



Climate change and evolution of the New World pitviper genus *Agkistrodon* (Viperidae)

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ABSTRACT

Aim We derived phylogenies, phylogeographies, and population demographics for two North American pitvipers, *Agkistrodon contortrix* (Linnaeus, 1766) and *A. piscivorus* (Lacépède, 1789) (Viperidae: Crotalinae), as a mechanism to evaluate the impact of rapid climatic change on these taxa.

Location Midwestern and eastern North America.

Methods We reconstructed maximum parsimony (MP) and maximum likelihood (ML) relationships based on 846 base pairs of mitochondrial DNA (mtDNA) ATPase 8 and ATPase 6 genes sequenced over 178 individuals. We quantified range expansions, demographic histories, divergence dates and potential size differences among clades since their last period of rapid expansion. We used the Shimodaira–Hasegawa (SH) test to compare our ML tree against three biogeographical hypotheses.

Results A significant SH test supported diversification of *A. contortrix* from northeastern Mexico into midwestern–eastern North America, where its trajectory was sundered by two vicariant events. The first (*c.* 5.1 Ma) segregated clades at 3.1% sequence divergence (SD) along a continental east–west moisture gradient. The second (*c.* 1.4 Ma) segregated clades at 2.4% SD along the Mississippi River, coincident with the formation of the modern Ohio River as a major meltwater tributary. A single glacial refugium was detected within the Apalachicola region of southeastern North America. Significant support was also found for a hypothesis of trans-Gulf rafting by the common ancestor of *A. piscivorus* from eastern Mexico (possibly the Yucatan Peninsula) to northern Florida. There, a Mid–Late Pliocene marine transgression separated it at 4.8% SD from mainland North America. Significant range expansions followed compressive glacial effects in three (of four) *A. contortrix* clades and in two (of three) *A. piscivorus* clades, with the Florida *A. piscivorus* clade exhibiting significant distributional stasis.

Main conclusions Pliocene glaciations, rapidly developing western aridity, and Pleistocene glacial meltwaters seemingly led to the diversification of *A. contortrix* and *A. piscivorus* in North America. Both species were pushed southwards by Pleistocene climate change, with subsequent northward expansions uninhibited topographically. The subspecific taxonomy used for *A. contortrix* and *A. piscivorus* today, however, appear non-representative. The monophyletic Florida subspecies of *A. piscivorus* may be a distinct species (at 4.8% SD), whereas two western subspecies of *A. contortrix* also appear to constitute a single distinct species, pending additional analyses. We conclude that both species of *Agkistrodon* can be used as suitable ectothermic models to gauge impacts of future climate change.

Keywords

Glaciation, mtDNA, North America, Okefenokee Trough, phylogeography, Pleistocene, Pliocene, Quaternary, refugia, subspecies.

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INTRODUCTION

Glacial–interglacial transitions, a major factor in global climate change (Adams *et al.*, 1999), are driven by the Earth's orbital eccentricity, tilt and precession (i.e. the Milankovitch theory of climate; Berger, 1978). Nine transitions (Lüthi *et al.*, 2008) occurred during the last 800,000 years of the Quaternary [i.e. the previous 1.8 million years (Myr)], and these impacted global biodiversity by promoting extinction over speciation (Davis & Shaw, 2001; Jansson & Dynesius, 2002; Zink *et al.*, 2004). However, they were not the exclusive mediators of climate-induced biological turnover. Additional (but much smaller) transitions embedded within the larger Milankovitch periodicities also precipitated abrupt initiations or terminations, and these in turn had a major impact on global biodiversity (Taberlet & Cheddadi, 2002).

Abrupt climatic transitions have been recorded earlier in the Quaternary, but are best documented within the last glacial–interglacial period [i.e. 110 thousand years ago (ka) to present] owing to well-studied Greenland ice cores (Alley, 2000; Mayewski & White, 2002). Recent data have extended our perspectives beyond 800 ka (Loulergue *et al.*, 2008; Lüthi *et al.*, 2008), and with unexpected results. From a contemporary stance, current greenhouse-gas concentrations are demonstrably greater than at any point in hundreds of millennia. From a deep historical perspective, the idea of altered ocean currents as drivers of rapid warming and cooling has been broadened to include earlier glacial periods. Taken as a whole, ice core data have consolidated our knowledge of previous environmental conditions, but have led to the unexpected discovery of just how rapidly global climate can change.

An excellent – and relatively proximate – demonstration of this point is the 12-kyr span at the Pleistocene–Holocene interface (i.e. from 18 to 6 ka). At the peak of the Wisconsin or Würm glaciation (20 ka; Saltzman, 2002), ice sheets in the Northern Hemisphere occasionally exceeded 3 km in depth. They dissipated in two main pulses (i.e. 14.5 and 11.5 ka; Fairbanks, 1989), but the subsequent rerouting of meltwater from the Laurentide Ice Sheet (Broecker *et al.*, 1989) triggered a thousand-year return to near-glacial conditions (the Younger Dryas: Dansgaard *et al.*, 1989; Alley *et al.*, 1993). At *c.* 10 ka (onset of the Holocene interglacial), the last ice sheets disappeared, and by the Mid-Holocene (8–6 ka) a period of tremendous warmth and dryness had developed (the Altithermal) (Frakes *et al.*, 1992). On five separate occasions during this 12-kyr span, 'rapid climate change events' (RCCEs; Mayewski & White, 2002) shifted global temperatures from warm to cold (or vice versa) in less than a decade. At the terminus of the Younger Dryas, temperatures in Greenland rose *c.* 5°C in less than 4 years (Alley, 2000). RCCEs not only exacerbated the tremendous climatic variability of this period, but they also impacted both physical (Reusser *et al.*, 2004) and biological (Douglas *et al.*, 2003, 2006) components of the landscape.

THE IMPACTS OF CLIMATE CHANGE ON BIODIVERSITY

In a series of increasingly more focused papers, Hewitt (1996, 1999, 2000, 2004a) examined the impacts of Pleistocene and Holocene climate shifts on the genetic variability of (primarily) European biota. He provided not only a compendium of case studies across a variety of taxa, but also several testable hypotheses. For example, Hewitt (1996) postulated that sudden and major climate reversals would eliminate species over large parts of their northern and southern ranges – even if reversals were 100 years or less in duration – resulting in populations that were centrally distributed but capable of dispersing once unfavourable conditions ameliorated. Hewitt (1999) also noted that rapid warming would allow refugial populations at the northern extent of their ranges to expand quickly, and that genes from these pioneering individuals would dominate in the new populations. Furthermore, such expansions would occur iteratively as newer terrain opened, but with an accompanying loss of heterozygosity and rare alleles via founder effects. Smaller and interspersed climatic oscillations would exacerbate this effect (Ibrahim *et al.*, 1996) because they would shrink, fracture and temporarily halt the once-advancing edge so as to yield smaller, bottlenecked populations that become vanguards for subsequent expansion.

For most organisms, the result of such a range expansion would be reduced genetic variability across large geographic expanses, whereas slower and more measured extensions involving individuals from different refugia would yield a much larger effective population size and a concomitantly elevated genetic variability. Finally, Hewitt (1999) hypothesized that populations farther south in a distribution would be blocked from advancing by more northern populations, and if the habitats of these southern populations were suitably heterogeneous and embedded within a variable climate, then a number of different haplotypes (or even subspecies) would remain *in situ*. The boundaries between these 'expanded edge' vs. 'blocked interior' genomes should persist and be detectable over time (Hewitt, 1996, 2000).

Given these considerations, it seemed prudent to test the paradigm outlined by Hewitt (1996, 1999, 2000, 2004a,b) through an evaluation of North American taxa with overlapping distributions, particularly given that separate taxa may have been differentially impacted by the same environmental event (Zink *et al.*, 2004). Similarly, it would be useful to employ relatively broad-ranging taxa that currently occupy both previously glaciated and previously unglaciated terrain so that potential range shifts and phylogeographic fragmentation can be deduced. To this end, mtDNA data can be used in combination with geographic information and appropriate analytical tools to assess the timing and extent of clade diversification, and the interruptions that resulted when clades were isolated by past and present geographic barriers (Douglas *et al.*, 2006).

Here, we evaluate two species of the genus *Agkistrodon* with four main questions in mind: (1) How has the distribution of

genetic variation in *A. contortrix* (Linnaeus, 1766) and *A. piscivorus* (Lacépède, 1789) been shaped by the climatic history of North America? (2) Can the timing of origination of major clades be identified and, if so, what are the most likely causative factors? (3) Can detectable (i.e. historic) imprints of refugia and subsequent recolonizations be identified within the distributions of these two species? (4) Is genetic variation in *A. contortrix* and *A. piscivorus* congruent with their current subspecific taxonomies?

MATERIALS AND METHODS

Study organisms

Agkistrodon is composed of four New World species (Gutberlet & Harvey, 2004): cantil, *Agkistrodon bilineatus* (with three subspecies; Fig. 1); cottonmouth, *A. piscivorus* (with three subspecies; Fig. 1); copperhead, *A. contortrix* (with five subspecies; Fig. 1); and Taylor's cantil, *A. taylori*. Van Devender & Conant (1990) suggested that the most recent common ancestor (MRCA) of modern *Agkistrodon* diversified as a result of large-scale vicariance and alterations of climate, with cottonmouths as sister to cantils + copperheads. Knight *et al.* (1992) used mtDNA to recover copperheads as sister to a cantil + cottonmouth clade, a topology first proposed by Brattstrom (1964). More recently, Parkinson *et al.* (2000) applied multiple mtDNA markers to recover the same topology as Knight *et al.* (1992), but with the suggestion that *Agkistrodon* originated in eastern North America and diversified westwards (i.e. trans-continental hypothesis; see their Fig. 1), with the MRCA of *A. bilineatus* eventually dispersing across the Isthmus of Tehuantepec and into the Pacific lowlands of Middle America.

Sampling, amplification and sequencing of mtDNA

Our samples were primarily aliquots of blood taken by syringe from the caudal vein of live specimens and preserved in 100% ethanol. Liver and/or muscle tissues were obtained from recent road kills or voucher specimens, and either frozen or preserved in 100% ethanol. Shed skins or clipped ventral scales also served as a source of DNA. Total genomic DNA was isolated using the PureGene DNA Isolation Kit (D-70KB; Gentra Systems, Inc., Minneapolis, MN, USA) and stored in DNA hydrating solution (same kit).

We amplified mitochondrial (mt) DNA ATPase 8 and ATPase 6 genes (ATP8, ATP6) in our samples using primers and conditions specified in Holycross & Douglas (2006). The specimens of *Agkistrodon* ($n = 158$) analysed were: 100 individuals of *A. contortrix*, 55 of *A. piscivorus*, one of *A. b. bilineatus*, one of *A. b. howardgloydi*, and one of *A. taylori*. Other New World and Old World crotalines (Viperidae: Crotalinae) were sequenced, namely two individuals of *Bothrops moojeni*, one of *Deinagkistrodon acutus*, two of *Gloydium blomhoffii*, one of *Ovophis okinavensis*, one of *Porthidium yucatanicum*, one of *Protobothrops mucrosquamatus*, one of

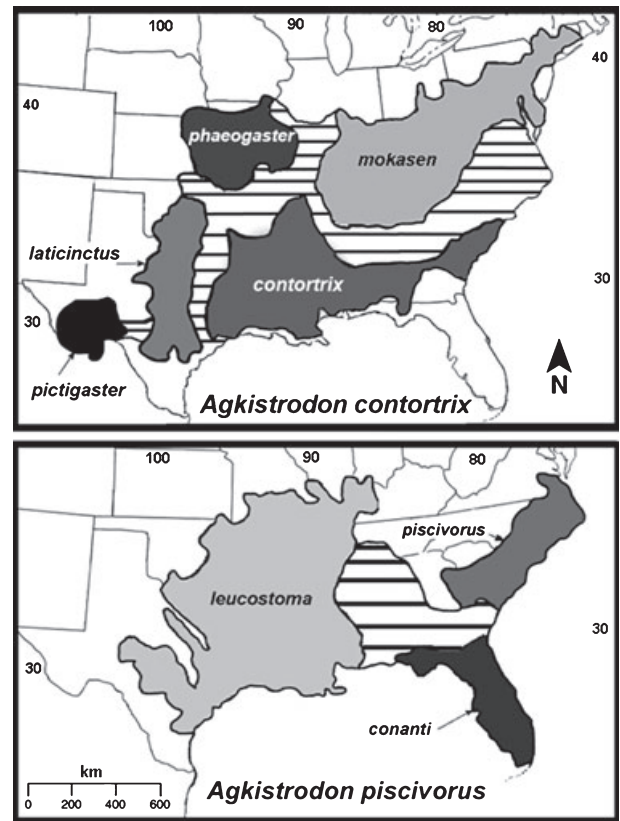


Figure 1 Geographical distributions of *Agkistrodon contortrix* (five subspecies) and *A. piscivorus* (three subspecies) in eastern North America. Striped areas among subspecies represents putative regions of morphological ambiguity (following Campbell & Lamar, 2004).

Trimeresurus gracilis, and five species of rattlesnakes (two individuals of *Sistrurus catenatus*, one of *S. miliarius*, one of *Crotalus horridus*, one of *C. intermedius*, and one of *C. pricei*). A single individual of *Azemiops feae* (Azemiopinae) was used as sister to Crotalinae. Single representatives of Old World vipers (Viperidae: Viperinae) were: *Atheris squamigera*, *Daboia russelii* and *Echis carinatus*. The sister lineage to all advanced snakes (*Acrochordus granulatus*, Acrochordidae; Greene, 1997; Lawson *et al.*, 2005) represented the outgroup. We analysed a total of 178 individuals (158 ingroup, 20 outgroup). Sampling localities for *Agkistrodon* are provided in Appendix S1 (see Supporting Information).

Double-stranded sequencing reactions were conducted with fluorescently labelled dideoxy terminators according to the manufacturer's recommendations [Applied Biosystems Inc. (ABI), Forest City, CA, USA]. Labelled extension products were analysed with an automated DNA sequencer (ABI Prism 3100). Sequences were aligned automatically using Sequencher (Gene Codes, Ann Arbor, MI), and the effectiveness of combining the two gene sequences for analysis was tested with the partition homogeneity test (Farris *et al.*, 1994) implemented in PAUP* (ver. 4.04b10, D.L. Swofford, <http://paup.csit.fsu.edu/>).

Sequence evolution and neutrality

To ensure selective neutrality of our ATP sequences, we applied the Hudson-Kreitman-Aguade (HKA) test (Hudson *et al.*, 1987) based on the fact that both ATP regions are found on the same chromosome. We tested neutrality by using the McDonald-Kreitman (MK) test (McDonald & Kreitman, 1991), as implemented in DnaSP (Rozas *et al.*, 2003). We then determined the model of sequence evolution that best fit each study species by using the program Modeltest (Posada & Crandall, 1998).

Phylogenetic analyses and sequence divergence

We performed a maximum likelihood (ML) analysis using PhyML (ver. 2.4.4: Guindon & Gascuel, 2003; <http://atgc.lirmm.fr/phyml/>), and executed a parsimony analysis using TNT (ver.1: P. Goloboff, S. Farris, K. Nixon: <http://www.cladistics.com/aboutTNT.html>), with minimum length trees derived using the following parameters: Random Sectorial Searches (RSS) = 15/35/3/5; Consensus-based Sectorial Searches (CSS) = same as above; Tree-Drifting (DFT) = 30/4/0/20/0; and Tree-Fusing (TF) = 5 (see Goloboff, 1999).

We generated sequence divergence (p) values, corrected for within-group variance, for each defined clade based on 1,000 bootstrapped sequences and employing MEGA3 (Kumar *et al.*, 2004). To ascertain clock-like behaviour in our sequences, we applied Tajima's (1993) test to compare a study species vs. a recent outgroup, with five random evaluations per clade, each involving three comparisons.

Timing of cladogenetic events

To determine the timing of cladogenesis, we identified five calibration points from the literature to anchor our divergence estimates (following Douglas *et al.*, 2006). Next, we utilized a semi-parametric approach as implemented in r8s (ver. 1.71; Sanderson, 2003) to calculate divergence dates across the ML tree, using penalized likelihood and the truncated Newton algorithm. This approach has been used successfully in a variety of applications (Douglas *et al.*, 2006; Burbrink & Lawson, 2007) and is recognized for its reliability and consistency (Renner, 2005).

Our calibrations represented one fixed, two maximum and three minimum constraints. These anchored specific nodes within the tree, which allowed the software to derive divergences for the remainder of our nodes based upon branch lengths. The node representing the MRCA of *Acrochordus* was fixed at a conserved 70 million years (Ma) (Rage, 1987; Greene, 1997). The node representing the MRCA of pitvipers (= *Azemiops*) was constrained conservatively to a maximum 40 Ma, and that for Crotalines (= *Deinagkistrodon*) to a maximum 34 Ma (Greene, 1992; Szyndlar & Rage, 2002). The MRCA node for North American *Agkistrodon* was constrained maximally at 22 Ma (Early Miocene, Late Arikarean; Holman, 2000; Parmley & Holman, 2007). The

latter date was based upon an unidentified North American viperid fossil, but, given that viperines are absent from the New World, we interpret this fossil to represent a pitviper (Crotalinae). Finally, the node representing the MRCA of *Sistrurus* was constrained minimally at 9 Ma (Parmley & Holman, 2007), and nodes representing MRCAs of *A. contortrix* and *C. horridus* each were constrained minimally at 7 Ma (Late Miocene, Middle Hemphillian; Holman, 2000: p. 297).

To minimize the effects of rate variation across different branches of the tree, we estimated a smoothing parameter using the cross-validation process of the software, and applied a log penalty function to effectively correct for fractional rather than absolute rate changes. Error estimates were derived using the r8s bootstrap kit (Torsten Eriksson: http://www.bergianska.se/index_forskning_soft.html).

Range expansions, demographic histories, regional molecular diversities

To examine broad-scale patterns of regional biodiversity, we pooled samples by species within those geographic regions identified as having a common history. For each such clade, we first calculated the haplotype (h) and nucleotide (π) diversities, with the latter serving as an estimate of Θ in a population of constant size. This estimate, however, is more sensitive to changes in effective population size than one based on segregating sites (Tajima, 1989a). We estimated the manner in which these two differed using Tajima's D -statistic and, if neutrality was sustained, applied Tajima's D to infer demographic history (Tajima, 1989b). For additional clarification, we computed Fu's F_s (Fu, 1997), which is particularly efficient at detecting population expansion. We also contrasted our estimates with values for Fu and Li's F^* and D^* (Fu & Li, 1993), which provide additional information regarding expansion vs. background selection, particularly when calculated in tandem with Fu's F_s . We derived h , π , Tajima's D , Fu's F_s , and Fu and Li's F^* and D^* in DnaSP using the coalescent with 1000 replications.

As a fifth test, we used DnaSP to conduct mismatch distribution analyses (MDA: Rogers & Harpending, 1992), defined as the number of nucleotide differences between all pairs of individuals in a region. We employed the R^2 statistic (Ramos-Onsins & Rozas, 2002) to assess the statistical significance of our MDAs.

Gene flow among regions and biogeographical tests

We utilized an 'isolation-with-migration' model (program IMA; Hey & Nielsen, 2007) to judge potential size differences in clades at their inception, or since their last period of rapid expansion. This program generates samples of genealogies from closely related populations and then builds from these trees a joint-posterior probability density function (PDF) and a description of its concomitant peaks. Each genealogy sampled by the IMA program yields a contribution to the overall

probability of the population mutation rate parameter Θ (where $\Theta = 2Nu$). Sampling a large number of genealogies from a Markov chain Monte Carlo (MCMC) process that has reached stationarity will provide good estimates of the posterior densities [i.e. $p(\Theta|X)$] for any Θ . These can then be maximized to find the highest probabilities and associated parameter values for Θ .

We derived estimates of Θ for each clade or region and converted them to an effective population size by multiplying with an estimate of the mutation rate ascertained on the scale of generations. We accomplished this by calculating the mutation rate for *A. contortrix* from the sequence divergences separating clades AC1 and AP1 from *Bothriechis schlegelii* (following Castoe & Parkinson, 2006, their Fig. 3). These *p*-distances are 19.928 and 18.848, respectively. We divided them by twice the temporal value of their MRCA node ($Y = 24$ Ma), so as to represent both branches extending from that node. The resulting values (i.e. 8.3033×10^{-7} and 7.8533×10^{-7}) represent mutation events locus⁻¹ year⁻¹. We multiplied these values by the generation time for *A. contortrix* and *A. piscivorus* (i.e. 3 years; Fitch, 1960) to yield a mutation rate on the scale of generations.

We constructed three trees to represent biogeographical hypotheses for the evolution of *Agkistrodon* in North America, and tested them against our ML tree using the Shimodaira–Hasegawa (SH) test (Shimodaira & Hasegawa, 1999), as implemented in the program SHTESTS (ver.1, A. Rambaut: <http://evolve.zoo.ox.ac.uk/software.html>). Topologies included the outgroup (*P. yucatanicum*) plus nine lineages distributed across three clades (three within the *A. bilineatus/A. taylori* clade, three within the *A. contortrix* clade, and three within *A. piscivorus*), and our null hypothesis was that all depicted trees were equally good explanations of our data. SH tests simultaneously compared all topologies and adjusted for multiple comparisons by re-optimizing branch lengths and other tree parameters over each of 1,000 bootstrap replicates.

The SH test is an improvement over the Kishino–Hasegawa (KH) test (Kishino & Hasegawa, 1989; implemented in Parkinson *et al.*, 2000), as the latter cannot be validly applied to compare two *a posteriori* trees, or even an optimal *a posteriori* tree with one constrained *a priori* (Goldman *et al.*, 2000). The SH test not only compares multiple trees but also tolerates reasonable violations of *a priori* tree selection by utilizing a one-tailed test and by employing an additional methodological step that relates the contribution of the ML tree to the null distribution (Planet, 2006).

Two factors, however, that can compound the SH test are an inappropriate model of sequence evolution and a large number of constraint trees being tested (Buckley, 2002). To address these issues, we applied Modeltest to re-evaluate sequence evolution within our abbreviated (10-lineage) ML tree, and we limited the number of constraint trees while simultaneously ensuring that every reasonable evolutionary topology was available and not *a priori* selected. This was achieved by alternating the direction of transcontinental evolution within *Agkistrodon* from east to west (and vice versa), and by rotating

each of the three *Agkistrodon* clades as sister to the remaining two, with a specific regional lineage designated as sister within each clade so as to correspond to the resulting geographic trajectory of transcontinental evolution in the hypothesized MRCA.

As an additional measure of confidence in branch support in our ML tree, we generated 1,000 bootstrap pseudoreplicates using the program GARLI (Genetic Algorithm for Rapid Likelihood Inference, ver. 0.951; D.J. Zwickl, <http://www.nes-cent.org/informatics/software.php>), and used these to derive a majority rule consensus tree in PAUP*.

RESULTS

MtDNA sequencing and sequence evolution/ neutrality

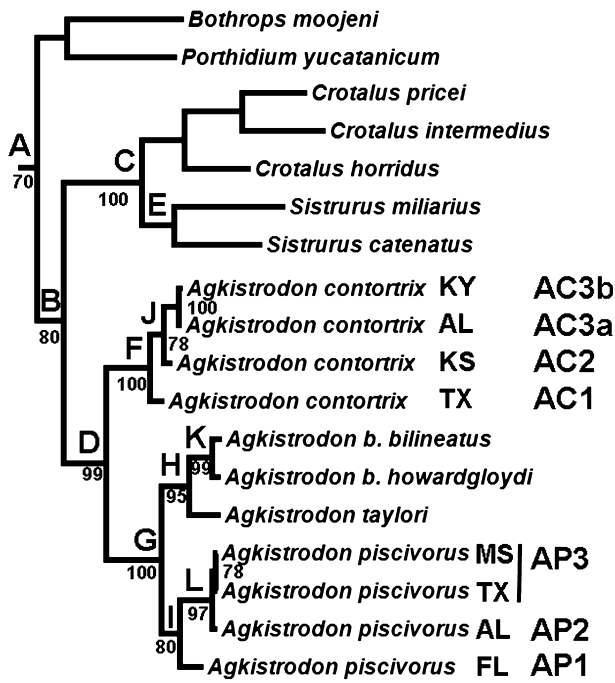
Polymerase chain reaction (PCR) amplifications and automated sequencing of ATP8 and ATP6 resulted in 849 base pairs (bp) of unambiguously readable sequence with no indels. Combining these two gene sequences was supported by a non-significant partition homogeneity test (PAUP*: $P > 0.32$). We found all sequences to be evolving neutrally (HKA: $0.25 < P < 0.90$; MK: $0.35 < P < 0.78$) and in a rate-uniform manner (non-significant Tajima's test). The best-fitting model of sequence evolution for all clades and outgroups was 'general time-reversible' (GTR).

Phylogenetic analyses and sequence divergences

Using ML and all species, an optimized tree was obtained that served as input for a second ML run that yielded a tree with log likelihood = -8594.441 and a gamma shape parameter = 0.997, with the proportion of invariant sites = 0.306 and nucleotide frequencies of (A) = 0.324; (C) = 0.324; (G) = 0.098; (T) = 0.254. The ML tree was used to derive divergence times. Monophyletic groups in the latter coincided with those delimited from four MP trees, each with length (L) = 1852, compiled into a single majority-rule consensus tree.

Our ML tree, rooted at *Acrochordus*, depicts the Viperinae as sister to *Azemiops feae* (Azemiopinae) + Crotalinae (pitvipers). Short branches within the Crotalinae (not shown) support arguments that the pitviper clade evolved quite recently (Kraus *et al.*, 1996). A *Porthidium* + *Bothrops* clade was sister to the remainder of the pitvipers, with copperheads (*A. contortrix*) forming a monophyletic group composed of four regional subclades (Fig. 2): a western clade (AC1) is sister to all remaining clades [i.e. a midwestern-southern clade (AC2), an eastern-southeastern clade (AC3b), and a smaller, more regional, clade (AC3a)].

Cantils (*A. bilineatus*, *A. taylori*) + cottonmouths (*A. piscivorus*) also formed a monophyletic group (Fig. 2), with cantils sister to a subdivided *A. piscivorus*. The *A. piscivorus* clade was composed of three geographically defined subgroups: AP1 (Florida clade) was sister to AP2 (south-eastern clade) and AP3



ML Bootstrap

Figure 2 Maximum likelihood tree depicting relationships among clades sister to crotaline genera *Porthidium* and *Bothrops*. *Sistrurus* and *Crotalus* are rattlesnake genera. The abbreviations following specific names for *Agkistrodon contortrix* and *A. piscivorus* denote state names: AL, Alabama; FL, Florida; KS, Kansas; KY, Kentucky; MS, Mississippi; TX, Texas. Molecular clades (as depicted in Fig. 3) are represented for *A. contortrix* as: Western, AC1; Midwest-South, AC2; East-Southeast, AC3a, AC3b. For *A. piscivorus* they are depicted as: Florida, AP1; Southeastern, AP2; Midwestern-Western, AP3. Letters situated above nodes are dated in Table 3, and numbers below (or to the right of) nodes represent bootstrap support values based on 100 pseudo-replications.

Table 1 Pairwise net sequence divergence (%) derived between clades of *Agkistrodon* (below the diagonal) from 849 base pairs of mtDNA ATPase 8 and ATPase 6 genes and corrected for within-clade variability.

	AP3	AP2	AP1	AC3b	AC3a	AC2	AC1
AP3	X	0.3	0.7	1.1	1.1	1.1	1.1
AP2	0.6	X	0.7	1.1	1.1	1.1	1.1
AP1	4.7	4.8	X	1.1	1.1	1.1	1.1
AC3b	12.7	12.8	12	X	0.2	0.5	0.7
AC3a	12.4	12.5	12.1	0.5	X	0.5	0.6
AC2	12.6	12.7	11.8	2.4	2.2	X	0.6
AC1	11.7	11.8	11.6	4	3.6	3.1	X

Standard errors derived by bootstrap (1000 replicates) are represented above the diagonal.

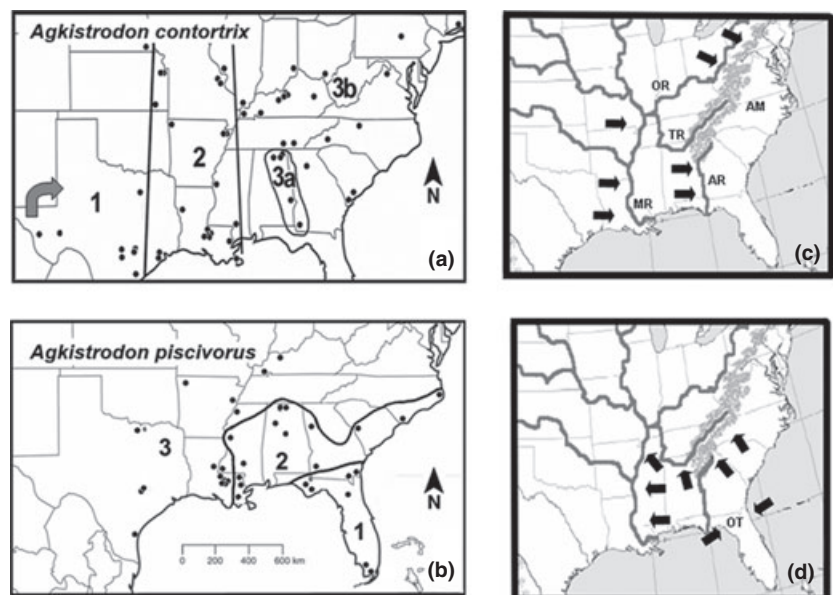
(midwestern-western clade). Clades recovered for *A. contortrix* and *A. piscivorus* are delineated cartographically in Fig. 3, along with the geographic barriers that potentially disrupted their hypothesized expansions.

Sequence divergences (SD) within *A. contortrix* and *A. piscivorus* are pronounced (Table 1). For the former, clade AC1 (western clade) differed from all other clades at 3.1–4% SD. The midwestern-southern clade (AC2) differed from AC3b and AC3a by 2.4 and 2.2% SD, respectively, whereas AC3a differed from AC3b by 0.5% SD. Within the *A. piscivorus* clade, AP1 (Florida clade) differed by 4.7 and 4.8% SD from the other two clades, whereas AP2 (south-eastern clade) and AP3 (western clade) differed by 0.6% SD.

Demographic histories, range expansions and timing of cladogenesis

Tajima’s *D*-statistic was negative in six (of seven) regions (86%), and significantly so in five (71%), indicating popula-

Figure 3 Geographic extent of regional clades in our two study species as determined using 849 base pairs of mtDNA ATPase 8 and ATPase 6 sequences, and geographic discontinuities influencing their diversification. Individual sampling locations are represented on the map by circles: (a) *Agkistrodon contortrix* (AC1, AC2, AC3a, AC3b); (b) *A. piscivorus* (AP1, AP2, AP3); (c) *A. contortrix*; and (d) *A. piscivorus*. AM, Appalachian Mountains; AR, Apalachicola River; MR, Mississippi River; OR, Ohio River; OT, Okefenokee Trough; TR, Tennessee River.



tion growth (Table 2). The only region with a positive Tajima's *D* was AP1. Four (of five) clades with a significantly negative Tajima's *D*-statistic also reflected significantly negative Fu's *F_s* values, whereas the fifth (although negative) was non-significant owing to its restricted geographic distribution. Of the two regional clades with non-significant but negative Tajima's *D*-statistics, one (i.e. AC1) also projected a significantly negative Fu's *F_s* value. Three (of five) regional clades with significantly negative Tajima's *D* and Fu's *F_s* values also demonstrated non-significant Fu's *F** and Fu and Li's *D** (Table 2).

MDA, another approach to assessing historical changes in population size, projected relatively smooth Poisson mismatch curves for all four regional clades in *A. contortrix* (Fig. 4), and for two (of three) in *A. piscivorus* (Fig. 5). Five of these seven regional clades (71%) are significantly unimodal, a characteristic of rapid demographic expansion (Table 2, *R*² statistic: Ramos-Onsins & Rozas, 2002). Of the remaining two clades, one (AC3a) reflected a relatively smooth curve that lacked statistical significance because of low sample size and restricted geographic distribution. The second clade (AP1; Fig. 5) displayed a significant multimodal signature of a population at constant size.

To generate dates for the inception of our recovered clades, we employed five iterations of the Truncated Newton (TN) method of r8s to establish an optimal smoothing parameter of 4.18 (chi-square = 1520.18). Utilizing this and the gamma value in concert with method TN yielded the divergence times given in Table 3. We also present confidence limits around these divergence times, as well as the status of the nodes labelled in Fig. 2. Hypothesized dates for other cladogenic events are provided in Table 3.

Gene flow among regions and biogeographical tests

Fig. 6 shows pairwise comparisons of Θ vs. the posterior probability densities of Θ [i.e. $p(\Theta|X)$, depicted as *P*] for each clade of *A. contortrix* and *A. piscivorus*. Solving for *N_e* derives the effective population sizes for these clades. In a relative sense, this computation differs among, but not within, species.

Thus, the western clade of *A. contortrix* (AC1) has a posterior probability of Θ that is 2.6 times smaller than that of AC2 (the midwestern clade), whereas clades AC2 and AC3b have relatively similar values, suggesting comparable demographic scenarios. Likewise, all three clades of *A. piscivorus* have similar probability densities, again suggesting parallel demographic responses across clades.

Fig. 7 depicts our abbreviated 10-lineage ML tree, as derived under the HKY model of sequence evolution and with non-parametric bootstrap support values for branches. We also present three alternative biogeographical hypotheses representing different evolutionary scenarios for *Agkistrodon* in North America.

Our first hypothesis (H1: AC-ENA) denotes *A. contortrix* as the sister group to cantils + cottonmouths, with the MRCA of *Agkistrodon* originating in eastern North America and diversifying south- and westwards. Here, the easternmost clade of *A. contortrix* (namely AC3b) is sister to all remaining copperhead clades. Furthermore, the eastern clade of *A. piscivorus* (AP2) is sister to all the remaining cottonmouth clades, and *A. b. bilineatus* is sister to all remaining cantil clades (see Werman, 2005).

Our second hypothesis (H2: AP-ENA) places *A. piscivorus* as the sister group to cantils + copperheads, with the MRCA of *Agkistrodon* originating in eastern North America and diversifying south- and westwards. Here, as in H1, the eastern clade of *A. piscivorus* (AP2) is sister to all remaining cottonmouth clades, and *A. b. bilineatus* is sister to all remaining cantil clades.

The third hypothesis (H3: AB-WNA) denotes the cantils as the sister group to copperheads + cottonmouths, with the MRCA of *Agkistrodon* originating in western North America (presumably Mexico) and diversifying north- and eastwards. Here, the westernmost clade of *A. piscivorus* (AP3) is sister to all remaining cottonmouth clades, and the westernmost clade of *A. contortrix* (AC1) is sister to all remaining copperhead clades.

The SH test rejected our null hypothesis that all trees in Fig. 7 (H1–H3) were statistically valid explanations of our data, thus arguing against the three trans-continental bioge-

Table 2 Molecular diversity in regional clades of *Agkistrodon piscivorus* and *A. contortrix* derived from 849 base pairs of mtDNA ATPase 8 and ATPase 6. Regions are depicted in Fig. 3.

Region	<i>n</i>	<i>H</i>	<i>h</i>	Pi	T-D	Fu-Fs	F-L-D	F-L-F	<i>R</i> ²
AP3	27	8	0.56 (0.111)	0.0009 (0.0003)	-1.71 (<i>P</i> < 0.019)	-5.333 (<i>P</i> < 0.00000)	-1.43	-1.77	0.062 (<i>P</i> < 0.00000)
AP2	18	8	0.745 (0.102)	0.0012 (0.0012)	-1.79 (<i>P</i> < 0.011)	-5.554 (<i>P</i> < 0.001)	-2.33 (<i>P</i> < 0.006)	-2.52 (<i>P</i> < 0.01)	0.071 (<i>P</i> < 0.00000)
AP1	9	4	0.69 (0.021)	0.004 (0.00001)	0.03	1.546	0.67	0.58	0.175 (<i>P</i> > 0.44)
AC3b	44	11	0.71 (0.09)	0.002 (0.0003)	-1.72 (<i>P</i> < 0.02)	-4.12 (<i>P</i> < 0.006)	-1.92	-2.17	0.066 (<i>P</i> < 0.003)
AC3a	5	3	0.111 (0.0003)	0.0004 (0.0003)	-1.71 (<i>P</i> < 0.01)	-0.829	-2.30	-2.46	0.245 (<i>P</i> > 0.18)
AC2	33	13	0.797 (0.057)	0.001 (0.003)	-1.85 (<i>P</i> < 0.01)	-10.13 (<i>P</i> < 0.00000)	-2.87 (<i>P</i> < 0.008)	-2.99 (<i>P</i> < 0.006)	0.047 (<i>P</i> < 0.0000)
AC1	22	6	0.537 (0.123)	0.001 (0.0002)	-1.152	-2.649 (<i>P</i> < 0.015)	-0.461	-0.759	0.087 (<i>P</i> < 0.045)

n, sample size; *H*, number of haplotypes; *h*, haplotype diversity (standard deviation in parentheses); Pi, nucleotide diversity (standard deviation in parentheses); T-D, Tajima's *D*; Fu-Fs, Fu's *F_s*; F-L-D, Fu & Li's *D*; F-L-F, Fu & Li's *F*; *R*², Ramos-Onsins and Rozas's *R*² (significant values in bold).

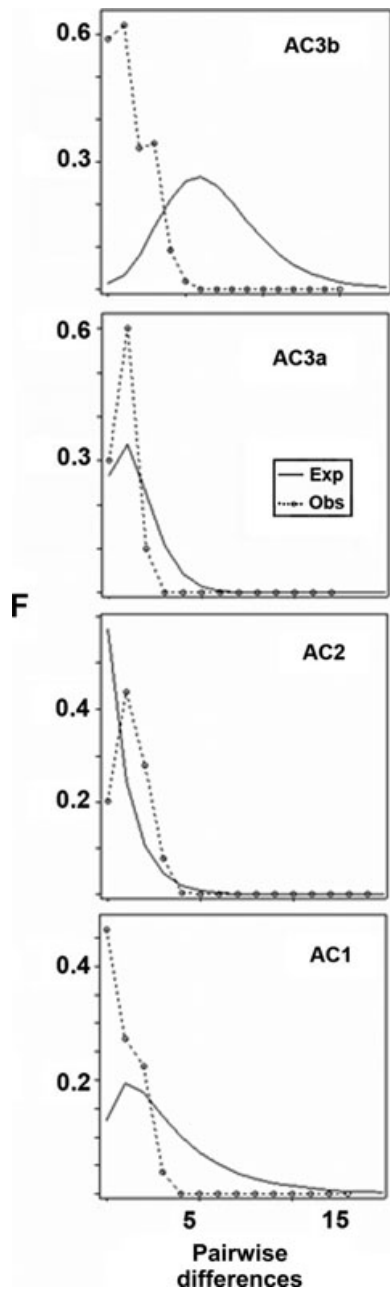


Figure 4 Plots depicting mismatch distribution analyses for regional clades (AC1, AC2, AC3a, AC3b) in *Agkistrodon contortrix*, as determined from 849 base pairs of mtDNA ATPase 8 and ATPase 6 sequences. F, frequency; Pairwise differences, number of nucleotide differences between all pairs of individuals in the clade.

graphical hypotheses they represent. Instead, our results showed that copperheads are the sister group to cantils + cottonmouths, and the direction of evolution for copperheads is from west to east. However, in the case of *A. piscivorus*, our basal-most (and presumably oldest) population occurred in northern Florida, thus rendering problematic the mechanism by which the MRCA of *A. piscivorus* accessed the Florida peninsula. None of the hypotheses presented by earlier researchers (Van Devender & Conant, 1990; Parkinson *et al.*,

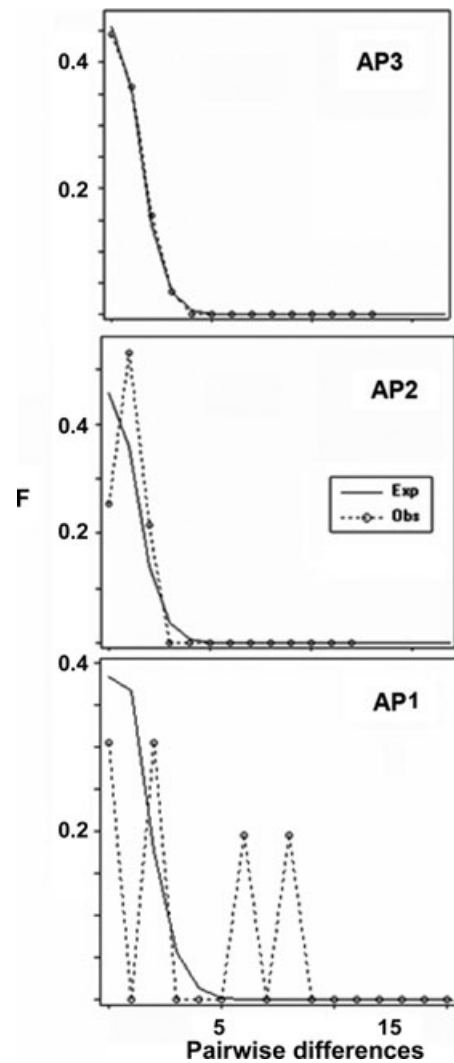


Figure 5 Plots depicting mismatch distribution analyses for regional clades (AP1, AP2, AP3) of *Agkistrodon piscivorus*, as determined using 849 base pairs of mtDNA ATPase 8 and ATPase 6 sequence data. F, frequency; Pairwise differences, number of nucleotide differences between all pairs of individuals in the clade.

2000) either explains this result or fully agrees with our phylogeographic results. Our hypothesis (i.e. trans-gulf rafting) does both, and is discussed below.

DISCUSSION

Historically, researchers have argued that Pleistocene glaciations in eastern North America compressed biotic distributions to southern continental extremes, with post-glacial trajectories occurring northwards along routes made relatively predictable by topographic features (Hewitt, 1996; reviewed by Soltis *et al.*, 2006). Many of these topographic features are riverine systems with headwaters in mountains trending northeast to southwest, and which run directly south into the Gulf (i.e. Mississippi, Tombigbee, Apalachicola, Suwanee

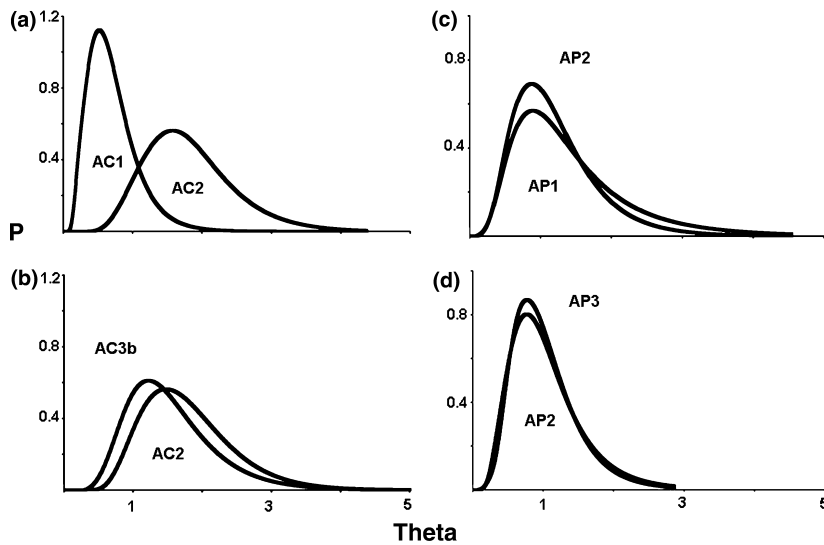


Figure 6 Pairwise comparisons of Theta (where $\Theta = 2N_e u$) vs. P [the posterior probability densities of Θ , i.e. $p(\Theta|X)$] for each geographic clade of *Agkistrodon contortrix* (AC1, AC2, AC3a, AC3b) and *A. piscivorus* (AP1, AP2, AP3), as determined from 849 base pairs of mtDNA ATPase 8 and ATPase 6 sequence data (see text for formulation).

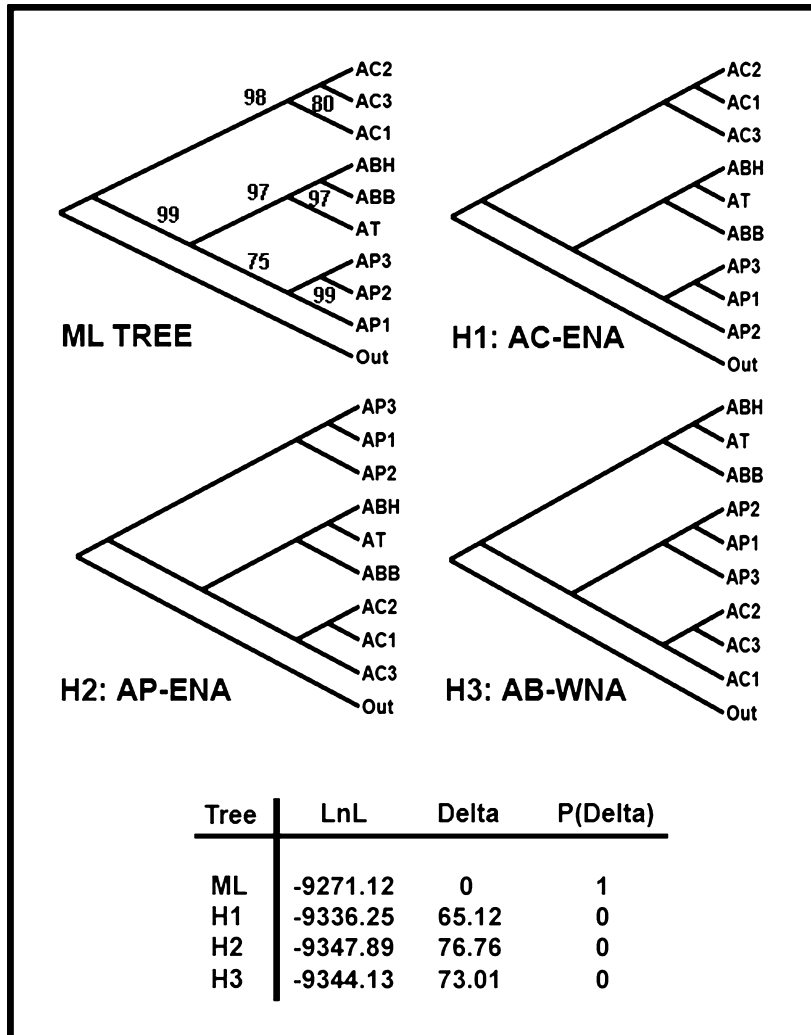


Figure 7 Constraint trees representing alternative biogeographical hypotheses for the evolution of *Agkistrodon* in North America. ML tree – maximum likelihood tree based upon 849 base pairs of mtDNA ATPase 8 and ATPase 6 sequence data demonstrating relationships among geographic clades in *Agkistrodon contortrix* (AC1, AC2, AC3), *Agkistrodon piscivorus* (AP1, AP2, AP3), and *A. taylori/A. bilineatus* (AT, *A. taylori*; AB, *A. b. bilineatus*; ABH, *A. b. howardgloydii*). OUT, outgroup *Porthidium yucatanicum*. Bootstrap support of nodes is depicted. H1: AC-ENA – transcontinental evolution of *Agkistrodon* from eastern North America westwards to Mexico with *A. contortrix* as sister to all other clades; H2: AP-ENA – transcontinental evolution of *Agkistrodon* from eastern North America westwards to Mexico with *A. piscivorus* as sister to all other clades; and H3: AB-WNA – transcontinental evolution of *Agkistrodon* from Mexico north- and eastwards to eastern North America, with *A. bilineatus* as sister to all other clades. The table depicts results of the Shinodaira–Hasegawa (SH) test of the null hypothesis that the constraint trees are statistically valid representations of the data. LnL, log-likelihood; Delta, difference in LnL between ML and constraint tree; P(Delta), probability of Delta.

rivers) or obliquely southeast to the Atlantic Ocean. These fluvial barriers were dramatically accentuated by eustatic changes in sea level during the Quaternary. However, one

salient aspect of our research is that, although both *A. contortrix* and *A. piscivorus* display post-glacial dispersal, their phylogeographic patterns are demonstrably inconsistent,

with separations along north-trending topographic features. Rather, vicariant breaks in *Agkistrodon* often pre-date the Quaternary, and when they occur within the Pleistocene, they fall outside the European paradigm in which phylogeographies reflect vicariant breaks (Hewitt, 2000). This possibly results from a lack of east–west-trending mountain ranges in eastern North America that would effectively block the northward rebound of taxa initially pushed southwards by glacial conditions. Below, we discuss the phylogeographic signatures of regional clades depicted in our study species, and relate their timelines to potential causative factors as deduced from our knowledge of North American climatic and geological history.

Vicariant evolution of *Agkistrodon contortrix*

Our analyses support the hypothesis (Van Devender & Conant, 1990) that the MRCA of *A. contortrix* evolved in Mexico and dispersed north- and eastwards into the United States along a Late Miocene [8.24 Ma (7.33–9.14)] mesic corridor (as per Martin & Harrell, 1957). Subsequent vicariant events, however, sundered it on at least two occasions.

Vicariant event 1

The Pliocene in the western United States was characterized by the onset of glacial cycles, increasing levels of aridity and seasonality, as well as by rapidly developing prairie-like grasslands east of the Rocky Mountains. These events not only isolated and reduced the resident Tertiary floras, which allowed for the rapid development of modern plant communities, but they also catalyzed species differentiation in a variety of vertebrates (Axelrod, 1948).

AC1 (sister clade to all other members of *A. contortrix*) separated from the more eastern clades AC2/AC3b at 5.1 Ma along a major east–west moisture gradient long recognized for a variety of organisms (albeit with slight variations and under different labels; Allen, 1892; Dice, 1943; Savage, 1960; Gleason & Cronquist, 1964; Hagmeier, 1966; Weaver & Clements, 1966). This continental-level separation represents the leading edge of a widespread western grassland/sub-desert environment in the Early Pliocene, and is concomitantly associated with expanding aridity. It is recovered in every one of our biogeographical interpretations, and provides an explanation for the 3.1% SD separating AC1 from AC2.

Vicariant event 2

The biogeographical separation of clade AC2 from AC3b occurred c. 1.4 (1.79–1.01) Ma along the Mississippi River, a faunal break well recognized in the biological literature (Soltis *et al.*, 2006). This river received > 50% of all North American glacial meltwater drainage and, as a result, provided 1.5+ Myr of sediment transport to the northern

Gulf of Mexico (Pulham, 1993). The size of this river, and thus its vicariant impact upon the evolution of clades AC2 and AC3b, can be gauged from an analysis of Quaternary fluvial sediments.

During the Plio-Pleistocene transition, RCCEs alternately reconfigured this river channel from a braided form indicating colder periods into much broader meltwater meanders characteristic of deglaciation (Rittenour *et al.*, 2007). The latter provoke our interest because they deposited distinct sedimentation signatures within the channel and/or the northern Gulf of Mexico (Winn *et al.*, 1998). Often, these meanders contain clay-rich soils from mid-continental terraces rather than glacial till from the Laurentide Ice Sheet (Brown & Kennett, 1998), which underscores the erosive power exhibited by these flows. Considerable variation in meltwater discharge was provoked by the onset, duration, and cessation of RCCEs, with resulting flows in the Late Pleistocene fluctuating by a factor > 5 (Teller, 1990).

Several lines of independent information establish 1.5–1.4 Ma as a period when the discharge of the Mississippi River was enormous. For example, sedimentation onto the Gulf's Mississippi Fan increased dramatically during this period (Pulham, 1993; Villamil *et al.*, 1998), consistent with the formation of the modern Ohio River as a major meltwater tributary to the Mississippi. Similarly, subterranean Appalachian cave deposits (Anthony & Granger, 2007) pinpoint the glacial reorganization of the Ohio River Basin as occurring c. 1.5 Ma. Finally, the community composition of benthic foraminifera in the Gulf firmly established glacial RCCEs as occurring from 1.6 to 1.2 Ma (Schnitker, 1993). These disparate data support our contention that the Lower Mississippi River was a broad and formidable vicariant barrier during the approximate time at which the MRCAs of AC2 and AC3b separated.

Vicariant evolution of *Agkistrodon piscivorus*

Given the phylogeny we recovered for *Agkistrodon*, and specifically for *A. piscivorus* (Fig. 2), we were unable to confirm a terrestrial (i.e. trans-continental) dispersal route based on a western (Mexican) origin of the MRCA. Accordingly, we hypothesize that the MRCA of *A. piscivorus* dispersed from eastern mainland Mexico (possibly the region of Yucatán) into southeastern North America (northern Florida) via trans-Gulf rafting (see Rosen, 1978). Although direct observations of rafting vertebrates are scant (Thiel & Gutow, 2005), available evidence (e.g. Raxworthy *et al.*, 2002; Calsbeek & Smith, 2003; Heinicke *et al.*, 2007; Measey *et al.*, 2007; Waters, 2008) validates long-distance dispersal (LDD) as an important factor in the evolution of many terrestrial species. The quantification, prediction and interpretation of LDD now constitutes a recognized research programme (Nathan, 2005), with episodic dispersals depicted as random, independent events promoting allopatric speciation.

Table 3 Divergence dates and confidence intervals for nodes identified with letters in Fig. 2.

Node	Mean age (CI values)	Configuration
<i>Acrochordus granulosus</i>	70 (0.0–0.0)	Fix (70)
<i>Azemiops feae</i>	15.78 (12.68–18.88)	Max (40)
<i>Gloydus blomhoffii</i>	12.34 (10.41–14.27)	Max (34)
A	12.0 (10.38–13.63)	E
B	11.69 (10.04–13.34)	Min (22)
C	8.82 (7.3–10.35)	E
D	8.24 (7.33–9.14)	Min (7)
E	2.69 (0.09–4.27)	Min (9)
F	5.1 (5.0–5.2)	Min (5)
G	3.64 (2.97–4.3)	E
H	1.83 (1.43–2.23)	E
I	1.88 (1.46–2.31)	E
J	1.4 (1.01–1.79)	E
K	0.51 (0.36–0.67)	E
L	0.20 (0.11–0.29)	E

Mean age is the time in millions of years since the most recent common ancestor. Configuration of the node is specified: Fix, fixed; Max, maximum; Min, minimum; E, estimated (with numbers in parentheses representing Ma); CI, confidence interval.

Florida and the Gulf of Mexico during the Miocene–Pliocene

From the Mid–Late Miocene to the Early Pliocene, northern Florida appeared first as a small peninsula, then as a peninsula/archipelago complex, and then developed into a narrowly reduced and compressed peninsula. Based on our phylogenetic and biogeographical analyses, we propose that the MRCA of *A. piscivorus* arrived onto this reduced landmass in the Early–Mid Pliocene (4.13–2.97 Ma; Table 3). Specifically, support for this scenario is our evidence that the northern populations of *A. p. conanti* are sister to all other populations in Florida, and basal to *A. piscivorus*. This suggests that the MRCA of *A. piscivorus* initially colonized the northern region of Florida, and subsequently dispersed southwards as the peninsula increased in size. A large interior continental ice mass and the cooler temperatures of a reduced Gulf of Mexico produced raft-friendly winds that were possible drivers of trans-Gulf rafting. These glacial characteristics were present in the Mid–Late Pliocene (3.6–2.4 Ma) (Barendregt & Duk-Rodkin, 2004; their Fig. 1; Mudelsee & Raymo, 2005) and may also have facilitated the onshore winds important for rafting. For example, full glacial conditions during the Pleistocene promoted strong onshore winds, which deposited shore-parallel sand dunes along the southern coasts of Alabama and the panhandle of Florida (Otvos, 2004).

Evolution of Agkistrodon piscivorus in Florida

During the Miocene, the development of a swift-flowing and unidirectional deepwater channel (Okfenokee Trough) connecting the northwestern Gulf of Mexico with the Atlantic Ocean effectively separated Florida from mainland North

America (Riggs, 1979, 1984: their Fig. 1). This broad and relatively sediment-free channel closed at the end of the Miocene when sea levels dropped precipitously, only to re-initiate from the Mid to Late Pliocene as a prolonged marine transgression (Olsen, 1968; reviewed by Bert, 1986). It proved to be a significant vicariant barrier for a variety of marine, freshwater and terrestrial taxa (algae: Zuccarello *et al.*, 2006; amphibians: Liu *et al.*, 2006; crustaceans: Bert, 1986; Felder & Staton, 1994; mussels: Lee & Ó Foighil, 2004; reptiles: Auffenberg & Milstead, 1965; Walker & Avise, 1998; Roman *et al.*, 1999; Weisrock & Janzen, 2000; woodrats: Hayes & Harrison, 1992). These conditions would have similarly sundered *A. piscivorus*, with one clade constrained within the Florida peninsula/archipelago, and a second north of the Okfenokee Trough.

Interestingly, Remington (1968) recognized this odd juxtaposition of resident Floridian and Gulf forms in northwestern Florida and identified this region as one of five major terrestrial suture zones in North America. Remington designated north Florida as distinctive simply because he judged that two biotic regions coalesced there. Actually, the extensive development of the Okfenokee Trough as a vicariant divisor through the Pliocene was the cause (Bert, 1986). We hypothesize that *A. p. conanti* evolved in relative isolation within peninsular Florida for *c.* 1 Myr and that this temporal duration allowed it to assume an evolutionary trajectory independent from those of all other populations of *A. piscivorus* in southeastern North America.

Glaciations, re-colonizations and *Agkistrodon* in North America

Agkistrodon contortrix

Two of four regional copperhead clades (i.e. AC1 and AC3b) showed dramatic and statistically significant evidence of range expansion when tested across a series of molecular genetic statistics. A third (AC2) offered mixed signals (i.e. three affirmative tests out of five approximating expansion), and a fourth (AC3a) provided only a single positive result for range expansion, probably owing to its localized and reduced nature. Clade AC1 seemingly evolved as a small population with reduced variability [as suggested by $p(\Theta|X)$], and then dispersed as a leading edge (Hewitt, 1999) north- and eastwards from Mexico into North America during the Late Miocene. Clades AC2 and AC3b are relatively similar with regard to $p(\Theta|X)$ values, which suggests a congruent demographic scenario. Both show significant expansionist properties, with those of AC3b somewhat more prominent. Furthermore, their expansions were rapid, as evidenced by the reduced genetic variability found across their broad geographic distributions. If a slower and more evenly distributed expansion had occurred, perhaps even integrating across distinct refugia, then a much larger effective population size would be evident, as would an elevated level of genetic variability. This clearly is not the case, and our analyses instead

corroborate Hewitt's (1999) hypotheses outlined above. Furthermore, *A. contortrix* had established a west-to-east expansion prior to the onset of the Pleistocene, and subsequent glaciations merely pushed these already-differentiated clades farther south. They rebounded northwards on a broad basis during interglacials, with one refugial clade still evident.

Agkistrodon piscivorus

During the last glaciation, south-eastern Georgia was $4.0 \pm 0.6^\circ\text{C}$ cooler than it is today, with similar temperatures occurring in southern Texas and northern New Mexico, suggesting that the southern United States cooled uniformly during this period (Clark *et al.*, 1997). These environmental conditions apparently impacted *A. piscivorus* more than *A. contortrix*. When judged in pairwise comparisons, the three geographic clades of *A. piscivorus* have similar probability densities of Θ , again suggesting a reasonably similar demographic response across clades. Interestingly, AP1 shows statistical characteristics of a clade at constant size, whereas both AP2 and AP3 are clades in expansion. We argue that AP1 reflects stasis because it was geographically limited with regard to its capability for expansion, being blocked by the establishment of AP2 in southeastern North America (congruent with the hypothesis of Hewitt, 1999). However, range expansions for both AP2 and AP3 were relatively unrestricted, occurring predominantly in a northeastern/northcentral direction.

Quaternary refugia in eastern North America

Eastern North America harbours geographic features that are now recognized as barriers to vertebrate gene flow (Avice, 1996, 2000; synopsis by Soltis *et al.*, 2006). One of these is the Apalachicola River (AR; Fig. 4c). During the interglacials of the Plio-Pleistocene, this river formed a large, intrusive salt-water channel extending from the Gulf Coast north- and eastwards into Georgia and Alabama, apparently to the north Georgia fall line (i.e. the Mesozoic shoreline of the Atlantic Ocean, depicted as an *c.* 30-km geological boundary separating sedimentary rocks of the Upper Coastal Plain from crystalline rocks of the Piedmont) (Blainey, 1971; Pauly *et al.*, 2007; and references therein). This river probably served as a demarcation for eastern and central black rat snake clades (Burbrink *et al.*, 2000). In this study, however, the Apalachicola circumscribed a small cluster of unique haplotypes (i.e. AC3a) embedded within a larger and geographically more expansive AC3b clade. Thus, rather than serving as a vicariant break, the bluffs of the Apalachicola River harboured a relictual clade of copperheads. These bluffs demonstrate their historical legacy as a glacial refugium by bearing additional relictual taxa: a differentiated clade of eastern North American deciduous tulip poplar (*Liriodendron tulipifera*; Parks *et al.*, 1994; Soltis *et al.*, 2006), and an understorey shrub, the Florida yew (*Taxus floridana*; Kwit *et al.*, 2004).

Following Hewitt's (1999) logic, AC3a represents a clade prevented from advancing by populations of AC3b further

north. Again (as per Hewitt, 1996, 2000), the boundaries separating an 'expanded edge' clade (AC3b) from a 'blocked interior' clade (AC3a) persist and are genetically detectable today.

A non-representative taxonomy for North American *Agkistrodon*

Although the two species of *Agkistrodon* are distinct (11.7–12.8% SD), only one of eight subspecies (12.5%) is supported at the molecular level. AP1 (*A. p. conanti*) separates from conspecifics at 4.7–4.8% SD and could represent another example of a biological species buried within the thicket of infraspecific taxonomy (Douglas *et al.*, 2007). AC1, a composite of the two westernmost *A. contortrix* subspecies (*A. c. laticinctus*, *A. c. pictigaster*), could represent a similar situation.

These data point to a larger issue. Much subspecific (and often species-level) taxonomy developed during an earlier generation of biological research does not seem to stand up well to modern scrutiny. Here, our objective is not to criticize but to focus attention on the premise that all taxonomies, by their very nature, are hypothetical and thus transitory (Gould, 1982; Sites & Marshall, 2003). An inability to fully grasp this concept is particularly damaging when superimposed onto the realities of conservation biology. A taxon-centric biodiversity perspective agrees with static restoration targets (e.g. 'save the panda'), but it becomes increasingly difficult to reconcile when pitted against the dualities of escalating conservation costs and diminishing inputs (< US \$2 billion/annum worldwide by non-governmental organizations, Halpern *et al.*, 2006). A more appropriate path may be to prioritize regions for conservation by using a deep historical approach (Douglas *et al.*, 2006; Willis & Birks, 2006), with the employment of molecular characters to re-frame the hierarchy of biodiversity and to identify its causation (Douglas *et al.*, 2002). In this sense, our phylogeographies contribute to a developing baseline of taxa within eastern North America (Soltis *et al.*, 2006) that may promote such a regional conservation and management perspective.

Global climate change: past and future

The analysis of pollen from lake/wetland sediments indicates that many Pleistocene plant communities were composed of species that, although extant today, no longer co-mingle as a biological community. These disparate associations, dubbed 'no-analogue' communities owing to their lack of antecedents (Jackson & Williams, 2004), developed in response to climatic conditions specific for a particular time and place. Their subsequent disintegration during the Holocene was concomitant with the loss of unique and specialized habitats. The inability of these species to demonstrate either previous or present communal relationships was a serious and unsettling issue for an earlier generation of palaeobiogeographers.

Williams *et al.* (2007) extended earlier observations to predict that no-analogue communities will result from ongoing global climate change. In fact, numerous no-analogue communities

might evolve, and iteratively, as the Earth progresses along a series of increasingly more abrupt RCCEs. The prediction of likely range shifts will be extremely important within this new adaptive landscape, as will the determination of evolutionary 'hotspots' using molecular characteristics. An understanding of connectivity among populations within these heterogeneous landscapes will require data on both historical and on-going gene flow (Holycross & Douglas, 2006).

This approach fits well with contemporary perspectives that depict global hotspots as the transitory effects of ecological processes acting over millennia (Renema *et al.*, 2008). Today, however, environmental change operates on a scale immensely more compressed than when biodiversity first evolved, and this will accelerate the natural senescence of regional biodiversities to the point where premature termination is instead promoted. The manner in which a species can adjust to newly developing selective pressures will be dictated by the amount and distribution of genetic variability it possesses. In this regard, a lack of genetic variability in most southeastern to midwestern regional clades of North American *Agkistrodon* (as measured by a single-locus approach) is somewhat disconcerting. Although North American *Agkistrodon* have successfully withstood a relatively recent extinction event (the Pleistocene), they do not seem to have rebounded adequately with regard to genetic variability and thus might not adapt quickly to global warming, particularly given the hypothesis (Davis & Shaw, 2001) that RCCEs will overwhelm the capacity of most populations to adapt. Here, rapid reductions in habitat coupled with truncated heterozygosity will yield negative effects (Bouzat *et al.*, 1998; Holycross & Douglas, 2006), and conservation biologists must be primed for these eventualities. Global climate change in the coming decades will pare not only historical legacy but also future evolution, while concomitantly spawning intense conservation conflicts.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Specimens employed in this study.

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BIOSKETCHES

The authors share a common interest in deciphering the evolution of biological communities in North America, with particular focus on endemic amphibians, reptiles and fishes. M.E.D. and M.R.D. jointly run the Conservation and Molecular Ecology Laboratory (CAMEL) at the Illinois Natural History Survey (Institute for Natural Resource Sustainability, University of Illinois), while the research interests of G.W.S. focus on a phylogenetic comparative approach to the behavioural ecology and mating systems of reptiles, particularly pitvipers. L.W.P. is proprietor of Eagle Mountain Publishing, LC, specializing in biological titles, and his research interests focus on the natural history of New World herpetofauna.

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