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PHYLOGENETIC ANALYSIS OF COMMON GARTER SNAKE (*THAMNOPHIS SIRTALIS*) STOMACH CONTENTS DETECTS CRYPTIC RANGE OF A SECRETIVE SALAMANDER (*ENSATINA ESCHSCHOLTZII OREGONENSIS*)

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***Abstract.*—Given the current global amphibian decline, it is crucial to obtain accurate and current information regarding species distributions. Secretive amphibians such as plethodontid salamanders can be difficult to detect in many cases, especially in remote, high elevation areas. We used molecular phylogenetic analyses to identify three partially digested salamanders palped from the stomachs of three Common Garter Snakes (*Thamnophis sirtalis*) from the Klamath Mountains in northern California. Our results conclusively show that the salamanders were all individuals of *Ensatina eschscholtzii oregonensis*, revealing a substantial vertical range extension for this sub-species, and documenting the first terrestrial breeding salamander living in the sub-alpine zone of the Klamath Mountains.**

Key Words.—Common Garter Snake, distribution, *Ensatina, Ensatina eschscholtzii*, Klamath Mountains, mitochondrial DNA, *Thamnophis sirtalis*

INTRODUCTION

One of the most alarming aspects of the ongoing global decline in amphibian species concerns the disappearance of amphibians from relatively pristine ecosystems (Wake 1991). A well-documented example comes from the high elevations of the Sierra Nevada Mountains in California, where two species of frogs, Yosemite Toads (*Bufo canorus*) and Mountain Yellow-Legged Frogs (*Rana muscosa*), have been extirpated from much of their historic ranges in the past few decades (Bradford et al. 1994; Knapp and Matthews 2000; Davidson and Fellers 2005; Vredenberg et al. 2007). The substantial distributional database for each species, which helped bring these declines to light, was largely made possible by one life-history trait of these particular amphibian species: their aquatic breeding nature. Biologists conducting surveys during the summer season can easily detect the presence of all life stages of aquatic breeding amphibians by searching the meadows and edges of streams, ponds, and lakes in the region. By contrast, plethodontid salamanders do not have an aquatic-larval stage in their life history, which makes them more difficult to detect than aquatic breeders. The low detectability of terrestrial salamanders is because a significant proportion of populations are subterranean (Bailey et al. 2004). For example, Grinnell and Storer (1924) noted that the most scientifically exciting discovery of their survey of vertebrates in the Sierra Nevada occurred when two individuals of a previously unknown species of plethodontid salamander (today known as *Hydromantes platycephalus*) were caught in a trap set for mammals at an elevation of 3292 m. This illustrates the existence of a field-detection bias among amphibian species. To counter this conservation challenge, alternative methods must be employed to discover cryptic populations of amphibians.

The Klamath Mountains of northwestern California are located within a region renowned for its high amphibian diversity (Bury and Pearl 1999). This region is a diversity hotspot for plethodontid salamanders, with at least eight species known to occur there (Stebbins 2003), some of which were only recently described (e.g., *Plethodon asupak* and *P. stormi*). Recent field studies in the sub-alpine portion of the Klamath Mountains have investigated the distributional ecology of amphibians and reptiles in the area (Welsh et al. 2006; Pope et al.

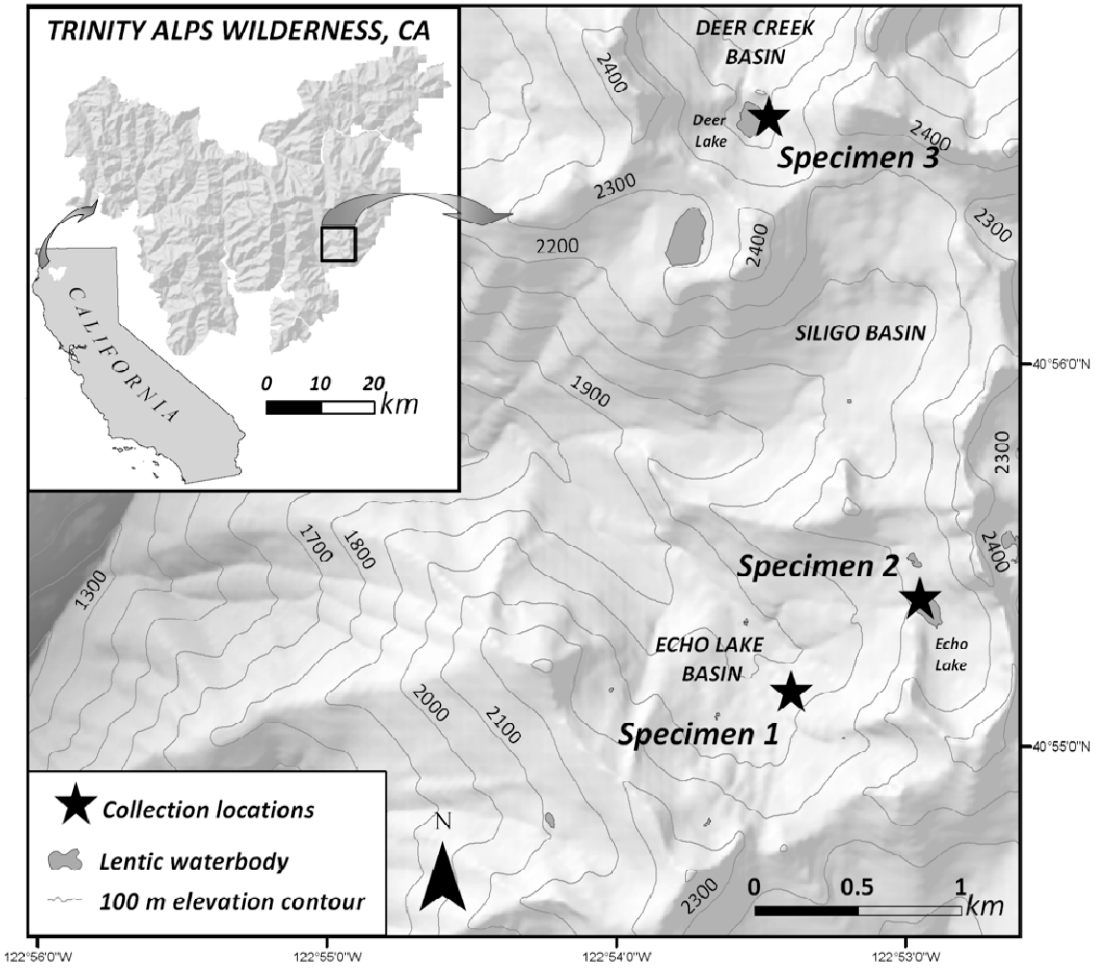

FIGURE 1. Map of the study site in the Trinity Alps Wilderness of northwestern California, USA.

2008). As part of these surveys, data on the dietary habits of two locally common amphibian predators, the Common Garter Snake (*Thamnophis sirtalis*) and the Aquatic Garter Snake (*T. atratus*), were obtained whenever possible (Pope et al. 2008). Among the food items recovered from the stomachs of three *T. sirtalis* were three partially digested salamanders. A number of regional salamander experts examined the specimens and were unable to identify them to species level based on morphological characteristics.

Wildlife forensics represents another application of molecular phylogenetic methods. For example, studies have used these powerful techniques to establish the true species identities of putative whale meat purchased from meat markets (Baker and Palumbi 1994), and fish offered as sushi in New York City restaurants (Lowenstein et al. 2009). In these studies mitochondrial DNA is preferable over nuclear DNA because of its lack of introns, high substitution rate, limited exposure to recombination, and haploid mode of inheritance (Hebert et al. 2003). The salamander tissues examined in this case were degraded, making longer fragments more difficult to amplify and sequence. Therefore, we sequenced a short fragment of the cytochrome b gene due to its high proportion of phylogenetically informative sites and the availability of homologous sequences from other salamanders in the region. Here, we used this methodology to conclusively establish the species identities of three salamanders from the sub-alpine zone of the Klamath Mountains, California.

MATERIALS AND METHODS

Study site.—We collected the salamander specimens from the sub-alpine zone of the Trinity Alps Wilderness, located in the Klamath Mountains of California (Fig. 1). The topography of the region consists mainly of steep elevational gradients on ridge slopes composed of bare rock outcrops and expansive talus fields (Fig. 2). Floristic zones in the study area include sub-alpine forest, montane chaparral, and sub-alpine meadow, resulting in a sparse vegetation canopy (Ferlatte 1974).

FIGURE 2. Echo Lake basin, Trinity Alps Wilderness, California, USA. (Photographed by Justin Garwood).

Precipitation in the region primarily falls as snow from November to May, followed by sparse rain from localized thunderstorms between June and October.

Genetic samples.—We obtained the salamander tissue samples from a garter snake (*Thamnophis spp.*) dietary study that occurred between 2003 and 2006 (Pope et al. 2008). We performed a palping procedure (Pope et al. 2008) on three individual *T. sirtalis*, found at elevations of 2,065 m, 2,215 m, and 2,177 m (Fig 1). Each snake regurgitated a partially digested, unidentifiable salamander; these specimens are hereafter referred to as Specimen 1, Specimen 2, and Specimen 3. We preserved the specimens in 95% ethanol. We extracted DNA using the Phenol-Chloroform-Isoamyl Alcohol method (Sambrook et al. 1989), and we used the Polymerase Chain Reaction (PCR) to amplify a portion of the cytochrome b region of the mitochondrial genome using primers MVZ 15 (Kocher et al. 1989) and CytB2 (Moritz et al. 1992). Associated Genbank accession numbers and museum voucher numbers can be found in Table 1.

Data analyses.—We first used NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST) to determine the likely identity of our unknown specimens. BLAST is an algorithm that matches DNA sequences based solely on their raw similarity, thus it is possible that our sequences could match with a GenBank sequence due to homoplasies in the data rather than homologies. Therefore, we also employed Bayesian and Maximum Likelihood (ML) phylogenetic methods, which explicitly account for any homoplasies in the data, to confirm the identity of the unknown specimens. In other words, a phylogenetic approach is needed to determine which of the known salamander species living in northwestern California is most closely related to our unknown specimens.

We used Genbank to download cytochrome b sequences from all known salamander species native to northwestern California, and unambiguously aligned these sequences by eye with those of our unknown specimens. We separated the data into three partitions corresponding to the first, second, and third codon positions and used the Akaike information criterion (AIC) in MrModeltest v2.2 (Nylander, J.A.A. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden) to determine the best-fit nucleotide substitution model for each data partition. We then

TABLE 1. Sample names and associated Genbank and museum voucher numbers for salamanders used in our study.

Sample	Genbank Accession No.	Museum Voucher No.
Ensatina eschscholtzii oregonensis	FJ151686.1	MVZ S10793
Ensatina eschscholtzii platensis	FJ151980.1	MVZ 237157
Ensatina eschscholtzii picta	FJ151674.1	MVZ 220597
Aneides flavipunctatus	AY728214.1	MVZ 219973
Ambystoma macrodactylum	EF036634.1	JPB 21448
Hydromantes shastae	U89611.1	MVZ202326
Plethodon elongatus	AY728223.1	MVZ 220003
Dicamptodon tenebrosus	AY734593.1	MVZ 192640
Batrachoseps attenuatus	AY728228.1	MVZ 230761
Taricha granulosa	EU880333.1	MVZ 225502
Specimen 1	HM185820	HSU 726
Specimen 2	HM185821	HSU 727
Specimen 3	HM185822	HSU 728

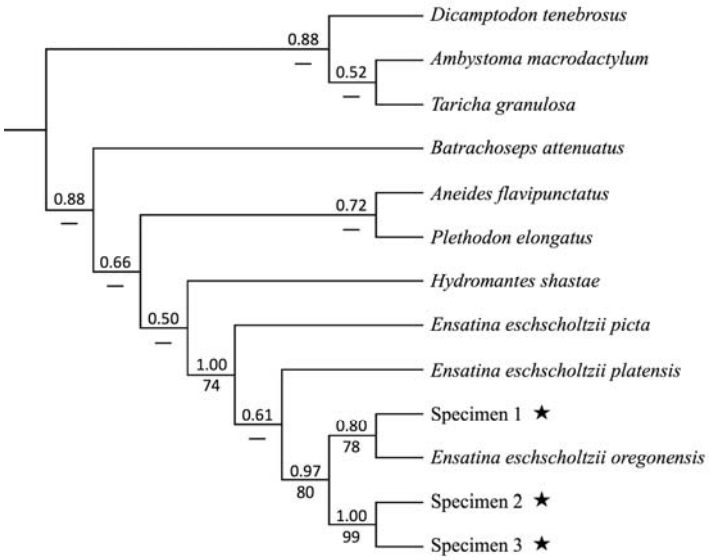

FIGURE 3. Phylogenetic tree of the known salamander species from northwestern California plus the unknown specimens (stars), based on Bayesian and Maximum Likelihood analyses of the cytochrome b gene. The numbers above nodes are Bayesian posterior probabilities while the numbers below nodes represent bootstrap proportion values. Only bootstrap proportion values for nodes that agree with our Bayesian analysis are shown.

analyzed these sequence data with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). We ran two analyses with four chains for 20 million generations, sampling every 1000 generations to produce 20,000 trees per run. After removing the first 5,000 trees of each run, we combined the remaining 30,000 trees to produce a 50% Majority Rule tree. Bayesian posterior probability values of ≥ 0.95 indicate strong statistical support for a clade's existence. We also analyzed these sequence data using a ML method as implemented in the software GARLI (Zwickl 2006), with 1000 bootstrap replicates (Felsenstein 1985). Following Hillis and Bull (1993) we consider bootstrap values ≥ 70 % as constituting strong statistical support for a clade. We rooted our phylogenetic tree using the midpoint method.

RESULTS

On the basis of raw sequence similarity, the results of the BLAST search suggested that the unknown samples were *Ensatina eschscholtzii oregonensis*. The aligned sequence data used in the phylogenetic analyses consisted of 362 unambiguously aligned base pairs of which 164 nucleotide positions were variable and 111 of which were parsimony informative. The nucleotide substitution models chosen for codons 1, 2, and 3 were SYM+G, HKY+G, and GTR+G respectively. Both Bayesian and ML analyses reveal that all three unidentified salamanders are nested within the *Ensatina eschscholtzii* clade with a posterior probability of 1.0 and a bootstrap proportion of 74 supporting the grouping (Fig. 3). The unknown samples within this *Ensatina*

FIGURE 4. An *Ensatina eschscholtzii oregonensis* found on 6 October 2007 at 964 m in the Stuarts Fork drainage, Trinity Alps Wilderness, California, USA. (Photographed by Justin Garwood).

clade are most closely related to the individuals of *E. e. oregonensis*, a relationship supported by a posterior probability of 0.97 and a bootstrap proportion of 80. The haplotypes for Specimens 2 and 3 were identical and differed from the known *E. e. oregonensis* haplotype by nine base pair mutations for a sequence divergence of 2.48%. The haplotype for Specimen 1 differed from the known *E. e. oregonensis* haplotype by one base pair mutation for a sequence divergence of 0.28%.

DISCUSSION

The Klamath Mountains, the region of the United States that encompasses the northwestern corner of California and adjacent Oregon, is considered to be a diversity hotspot for amphibians. Much of this species richness is due to the great variety of plethodontid salamanders found in the region. Although the distributions of some amphibian populations range into the highest elevations, or sub-alpine zone, of the Klamath Mountains, all species known from high elevations of this range are aquatic breeders (Welsh et al. 2006). Thus, the unexpected finding that several plethodontid salamanders were consumed by garter snakes represented a significant discovery despite the fact that the partially digested specimens were not identifiable to species using morphological criteria.

Although some research has indicated that a single locus approach can be unreliable for identifying plethodontid salamanders (Vences et al. 2005), our analysis of mtDNA sequences confirmed that each of the salamanders belong to the same species (*E. e. oregonensis*; Fig. 4). Our findings not only represent a new distributional record for the sub-alpine zone of the Klamath Mountains, but also increase the known elevational limit of this subspecies from 1676 m (Stebbins 1949) to 2215 m. Is it possible that the snakes consumed the salamanders below 1676 m elevation and subsequently moved uphill? Several lines of evidence suggest that this scenario is very unlikely. Under ideal thermal conditions (25–35° C), garter snakes are able to digest approximately one half of an adult mouse per day (Stevenson et al. 1985). Because an adult *Ensatina* and an adult mouse are similar in size, an active garter snake would digest the salamander in no more than a few days in warm summer conditions. Radiotelemetry studies of *T. sirtalis* revealed average daily movements of only 8 m, and no daily movements greater than 150 m were observed (Fitch and Shirer 1971). Therefore, during the time it would take to fully digest an *Ensatina* salamander, a garter snake is unlikely to travel more than a few hundred meters from where it consumed its meal. In order to ascend from the previously known elevational range of *Ensatina* (< 1676 m) to the point of capture, each of the snakes would have had to travel at least 2 km and climb nearly 500 m in elevation (Fig. 1).

Despite sampling the stomachs of over 400 garter snakes in the Trinity Alps (Pope et al. 2008), instances of salamander predation seem to be a relatively rare occurrence. In addition to the three *Ensatina* identified in this study, garter snakes in the Trinity Alps study area are rarely observed consuming Rough-skinned Newts (*Taricha granulosa*) and Long-toed Salamanders (*Ambystoma macrodactylum*). In contrast, Cascades Frogs (*Rana cascadae*) and Pacific Tree Frogs (*Pseudacris regilla*) were the primary amphibian food items for *T. sirtalis* and *T. atratus* (Pope et al. 2008). We speculate that the snakes' preference for frogs may simply be a function of both species being diurnally active, whereas *Ensatina* may not generally be available to the snakes owing to their nocturnal activity and use of subterranean daytime retreats in forested regions (Stebbins 1954). Moreover, this sub-alpine region has shallow and seasonally dry serpentine soils, resulting in sparse vegetation cover and scant downed wood. We suspect the region's expansive talus fields may support populations of *Ensatina* by providing moist microclimates year-round suitable for terrestrial salamanders but not for active garter snakes.

A precedent does exist for the discovery of a salamander known from an unlikely area from the stomach of a snake predator. Wall (1911) reported finding a species of salamander from the family Salamandridae inside the stomach of a snake in the remote high elevation Hindu Kush Mountains of Pakistan, an area from where salamanders are not known to this day. This salamander specimen was never described, and apparently was not preserved, but due to its extreme isolation from other salamanders it likely represents a new species. Similarly, examination of the stomach contents of Sperm Whales (*Physeter macrocephalus*) has revealed the existence of new squid species (Verrill 1879; Joubin 1900). We propose that phylogenetic analysis of stomach contents could be employed throughout the world to increase the detection probability of small vertebrates. Although the salamanders in this study were found to be representatives of a known species, the approach we employed here conceivably could result in the discovery of new amphibian species.

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