.13	ns
I–T		465.98	+0.008	62.77	ns
1–2–3–4: IBD					
Clade *1–14*					
9	Interior	280.89	−0.0008	322.21	−0.005
16	Tip	0.00	−0.0004	242.40	ns
17	Tip	87.76	−0.0044	599.51	+0.007
33	Tip	0.00	ns	440.70	ns
34	Tip	0.00	ns	1404.72	+0.018
35	Tip	0.00	ns	414.86	ns
37	Tip	0.00	ns	284.14	ns
38	Tip	131.75	ns	240.70	ns
39	Tip	0.00	ns	297.54	ns
I–T		237.00	ns	−131.92	−0.016
1–2–11–12–13: LRE					
Clade *2–1*					
1–1	Tip	444.08	ns	501.66	ns
1–2	Interior	418.40	ns	482.79	ns
1–3	Tip	0.00	−0.004	163.63	−0.001
I–T		34.93	ns	27.22	ns
1–2–3–4: IBD					
Clade *2–3*					
1–6	Tip	189.31	−0.000	361.49	−0.000
1–7	Tip	147.79	−0.040	537.79	ns
1–8	Interior	569.52	ns	585.46	+0.002
1–11	Tip	93.29	−0.011	616.17	ns
I–T		403.85	+0.000	168.12	+0.001
1–2–3–4: IBD					
Clade *2–5*					
1–12	Tip	167.87	ns	356.33	ns
1–13	Tip	0.00	ns	1314.12	+0.002
1–14	Interior	370.11	−0.006	387.36	−0.006
I–T		258.20	ns	−288.24	−0.029
1–2–11–12: CRE					
Clade *3–1*					
2–1	Tip	473.07	ns	467.30	ns
2–2	Interior	90.63	−0.000	482.15	ns
I–T		−382.44	−0.000	14.85	ns
1–2–11–12: CRE					
Clade *3–2*					
2–3	Interior	546.87	−0.008	614.32	ns
2–4	Tip	257.96	−0.000	513.96	ns
2–5	Tip	441.07	−0.000	624.89	ns
I–T		140.59	+0.001	9.91	ns
1–2–3–4: IBD					
Total cladogram					
3–1	Tip	468.97	−0.000	765.76	+0.003
3–2	Interior	604.32	−0.000	642.13	−0.002

Table 2 (*continued*)

	Position	D_c	P	D_n	P
I–T		135.35	+0.002	−123.63	−0.001
1–2–3–5–15: PF					

For clade-groups with significant geographical associations we present the clade distance (D_c), nested-clade distance (D_n), and interior– tip (I–T) contrasts. Group designations correspond to those in Fig. 8. Probability values for significantly larger (+) or smaller (−) than expected based on the null hypothesis of no geographical association are based on 10,000 permutations. Isolation-by-distance (IBD), contiguous range expansion (CRE), past fragmentation (PF), or long-distance range expansion (LRE).

Table 3
Results of nested-clade analysis for *P. crucifer*

	Position	D_c	P	D_n	P
Clade *1–3*					
8	Interior	282.68	−0.0016	324.76	ns
10	Interior	0.00	−0.0457	454.84	ns
19	Interior	0.00	ns	387.38	ns
30	Tip	139.72	ns	478.96	+0.0257
31	Tip	0.00	ns	477.50	ns
43	Tip	194.16	ns	364.71	ns
44	Interior	0.00	ns	273.90	ns
45	Tip	0.00	ns	273.90	ns
58	Tip	0.00	ns	477.49	ns
59	Interior	113.37	−0.003	357.78	ns
60	Interior	0.00	−0.045	459.01	ns
61	Interior	0.00	ns	275.70	ns
I–T		81.29	ns	−28.12	ns
1–2–3–5–15: PF/RE					
Clade *1–10*					
6	Interior	303.69	ns	302.14	ns
15	Tip	0.00	ns	973.05	ns
16	Tip	0.00	ns	212.97	ns
20	Tip	0.00	ns	15.95	ns
25	Interior	0.00	+0.050	247.90	ns
26	Tip	0.00	ns	314.93	ns
28	Interior	0.00	ns	268.56	ns
I–T		279.07	ns	−80.93	ns
1–2–3–4: IBD					
Clade *1–18*					
2	Tip	138.26	ns	117.84	ns
3	Interior	85.07	ns	92.44	ns
5	Tip	0.00	+0.002	195.53	ns
34	Tip	0.00	ns	278.06	ns
38	Tip	0.00	ns	215.54	ns
I–T		20.55	ns	-73.67	ns
1–2–11–12: CRE					
Clade *2–4*					
1–9	Interior	458.21	−0.000	481.32	−0.000
1–10	Interior	63.89	−0.004	1031.39	+0.000
I–T	No I–T contrasts: inconclusive				
Clade *2–9*					
1–5	Tip	94.01	−0.000	183.04	−0.000
1–6	Interior	244.24	ns	329.62	+0.000
I–T		150.23	+0.000	146.58	+0.000
1–19–20: Inadequate sampling					
Clade *2–10*					
1–18	Interior	174.09	−0.003	200.84	−0.002
1–19	Tip	247.83	ns	465.15	+0.001
I–T		−73.74	ns	264.31	−0.001
1–2–11–12: CRE					
Clade *2–11*					
1–20	Interior	197.25	−0.038	208.63	ns

Table 3 (*continued*)

	Position	D_c	P	D_n	P
1–21	Tip	0.00	ns	392.72	ns
I–T		197.25	ns	−184.09	ns
1–2–11–12: CRE					
Clade *3–3*					
2–5	Tip	313.38	−0.000	365.40	−0.024
2–6	Interior	183.09	−0.002	546.57	+0.036
I–T		−130.29	ns	181.17	+0.031
1–2–11–12–13–14: RE					
Clade *3–6*					
2–3	Tip	155.27	−0.000	343.77	−0.000
2–4	Interior	551.62	+0.047	610.87	+0.000
I –T		396.35	+0.000	267.09	+0.000
1–2–3–4: IBD					
Clade *4–1*					
2–1	Tip	355.29	−0.003	358.39	−0.007
2–2	Interior	186.65	ns	569.40	+0.028
I–T		−168.64	ns	211.01	+0.020
1–19–20–2–3–4–9: PF					
Clade *4–2*					
3–3	Interior	412.21	−0.000	595.25	ns
3–4	Tip	144.86	−0.012	484.83	ns
3–5	Tip	252.47	−0.000	1032.60	+0.000
3–6	Tip	489.67	−0.000	583.63	ns
I–T		5.83	ns	−115.39	−0.042
1–11–12–13–14: RE					
Clade *5–1*					
4–1	Interior	371.25	−0.000	417.20	−0.000
4–2	Interior	678.97	+0.000	692.49	+0.000
I –T	No I–T contrasts: inconclusive				
Clade *6–1*					
5–1	Interior	625.23	−0.000	637.74	−0.000
5–2	Interior	237.85	−0.000	1175.29	+0.000
I–T	No I–T contrasts: inconclusive				

Clade distance (D_c), nested-clade distance (D_n), and interior–tip (I–T) contrasts are shown for those groups with significant geographical association (>0.05). Group designations correspond to those in Fig. 9. Probability values for significantly larger (+) or smaller (−) than expected based on the null hypothesis of no geographical association are based on 10,000 permutations. Isolation-by-distance (IBD), range expansion (RE), contiguous range expansion (CRE), and past fragmentation (PF).

sampled populations (12 of 15 individuals were haplotype 1).

4. Discussion

4.1. Refugial models and secondary contact

The patterns of diversification in both *R. catesbeiana* and *P. crucifer* suggest complex histories involving both allopatric isolation among refugial areas and prominent patterns of dispersal, albeit over different spatial and temporal scales. A number of characteristics of our data support the inference of allopatric divergence in *R. catesbeiana* and *P. crucifer*. The topological position of the monophyletic 'western' *R. catesbeiana* lineage in our ML tree, together with biogeographic evidence from the *R. catesbeiana* species group (Austin et al., 2003), and fossil evidence (Fig. 1) support our interpretation that the western lineage is derived from an eastern one by range expansion, and that it was present, and likely became isolated, west of the Mississippi River early during the Pleistocene. The limited geographic distribution of the ancestral western haplotypes (group *2–2*) suggests that the Gulf Coastal Plain (west of the Mississippi) was a likely refugial area. Further sampling from the Gulf Coastal Plain region would clarify the distribution and abundance of this ancestral group. In contrast, the widespread overlap of major haplotype groups in the east precludes a definitive identification of refugial areas, although the distribution of the ancestral eastern group (group *2–3*), together with the aquatic nature of this species and relatively xeric habitat at higher elevations during the early Pleistocene (i.e., southern Appalachians, Delcourt and Delcourt, 1998; Highton, 1995), suggests Atlantic and/or Gulf Coastal Plain refugia. In *P. crucifer* the large genetic distance separating the

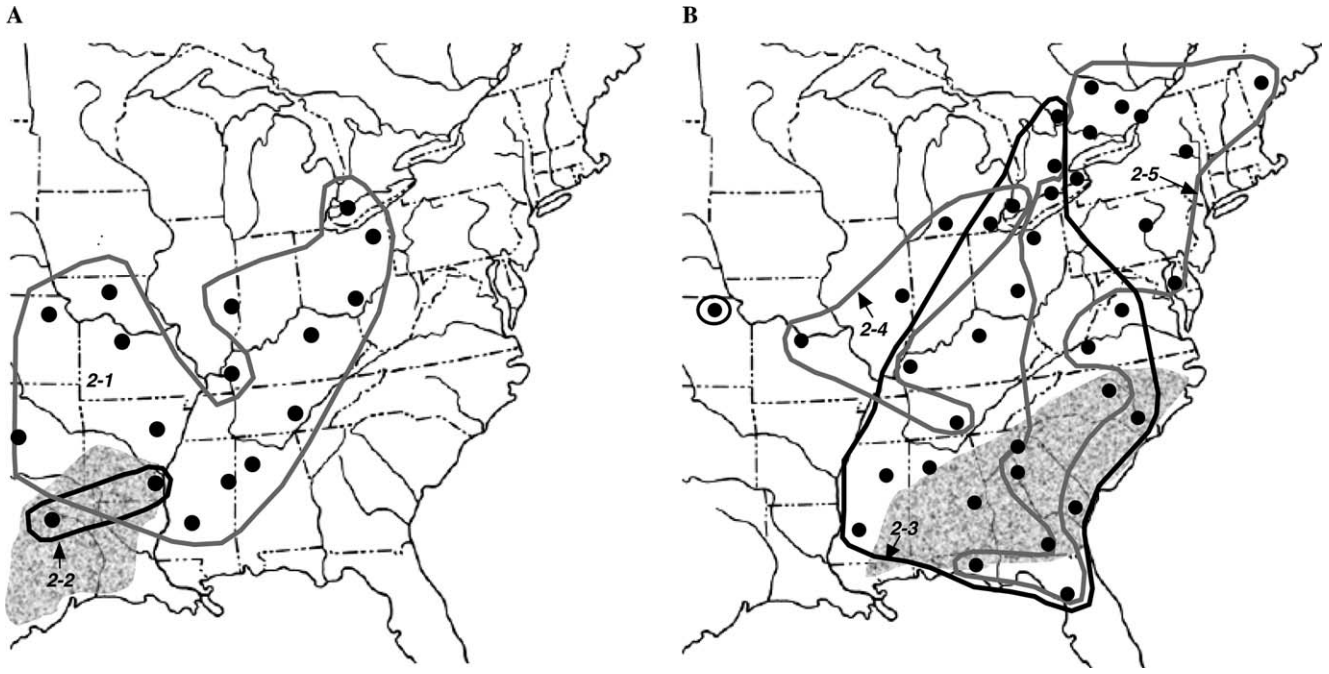

Fig. 8. Geographic distribution of some *R. catesbeiana* cytochrome *b* clade-groups identified through the nesting procedure of Templeton et al. (1987). Grey areas represent proposed major Pleistocene refugal areas. Group numbers correspond those in Fig. 3 and Table 2. (A) The western group *3–1* consisting of groups *2 –1* and *2–2*. (B) Eastern group *3–2* partitioned among its nested *2*-step groups. Note the single individual representing a common haplotype (8) from group *2–3* was found in Kansas (see Section 4).

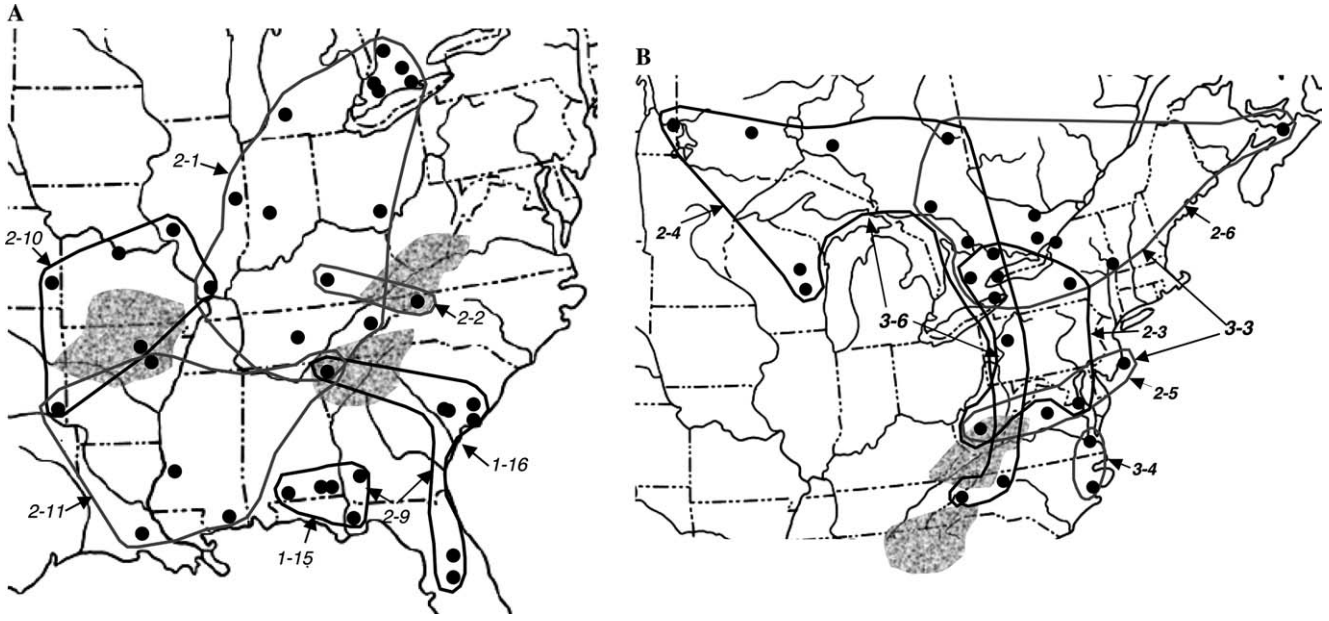

Fig. 9. Geographic distribution of some *P. crucifer* cytochrome *b* clade-groups identified through the nesting procedure of Templeton et al. (1987). Group numbers correspond to those in Fig. 4 and Table 3. (A) *1*-step and *2*-step clades from western, southwestern, central and southeastern regions. (B) *2*-step and *3*-step clades from the eastern group.

western group from, and large genetic distances within eastern lineages reflects allopatric differentiation, with an Ozark Central Highlands refuge and multiple southern Appalachian refugia.

Key points from our analyses support the inference of allopatric fragmentation. First, in both *R. catesbeiana* and *P. crucifer* we have, within each divergent clade, widespread haplotypes, suggesting at least moderate historical levels of gene flow. Second, we see similar longitudinal genetic breaks associated with the Mississippi River and the Appalachians (e.g., Brant and Orti, 2003; Burbrink et al., 2000; Walker et al., 1998) and

widespread general phylogeographic concordance (particularly in *P. crucifer*) with other taxa (see below). Although simulations have demonstrated the development of large phylogenetic breaks in the absence of barriers (Hoelzer, 2001; Irwin, 2002; but see Templeton, 2004), phylogeographic patterns that fit expectations based on biogeographic evidence (Hocutt and Wiley, 1986; Mayden, 1985, 1988; Near et al., 2001; Zamudio and Savage, 2003) and a general concordance across an increasing number of taxa supports a pattern derived from allopatry.

An increasingly common trend among eastern North American terrestrial vertebrates is a pattern of longitudinal genetic diversity (Burbrink, 2002; Burbrink et al., 2000; Church et al., 2003; Clark et al., 2003; Zamudio and Savage, 2003), where much of the variation in diversity is detected along an east-to-west axis. This pattern of diversity is greatest across 'longitudinal zones' within which admixture of divergent lineages occurs as populations rapidly spread north (or south) from refugial areas (Hewitt and Ibrahim, 2001). This often results in greater diversity occurring in areas of admixture rather than refugial areas (e.g., Petit et al., 2003). Some of the highest diversity measures in both *R. catesbeiana* and *P. crucifer* were detected in populations as far north as Ontario (data not shown) where central and eastern lineages have apparently introgressed. The Mississippi River has been noted as a common barrier to gene flow in a number of less vagile vertebrate species (Blair, 1958; Burbrink, 2002; Burbrink et al., 2000; Templeton et al., 1995; Walker et al., 1998; Wiley and Mayden, 1985). However, the large geographic overlap of eastern and western clades in *R. catesbeiana* (Fig. 8), and the presence of the *P. crucifer* southwestern group widely dispersed on both sides of the lower Mississippi (clade *2–11*, Fig. 9), together with detected gene flow across the southern Mississippi River in *Ambystoma maculatum* (Zamudio and Savage, 2003) suggests that the Mississippi River has not acted as a continuous barrier. Absolute barriers to gene flow may have been restricted to periods of high sea levels that inundated the Mississippi delta and Pleistocene interglacial alluviation that widely broadened the Mississippi River floodplain (Thornbury, 1965).

Range expansion was detected through NCA in both *R. catesbeiana* and *P. crucifer*, in groups that are at least partially located in previously glaciated zones. Within *P. crucifer*, group *3–6* (Fig. 9) has a pattern suggested by NCA to reflect isolation-by-distance, despite its widespread distribution across northern Ontario. This is the result of `GEODIS` using greater circle distances when, in this case, range expansion of group *2–4* (a sub-group of *3–6*) better fits a linear pattern (i.e., extending linearly north then west around the northern Great Lakes). Ecologically divergent species that have expanded their geographic ranges into previously glaciated regions provide robust tests of the NCA methodology, as the ability of NCA to identify contiguous range expansion is the least theoretically justified (Templeton, 1998).

The depth of divergence and topological position of the *P. crucifer* western group relative to the remaining lineages (Figs. 4 and 6), together with the deep pairwise divergence among lineages (Fig. 7), suggests that a possible Pliocene vicariant event separated these early lineages into Eastern and Interior Highland regions (i.e., the pre-Pleistocene vicariance hypothesis, Mayden, 1985; Pfliefer, 1971), with subsequent (i.e., lower nesting level) patterns of dispersal of eastern lineages from southern Appalachian refugia. This is supported by the overlap of divergent clades within the southern Appalachian region, and the pattern of contiguous range expansion following deglaciation (groups *2–11*, *2–10*, and *3–3*); Fig. 9, Table 3; see also Zamudio and Savage, 2003). Again, the depths of divergence among southwestern, central and eastern lineages reflect a late Pliocene origin, possibly diverging in mountain forest refugia during arid conditions as has been hypothesized for plethodontid salamanders (Highton, 1995). Other widespread eastern North American poikilotherms have demonstrated similar or greater levels of inter-clade divergence in cytochrome *b* (up to 16.9%, Burbrink et al., 2000) or other mtDNA genes (up to 7.7%, Zamudio and Savage, 2003).

4.2. Ecological and anthropogenic influences on phylogeographic structure

Is congruence (or lack thereof) in phylogeographic pattern influenced by the ecology of the organisms? Within the context of previous phylogeographic studies of taxa from eastern North America (e.g., rat snake (*Elaphe obsoleta*), Burbrink et al. (2000); short-tailed shrew (*Blarina brevicauda*), Brant and Orti (2003); tiger salamander (*Ambystoma tigrinum*), Church et al. (2003), spotted salamander (*Ambystoma maculatum*), Zamudio and Savage (2003)), it is apparent that there is wide-scale congruence among many species as determined by: (1) allopatric breaks corresponding to the present day Mississippi River region, and (2) post-glacial expansion of distinct lineages isolated in various Appalachian or Coastal Plain refugia, in particular a common pattern of central (i.e., bounded by the Mississippi River and Appalachian Mountains) and eastern (east of Appalachian and Coastal Plain) lineages. This emerging pattern, from such a diverse group of vertebrates, suggests that many ecological characteristics may not play a prominent role in shaping gross phylogeographic patterns.

Despite some general similarities, the overall phylogeographic pattern in *R. catesbeiana* is substantially less structured than in *P. crucifer* and the taxa discussed previously. The dominant pattern of recurrent gene flow restricted by isolation-by-distance and the large

geographic overlap among lineages in *R. catesbeiana* (Table 3; and Fig. 8) suggests that major physiographic elements (e.g., the Appalachian Mountains and Mississippi River) have not (recently) been major barriers to gene flow in this species. This may not be surprising considering the dispersal potential of *R. catesbeiana* and the species' broad niche tolerance (relative to *P. crucifer*). The validity of this assumption is evident from the success the species has had following introductions into diverse regions such as the western North America, Europe, South America and elsewhere (e.g., Gasc et al., 1997; Hedges, 1999; Lanza, 1962; Mahon and Aiken, 1977; Perez, 1951; Telford, 1960).

Little is known about the scale of gene flow in either species, although maximum daily and seasonal dispersal estimates differ considerably (Davis, 1999; *P. crucifer*, Delzell, 1958; Ireland, 2003; *R. catesbeiana*, Willis et al., 1956). Gene flow estimates from microsatellite DNA among populations of *R. catesbeiana* in Ontario are high at the inter-population scale of 10's of kms (Austin, unpublished data). Human induced dispersal may be a confounding factor. Although the frequency and geographic scale of such artificial movement is not well known, frequent anthropogenic dispersal could result in either: (1) a lack of genetic structure at the fine geographic scales of dispersal (lower clade levels) assuming artificial dispersal is predominantly at this scale, or (2) if frequent enough, larger geographic scale movements may be detected as long-distance dispersal by the NCA. The proportion of non-significant geographic structure is frequently greater in lower level clades, primarily where sample sizes are small (e.g., Durand et al., 1999; Hurwood and Hughes, 2001; Templeton et al., 1995), as is the case here. However, long-distance colonization was detected in the *R. catesbeiana* clade *1–14*, and one individual with an eastern haplotype was detected in Kansas (Fig. 8). Given the nature of dispersal in amphibians in general, these large-scale patterns are likely the result of insufficient sampling at fine geographic scales or a signature of human-induced gene flow, rather than the retention of ancestral polymorphism. However, phylogenetic structure is apparent at higher clade levels suggesting that, if artificial gene flow is present, it is insufficient to have a marked effect at the continental scale.

In summary, a wide array of species appear to have responded similarly to climatic fluctuations of the Pleistocene, and range-expansion patterns may reflect species attributes such as dispersal potential and habitat associations over other basic life-history characteristics. If one assumes that these phylogeographic patterns reflect temporal congruence (i.e., lineage sundering occurred at approximately the same time), then significant rate heterogeneity exists among species. Such differences in levels of divergence may be partially attributable to generation times, or metabolic rates, and this represents an interesting future area of inquiry. However, assuming relative rate homogeneity, it appears that a widespread *R. catesbeiana* ancestral population diverged into eastern and western lineages over the repeated glacial periods of the Pleistocene. Similar dynamics shaped a wide variety of taxa including *P. crucifer*, although it appears that much of this diversity predates the Pleistocene. For many species discussed above, and including *P. crucifer*, it is possible that the Pleistocene refugia served as 'finishing schools,' allowing lineages originating in the Pliocene to diverge through the repeated glacial cycles of the Pleistocene. However, whether 'neutral' phylogeographic divergence reflects adaptive divergence in these and other widespread species requires further attention.

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