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Author(s) :Lorin A. Neuman-Lee, Suzanne E. McGaugh, Michael Pfrender, and Fredric J. Janzen

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## Using Mitochondrial DNA to Determine the Identity and Origin of a Gartersnake Found in Alaska

LORIN A. NEUMAN-LEE,<sup>1,2</sup> SUZANNE E. MCGAUGH,<sup>3</sup> MICHAEL PFRENDER,<sup>4</sup> AND FREDRIC J. JANZEN<sup>1</sup>

<sup>1</sup>Department of Ecology, Evolution and Organismal Biology, Iowa State University, Ames, Iowa 50011 USA

<sup>3</sup>Department of Biology, Duke University, Durham, North Carolina 27708 USA

<sup>4</sup>Department of Biological Sciences, Notre Dame University, Notre Dame, Indiana 46556 USA

**ABSTRACT.**—In an era of rapidly changing environments and greater human mobility and penetration into wild areas, organisms are being discovered in increasingly unexpected places. One such finding is a road-killed juvenile gartersnake (*Thamnophis*) outside of Haines, Alaska, in August 2005. The poor condition of the specimen prevented a positive identification based on morphology alone. Furthermore, no snakes are known to be native to this region. We therefore undertook a molecular approach to determine the species and geographic origin of the individual. We sequenced two partial loci of mitochondrial DNA (cytochrome *b* and NADH subunit 2) from the Alaska specimen and seven specimens from localities in the lower northwestern United States. Phylogenetic reconstruction using our sequences and additional GenBank samples unambiguously revealed that the Alaska specimen is *Thamnophis ordinoides* and that it shares a haplotype with the northernmost sampled Washington population of *T. ordinoides*. In light of these analyses, we assess the likelihood that the specimen represents a relict population, a recent natural colonization, or a fresh introduction.

Mitochondrial DNA (mtDNA) holds insight into the evolutionary history of an organism. Phylogenetic analyses of mtDNA can be used to determine the taxonomic identity and geographic origin of an individual. To that end, mtDNA sequence data can be used forensically to determine the original location of individuals displaced by natural or human-mediated causes (Rowe et al., 1998; Hua et al., 2005; Pauly et al., 2008). For example, illegal harvesting and trading of regulated animal products, such as sea turtle eggs and elephant ivory, have been monitored using mtDNA (Moore et al., 2003; Withler et al., 2004). Moreover, Pauly et al. (2008) sequenced the cytochrome *b* region of mtDNA from *Rana aurora* specimens from extralimital populations in northwestern North America, finding that these frogs are most closely related to *R. aurora* from populations in the northernmost clade of this species.

A badly damaged road-killed snake was found in southeastern Alaska and identified based on superficial morphology to be a gartersnake (*Thamnophis*). Based on their natural histories and geographic distributions, both *Thamnophis sirtalis* and *Thamnophis elegans* have been considered likely candidates to occur naturally in Alaska (Hodge, 1976). Despite numerous anecdotal accounts of *Thamnophis* occurring in Alaska, no photographs or voucher specimens existed until now.

The discovery of this road-killed snake in Alaska could indicate the detection of a previously unknown population, a major range expansion, or a recently introduced individual. To help distinguish among these hypotheses, we sequenced and analyzed parts of two mitochondrial genes of this specimen. We combined this sequence information with mtDNA sequences that we generated from additional *Thamnophis* specimens in the lower northwestern United States and those obtained from GenBank, and then we subjected these data to phylogenetic analysis to ascertain the taxonomic identity of the Alaska specimen and to determine its closest likely geographic origin relative to the available set of reference samples.

### MATERIALS AND METHODS

**The Specimen.**—The snake specimen (UAM Herpetology 314), roughly 200 mm in total length, was found as a flattened, dry road-kill approximately 1.5 km south of Haines, Alaska, on 21 August 2005 (Fig. 1). The location was described as “the shoulder of a paved road through a low density residential area along a stretch of the road abutting a wetland area stretching unbroken to the east.” Superficial examination of the specimen suggested that it likely was a gartersnake (*Thamnophis*). Thus, we targeted our molecular efforts accordingly.

**DNA Extracting and Sequencing.**—Tissue was obtained from the dorsal scales of the focal specimen and was stored in 95% ethanol. Genomic DNA was extracted using the DNeasy extraction kit (QIAGEN). Partial mitochondrial loci NADH subunit 2 (ND2) and cytochrome *b* (cyt *b*) were amplified using a slightly modified protocol from Janzen et al. (2002). For ND2, 25  $\mu$ L contained 1 $\times$  buffer (Bioline), 0.8 mM dNTP, 3 mM MgCl<sub>2</sub>, 0.25  $\mu$ M of each primer, 0.5  $\mu$ L of Taq, and

4  $\mu$ L of DNA. The amplification conditions consisted of 30 sec of denaturing at 94°C, 30 sec of primer annealing at 48°C, and 90 sec of extension at 72°C for 30 cycles. For cyt *b*, reactions contained 1 $\times$  buffer, 0.8 mM dNTP, 3 mM MgCl<sub>2</sub>, 0.25  $\mu$ M of each primer, 0.5  $\mu$ L of Taq, and 4  $\mu$ L of DNA, brought to a total volume of 25  $\mu$ L with distilled H<sub>2</sub>O. The amplification conditions consisted of 60 sec of denaturing at 94°C, 90 sec of primer annealing at 50°C, and 120 sec of extension at 72°C for 40 cycles. All PCR reactions were conducted using Techne TC-412 and Eppendorf Mastercycler gradient thermocyclers. Primer sequences for ND2 (CE2230 and H5051) and cyt *b* (LGLU and H15544) are described in Janzen et al. (2002).

For ND2 and cyt *b*, the PCR product was run on 1.5% low-melt agarose Tris-borate-EDTA gel, and the target DNA fragment with the expected size was excised. The fragment was extracted using the QIAquick gel extraction kit (QIAGEN). The samples were sequenced using an automated sequencer (3730xl DNA Analyzer, Applied Biosystems) using BigDye terminator chemistry (vers. 3.) per the manufacturer’s instructions (Applied Biosystems).

**Phylogenetic Analyses.**—Partial sequence data from ND2 (512 bp) and cyt *b* (593 bp) were combined to produce an alignment of 1105 bp for the unknown specimen. To determine the closest relative of this specimen, we downloaded homologous sequences from 12 species of *Thamnophis*, as well as the outgroup *Nerodia fasciata*, from GenBank (ND2: AF420171.1, AF420195.1, AF420119.1, AF420087.1, AF420115.1, AF420105.1, AF420209.1, AF420141.1, AF420091.1, AF420163.1, AF420145.1, AF420094.1, AY870612.1; and cyt *b*: AF420169.1, AF420193.1, AF420117.1, AF420085.1, AF420113.1, AF420103.1, AF420133.1, AF420139.1, AF420089.1, AF420161.1, AF420143.1, AF420107.1, AY866529.1). The targeted species of *Thamnophis* (*atratus*, *brachystoma*, *butleri*, *couchii*, *elegans*, *eques*, *gigas*, *hammondi*, *marcianus*, *proximus*, *radix*, and *sirtalis*) were included in the analyses because they were western North American species or provided an additional level of phylogenetic reference (de Queiroz et al., 2002). *Nerodia fasciata* was used as an outgroup because of the close morphological and phylogenetic relationship between the genera *Thamnophis* and *Nerodia* (Alfaro and Arnold, 2001). Initial nucleotide BLAST searches using ND2 and cyt *b* information from the focal specimen suggested that *Thamnophis ordinoides* was a close relative. Consequently, homologous mtDNA sequences were generated from seven specimens of *T. ordinoides* from Del Norte County, California; Benton County, Oregon; and Pierce and Thurston counties, Washington (Appendix 1) for inclusion in the final analyses.

Sequences from the two mitochondrial genes were concatenated for each of the 21 sequences, and alignments were performed with Clustal W (Thompson et al., 1994). Alignments were visually reviewed and corrected in BioEdit 7.0.0 (Hall, 1999). Parsimony analysis was conducted for the combined loci with PAUP\*4.0b10 (Swofford, 2001) using a heuristic analysis with 10 random taxon addition replicates and the tree-bisection and reconnection branch swapping algorithm. Nodal support was assessed with nonparametric bootstrapping using 1,000 pseudoreplicates. The maximum likelihood analysis also was constructed in PAUP\*4.0b10 (Swofford, 2001) using parameters estimated with respect to Akaike’s Information Criterion in Modeltest 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). For the combined data, the model of Hasegawa-Kishino-Yano + invariant sites + gamma (HKY

<sup>2</sup>Corresponding Author. Present address: Department of Biology, Utah State University, Logan, Utah 84341 USA; E-mail: lorin215@gmail.com



FIG. 1. Dorsal (A) and ventral (B) views of specimen from Alaska (UAM 314). Each black and yellow square beneath specimen = 1 cm.

+ I + G) sequence evolution with a gamma shape parameter of 1.1256 and 66.99% invariant sites was identified. Nodal support was assessed using a nonparametric bootstrap with 100 pseudoreplicates and one random sequence addition per replicate, and these were condensed into a majority rule consensus tree.

#### RESULTS

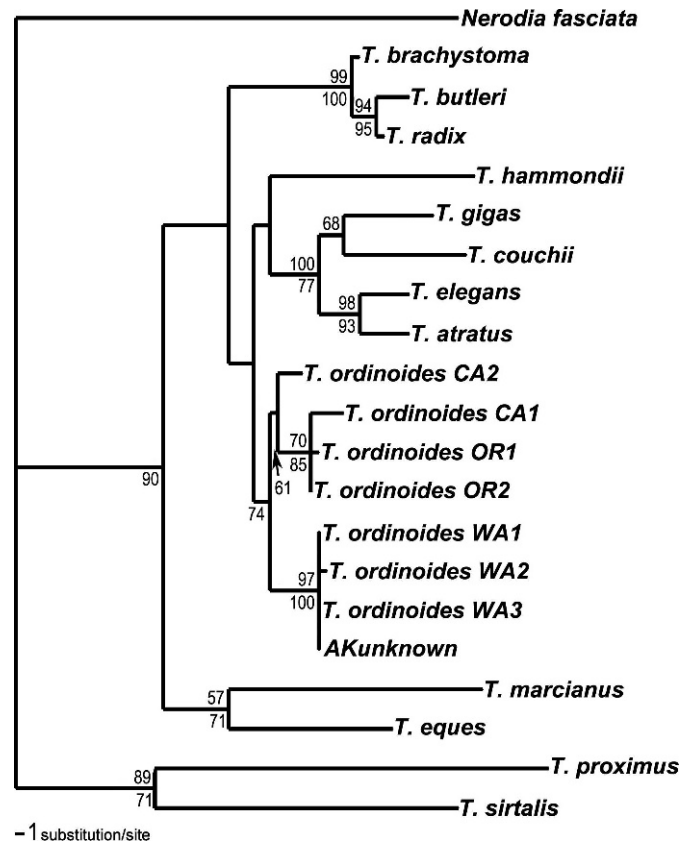
Of the 1,105 aligned characters (1–593 *cyt b*; 594–1,105 ND2) between the 20 *Thamnophis* samples and the outgroup, 117 were parsimony informative (*cyt b*, 64; ND2, 53), whereas another 102 characters were variable but parsimony uninformative (*cyt b*, 50; ND2, 52). Within *Thamnophis*, 104 characters were parsimony informative (*cyt b*, 60; ND2, 44), whereas another 90 were variable but parsimony uninformative (*cyt b*, 50; ND2, 40).

The Alaskan specimen grouped with *T. ordinoides* with extremely high bootstrap support and was most similar to the samples from Washington (Fig. 2). The Alaska snake was identical in *cyt b* and ND2 sequence to the two northernmost Washington specimens (Pierce Co.) of *T. ordinoides*. The other Washington sample (Thurston Co.) differed by a single autapomorphy, an A→T transition in ND2.

#### DISCUSSION

The mtDNA sequencing and phylogenetic analyses identified the unknown snake (Fig. 1) as *T. ordinoides*. The natural range of *T. ordinoides* encompasses the Pacific Coast from northern California to southern British Columbia, Canada (including Vancouver Island) (Fig. 3; Stebbins, 2003). Similar to the findings of Pauly et al. (2008) for introduced Alaska specimens of *R. aurora*, the Alaskan snake that we analyzed was most closely related to reference specimens from the northernmost sampling point in Washington (Figs. 2 and 3). Notably, the northernmost known population of *T. ordinoides* is >1,000 km south of Haines, Alaska.

Three hypotheses may explain the presence of *T. ordinoides* in Alaska. First, environmental conditions in some areas could conceivably permit populations of such taxa to have thrived as undiscovered relicts, separated by geological events such as volcanoes and glaciers (Inger and Voris, 2001). This pattern has been noted in a wide variety of taxa, such as plants, reptiles, amphibians, and fish (e.g., Dowling, 1956). Our genetic data, as well as a lack of *Thamnophis* specimens being found previously, suggest that this hypothesis is unlikely.



—1 substitution/site

FIG. 2. Phylogenetic tree topology resulting from parsimony and maximum likelihood analyses of 1105 bp of the mitochondrial genes cytochrome *b* (593 bp) and NADH subunit 2 (512 bp) for *Thamnophis* spp. Only this figure is presented because the individual topologies from each approach were essentially the same. The most parsimonious tree length = 388, consistency index (CI) = 0.65, retention index = 0.57, and rescaled CI = 0.37, whereas the HKY + I + G model of sequence evolution was fitted for the maximum likelihood analyses. The proportion of bootstrap pseudoreplicates supporting a particular node is given above the node for maximum likelihood and below the node for parsimony analyses. Note that the Alaska specimen (AKunknown) is well supported as *T. ordinoides*.



FIG. 3. Map of the presently known range of *Thamnophis ordinoides* (hatched), with location of Alaska individual indicated by the star.

Second, the area near Haines could have been colonized recently as part of a natural range expansion by *T. ordinoides*. Well-documented cases exist of range expansions to higher latitudes as climates in those areas become more habitable (Parmesan and Yohe, 2003; Crozier, 2004). *Thamnophis* is an excellent candidate for this type of range expansion, because species in this genus are widespread throughout most of North America, and many are habitat and dietary generalists (Rossman et al., 1996). Furthermore, parts of southern Alaska may be suitable (Hodge, 1976), although no vouchered specimens exist for snakes anywhere in Alaska (Jarrell, pers. comm.). Nonetheless, the sample was found >1,000 km north of the nearest known population of *T. ordinoides* in British Columbia, so natural movement of this species northward presumably would have been documented before our study.

The third, and seemingly most likely, hypothesis is that the snake was an accidental or intentional transport and release. Intentional release of pet snakes is well documented (e.g., Rodda et al., 2009). An unintentional introduction is also possible, because a well-traveled ferry system (the Alaska Marine Highway) connects the city of Haines, Alaska (near the site of the discovered specimen), to its southernmost port of Bellingham, Washington, which is within the known range of *T. ordinoides* (Fig. 3). Novel direct connectivity between previously separated habitats has been linked to other biological invasions, such as the sea lamprey (*Petromyzon marinus*) in the U.S. Great Lakes and the water hyacinth (*Eichhornia crassipes*) throughout most of the tropical and subtropical areas of the world (Mooney and Drake, 1987). *Thamnophis ordinoides* also has been recorded in other nonnative geographic locations, such as Guam, where it arrived via the Christmas tree trade (Reed, pers. comm.). If the Alaska specimen is a result of an accidental or intentional transport and release, additional surveys may be necessary to monitor the area, because Alaskan habitat fulfills two of the three predictors of biological invasion (Moulton and Pimm, 1986): relatively few species and no additional members of the same taxonomic group.

Future work, including additional, faster evolving markers, and samples at the historical northern edge of the range for *T. ordinoides* (Fig. 3), will be able to more precisely exclude one or more hypotheses regarding the natural provenance of the Alaska specimen. However, the discovery of this snake so far outside of its known range raises important issues regarding undiscovered populations and the ever-increasing accessibility to species of novel environments through climate alteration and human transport.

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APPENDIX 1. Locality information for the eight specimens of *T. ordinoides* sequenced for the mtDNA analysis. Latitude (N) and longitude (W) are in decimal degrees. SJA = Stevan J. Arnold, MVZ = Museum of Vertebrate Zoology, HIG = Richard Highton, UAM = University of Alaska Museum.

Collection	State	County	N	W	Locality name	Museum no.	Notation
SJA	California	Del Norte	41.8718	123.8447	Patrick Creek	9008	CA1
SJA	California	Del Norte	41.5492	124.0612	Requa	9161	CA2
SJA	Oregon	Benton	44.566	123.334	Bald Hill	17129	OR1
SJA	Oregon	Benton	44.566	123.334	Bald Hill	17134	OR2
MVZ	Washington	Pierce	47.0969	122.63	Unknown	228283	WA1
MVZ	Washington	Thurston	47.0544	122.9483	Unknown	228286	WA2
HIG	Washington	Pierce	Unknown	Unknown	Unknown	48465	WA3
UAM	Alaska	Haines	59.20904	135.43719	Haines, Alaska	Herp. 314	AKunk.