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Post-glacial recolonization of the Great Lakes region by the common garter snake (*Thamnophis sirtalis*) inferred from mtDNA sequences

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Abstract

Pleistocene events played an important role in the differentiation of North American vertebrate populations. Michigan, in particular, and the Great Lakes region, in general, were greatly influenced by the last glaciation. While several hypotheses regarding the recolonization of this region have been advanced, none have been strongly supported. We generated 148 complete ND2 mitochondrial DNA (mtDNA) sequences from common garter snake (*Thamnophis sirtalis*) populations throughout the Great Lakes region to evaluate phylogeographic patterns and population structure and to determine whether the distribution of haplotypic variants is related to the post-Pleistocene retreat of the Wisconsinan glacier. The common garter snake was utilized, as it is believed to have been one of the primary vertebrate invaders of the Great Lakes region following the most recent period of glacial retreat and because it has been a model species for a variety of evolutionary, ecological, behavioral, and physiological studies. Several genetically distinct evolutionary lineages were supported by both genealogical and molecular population genetic analyses, although to different degrees. The geographic distribution of the majority of these lineages is interpreted as reflecting post-glacial recolonization dynamics during the late Pleistocene. These findings generally support previous hypotheses of range expansion in this region.

Keywords: Glaciation; Great Lakes; Michigan; Mitochondrial DNA; Molecular phylogeography; Natricine; Nested clade analysis; Recolonization; *Thamnophis*; *Thamnophis sirtalis*

1. Introduction

Thamnophis sirtalis is the widest ranging reptile in North America (Rossman et al., 1996) due, in part, to its generalist nature. Populations of *T. sirtalis* are known from nearly every natural habitat across its range, except for deserts, as well as from many urban areas. Within those habitats *T. sirtalis* preys on earthworms, leeches, fish, amphibians, small mammals, birds, and even roadkill (Harding, 1996; Rossman et al., 1996). *Thamnophis sirtalis* is arguably the most researched snake in the world, being used for short- and long- term studies on behavior, physiology, ecology, and evolution for more than fifty years (e.g., Burghardt, 1969; Carpenter, 1952; de Queiroz et al., 2002; Fitch, 1965; Janzen et al., 2002; Rossman et al., 1996; Seigel et al., 1987; Seigel and Collins, 1993). It is also the only snake species to be nominated to have its whole genome sequenced. While phylogeographic work has been performed with *T. sirtalis* subspecies found on the west coast of the USA (Janzen et al., 2002) and with some Canadian populations (Rye, 2000), virtually nothing is known about the phylogeography of *T. sirtalis* distributed east of the Rocky Mountains in the United States or for the genus in general (Alfaro and Arnold, 2001). Given the research popularity of this species,

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it seems timely to examine the phylogeography of *T. sirtalis* in the more central and eastern parts of its vast range. We chose to examine the phylogeography of the widest ranging subspecies of *T. sirtalis*, the eastern garter snake, *T. s. sirtalis*, with an emphasis on the recolonization of Michigan and the Beaver Archipelago of northeastern Lake Michigan, following the last glaciation of the Pleistocene.

During the Pleistocene, climatic changes associated with glacial advances and retreats resulted in range reductions for a variety of taxa worldwide and most likely influenced the current genetic composition and diversification of those taxa (Hewitt, 1996, 2000, 2001). The Great Lakes region of North America, specifically, was marked by dramatic climate change, fluctuating lake levels, and isostatic rebound resulting from a series of glacial and interglacial oscillations (Petty et al., 1996). As recent as 18,000 years before present (ybp), the Wisconsinan glacier covered the majority of the area occupied by the Midwest region of the United States of America, not fully retreating until ca. 10,000 ybp (Anderson and Lewis, 1992; Larsen, 1987; Webb et al., 1993). These events had a dramatic effect on the distribution of taxa in this region, with the current inhabitants recolonizing only relatively recently after the retreat of the glaciers (e.g., Holman, 1992, 1998). Without taking into account these historical events, the current distributions and underlying genetics of taxa in this region and other areas influenced by the events of the Pleistocene cannot be fully understood. To date, most studies on the phylogeographic impact of Pleistocene events on North American taxa have been conducted on southern or western continental species, with few such studies focusing on taxa that have recolonized previously glaciated regions (Austin et al., 2002; Ayoub and Reichert, 2004; Bernatchez and Dodson, 1991; Billington et al., 1992; Fuerst and Austin, 2004; Green et al., 1996; Hewitt, 1999, 2000, 2001; Janzen et al., 2002; Smith and Green, 2004; Zamudio and Savage, 2003). Therefore, our study not only adds to our knowledge of *T. sirtalis* ecology and evolution, but also provides us with one of few phylogeographic studies on a member of the Midwest fauna.

A hypothetical pattern of colonization in which primary amphibian and reptilian invaders closely followed the non-uniform retreat of the ice sheet into the areas surrounding the Great Lakes has been proposed based on geological data, paleobotanical and paleovertebrate assemblages, and ecological tolerances of modern reptilian and amphibian fauna (Holman, 1992). The ice sheet extended well into Illinois, Indiana, Ohio, and Wisconsin, and completely covered Michigan during its greatest extent (Fig. 1). Since primary reptile and amphibian invaders are believed to have stayed close to the margins of the ice sheet, they probably never completely moved out of Illinois, Indiana, Ohio,

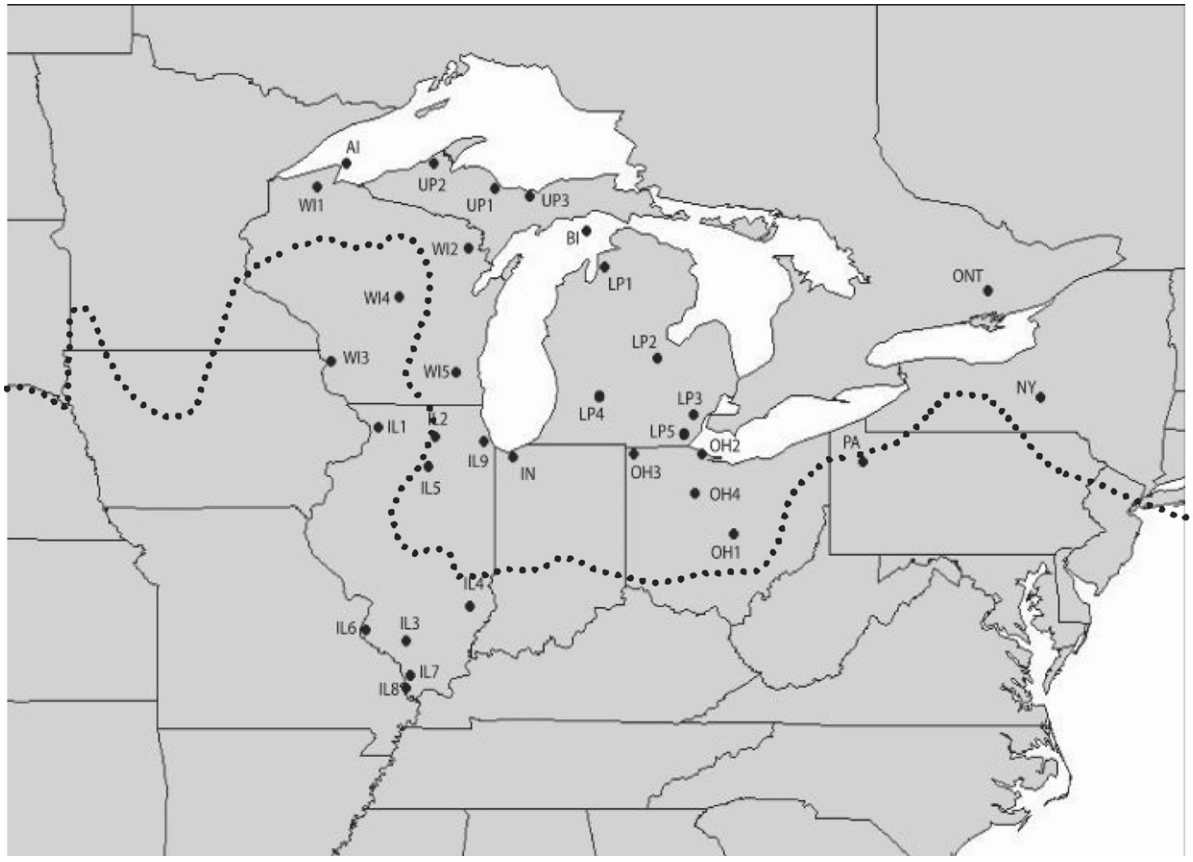

Fig. 1. Collection localities for *Thamnophis sirtalis* populations sampled for this study. Exact localities for all collection sites are listed in Appendix A, and are identified by alphanumeric characters. Dotted line represents the maximum southern extent of Wisconsinan ice sheets (modified from Holman, 1992).

and Wisconsin. However, Michigan populations must have either become locally extinct or retreated to more southerly states not affected by the ice sheet. As the ice sheet retreated and Michigan became exposed, invaders are believed to have entered the lower peninsula of Michigan from Indiana and Ohio, and the upper peninsula of Michigan from Wisconsin. Individuals are also believed to have colonized southern Ontario, Canada through the gap between Lake Erie and Lake Huron via Michigan. In addition to the hypothesized patterns of mainland recolonization, hypotheses regarding the recolonization of the archipelagos found in each lake have also been generated and vary from island system to island system. For our study, we not only sampled from mainland sites throughout the Great Lakes region, but also from an archipelago in Lake Michigan that may provide further support for the two-front Michigan recolonization hypothesis.

Great Lakes island age and mode of origin are variable, ranging from 2000 to 10,000 years and including both land bridge islands once part of the mainland and 'oceanic' islands that emerged as lake levels declined (Karrow and Calkin, 1985; Nuhfer and Dalles, 1987; Petty et al., 1996). We focused on the Beaver Archipelago (Fig. 2), which is located in northeastern Lake Michigan ca. 25–30 km from both the lower and upper peninsula of Michigan. It is the largest archipelago in Lake Michigan, and it is located in an area that may act as a secondary contact point for upper and lower peninsula *T. sirtalis* lineages. The archipelago consists of one main island (Beaver, 15130.0 hectares (ha)), three moderately large islands (Garden, High, and Hog, 1023.9–1989.0 ha), and six smaller islands (Hat, Pismire, Shoe, Squaw, Trout, and Whiskey, 1.0–52.3 ha). All but the smallest islands (i.e., Hat, Pismire, and Shoe) are thought to support, or historically to have supported, populations of *T. sirtalis* (Hatt et al., 1948; Placyk and Gillingham, 2002). It has been hypothesized that since the archipelago was at one time connected by a land bridge to the lower peninsula of Michigan (Dietrich, 1988; Hough, 1958; Kapp et al., 1969), the taxa found on the islands are descendents of lower peninsula populations. Further support for this view is that some species found in the Beaver Archipelago are rare (eastern milksnake, *Lampropeltis t. triangulum*) or absent (northern ribbonsnake, *T. sauritus septentrionalis*) from the upper peninsula of Michigan but common in the lower peninsula. However, many reptiles and amphibians, including gartersnakes, are adept swimmers and may have been able to colonize the archipelago at any point (as indicated by their presence on oceanic islands in the upper Great Lakes, King, 1988). In fact in the last 10,000 years the archipelago was separated from Michigan's upper peninsula by the Mackinac River, which may have only been 1.6 km wide in some areas (Hough, 1958). By sampling these islands, we hope to shed light on the evolutionary history of Beaver Archipelago *T. sirtalis* populations and more fully understand the phylogeography of *T. sirtalis* in the Great Lakes region, especially in Michigan.

Thamnophis sirtalis is a model species for the examination of phylogeographic patterns in the Great Lakes region for several reasons. First, evidence from fossil deposits of ca. 15,000–14,000 ybp indicate that *T. sirtalis* was present in the Midwest during the last glaciation and that it stayed

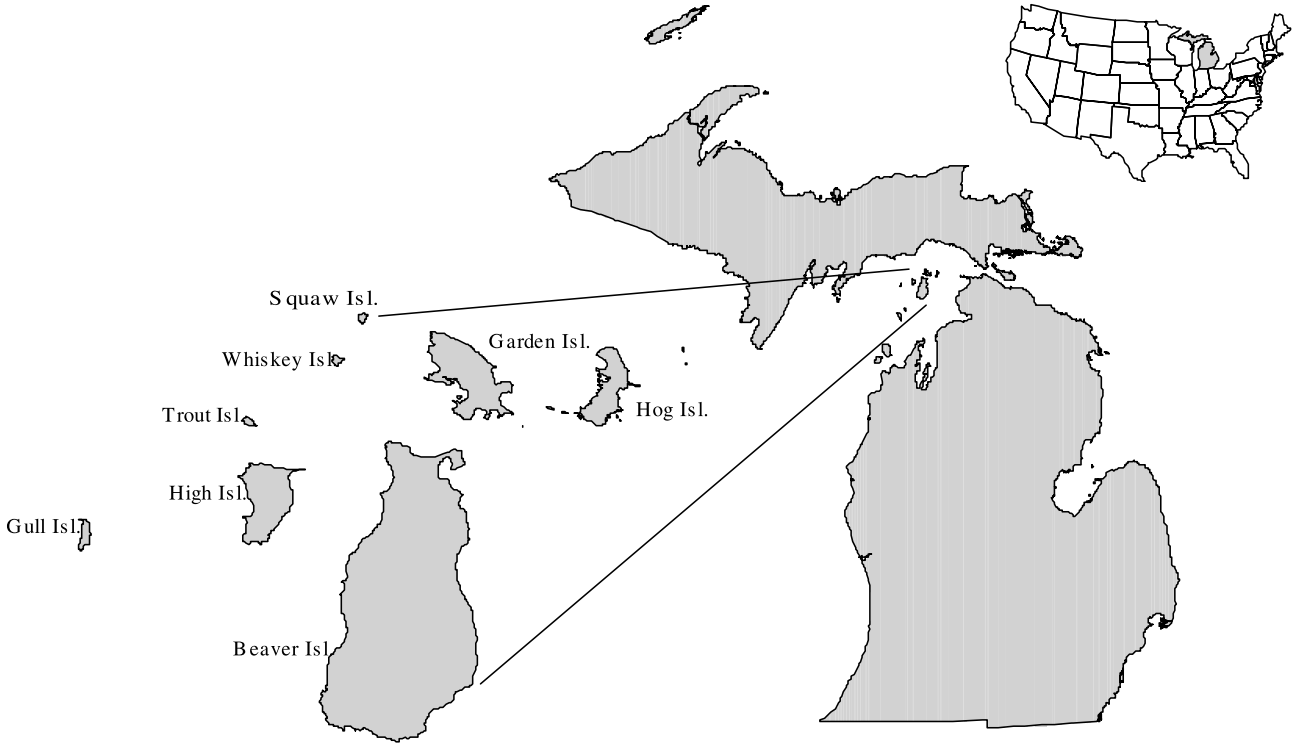

Fig. 2. The Beaver Archipelago of Lake Michigan located in Charlevoix County, Michigan, USA (modified from Hansknecht, 2003).

close to the margins of the retreating glacier (Holman, 2000). Holman (1992) considers it to be one of the primary vertebrate invaders of the postglacial Midwest. Second, *T. sirtalis* is currently considered the most common reptile species in the region, occurring in almost every county and on every Great Lakes archipelago (Harding, 1996) making the collection of specimens and tissue samples for our study relatively simple. Finally, molecular resources (e.g., mtDNA and microsatellite primers) have been developed for this species and are readily available (e.g., Garner et al., 2002, 2004; Garner and Larsen, 2005; Kumazawa et al., 1996; McCracken et al., 1999).

While the hypotheses on the postglacial recolonization of the Great Lakes region discussed above are based on fossil and other geological data, they have not yet been specifically tested with any other type of data with few exceptions (e.g., Smith and Green (2004) used mitochondrial DNA sequences with toad, *Bufo fowleri*, populations located around Lake Erie). Our goal is to use phylogeographic and population genetic analysis of mtDNA sequence data to test hypotheses regarding the recolonization of the Great Lakes region and specifically of Michigan and the Beaver Archipelago following the last glaciation. Michigan serves as the focal point for this work, because this study is part of a broader project that is examining geographic variation in the phenotypic traits of Beaver Archipelago *T. sirtalis* populations and populations from the surrounding mainland (Placyk, 2006). Using mitochondrial DNA (mtDNA) sequences to reconstruct phylogenetic relationships among *T. sirtalis* from the Great Lakes region, we address several important evolutionary issues to help understand recolonization following the last glaciation. In particular, we are interested in (I) how populations are genetically structured within the Great Lakes region, especially in Michigan, and (II) if this structuring and the pattern of haplotypic variation are consistent with historical geography. In addition, our work will provide the first such study on a reptile from this region, as well as much needed data on recolonization of previously glaciated areas in the Great Lakes region in general.

2. Methods

2.1. Taxon sampling

Mitochondrial haplotypic variation was examined in 148 individuals of *T. s. sirtalis* from 37 locations (Fig. 1, Appendix A). Sampling was concentrated in sites surrounding and within Lake Michigan to examine fine scale patterns of haplotypic variation with additional sites examined from across much of the eastern Midwest states (i.e., Illinois, Indiana, Michigan, Ohio, and Wisconsin), Pennsylvania, and Ontario, Canada to begin to resolve larger geographic patterns of haplotype radiation following the Wisconsinan glaciation. We also heavily focused sampling efforts in the Beaver Archipelago of northeastern Lake Michigan as the evolutionary history of taxa on these islands is a point of some controversy (Hatt et al., 1948) and it may act as a point of secondary contact for two lineages. Samples were obtained from numerous sources as frozen muscle tissue or tail tips from live specimens and as previously extracted DNA (see Appendix A for sources). Many times voucher specimens were collected, but a small fraction of our sources did not have vouchers available (Appendix A). This was deemed appropriate, as the mtDNA region we amplified (i.e., ND2) is known to differ significantly between *T. sirtalis* and the five other *Thamnophis* species (i.e., *T. butleri*, *T. brachystoma*, *T. radix*, *T. sauritus*, *T. proximus*) that may occur sympatrically with *T. sirtalis* throughout our sampling range (Alfaro and Arnold, 2001; Placyk, unpub. data) and as a result, could easily be eliminated from our alignment.

2.2. DNA extraction and sequencing

For most individuals, we used tail tips to obtain total genomic DNA with the DNeasy® Tissue Kit (Qiagen). The entire ~1020 bases of ND2 were PCR-amplified using the forward primer L4437b (5′-CAG CTA AAA AAG CTA TCG GGC CCA TAC C-3′; Kumazawa et al., 1996), which lies in the tRNA-Met upstream of ND2, and the reverse primer Sn-ND2r (5′-GGC TTT GAA GGC TMC TAG TTT-3′; R. Lawson, pers. comm.), which lies in the tRNA-Trp downstream of ND2. Polymerase chain reactions (PCR) were conducted in 25-μL volumes with 1.0 μL DNA, 1× ExTaq PCR buffer (PanVera/TaKaRa), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μg/μL bovine serum albumin, 0.1 mM each primer, and 1.25 U of ExTaq polymerase (Panvera/TaKaRa). Amplification conditions involved 30 cycles each consisting of 1 min of denaturing at 94 °C, 1 min of primer annealing at 55 °C, and 1.5 min of extension at 72 °C. A negative control was included for all PCRs. PCR products were purified prior to sequencing using ExoSAP-IT™ (USB Corporation).

Sequencing reactions were carried out using the internal primers H5382 (5′-GTG TGG GCR ATT CAT GA-3′) and L5238 (5′-ACM TGA CAA AAA ATY GC-3′) (de Queiroz et al., 2002) and Big Dye® Terminator v3.1 Cycle Sequencing kits (Applied Biosystems), and read on an automated sequencer (Applied Biosystems 3100, University of Tennessee Molecular Biology Resource Facility). Sequences were edited using the program Sequencher 3.1.1 (Gene Codes Corporation, Ann Arbor, MI). Alignments were performed initially using Clustal X (Thompson et al., 1997) and subsequently manually refined. The sequence alignment is available either from the corresponding author or from TreeBase (Study Accession No. S1622; Matrix Accession No. M2922).

2.3. Data analyses

Network analysis to estimate a gene genealogy was carried out using TCS 1.13 (Clement et al., 2000) which implements the Templeton et al. (1992) statistical parsimony

procedure. The haplotype network was then converted into a series of nested clades (Templeton, 1998) for interpretation and for use in statistical analyses of geographic vs. genetic variation (see below). Ambiguous connections (loops or reticulations) in the haplotype network were resolved using approaches from coalescent theory (see Crandall, 1994). In the case of DNA sequence data, this resolution generally involves a comparison of the probabilities of whether a haplotype arose via mutation from either a high- or low- frequency haplotype. Coalescent theory would suggest that, based on these probabilities, the new haplotype arose from the higher-frequency haplotype. In addition to the ND2 sequences generated for this study, one from Carroll County, IL (Janzen et al., 2002; GenBank Accession No. AY136236) and one from Cortland County, NY (Janzen et al., 2002; GenBank Accession No. AY136237) were included in our network analysis.

Statistical analyses of geographic/genealogical associations were conducted using GeoDis ver. 2.0 (Posada et al., 2000). All statistical analyses in GeoDis were performed using 10,000 (Monte Carlo) replications. Results obtained from GeoDis were then interpreted using the revised inference key of Templeton (1998, 2004).

The population genetic structure elucidated by our network analysis was further assessed and supported by performing analysis of molecular variance (AMOVA). Φ statistics (analogous to the *F*-statistics of Wright, 1965), were calculated using ARLEQUIN ver. 2.000 (Excoffier et al., 1992; Schneider et al., 2000). Two sets of analyses were performed by grouping individuals. These groups represent the highest level of genetic apportionment. The first analysis grouped individuals into clades based on our nested clade analysis (see below), while our second analysis divided individuals into seven geographic groups (Ohio, Indiana, Illinois, Wisconsin, lower peninsula of Michigan, upper peninsula of Michigan, and the Beaver Archipelago of Lake Michigan). Michigan was split into three separate geographic groups, as none of the three are currently directly connected to each other. The group apportionment of variation with respect to all haplotypes is described by Φ_{CT}, while Φ_{SC} describes the apportionment within populations of the defined groups, and Φ_{ST} refers to the variation in a single population relative to all haplotypes. The AMOVA assumes that groupings represent populations and that the populations are in drift-migration equilibrium, conditions that may be unrealistic for combined population groupings. Levels of significance of the Φ statistics were determined through 1000 permutation replicates.

In addition to employing a gene genealogy approach, we performed phylogenetic analyses of the ND2 data under the criterion of maximum likelihood (ML) as implemented in PAUP* (Swofford, 2002). Gene genealogical approaches are usually used for intraspecific analyses because they allow ancestral haplotypes to exist at internal nodes, which more accurately reflects haplotype relationships. Genealogical approaches such as statistical parsimony implemented in TCS (Clement et al., 2000), however, may be unable to connect all sequences into a single network if divergence between sequences is large. Thus we used ML analysis to complement the genealogical analysis, assess relationships among major clades, and root the tree. For our ML analysis, we used the hierarchical likelihood ratio test from Modeltest (Posada and Crandall, 1998) to determine the appropriate ML model for the analysis. The model we used was the HKY + G. ML analyses were conducted with 100 random sequence addition heuristic search replicates with tree-bisection-reconnection (TBR) branch swapping. Bootstrap analysis was employed to assess internal support for the inferred phylogeny using 100 bootstrap replicates with simple taxon addition heuristic searches and TBR branch swapping.

In addition to the sequences used in the network analysis we included in the ML analysis sequences of *T. sirtalis* from western N. America reported by Alfaro and Arnold (2001) ($n=1$), de Queiroz et al. (2002) ($n=1$), and Janzen et al. (2002) ($n=32$) to assess the relationship among Midwest/eastern and western N. American *T. sirtalis* populations. To root the ML tree we included sequences of *T. elegans* (Janzen et al., 2002; GenBank Accession No. AY136238), *T. proximus* (Alfaro and Arnold, 2001; de Queiroz et al., 2002; GenBank Accession No. AF383847, AF420163) and *T. sauritus* (de Queiroz et al., 2002; GenBank no. AF420179). These taxa were chosen based on broad-scale phylogenetic analyses of *Thamnophis* phylogeny (Alfaro and Arnold, 2001; de Queiroz et al., 2002) that show *T. proximus* and *T. sauritus* as sister to *T. sirtalis*, with *T. elegans* representing a more distant outgroup.

3. Results

3.1. Sequence variation

We obtained complete ND2 sequences for 148 *T. sirtalis* individuals from 37 populations. Thirty six unique haplotypes (GenBank Accession No. DQ995362–DQ995397) were detected in these 148 sequences. Of the 1101 bp used in analyses, 72 were variable, and 56 were phylogenetically informative. With up to 6.5% divergence between sequences, the populations used in our study express more intraspecific mtDNA sequence divergence than many other species of gartersnake (2.5% in the Mexican gartersnake (*T. eques*); de Queiroz and Lawson, 1994; 5.5% in the terrestrial gartersnake (*T. elegans*); de Queiroz and Lawson, 1994) including previous estimates for *T. sirtalis* (2.5%; Janzen et al., 2002). No stop codons or indels were detected in any of our sequences indicating the functionality of the ND2 gene. Using the program DnaSP (Rozas and Rozas, 1999), we found that 19 of the 72 variable sites represented nonsynonymous substitutions, which may be under selection (Zink, 2005), and thus may confound our geneaological and phylogenetic analyses. However, only nine of these were phylogenetically informative sites (of 56 total phylogenetically informative sites) and of those only two were distributed in populations that were geographically isolated from each other. Removing these two sites from our analyses resulted in no difference in the structure of either our

nested cladogram or our maximum likelihood tree; therefore, they were kept in the data set for all analyses, as it is possible that they resulted from geographical isolation rather than selection (Zink, 2005). In addition, a Tajima's Test (Tajima's D = −1.07, $P > 0.10$), Fu and Li's Tests (Fu and Li's D* test statistic = −1.22, Fu and Li's F* test statistic = −1.40, $P > 0.10$), and a McDonald and Kreitman Test (Neutrality Index = 1.46, Fisher's exact test two-tailed P-value = 0.42), all conducted using DnaSP (Rozas and Rozas, 1999), support the neutrality of our data. Therefore, if selection is acting on these sites, it appears to be negligibly influencing our phylogeographic hypotheses.

3.2. Nested clade analyses

Gene genealogy reconstruction resulted in two independent networks that could not be connected with 95% confidence levels by the statistical parsimony approach implemented in TCS (Templeton et al., 1987). The smaller network, which includes six haplotypes from localities in Ohio, Illinois, Pennsylvania, southern Michigan, and Ontario, Canada, is far removed from the remaining network (over 25 mutational steps) and mostly includes a small number of sequences from poorly sampled locations from the outskirts of the area of interest for this study. As a result, it and the haplotypes within it were not used in our statistical analyses of geographic/genealogical associations or our AMOVA analyses. These haplotypes are, however, utilized in our maximum likelihood analysis to help understand the more broad-scale implications of our data. The larger network includes 30 haplotypes from localities throughout our entire sampling range, except Ontario. Each of the 37 haplotypes from both networks were assigned a number, 1 to 37, and the localities they represent are detailed in the Appendix A. The nested cladogram representing our second network (Fig. 3), as well as or maximum likelihood tree also utilize these numbers (Fig. 4).

Nesting procedures resulted in three levels of nested clades (Fig. 3). At the highest level of nesting, three clades were recognized (3.1, 3.2, 3.3). The first and largest (3.1, 15 haplotypes, 87 individuals with 38 from the Beaver Archipelago) is split into three main clades (2.1, 2.2, 2.3). Clade 2.1 is the most restricted, including only individuals from the Beaver Archipelago; clade 2.2 is comprised of individuals from Wisconsin and the Beaver Archipelago, and clade 2.3, which is basal to clades 2.1 and 2.2, is the most widespread including individuals from the upper peninsula of Michigan, Wisconsin, the Apostle Islands of Lake Superior, Indiana, Illinois, and Ohio. The second largest level three clade (3.2, 10 haplotypes, 28 individuals with nine from the Beaver Archipelago) is also split into three smaller clades (2.4, 2.5, 2.6). Clade 2.6 is the most restricted, represented by only one individual from Indiana, clade 2.5 includes individuals from the Beaver Archipelago and the lower peninsula of Michigan, and clade 2.4, which is basal to clades 2.5 and 2.6, is the most widespread with individuals from Indiana, the Beaver Archipelago, and the lower peninsula of Michigan. The third and smallest level three clade (3.3, 5 haplotypes, 5 individuals) is further broken down into two main clades (2.7, 2.8) both of which include sequences from Illinois individuals only.

Our haplotype network illustrates that tip haplotypes ($n = 20$) are more widespread geographically than ancestral (internal) haplotypes ($n = 11$), an expectation of range expansion, and this is supported by our nested distance analysis of the dispersion of the clades. The analysis suggests that four nested groups in the overall nested cladogram show geographic distributions that are significantly different from random expectations (Table 1). We infer from these patterns of genetic variation that past fragmentation, followed by both contiguous range expansion and long distance colonization has occurred throughout the Midwest (Table 2). In addition, patterns of isolation by distance, suggesting reduced gene flow, occur between the Beaver Archipelago and mainland populations (Table 2). In particular, clade 1.5 (with individuals from the upper peninsula of Michigan, Wisconsin, and the Apostle Islands) and clade 2.4 (with individuals from Indiana, the Beaver Archipelago, and the lower peninsula of Michigan) both exhibit patterns of genetic differentiation that indicate past fragmentation followed by range expansion. Clade 2.3 (upper peninsula of Michigan, Wisconsin, Apostle Islands, Indiana, Illinois, Ohio) shows evidence of long-distance colonization. Genetic variation in clade 3.1, which includes the majority of sequences from the Beaver Archipelago populations (as well as Illinois, Wisconsin, Indiana, and Ohio), is characterized by restricted gene flow with isolation by distance.

3.3. Analyses of molecular variance

The AMOVA conducted with the nested clade clusters revealed significant genetic structuring across all hierarchical levels (all $P < 0.0001$). Overall, 70.53% of the variation was a result of differences among the three clusters (i.e., clades 3.1–3.3) ($\Phi_{CT} = 0.71$). 13.51% of the total variation resulted from differences among populations within these groups ($\Phi_{SC} = 0.46$) and 15.95% was due to variation within populations ($\Phi_{ST} = 0.84$). When individuals were grouped into the seven geographic regions defined in the methods, significant genetic structuring existed across all hierarchical levels (all $P < 0.05$), but only 16.23% of the variation was a result of differences among the groupings ($\Phi_{CT} = 0.16$). The remainder of the total variation resulted from differences among populations of these groups (42.81%, $\Phi_{SC} = 0.51$) and within populations (40.95%, $\Phi_{ST} = 0.59$).

3.4. Phylogenetic analyses

To reduce the computational time, maximum likelihood analysis was performed on the 37 unique haplotypes rather than on all 148 individual sequences (Fig. 3). In addition, as noted above, all sequences used by Janzen et al. (2002) were also included in this analysis, as well as one sequence each from Alfaro and Arnold (2001) and de Queiroz et al. (2002). Note that the very short branch lengths of our

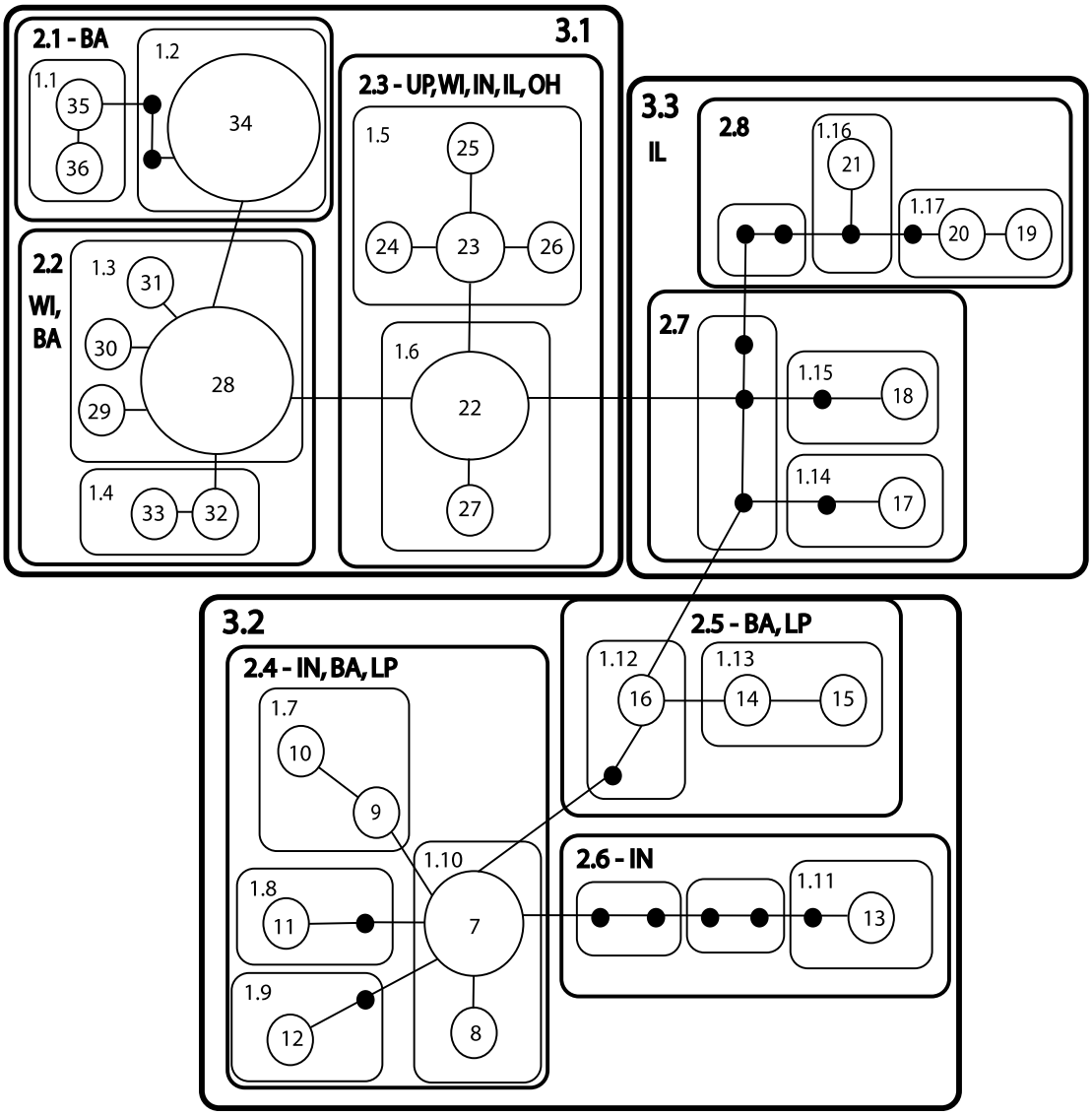

Fig. 3. Nested arrangement of common gartersnake, *Thamnophis siratlis*, ND2 haplotype network following the procedure given by Templeton et al. (1987, 1992). Haplotypes are labeled as in Appendix A. Solid lines connect haplotypes with a single step. Missing intermediates are indicated by closed circles. Letters indicate the collection origin of haplotypes in each clade. Points of origin include the Beaver Archipelago (BA), the lower peninsula of Michigan (LP), the upper peninsula of Michigan (UP), Wisconsin (WI), Indiana (IN), Ohio (OH), and Illinois (IL).

maximum likelihood tree (Fig. 4) indicate very little divergence between most haplotypes. In addition, while the results of the phylogenetic analysis relating to our sequences are topologically concordant with our network analysis, important differences are highlighted in interpretation between phylogenetic and genealogical approaches. Rather than forming distinct clades (as with our network analysis), our haplotypes form two grades. The first grade represents the more distantly related network of haplotypes that was detected in our nested analysis, but that was not used in our AMOVA analysis or our statistical analyses of geographic/genealogical associations. The second grade represents the larger network (Fig. 3) which includes the remainder of our sequences. This grade also includes all of the western *T. sirtalis* sequences and indicates that the haplotypes found in our larger network are actually more closely related to the western haplotypes than they are to haplotypes found in our smaller network. Thus nested clade analysis defines clades based on dissection of existing and inferred haplotypic variation into groups for further analysis of geographic vs. genetic variation without respect to monophyly vs. paraphyly, and importantly, allows observed haplotypes to occupy internal nodes (i.e., ancestral haplotypes). Traditional phylogenetic analysis, on the other hand, infers relationships under the assumption that all haplotypes occupy terminal positions, and does not allow existing haplotypes to occupy internal node positions.

The phylogeny inferred from maximum likelihood analysis (Fig. 4) depicts an initial divergence in our sample between two well-supported haplotype groups corresponding to a more eastern grade vs. clades 3.1, 3.2, and 3.3 of the

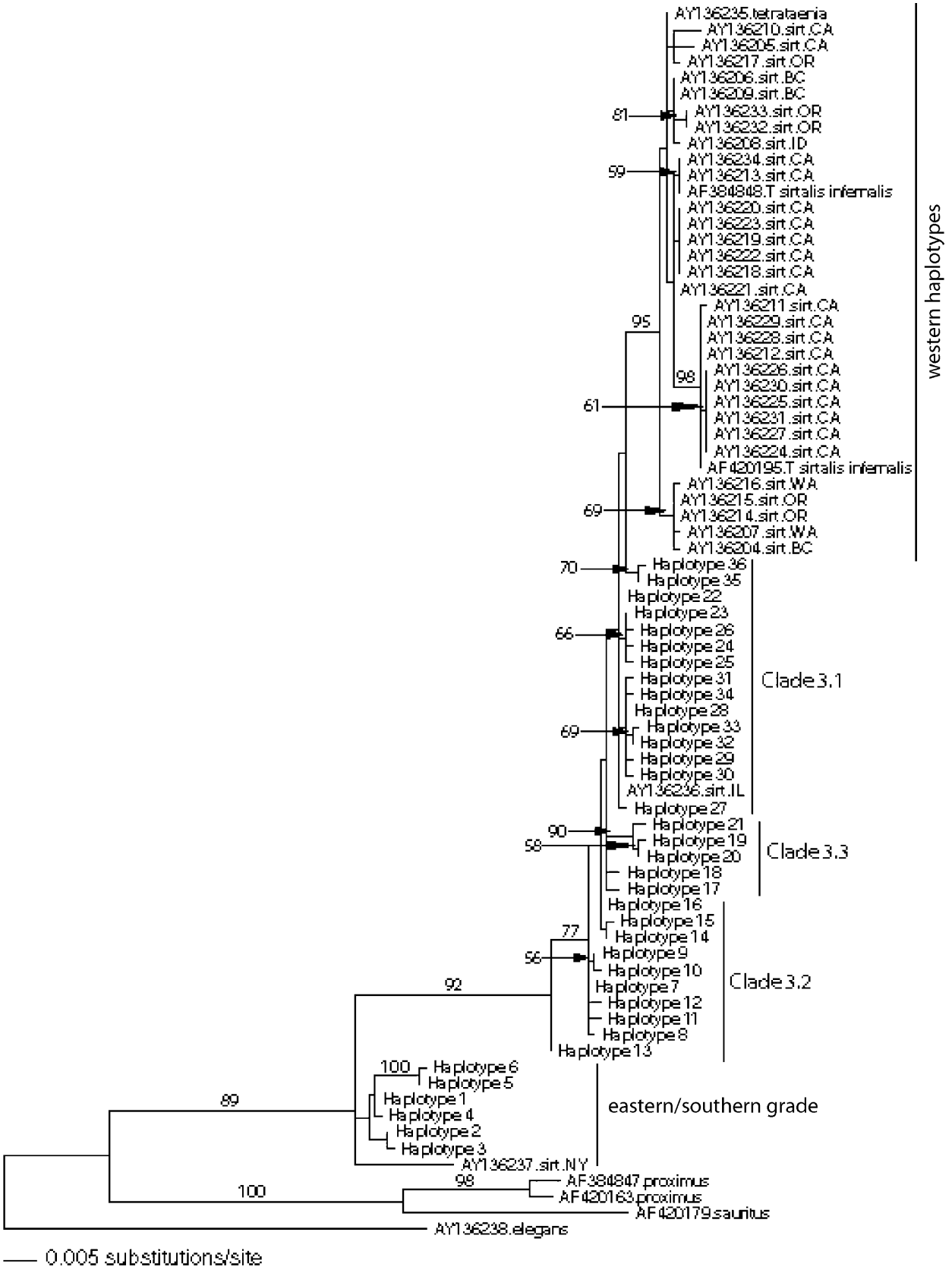

Fig. 4. Maximum likelihood tree recovered from all 37 unique *Thamnophis sirtalis* ND2 haplotypes. Numbers above or to the left of branches are bootstrap values (>50%, 100 random sequence addition heuristic search replicates with tree-bisection-reconnection [TBR] branch swapping) for those nodes recovered in the maximum likelihood analysis. Wording to the right of the tree indicates either (1) which of the three highest level nested clade analysis clusters particular haplotypes belong to, (2) haplotypes from a smaller more distant network detected by our nested analysis (i.e., the eastern/southern grad), or (3) western haplotypes from previous studies.

Table 1
Results of the nested geographic distance analysis for *Thamnophis sirtalis* populations from the Great Lakes region

	Position	D_C	$P<=$	$P>=$	D_N	$P<=$	$P>=$
Clade 1–5							
Haplotype 23	Interior	58.9432	0.0160*	0.9950	62.0556	0.0110*	1.0000
Haplotype 26	Tip	0.0000	1.0000	1.0000	252.0394	1.0000	0.0860
Haplotype 25	Tip	0.0000	0.2560	1.0000	81.5227	0.5050	0.5090
Haplotype 24	Tip	0.0000	1.0000	1.0000	109.7750	0.9160	0.5090
I-T clades	—	58.9432	0.6670	0.3340	−69.1594	0.0430*	0.9580
Clade 2–3							
Clade 1–6	Interior	264.1740	0.0000*	1.0000	319.3028	0.0850	0.9150
Clade 1–5	Tip	77.5425	0.0000*	1.0000	445.2534	0.9380	0.0620
I-T clades	—	186.6316	0.9950	0.0050*	−125.9506	0.0690	0.9310
Clade 2–4							
Clade 1–10	Interior	186.5159	0.3960	0.6160	189.3933	0.3040	0.7080
Clade 1–7	Tip	239.8420	0.9720	0.0380*	247.5812	0.7860	0.2240
Clade 1-8	Tip	0.0000	1.0000	1.0000	343.8525	1.0000	0.2950
Clade 1-9	Tip	0.0000	1.0000	1.0000	74.3821	0.3850	1.0000
I-T clades	—	42.6106	0.0450*	0.9560	−42.8023	0.2340	0.7670
Clade 3–1							
Clade 2–1	Tip	13.9998	0.0000*	1.0000	260.4246	0.0580	0.9420
Clade 2–2	Interior	170.0864	0.0050*	0.9950	205.1254	0.0000*	1.0000
Clade 2–3	Interior	362.4726	0.8920	0.1080	386.5684	0.9820	0.0180*
I-T clades	—	258.9137	1.0000	0.0000*	41.6789	0.7700	0.2300

For each nested clade with significant geographical associations we report the levels of clade/haplotype dispersion (D_C), displacement from clades at higher nesting levels (D_N), tip-interior contrasts (I-T), and probabilities for larger ($<=$) and smaller ($>=$) than expected distance values based on comparisons with the null hypothesis of no geographical association. Significant probabilities ($P < 0.05$) are indicated by an asterisk.

Table 2
Demographic inferences from nested geographical distance analysis for the Great Lakes region *Thamnophis sirtalis* clades (after Templeton, 2004)

Clade	Inference chain	Inferred pattern
Clade 1–5	1-2-11-12-13-Yes	Past fragmentation followed by range expansion
Clade 2–3	1-2-3-5-15-No	Long-distance colonization
Clade 2–4	1-2-11-12-13-Yes	Past fragmentation followed by range expansion
Clade 3–1	1-2-3-4-No	Restricted gene flow with isolation by distance

network analysis (Fig. 3). Geographically this indicates a primary divergence between a primarily eastern/southern clade (Ontario, Pennsylvania, Ohio, lower peninsula of Michigan, and one Illinois population) vs. a primarily northern/western clade (Illinois, Indiana, Wisconsin, Michigan lower and upper peninsula, and Beaver and Apostle Islands) (Fig. 1). Within this latter group one of the clades delimited by nested clade analysis is monophyletic (3.1), one is unresolved, but its monophyly is not directly contradicted (3.3), and one is paraphyletic to the other two (3.2). Geographically, the paraphyletic grade of 3.2 includes individuals from the north-central portion of the range (northern Indiana, lower peninsula of Michigan, and the Beaver Islands) with the southernmost population (Indiana, haplotype 13) occupying the basalmost position. Clade 3.3 consists entirely of individuals from Illinois, primarily from the southwestern portion of the state. Finally, clade 3.1 is resolved as monophyletic group although not with strong bootstrap support (<50%). Geographically this clade is principally confined to the western portion of our sample range with individuals from Illinois, Wisconsin, the upper peninsula of Michigan, the Beaver and Apostle Islands. One population from northwestern Ohio (OH2) is also included in this clade. In addition, two haplotypes from this more western distributed group appear to be the sister group for all of the western haplotypes. The distribution of the western haplotypes is concordant with the findings of Janzen et al. (2002) with only very minor differences (e.g., a sequence from an Oregon *T. sirtalis*, AY136317, is more closely related to several Calfornia *T. sirtalis* sequences than to other Oregon sequences, as originally indicated by Janzen et al. (2002)). The sequence from the Alfaro and Arnold (2001) study (AF383838), which represents a Humboldt County, California *T. sirtalis*, falls out with the Janzen et al. (2002) Humboldt County *T. sirtalis* sequence (AY136213). The sequence produced for the de Queiroz et al. (2002) study (AF420195) was from Santa Clara County, California, which was not sampled by Janzen et al. (2002); however, it is most similar to *T. sirtalis* sequences from Sonoma County, California, which is in the general vicinity as Santa Clara County.

4. Discussion

4.1. General phylogeographic patterns

The goal of this study was to reconstruct the phylogeographic patterns and to evaluate the role of Pleistocene glaciations in the Great Lakes region of North America on the evolutionary history of the common gartersnake, *T. sirtalis*, especially in Michigan. 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., Arbogast, 1999; Austin et al., 2002; Burbrink et al., 2000; Brant and Orti, 2003; Byun et al., 1997; Conroy and Cook, 2000; Demboski and Cook, 2001; Hays and Harrison, 1992; Klicka and Zink, 1997; Zamudio and Savage, 2003). The majority of the results presented herein are in agreement with earlier hypotheses related to the recolonization of the Great Lakes region by reptiles and amphibians (e.g., two main recolonization pathways exist with one following along the east coast of Lake Michigan and another along the west coast), but at least one earlier hypothesis is rejected (i.e., the Beaver Archipelago was colonized solely by snakes from the lower peninsula of Michigan), with an alternate hypothesis suggested (i.e., the Beaver Archipelago was colonized by snakes from both the upper and lower peninsula of Michigan and acts as a secondary contact point for the east coast/west coast lineages discussed above).

Both our nested clade analysis and a standard phylogenetic analysis resulted in similar tree topologies, with strong support for two distinct lineages. Nested clade analysis also suggests that one of these lineages is split into three main clades, some of which are supported by our phylogenetic analysis. AMOVA results strongly support the genetic distinctiveness of these three clades, which better explain the genetic variation displayed by our data than grouping snakes into their respective states. From these analyses, two important patterns emerge. First, Great Lakes populations recolonized areas that were covered by the glacier via two routes: (1) from Indiana along the eastern coast of Lake Michigan into the lower peninsula of Michigan, and (2) from Illinois and Indiana into Wisconsin and the upper peninsula of Michigan along the western coast of Lake Michigan. Second, the Beaver Archipelago in northeastern Lake Michigan is an area of secondary contact for the two lineages that diverged around either side of Lake Michigan. In addition, although not explicitly examined here, there is some evidence that the more southern and eastern states we sampled may have acted as refugia for *T. sirtalis* during the glaciation and that Ontario, Canada was recolonized by Ohio and Michigan populations, presumably via the gap between Lake Huron and Lake Erie. More sampling along the east coast and states south of Illinois, Indiana, and Ohio are needed to more fully support such hypotheses.

In general, however, our data support hypotheses concerning the recolonization of the Great Lakes region following the Wisconsinan glaciation (Fig. 5), especially the recolonization of Michigan. Primary reptile and amphibian invaders of the region are believed to have stayed close to the margins of the retreating ice sheet and quickly recolonized the region via two routes located to the east and west of Lake Michigan. Our data agree with past hypotheses that the upper peninsula of Michigan was colonized via Wisconsin and a route west of Lake Michigan, while the lower peninsula of Michigan was recolonized by animals from Indiana and Ohio. In addition, we found an enigmatic group of haplotypes that occur only in Illinois populations. Suggestions for the origin of this group are discussed below. We rejected a long standing hypothesis that the Beaver Archipelago in northeastern Lake Michigan was recolonized solely by lower peninsula populations. Our data indicate that the Beaver Archipelago acts as a secondary contact point between the east and west coast Lake Michigan lineages, although whether the contact was natural dispersion or human assisted remains unknown. Interestingly, 81% of the Beaver Archipelago snakes we sampled are found in the western group of haplotypes. Most hypotheses regarding the recolonization of the Beaver Archipelago suggest that island populations most likely originated from the lower peninsula of Michigan. If any animals colonized from the west or from the upper peninsula of Michigan, their contribution to the genetic make-up of the island populations was believed to be minimal at best. Our results suggest the opposite. The Beaver Archipelago is also characterized by several unique haplotypes not found on the mainland (haplotypes 8, 9, 14, 15, 34, 35, and 36) indicating that genetic divergence and evolution may be ongoing on these islands.

In addition to testing hypotheses regarding the paths animals took when recolonizing the Great Lakes region, our data also allow us to infer specific patterns of recolonization, as 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). Knowles and Maddison (2002) have criticized the methods used by Templeton et al. (1987) to examine such associations, citing the frequent failure of nested clade analysis to recover the true history of simulated data sets. In response, Templeton (2004) modified the nested clade analysis inference tree, resulting in improved performance with simulated data. Further validation comes from analysis of 42 molecular data sets for which strong *a prior* phylogeographic expectations could be inferred (Templeton, 2004).

There are three major biological factors that may cause spatial or temporal associations of haplotype variation: (1) restricted gene flow, (2) past fragmentation, and (3) range expansion (Templeton et al., 1995). Our analyses indicate that all three of these factors are or have been at work among the sampled populations, but that recurrent gene flow is also common, which may explain the short branch lengths of our maximum likelihood tree. Restricted gene flow (due to isolation by distance) led to the nonrandom geographic/genealogical associations between the Beaver Archipelago and the surrounding mainland, especially Wisconsin and the upper peninsula of Michigan. Given that the islands are currently separated from the mainland by ca. 25–30 km of water, restricted gene flow between the populations found on these islands and the mainland are to be expected. Past fragmentation followed by range expansion is seen throughout the data set.

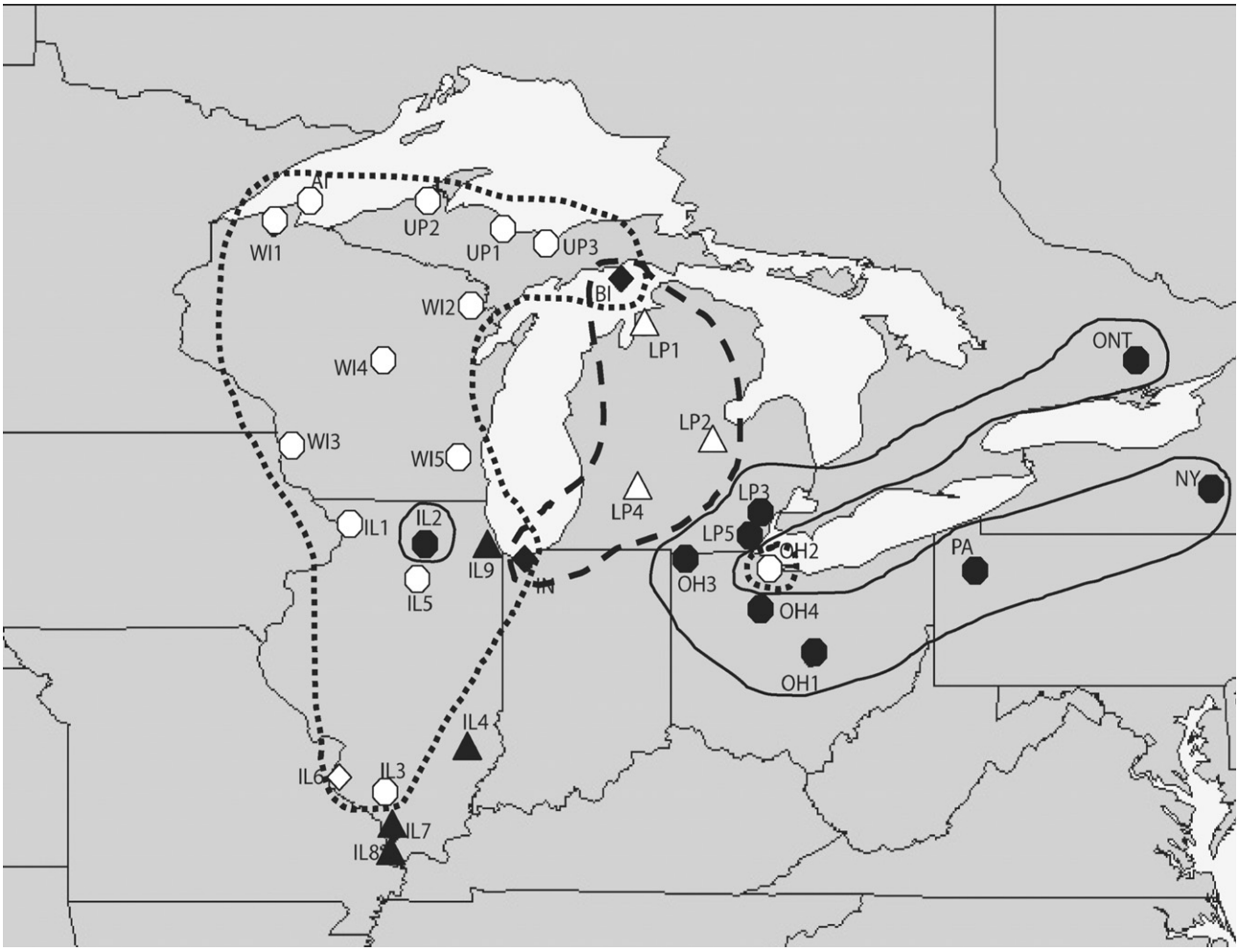

Fig. 5. Map showing the current distribution of *Thamnophis sirtalis* mtDNA clades (as inferred from nested clade analysis) following glacial retreat. Symbols represent the geographic locations of the identified clades: open circles, 3.1; open triangles, 3.2; closed triangles, 3.3; closed circles, eastern/southern grade; open diamonds, 3.1 & 3.3; closed diamonds, 3.1 & 3.2. Current distribution of clade 3.1 is represented by a dotted line, current distribution of clade 3.2 is represented by a dashed line, and current distribution of the eastern/southern grade is represented by a solid line.

4.2. Phylogeography of Great Lakes region taxa

In general, our data support past hypotheses regarding recolonization of the Great Lakes region following the retreat of the Wisconsinan glacier, and provide one of the most extensive sampling of any taxon in this region with the exception of Smith and Green's (2004) work with the toad, *B. fowleri*, (158 individuals sequenced from 21 populations). However, their focus was specifically on populations surrounding Lake Erie, not Lake Michigan; therefore, their results are generally not comparable to ours with one exception. Smith and Green (2004) found two distinct lineages of *B. fowleri* surrounding Lake Erie with one lineage linking populations from the northwestern shore of Lake Erie in Ontario to populations in Indiana indicating a possible colonization route that followed thru southern Michigan and the gap between Lake Huron and Lake Erie with the other lineage extending from Ohio and Pennsylvania and wrapping around the east coast of Lake Erie. While, Smith and Green (2004) attribute the differences they detected between the two lineages to stochastic postfounder hybridization with another species of toad, *Bufo americanus*, and fixation, our eastern/southern grade supports the hypothesis (Holman, 1992) that a major colonization route via the gap between Lake Huron and Lake Erie existed between the Midwestern United States and Ontario; however, more sampling of *T. sirtalis* and other species is needed in Pennsylvania, New York, and Ontario to further understand patterns of colonization in the southeastern Great Lakes region. Others have also sampled within this region on a smaller scale.

Rye (2000) provides evidence from Canadian gartersnake population allozyme and mtDNA cytochrome *b* data that support our hypothesis for multiple fronts of post-Pleistocene colonization within the Great Lakes region. Samples from an east–west transect reveal two genetic lineages that meet north of Lake Superior (Rye, 2000). A western lineage occurs in western Ontario and Manitoba and an eastern lineage occurs in central and eastern Ontario. The location of contact between lineages is consistent with two fronts of colonization diverging around the Great Lakes and meeting secondarily. Zamudio and Savage (2003) found that spotted

salamander (*Ambystoma maculatum*) haplotypes from individuals collected in Indiana were basal to two separate Midwest lineages. One of these lineages extends up the west coast of Lake Michigan into the upper peninsula of Michigan, while the other extends from the southernmost counties of the lower peninsula of Michigan and gave rise to southern Ontario, Canada populations. While these results mirror ours, salamander populations in the central and northern portions of the lower peninsula of Michigan, the Beaver Archipelago, and Ohio were not sampled. Therefore, the complete evolutionary history of Ohio and Michigan populations, and the origin of the Beaver Archipelago populations, were not elucidated.

Analysis of mtDNA from the spring peeper, *Pseudacris crucifer*, (a small frog) also suggests multiple fronts of colonization within the Great Lakes region (Austin et al., 2002). Different lineages occur east and west of Lake Michigan, consistent with the hypothesis that the lake has functioned as a barrier to gene flow. However, routes of colonization differ from those hypothesized here and in the past. The southern Great Lakes region was apparently colonized from unglaciated areas to the south whereas the northern Great Lakes region was colonized by a lineage originating east of the Appalachian Mountains that expanded counterclockwise around the Great Lakes into Minnesota and northern Wisconsin (Fig. 6 in Austin et al., 2002). During the Xerothermic period (4–6,000 ybp), contact between lineages was prevented by the prairie peninsula, which extended eastward across N Indiana, NW Ohio, and SW Ontario. Once this barrier disappeared, secondary contact occurred in SW Ontario. Even though Holman (1992) considers both *T. sirtalis* and *P. crucifer* primary invaders, it is possible that they recolonized the Midwest differently, given that differences in behavior, ecology, and physiology exist between them. More sampling of *T. sirtalis* populations on the east coast of the United States and Canada are needed to further understand these distinct recolonization patterns.

For mammals, Brant and Orti (2003) recently examined the postglacial recolonization of the eastern United States by the Northern short-tailed shrew (*Blarina brevicauda*), and while they did not extensively sample in the Great Lakes region, they did designate a phylogroup of east-central haplotypes that includes populations from Indiana and Wisconsin, indicating a possible west coast of Lake Michigan migration route. Several other studies have been conducted in the general vicinity of the Great Lakes region, but they do not focus on the recolonization of that area specifically (e.g., Fuerst and Austin, 2004; Starkey et al., 2003) and are not as detailed as those discussed above. In addition, we have found no other studies on any taxa that have specifically examined the molecular phylogeography of Beaver Archipelago populations, so our data on this unique system cannot be compared to any other study.

The Beaver Archipelago was traditionally thought to have been colonized solely by lower peninsula Michigan populations. This hypothesis was based on two main facts: (1) in the last 10,000 years, the islands of the Beaver Archipelago were connected to the lower peninsula of Michigan by a land bridge, and (2) at least one species currently found on the islands occurs in the lower peninsula but not in the upper peninsula of Michigan and several species that are rare in the upper peninsula of Michigan are common on the islands and the lower peninsula. However, while the islands were connected to the lower peninsula, they were only separated from the upper peninsula by the Mackinac River, which may have been only 1.6 km wide in some areas. The common gartersnake is an excellent swimmer and often can be found in logs; therefore, it could have easily crossed the river via swimming or rafting. In addition, Native Americans and European settlers often traveled from the upper peninsula to the islands carrying with them supplies that gartersnakes and other small animals may have had access to (e.g., straw bales for livestock). As a result, animals may have migrated to the islands from the upper peninsula as stowaways on human vessels at any time in the last 14,000 years (Tanner, 1986). Our data indicate that the islands have been colonized by populations from both the lower and upper peninsula of Michigan and that the islands act as a secondary contact point for the east and west coast Lake Michigan lineages. Therefore, any future hypotheses regarding the origin and divergence of Beaver Archipelago taxa should take into account the underlying genetics of the populations.

The third distinct clade detected in our analyses is found only in Illinois. Based on our phylogenetic analysis this group appears to be an offshoot of the clade that is found along the west coast of Lake Michigan; therefore, it may be a more recently derived lineage arising from that group (e.g., *T. s. semifasciatus*), or it may be a precursor to that group. In terms of the former hypothesis, the range of *T. s. semifasciatus*, the Chicago gartersnake, overlaps with that of *T. s. sirtalis* in northeastern Illinois. It is possible that snakes that were identified as *T. s. sirtalis* were actually *T. s. semifasciatus*, as the two are very similar morphologically (Rossman et al., 1996). However, only one county (Cook County, IL 9 from Fig. 1) in this third clade is found within the known range of *T. s. semifasciatus*, which leads us to believe some other phenomenon may be responsible for the third clade; however, it may also be that the range of *T. s. semifasciatus* is expanding southward or interbreeding between the two subspecies may be producing a hybrid that is found throughout Illinois. A second more likely explanation requires further sampling of the states south and west of Illinois, as gartersnake populations from states not sampled (e.g., Iowa, Missouri, Arkansas) may be ancestral to lineages found in the Great Lakes region and our enigmatic Illinois group may be related to them. For example, Austin et al. (2002) indicated that southern Illinois spring peeper populations may have been colonized by populations from Missouri, Kansas, and Arkansas, and this may also be the case for *T. sirtalis*.

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Appendix A

Collection data for *Thamnophis sirtalis* included in this study. For each population sampled (alphanumeric locality codes refer to those in Fig. 1) we list collection locality (state, county, and ca. coordinates), sample sizes (n), the unique mtDNA haplotypes present, the nested clade analysis level 3 grouping (see Fig. 3; all those marked NCA for this category are found in the eastern grade, see Fig. 4), and the source of the specimen. Tissue sample sources included John S. Placyk, Jr. (JSP), Gordon M. Burghardt (GMB), Richard B. King (RBK), Gary S. Casper (GSC), Jace W. Robinson (JWR), Ronald K. Gratz (RKG), Briar J. Howes (BJH), Erik R. Wild (ERW), Alan Resetar (AR), Paul T. Andreadis (PTA), John Marshall (JM), Scott R. Ballard (SRB), and Harlan D. Walley (HDW).

Locality	State/province	County	Coordinates	*n*	Haplotype no.	NCA group	Source
BA[a] (Beaver Isl. 1)	Michigan (MI)	Charlevoix	46°03′N, 85°58′W	10	7, 9, 28, 34	3.1, 3.2	JSP
BA[a] (Beaver Isl. 2)	MI	Charlevoix	46°04′N, 85°59′W	11	14, 15, 28, 34	3.1, 3.2	JSP
BA[a] (Garden Isl.)	MI	Charlevoix	46°19′N, 85°49′W	11	7, 9, 28, 34, 36	3.1, 3.2	JSP
BA[a] (High Isl.)	MI	Charlevoix	46°13′N, 86°05′W	12	7, 28, 34, 35	3.1, 3.2	JSP
BA[a] (Squaw Isl.)	MI	Charlevoix	46°23′N, 85°59′W	1	8	3.2	JSP
BA[a] (Trout Isl.)	MI	Charlevoix	46°17′N, 86°09′W	2	34	3.1	JSP
LP1[a]	MI	Antrim	45°03′N, 84°57′W	9	7, 12	3.2	JSP
LP2	MI	Saginaw	43°19′N, 84°02′W	1	7	3.2	GMB
LP3	MI	Wayne	42°15′N, 83°17′W	3	1, 2	N/A	GMB
LP4[a]	MI	Barry	42°36′N, 85°18′W	1	16	3.2	A.R.
LP5	MI	Monroe	41°55′N, 83°30′W	1	1	N/A	GMB
UP1[a]	MI	Marquette	46°49′N, 87°58′W	4	23	3.1	JSP
UP2[a]	MI	Houghton	47°11′N, 88°57′W	2	25	3.1	RKG
UP3[a]	MI	Alger	47°09′N, 86°28′W	5	23, 24	3.1	GSC
AI (Apostle Islands)	Wisconsin (WI)	Bayfield	46°95′N, 90°63′W	1	26	3.1	GMB
WI1[a]	WI	Bayfield	46°37′N, 91°10′W	9	28, 31, 32, 33	3.1	ERW, GSC
WI2	WI	Marinette	45°20′N, 88°00′W	7	28, 30	3.1	GSC
WI3[a]	WI	Crawford	43°13′N, 90°55′W	3	22, 28	3.1	HDW
WI4[a]	WI	Portage	44°28′N, 89°30′W	1	28	3.1	ERW
WI5	WI	Waukesha	43°00′N, 88°14′W	3	22, 29	3.1	GMB
PA[a]	Pennsylvania (PA)	Venango	41°23′N, 79°45′W	1	1	N/A	GMB
IN[a]	Indiana (IN)	Porter	41°37′N, 87°04′W	9	7, 10, 11, 13, 22	3.1, 3.2	RBK
IL1	Illinois (IL)	Carroll	42°05′N, 90°08′W	1	22	3.1	GenBank
IL2[a]	IL	Dekalb	41°58′N, 88°41′W	8	1	N/A	RBK
IL3[a]	IL	Perry	38°05′N, 89°22′W	2	22	3.1	AR, SRB
IL4	IL	Richland	38°42′N, 88°05′W	1	17	3.3	AR
IL5[a]	IL	La Salle	41°20′N, 88°52′W	2	22	3.1	JWR
IL6[a]	IL	Monroe	38°16′N, 90°10′W	2	21, 22	3.1, 3.3	SRB
IL7[a]	IL	Union	37°27′N, 89°15′W	1	20	3.3	SRB
IL8[a]	IL	Alexander	37°11′N, 89°20′W	1	19	3.3	SRB
IL9[a]	IL	Cook	41°53′N, 87°39′W	1	18	3.3	AR

(*continued on next page*)

Appendix A (*continued*)

Locality	State/province	County	Coordinates	*n*	Haplotype no.	NCA group	Source
OH1	Ohio (OH)	Licking	40°04′N, 82°25′W	2	5, 6	N/A	PTA
OH1	Ohio (OH)	Licking	40°04′N, 82°25′W	2	5, 6	N/A	PTA
OH2[a]	OH	Ottawa	41°30′N, 82°56′W	8	22, 27	3.1	RBK
OH3	OH	Williams	41°40′N, 84°33′W	6	1, 4	N/A	RBK, JM
OH4	OH	Wyandot	40°51′N, 83°17′W	2	1	N/A	RBK
ONT	Ontario	Addington	45°00′N, 77°17′W	4	2, 3	N/A	BJH
NY	New York	Cortland	42°40′, 76°13′W	1	37	N/A	GenBank

[a] Voucher numbers: Beaver Isl. 1 (University of Tennessee Reptile Ethology Lab (UTKREL) 01-MM-0001–0010), Beaver Isl. 2 (UTKREL 01-SM-001–0011), Garden Isl. (UTKREL 01-GI-0001–0011), High Isl. (UTKREL 01-HI-0001–0012), Squaw Isl. (UTKREL 01-SI-0001), Trout Isl. (UTKREL 01-TI-0001–0002), LP1 (UTKREL 01-LP-0001–0009), LP4 (Thsi 505-01), UP1 (UTKREL 01-UP-0001–0004), UP2 (UTKREL 01-UP-0005–0006), UP3 (Milwaukee Public Museum (MPM) 33436–33440), WI1 (Thsi 204-01–09), WI3 (Thsi 200-05–07), WI4 (Thsi 201-02), PA (UTKREL 86-PA-1684), IN (Thsi 300-01–09), IL2 (This 100-02–09), IL3 (Thsi 111-02), IL5 (Thsi 107-01–02), IL6 (Thsi 111-03–04), IL7 (Thsi 111-05), IL8 (Thsi 111-01), IL9 (Thsi 114-01), and OH2 (Thsi 403-01–08). Vouchers beginning with "Thsi" are located at the King Lab at Northern Illinois University. Tissue samples for which vouchers are not available were donated as tail tips or extractions.

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