

# EVIDENCE FOR MULTIPLE PLEISTOCENE REFUGIA IN THE POSTGLACIAL EXPANSION OF THE EASTERN TIGER SALAMANDER, *AMBYSTOMA TIGRINUM TIGRINUM*

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**Abstract.**—Pleistocene glaciations were important determinants of historical migration and, hence, current levels of genetic diversity within and among populations. In many cases, these historical migrations led to the existence of disjunct populations of plants and animals. However, the origin and timing of arrival of these disjunct populations is often debated. In the current study, we identify potential refugia and estimate the timing of vicariance events of the eastern tiger salamander, *Ambystoma tigrinum tigrinum*, using mitochondrial sequence data. The results suggest a vicariant event 0.75–2 million years ago, separating the tiger salamanders to the east and west of the Appalachian River Basin. East of the Appalachians, there appear to be multiple independent refugia with little migration among the remaining populations. In particular, populations along the Atlantic Coastal Plain were likely isolated in a coastal plain refugium in the Carolinas. Migrants from this refugium were the likely source of colonists for populations occupying previously glaciated areas along the northeastern Atlantic Coast. A second potential refugium occurs in the Blue Ridge Mountains of western Virginia. This refugium contains a disjunct population of the eastern tiger salamander, as well as a community of nearly 70 other disjunct plant and animal species. The tiger salamanders here have been isolated from other populations for 200,000–500,000 years. These results suggest that disjunct mountain populations of Coastal Plain species may have existed in situ throughout the Pleistocene in Appalachian refugia. Therefore, these disjunct populations are not of recent origin, but rather exist as relicts of a warmer, more widespread fauna and flora that is now restricted to the Coastal Plain.

**Key words.**—Appalachian, disjunct, migration, mitochondrial DNA, phylogeography.

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Recently, there have been an increasing number of studies using molecular techniques to infer migration patterns of plants and animals (Green et al. 1996; Hewitt 1996, 1999; Soltis et al. 1997; King and Ferris 1998; Taberlet et al. 1998; Tremblay and Schoen 1999; Abbott et al. 2000; Burbrink et al. 2000; Maskas and Cruzan 2000; Riddle et al. 2000; Nielson et al. 2001). From this work, it has become clear that the cycles of Pleistocene glaciations were major determinants of historical migration and, hence, current genetic diversity in Europe and North America (Bermingham et al. 1992; Strange and Burr 1997; Comes and Abbott 1998; Walker and Avise 1998; Riddle et al. 2000; for a review, see Avise 1992). These studies have contributed to our understanding of community compositions prior to, as well as throughout, the Pleistocene glacial cycles (Byun et al. 1997; Holder et al. 1999; Abbott et al. 2000; Riddle et al. 2000). Furthermore, such studies can help to target historically unique populations or lineages for conservation and preservation efforts (Ellsworth et al. 1994; Nielson et al. 2001).

Despite this recent work, gaps remain in our knowledge of the Pleistocene history, particularly of eastern North America. This is due in part to the relatively low abundance of fossil data from the Late Tertiary and Early to Middle Pleistocene of this region (Graham 1999). For instance, there are competing hypotheses for the origins of disjunct populations of coastal plain plants and animals in the mountainous regions of the Southeast (Braun 1937, 1947; Carr 1938; Harvill 1973, 1992; Jackson and Singer 1997). These disjunct populations

may be the remnants of a widespread Late Tertiary flora and fauna, when warmer conditions prevailed (Braun 1937, 1947; Carr 1938; Pittillo et al. 1998). If this were the case, these populations would have existed in situ throughout the Pleistocene, with these disjunct populations representing Pleistocene refugia (Braun 1937, 1947; Smith et al. 2000). Alternatively, disjunct populations may reflect relatively recent postglacial migrations from alternative refugia (Harvill 1973, 1992; Reznicek 1994; Wisheu et al. 1994; Fleming and Van Alstine 1999).

The eastern tiger salamander, *Ambystoma tigrinum tigrinum*, is representative of many plants and animals in eastern North America that have been influenced by the Pleistocene glaciations. Tiger salamanders exist in southeastern North America in areas that have historically remained free from ice, but they are also abundant in the Midwest and northern areas that were ice-covered during the most recent glaciation (Conant and Collins 1998; see Fig. 1). However, their distribution along the East Coast is patchy, with several populations being relatively isolated from the more continuously distributed populations in the South and Midwest. In particular, a single population exists in the Blue Ridge Mountains, where it is disjunct from the more continuous distributions to the east and west of the mountains. The origins of this disjunct population are not known and, more broadly, the patterns of migration among the remaining patchily distributed populations are unclear.

In this study, we use molecular data to show that these isolated populations harbor historically unique lineages of *A. t. tigrinum*. In particular, there is evidence that tiger salamanders existed in at least two independent refugia in the

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eastern United States and remained isolated throughout much of the Pleistocene. After the Pleistocene, one refugium likely served as the source of colonists for populations in the Northeast. The second refugium existed in the Appalachian Mountains, where that population has remained isolated, without emigration or immigration. This disjunct mountain population of tiger salamanders may be representative of a preglacial community that existed throughout the Pleistocene. More broadly, this evidence suggests the need for a re-evaluation of the origins of disjunct communities and not simply attributing them to postglacial expansion.

#### *Study System*

Tiger salamanders are widely distributed from the Gulf Coastal Plain throughout the Plains of the Midwest as far north as Ontario and Manitoba (Fig. 1). East of the Appalachian Mountains, tiger salamanders exist in only a few locations within the Piedmont Physiographic Province and as fragmented populations along the Coastal Plain (Fig. 1). Only one population exists in the Appalachian region, with the nearest population occurring 161 km to the southeast in the Coastal Plain of Virginia (Mitchell and Reay 1999). This disjunct mountain population is in southern Augusta County, Virginia, along the western edge of the Blue Ridge Province in a community composed of nearly 70 other Coastal Plain disjuncts (Buhlmann and Hoffman 1990; Flemming and Van Alstine 1999; Roble 1999).

The distribution of the eastern tiger salamander was certainly influenced by the Pleistocene glaciations. Fossil evidence suggests that tiger salamanders existed in the Appalachian Mountain regions of West Virginia and Maryland in the early Pleistocene (~600,000 years ago; Holman 1999). However, these populations went extinct before the end of the Pleistocene (Holman 1999). The onset of the Pleistocene was characterized by a general cooling trend (Pittillo et al. 1998; Graham 1999), and glacial advances resulted in the migration or extinction of many populations (Whitehead 1973; Savin et al. 1975; Tallis 1991; Hewitt 1996). The most recent, the Wisconsin glaciation, reached its southern limit in North America 18,000–14,000 years ago at about 41°N latitude (Fig. 1; see Pielou 1991). Many populations that had once inhabited areas north of the glacial boundary were restricted to smaller ranges south of the ice or to hospitable refugia (Delcourt and Delcourt 1987; Cogbill et al. 1997). Overall climatic cooling affected unglaciated areas as well, resulting in dramatic habitat changes throughout much of the Mid-Atlantic (Whitehead 1973; Delcourt and Delcourt 1987). For instance, spruce-fir forest grew as far south as the Carolinas (Whitehead 1973), an area now inhabited by the eastern tiger salamander.

The current distribution of the tiger salamander in areas where the climate was significantly cooler during the Pleistocene glaciation (the Blue Ridge Mountain population as well as the Mid-Atlantic Coastal Plain populations) may be due to post-Pleistocene range expansion, or they may have existed in situ in several refugia. At the end of the Pleistocene (18,000–10,000 years ago), the retreat of the Wisconsin ice sheets exposed areas that were rapidly recolonized (Davis 1976, 1981; Huntley and Birks 1981; Webb 1988; Woods

and Davis 1989). Furthermore, the glacial retreat was accompanied by a considerable warming of the climate (beyond present levels), which may have presented an opportunity for organisms adapted to warmer climates to extend their ranges (Jackson and Singer 1997; Williams et al. 2000), possibly allowing tiger salamanders and other Coastal Plain species to migrate into the mountains. Alternatively, populations may have existed throughout the Pleistocene in mountain refugia. There is some evidence that the latter hypothesis may be a more likely scenario for the eastern tiger salamander. Tiger salamanders, as well as several other disjunct amphibian and reptile species, can be found in the late Pleistocene (20,000–10,000 years ago) fossil record of western Virginia (Guilday 1962; Holman 1986; Fay 1988), suggesting that the mountain populations may have existed concurrently with the end of the Pleistocene epoch. However, it is unclear from the fossil record if these species existed in situ throughout the Wisconsin glaciation.

#### MATERIALS AND METHODS

##### *Tissue Samples*

Tissue collections were made from individuals representing most of the known populations of the eastern tiger salamander throughout the Northeast, the Appalachians, and the Southeast. Tissue was also collected from several locations in the more contiguous range of the tiger salamander south and west of the Appalachians (Fig. 1, Appendix). Toe, tail, or larval tissues were used as sources of DNA (Appendix). For all field-collected samples, at least two individuals were sampled per population for a total of 54 individuals. Several sequences were also obtained from the Genbank database, including eight tiger salamander sequences, one *Ambystoma mexicanum* sequence, and one *A. californiense* sequence. Only one individual per population was available for the sequences obtained from Genbank, although three of the tiger salamander sequences in Genbank were from populations that were resampled for the current study.

##### *DNA Isolation, Amplification, and Sequencing*

Total DNA was isolated from tissue using DNeasy animal mini kits as specified by the manufacturer (Qiagen, Inc., Valencia, CA). The mitochondrial D-loop and adjacent intron were amplified via polymerase chain reaction (PCR). This region has been shown to be useful in determining relationships within and among species of many amphibians, including species in the *Ambystoma tigrinum* complex (Shaffer and McKnight 1996). Each PCR reaction consisted of 2.5  $\mu$ l of 10 $\times$  reaction buffer (Applied Biosystems, Foster City, CA), 1.5  $\mu$ l of 25 mM MgCl<sub>2</sub>, 1.0  $\mu$ l of each 10  $\mu$ M primer in a pair, 0.5  $\mu$ l of a 2.5 mM dNTP solution in equimolar ratio, 0.1  $\mu$ l of Taq polymerase, and ~40 ng of template DNA for a total volume of 25  $\mu$ l.

Double stranded PCR products were produced for the D-loop and adjacent intron using primers designed by Shaffer and McKnight (1996) as well as two internal primers designed by the authors based on preliminary data. Primers DL3 and THR (Shaffer and McKnight 1996) were used to amplify 350 bp of the intron and adjacent D-loop region. The uni-

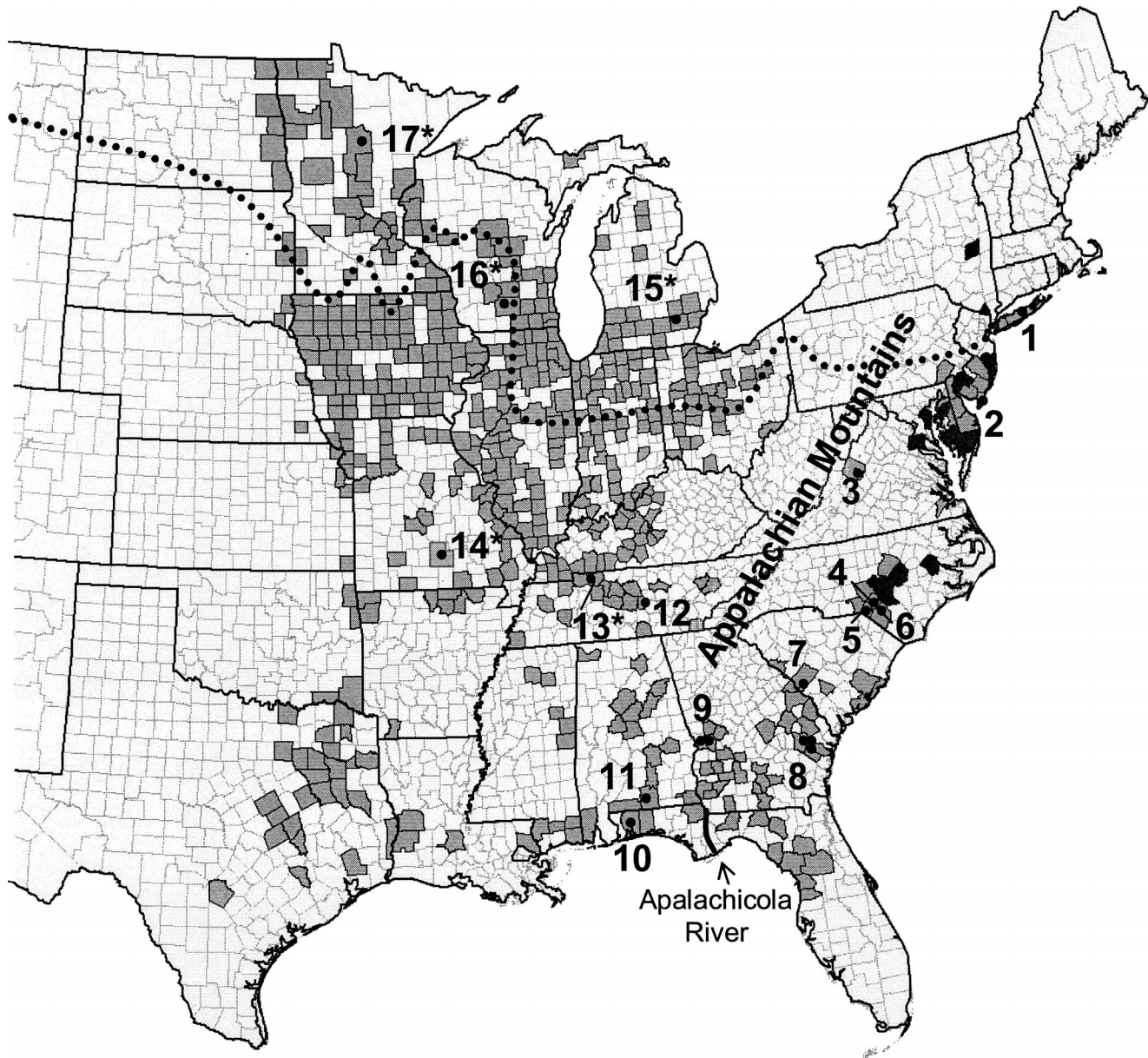


FIG. 1. Range map of the eastern tiger salamander, *Ambystoma tigrinum tigrinum*, based on county locality records. Extant populations are highlighted in light gray, and populations known to be extinct are highlighted in dark gray. It is likely that many more populations have gone extinct, although this has not been verified in most cases (e.g., See Pflingsten and Downs 1989). Populations sampled for the current (●) study are numbered. Sequence data obtained from Genbank (Shaffer and McKnight 1996) are denoted by asterisks. The dotted line represents the approximate southern limit of glacial ice during the Pleistocene (see Pielou 1991). County localities were identified from individual state records from the following sources: Breckenridge (1944), Wheeler and Wheeler (1966), Collins (1974), Johnson (1977), Vogt (1981), Stine (1984), Lynch (1985), Arndt (1989), Pflingsten and Downs (1989), Harding and Holman (1992), Mount (1996), Fischer et al. (1999), Redmond and Scott (1996), Mitchell and Reay (1999), Phillips et al. (1999), Dixon (2000), Hulse et al. (2001), Minton (2001), as well as S. Bennett, K. Irwin, J. Jensen, S. Smith, and R. Zappalorti (pers. comm.).

versal frog primer (12SAR; Kocher et al. 1989) and DL4 (Shaffer and McKnight 1996) were used to amplify the remainder of the D-loop and a portion of the 12S. The 12S region is not variable enough for population-level studies in tiger salamanders and was only amplified to complete D-loop amplification. Internal primers Dint (AACCGCTCTT-GAGCCGTTG) and Fint (TAAGTTAACCCCTACCC) were designed to amplify the interior portion of the 12SAR to DL4 fragment. All PCR reactions were performed on a

Applied Biosystems GeneAmp 9700 thermal-cycler (Perkin-Elmer) with 40 cycles consisting of 30 sec at 94°C, 30 sec at 48°C, followed by 90 sec at 72°C.

Double-stranded PCR products of each region were cleaned using Qiaquick PCR purification kits according to the manufacturers' instructions (Qiagen, Inc.). The cleaned product was dried completely using a speed-vac and resuspended to yield 5–10 ng of DNA per microliter of water for use in subsequent cycle sequencing reactions. The cycle se-

quencing reaction consisted of 2  $\mu$ l of Big Dye terminator ready reaction mix (Applied Biosystems, Inc., Foster City, CA), 2  $\mu$ l dilution buffer (159:40:1; H<sub>2</sub>O: Tris HCl [pH 9.0]: 1 M MgCl), 1  $\mu$ l of a single 10- $\mu$ M primer, and 50–100 ng double-stranded DNA, for a total of 10  $\mu$ l per reaction. Both fragments were sequenced in two directions using the same primers used to produce the double stranded PCR products.

Single-stranded PCR products were purified using Centri-sep spin columns (Princeton Separations, Adelphia, NJ) according to the manufacturer's instructions. The cleaned product was sequenced using an ABI 377 automated sequencer and the sequences were analyzed using the Genescan software (ver. 3.1, Perkin-Elmer). Ambiguous base calls were verified manually and consensus sequences were assembled using the computer program Sequencher (Gene Codes Corporation, Inc., Ann Arbor, MI). Alignments were made using the computer software package GCG (Wisconsin Package, ver. 10.0, Genetics Computer Group Inc., Madison, WI) and corrected manually. The two fragments were subsequently assembled to form a single contiguous sequence per individual. Identical sequences across individuals were merged into one sequence for phylogenetic analyses using the program MacClade (Madison and Maddison 1992) and subsequently deposited in GenBank.

#### Phylogenetic Analysis

*Ambystoma californiense* and *A. mexicanum* were designated as outgroups in the analyses. *Ambystoma californiense* has been shown to be a distinct species, possibly sister species to the *A. tigrinum* complex, based on both morphological (Kraus 1988) and molecular data (Shaffer et al. 1991; Shaffer and McKnight 1996). *Ambystoma mexicanum* is a species within the *A. tigrinum* complex; however, it has been distinct from *A. t. tigrinum* for several million years (Shaffer 1984; Shaffer and McKnight 1996).

Genetic variation within populations and regions was estimated by calculating the average number of nucleotide differences among individuals ( $k$ ; Tajima 1983) and genetic distances corrected for the most likely model of sequence evolution. The average number of nucleotide differences was calculated using the program DnaSP ver. 3 (Rozas and Rozas 1999). To estimate genetic distances, we first determined the best model of nucleotide evolution using likelihood-ratio tests. We began with the most complex model of evolution (general time reversible with gamma distributed rate variation and estimating the proportion of invariant sites) then compared this to ever-simpler models in a stepwise fashion. The best model of evolution was the simplest model that did not significantly reduce the likelihood of the data according to the likelihood-ratio test. This model was used in subsequent phylogenetic analyses in PAUP\* (Swofford 1999) as well as in calculating genetic distances within and among populations using the program MEGA version 2.1 (Kumar et al. 2001).

Maximum likelihood (ML) analyses were performed in PAUP\* 4.0b4 (Swofford 1999) using the model of evolution as determined above. The analyses were performed with 10 random addition sequences and tree bisection-reconnection (TBR) branch swapping algorithms in a heuristic search.

Maximum-parsimony and neighbor-joining analyses were also performed using 10 random addition sequences. Bootstrap analyses with 1000 replicates were performed with both neighbor-joining and parsimony methods to determine support for the tree topology.

#### Estimating Divergence Times

We estimated divergence times using two methods, enforcing a molecular clock on our phylogenetic reconstructions and by simulating the coalescent process. For the first method, we determined if the application of a molecular clock was valid by enforcing the molecular clock assumption on our data using PAUP\*, while maintaining all other parameters used in finding the maximum likelihood tree (Felsenstein 1981; Huelsenbeck and Crandall 1997; Huelsenbeck and Rannala 1997). We then estimated the likelihood score of the tree with the clock enforced and compared that value to the maximum likelihood score without the clock enforced using a likelihood ratio test. These comparisons were done over the entire tree as well as for various subsets of the data in order to determine which clades or individual sequences violated the molecular clock assumption. For those clades that did not violate the clock, a divergence rate of 1–1.5% per million years was assumed based on previous analyses of this group (Shaffer and McKnight 1996).

To estimate divergence times using coalescent theory, we simulated the coalescent process using the program Genetree by R. C. Griffiths (see Griffiths and Tavaré 1994; available via <http://www.maths.monash.edu.au/~mbahlo/mpg/gtree.html>). We excluded all sites that had gaps due to insertion/deletion polymorphisms. To estimate  $T_{MRCA}$ , we used Genetree to estimate the most likely value of  $\theta$  based on the distribution of mutations on the genealogies ( $=2N_f\mu$  for mitochondrial loci, where  $N_f$  is the effective population size of females and  $\mu$  is the mutation rate per gene per generation). We used a conservative per base pair mutation rate from the divergence rate estimate of 1% divergence per million years (Shaffer and McKnight 1996) to estimate  $N_f$  from  $\theta$ . Using our estimate of  $\theta$ , we ran 1 million simulations of the coalescent process. The simulation estimated the  $T_{MRCA}$  in coalescent units,  $T$ . Genetree also calculated confidence intervals for  $T_{MRCA}$ , but they should be interpreted with caution because we did not incorporate the uncertainty in our estimates of  $\theta$ . Because mitochondria are haploid, we calculated the coalescence time in years,  $t = TN_f g$ , where  $g$  is the generation time in years. Having estimated  $\theta$  and the per year substitution rate, effective population size and generation time do not factor into the calculations of coalescence times. To see this, notice that  $N_f$  can be expressed as  $\theta/2vg$ , where  $v$  is the mutation rate per gene per year and  $g$  is the number of years per generation. Therefore  $t = T\theta/2v$ .

## RESULTS

### Sequence Variation

We sequenced 1006 base pairs from 54 individuals. The D-loop and adjacent intron comprised 750 bp of the sequence region, and the remaining base pairs were part of the 12S region or intervening spacers. There were 37 distinct tiger

TABLE 1. Genetic distances within and among populations sampled for the current study. With the exception of Robeson County, North Carolina, genetic distances were only calculated for populations from which more than one individual was sequenced. A total of 54 individuals were included in the analyses for 750 bp of the D-loop region and adjacent intron. n/c, no comparison; only a single individual in the population.

| Location                     | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    |
|------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1. New York                  | 0.004 |       |       |       |       |       |       |       |       |       |
| 2. New Jersey                | 0.003 | 0.000 |       |       |       |       |       |       |       |       |
| 3. Virginia                  | 0.013 | 0.010 | 0.001 |       |       |       |       |       |       |       |
| 4. North Carolina (Hoke)     | 0.008 | 0.007 | 0.016 | 0.003 |       |       |       |       |       |       |
| 5. North Carolina (Scotland) | 0.006 | 0.003 | 0.011 | 0.006 | 0.004 |       |       |       |       |       |
| 6. North Carolina (Robeson)  | 0.008 | 0.005 | 0.015 | 0.001 | 0.004 | n/c   |       |       |       |       |
| 7. South Carolina            | 0.012 | 0.010 | 0.006 | 0.016 | 0.011 | 0.015 | 0.005 |       |       |       |
| 8. Eastern Georgia           | 0.009 | 0.006 | 0.004 | 0.013 | 0.007 | 0.011 | 0.006 | 0.002 |       |       |
| 9. Western Georgia           | 0.013 | 0.010 | 0.011 | 0.017 | 0.011 | 0.015 | 0.011 | 0.008 | 0.008 |       |
| 10. Florida                  | 0.024 | 0.020 | 0.021 | 0.027 | 0.022 | 0.026 | 0.022 | 0.019 | 0.015 | 0.004 |

salamander haplotypes. For these sequences, excluding outgroups, there were 56 segregating sites, 36 of which were parsimony informative. Most of this variation occurred within the D-loop and adjacent intron (48 segregating sites, 34 parsimony-informative sites); therefore, the following results are based on data from the D-loop and adjacent intron only. Neither the 12S region nor the intervening spacers were analyzed due to extremely low levels of sequence variation in the study taxa. Most of the genetic variation occurred among populations, rather than within. The average genetic distance among populations was 0.014, whereas the average genetic distance within populations was 0.002 (Table 1). Analysis of molecular variance (Excoffier 1992) generally confirms this pattern of strong phylogeographic structure; a relatively small proportion of the variation occurred within populations (9.97%), but substantial variation occurred both among populations within regions (31%) and between eastern and western regions (59.03%) as defined by the Apalachicola River basin. The highest levels of genetic variation were found in the southern populations in Georgia, South Carolina, and North Carolina (populations 4–9;  $k = 4.988$ ). Conversely, northern populations showed lower levels of genetic variation within populations (populations 1–3,  $k = 3.253$ ). Most of this variation in northern populations was between Virginia (population 3) and the Northeastern (populations 1, 2) regions, both of which had low diversity within regions ( $k = 0.533$  and 1.8, respectively).

#### Phylogenetic Analyses

The model of evolution used for the ML analyses corresponded to the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) + invariant sites (0.8) with gamma distributed rate variation (0.78) (HKY + I +  $\Gamma$ ). The resulting maximum likelihood tree had a  $-\ln L = 1709.75$  (Fig. 2). Neighbor-joining and parsimony trees were essentially identical in topology to the ML tree, particularly in clades with bootstrap support above 55% (Fig. 2). The results of the neighbor-joining and parsimony bootstrap analyses were also nearly identical, so we report only the neighbor-joining bootstrap values in Figure 2.

The two most distinct clades can be separated based on geography into an eastern clade and a western clade (average genetic distance = 0.021). The eastern clade contains only individuals from east of the Apalachicola River as well as

the population from the Blue Ridge Mountains. The western clade contains populations from Florida and states north and west of the Appalachian Mountains. The one exception to this is individual 45, which was sampled from Georgia but had a distinctly western mitochondrial DNA haplotype. We think this could be due either to contamination in the laboratory or human-mediated migration (see below), given the large genetic distances between the eastern and western clades. To be conservative in estimating the magnitude of genetic diversity within the eastern lineage, we chose to exclude this individual when calculating genetic distances or nucleotide diversity within this lineage.

Within the eastern clade, several smaller clades exist, corresponding to different geographic regions. The more distinct clades include the western Georgia population (9), the South Carolina population (7), the disjunct Blue Ridge Mountain population (3), and the North Carolina populations (4–6) together with the northeastern Atlantic Coastal Plain populations (1, 2). Members of the eastern Georgia population (8) do not form a distinct clade, and the relationships among clades are not well resolved. However, genetic distances among clades suggest that the Blue Ridge Mountain population (3) is nearly equidistant from both the South Carolina (7) and eastern Georgia populations (8; Table 1, average genetic distance = 0.005). It is also clear that the clade containing the North Carolina and Northeastern populations (populations 1, 2 and 4–6, respectively) is distinct from the remaining eastern populations (3, 7–9; average genetic distance = 0.012). Sampling in the western clade does not allow such phylogeographic comparisons.

#### Divergence Times

Both the coalescent simulations as well as the molecular clock analyses suggest similar divergence times for the major lineages within the tiger salamander clade. A previous molecular phylogenetic study of the tiger salamander complex suggested an approximate rate for mitochondrial D-loop evolution in tiger salamanders at 1–1.5% per million years (Shaffer and McKnight 1996). We used this rate to approximate the divergence dates among clades. The dataset analyzed as a whole violated the molecular clock assumption; accordingly, we used a selective omission process wherein taxa were pruned from the tree to meet the assumptions of the molecular clock (data not shown). As a result, all divergence time es-

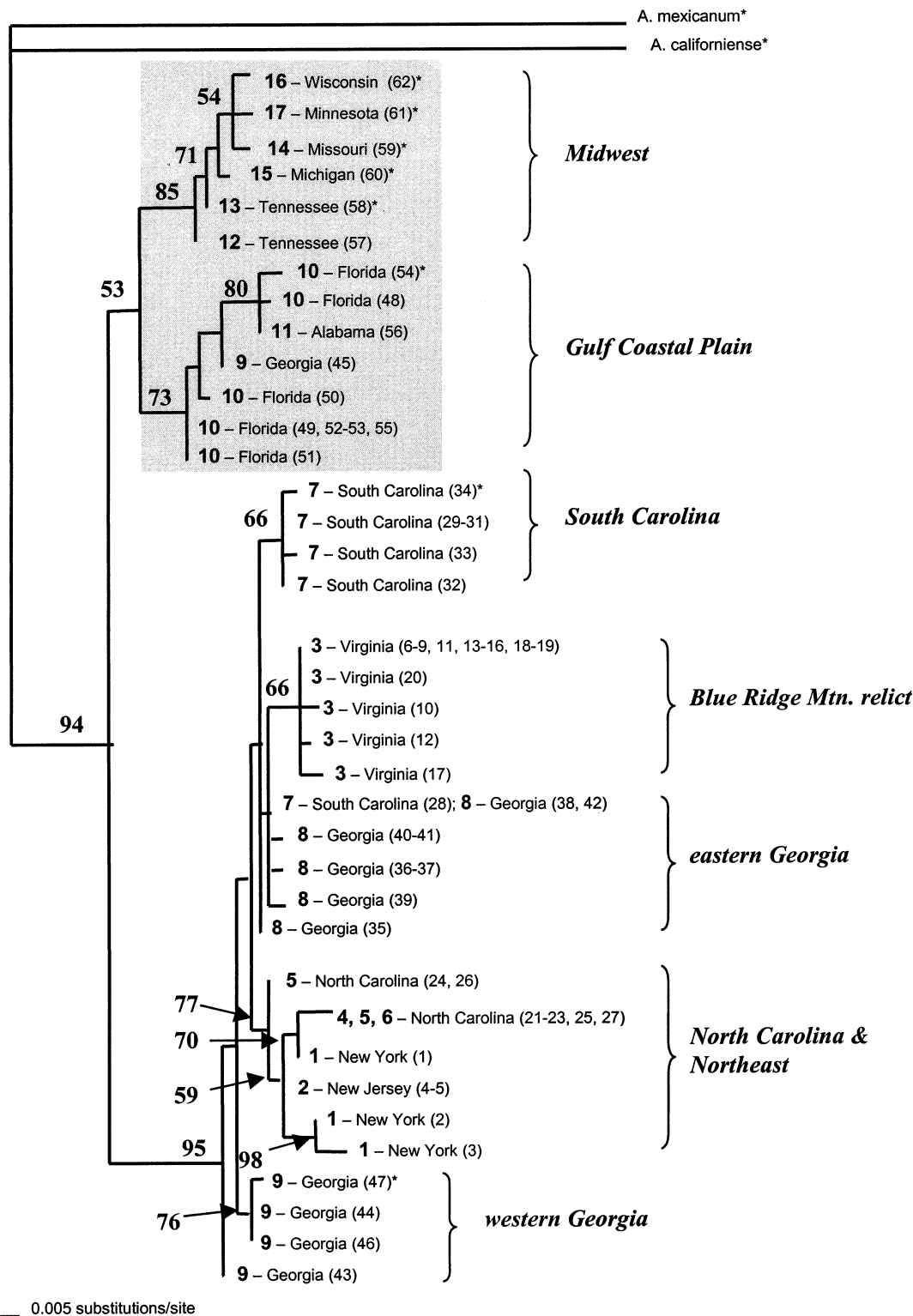


FIG. 2. Maximum-likelihood phylogeny based on sequences of 750 bp of the mitochondrial D-loop region and adjacent intron. Bootstrap values of 1000 neighbor-joining replicates are listed above the nodes. Branches are labeled with population number (corresponding to Fig. 1 and Appendix), state location, and individual collection numbers (corresponding to Appendix). The western clade (including all individuals from west of the Apalachicola River Basin) is distinguished from the eastern clade by a gray box. Major clades within each region are bracketed and labeled.

timates were made among lineages that did not have significant rate heterogeneity. The most ancient split lies between the western and eastern clades, with an average genetic distance of 2.1% and an estimated divergence time of 1.4–2.1 million years. Within the eastern clade, the southern lineages from South Carolina and Georgia, as well as the isolated Virginia mountain population, differ from one another by about 0.5%, translating into a divergence time between 333,000 and 500,000 years. Conversely, the more northern lineage including all Coastal Plain populations from North Carolina to New York, differs from the southern and mountain lineages by about 1.0% on average, indicating that the North Carolina–Northeastern lineage became isolated from the southern lineages between 666,000 and 1 million years ago. However, divergence times among populations in the Northeast are as recent as 13,000–200,000 years ago.

The coalescent simulation resulted in slightly more recent, although similar, divergence times. Incorporating population growth into the coalescent simulation did not significantly increase the likelihood of the data fitting the model, meaning that the distribution of mutations throughout the tree was effectively neutral (Tajima's  $D$  not significantly different from zero). The entire dataset violated the infinite sites model, so we estimated the time since the most recent common ancestor ( $T_{\text{MRCA}}$ ) for a subset of the data including only samples from eastern North America. Even within those data, there was some violation of the infinite alleles assumption that led to ambiguities with respect to the ancestors of the Virginia population. Specifically, there was one segregating site that suggested the Virginia haplotypes were descended from an ancestor in South Carolina, and another that suggested the Virginia haplotypes were descended from an ancestor in Georgia. We therefore estimated the age of the divergence of the Virginia haplotypes by estimating the most likely age of each of these mutations when the other of the characters in question was omitted.

For the data supporting a Georgian origin of the Virginia populations,  $\theta = 5.4$ . Knowing the mutation rate, we solved for the effective population size of females ( $\sim 144,000$ ). For the entire genealogy of the eastern lineages,  $T = 1.09$  (standard deviation  $\pm 0.251$ ). This translates to a  $T_{\text{MRCA}}$  for all eastern populations of 784,000 ( $\pm 180,000$ ) years ago. The likely age of the Virginia population is bracketed by the age of two mutations. One mutation was shared by all Virginia haplotypes and the most closely related non-Virginian haplotype; it most likely predates the origin of the Virginia population. Another mutation was unique to the Virginian population and likely postdates the origin of the Virginia population. The  $T_{\text{MRCA}}$  for these mutations were 0.391 ( $\pm 0.125$ ) and 0.250 ( $\pm 0.093$ ), respectively, which translates to an origin for this population between 282,000 and 180,000 years ago.

Analyzing the same data, but excluding the mutation that connects the Virginia population to the Georgia populations and including the mutation that connects it to the South Carolina populations, gives an almost identical result:  $\theta = 5.0$  ( $N_f \approx 134,000$ ),  $T_{\text{MRCA}}$  (in years) for the eastern populations was 759,000 ( $\pm 181,000$ ) years ago,  $T_{\text{MRCA}}$  (in years) of the two mutations bracketing the origin of the Virginia popu-

lation was 307,000 ( $\pm 89,000$ ) and 180,000 ( $\pm 69,000$ ) years ago.

The maximum age of the lineage containing individuals from North Carolina and the Northeast was more difficult to resolve because no single mutation marked the divergence between this lineage and the remaining populations from Georgia, South Carolina, and Virginia. However, one substitution unique to the North Carolina–Northeastern lineage had a  $T_{\text{MRCA}}$  of 0.566 ( $\pm 0.233$ ), suggesting a minimum age of 388,000 ( $\pm 158,000$ ) years ago for the common ancestor of these populations.

## DISCUSSION

The results of the current study show that the eastern and western populations of the eastern tiger salamander have been isolated from one another since the early Pleistocene. More significantly, the eastern lineage is composed of several distinct clades, each of which may have had unique histories during the Pleistocene glaciations. The most interesting result is that there were at least two distinct refugia in eastern North America (the Blue Ridge Mountains of Virginia and the mid-Atlantic Coastal Plain) that remained isolated throughout much of the Pleistocene. The population from the mountain refugium has remained isolated even during the post-Pleistocene, while emigrants from the Mid-Atlantic populations swept northward, colonizing the previously glaciated regions of the Northeast. This evidence of Coastal Plain disjuncts surviving throughout the Pleistocene in an isolated mountain refugium is contrary to the prevailing hypothesis that disjuncts spread from the Coastal Plain into the mountains during the postglacial warm interval 7,000–10,000 years ago.

The most ancient divergence within the eastern tiger salamander lineage is between the western and eastern clades (Fig. 2). The average level of sequence divergence between these two lineages is comparable to or greater than that found between other subspecies of the *Ambystoma tigrinum* complex (see Shaffer and McKnight 1996). These results suggest that the eastern and western lineages of *A. t. tigrinum* have been isolated for 0.75–2.1 million years, corresponding to the Late Tertiary or onset of the Pleistocene (Graham 1999). The most likely geographic barrier isolating these two lineages is the Apalachicola River Basin (Fig. 1). This is consistent with several other studies of aquatic as well as terrestrial species in eastern North America (Avise et al. 1979; Ellsworth et al. 1994; Burbrink et al. 2000; but see Phillips 1994; Donovan et al. 2000).

Within the eastern lineage, the southernmost populations appear to contain more genetic diversity than the northern populations (Table 1,  $k = 4.988$  in the south,  $k = 3.253$  in the north). This is consistent with the idea that the southern populations were likely the pool from which the northern migrants originated (Ibrahim et al. 1995; Hewitt 1996). However, one sampled individual from Chattahoochee County, Georgia (east of the Apalachicola River) appears to be most closely related to individuals in the western clade from Florida. This would suggest recent migration, either naturally or through use of the salamanders as fish bait. Selling larval tiger salamanders, often called waterdogs, as fish bait is common in the Midwest; although it is outlawed in several states

due to the states' endangered status of the eastern tiger salamander (Pague and Buhlmann 1991). Despite this potential migration, it has apparently not had a large effect on current levels of diversity in the eastern lineage.

#### *Migration along the Eastern Seaboard*

Although all populations in the eastern lineage appear to have a common ancestor near 1 million years ago, recent migrations are more complex. It appears that the colonization of the northernmost populations (1–3) occurred from at least two pools of individuals at separate times in the past. That is, the Blue Ridge Mountain population (3) has existed in situ throughout at least a portion of the Pleistocene, whereas the northeastern populations (1, 2) have a post-Pleistocene origin probably from somewhere in the Mid-Atlantic coastal plain.

The most diverse population east of the Apalachicola River occurs in western Georgia (Table 1). This population is as distinct from the eastern Georgia populations as it is from the geographically more remote South Carolina and Virginia populations (Table 1). Divergence among the Georgia populations may be due to their existence on separate river drainages (western Georgia rivers drain to the Gulf of Mexico, whereas eastern rivers drain to the Atlantic Ocean). However, given that tiger salamanders are mostly terrestrial, only returning to ponds to breed (generally not river floodplains), it may also be that the large Altamaha River and its tributaries have acted as barriers to frequent migration across central Georgia.

In contrast, the eastern Georgia populations are genetically equidistant from the South Carolina and Blue Ridge Mountain populations (Table 1). The estimated divergence dates for these populations are about 200,000–500,000 years ago. This close relationship between the mountain population in Virginia and the more southern populations in Georgia and South Carolina (average genetic distance = 0.005) suggests that colonists of the Blue Ridge Mountain population (Virginia) did not come directly west from the Coastal Plain populations of North Carolina (average genetic distance = 0.014). Instead, it is more likely that colonists of the montane Virginia population originated from other inland populations or perhaps now extinct Appalachian Mountain populations (see Holman 1999).

#### *Alternative Pleistocene Refugia*

Populations from the Coastal Plain of North Carolina and north to New York are distinct from more southern populations as well as the population in the Blue Ridge Mountains. Surprisingly, the Coastal Plain populations appear to have diverged from the remaining eastern populations between 400,000 and 1 million years ago, coinciding with the onset or early cycles of the Pleistocene glaciations. This suggests that at least some coastal populations existed throughout repeated glacial advances, including the largest and most recent Wisconsin glaciation during which the Atlantic Coastal Plain was spruce parkland as far south as the Carolinas (Whitehead 1973). These Coastal Plain refugia were then the likely source of migrants expanding north through Maryland and Pennsylvania into New York and New Jersey at the end of the

most recent glaciation (10,000–20,000 years ago). Unfortunately, many of these populations have recently become extinct due to human impact (Fig. 1).

Migrants out of Coastal Plain refugia likely colonized northeastern North America; however, these migrations apparently did not reach inland to the Appalachian Mountains. Instead, the mountain population existed in an alternative Pleistocene refugium, where it remains isolated to this day. The occurrence of plants and animals with northern affinities found in disjunct Appalachian pockets is often attributed to the existence of isolated Pleistocene refugia in this region (Harvill 1973). However, it is generally assumed that the occurrence of Coastal Plain disjuncts in the southern Appalachians is due to post-Pleistocene migrations during the warm period at the end of the last glaciation (Harvill 1973, 1992; Reznicek 1994; Wisheu et al. 1994). The population of tiger salamanders in the Blue Ridge Mountains of Virginia is geographically nearest to sampled populations in North Carolina; however, it is genetically more similar to populations in South Carolina and Georgia. These results, along with the timing of divergence of these populations (250,000–500,000 years ago), rule out the often-invoked theory of post-Pleistocene dispersal for the existence of Coastal Plain disjuncts in this region. Instead, this region appears to have been a refugium for tiger salamanders and other organisms during much of the Pleistocene.

The Blue Ridge Mountain population of tiger salamanders is part of a larger community of disjunct species. Nearly 70 species of disjunct plants and animals occupy the area in and around the sinkhole ponds in which the tiger salamanders breed (Buhlmann and Mitchell 1999; Fleming and Van Alstine 1999; Mitchell and Buhlmann 1999; Roble 1999). These disjunct plants and animals generally have northern, Coastal Plain, or even Midwestern affinities; they include one additional amphibian species, one reptile, 10 odonates, and 56 plants (both monocots and dicots; Buhlmann and Mitchell 1999; Fleming and Van Alstine 1999; Mitchell and Buhlmann 1999; Roble 1999). What makes the existence of this community even more surprising is the relatively small area it occupies; approximately 1350 ha in the southeastern corner of one county in Virginia. The unique climate of this area may be part of the reason that disjuncts have persisted here (Woodward 1990). Although the climate of this region is complex, it is generally both cooler and wetter than surrounding areas in Virginia (Klopper 1999). Preliminary studies of plant disjuncts in this community show phylogeographic patterns similar to those seen in the tiger salamander, with plants from this disjunct community being genetically more similar to populations of the same species in Georgia rather than coastal North Carolina or Virginia (D. R. Church, unpubl. data). Furthermore, there are several known and possible endemic species in this community that may be sufficiently diverged to warrant species status (Simurda and Knox 2000; J. Townsend, pers. comm.). The extant flora and fauna of this unique region provide an opportunity to examine the community composition of Pleistocene refugia, given that our analyses suggest that this community of disjuncts existed in situ as other populations to the east expanded and contracted throughout the many glacial advances and retreats of the Pleistocene epoch.

### Conclusions and Conservation Implications

Our study identifies several unique lineages and populations of the eastern tiger salamander. The lineage in the Coastal Plain from North Carolina to New York has remained isolated for 0.4–1 million years, while the only known mountain population (Virginia) has also existed *in situ* throughout much of the Pleistocene. The montane tiger salamander population is also part of a larger community that is home to many other disjunct species, which may have similar histories. More generally, the eastern tiger salamander is clearly separated into two distinct lineages, east and west of the Apalachicola River. These lineages have been distinct from one another since the early Pleistocene. This level of divergence is similar to that between distinct species or subspecies in the *Ambystoma tigrinum* complex (Shaffer and McKnight 1996). The genetic distinction of the eastern and western lineages as well as the existence of a relict Pleistocene population present an excellent opportunity for further study of the variation in other characters among these populations, including life history, adult morphology, behavior, and development.

The distribution of the eastern tiger salamander is fragmented (e.g., Stine 1984; Arndt 1989; Mitchell and Reay 1999) and many populations have been driven to extinction in the last century (Hulse et al. 2001). Habitat loss has effectively isolated many remaining populations (Pague and Buhlmann 1991) and, although the tiger salamander is listed as endangered in Virginia and the northeastern states, this status does little to protect remaining upland forest and ephemeral wetlands critical to the species survival. This study identifies genetically distinct populations throughout eastern North America that should be given special conservation consideration, in particular, the Blue Ridge Mountain population, which is likely to be a Pleistocene relict.

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## APPENDIX

| Population | Specimen             | Locality   | Collectors                                  |
|------------|----------------------|--|---|
| 1          | 1                    | NY, Suffolk Co. Riverhead, Sand Pits   | N. Soule                                    |
| 1          | 2                    | NY, Suffolk Co. Manorville, Sand Pits  | N. Soule                                    |
| 1          | 3                    | NY Suffolk Co. Horn Pond, ~5 mi NW of Sand Pits  | N. Soule                                    |
| 2          | 4, 5                 | NY, Cape May Co., Bayshore Mall area off Rt. 9. Lower Tap, known as Cold Spring                          | R. Zappalorti                               |
| 3          | 6–9                  | VA, Augusta Co., George Washington and Jefferson National Forest, pond 2                                 | D. R. Church                                |
| 3          | 10–13                | VA, Augusta Co., George Washington and Jefferson National Forest, pond 17                                | D. R. Church                                |
| 3          | 14–16                | VA, Augusta Co., George Washington and Jefferson National Forest, pond 13                                | D. R. Church                                |
| 3          | 17–20                | VA, Augusta Co., George Washington and Jefferson National Forest, pond 11                                | D. R. Church                                |
| 4          | 21–23                | NC, Hoke Co., 1.5 mi. E of Antioch, Hamby's Bay off N side SR 1448, 1.2 rdmi SE jct SR 1105              | J. C. Beane, S. L. Alford                   |
| 5          | 24                   | NC, Scotland Co., ~11 mi NW Wagram, Seventeen Frog Pond, Sandhill Game Lands off W side Strausburg Rd.   | J. C. Beane, S. L. Alford, D. S. Dombrowski |
| 5          | 25, 26               | NC, Scotland Co., ~11 mi NW Wagram, Seventeen Frog Pond, Sandhill Game Lands off W side Strausburg Rd.   | J. C. Beane, J. T. Finnegam, S. J. Horton   |
| 6          | 27                   | NC, Robeson Co., 1.75 mi WSW Lumber Bridge, along SR 1704, 0.5 rdmi W jct. SR 1705, near Greenspond Bay  | J. C. Beane, S. L. Alford                   |
| 7          | 28–33                | SC, Aiken Co., Savannah River Site, Ellenton Bay   | D. Scott                                    |
| 7          | 34 (42) <sup>1</sup> | SC, Aiken Co., Savannah River Site, Ellenton Bay   | R. Semlitsch                                |
| 8          | 35–38                | GA, Evans Co., 7.5 km SSE of Groveland, Fort Stewart, very near Salem Cemetary and Liberty Co. line      | D. Stevenson, K. Dyer, J. Palis             |
| 8          | 39                   | GA, Liberty Co., Alpha 6 borrow pit, W and adjacent FS Rd 57, 3 km S jct 57 and Hwy 144, Fort Stewart    | D. Stevenson                                |
| 8          | 40–42                | GA, Long Co., Echo 12, Cypress Pond, 4.2 km E Oak Grove Cemetary, 31°56'20"N, 81°49'20"W                 | D. Stevenson                                |
| 9          | 43–45                | GA, Chattahoochee Co., K–12, Fort Benning  | L. Andrews                                  |
| 9          | 46                   | GA, Chattahoochee Co., K–12, Fort Benning  | L. Andrews, J. Jensen                       |
| 9          | 47 (43) <sup>1</sup> | GA, Marion-Chatahoochee Co. border, approximately 7.5 mi S of Box Springs                                | H. B. Shaffer                               |
| 10         | 48                   | FL, Santa Rosa Co., Blackwater River State Forest, Goose Pond N of Munson                                | B. Kemker, P. Moler, B. Mansell             |
| 10         | 49–53                | FL, Santa Rosa Co., Blackwater River State Forest, 13.6 km N of Harold                                   | P. Moler, K. Enge, et al.                   |
| 10         | 54 (44) <sup>1</sup> | FL, Santa Rosa Co., approximately 4 mi E of Jay  | P. Moler                                    |
| 10         | 55                   | FL, Santa Rosa Co., Pleasant Home Pond   | K. Enge                                     |
| 11         | 56                   | AL, Covington Co., Conecuh National Forest, 31°05'04"N, 86°39'36"W                                       | M. Bailey                                   |
| 12         | 57                   | TN, Cannon Co., Burton-Burgen Rd., 700 m S of Bradyville Hill Rd   | B. T. Miller                                |
| 13         | 58 (41) <sup>1</sup> | TN, Montgomery Co., cattle tank of Mr. Culpepper, 0.25 mi S of junction of old Hwy. 149 at Oak Ridge Rd. | H. B. Shaffer                               |
| 14         | 59 (40) <sup>1</sup> | MO, Texas Co., 4 mi S of Cabool, along Hwy 63  | R. Altig                                    |
| 15         | 60 (38) <sup>1</sup> | MI, Washtenaw Co., Goss Pond, between Goss Rd. and U.S. 23   | F. Kraus                                    |
| 16         | 61 (39) <sup>1</sup> | MN, Cass Co., along Hwy. 64, 6.7 mi N of intersection of Hwy. 64 and Hwy. 210                            | H. B. Shaffer                               |
| 17         | 62 (37) <sup>1</sup> | WI, Dane Co., along Hwy. 12, approximately 2 mi N (by road) of Oregon                                    | H. B. Shaffer                               |

<sup>1</sup> Specimens sequence by H. B. Shaffer and M. L. McKnight (1996). Numbers in parentheses indicate original population numbers assigned by Shaffer and McKnight (1996).