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Cytochrome *b* Phylogeny Does Not Match Subspecific Classification in the Western Terrestrial Garter Snake, *Thamnophis elegans*

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We sequenced a 307-bp fragment of the mitochondrial cytochrome *b* gene from 42 individuals representing 14 populations of the western terrestrial garter snake, *Thamnophis elegans*. Current taxonomy recognizes either five or six subspecies of *T. elegans* based on color and scale morphology, but all agree on three major geographic races (*T. e. elegans*, *terrestris*, and *vagrans*). Although the cytochrome *b* phylogeny did not match subspecific classification of the populations, it did yield geographically proximate groups. Populations from the Sierra Nevada range and Monterey, California, formed one monophyletic group of *T. e. elegans* and *T. e. terrestris*. This Sierran/Monterey group was included in a larger group with eastern populations from the Great Basin (*T. e. vagrans*). The other well-supported group was comprised of populations from the western Great Basin (*T. e. vagrans*). One population from the northern California coast (*T. e. terrestris*) was basal to both groups of populations. Thus, neither *T. e. vagrans* nor *T. e. terrestris* formed monophyletic groups. Average percent sequence divergence between the outgroup (*T. sirtalis*) and *T. elegans* was 7.9–12%. Within *T. elegans*, divergence among populations ranged from 0.3–7.7%.

THE modern concept of *Thamnophis elegans* and its array of geographic races is based on morphological analyses (e.g., Johnson, 1947; Fox, 1951; Rossman, 1979). Six subspecies were recognized in the most recent review (Rossman et al., 1996). Three occur over extensive geographic areas: *T. e. elegans* in the Sierra Nevada and Inner Coast Range of northern California and western Oregon; *T. e. terrestris* in coastal California and Oregon; and *T. e. vagrans* in the Great Basin and Rocky Mountains from Arizona and New Mexico northward to British Columbia, Alberta, and Saskatchewan and eastward to the Black Hills of South Dakota. The other three subspecies are found in much smaller areas: *T. e. arizonae* in eastern Arizona and western New Mexico; *T. e. hueyi* in the Sierra San Pedro Martir of northern Baja California; and *T. e. vascotanneri* in southeastern Utah. In addition, two other subspecies have been previously recognized: *T. e. biscutatus* in the Klamath Lake Basin of southern Oregon; and *T. e. nigrescens* in the Puget Sound drainage and coastal British Columbia (Fitch, 1980, 1983; Fox, 1951). Although some of the characters that define subspecies (e.g., dorsal stripe patterns) appear to be minor distinctions, many of them are supported by substantial statistical differences in scalation and dentition characters. In addition, many of these traits are heritable (Arnold, 1988; Arnold and Phillips, 1999; Bronikowski and Arnold, 1999). Thus, these characters may be markers for important events in the genealogy of this species. As Rossman et al. (1996) point out, a study of geographic variation in the phenotypic characters throughout the range of *T. elegans* is badly needed.

Although the subspecies of *T. elegans* might represent monophyletic groups, a limitation of the morphological work is that no comprehensive statistical analyses have been conducted across the entire species range. In the most recent analysis of the subspecies with the widest range (*T. e. vagrans*), for example, populations were pooled over large geographic areas (Fitch, 1940). An electrophoretic study that included nearly all species in the genus *Thamnophis* by de Queiroz and Lawson (1994) indicated that *T. elegans* was part of a group that included *T. eques, T. marcianus,* and *T. sirtalis*. These authors report that shared derived alleles in all sampled populations of *T. elegans* support a concept of monophyly for this species. However, their analysis of cytochrome *b* sequence data presents a different picture. The sequence data suggested that *T. elegans* was part of a group that included

T. brachystoma, T. butleri, and *T. radix*. A sample of three sequences representing *T. elegans* from Arizona, California, and Washington suggested that this species was paraphyletic with respect to *T. radix*. The authors suggested that *T. elegans* might be monophyletic and the sequence results might reflect incomplete lineage sorting following speciation. Because two of the three sequences in question contained many gaps, the results might also be a sampling artifact. A limitation of the published sequence work is its reliance on extremely small samples from widely scattered sites in the range of *T. elegans*.

The goal of this paper is to enlarge the molecular view of relationships among the populations currently recognized as *T. elegans* by analyzing additional sequences of cytochrome *b* and comparing this mitochondrial gene tree to subspecies designations. Our focus is on the monophyly and relationships of the three main subspecies (*T. e. elegans, T. e. terrestris,* and *T. e. vagrans*; Rossman et al., 1996), rather than on the monophyly of *T. elegans* itself. Recent articles debate the potential pros and cons of using mitochondrial DNA to construct phylogenetic hypotheses (e.g., Avise, 1994; Rand, 1994; Wallis, 1999), and this debate is far from being resolved. Notwithstanding, this is the first extensive mitochondrial DNA treatment of the diversity within *T. elegans*.

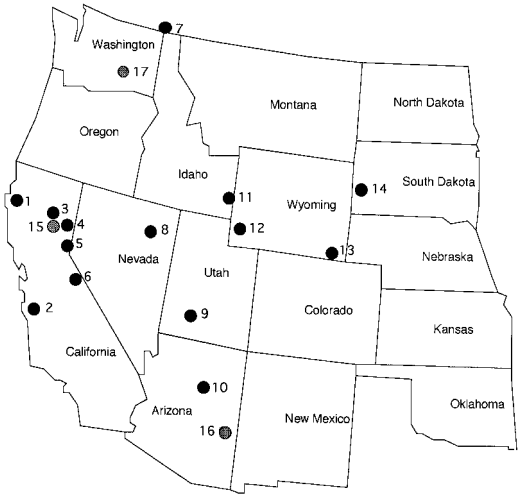

Fig. 1. Collection localities of *Thamnophis elegans* from the United States and British Columbia. We collected at populations 1–14 (for descriptions, see Appendix). Populations 15–17 were reported by de Queiroz and Lawson (1994). Their locations on this map are approximate. Populations 1 and 2 are *T. e. terrestris*; populations 3–6 and 15 are *T. e. elegans*; populations 4–14 and 17 are *T. e. vagrans*; population 16 was reported as *T. e. vagrans* but occurs in the range of the new subspecies *T. e. arizonae* (after Rossman et al. 1996).

Materials and Methods

Populations.—Several snakes from each of 14 populations of *T. elegans* were sampled from 1990–1997 (Fig. 1). Liver samples were obtained from live animals in the field and were frozen in liquid nitrogen until transport to the laboratory where they were placed in a −80 C freezer. (See the last column of the Appendix for specimen voucher number.) These populations were chosen to sample the geographic range of *T. elegans*. The populations included four that represented the mountain subspecies (*T. e. elegans*), two populations of the coastal subspecies (*T. e. terrestris*), and eight populations of the Great Basin subspecies (*T. e. vagrans*; Appendix). These are the three main forms of *T. elegans* (Rossman et al., 1996). In addition to our sampling, we included the three haplotypes reported for *T. elegans* in de Queiroz and Lawson (1994), which indlucded one *T. e. elegans,* one *T. e. vagrans*, and one individual representing *T. e. arizonae* (Tanner and Lowe, 1989). De Queiroz and Lawson (1994: fig. 2) found *T. sirtalis* to be in a separate monophyletic group than that containing these *T. elegans* haplotypes. Therefore, we sampled *T. sirtalis* for use as an outgroup from Whiteside County, Illinois; Pacific and Jefferson Counties, Washington; Lassen County, California; and Benton County, Oregon (Appendix).

Laboratory procedures.—A total of 307 base pairs of the mitochondrial cytochrome *b* gene were amplified and sequenced using the PCR technique and automated sequencing. Total cellular DNA was extracted from either a single scale or a 10-mg liver slice using a standard phenol/chloroform extraction method (Protocol 1 in Hillis et al., 1996). We used PCR primers that were shortened universal cytochrome *b* primers from Kocher et al. (1989; Light strand: 23 bp: 5′–CCATCCAACATCTCAGCATGATGAAA, Heavy strand: 25 bp: 5′–CCCCTCAGAATGATATTTGTCCTCA). Two μl of resuspended DNA were amplified in 50 μl reactions containing: 1x Taq buffer, 4 mM MgCl2, 1 μM of each primer, 0.1 mM dNTP, and 1 unit of Taq polymerase (Boehringer Mannheim, Indianapolis, IN) in a Perkin Elmer 2400 thermal cycler for 30 cycles of 94 C: 1 min, 45 C: 2 min, 72 C: 2 min. Double-stranded amplified products were gel purified on lowmelt agarose, and plugs were suspended in 100 μl dH2O and reamplified under the same conditions. Reamplification prod-

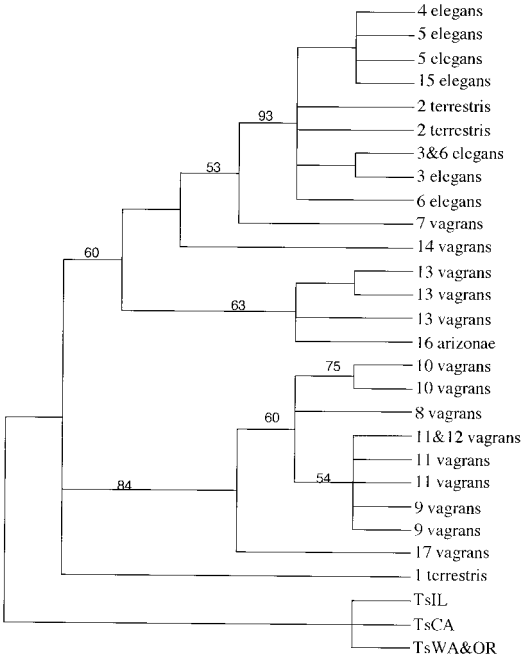

Fig. 2. Strict consensus of six most-parsimonious cytochrome *b* gene trees. Imposed on the consensus tree are values from 200 bootstrap replicates (i.e., the proportion of trees in which that group was supported). Consistency Index = 0.734. Taxa are labeled with population number and subspecies designations (see Appendix) for *T. elegans*, and with "Ts" for the *T. sirtalis* outgroup populations.

ucts were purified and concentrated with Microcon-22 purifiers and Microcon-100 filters (Amicon, Inc., Beverly, MA).

For each sample, both the heavy and light strands were sequenced following the procedure for use with the Applied Biosystems, Inc., Prism 377 fluorescent dye-based automated sequencer (Perkin Elmer ABI, Foster City CA). ABI software was used to resolve ambiguous bases where possible (AutoAssembler 1.4) and to align the sequences (Sequence Navigator 1.0.1).

Phylogenetic analysis.—All analyses were performed in program PAUP (Phylogenetic Analysis Using Parsimony, v4.0b3, D. L. Swofford, 2000, unpubl.). We used the parsimony optimality criterion that chooses trees of minimum length, and assumes that simpler trees (those with the fewest number of character changes) better reflect the true relationship among taxa than more complex trees (Swofford et al., 2000). For the parsimony analysis, we performed a heuristic search using random stepwise addition of 10 replicates each and a branch-swapping algorithm of tree-bisection-reconnection, and ignored uninformative characters. In addition, 200 bootstrap replicates (random resampling of variable characters) were calculated with the same criterion. Concerning character weighting, because of the low maximum genetic difference between the outgroup (*T. sirtalis*) and all ingroup taxa, it was unlikely that multiple substitutions at single sites had occurred (Hackett, 1996). In addition, scatter plots of genomic divergence resulting from transitions and transversions versus total genomic divergence (uncorrected) revealed linear relationships which supports this assumption.

To test whether the resulting gene tree differed significantly from one in which all populations and subspecies were constrained to be monophyletic, we performed a permutation test in PAUP. This test (t-ptp, topology-dependent permutation tail probability) calculates a probability value that the monophyletic tree is not different from the tree of shortest length (see also Swofford et al., 1996).

Results

We found 22 haplotypes of the cytochrome *b* fragment from the 42 individuals sequenced from our 14 *T. elegans* populations (Appendix). One additional haplotype was found by including the three individuals from de Queiroz and Lawson (1994; Appendix). The six *T. sirtalis* individuals yielded three additional haplotypes. Overall, 57 of 307 bases were variable, which translated into three variable amino acids. These 57 variable nucleotides resulted in genetic distances between the outgroup (*T. sirtalis*) and the populations of *T. elegans* that ranged from 7.9–12%. Within *T. elegans*, sequence divergence among populations ranged from 0.3–7.7%, whereas that within *T. sirtalis* ranged from 0.3–0.6%. Forty-two variable sites were informative for phylogenetic reconstruction.

When these DNA sequences were subjected to parsimony analysis, six most-parsimonious trees of length 64 steps resulted (Fig. 2). This analysis indicates paraphyletic relationships for two of three subspecies of *T. elegans* examined (*T. e. terrestris* and *T. e. vagrans*). The consistency index (CI) for each of the six most-parsimonious trees was 0.734, (rescaled = 0.670), which indicates that the data fit these trees well.

To test whether the resulting consensus phylogeny was significantly different from one in which all populations and subspecies were monophyletic, a permutation test was performed (with the three de Queiroz and Lawson, 1994, taxa deleted). In 100 replicates, the dif-

ference between the unpermuted data and the monophyletic constraint tree was nine steps. The minimum and maximum differences between the constraint tree and the observed replicate tree were 26 and 48 steps, respectively. The overall probability that the tree constrained to be monophyletic was not different from the shortest trees observed in all replicates was $P = 0.01$. Thus, the null hypothesis of monophyly was rejected, that is, the paraphyletic subspecies revealed in the parsimony analysis fit the data significantly better than a monophyletic tree.

Discussion

In only one case was a population with multiple haplotypes monophyletic (population 10 from Arizona). When multihaplotype populations were not strictly monophyletic, the populations formed monophyletic groupings with nearby populations. For example, although the Sierran populations (*T. e. elegans*) did not form individual groups (collection localities 3, 5, and 6), they grouped with the other populations of *T. e. elegans* (4 and 15) as well as with a population of *T. e. terrestris* from Monterey County, California (Fig. 2). Monterey snakes and snakes from Humboldt County, California, belong to the coastal subspecies (*T. e. terrestris*); however, these two populations did not group together. In fact, snakes from Humboldt County (locale 1) were not included in any population groupings in this analysis (Fig. 2). Thus, *T. e. terrestris* appears to be an unsupported subspecific designation according to these molecular data, whereas *T. e. elegans* may extend beyond its Sierran range. The other recognized subspecies (*T. e. vagrans*) was also found to be paraphyletic. Snakes from British Columbia, South Dakota, and eastern Wyoming were more closely related to Sierran and Monterey snakes than to those from the western Great Basin. In addition, the *T. e. arizonae* sample from de Queiroz and Lawson (1994) (locale 16) was indistinguishable from the eastern Wyoming population and was distinct from our Arizona population. The other main group included the western Great Basin populations.

Distances in this study were similar to those reported in a thamnophine molecular phylogeny that sampled two or three populations of each *Thamnophis* species. De Queiroz and Lawson (1994) reported 9.3% divergence between *T. elegans* and *T. sirtalis* versus 7.9–12.0% in this study. And they reported 2.6–5.5% sequence divergence among three individuals of *T. elegans* (0.3–7.7% in this study). Furthermore, in our study, *T. elegans* exhibited substantially more sequence divergence among populations than *T. sirtalis*. Populations of *T. elegans* had up to 7.7% sequence divergence, but the populations of *T. sirtalis* had only 0.6% divergence or less even though populations were sampled from Illinois to Washington, representing multiple subspecies. This discrepancy between *T. elegans* and *T. sirtalis*, together with statistical results indicating paraphyly of *T. elegans* subspecies, suggests that deep lineages are included within the set of populations currently recognized as *T. elegans*.

The results of this mitochondrial study do not agree with a current subspecific classification for *T. elegans* based on scalation, dentition, and coloration characters (reviewed in Rossman et al., 1996). The mitochondrial analysis may have identified real clades that were not apparent to workers using phenotypic characters. Under this interpretation, multiple cryptic subspecies species may reside within *T. e. terrestris* and *T. e. vagrans*. Alternatively, the mitochondrial results may represent a gene genealogy that is at odds with the major features of genome evolution in *T. elegans*. De Queiroz and Lawson's (1994) study, in which allozymes and mitochondrial sequences gave conflicting results for *T. elegans*, also underscores the need for a comprehensive study of nuclear and mitochondrial sequences, as well as morphological characters, throughout the range of *T. elegans*.

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APPENDIX. POPULATION (NUMBER FROM FIGURE 1), SUBSPECIES, COUNTY (STATE), COORDINATES, SAMPLE SIZE (*n*), HAPLOTYPE, AND SPECIMEN.

Population	Subspecies[a]	County (State)	Latitude (N), Longitude (W)	*n*	Haplotype #	Specimen #[b]
1	terrestris	Humboldt (CA)	40.9299, 124.1301	3	9	SJA 19858, 20049, 21503
2	terrestris	Monterey (CA)	36.5298, 121.9230	4	7, 8	SJA 9953-6
3	elegans	Shasta (CA)	41.0683, 121.6957	4	4, 6	SJA 3438, 3465, 3589, 3593
4	elegans	Lassen (CA)	40.6997, 120.7407	4	1	SJA 22579, 22596-8
5	elegans	Sierra (CA)	39.4998, 120.2363	2	2, 3	SJA 22477, 22312
6	elegans	Tuolumne (CA)	38.4107, 120.0457	4	4, 5	SJA 3494, 3506, 3510, 3521
7	vagrans	Creston (BC)[c]	49.1000, 116.5167	3	22	SJA 9947-9
8	vagrans	Elko (NV)	40.8772, 115.7007	1	21	SJA 21563
9	vagrans	Garfield (UT)	37.6230, 112.4717	2	17, 18	SJA 23092-3
10	vagrans	Coconino (AZ)	34.9370, 111.4785	2	19, 20	SJA 9957-8
11	vagrans	Caribou (ID)	43.2553, 112.2658	3	14, 15, 16	SJA 21550-2
12	vagrans	Lincoln (WY)	41.7763, 110.5341	3	14	SJA 21533, 21536, 21539
13	vagrans	Laramie (WY)	41.3280, 105.6044	3	11, 12, 13	SJA 21519-21
14	vagrans	Lawrence (SD)	43.9804, 103.4788	4	10	SJA 21507, 21511-2, 21515
15	elegans	Plumas (CA)	40.0682, 120.9458	1	1	CU 12483
16	arizonae	Apache (AZ)	33.8319, 109.1025	1	13	CU 12481
17	vagrans	Kittitas (WA)	46.9152, 120.6941	1	23	CU 12479
	T. sirtalis sirtalis	Whiteside (IL)	41.9500, 90.1167	1	24	FJJ 521141
	T. sirtalis concinnus	Pacific (WA)	46.3000, 123.9667	1	25	UTA-R43371
	T. sirtalis concinnus	Jefferson (WA)	47.5167, 124.0167	1	25	UTA-R43075
	T. sirtalis concinnus	Benton (OR)	44.5500, 123.3667	1	25	UTA-R43309
	T. sirtalis fitchi	Lassen (CA)	40.5167, 120.7500	1	26	UTA-R43457
	T. sirtalis fitchi	Humboldt (CA)	41.2167, 124.1000	1	26	UTA-R46968

[a] Rossman et al., 1996.

[b] Specimens are located in the laboratories of S. J. Arnold, Dept. of Zoology, Oregon State University (SJA#); E. D. Brodie Jr., Dept. of Biology, Utah State University (UTA#); or F. J. Janzen, Dept. of Zoology, Iowa State University (FJJ#); CU specimens are from deQueiroz and Lawson (1994).

[c] City, Province.