

# Phylogeography of the Northern short-tailed shrew, *Blarina brevicauda* (Insectivora: Soricidae): past fragmentation and postglacial recolonization

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## Abstract

*Blarina brevicauda* is distributed across the northeastern region of North America, in areas previously covered by Pleistocene glaciers. Previous molecular systematic study of the species in the genus *Blarina* suggested the presence of two distinct eastern and western phylogroups within *B. brevicauda*, in agreement with traditionally recognized semi-species. To expand the previous work, a collection of 76 individuals from 14 localities collected throughout the range of *B. brevicauda* was used to assess the mitochondrial (mt) cytochrome *b* genealogy for this species. Minimum evolution, maximum parsimony, analysis of molecular variance and nested clade analysis each supported the same conclusions of two well-differentiated and monophyletic east–west groups, separated by the Mississippi River. Denser sampling in areas immediately East of the Mississippi basin revealed further subdivision within the eastern phylogroup into an East-Central and an Appalachian clade. The western phylogroup differed from the eastern phylogroup by 2.5% mean absolute DNA sequence difference. About 65% of the genetic variance among samples was explained by the east–west subdivision alone. High haplotype diversities, low nucleotide diversities and unimodal mismatch distributions within subclades suggest recent expansion or diversification within each group. No phylogeographic structure was found within the western phylogroup, but genetic structure because of restricted gene flow and isolation by distance was inferred for the eastern group. The present distribution of *B. brevicauda* is best explained by past fragmentation and range expansion events during and following the Pleistocene glacial cycles.

*Keywords:* *Blarina*, cytochrome *b*, mitochondrial DNA, nested clade analysis, refugia, shrews

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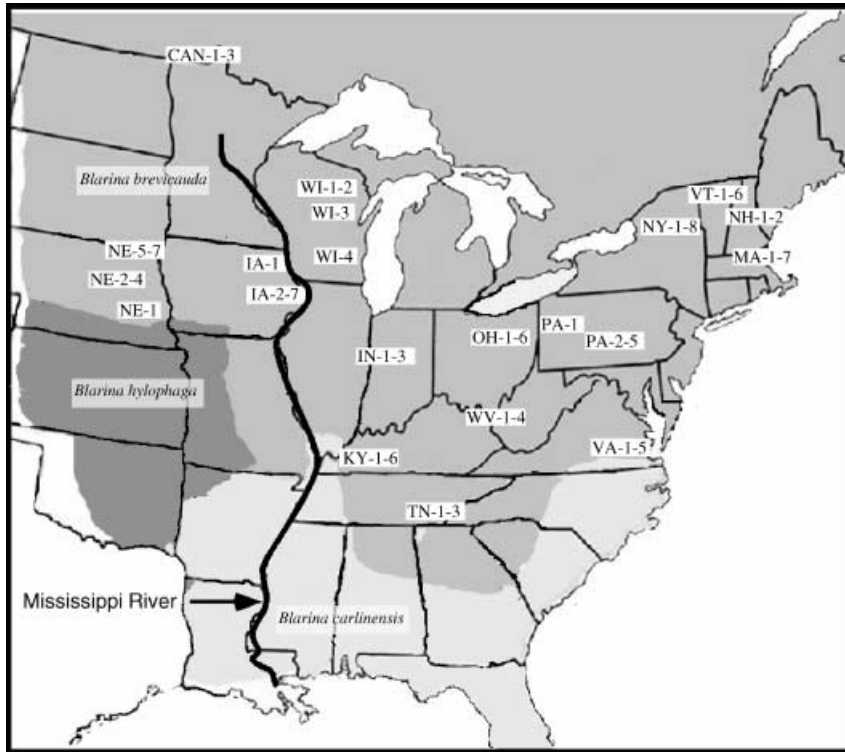
## Introduction

Molecular markers coupled with sophisticated analytical tools have contributed significantly to our understanding of the complex interplay between historical and contemporary processes shaping intraspecific diversification (Avice 1994, 2000; Templeton 1998; Posada & Crandall 2001). Among historical factors, Pleistocene glaciations had a profound impact on the north temperate biota and have been ascribed a dominant role in determining genetic population structure (Pielou 1991; Klicka & Zink 1997; Avice & Walker 1998; Avice 2000; Knowles 2001). During the last 18 000 years,

beginning with the retreat of the ice sheets, many organisms expanded their ranges northwards into recently de-glaciated areas (Pielou 1991). Comparisons of mitochondrial DNA (mtDNA) sequence divergence among populations and species have been used to test hypotheses of vertebrate evolution during the Pleistocene (Klicka & Zink 1997; Avice & Walker 1998; Avice *et al.* 1998; Milá *et al.* 2000; Knowles 2001). In this study, we use this approach to expand previous work (Brant & Ortí 2002) to investigate the phylogeographic history of the northern short-tailed shrew, *Blarina brevicauda*.

The genus *Blarina* Gray, 1823 is composed of three species distributed parapatrically throughout the eastern half of North America (Fig. 1; George *et al.* 1981, 1986; Genoways & Choate 1998; Brant & Ortí 2002). Northern short-tailed shrews (*B. brevicauda*) are relatively large, robust, semi-fossorial shrews most active in the early morning. Their

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**Fig. 1** Map showing the distribution of the three species of *Blarina* in different shades of grey and the locations of sites used for sampling individuals of *B. brevicauda*. Locality and label designations are given in the Appendix.

success in colder latitudes has been attributed to a diverse diet that includes earthworms, millipedes, molluscs, small vertebrates and insects (Martinsen 1969; George *et al.* 1986). These shrews are found almost continuously from north-central to northeastern United States and the southern regions of adjacent Canadian provinces (George *et al.* 1986; Fig. 1). Although they are ubiquitous throughout most of their range, regional distribution is limited by moisture and, secondarily, temperature and ground cover (George *et al.* 1986).

Brant & Ortí (2002), based on phylogenetic analysis of mtDNA sequences, reconstructed the relationships among the three species of *Blarina* and showed a clear separation of eastern and western mtDNA clades within *B. brevicauda* and *B. carolinensis*. Albeit based on limited population samples, the putative barrier within species was mapped to the Mississippi basin. This and other studies hypothesized that populations of *B. brevicauda* were most likely affected by fragmentation events during the Pleistocene, followed by range expansion into formerly glaciated areas, probably combined with isolation by distance caused by limited dispersal capabilities (Jones *et al.* 1984; Brant & Ortí 2002). The goal of this study is to examine in detail the pattern of mtDNA haplotype variation across the range of *B. brevicauda*, and to test hypotheses of differentiation in isolated glacial refugia and subsequent expansion. We expand previous work (Brant & Ortí 2002) with the addition of population samples across the eastern half of the distribution of the northern short-tailed shrew, *B. brevicauda*.

## Materials and methods

### *Specimens examined*

Samples of individuals of *Blarina brevicauda* were obtained from areas not included in the study of Brant & Ortí (2002) (Fig. 1). Data were collected for 76 individuals of *B. brevicauda* and two outgroup *B. carolinensis*, representing a total of 14 populations. Although voucher specimens were saved for all individuals collected, not all have been deposited in museum collections. Presently, all samples can be traced by field number and are available from the senior author (see Appendix). Northern short-tailed shrews were trapped in Sherman live traps, baited with peanut butter, set along small mammal runs in roadside ditches with dense grass or in mixed deciduous forest along fallen logs, and/or mole holes. Abdominal tissue was removed from trapped animals and placed immediately in 95% ethanol and stored at  $-20^{\circ}\text{C}$  for subsequent analysis.

### *Laboratory procedures*

Methods for DNA extraction and polymerase chain reaction (PCR) are as described in Brant & Ortí (2002). Approximately 50 ng of the purified PCR product was used for sequencing with each primer using the BigDye terminator kit (Applied Biosystems) and determined directly with a Base Station Sequencer (MJ Research). Forward and reverse strands were determined for consistency and the nucleotide

sequence data obtained have been deposited in GenBank (see Appendix).

### Data analysis

The cytochrome *b* sequences were aligned by eye based on the amino acid sequences because no indels were observed. The final alignment included 1131 base pairs (bp), two amino acids short of the complete sequence. Phylogenetic analyses were conducted using PAUP\* version 4.0b4 (Swofford 2000). Maximum parsimony analyses were performed using heuristic searches starting with stepwise addition trees and replicating 10 times, with each replicate using a random input order of sequences to get the initial tree. Branch swapping was performed by tree-bisection-reconnection. Bootstrap analysis (Felsenstein 1985) with 100 pseudo-replicates was used to measure support of the resulting topologies; each pseudoreplicate used a heuristic search with a single replication, based on random stepwise addition of taxa and tree-bisection-reconnection branch swapping. An optimal model of nucleotide evolution for maximum likelihood and minimum evolution analyses was determined by MODELTEST 3.0 (Posada & Crandall 1998). Minimum evolution analyses were performed using the maximum likelihood parameter values obtained by MODELTEST, heuristic searches starting with stepwise addition trees and replicated 10 times, with each replicate starting with a random input order of sequences. Branch swapping was performed by tree-bisection-reconnection. Bootstrap analyses with 500 pseudoreplicates were used to measure support of the resulting maximum likelihood and minimum evolution topologies; each pseudoreplicate used heuristic searches with a starting tree obtained by neighbour joining and nearest neighbour interchange branch swapping.

Haplotype and nucleotide diversity ( $\pi$ ) values were calculated for groups of haplotypes to measure DNA polymorphism using the DNASP software v3.53 (Roza & Roza 1999). These measures are appropriate for this type of study because they do not depend on length of DNA or sample size (Nei & Li 1979; Nei 1987).

Analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) was conducted to summarize variation among and within populations with the computer program ARLEQUIN (Schneider *et al.* 2000). Genetic diversity was subdivided into hierarchical components based on geographical location of the samples (states) and on results of the genealogical analysis, following phylogroup identification. Mismatch distributions were calculated for each of the groups to assess graphically the changes in population size inferred by other analyses (see nested clade analysis below). Assuming the infinite sites model, the mismatch distribution is smooth and often unimodal as a result of population expansion whereas for stationary populations the distribution is reliably ragged and often multimodal

(Harpending *et al.* 1998). Finally, Fu's  $F_s$  neutrality test (Fu 1997) was applied as an additional assessment of possible population expansion. Under the assumption of neutrality, a population expansion produces large negative value of  $F_s$  (Fu 1997).

### Nested clade analysis

Nested clade analysis was based on an intraspecific cladogram estimation procedure designed to address population level phenomena, which are not accounted for in traditional phylogenetic methods (Templeton *et al.* 1992; Posada & Crandall 2001). The cladogram estimation procedure and nesting rules described by Templeton *et al.* (1992, 1995), and their extension for DNA sequence data by Crandall (1996), were used to construct the nested cladogram network. Estimation of the 0.95 probability limit for parsimony and the network connections were obtained with the program tcs v1.0 (Clement *et al.* 2000). The resulting genealogical links were checked manually against a distance matrix and errors were corrected by hand. Nested clade analysis uses this haplotype network to define a series of nested sets or 'clades' (Templeton *et al.* 1987; Templeton & Sing 1993).

This nesting structure, together with information on the geographical distribution of the haplotypes, is used to estimate two geographical measures for each clade, the clade distance ( $D_c$ ) and the nested clade distance ( $D_n$ ).  $D_c$  is a measure of the geographical extent of a given clade, while  $D_n$  is a measure of the average geographical distance of individuals in a clade from all individuals that belong to the next higher-level nesting clade within which it is contained (Templeton 1998). Geographical analyses were performed using GEODIS 2.0 (Posada *et al.* 2000). Significant values of  $D_c$  and  $D_n$  were interpreted with the most recent inference key ([http://zoology.byu.edu/crandall\\_lab/geodis.htm](http://zoology.byu.edu/crandall_lab/geodis.htm)) modified from Templeton (1998).

## Results

### General pattern of sequence variation

A total of 1131 bp of cytochrome *b* was analysed for all 76 individuals of *Blarina brevicauda* plus two outgroup taxa. Among the sequences analysed for the 76 *B. brevicauda*, 1000 sites were constant and 131 were variable. Of the variable sites, 104 (80%) were polymorphic sites at the third codon position, 20 (15%) were polymorphic sites at the first codon position and seven (5%) were polymorphic sites at the second codon position. A plot of the substitution pattern of cytochrome *b* gene for transitions and transversions at the third codon position did not show saturation for *B. brevicauda* (Brant & Ortí 2002). This variation defined 61 distinct haplotypes among the 76 individuals examined. The HKY + I + G model (Hasegawa *et al.* 1985; Yang 1993;

**Table 1** Sample sizes, number of unique haplotypes observed, absolute (%) genetic differences, haplotype diversity and nucleotide diversity (Nei 1987) for *Blarina brevicauda* mtDNA cytochrome *b* sequences

Comparisons	n	No. of unique haplotypes	Percentage sequence difference		Haplotype diversity	Nucleotide diversity
			Range	Mean		
Within Western phylogroup*	17	13	0–1.1	0.5	0.963	0.005
Within Appalachian (APP)	42	29	0–1.6	0.6	0.959	0.006
Within East Central	17	15	0.09–1.9	0.8	0.985	0.01
Between APP/East Central	59	44	0.7–2.4	1.5	0.978	0.01
Between Western/Eastern	76	57	1.7–3.2	2.5	0.985	0.015
<i>B. brevicauda</i> / <i>B. carolinensis</i>	78	59	6.5–8.0	7.2	NR	NR

\*Individuals are grouped according to the phylogenetic results (Fig. 2).

NR = not reported.

Gu *et al.* 1995) was selected by MODELTEST (Posada & Crandall 1998) as the best fit for the cytochrome *b* data. Parameters estimated for this model were:  $Ti : Tv$  ratio = 7.2284, gamma shape parameter = 0.7667, base frequencies  $A = 0.2881$   $C = 0.2914$   $G = 0.1422$   $T = 0.2783$ , and proportion of invariable sites (pinvar) = 0.5226. Maximum per cent sequence difference (uncorrected) among haplotypes was 3.2%. Several measures of genetic variation are given in Table 1.

#### Phylogenetic inference

Maximum parsimony analysis of the shrew cytochrome *b* resulted in 432 equally parsimonious trees (length 265, consistency index (CI) = 0.762, retention index (RI) = 0.897). Areas of incongruence between the 432 equally parsimonious trees comprised relationships among terminal taxa within major haplotype groups. One of the shortest trees was chosen arbitrarily to represent this result (Fig. 2). The maximum parsimony analysis supported the following haplotype groups: the 'Western' haplotypes (from 17 individuals collected in IA, NE, CAN), 'East Central' haplotypes (from 14 individuals collected in KY, IN, WI, OH), and the 'Appalachian' haplotypes (from 45 individuals collected in TN, WV, OH, PA, NY, NH, VT, MA, VA). The minimum evolution and maximum likelihood analyses of cytochrome *b* also supported the monophyly of and the same relationships among the major groups shown in the maximum parsimony tree (see bootstrap values in Fig. 2).

#### Nested clade analysis

The nested haplotype network is an alternative graphic method with which to illustrate the genetic diversity and population structure of a species. A network of most parsimonious connections (Fig. 3) was constructed for the cytochrome *b* haplotypes of *B. brevicauda* to test for significant associations of haplotype distribution and geographical location. Mitochondrial DNA haplotypes separated by up

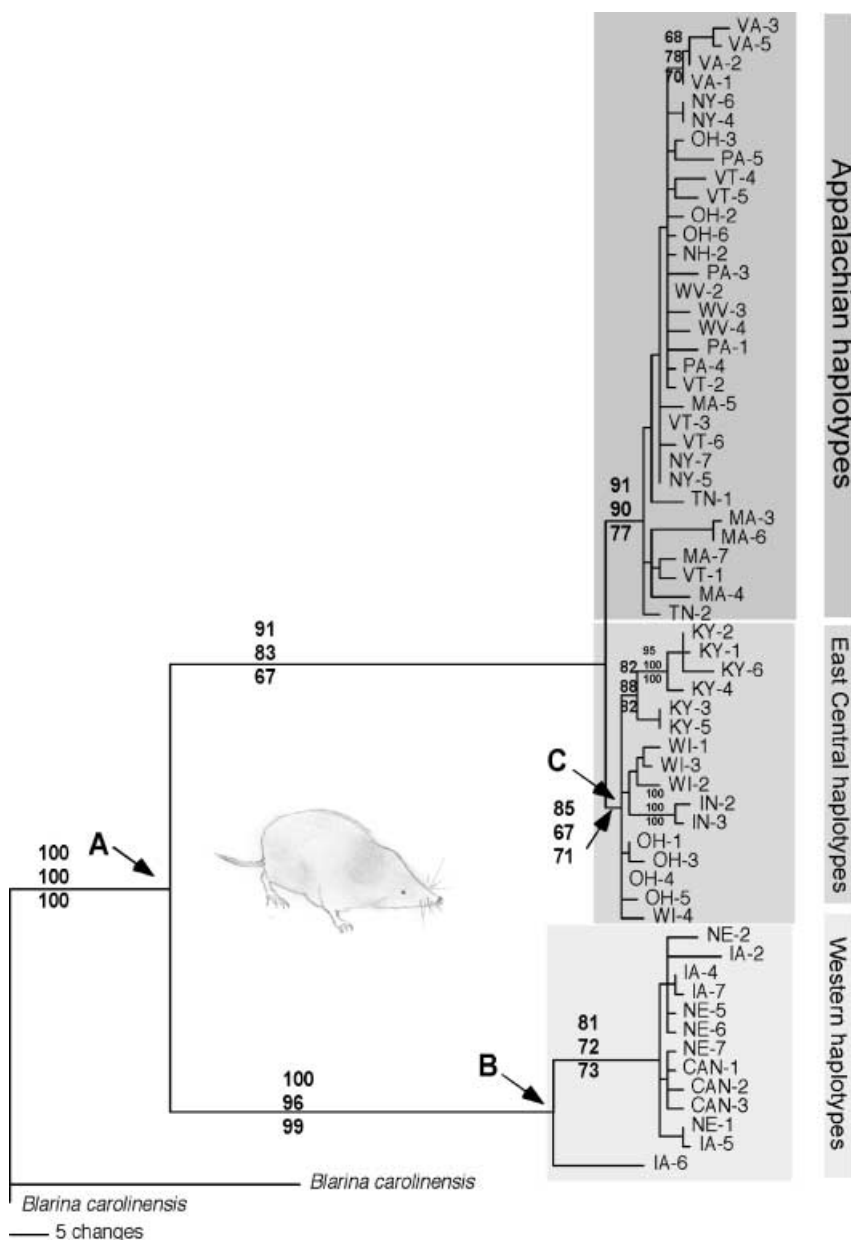
to 14 mutational steps have a probability > 0.95 of being connected in a parsimonious fashion. The nesting algorithm was terminated with the nonparsimonious union of clade 5-1 and clade 4-3 (19 mutational steps between haplotypes OH-4 and NE-6; Fig. 3). Nineteen mutational steps connect these two haplotype networks, a connection beyond the confidence limits of parsimony (Templeton *et al.* 1995). Although there are some ambiguities or loops within the joined networks, given the nesting rules in Templeton *et al.* (1987, 1992) and Templeton & Sing (1993) these loops do not affect the nested design or the conclusions drawn from the clade analysis.

In the western phylogroup, the null hypothesis of no association between haplotype distribution and geographical location could not be rejected at any of the clade levels except the total cladogram (Table 2). At the total cladogram level for the western phylogroup, a conclusion of contiguous range expansion defined the geographical distribution

**Table 2** Inference chain results of geographical distance analysis from Fig. 4

Clade	Chain of inference	Inference
West phylogroup		
West total cladogram	1-2-3-11-12	Contiguous range expansion
East phylogroup		
Clade 3-2	1-2-11-17-4	Restricted gene flow with isolation by distance
Clade 3-8	1-2-3-4	Restricted gene flow with isolation by distance
Clade 4-1	1-2-3-4	Restricted gene flow with isolation by distance
East total cladogram	1-2-11-17	Inconclusive outcome
East + West clades	1-2-3-4-9	Past fragmentation*

\*Confirmed as clades that were connected mutationally by larger than average steps.



**Fig. 2** One of the 432 most parsimonious trees based on cytochrome *b* sequences of *Blarina brevicauda*. The trees differ only in alternative placements of haplotypes within each of the three groups of haplotypes (Western, East Central, and Appalachian). Bootstrap values (50% majority rule) of the maximum parsimony and maximum likelihood analyses are shown above and the minimum evolution analyses are below the branches. Letters and numbers follow the locality and individual as in the Appendix. A = past fragmentation, B = contiguous range expansion, and C = restricted gene flow with isolation by distance, following the inference key of nested clade analysis (Table 2).

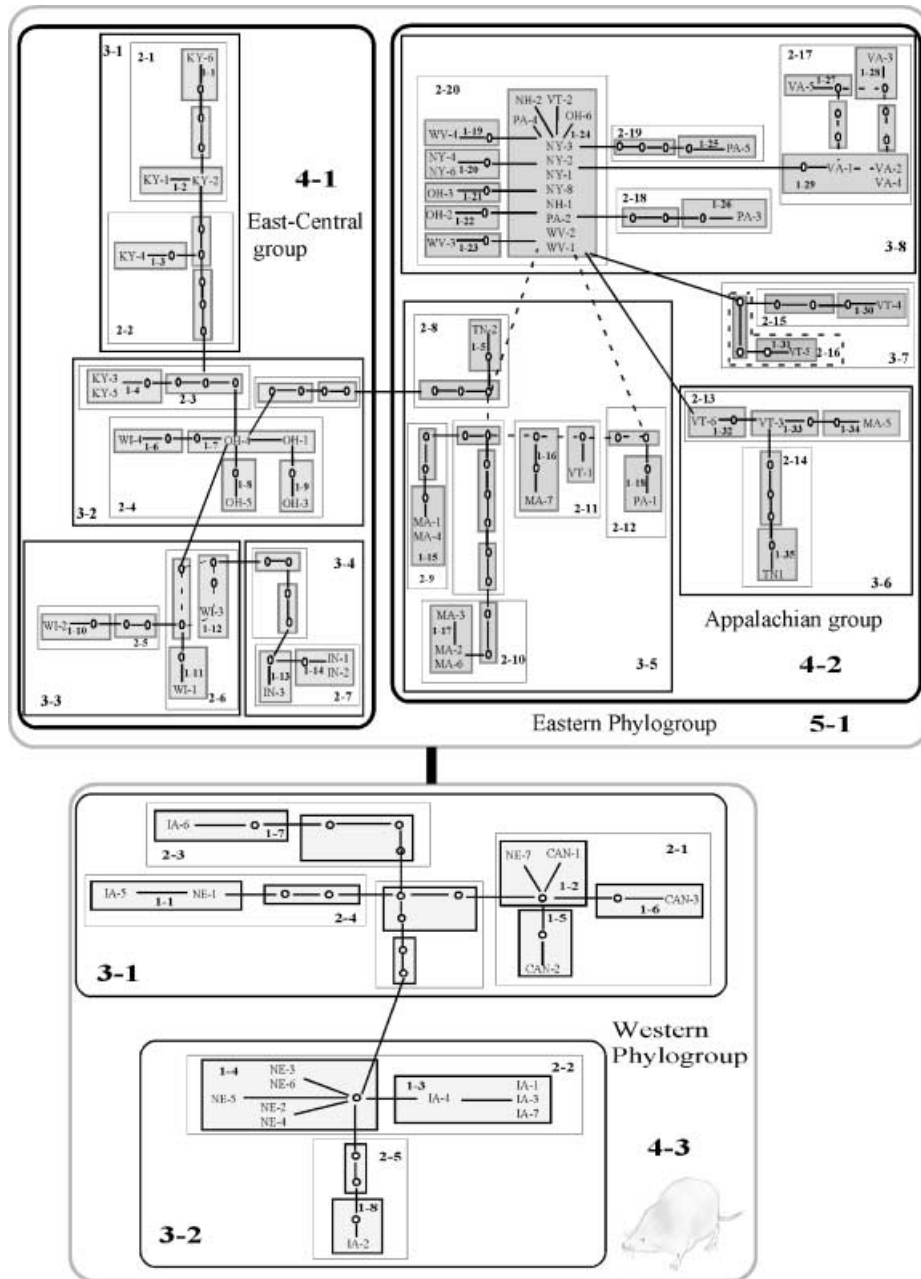
of haplotypes (Fig. 3). Within the eastern phylogroup, the nested contingency analysis detected significant geographical associations within three individual clades (Table 2). These associations (clades 3-2, 3-8 and 4-1) were all defined by restricted gene flow with isolation by distance. Clade 4-2 contained the most widespread haplotypes (Fig. 3). The total cladogram for the eastern phylogroup had an inconclusive outcome for the process responsible for separation of the East Central and Appalachian networks. The relationship between the western and eastern groups was defined by past fragmentation events (Table 2). Marked differences in genetic diversity were found between the western and eastern phylogroups. AMOVA (Table 3) revealed that more than 67% of the variation among haplo-

types is explained by the west–east split. The average nucleotide diversity value estimated within the eastern phylogroup ( $\pi = 0.01$ ) was twice the value estimated for the western phylogroup ( $\pi = 0.005$ ; Table 1).

Historical expansion of each group of populations is suggested by mostly unimodal mismatch distributions (Fig. 4) and large negative values of  $F_s$  (Fu 1997). These were  $F_s = -13.8$ ,  $F_s = -6.8$ , and  $F_s = -24.7$  for West, East Central, and Appalachian populations, respectively.

## Discussion

The goal of this study was to reconstruct the phylogeographic pattern and to evaluate the role of Pleistocene



**Fig. 3** The cytochrome *b* haplotype network (estimated cladogram) for *Blarina brevicauda* following the procedure given by Templeton *et al.* (1987, 1992). Connections < 14 mutational steps have a 0.95 probability of being parsimonious. Haplotypes are labelled as in Appendix. Solid lines connect haplotypes with a single step, boxes indicate nested clades of increasing steps; missing intermediates are indicated by an open circle; ambiguity is indicated by a dashed line; the thick solid line connecting clades 5-1 to 4-3 indicates a nonparsimonious connection of 19 steps. The western haplotypes are included in clade 4-3, the East Central haplotype are included in clade 4-1 and the Appalachian haplotypes are included in clade 4-2.

glaciations in eastern North America on the evolutionary history of *Blarina brevicauda*. The data collected contribute to a growing body of evidence suggesting that Pleistocene events played an important role in the differentiation of North American vertebrate populations (e.g. Hays & Harrison 1992; Byun *et al.* 1997; Klicka & Zink 1997;

Arbogast 1999; Burbrink *et al.* 2000; Conroy & Cook 2000; Milá *et al.* 2000; Demboski & Cook 2001; Austin *et al.* 2002). The results presented herein are in agreement with an earlier analysis of phylogenetic relationships among short-tailed shrews (Brant & Ortí 2002). Both standard phylogenetic analyses and nested clad analysis resulted

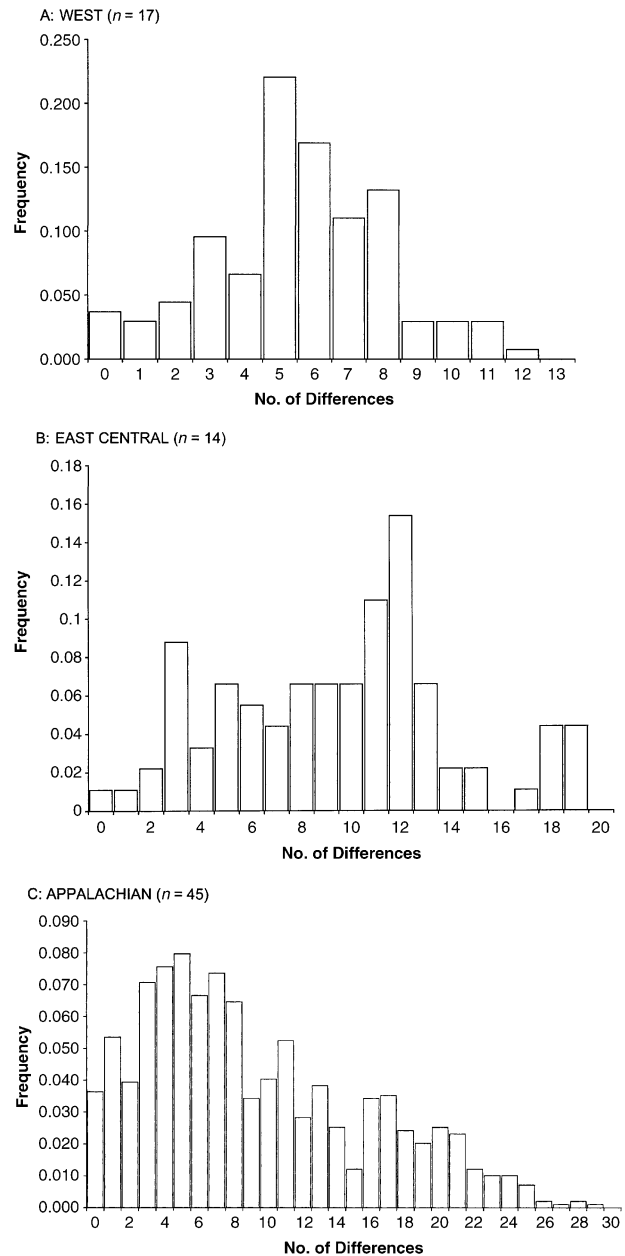
**Table 3** Results of hierarchical analysis of genetic variance

Subdivision	% variance component	
	Within populations	Between populations
1. West–East	32.8	67.2
2. West–East central–Appalachian	37.9	62.1

in similar tree topologies, with strong support for distinct western and eastern phylogroups (Fig. 2; 3). One of the advantages of using nested clade analysis is to discriminate statistically among various biological explanations for significant associations between geography and genetic data (Templeton *et al.* 1987). The biological explanations provided by this approach plus habitat requirements necessary for these shrews seem to be compatible with what is known about glacial and climatic fluctuations of the Pleistocene epoch. Much of the current distribution of *B. brevicauda* in eastern North America was covered by ice sheets during the Pleistocene glacial cycles, such that current distributions of the northwestern and northeastern phylogroups are a result of postglacial expansion in the north.

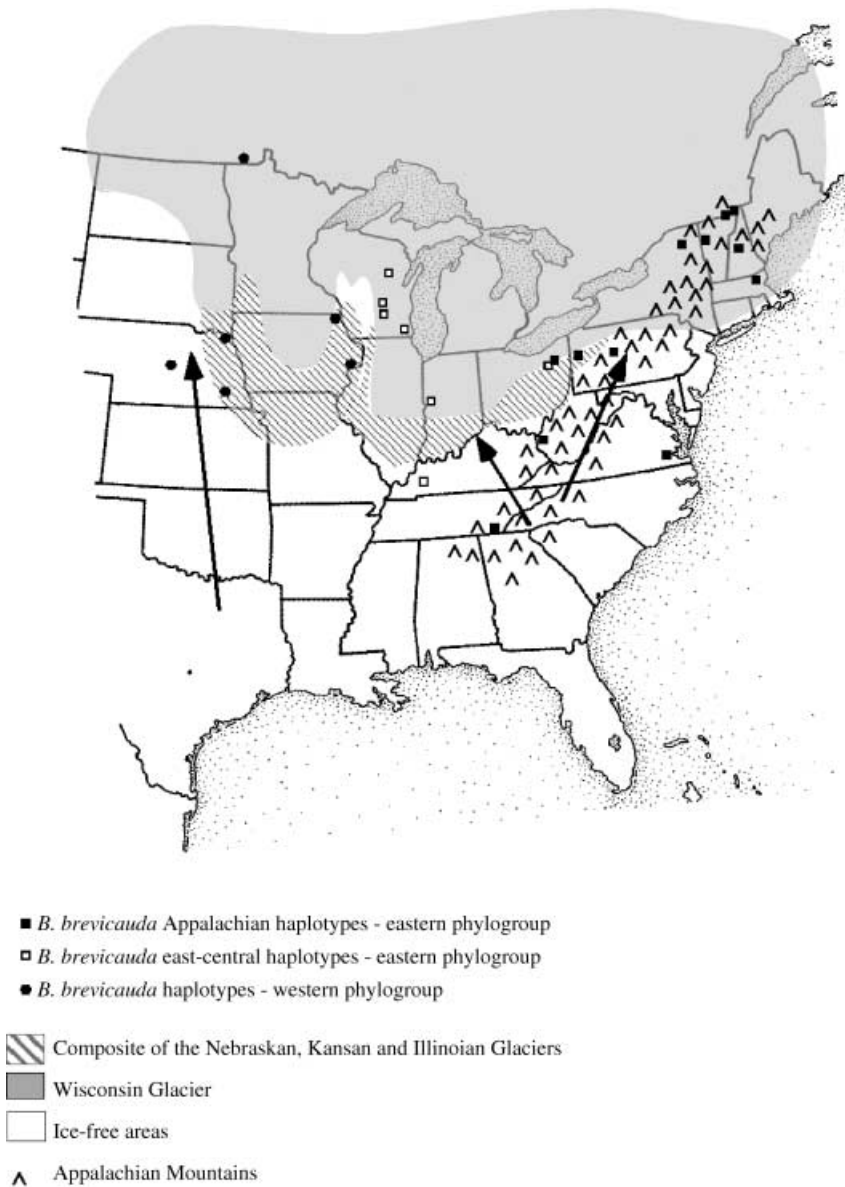
#### Phylogeography of *B. brevicauda*

The distribution of mtDNA haplotypes in *B. brevicauda* is consistent with a Category I phylogeographic hypothesis (Avice 2000). Prominent genetic gaps define deep allopatric lineages, probably originated from long-term extrinsic barriers to gene exchange (Avice 2000). The western and eastern phylogroups of *B. brevicauda* are separated by the Mississippi River, a pattern found most commonly in many 'highland' fishes, amphibians and reptiles in central North America (e.g. Blair 1958, 1965; Wiley & Mayden 1985; Robinson 1986; Mayden 1988; Walker *et al.* 1998; Burbrink *et al.* 2000; Near *et al.* 2001). These phylogroups are maintained as disjoint populations yet they still occupy relatively similar environments on opposite sides of the Mississippi drainage (e.g. Blair 1965). The high haplotype and low nucleotide diversity values obtained among all samples within each phylogroup are consistent with expectations of a Pleistocene population expansion over formerly glaciated areas (Table 1). Mismatch distributions (Fig. 4) and  $F_s$  values seem to support this scenario. This pattern is expected for recently expanding populations, each composed of several subpopulations with multiple haplotypes. Recent diversification within each population constrains the amount of variation among haplotypes. Population expansion from restricted areas to newly available habitat is consistent with Pleistocene dispersal



**Fig. 4** Mismatch distributions for each group of populations. (A) Western phylogroup ( $n = 17$  individuals), (B) East Central phylogroup ( $n = 14$  individuals); (C) Appalachian phylogroup ( $n = 45$  individuals).

from southern refugia into northern areas as glaciers retreated. Likewise, the tree topology with shallow, largely unresolved terminals within each of the two phylogroups (western and eastern) and a longer branch leading to the western phylogroup also supports the proposed scenario. The time of separation between the eastern and western clades was estimated at about 1.2 million years ago (Ma), following the onset of the Pleistocene, about 2.5–3.0 Ma (Brant & Ortí 2002). The nested clade statistical inferences



**Fig. 5** Map showing the partial extent of Pleistocene glaciation and the hypothesized northern dispersal routes of *Blarina brevicauda* mitochondrial clades from southern refugia following glacial retreat (glacial extent after Fenneman 1938).

are consistent with differentiation in isolated glacial refugia and subsequent expansion into ice-free areas.

It is also possible that before the Pleistocene, populations of *B. brevicauda* were less structured because of the high east-west gene flow across their northern distribution in Canada where the Mississippi River was not a severe barrier. The molecular evidence suggests that the two phylogroups of *B. brevicauda* became isolated probably some time during the onset of the earliest Pleistocene glaciers (Nebraskan) and that current patterns of genetic variation reflect postglacial redistribution of lineages with limited gene flow among them (Fig. 5). During each interglacial period of the Pleistocene, the Mississippi River experienced alluviation of valleys cut during the previous glacial stages, during which the flood plain extended well beyond

its current boundaries (Thornbury 1965; Burr & Page 1986) fed by cold water influx from the melting glaciers. These river conditions would have easily prevented the dispersal of *B. brevicauda*, thus maintaining a barrier between the western and eastern phylogroups. Furthermore, as the ice began to shrink, expanding areas of open ground became available, but until vegetation migrated to fill those areas, there was no habitat for animal migrants (Pielou 1991). The dynamics of the moving waters and desert-like habitat during the interglacials, and valleys with low moisture during the glacial periods would have prevented the movement of large numbers of shrews across the Mississippi River valley. The high metabolism, low vagility and moisture requirements of these shrews would have made it nearly impossible for individuals to migrate across this barrier.

### Phylogeography of the western phylogroup

The phylogeographic pattern of the western haplotypes in the nested clade analysis was described at the total cladogram level because of contiguous range expansion events (Table 2). There was no significant geographical structuring of haplotypes within the network, despite haplotypes being separated geographically by more than 400 km (IA, NE and CAN; Fig. 3). The results of nested clade analysis are consistent with fluctuations in habitat availability in eastern North America during Pleistocene glacial cycles. Presence of shrews in these habitats could only have become possible once the glaciers retreated. The location of a glacial refugium for the western phylogroup is unclear but fossil and climate evidence suggest it was most likely in or around Oklahoma, Texas, and/or southern Kansas (Jones *et al.* 1984). The climate in these southern areas during the glacial periods would have been more similar to the current higher latitude climate (Pielou 1991), and thus would have been able to support the short-tailed shrews. The formidable barriers of the Mississippi River and the Rocky Mountains would have prevented the shrews from migrating too far to the east or west.

The North American Midwest region has relatively little topographic and climatic diversity. As a result, there has been relatively little environmental basis for local differentiation among species in these regions (Stein *et al.* 2000). Similarly, the tree topology of the western phylogroup (Fig. 2), with shallow branches, little internal resolution, bootstrap support (Fig. 2), and the low sequence differences (Table 1) are consistent with an expanding population, after a colonization event into the previously glaciated areas. The average nucleotide diversity of the western phylogroup was half that reported for the eastern phylogroup. This difference in nucleotide diversity values, the unimodal mismatch distribution (Fig. 4A) and the large negative value of  $F_s$  (-13.8) all lend support to the idea that this group has undergone a demographic expansion more recently than the eastern phylogroup.

### Phylogeography of the eastern phylogroup

The phylogeographic pattern of the eastern haplotypes in the nested clade analysis was described mostly by restricted gene flow and isolation by distance (clades 3-2, 3-8 and 4-1; Table 2). However, at the higher nesting level (clades 4-1 vs. 4-2; Fig. 3) application of the inference key did not produce a biologically significant inference (Table 2). The result of 'inconclusive outcome' suggests inadequate sampling; therefore, future sampling should be directed towards the gaps in sampling locations between the two clades (Table 2; Templeton *et al.* 1995; Templeton 1998). The inference of restricted gene flow and isolation by distance is consistent with cycles of southerly retreat

followed by northerly advances of these shrews during the glacial cycles of the Pleistocene, confining the shrews to areas with sufficient moisture.

Pleistocene fossils suggest that many groups of animals tracked the movement of their preferred vegetation types closely (Hibbard *et al.* 1965). Therefore, it is possible that the southern glacial refugia for the eastern clade of *B. brevicauda* haplotypes were located in isolated unglaciated areas of the Appalachian Mountains. Support for the above proposal comes from palaeobotanical evidence that species of eastern white pine and eastern hemlock survived the Wisconsin glaciation in refugia in the eastern foothills of the Appalachians and on the adjacent coastal plain (Davis 1981; Pielou 1991). These trees seem to have habitat requirements similar to species of *Blarina*. Miller & Getz (1977) reported that short-tailed shrews have broad habitat requirements but that they were most common in areas with more than 50% herbaceous cover. In agricultural areas of Great Plains habitat, shrews are confined to riparian or other mesic habitats, such as grassy roadside ditches and fences with ample ground cover and litter (Genoways & Choate 1972; Choate & Fleharty 1973). Generally, shrews are not found in areas where moisture content of the soil is insufficient to keep the air in the soil and litter saturated (Pruitt 1953, 1959; Getz 1961). Eastern hemlock requires rich, moist soil and shaded habitat conditions, also preferred by *B. brevicauda* (Hibbard 1963; Hibbard *et al.* 1965; George *et al.* 1986), suggesting that climate during the glacial advances in the Appalachians must have been adequate to support both species of trees and shrews. Therefore, possible glacial refugia in the southern Appalachian by the eastern phylogroup of *B. brevicauda* is supported by the palaeobotanical data (Davis 1981).

A study of the North American rat snake (Burbrink *et al.* 2000) found that the Appalachian Mountains are a significant barrier to gene flow, leading to the isolation of an eastern clade of snake haplotypes. This result was not found for *B. brevicauda*, indicating that although heterogeneous habitat across the Appalachians may restrict gene flow, these mountains do not constitute an insurmountable barrier for shrews.

The eastern and western phylogroups of *B. brevicauda* should both exhibit signs of range expansion events, because each phylogroup now occupies habitat previously covered by glaciers. Climatic and topographic differences (Stein *et al.* 2000) between western and eastern areas might explain the different processes inferred for each phylogroup by nested clade analysis. Most of the Central Lowlands (Fenneman 1938) has relatively little topographic diversity, and climatic zones generally grade subtly from one area to another. Consequently, there has been little environmental basis for local differentiation among species in the Central Lowlands, relative to

species that occupy areas to the east (e.g. the Appalachians), where regional geomorphology is very diverse (Thornbury 1965; Stein *et al.* 2000). Vegetation and topographic diversity, as well as practice of agriculture and road building, probably have contributed to the current pattern of restricted gene flow in the east, because shrews are not found commonly in dry, exposed habitat, dispersal is limited, and roads and streams can form formidable barriers to dispersal (Genoways & Choate 1972; Choate & Fleharty 1973; Benedict 1999). The relative topographic and climatic uniformity throughout the Central Lowlands (Fenneman 1938) plus a tolerance for drier conditions in the western phylogroup have probably contributed to the lack of structure in the western phylogroup (Graham & Semken 1976; Graham 1976). To understand further the processes responsible for the observed pattern, more collections from areas not covered by the glaciers will be necessary.

#### Taxonomy of *B. breviceauda*

There are 11 subspecies of *B. breviceauda* recognized on the basis of morphology (Hall 1981; Jones *et al.* 1984; George *et al.* 1986) that have been divided into two semi-species. The *brevicauda* semi-species and the *talpoides* semi-species (Jones *et al.* 1984; George *et al.* 1986) correspond exactly to the western and eastern phylogroups, respectively. The larger (western) and smaller (eastern) phenotypes have been recognized in the fossil record from at least the early Pleistocene (Jones *et al.* 1984), results that correspond roughly to their time since divergence around 1.2 Ma (Brant & Ortí 2002).

If populations have been separated long enough by historical barriers to gene flow, geographical segregation of lineages along recognized boundaries based on morphological variation could be expected (Avise 1994). Thus, the concordance between the two phenotypes of shrews constituting semi-species (*talpoides* and *brevicauda* semi-species) and the cytochrome *b* partition of variation (western and eastern phylogroups) suggests that this gene tree correctly identifies the phylogenetic history of shrews in this region. Detailed analyses of the palaeoecology of Pleistocene *Blarina* suggested that the two semi-species of *B. breviceauda* were separated by moisture extremes (Graham & Semken 1976; Graham 1976). It is worthwhile noting that the southern boundary for *B. breviceauda* (northern boundary of *B. carolinensis*; Genoways & Choate 1998) corresponds roughly to the northern extent of the coastal plains (Fenneman 1938; Thornbury 1965; Robinson 1986). Hibbard *et al.* (1965) described two 'evolving' lineages (*B. carolinensis* and *B. breviceauda*) and associated fauna, supporting an interpretation that the observed morphological differences in these two shrews are the result of shifts in ecology. A coastal plains boundary seems common

for populations that might have survived the Wisconsin glacial stage in a Florida refugium (Blair 1965).

It has been suggested that chromosomal rearrangements may result in diversification or speciation in some groups of shrews (e.g. Zima *et al.* 1998). However, the absence of eastern and western chromosomal races of *B. breviceauda* does not support the eastern and western mtDNA phylogroups found in this study (Meylan 1967; Genoways *et al.* 1977; George *et al.* 1982). Consequently, karyotypic differences between populations of shrews as reported in the literature are not a likely explanation responsible for, or a consequence of, the two well-supported phylogroups. However, because chromosome number is only one type of the possible karyotypic diversity, more studies are needed to verify the significance of chromosomal evolution in the diversification of the short-tailed shrews.

The Pleistocene glacial cycles of North America and Eurasia were once thought to be responsible for the speciation of many groups of organisms (Hewitt 1996) but growing evidence from several phylogeographic studies are defining an earlier time of diversification, during the Pliocene or Miocene (Zink & Slowinski 1995; Klicka & Zink 1997) while the Pleistocene glacial cycles have contributed to diversification at the intraspecific level. Phylogeographic studies in other regions of North America show the genetic impact of the regional Pleistocene history on small mammals, suggesting that the Pleistocene events are responsible for recent population structure, but not for speciation of these groups. This seems to be the case for flying squirrels (Arbogast 1999), black bears (Wooding & Ward 1997), red-tailed chipmunks (Good & Sullivan 2001), and dusky shrews (Demboski & Cook 2001) in the Pacific Northwest and Rocky Mountain areas.

Among other mammalian taxa a similar pattern to the one found in this paper was described by Hays & Harrison (1992) for the woodrat, *Neotoma floridana*, in eastern North America. They reported distinct western and northeastern mtDNA clades. Within the latter, they found low mtDNA sequence differences and hypothesized a recent range expansion, after the glaciers retreated, from refugial populations in southeastern North America to New York, northern New Jersey and northeastern Pennsylvania, starting about 15 000 years ago. Likewise, the western clade of *N. floridana* haplotypes had low sequence differences, indicating a recent range expansion into the Great Plains. As there was a large sequence difference between the western and the eastern clades, they hypothesized separate Pleistocene refugia during the glacial advances. Future studies of co-distributed taxa in eastern North America will test the generality of this pattern. With additional analyses of comparative phylogeographic patterns of organisms distributed across this region, the effect of Pleistocene glaciations and the Mississippi River basin on population structure of tetrapods can be tested rigorously.

## Conclusions

The cytochrome *b* sequence data presented here were used to produce the first comprehensive phylogeographic analysis for a species of short-tailed shrews. Major findings of this research includes (i) strong support for an eastern and western clade of populations of *B. brevicauda*, found in Brant & Ortí (2002), (ii) evidence that the glaciated parts of eastern North America were colonized by a minimum of two sets of invading populations or phylogroups, and (iii) results suggesting refugia around Oklahoma, Texas, and/or southern Kansas for the western phylogroup, southern Appalachians for the eastern phylogroup, then subsequent range expansion of both western and eastern phylogroups into the formerly glaciated areas (Fig. 5). The habitat requirements of the species, the climatic fluctuations, and the formidable barrier of the Mississippi River are proposed to have been significant in maintaining the separation of the two phylogroups during the Pleistocene.

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This research comprises part of SB's PhD thesis, which was concerned with examining the micro- and macroevolutionary patterns and processes affecting the genetic structure of short-tailed shrews and their nematode parasites. S.B. is currently a postdoctoral fellow at Louisiana State University examining co-evolutionary relationships among Mexican gophers and their lice. GO is the advisor for SB's PhD thesis and his research focuses on using molecular markers to reconstruct the gene genealogies ranging from paternity analysis to deep phylogenies, focusing mainly on the molecular systematics of fishes. For more information, see <http://golab.unl.edu/>.

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## Appendix

Individuals of *Blarina brevicauda* included in this study.

Collection locality	Spec.†	Field ID no.	Latitude; longitude	GenBank accession number
Iowa	IA-1	1132	43.1000; 91.1833	AF395466*
	IA-2	1385	41.5844; 90.8999	AF533605
	IA-3	1386	41.5844; 90.8999	AF533607
	IA-4	1387	41.5844; 90.8999	AF533606
	IA-5	1388	41.5844; 90.8999	AF533608
	IA-6	1390	41.5844; 90.8999	AF533610
	IA-7	1391	41.5844; 90.8999	AF533609
Nebraska	NE-1	1007	40.8585; 96.8573	AF395464*
	NE-2	1023	42.5909; 96.7537	AF533602
	NE-3	1025	42.5909; 96.7537	AF533601
	NE-4	1027	42.5909; 96.7537	AF395465*
	NE-5	1037	41.6856; 98.9256	AF533603
	NE-6	1040	41.6856; 98.9256	AF533604
	NE-7	1042	41.6856; 98.9256	AF395461*
Manitoba Canada	CAN-1	1096	49.1000; 95.9833	AF395462*
	CAN-2	1097	49.1000; 95.9833	AF395463*
	CAN-3	1098	49.1000; 95.9833	AY138971
Kentucky	KY-1	1209	36.7452; 87.7745	AF534121
	KY-2	1220	36.7452; 87.7745	AF533632
	KY-3	1221	36.7452; 87.7745	AF395467*
	KY-6	1227	36.7452; 87.7745	AF533633
Indiana	IN-1	1360	39.9785; 87.1493	AF533634
	IN-2	1361	39.9785; 87.1493	AF533635
	IN-3	1362	39.9785; 87.1493	AF533636
Wisconsin	WI-1	1402	43.19; 89.40	AF533611
	WI-2	1403	43.6076; 89.3907	AF533642
	WI-3	1407	42.821; 88.587	AF533637
	WI-4	1422	44.8847; 89.2633	AF533643
Ohio	OH-1	1091	40.8045; 81.9834	AF395469*
	OH-2	1093	40.8045; 81.9834	AF533613
	OH-3	1094	40.8045; 81.9834	AF395472*
	OH-3	1338	41.1768; 81.6493	AF533629
	OH-4	1340	41.1768; 81.6493	AF533630
	OH-5	1341	41.1768; 81.6493	AF533631
Massachusetts	OH-6	1342	41.1768; 81.6493	AF533614
	MA-1	1363	42.5072; 71.2930	AY138978
	MA-2	1367	42.5072; 71.2930	AF533638
	MA-3	1368	42.5072; 71.2930	AF534122
	MA-4	1369	42.5072; 71.2930	AF533640
	MA-5	1370	42.5072; 71.2930	AY138973
	MA-6	1374	42.5072; 71.2930	AF533639
Tennessee	MA-7	1375	42.5072; 71.2930	AY138979
	TN-1	1379	35.3468; 84.0612	AF534123
Vermont	TN-2	1380	35.3468; 84.0612	AF533641
	VT-1	1398	44.148; 73.0179	AY138980
	VT-2	1399	44.148; 73.0179	AF534118
	VT-3	1400	44.148; 73.0179	AF533623
	VT-4	1436	44.965; 71.7383	AY138975
	VT-5	1437	44.82; 71.91	AF533624
	VT-6	1439	44.82; 71.91	AF534119

Appendix *Continued*

Collection locality	Spec.†	Field ID no.	Latitude; longitude	GenBank accession number
New York	NY-1	1392	44.07; 74.28	AF533625
	NY-2	1393	44.07; 74.28	AY138976
	NY-3	1395	44.07; 74.28	AF533626
	NY-4	1396	44.07; 74.28	AF533612
	NY-5	1426	44.07; 74.28	AY138977
	NY-6	1427	44.07; 74.28	AF534115
	NY-7	1428	44.07; 74.28	AF534120
	NY-8	1429	44.07; 74.28	AF533627
Virginia	VA-1	1315	37.2301; 76.6230	AY138972
	VA-2	1317	37.2301; 76.6230	AF534116
	VA-3	1318	37.2301; 76.6230	AF395473*
	VA-4	1319	37.2301; 76.6230	AF533615
	VA-5	1320	37.2301; 76.6230	AF395474*
West Virginia	WV-1	1376	38.28; 82.35	AF534117
	WV-2	1377	38.28; 82.35	AF533617
	WV-3	1378	38.28; 82.35	AF533618
	WV-4	1420	38.28; 82.35	AY138974
Pennsylvania	PA-1	1365	40.8531; 80.3994	AF533619
	PA-2	1410	40.5683; 79.02667	AF533621
	PA-3	1411	40.5683; 79.02667	AF533616
	PA-4	1412	40.5683; 79.02667	AF533622
	PA-5	1414	40.5683; 79.02667	AF533620
New Hampshire	NH-1	1129	43.6761; 72.0610	AF395470*
	NH-2	1152	43.6761; 72.0610	AF395471*

\*GenBank accession numbers from Brant & Ortí (2002).

†Spec., specimen.