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Molecular phylogeography of common garter snakes (*Thamnophis sirtalis*) in western North America: implications for regional historical forces

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Abstract

Complete ND2 and partial ND4 and cytochrome *b* mitochondrial DNA (mtDNA) sequences were analysed to evaluate the phylogeographic patterns of common garter snakes (*Thamnophis sirtalis*) in western North America. This species is widely distributed throughout North America, and exhibits extensive phenotypic variation in the westernmost part of its range. The overall phylogeographic pattern based on mtDNA sequences is concordant with results from studies of other species in this region, implicating historical vicariant processes during the Pleistocene and indicating bottleneck effects of recent dispersal into postglacial habitat. Indeed, the topology is statistically consistent with the hypothesis of both southern (Great Basin and California) and northern (Haida Gwaii) refugia. Specifically, we identified genetic breaks among three major clades: Northwest Coastal populations, Intermountain populations, and all California populations. The California clade contained the only other well-supported branching patterns detected; relationships among populations within the two northern clades were indistinguishable. These molecular splits contrast sharply with all prior geographical analyses of phenotypic variation in *T. sirtalis* in this region. Our results suggest that the extensive phenotypic variation in western *T. sirtalis* has been shaped more by local evolutionary forces than by shared common ancestry. Consequently, we consider all morphologically based subspecies designations of *T. sirtalis* in this region invalid because they do not reflect reciprocal monophyly of the mtDNA sequences.

Keywords: molecular phylogeography, snakes, subspecies, *Thamnophis sirtalis*

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Introduction

Molecular phylogeography is a powerful concept that has linked biogeography with the genetics of populations (for review see Avise 2000). As a consequence, this nascent field is providing tremendous insight into numerous fundamental evolutionary issues (Avise 1994). For example, comparative phylogeography is increasingly enlightening our understanding of adaptive radiation and patterns of diversity (e.g. Bermingham & Moritz 1998; Moritz & Faith 1998). A molecular approach to comparative phylogeography can provide an especially useful understanding of these problems when biogeographic history and evolutionary relationships among morphologically designated populations or taxa are unknown (e.g. Avise *et al*. 1987).

The intraspecific molecular phylogeography has been characterized for numerous North American vertebrates (for review see Avise 2000). There is an especially fast growing literature on phylogeographic patterns of taxa in western North America (e.g. Gray 1995; McKnight 1995;

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Tan & Wake 1995; Green *et al*. 1996; Byun *et al*. 1997; Janzen *et al*. 1997; Soltis *et al*. 1997; Wake 1997; Zamudio *et al*. 1997; Rodriguez-Robles *et al*. 1999; Pook *et al*. 2000; Rodriguez-Robles & De Jesus-Escobar 2000; Nielson *et al*. 2001; Rodriguez-Robles *et al*. 2001). This geographical region is of particular interest because it has experienced a series of powerful geological events, including repeated marine incursions, glaciation and postglacial flooding, active tectonic movement, and volcanism (Yanev 1980; Barnosky *et al*. 1987; Alt & Hyndman 1995; Josenhans *et al*. 1995). This panoply of geological activity has undoubtedly affected the geographical distribution of the biota in western North America, but remains to be examined thoroughly and rigorously. Indeed, some research has yielded conflicting results regarding the causes of certain phylogeographic patterns (e.g. Demboski *et al*. 1999 vs. Byun *et al*. 1999).

Common garter snakes (*Thamnophis sirtalis*) provide an excellent system to evaluate phylogeographic patterns in western North America. This species is broadly distributed, ranging continent-wide south from central Canada to the Gulf Coast, but excluding much of the southwestern United States (Stebbins 1985; Conant & Collins 1991). *Thamnophis sirtalis* currently consists of 12 recognized subspecies based primarily on colour pattern variation (Rossman *et al*. 1996, plus *T. s. tetrataenia*), including five along the west coast of North America. Indeed, *T. sirtalis* has been the subject of classic (and deeply conflicting) systematic studies involving geographical variation in morphology (e.g. Boulenger 1893; Cope 1900; Brown 1901; Ruthven 1908). The phylogeography of the entire genus *Thamnophis*, as well as that of individual species, is virtually unstudied (Alfaro & Arnold 2001).

Using mitochondrial DNA (mtDNA) sequences to reconstruct phylogenetic relationships among western *T. sirtalis*, we address several important evolutionary issues: (i) how are populations genetically structured within western North America, and (ii) using a statistical approach, what historical mechanisms can be invoked to explain this biogeographic pattern? Our study is thus focused on historical issues and is not designed to examine the role of continuing gene flow. We also briefly comment on taxonomic issues regarding the validity of currently recognized morphologically based subspecies of *T. sirtalis*.

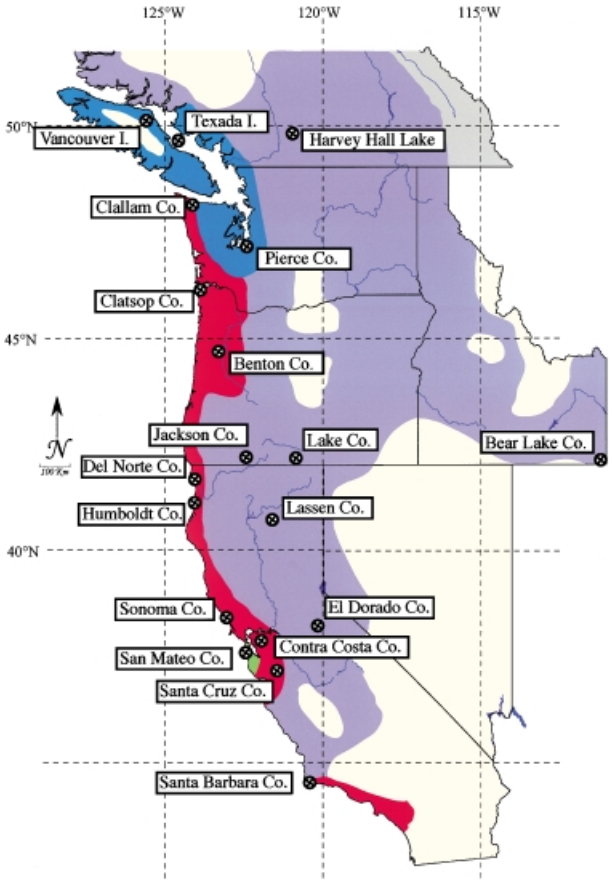

Fig. 1 Sampling scheme and morphology-based subspecies ranges for *Thamnophis sirtalis* in western North America (see Appendix 1; Rossman *et al*. 1996). Red, *T. s. concinnus*; purple, *T. s. fitchi*; green, *T. s. infernalis*; blue, *T. s. pickeringi*; grey, *T. s. parietalis* (not sampled).

Materials and methods

DNA extraction and sequencing

We obtained tissue from 32 field-collected snakes from 19 carefully chosen populations in the westernmost portion of the distribution of *Thamnophis sirtalis* in North America (Appendix 1; Fig. 1). We focused on these 19 populations to characterize and sample adequately the five subspecies in this geographical area and to provide an appropriate historical context to related studies involving the evolution of resistance to tetrodotoxin (Brodie & Brodie 1999). We also obtained tissue from populations of *T. sirtalis* from Illinois and New York, *T. proximus* from Arkansas, and *T. elegans* from California. In most cases, animals were pregnant females who were returned to the laboratory for experiments to be reported elsewhere (see also Brodie & Brodie 1999). Tissue samples consisted of ventral scale clips stored in 95% ethanol. Genomic DNA was isolated from the samples using a Proteinase K/sodium dodecyl sulphate digestion at 37 °C for 2 h and a standard phenol–chloroform extraction method (Hillis & Moritz 1990).

From this genomic DNA, we examined most or all of three separate mitochondrial genes. We amplified and sequenced an ~600-base pair (bp) fragment of cytochrome *b*, an ~600-bp fragment of ND4, and the entire ~1050 bases of ND2. Polymerase chain reaction (PCR) was conducted in 25-μL volumes with 0.5–1.0 μg of purified DNA, 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, and 0.1% Triton

X-100) (Promega), 0.1 mM dNTPs, and 0.5 units of *Taq* polymerase (Boehringer Mannheim) in each case. However, primers and most PCR conditions for optimal initial amplification of each gene differed. For cytochrome *b*, we used 2 mM of $MgCl_2$ and 0.4 μM of each primer [5′–3′: (LGLU) TGA TCT GAA AAA CCA CCG TTG TA and (H15544) AAT GGG ATT TTG TCA ATG TCT GA]. Amplification conditions involved 1 min of denaturing at 94 °C for 40 cycles, 1.5 min of primer annealing at 50 °C, and 2 min of extension at 72 °C. For ND4, we used 1.5 mM of $MgCl_2$ and 1 μM of each primer [5′–3′: (DW1641) TGA CTA CCA AAA GCT CAT GTA GAA GC and (DW1642) TAT TAG TAG GTG TTC TCG]. Amplification conditions involved 0.5 min of denaturing at 94 °C for 35 cycles, 1 min of primer annealing at 50 °C, and 1 min of extension at 72 °C. For ND2, we used 1.5 mM of $MgCl_2$ and 0.4 μM of each primer [5′–3′: (CE2330) CTA ATA AAG CTT TCG GGC CCA TAC, (H5051) TCG GTG CTA TTT TTA GTG TTG CTA, (CE2331) TTC TAC TTA AGG CTT TGA AGG C, and (L4956) CTA TTA TGC GCC ACC CTA TCA AT]. Amplification conditions involved 0.5 min of denaturing at 94 °C for 30 cycles, 0.5 min of primer annealing at 48 °C, and 1.5 min of extension at 72 °C.

We ran the PCR product on a 1.5% low-melt agarose tris-borate-EDTA (TBE) gel and then excised the target DNA fragment. The fragment was suspended in 500 μL deionized H_2O and heated at 95 °C for 5 min. This mixture was used as the template in a second PCR (run in triplicate) to generate double-stranded DNA for sequencing. When necessary, this product was run on a 1% TBE agarose gel and the band was excised. DNA was purified from the gel slice with a 0.22 Micropure separator (Amicon) and concentrated in an M-100 microconcentrator (Amicon). Template was sequenced in both directions at the Iowa State University DNA Sequencing Facility on an ABI PRISM model 377 automated sequencer. The region of overlap between the pair of sequences for each individual was evaluated to verify the integrity of the sequencing.

Phylogenetic analysis

We assembled forward and reverse sequences for each gene into a contiguous fragment with SEQUENCE NAVIGATOR version 1.0.1 (©Applied Biosystems 1994). We then aligned all sequences manually using SEQUENCE ALIGNMENT PROGRAM (SE-AL) version 1.d1 (Rambaut 1995). To evaluate the phylogenetic content of the sequences, we performed the *g*-test (Hillis & Huelsenbeck 1992) with 100 000 trees randomly generated by version 4.0b3a of PAUP*, written by Swofford (2000). We then used PAUP* to conduct phylogenetic parsimony and maximum likelihood analyses and to calculate the observed proportional sequence divergences (*p*-distances) between all pairwise comparisons of individual snakes. In initial phylogenetic analyses, we rooted all trees with the homologous sequences from a western ribbon snake (*Thamnophis proximus*) and a mountain garter snake (*T. elegans*). Previous molecular studies hypothesize *T. proximus* to be a member of a clade closely related to *T. sirtalis* and *T. elegans* to be a member of a deeply divergent sister clade to the clade including *T. proximus* and *T. sirtalis* (de Queiroz & Lawson 1994; Alfaro & Arnold 2001). However, using both *T. proximus* and *T. elegans* as out-groups produced the same phylogenetic outcomes as using only *T. elegans* as an out-group. We therefore present the latter results for simplicity.

We conducted analyses of phylogenetic patterns among western populations of *T. sirtalis* using all *T. sirtalis* samples, including one from Illinois and one from New York, with *T. elegans* as an out-group. To account for possible intrapopulation variance, we analysed multiple individuals (from different kin groups) from three populations (Appendix 1). In the parsimony analyses, we performed a heuristic search with 10 replicates of random addition because of the large number of samples involved. Sequence data were unweighted in these analyses. We also employed a maximum likelihood approach (HKY85 + gamma model; Hasegawa *et al*. 1985) to estimate the phylogeny with empirical base frequencies and estimated transition : transversion ratios. The HKY85 + gamma model was determined to be the best description of the data according to MODELTEST (Posada & Crandall 1998). We used bootstrapping (Felsenstein 1985) with 1000 replicates and decay analyses (Bremer 1996) to test the reliability of the data in finding the best tree and to test the robustness of clades.

We used the resulting phylogenetic topologies to conduct statistical tests of several alternative biogeographic hypotheses with three different methods: COMPONENT LITE (Page 1997), and Wilcoxon signed-ranks (Templeton 1983) and Shimodaira–Hasegawa (Shimodaira & Hasegawa 1999) tests available in PAUP* (Swofford 2000). All three methods produced qualitatively similar results. However, we report only the specific results of the COMPONENT LITE analyses in this paper because that approach deals strictly with the branching pattern (without reference to branch lengths) among the populations. In other words, COMPONENT LITE does not consider the genetic distance between populations, only their topological relationship, which is the key issue for testing biogeographic hypotheses.

These statistical tests involved comparing the topologies of the maximum parsimony tree and the maximum likelihood tree to several hypothetical tree topologies to help distinguish among hypotheses of range expansion following the end of Pleistocene glaciation (Stebbins 1949; Soltis *et al*. 1997). We constructed hypothetical trees taking into account only latitude and major geographical features because we sought to test only the broad-scale patterns of geographical expansion. Because common garter snakes

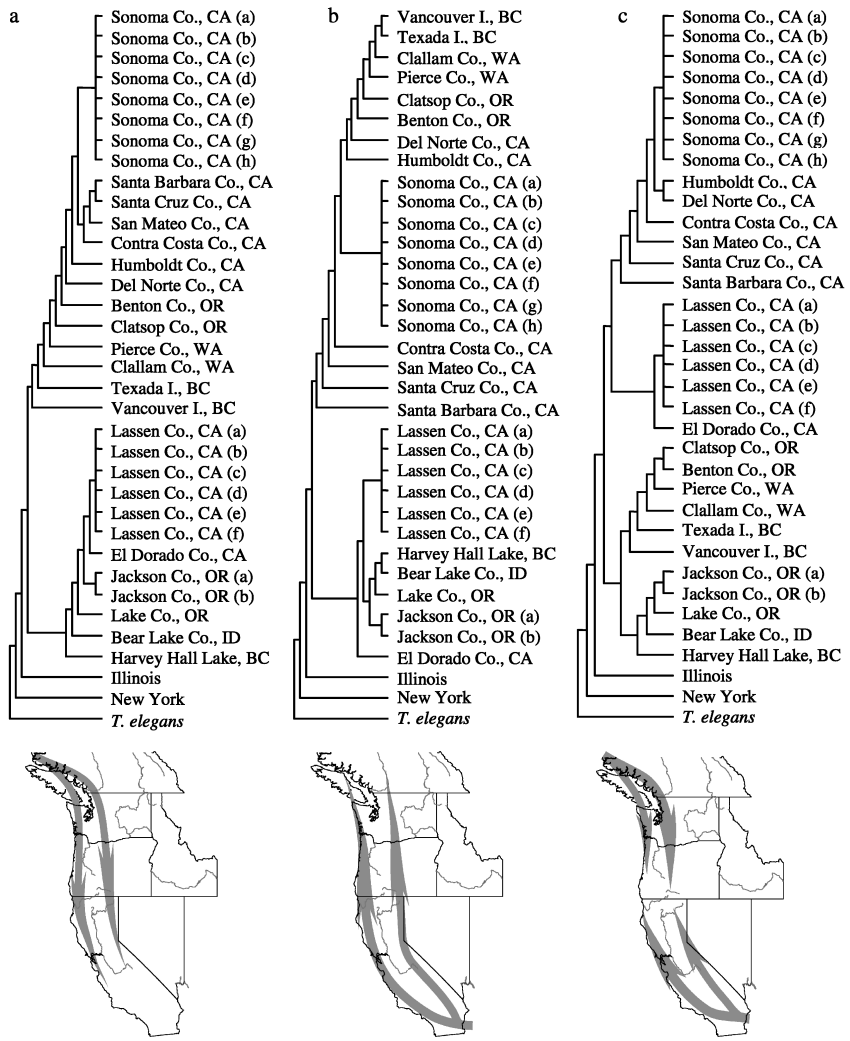

Fig. 2 Phylogenetic trees and pictorial scenarios representing three biogeographic hypotheses of postglacial expansion by *Thamnophis sirtalis* in western North America: (a) colonization from a northern refugium only, (b) colonization from a southern refugium only, and (c) colonization from concurrent northern and southern refugia. Taxon labels are as listed in Appendix 1.

are remarkably mobile and tolerant of a wide variety of environmental conditions, for the populations we examined we considered only the Cascade Mountains and the California Coast Range as significant barriers to east–west migration in all biogeographic hypotheses (Fig. 2a–c) and the Klamath Mountains as a significant barrier to north–south migration in one hypothesis (Fig. 2c). Thus, we generated latitudinally linked populations (see Appendix 1 for geographical coordinates) within each of the coastal and inland clades as described below. Specifically, we modelled (i) southward expansion along both the Pacific coast and the Great Basin from a northern refugium in southwestern Canada; (ii) northward expansion from a southern refugium in southern California along both the Pacific coast and the Sierra Nevada Mountains; and (iii) both southward and northward expansion from concurrent northern and southern refugia, respectively, as in (i) and (ii), meeting in the vicinity of the Klamath Mountains. Testing more refined biogeographic hypotheses than these three must await future fine-scale sampling of *T. sirtalis* populations.

Results

Sequence variation

We obtained a data set 2217 bases in length; 576 bases were collected from cytochrome *b*, 1050 bases from ND2, and 591 bases from ND4. There were 273 variable characters in the total combined data set, 89 of which were parsimony informative. However, we found only 55 variable characters within the western North American *Thamnophis sirtalis* populations (i.e. the in-group), 30 of which were parsimony informative. Within the in-group, we detected three parsimony informative characters at first codon positions, four at second positions, and 23 at third positions. Despite these modest numbers, the distribution of 10^6 trees generated randomly from the complete data set was

significantly left skewed ($g_1 = -1.6907$, $P < 0.01$, mean ± SD tree length = 513.1 ± 12.0, range = 416–538), strongly suggesting the presence of a phylogenetic signal in the data. This result persisted when only in-group taxa were included in the test ($g_1 = -0.5586$, $P << 0.01$; mean ± SD tree length = 203.8 ± 10.0, range = 151–224), revealing the appropriateness of phylogenetic analysis even at this restricted level.

We sequenced and analysed multiple snakes from Lassen County (Co.), CA (six individuals), Sonoma Co., CA (eight individuals), and Jackson Co., OR (two individuals) to assess the degree of sequence variability at the focal mtDNA loci for these populations. Variation among individuals within a population was very low, if any was present at all; *p*-distances between individuals ranged from 0 to 0.0018 for both Sonoma Co., CA and Lassen Co., CA populations, and no sequence variation was found between the two Jackson Co., OR individuals. With respect to comparisons between in-group populations only (i.e. disregarding comparisons between individuals within a population), *p*-distances ranged from 0 (e.g. Benton Co., OR and Clatsop Co., OR) to 0.0090 (e.g. Sonoma Co., CA and Vancouver Island, BC). Despite the low variation present between some populations, the variation that we detected within populations was concordant with the high level of similarity expected between individuals within a population; that is, multiple individuals grouped closely together in any phylogenetic analyses (see below).

Phylogenetic relationships

Maximum parsimony analysis produced a single most-parsimonious tree 309 steps in length (disregarding branches with 0 length) with a consistency index of 0.90 (Fig. 3). A more conservative analysis retaining only those branches with ≥ 80% bootstrap support revealed three major geographical clades: a Northwest Coastal clade, an Intermountain clade, and a California clade (Fig. 4). Maximum parsimony did not detect well-supported relationships among these three clades; the three formed a polytomy rising from the out-group taxa. Interestingly, the geographical proximity of the Vancouver Island, BC population and the nearby (separated by ~90 km and the Strait of Georgia) Texada Island, BC population was not reflected as genetic similarity of their mtDNA. Instead,

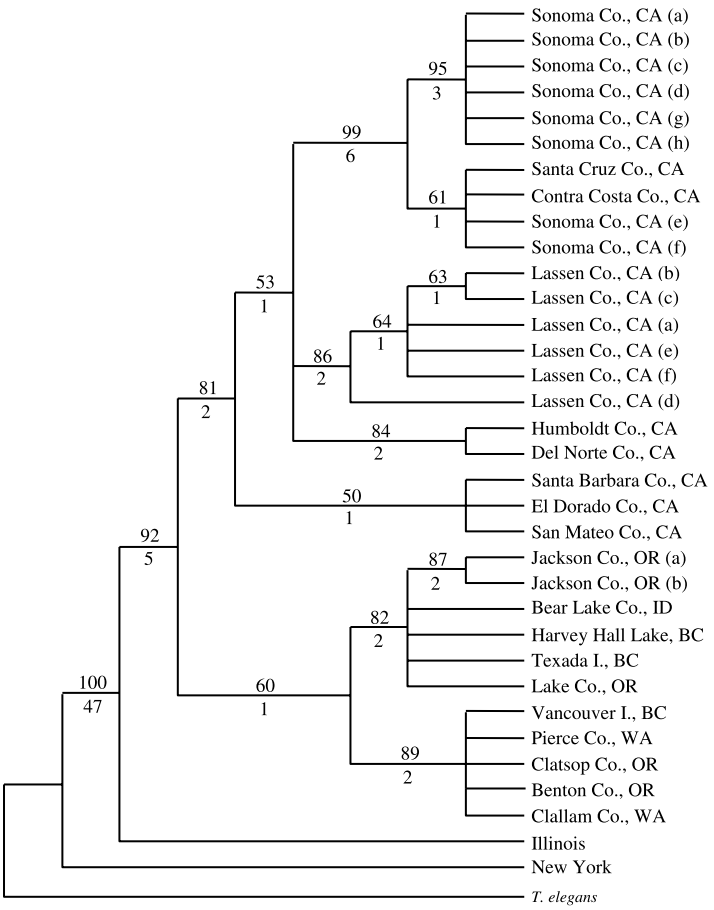

Fig. 3 Maximum parsimony tree for 32 individuals representing 19 populations of *Thamnophis sirtalis* in western North America. Taxon labels are as listed in Appendix 1. Numbers above branches indicate percentage support based on 1000 bootstrap replicates; numbers below branches are decay indices.

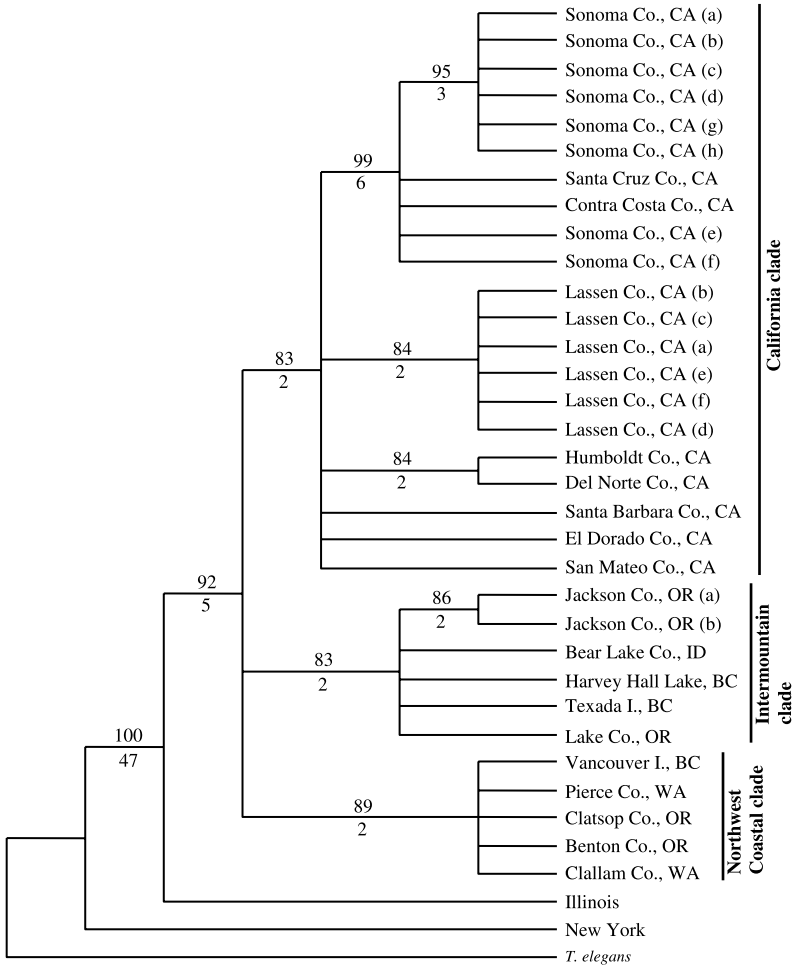

Fig. 4 Conservative maximum parsimony tree illustrating only branches with relatively strong support (i.e. ≥ 80% bootstrap support). Taxon labels are as listed in Appendix 1. Numbers above branches indicate percentage support based on 1000 bootstrap replicates; numbers below branches are decay indices. The labelled vertical bars on the right delineate regional clades described in the Results.

Vancouver Island, BC and Texada Island, BC grouped with the Northwest Coastal clade and the Intermountain clade, respectively.

Phylogenetic structure within each major geographical clade was minimal (Figs 3 and 4). No phylogenetic structure was evident among populations within the two northern clades. Much of the California clade was also part of a polytomy; two northern California populations, Del Norte Co. and Humboldt Co., formed a slightly divergent clade with relatively strong bootstrap support (84%). Also within the California clade was a relatively divergent group of three populations composed of Sonoma Co., Santa Cruz Co., and Contra Costa Co. This group is especially remarkable in that its bootstrap support is 99% and its decay index is 6; this level of support is comparable to that obtained for the branch separating the in-group taxa from the nearest out-group *T. sirtalis* population from Illinois (bootstrap support = 92%, decay index = 5). Also remarkable is that the populations in this group are only ~25–125 km away from another California population, San Mateo Co., yet genetic distinctness from this nearby population is readily apparent.

The maximum likelihood analysis recovered more detailed phylogeographic structure among the major clades (Fig. 5). In this analysis, the Northwest Coastal clade was sister to the Intermountain and California clades. Although more structure was recovered with maximum likelihood, most branch lengths were short, reflecting the overall low level of mtDNA divergence among these populations. Sonoma Co., Santa Cruz Co., and Contra Costa Co. maintained their divergent status in this analysis, exhibiting a clade branch length noticeably longer than any other branch length detected among the in-group populations except for the El Dorado Co. population (even greater than those between the three major geographical clades).

Biogeographic and systematic assessments

We used several methods, focusing on the path difference metric implemented in COMPONENT LITE (Page 1997), to assess objectively the three competing biogeographic hypotheses regarding historical relationships among the in-group populations (Fig. 2). After the hypothetical trees were constructed, we then employed COMPONENT LITE to

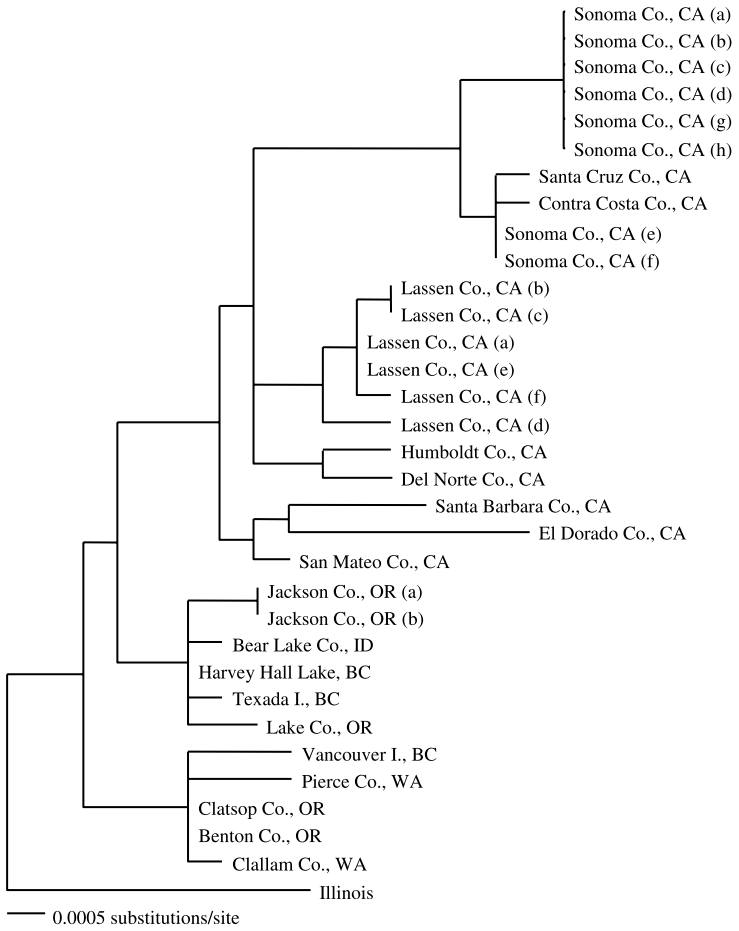

Fig. 5 Maximum likelihood tree for all individuals used in this study. Taxon labels are as listed in Appendix 1. Branches are drawn proportional to branch lengths estimated by the maximum likelihood model. Part of the out-group (i.e. *Thamnophis elegans* and *T. sirtalis* from New York) was pruned from the tree to emphasize branch lengths within the in-group.

compare the path difference for each test to a random distribution of 10^4 random trees generated by a Markov process. None of the hypothetical trees was significantly similar to the maximum parsimony tree ($P > 0.70$ in all three instances). This is a result of the large number of polytomies (and thus a reduced number of 'edges'; see Steel & Penny 1993) in the maximum parsimony tree relative to the more resolved hypothetical trees. The more resolved topology of the maximum likelihood tree was very different from the topologies modelling northward expansion from a southern refugium ($P = 0.6970$) and southward expansion from a northern refugium ($P = 0.9990$). However, the topology modelling expansion from concurrent northern and southern refugia was significantly similar to the maximum likelihood topology ($P = 0.0010$). These findings are mirrored qualitatively by the results obtained from similar analyses (not shown) using Wilcoxon signed-rank tests (Templeton 1983) and Shimodaira–Hasegawa tests (Shimodaira & Hasegawa 1999). Thus, conclusions drawn from this series of concordant independent analyses can be considered robust.

To evaluate the taxonomy of *T. sirtalis* in western North America, the subspecific designations of Rossman *et al.* (1996) were mapped onto the conservatively drawn maximum parsimony tree in place of their appropriate localities (Fig. 6). Under the topology provided by a conservative analysis of the focal mtDNA sequences, none of the morphologically defined subspecies appeared to be monophyletic. In the same regard, almost all of the in-group branch lengths calculated under maximum likelihood were very small. The only highly genetically divergent group is the previously mentioned California clade composed of Sonoma Co., Santa Cruz Co., and Contra Costa Co. populations (Fig. 5). All three of these populations fall in the range of *T. s. concinnus*; however, other populations that are not a part of this divergent group (e.g. Benton Co., OR and Del Norte Co., CA, among others) are also considered *T. s. concinnus* (Fig. 6). Finally, perhaps the most morphologically striking and divergent population (*T. s. infernalis* from San Mateo Co., CA) was only marginally divergent genetically from other populations examined (e.g. El Dorado Co., CA) and did not exhibit an elevated rate of molecular evolution (Fig. 5).

Discussion

Our research provides important insights into the phylogeography, mechanisms of genetic structuring, and

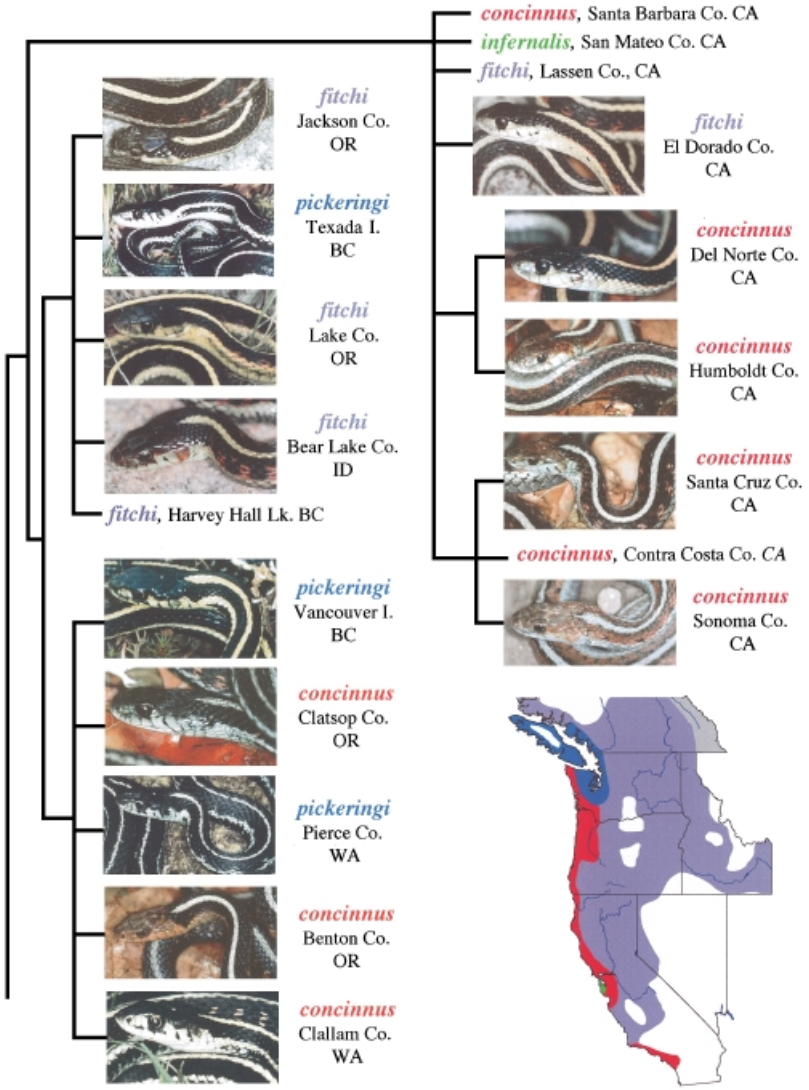

Fig. 6 Conservative maximum parsimony tree illustrating variation in colour pattern characters and indicating subspecies designations of *Thamnophis sirtalis* based on Rossman *et al*. (1996). Subspecies of *Thamnophis* are classified largely on the basis of head colour, the width and colour of dorsal stripes, background colour and the presence of lateral stripes. For simplicity, only one branch per locality is shown and the order of presentation, but not the topological structure, is modified from Fig. 4. Subspecies names are colour coded to match their geographical distribution in the range map at lower right (*sensu* Fig. 1).

systematics of a morphologically and ecologically variable species. We find that a combination of vicariant forces and postglacial dispersal have strongly shaped the genetic structure among populations of *Thamnophis sirtalis* in western North America. We also find that the genetic relationships revealed among these populations are not concordant with any morphologically based subspecies designations in this species.

Phylogeography

We detected three genetically and biogeographically distinct clades of *Thamnophis sirtalis* in western North America (Figs 3, 4 and 5). The Northwest Coastal clade consisted of populations located west of the Cascade Mountains and north of the Klamath Mountains near the Oregon–California border. A second group, the Intermountain clade, contained mostly populations east of the Cascade Mountains, within or north of the Klamath Mountains, and west of the Rocky Mountains. The third clade encompassed the remaining populations examined, all of which occurred in California, the southern portion of the range of *T. sirtalis* in western North America. Although each clade is distinct and is supported by substantive bootstrap values, it should be noted that decay indices were small (~2 in each case), particularly given the number of characters examined (~2200 bases of mtDNA sequence).

Despite the distinctness of these three clades, their interrelationships and intraclade affinities were more ambiguous. Parsimony analyses did not reliably support any bifurcating topology among them (Figs 3 and 4). Maximum likelihood analysis suggested that the Northwest Coastal clade was sister to the clade containing the Intermountain and California lineages, although the branch lengths were short (Fig. 5). Furthermore, neither parsimony nor maximum likelihood detected structure within either

northern clade. Only slightly more structure was apparent within the California clade, with one divergent group of three populations found near the San Francisco Bay area. These results are not surprising given the small decay indices, and suggest one or more of several mechanisms causing such low genetic divergences: (i) constraints on molecular evolution; (ii) continuing gene flow; and (iii) recent geographical expansion.

We do not believe that constraints on the evolution of mtDNA explain our results for at least two reasons. First, our in-group populations have readily differentiated from conspecific populations in Illinois and New York at the loci we examined (Figs 3 and 4). Second, and perhaps even more convincing, the California subclade containing populations from Sonoma Co., Santa Cruz Co., and Contra Costa Co. has evolved a number of fixed differences at these same loci. This result is approximately equivalent in magnitude to the genetic divergence separating the entire lineage of *T. sirtalis* in western North America from its conspecifics in Illinois (Figs 3, 4 and 5). The reason for the apparently elevated rate of molecular evolution in this California subclade is unknown but warrants further study.

Detecting the impact of continuing gene flow in our study is more problematic. In fact, we did not design this study with that goal in mind. That said, we believe continuing gene flow may be an important force in at least some instances. For example, in examining eight unrelated snakes from Sonoma Co., CA, not only did we detect intrapopulation genetic differences, but also we found that two of these individuals grouped more closely to snakes from nearby Contra Costa Co., CA and Santa Cruz Co., CA (Figs 3, 4 and 5). This result indicates that the two divergent Sonoma Co. snakes contained haplotypes more genetically similar to snakes from other populations than to other individuals in their own population, possibly because of the retention of ancestral polymorphisms. Migration from morphologically divergent populations may be a more likely explanation however. Experimental studies reveal considerable variation in resistance to tetrodotoxin within some populations (including Sonoma Co.; Brodie *et al*. submitted for publication), suggesting an influence of immigration. Indeed, despite preferences for specific habitat characteristics in western North America (e.g. White & Kolb 1974), common garter snakes can exhibit long-range movements (e.g. Gregory & Stewart 1975). A clear understanding of the roles of continuing gene flow and lineage sorting in shaping the genetic structure of populations of *T. sirtalis* in western North America awaits more extensive within-population sampling, genetic assessment of nuclear loci (particularly fast-evolving loci like microsatellites), and long-term mark–recapture field studies.

We consider historical forces to be the best explanation of low genetic divergences and poorly resolved topological arrangements among populations of *T. sirtalis* in western North America. We believe that a combination of vicariant events followed by genetic fixation and subsequent recolonization best account for our results. This conclusion is grounded in the following observation: much of the current habitat occupied by *T. sirtalis* in northwestern North America was either under ice or tundra-like during the last glacial period in the Pleistocene (Barnosky *et al*. 1987; Josenhans *et al*. 1995). Consequently, *T. sirtalis* and other co-distributed biota must have colonized the majority of northwestern North America from glacial refugia within the last ~10 000 years.

The results of our maximum likelihood analysis are most in accord with a biogeographic hypothesis that postulates restriction of *T. sirtalis* to multiple glacial refugia, during which fixation of respective mtDNA variants occurred, followed by colonization of previously unsuitable habitat (*sensu* Byun *et al*. 1997, 1999 and references cited therein). The presence of biota south of the glacial ice sheets in northern North America is well accepted, but the existence of contemporaneous northern refugia is controversial (the Haida Gwaii refugium hypothesis, e.g. Demboski *et al*. 1999 vs. Byun *et al*. 1999). The Haida Gwaii archipelago (formerly the Queen Charlotte Islands) located off the west coast of Canada contains a host of endemic taxa, which has led to the hypothesis that the region provided a refugium during the height of glaciation during the Pleistocene (summarized in Byun *et al*. 1997). Previous evolutionary analyses of the Haida Gwaii refugium hypothesis have consisted primarily of marshalling phylogenies of diverse taxa and qualitatively comparing the topologies for congruence to infer the presence/absence of a northern refugium (e.g. Byun *et al*. 1997, 1999; Soltis *et al*. 1997; Demboski *et al*. 1999). As a consequence, controversy has erupted over how to interpret the biogeographic signature of these genetic patterns and, thus, over the validity of the Haida Gwaii refugium hypothesis. To our knowledge, our analyses of phylogenetic topologies of *T. sirtalis* offer the first explicit statistical tests of this and competing biogeographic hypotheses involving this region (but see Pook *et al*. 2000). Our findings largely reject the hypotheses of solely a southern or solely a northern refugium for *T. sirtalis* in western North America in favour of the hypothesis of both southern (Great Basin and California) and northern (Haida Gwaii) refugia. We emphasize, however, that some of the internode distances on our maximum likelihood tree were short and relatively poorly supported, despite the extensive molecular data set compiled. Further work is clearly warranted to evaluate more critically our mtDNA phylogeographic hypothesis for *T. sirtalis* in western North America.

Comparative molecular phylogeography (*sensu* Bermingham & Moritz 1998; Avise 2000), even in the absence of statistical analysis, can comprise strong evidence for major historical processes when a diversity of organisms

shares underlying patterns of genetic structuring. The abundance of concordant phylogeographic patterns of organisms in western North America is important in this regard. Detailed comparisons are beyond the scope of this paper, but suffice it to say that the pattern that we detected within *T. sirtalis*, supported as it is by statistical tests, strengthens evolutionary conclusions concerning vicariant forces (e.g. Byun *et al*. 1997) and post-Pleistocene expansion in western North America (e.g. Green *et al*. 1996; Shaffer & McKnight 1996; Janzen *et al*. 1997). Of course, not all species will share these patterns, as a result of ecological and historical differences, so further studies will continue to be important (Avise 1998).

Taxonomic implications

If we are to consider morphologically based subspecies as valid taxonomic entities, then it seems reasonable to expect that genetic differentiation underlies this morphological variation and exhibits concordant taxonomic signals reflecting evolutionary history (*sensu* Burbrink *et al*. 2000). None of the morphologically based subspecies designations of *T. sirtalis* evaluated in this study seem to be valid based on mtDNA sequences, which do not indicate any reciprocal monophyly. All five western subspecies (Rossman *et al*. 1996) exhibit geographical ranges that are in conflict with monophyletic lineages derived from our extensive molecular analyses (Figs 1 and 6). That is, some populations of a given morphologically based subspecies are more closely related to populations of another subspecies than they are to other populations of their own subspecies. Furthermore, even the colour pattern characters (e.g. head colour, the width and colour of dorsal stripes, background colour, the presence of lateral stripes, and ventral pigmentation) on which the subspecies descriptions are primarily based (Rossman *et al*. 1996) do not hold up under scrutiny, retaining little evidence of the phylogenetic history of *T. sirtalis* in western North America (Fig. 6). Our findings are consistent with similar molecular phylogenetic analyses of morphologically based subspecies of *Thamnophis elegans* (Bronikowski & Arnold 2001) and most other snake species in North America (Rodriguez-Robles *et al*. 1999; Burbrink *et al*. 2000; Rodriguez-Robles & De Jesus-Escobar 2000; but see Rodriguez-Robles *et al*. 2001). These results are not entirely unexpected because subspecies are assumed to be interbreeding taxonomic units. As a consequence, gene flow among subspecies will act to constrain the achievement of reciprocal monophyly. At the same time, the mtDNA findings point to substantial convergence in colour patterns among various populations, implying a significant role for local evolutionary forces (e.g. natural selection) in shaping morphological variation.

Our findings have important taxonomic implications however. For example, the results suggest that the California clade has attained the status of an independent evolutionary lineage relative to the northern clades (Figs 3, 4 and 5). Furthermore, a split between Northwest Coastal and Intermountain clades is well supported north of California. We nonetheless recommend that the taxonomic issues raised by our mtDNA results be pursued at the phenotypic level as well. Subsequent nomenclature changes could then be suggested based on multiple criteria to best reflect the evolutionary history of western populations of *T. sirtalis*.

One particularly sensitive issue involves the status of *T. sirtalis* on the San Francisco Peninsula of California. This population has been afforded federal endangered status (Rossman *et al*. 1996; http://ecos.fws.gov/species_profile/species_profile.html?spcode = C002), as *T. s. tetrataenia*, but was renamed as *T. s. infernalis* based on examination of the holotype specimen (Boundy & Rossman 1995). The populations on the north coast of California that were previously known as *T. s. infernalis* were subsumed into the subspecies *T. s. concinnus* (Rossman *et al*. 1996). Our molecular analyses suggest that *T. s. tetrataenia* (San Mateo Co.) is not genetically unique from other California populations. However, geographically nearby populations (Sonoma Co., Santa Cruz Co., and Contra Costa Co.) belong to a strongly supported clade that does not include San Mateo Co. Populations of *T. sirtalis* in the San Francisco Bay area are clearly under tremendous pressure caused by extreme habitat loss, but do they comprise a separate *taxonomic unit* reflecting evolutionary relationships that deserves special protection? The answer to this question must be re-evaluated in light of our molecular results. Still, a more refined answer, and more appropriate conservation decisions in general, would probably be forthcoming with detailed genetic studies of these populations.

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Fred Janzen has a longstanding interest in the evolutionary ecology and genetic structure of reptile populations. James Krenz, a former MS student in the Janzen laboratory, is presently a technician in Michael Nachman's laboratory at the University of Arizona. Tamara Haselkorn, a former undergraduate research assistant in the Janzen laboratory, is currently a technician in Daniel Promislow's laboratory at the University of Georgia. This study largely arose from a long-term collaboration between Edmund Brodie, Jr and Edmund Brodie III on co-evolution between these predatory garter snakes and their newt prey (*Taricha*), which contain an otherwise lethal chemical called tetrodotoxin.

Appendix I

Locality information for all *Thamnophis sirtalis* and *T. elegans* out-group samples included in the phylogenetic analyses

Sample	Subspecies	Voucher no.	Locality (Latitude, Longitude)
Out	*T. elegans*	UTA R-43458	USA: California, Lassen Co., Peony Springs
1	*T. s. sirtalis*	UTA R-46064	USA: New York, Cortland Co.
2	*T. s. sirtalis*	J52114	USA: Illinois, Carroll Co.
3	*T. s. concinnus*	UTA R-46968	USA: California, Humboldt Co., Dry Lagoon (41°13.28′ N, 124°06.45′ W)
4	*T. s. concinnus*	UTA R-50348	USA: California, Del Norte Co., Crescent City (41°48.56′ N, 124°10.12′ W)
5	*T. s. concinnus*	No vouchers	USA: California, Sonoma Co., Willow Creek (30°25.57′ N, 123°04.45′ W)
6a	*T. s. fitchi*	UTA R-43626,	USA: California, Lassen Co., McCumber (40°32.47′ N, 121°44.01′W)
6b		UTA R-43172,	
6c		UTA R-43187,	
6d		UTA R-43253,	
6e		UTA R-43210,	
6f		UTA R-43268	
7	*T. s. concinnus*	No voucher	USA: California, Contra Costa Co., East Bay (37°58.69′ N, 122°13.62′ W)
8	*T. s. concinnus*	UTA R-46970	USA: California, Santa Cruz Co., Gilroy (36°58.29′ N, 121°35.35′ W)
9	*T. s. infernalis*	CAS 208713	USA: California, San Mateo Co., San Bruno (37°37.86′ N, 122°24.60′ W)
10	*T. s. fitchi*	No voucher	USA: California, El Dorado Co., Omo (38°34.61′ N, 120°38.80′ W)
11	*T. s. fitchi*	UTA R-46960	USA: California, Santa Barbara Co., Vandenburg (34°44.59′ N, 120°33.12′ W)
12	*T. s. pickeringi*	UTA R-46939	Canada: British Columbia, Texada I., Priest Lake (49°44.82′ N, 124°33.80′ W)
13	*T. s. fitchi*	UTA R-43106	USA: Oregon, Lake Co., Lofton Lake (42°15.97′ N, 120°49.80′ W)
14a	*T. s. fitchi*	UTA R-47199,	USA: Oregon, Jackson Co., Parsnip (42°06.04′ N, 122°27.03′ W)
14b		UTA R-48257	
15	*T. s. fitchi*	UTA R-46924	Canada: British Columbia, Harvey Hall Lake (49°44.57′ N, 120°40.59′ W)
16	*T. s. fitchi*	UTA R-46945	USA: Idaho, Bear Lake Co., Bear Lake (42°13.92′ N, 111°21.06′ W)
17	*T. s. concinnus*	UTA R-43533	USA: Washington, Clallam Co., Clallam Bay (48°14.33′ N, 124°15.47′ W)
18	*T. s. concinnus*	UTA R-43309	USA: Oregon, Benton Co., Philomath (44°13.13′ N, 123°24.39′ W)
19	*T. s. concinnus*	UTA R-43932	USA: Oregon, Clatsop Co., Warrenton (46°09.87′ N, 123°56.94′ W)
20	*T. s. pickeringi*	No voucher	USA: Washington, Pierce Co., Dupont (47°06.17′ N, 122°38.05′ W)
21	*T. s. pickeringi*	UTA R-46958	Canada: British Columbia, Vancouver I., Campbell River (49°59.23′ N, 125°26.45′ W)

Subspecies designations are based on Rossman *et al*. (1996). Museum and collector abbreviations: J, Fredric J. Janzen; CAS, California Academy of Sciences; all other specimens exhibit voucher numbers from The University of Texas at Arlington, Collection of Vertebrates (UTA). mtDNA sequences based on these samples have been deposited in GenBank (Accession No. AY136169–136273).