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## Mitochondrial Variation in Sharp-Tailed Snakes (*Contia tenuis*): Evidence of a Cryptic Species

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**ABSTRACT.**—We examined genetic variation and structure in mitochondrial DNA sequences of sharp-tailed snakes (*Contia tenuis*) from California and southern Oregon. Maximum parsimony and maximum likelihood analyses distinguish two mitochondrial lineages: a north coast clade restricted to cool evergreen forest along the Pacific Coast; and an interior/south clade widespread throughout California. The southern limit of the north coast clade is congruent with that of several other vertebrate taxa, a historical pattern consistent with a long-term marine embayment. We interpret additional phylogeographic pattern as resulting from either gene flow or incomplete lineage sorting. Genetic, distributional, ecological, and morphological data suggest that north coast and interior/south mitochondrial lineages of *C. tenuis* are distinct at the species level.

Knowledge of genetic variation is critical to our understanding of population genetics, speciation, and historical biogeography. Examination of variation within species has led to the discovery of genetic diversity, geographic pattern, and cryptic species (e.g., Tilley et al., 1978;

Yanev, 1980; Good, 1989; Highton, 1989; Omland et al., 2000; Jockusch et al., 2001). One common approach to the study of genetic variation involves analysis of mitochondrial DNA collected at the population level and interpreted in a phylogenetic context. Resultant intraspecific phylogenies, or phylogeographies (Avice et al., 1987; Avice, 1989), allow inferences about historical patterns of vicariance, dispersal, gene flow, and population subdivision (Templeton et al., 1995; Walker and Avice, 1998). When unre-

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lated taxa with similar distributions and ecologies are examined via the phylogeographic method, congruent phylogeographic patterns often suggest shared geologic and evolutionary history (Avice, 2000).

The sharp-tailed snake (*Contia tenuis*) is one of North America's least studied ophidians; there is little natural history information available (Leonard and Ovaska, 1998), and its phylogenetic placement among xenodontine colubrids is uncertain (Cadle, 1984). One of the smallest western snakes (rarely attaining 40 cm), it is secretive, ground dwelling, and seasonally active (Cook, 1960; Leonard and Ovaska, 1998). *Contia* ranges from northern Oregon south into California with disjunct populations in British Columbia and Washington (Leonard and Ovaska, 1998; Fig. 1).

We characterized patterns of genetic variation and structure in this poorly studied snake to assess whether these patterns were consistent with geography and with patterns described in other taxa. Information on *Contia tenuis* mtDNA will be useful for testing the influence of common historical events on the evolution of other vertebrates sharing its habitat and distribution (Feldman, 2000).

#### MATERIALS AND METHODS

**Population Sampling.**—We collected mitochondrial DNA sequence data from 22 individuals representing 19 localities in the southern two-thirds of the geographic distribution of *C. tenuis* (Fig. 1; Table 1). We deposited voucher specimens in the Museum of Vertebrate Zoology (MVZ) and California Academy of Sciences (CAS; Table 1).

**Laboratory Protocols.**—We isolated genomic DNA from liver tissue, scales and/or tail tips by standard proteinase K digestion and phenol/chloroform purification (Maniatis et al., 1982). To amplify a 900 base pair region of the ND4 gene and flanking tRNA<sup>his</sup>, tRNA<sup>ser</sup>, and tRNA<sup>leu</sup>, we conducted PCR using primers ND4 (5'-CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC-3') and Leu (5'-ACC ACG TTT AGG TTC ATT TTC ATT AC-3'; Arevalo et al., 1994) with the following thermal cycle parameters: 35 cycles: 1 min 94°C; 1 min 52°C; 2 min 72°C. The 5' end of primers ND4 and Leu match nucleotide positions 11,671 and 12,594, respectively, of the heavy strand of the mitochondrial genome of the snake *Dinodon semicarinatus* (Kumazawa et al., 1998). We purified PCR products with the Wizard Prep Mini Column Purification Kit (Promega, Inc.) and used purified template in 10 µl dideoxy chain-termination sequencing reactions (Sanger et al., 1977) using ABI Big Dye (Perkin-Elmer Applied Biosystems, Inc.) and primers ND4 and Leu. Following an isopropanol/ethanol precipitation, we

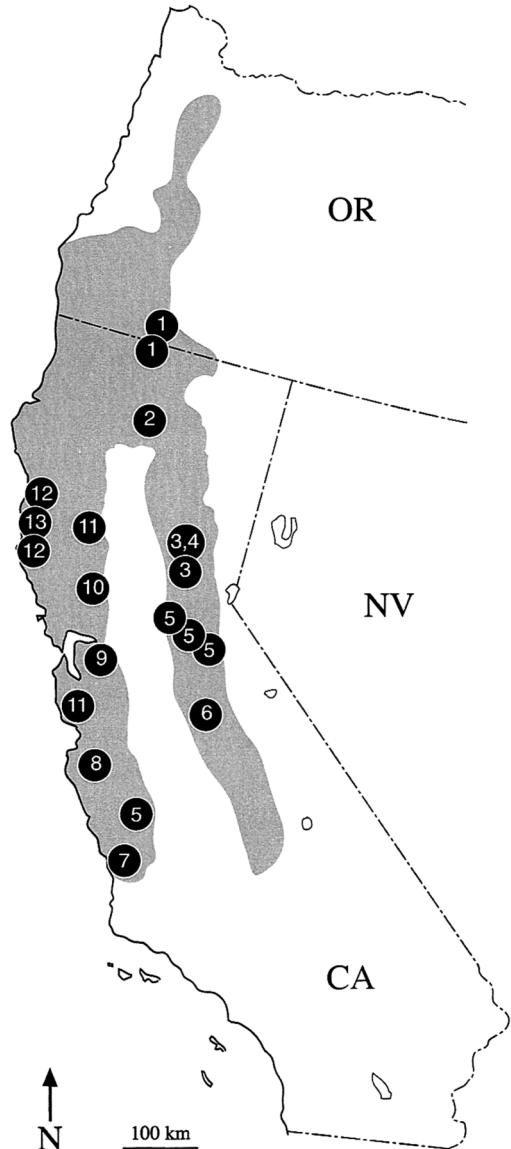


FIG. 1. Geographic range of *Contia tenuis* in California and Southern Oregon (after Stebbins, 1985). Dots indicate sample localities, numbers refer to unique mtDNA haplotypes.

ran cycle-sequenced products on an ABI 377 automated sequencer (Perkin-Elmer Applied Biosystems, Inc.).

**Sequence Analyses.**—We aligned DNA sequences with the program Sequencher™ 3.0 (Gene Codes Corp.) and translated protein coding DNA into amino acid sequences using MacClade 3.06 (W. P. Maddison and D. R. Maddison, Sinauer Assoc., Inc., Sunderland, MA, 1992, unpubl.). In addition, we identified tRNA

TABLE 1. Unique *Contia tenuis* and outgroup mtDNA haplotypes. CAS: California Academy of Sciences; MVZ: Museum of Vertebrate Zoology.

Haplotype	Locality	Museum	GenBank
1	N. of Hilt, Jackson Co., OR	no voucher	AF258879
	Hilt, Siskiyou Co., CA	CAS 210367	AF258879
2	Potter Creek, Shasta Co., CA	MVZ 164926	AF258880
3	Pike City, Sierra Co., CA	CAS 207044	AF402656
	Golden Trout, Butte Co., CA	CAS 205639	AF402656
4	Golden Trout, Butte Co., CA	CAS 205652	AF258881
5	S. of Georgetown, El Dorado Co., CA	CAS 208587	AF258882
	Rocklin, Placer Co., CA	CAS 210366	AF258882
	Chumash Circle, Calaveras Co., CA	MVZ 230096	AF258882
	Hwy 198, W. of Fresno Co. line, Monterey Co., CA	MVZ 208157	AF258882
6	Bear Valley, Mariposa Co., CA	CAS 205778	AF258883
7	Vineyard Drive, San Luis Obispo Co., CA	MVZ 208158	AF258884
	Vineyard Drive, San Luis Obispo Co., CA	MVZ 208160	AF258884
8	Hastings U.C. Reserve, Monterey Co., CA	CAS 205788	AF258885
9	Pleasant Hill, Contra Costa Co., CA	MVZ 232671	AF258886
10	Cache Creek, Yolo Co., CA	CAS 214873	AF402657
11	China Grade Rd., Santa Cruz Co., CA	CAS 205802	AF258887
	Brittan Ranch, Glenn Co., CA	CAS 202582	AF258887
12	Hwy 1 and 128 jct., Mendocino Co., CA	no voucher	AF258888
	Angelo Coast U.C. Reserve, Mendocino Co., CA	MVZ 230270	AF258888
13	Jackson State Forest, Mendocino Co., CA	MVZ 232650	AF402658
	Jackson State Forest, Mendocino Co., CA	MVZ 232651	AF402658
<i>D. punctatus</i>	Crystal Springs, San Mateo Co., CA	CAS 204287	AF258889
<i>H. platirhinos</i>	Southern Pines, Moore Co., NC	MVZ 175928	AF402659

genes by drawing their secondary structures following the criteria of Kumazawa and Nishida (1993). We deposited all sequences in GenBank (Table 1).

Using both uncorrected and maximum likelihood estimated distances, we computed pairwise sequence differences between haplotypes in PAUP\* 4.0b8 (D. L. Swofford, Sinauer Assoc., Inc., Sunderland, MA, 1998, unpubl.). To test for deviations from neutrality, we calculated Tajima's *D* (Tajima, 1989) using the program DnaSP 3 (Rozas and Rozas, 1999).

*Phylogenetic Analyses.*—We used molecular genetic data to determine evolutionary histories of populations by constructing intraspecific phylogenies. To infer haplotype relationships, we used maximum parsimony (MP; Swofford et al., 1996) and maximum likelihood (ML; Felsenstein, 1981) phylogenetic methods. We conducted all phylogenetic analyses in PAUP\* and coded tRNA in/dels as fifth character states. Last, we polarized the phylogeny via outgroup comparison (Maddison et al., 1984) using the xenodontine snakes *Diadophis punctatus* and *Heterodon platirhinos*.

We executed MP reconstructions with the branch-and-bound search algorithm (Hendy and Penny, 1982) using equally weighted characters. To assess nodal support, we performed a bootstrap analysis (Felsenstein, 1985) employing 1000 replicates of heuristic searches in

PAUP\*. Additionally, we calculated branch support (Bremer, 1988; 1994) for all nodes using the program TreeRot 2 (M. D. Sorenson, TreeRot, vers. 2, Boston, MA, 1999, unpubl.).

To estimate branch lengths and search for additional tree topologies, we performed ML analyses. To determine the most appropriate model of DNA substitution for reconstructing haplotype relationships under ML, we executed a hierarchical likelihood ratio test (LRT; J. Felsenstein, PHYLIP, vers. 3.5c, Seattle, WA, 1993, unpubl.; Goldman, 1993; Yang, 1996) via Modeltest 3.0 (Posada and Crandall, 1998). We then used a MP tree as our starting tree and estimated the ML tree successively until we obtained a stable topology (Wilgenbusch and de Queiroz, 2000).

*Estimating Divergence Times.*—We used gene trees, a molecular clock hypothesis, and geographic information to estimate evolutionary diversification and the degree of congruence between *C. tenuis* phylogeny and geologic events. To determine whether ND4 data are evolving in a clocklike fashion, we compared differences in log-likelihood scores for the same tree built under two different, nested models of molecular evolution (optimal model vs. molecular clock) using a LRT. Finally, we dated cladogenesis using uncorrected pairwise average distances between well-supported clades and a pairwise rate of sequence divergence of 1.3% per million years (reviewed in Macey et al., 1999, 2001).

RESULTS

*Genetic Variation.*—We did not find gene rearrangements in the sequenced region and ND4 appeared functional (Kumazawa and Nishida, 1995; Kumazawa et al., 1996; Macey and Verma, 1997; Macey et al., 1997). The final sequenced product was 860 bp; 694 bp of ND4 and 166 bp of tRNAs. Including outgroup, 229 nucleotide positions were variable and 101 were parsimony informative. Among *C. tenuis* samples, 73 nucleotide positions were variable, and 66 were parsimony informative. We found 13 unique mtDNA haplotypes in the 22 sharp-tailed snakes surveyed (Table 1). The model of DNA evolution that best fit these sequence data was the Hasegawa, Kishino and Yano model (HKY; Hasegawa et al., 1985) of nucleotide substitution in conjunction with gamma ( $\Gamma$ ; Yang, 1994a,b). This model accommodates unequal base composition, estimates transition and transversion substitution ratios, and accounts for heterogeneous rates of nucleotide substitutions across all sites. The ML HKY +  $\Gamma$  estimated pairwise distance comparisons between *C. tenuis* and outgroup taxa revealed sequence divergences ranging from 22.95% to 34.45%, whereas sequence divergence among *C. tenuis* haplotypes ranged from 0.12% (a single transition) to 8.97% (64 nucleotide differences; Table 2). The HKY +  $\Gamma$  model of DNA substitution estimated a ti:tv ratio of 5.38 for the ingroup, characteristic of ND4 sequence data for snakes (e.g., Zamudio and Greene, 1997; Kraus and Brown, 1998; Rodriguez-Robles and Jesus-Escobar, 1999, 2000). A high number of nonsynonymous substitutions have occurred in ND4 but mostly between two main clades of *Contia* (see below). Thus, we cannot reject the hypothesis of neutral evolution for haplotypes in either clade using Tajima's D statistic ( $D = 1.4427$ ;  $P > 0.10$ ; Tajima, 1989).

*Phylogenetic Relationships.*—The MP analysis produced nine shortest trees ( $L = 282$ ;  $CI = 0.933$ ;  $RI = 0.957$ ; Fig. 2A). The ML HKY+  $\Gamma$  search yielded one optimal tree ( $-\ln L = 2398.159$ ;  $\alpha = 0.391$ ; ti:tv = 2.81) identical to one of the nine most parsimonious trees (Fig. 2B).

Both MP and ML analyses reveal a basal divergence of *C. tenuis* populations in California into two major clades: a north coast clade and an interior/south clade (Fig. 2). *Contia tenuis* populations of the interior/south clade (Sierra Nevada Mtns., Cascade Range, Klamath Mtns., central and interior north coast of California; 100% bootstrap; decay index 24) are identified by haplotypes 1-10, whereas those of the north coast clade (Santa Cruz Mtns. and northern Coast Range; 100% bootstrap value; decay 26) are represented by haplotypes 11-13. The interior/south clade can be divided into subclades

TABLE 2. Pairwise comparisons of mtDNA sequences among all unique *Contia tenuis* and outgroup haplotypes. Uncorrected nucleotide differences (%) above diagonal. ML HKY +  $\Gamma$  corrected sequence divergences below diagonal.

Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	—	0.12	0.23	1.07	0.94	1.17	0.12	1.18	0.23	1.17	7.14	7.14	7.25	14.63	17.45
2	0.12	—	0.35	1.18	1.05	1.29	0.23	1.30	0.35	1.29	7.25	7.25	7.37	14.75	17.57
3	0.24	0.35	—	1.06	0.94	1.17	0.12	1.18	0.23	1.17	7.14	7.14	7.25	14.51	17.34
4	1.09	1.22	1.09	—	0.12	0.35	0.95	0.35	1.06	0.36	7.20	7.20	7.32	14.87	17.24
5	0.96	1.08	0.96	0.12	—	0.23	0.82	0.24	0.94	0.23	7.14	7.14	7.25	14.75	17.45
6	1.20	1.33	1.20	0.36	0.24	—	1.05	0.47	1.17	0.47	7.14	7.14	7.25	14.75	17.45
7	0.12	0.24	0.12	0.97	0.83	1.08	—	1.06	0.12	1.05	7.02	7.02	7.14	14.51	17.34
8	1.21	1.34	1.21	0.36	0.24	0.48	1.09	—	1.18	0.47	7.30	7.30	7.42	14.72	17.45
9	0.24	0.36	0.24	1.10	0.96	1.21	0.12	1.22	—	1.17	7.15	7.15	7.26	14.53	17.35
10	1.21	1.33	1.21	0.36	0.24	0.48	1.09	0.48	1.22	—	7.37	7.37	7.49	14.75	17.45
11	8.43	8.59	8.42	8.53	8.42	8.42	8.26	8.69	8.47	8.81	—	0.12	0.23	14.75	18.03
12	8.39	8.55	8.39	8.50	8.39	8.39	8.39	8.65	8.44	8.78	0.12	—	0.12	14.63	18.03
13	8.59	8.75	8.59	8.70	8.58	8.58	8.43	8.85	8.63	8.97	0.24	0.12	—	14.63	18.03
14 <i>D. punct</i>	23.19	23.43	22.95	23.80	23.42	23.43	22.95	23.22	23.09	23.42	24.48	24.12	24.24	—	16.71
15 <i>H. plat</i>	32.43	32.72	32.13	31.95	32.41	32.42	32.13	32.20	32.05	32.41	34.45	34.29	34.45	30.19	—

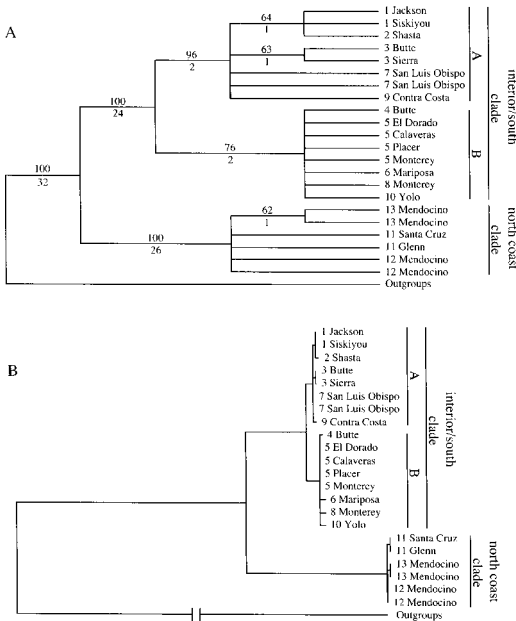


FIG. 2. Phylogenetic relationships of mtDNA lineages. (A) Strict consensus of nine equally parsimonious trees ( $L = 282$ ;  $CI = 0.933$ ;  $RI = 0.957$ ). Numbers above nodes indicate bootstrap support, numbers below denote decay indices. (B) Maximum likelihood tree constructed under the HKY +  $\Gamma$  model ( $-LnL = 2398.159$ ;  $\alpha = 0.391$ ;  $ti:tv = 2.81$ ). Branch lengths proportionate to ML estimates of genetic distances.

(A and B) that receive strong support but lack phylogeographic structure. Subclade A (Cascade and Klamath Mtns. and central coast; 96% bootstrap; decay 2) consists of haplotypes 1–3, 7, and 9, whereas subclade B (Sierra Nevada and Coast Ranges; 83% bootstrap; decay 2) contains haplotypes 4–6, 8, and 10.

**Divergence Times.**—The LRT could not reject a molecular clock hypothesis ( $P = 0.09$ ). The average uncorrected distances are 7.2% between the north coast clade and interior/south clade and 1.03% between the A and B subclades. The estimated mtDNA divergence rate of 1.3% sequence/million years (Macey et al., 1999, 2001) suggests that north coast and interior/south clades split roughly 5.5 million years ago, whereas subclades A and B split about 0.8 million years ago.

#### DISCUSSION

**Phylogeography.**—Sharp-tailed snakes belong to two major mitochondrial lineages: a north coast clade; and an interior/south clade (Fig. 2). Both clades, recovered by MP and ML methods, are strongly supported by bootstrap and decay analyses, and each is associated with different habitat types. Snakes of the north coast clade are restricted to wet Douglas fir and redwood forest

along the Pacific Coast, whereas interior/south clade *Contia* occupy drier woodland and forest characterized by grey pine, ponderosa pine, and oak. The north coast clade and interior/south clade differ by more than 7% sequence divergence (uncorrected), distance values that suggest a partition between clades over five million years old. The northern limit of each clade is unknown, but our sampling places the southern border of the north coast clade in the Monterey Bay region.

The Monterey Bay area is an important biogeographic region for many vertebrate species; it is the southern distributional limit for *Ambystoma macrodactylum*, *Aneides flavipunctatus*, *Batrachoseps attenuatus*, *Dicamptodon ensatus*, *Ensatina eschscholtzii xanthopicta*, and *Taricha granulosa*, and the northern distributional limit for *Batrachoseps luciae* and *Ensatina eschscholtzii eschscholtzii* (Yanev, 1980; Jockusch et al., 2001). Taxa distributed around Monterey Bay, such as *Taricha torosa* (Tan and Wake, 1995), *Elgaria multicarinata* (Feldman, 2000), *Thamnophis atratus* (Boundy, 1999), and *Strix occidentalis* (Aves: Strigiformes; Barrowclough et al., 1999), display genetic or morphological discontinuities across this area. Geologic evidence indicates that the Pacific Ocean invaded interior California around 5–24 million years ago through the present day Monterey Bay, and did not recede until about a million years ago (Oakshott, 1978; Howard, 1979; Dupre, 1990). Uncertainties involved with dating geologic events and calibrating molecular clocks, and confounding issues with plate tectonic data, prohibit our positively establishing the Monterey seaway as the causal factor in *Contia* cladeogenesis. Nevertheless, biogeographic congruence among vertebrates in this region, and the degree of concordance between *Contia tenuis* diversification and the Monterey embayment suggest this long-standing, marine barrier has played an important role in sharp-tailed snake evolution.

The distribution of genetic variation in *Contia* is not congruent with geography at lower levels in the phylogeny. The widespread interior/south clade displays internal haplotype structure inconsistent with geography as evidenced by the strongly supported subclades A and B. Three inner Coast Range populations group with Sierran populations (subclade B) and populations of sharp-tailed snakes from the Cascade, Klamath, and Sierra Nevada Mountains cluster with two coastal populations far to the south (subclade A). Additionally, one Sierran population (Butte Co.) contains members from subclades A and B. These data could indicate that *C. tenuis* disperses well and maintains gene flow throughout California. Sharp-tailed snakes are distributed along riparian corridors flanking

the American and Sacramento Rivers, and these and other drainages flow from the Sierra Nevada to the coast, possibly explaining the sharing of haplotype 5 in Sierran and coastal populations.

Although gene flow may link Sierran and coastal populations of *Contia*, long-term ecological and demographic processes may also explain intraclade patterns. Gene flow seems an unlikely explanation for the sharing of haplotype 11 between north coast populations on opposite sides of the San Francisco Bay and Sacramento-San Joaquin Delta. The Sacramento-San Joaquin Delta formed during the mid-Pleistocene (Dupre et al., 1991), influencing genetic and morphological evolution in *Thomomys bottae* (Mammalia: Rodentia; Patton and Smith, 1990), *Lampropeltis zonata* (Rodriguez-Robles et al., 1999), *Neotoma fuscipes* (Mammalia: Rodentia; Matocq, 2002) and *Sorex ornatus* (Mammalia: Insectivora; Maldonado et al., 2001). Taxa such as *Ensatina eschscholtzii* (Wake, 1997), *Diadophis punctatus*, *Elgaria multicarinata* (Feldman, 2000) and *Charina bottae* (Rodriguez-Robles et al., 2001) do not exhibit genetic subdivisions, however, indicating dispersal across the delta or maintenance of large effective population sizes in which reciprocal monophyly has not yet evolved. The San Francisco Bay and Sacramento-San Joaquin Delta effectively divide suitable habitat for the north coast clade; thus the sharing of haplotype 11 in *C. tenuis* across this region is likely caused by retained ancestral polymorphism. Sharp-tailed snakes probably maintain large effective population sizes, possibly because of their tendency to aggregate (Cook, 1960). Unfortunately, patterns of historical and contemporary gene flow and incomplete lineage sorting are difficult to distinguish (Matocq et al., 2000).

**Taxonomic Implications.**—Mitochondrial DNA evidence indicates that the north coast clade and interior/south clade of *C. tenuis* are exclusive lineages with separate and ancient evolutionary histories (i.e., phylogenetic species; Cracraft, 1983). Making species decisions based solely upon exclusivity is tenuous (de Queiroz, 1998), especially when those decisions are based entirely on a single molecular marker (Moritz et al., 1992; Wake and Schneider, 1998); multiple lines of evidence should be used to diagnose distinctness and permanence of independent units (de Queiroz, 1998). We recognize groups of populations as species if they are monophyletic and possess additional, independent characters suggestive of long-term evolutionary independence. Under these criteria, two species of *Contia* could be recognized.

Sequence divergence between north coast and interior/south clades of *C. tenuis* (Table 2) is equivalent or greater than genetic distances in the same mitochondrial region between *Lampropeltis zonata*

and *L. pyromelana* (Rodriguez-Robles and Jesus-Escobar, 1999), and various species of *Pituophis* (Rodriguez-Robles and Jesus-Escobar, 2000), *Lachesis* (Zamudio and Greene, 1997) and *Agkistrodon* (Parkinson et al., 2000). Members of the north coast clade occur in wet Douglas fir and redwood evergreen forest while populations of the interior/south clade occupy drier grey pine, ponderosa pine and oak habitat. North coast and interior/south clades are diagnosable by caudal scale counts, ventral scale counts and color differences (Hoyer, 2001). The two mtDNA clades appear parapatric along the inner Coast Range north of the Monterey Bay area, but more samples are needed to determine the integrity of these clades where their ranges meet.

Although we are confident that additional morphological, ecological and behavioral characters will further elucidate the two clades of *Contia*, we refrain from a taxonomic decision until a molecular or morphological survey throughout the entire range of *C. tenuis* delimits the geographical distribution of each clade. *Contia tenuis*, considered a morphologically uniform colubrid, now joins a growing list of Californian herpetofauna whose patterns of genetic variation and structure reveal more complex histories.

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