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Mitochondrial DNA-Based Phylogeography of North American Rubber Boas, *Charina bottae* (Serpentes: Boidae)

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We used 783 bp of mitochondrial DNA sequences to study the phylogeography of *Charina bottae* (rubber boa) in western North America, with an emphasis on populations from California (U.S.A.). Maximum-parsimony and maximum-likelihood methods identified a basal divergence within *C. bottae* that corresponds to southern and northern segments of its current distribution. These clades coincide with the ranges of the two recognized subspecies, *C. b. umbratica* in the south and *C. b. bottae* to the north. A subsequent cladogenetic event in the *C. b. bottae* clade resulted in two groupings, which we refer to as the Sierra Nevada and the Northwestern subclades, based on the geographic distribution of their constituent populations. The two subclades have completely allopatric distributions, with a genetic break in the vicinity of Lassen Volcanic National Park in northeastern California, an area that was subjected to glaciation during the Pleistocene and that has been volcanically active in the past 100 years. An earlier genetic study documented fixed differences between populations of *bottae* and *umbratica* in four of seven allozymes surveyed, and despite noticeable variation and overlap in the characters that define *C. b. bottae* and *C. b. umbratica*, the two forms still can be separated in most cases using a suite of morphological traits. All available evidence thus indicates that *C. b. umbratica* is a genetically cohesive, allopatric taxon that is morphologically diagnosable, and we conclude that it is an independent evolutionary unit that should be recognized as a distinct species, *Charina umbratica*.

Key Words: **biogeography; Boidae; *Charina;* mitochondrial DNA; phylogenetics; snakes; subspecies.**

INTRODUCTION

. . . as we have learned more and more about genetic cohesion . . . and more "polytypic" species have been revised taxonomically, we see that there are few examples of widespread genetically integrated and cohesive taxa. It seems that each time that a traditionally viewed polytypic species has been examined closely, it turns out that species . . . have been confounded . . . (D. R. Frost and D. M. Hillis, 1990, p. 93)

One common use of population genetic surveys is to study and quantify how genetic variation is distributed over geographic space within a species (Templeton, 1998). When the genetic variation is organized into an allele or haplotype genealogy, the resulting analysis of how allele genealogy correlates with geography is called *intraspecific phylogeography* (Avise, 2000). Phylogeographic studies of animal taxa predominantly rely on mitochondrial DNA (mtDNA) to assess the intra- and interpopulation spatial distribution of haplotypes. These genetic assessments have become a useful approach for evaluating patterns of speciation, extinction, vicariance, and dispersal in broadly distributed taxa and for associating these evolutionary processes with landscape evolution (e.g., Barber, 1999; Crandall and Templeton, 1999; Macey *et al.*, 1999; Patton *et al.*, 2000; Schneider *et al.*, 1998; Weisrock and Janzen, 2000).

Charina bottae (rubber boa) is widespread in western North America, ranging from southwestern Canada to southern California in the United States and eastward from the Pacific Coast to northern Wyoming and Colorado (Fig. 1). Rubber boas can attain maximum total lengths of approximately 83 cm and occur in grassland, broken chaparral, woodland, and forest, and although individuals are largely crepuscular and nocturnal, they can also be active during the daytime (Nussbaum *et al.*, 1983; Peterson and Dorcas, 1992; Ross, 1931; Stebbins, 1985). Based on differences in pattern of head scales and in number of dorsal scale rows and ventral scales, three subspecies of *C. bottae* have been described (*bottae*, *umbratica*, and *utahensis;* Cunningham, 1966; Klauber, 1943; Stebbins, 1985; Van Denburgh, 1920). Nevertheless, after studying in detail over 500 specimens from the northern part of the range of rubber boas, Nussbaum and Hoyer (1974)

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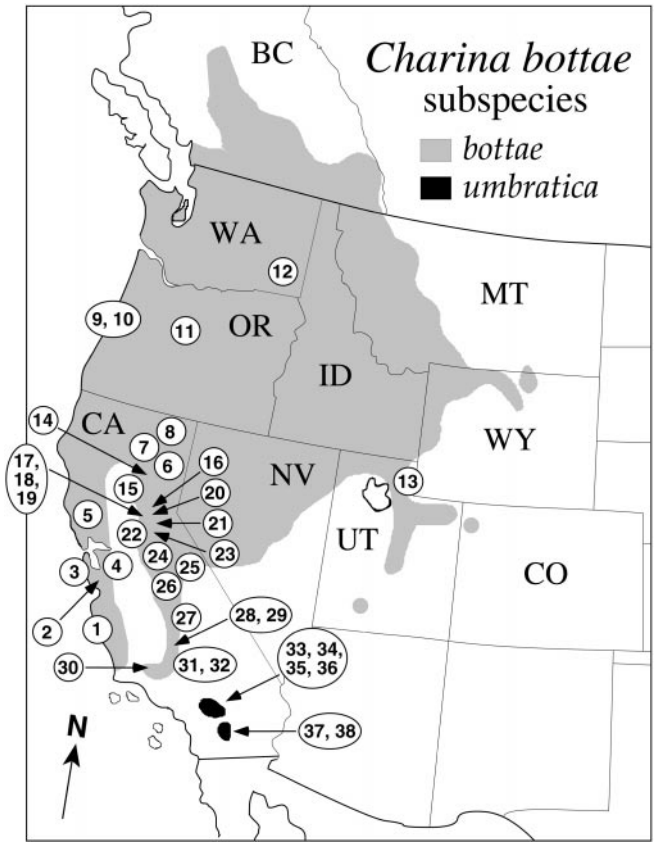

FIG. 1. Approximate distribution of the subspecies of *Charina bottae* in Canada and the United States (after Stebbins, 1985; Stewart, 1977). BC, British Columbia; CA, California; CO, Colorado; ID, Idaho; MT, Montana; NV, Nevada; OR, Oregon; UT, Utah; WA, Washington; WY, Wyoming. Numbers indicate the approximate localities of the specimens sequenced for this study.

convincingly demonstrated that there is no morphological basis for distinguishing *C. b. utahensis* as a taxon distinct from *C. b. bottae* (see also Ruthven, 1926; Tanner, 1933), a claim supported by our examination of the specimens included in this study. Therefore, we herein follow Stewart (1977) and recognize only two named population assemblages within *C. bottae, C. b. bottae* and *C. b. umbratica*.

An excellent fossil record indicates that Erycinae, the clade that includes rubber boas and their close relatives (Kluge, 1993), was present in North America 35 million years ago (mya) (Bell and Mead, 1996; Breithaupt and Duvall, 1986; Kluge, 1993), and molecular estimates derived from the data herein presented suggest that the ancestors of modern *C. bottae* evolved 29–10 mya. Rubber boas therefore offer an excellent opportunity to investigate to what extent the present phylogeographic structure of these snakes has been influenced by "deep" or older geological events versus "shallow" or more recent tectonic and climatic changes in northwestern North America (cf. Riddle, 1996; Walker and Avise, 1998). If deep phenomena (indicated by relatively high mtDNA sequence divergence) are mainly responsible for the present-day phylogeographic pattern of *C. bottae*, we expect that the genetic structure of this snake will predominantly consist of monophyletic groups separated by distinct phylogenetic gaps, which are suggestive of biogeographic barriers to gene flow. Alternatively, if the evolutionary history of *C. bottae* has been mainly shaped by shallower events (indicated by relatively low mtDNA sequence divergence), the populations of this species should not be clearly delimited by large genetic discontinuities, which would be an indication of ongoing or recent gene flow (Avise *et al.*, 1987; Riddle, 1996). Although these two scenarios perhaps represent endpoints of a continuum of possibilities, the dichotomy of deep versus shallow genetic divergences has practical value. Our goals herein are to use mtDNA sequences from specimens of *C. bottae* to infer evolutionary relationships among those populations (especially within California), to ascertain to what degree the phylogenetic pattern of these snakes is the product of responses to older geological events versus more recent phenomena, and to assess the implications of our findings for the taxonomy of rubber boas.

MATERIALS AND METHODS

Taxon Sampling, DNA Isolation, and Sequencing

We obtained tissue samples from 38 individuals of *C. bottae* belonging to the two subspecies of this taxon herein recognized, *bottae* (Pacific rubber boa) and *umbratica* (southern rubber boa; Table 1). We extracted total genomic DNA from tissue samples (liver and muscle) preserved in 95% ethanol or stored frozen at −70°C using the sodium dodecyl sulfate–proteinase K/phenol/RNAase method (Sambrook *et al.*, 1989). Using total cellular DNA as a template and primers designated ND4 and Leu (Arévalo *et al.*, 1994), we amplified (with the polymerase chain reaction, PCR) and used for phylogenetic analyses a 783-bp fragment of mtDNA that encompassed a 664-bp portion of the 3′ end of the nicotinamide adenine dinucleotide dehydrogenase subunit 4 (N*dh*4, or "ND4" gene) and a 119-bp section of two transfer ribonucleic acid (tRNA) genes ($tRNA^{His}$ and $tRNA^{Ser}$). ND4 is a reliable tracer of evolutionary history (Russo, 1997; Russo *et al.*, 1996; Zardoya and Meyer, 1996) and a relatively fast-evolving gene useful for resolving relationships among closely related taxa (e.g., Creer *et al.*, 1997; Liu *et al.*, 1999; Rodríguez-Robles and De Jesús-Escobar, 2000). PCR reactions were carried out in 25-μl volumes consisting of 12.5 μl of template DNA (1–2 ng DNA), 5.0 μl of primers (10 μM), 2.5 μl of 10× PCR reaction buffer (Boehringer Mannheim), 0.475 μl of deoxynucleoside triphosphates (40 mM), 0.125 μl of *Taq* DNA polymerase (5 U/μl), and 4.4 μl of H_2O. DNA was denatured initially at 94°C for

TABLE 1

Taxon, Sample Number, GenBank Accession Number, Voucher Number (If Available), and Locality of the Specimens Used in This Study

Taxon	Sample number	GenBank Accession No., voucher number, and locality
Outgroups		
Eryx miliaris	—	AF302942; MVZ 219298; Russia, Dagestan Autonomous Republic, Tarumovsky District, approximately 10 km south of Artezian on the Kizlyar to Astrakhan' Road
Charina reinhardtii	—	AF302943; CAS 207831; Equatorial Guinea, Bioko Island, ca. 15 km south of Malabo, along the coast road
Charina trivirgata	—	AF302944; CAS 200649; U.S.A., California, Riverside County, Diamond Valley
Charina bottae subspecies		
bottae	1	AF302945; MVZ 229876; U.S.A., California, Monterey County, Hwy. 1, 12 mi north of Nacimiento Road
bottae	2	AF302946; CAS 205801; U.S.A., California, Santa Cruz County, Santa Cruz Mountains
bottae	3	AF302947; CAS 204822; U.S.A., California, San Mateo County, San Francisco Watershed
bottae	4	AF302948; MVZ 164925; U.S.A., California, Alameda County, campus of University of California at Berkeley
bottae	5	AF302949; U.S.A., California, Napa County, Ida Clayton Road off Hwy. 128
bottae	6	AF302950; SJA 22800; U.S.A., California, Lassen County, Eagle Lake
bottae	7	AF302951; CSPU 2222; U.S.A., California, Shasta County, Rock Creek, 5 mi north of Pit River
bottae	8	AF302952; MVZ 162364; U.S.A., California, Modoc County, Cedar Canyon
bottae	9	AF302953; CSPU 2224; U.S.A., Oregon, Benton County, Corvallis
bottae	10	AF302954; CSPU 2226; U.S.A., Oregon, Benton County, Corvallis
bottae	11	AF302955; MVZ 162365; U.S.A., Oregon, Wheeler County, Hwy. 26, approximately 26 mi (by road) west of Grant County line
bottae	12	AF302956; MVZ 230472; U.S.A., Washington, Whitman County, 8.9 mi southwest of Pullman
bottae	13	AF302957; MVZ 230471; U.S.A., Utah, Cache County, Wasatch-Cache National Forest, Logan Canyon, Wood Camp Hollow
bottae	14	AF302958; CAS 209774; U.S.A., California, Nevada County, Tahoe National Forest, Forest Road 17, 0.6 mi (by road) of Forest Road 17-8
bottae	15	AF302959; CAS 206040; U.S.A., California, Plumas County, Plumas National Forest, Greenhorn Creek
bottae	16	AF302960; MVZ 162366; U.S.A., California, Nevada County, 3.5 mi northwest of Hobart Mills, Sagehen Research Station
bottae	17	AF302961; CAS 205637; U.S.A., California, Butte County, Plumas National Forest, Golden Trout Crossing Campground
bottae	18	AF302962; CAS 205988; U.S.A., California, Yuba County, Plumas National Forest, Gold Run Creek
bottae	19	AF302963; CAS 206320; U.S.A., California, Yuba County, Plumas National Forest, 1.8 mi south (by road) of Forest Road 690
bottae	20	AF302964; CSPU 2225; U.S.A., California, El Dorado County, Strawberry Creek, Hwy. 50
bottae	21	AF302965; MVZ 150180; U.S.A., California, Mono County, 0.5 mi east of Leavitt Meadows
bottae	22	AF302966; MVZ 197551; U.S.A., California, Tuolumne County, 0.7 mi southwest of Sourglass Crossing, Canyon of north Fork of Stanislaus River
bottae	23	AF302967; MVZ 230470; U.S.A., California, Tuolumne County, 10 mi south of Cherry Lake on Cherry Lake Road
bottae	24	AF302968; CSPU 2232; U.S.A., California, Mariposa County, south entrance of Yosemite National Park
bottae	25	AF302969; CAS 209228; U.S.A., California, Madera County, Sierra National Forest, North Fork Willow Creek
bottae	26	AF302970; CSPU 2223; U.S.A., California, Tulare County
bottae	27	AF302971; U.S.A., California, Kern County, Piute Mountains
bottae	28	AF302972; MVZ 229991; U.S.A., California, Kern County, summit of Breckenridge Mountain
bottae	29	AF302973; MVZ 229992; U.S.A., California, Kern County, summit of Breckenridge Mountain
bottae	30	AF302974; CSPU 2231; U.S.A., California, Kern County, Mount Pinos, McGill Campground
bottae	31	AF302975; U.S.A., California, Kern County, Tehachapi Mountains
bottae	32	AF302976; CSPU 2228; U.S.A., California, Kern County, Tehachapi Mountains, Camp Earl Anna
umbratica	33	AF302977; CSPU 2219; U.S.A., California, San Bernardino County, Green Valley Road
umbratica	34	AF302978; CSPU 2220; U.S.A., California, San Bernardino County, San Bernardino Mountains, Twin Peaks
umbratica	35	AF302979; CSPU 2227; U.S.A., California, San Bernardino County, San Bernardino Mountains, Heaps Peak Heliport
umbratica	36	AF302980; MVZ 230469; U.S.A., California, San Bernardino County, Baldwin Lake
umbratica	37	AF302981; CSPU 2229; U.S.A., California, Riverside County, San Jacinto Mountains, Idyllwild, Fern Valley
umbratica	38	AF302982; CSPU 2230; U.S.A., California, Riverside County, San Jacinto Mountains, Idyllwild, Humber Park

Note. Museum and Collector abbreviations are: CAS, California Academy of Sciences, San Francisco; CSPU, California State Polytechnic University, Pomona; MVZ, Museum of Vertebrate Zoology, University of California, Berkeley; SJA, Stevan J. Arnold.

5 min, and then 35 cycles of amplification were carried out under the following conditions: 94°C denaturation for 60 s, 55°C annealing for 60 s, and 72°C extension for 60 s, followed by a final 5-min extension at 72°C. Four microliters of the resulting PCR product was electrophoresed on a 1% agarose gel and stained with ethidium bromide to verify product band size.

We cleaned the double-stranded products with the QIAquick spin purification kit (Qiagen) and then cycle-sequenced them using fluorescent dye-labeled terminators (ABI Prism dye terminator cycle sequencing Ready Reaction kit with AmpliTaq DNA polymerase, FS; Perkin–Elmer). We performed sequencing reactions in 10-μl volumes (4 μl of template DNA, 3 μl of Ready Reaction, 1 μl of half term, 1 μl of primer, and 1 μl of H_2O) for 25 cycles using the following conditions: 30 s at 95°C, 15 s at 50°C, and 4 min at 60°C. After cycle sequencing, we ethanol precipitated the DNA, dried it, and resuspended it in formamide/blue dextran (5/1) by heating at 90°C for 2 min. We ran all samples for 8 h on a 4.8% Page Plus (Ameresco) acrylamide gel using an ABI Prism 377 automated sequencer and sequenced all PCR fragments in both directions.

Phylogenetic Analyses

Sequences from the light and heavy DNA strands were input into the Sequence Navigator (version 1.0.1) program and aligned to each other and to the reference sequence of the colubrid snake *Dinodon semicarinatus* (Kumazawa *et al.*, 1998). This initial alignment was refined with the MacDNASIS Pro software (version 1.0). Pairwise comparisons of observed proportional sequence divergence (*p*-distance), corrected sequence divergence (with the Tamura–Nei model; Tamura and Nei, 1993), and number of transitions and transversions by codon position were obtained using the computer program PAUP* 4.0b2a (Swofford, 2000). To estimate the phylogenetic information content of the mtDNA character matrix, we used the *g* test (Hillis and Huelsenbeck, 1992; Huelsenbeck, 1991) to assess the skewness of the tree length distribution of 100,000 trees randomly generated with PAUP*. Probability of phylogenetic structure was assessed using the values provided by Hillis and Huelsenbeck (1992).

We used two methods of phylogenetic reconstruction: maximum-parsimony (MP; Camin and Sokal, 1965; Swofford *et al.*, 1996) and maximum-likelihood (ML; Felsenstein, 1981; Rogers and Swofford, 1999; Steel and Penny, 2000), as implemented by PAUP*. For MP analyses, we used two character weighting schemes: equal weighting, where all nucleotide substitutions were weighted equally regardless of type or codon position, and differential codon position weighting, where we down-weighted third-position transitions (see below). Sites with insertion or deletion events were removed from the analyses. Each base position was treated as an unordered character with four alternative states. We used *Charina trivirgata*, *C. reinhardtii*, and *Eryx miliaris* as outgroups because previous systematic studies (Kluge, 1993) identified these taxa as close relatives of *C. bottae*.

We used heuristic search strategies for each tree-building methodology. We used 100 repeated randomized input orders of taxa for all MP analyses to minimize the effects of entry sequence on the topology of the resulting cladogram(s). MP analyses were conducted without the steepest descent option, and with accelerated character transformation (ACCTRAN) optimization, tree bisection–reconnection (TBR) branch swapping, save all minimal trees (MULPARS), and zero-length branches collapsed to yield polytomies settings in place. We used nonparametric bootstrapping (100 pseudoreplicates, 10 addition-sequence replicates for MP, 50% majority rule) to assess the stability of internal branches in cladograms (Berry and Gascuel, 1996; Felsenstein, 1985; Felsenstein and Kishino, 1993; Sanderson, 1995). Nonparametric bootstrap values generally are a conservative measure of the probability that a recovered group represents a true clade (Hillis and Bull, 1993; Zharkikh and Li, 1992).

For ML analyses we randomly selected as the starting tree one of the trees found during the MP searches. Using empirical nucleotide frequencies and five rate categories, we fixed the probabilities of the six possible nucleotide transformations (A $\leftrightarrow$ C, A $\leftrightarrow$ G, A $\leftrightarrow$ T, C $\leftrightarrow$ G, C $\leftrightarrow$ T, G $\leftrightarrow$ T), the proportion of invariable sites θ, and the α "shape" parameter of the gamma distribution of rate heterogeneity across nucleotide positions (Yang, 1996) to the empirical values calculated from the starting tree in a search for a better ML tree (a tree with a higher log-likelihood value), under the general time-reversible (GTR) model of nucleotide substitution (Gu *et al.*, 1995; Swofford *et al.*, 1996; Yang, 1994). If a tree of higher likelihood was found, we reoptimized and fixed the parameters for a subsequent ML search. We repeated this procedure until the same tree was found in successive iterations (Swofford *et al.*, 1996).

Because ND4 is a protein-coding gene, we plotted *p*-distance (y) versus corrected (with the Tamura–Nei model) estimates of proportional sequence divergence (x) for first, second, and third codon positions. This was done separately for transitions and transversions at protein-coding positions to test for the possibility that some types of nucleotide substitutions have become saturated. Points that fall along the $y = x$ line have the same observed and estimated numbers of changes and thus have not been subjected to multiple hits. Points that fall below the $y = x$ line indicate that multiple hits have occurred; saturation is reached when observed sequence divergence does not continue to increase, despite the fact that corrected estimates do. Conventional statistical tests of the relationship between estimated and observed sequence divergence are not appropriate because of nonindependence of the

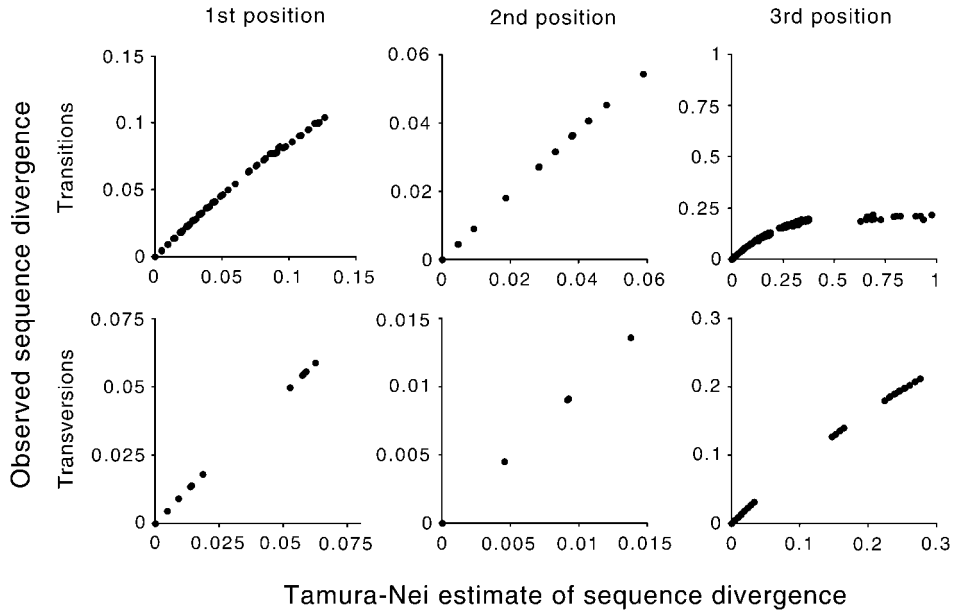

FIG. 2. Scatter plots of pairwise sequence differences (uncorrected) in transitions and transversions at first, second, and third codon positions versus Tamura–Nei estimates of pairwise divergence for the same class of substitutions.

data points due to the inclusion of each point in more than one pairwise comparison. Accordingly, the plots were used as heuristic devices to help identify classes of changes occurring at different rates, which should be weighted differently in phylogenetic analyses.

RESULTS

Sequence Variation

The 38 specimens of *C. bottae* included in this study yielded 33 unique mtDNA haplotypes. Including the outgroup taxa, there were 262 variable and 174 potentially phylogenetically informative characters in the 783-bp mtDNA data matrix. Of the informative characters, 27 were at first codon positions, 11 at second positions, 112 at third positions, and 24 at noncoding positions. Within *C. bottae*, there were 14, 2, 72, and 5 informative characters at first, second, third, and noncoding positions, respectively. Significant phylogenetic signal was present in the data set ($g_1 = -0.83$, $P <<< 0.01$; mean ± SD tree length 978.2 ± 31.5, range 719–1055). Thus, inferring cladograms was justified.

Scatter plots of observed versus estimated sequence divergences indicated that these relationships are linear for first and second position transitions and transversions and third position transversions (Fig. 2). Third position transitions deviated greatly from a linear pattern, suggesting that these mutations are saturated. To estimate the transition-to-transversion bias for third position transitions, we fitted a least-squares regression line, forced through the origin, to the part of the curve of third position transitions that is roughly linear. The slope of the regression line, 0.72, is an estimate of the transition-to-transversion ratio (Lara *et al.*, 1996; Moore and DeFilippis, 1997). Therefore, we down-weighted third codon transitional changes by a factor of 7 using a 7:7:1 codon position weighting (first, second, and third codon position, respectively) to correct for the biased substitution rates at this position.

Phylogenetic Relationships

Unique mtDNA haplotypes were used for our analyses, and consequently those of *C. b. bottae* samples 3, 10 and 12, and 18 and 19 were omitted, as they were identical to the haplotypes of *C. b. bottae* samples 2, 8, and 17, respectively. MP analyses with all characters weighted equally resulted in 42 most parsimonious trees 438 steps in length (L), a consistency index (CI) of 0.724, and a retention index (RI) of 0.838. Adjusting for the third position transitional bias in the coding region of the fragment of the ND4 gene sequenced also resulted in 42 equally parsimonious trees ($L = 1008$, CI = 0.761, RI = 0.827). The two MP bootstrap consensus trees from these analyses (Fig. 3) and the single ML tree obtained (log-likelihood score LnL = −3145.129; Fig. 4) recovered similar major groupings of rubber boas.

The most basal split within *C. bottae* corresponds to northern and southern segments of the current distribution of this snake (Figs. 3–5). The southern clade is formed exclusively by populations of *C. b. umbratica*. The northern clade is composed of two subclades, the "Sierra Nevada subclade," consisting of populations east of the Central Valley of California, and the "North-

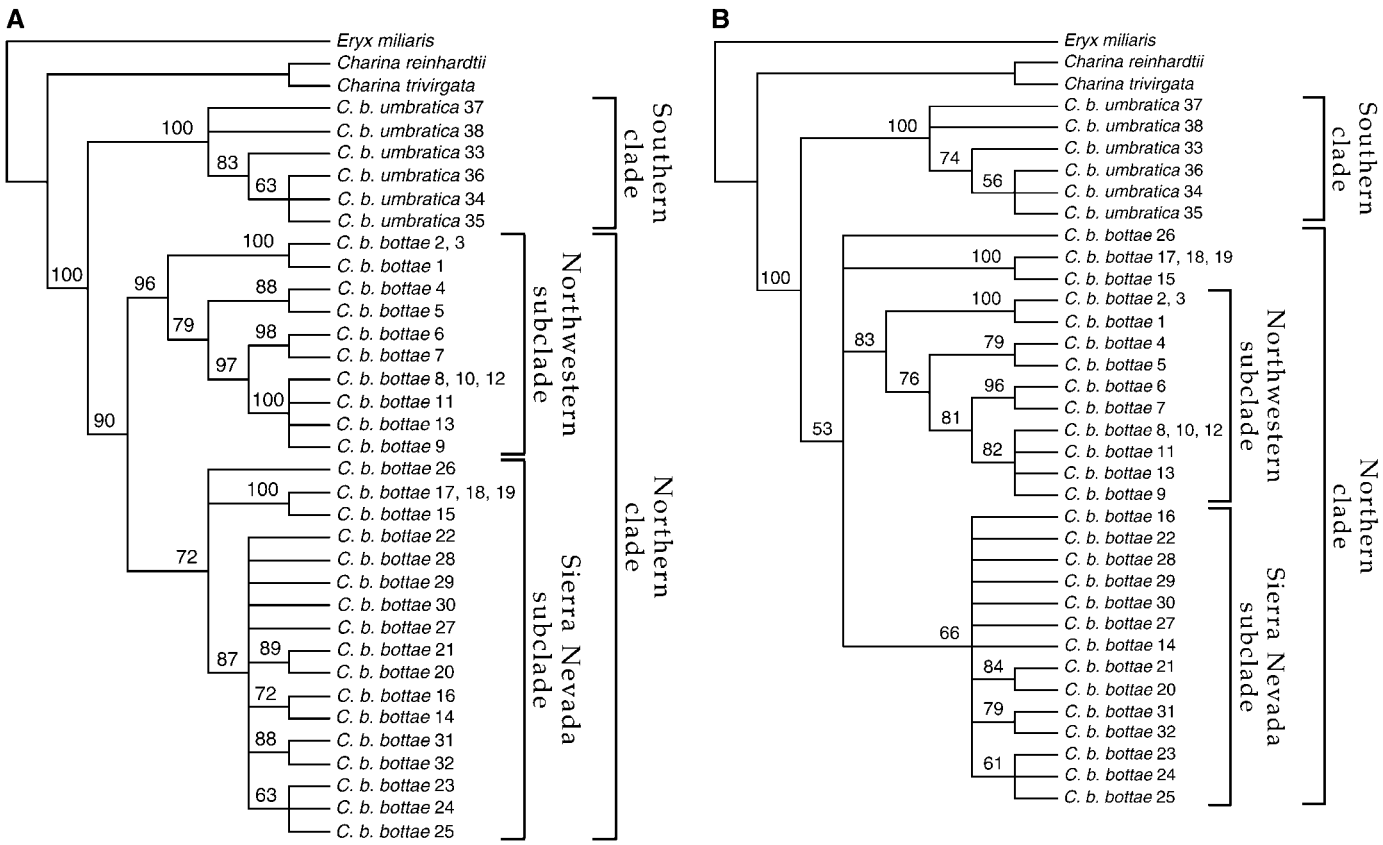

FIG. 3. Maximum-parsimony bootstrap consensus trees for 33 unique mtDNA haplotypes of *Charina bottae*. Numbers on the tree indicate percentage of nonparametric bootstrap support for nodes retained by more than 50% of bootstrap replicates: (A) with all characters weighted equally; and (B) with third position transitions down-weighted by a factor of 7:1.

western subclade," composed of specimens from coastal and northeastern California, Oregon, and Utah. The monophyly of the *umbratica* clade is unequivocal, and the Northwestern subclade is also well-supported (96 and 83% bootstrap support in equally and differentially weighted MP analyses, respectively), but the Sierra Nevada subclade is more weakly supported. Nevertheless, the topology of the ML tree agreed very well with those of the MP cladograms, suggesting that the three clusters of rubber boas represent true clades, irrespective of the level of bootstrap support.

DISCUSSION

Our phylogenetic analyses showed that the deepest split within *C. bottae* divided the populations of this snake into southern (*C. b. umbratica*) and northern (*C. b. bottae*) clades. Phylogeographic studies of *Lampropeltis zonata* (California mountain kingsnake) and *Diadophis punctatus* (ring-necked snake) in California also indicate that the most basal genetic break within these taxa corresponds to southern and northern segments of their distribution and occurs in the same general region as that of rubber boas (Rodríguez-Robles *et al.*, 1999; Feldman, 2000). Present-day southern California was separated from regions to the north by extensive, shallow inland seaways that mostly did not recede until the Pliocene (5–1.6 mya; Dupré *et al.*, 1991; Norris and Webb, 1990). These marine barriers were suggested to have played a role in the basal split of *L. zonata* (Rodríguez-Robles *et al.*, 1999), and perhaps they contributed to the current population genetic structure of *C. bottae* and *D. punctatus* as well.

A subsequent cladogenetic event in the *bottae* clade resulted in two groupings, which we refer to as the Sierra Nevada and the Northwestern subclades, based on the geographic distribution of their constituent populations. These two subclades have completely allopatric distributions, with a break that occurs somewhere in the vicinity of Lassen Volcanic National Park in northeastern California (between the localities of *C. b. bottae* samples 6 and 14, which lie about 120 (airline) km apart; Fig. 1). This region coincides geographically with an area in which a relatively large, localized genetic discontinuity is also found between *Ensatina eschscholtzii oregonensis* (Oregon ensatina salamander) and *E. e. platensis* (Sierra Nevada ensatina salamander; Jackman and Wake, 1994). As suggested for *E. eschscholtzii*, much local extinction

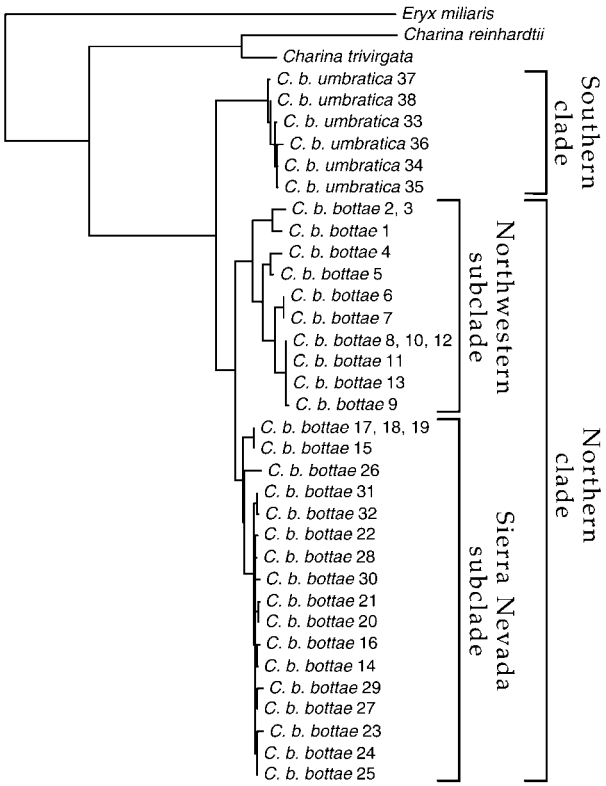

FIG. 4. Maximum-likelihood tree for 33 unique mtDNA haplotypes of *Charina bottae.* Branches are drawn proportional to branch lengths (expected amount of character change) estimated by the maximum-likelihood algorithm.

and recolonization has probably occurred in the Lassen region due to at least two main factors: extensive volcanism (Lassen Peak has been volcanically active in the last 100 years; Clynne, 1999; Norris *et al.,* 1997) and the fact that this upland area was subjected to glaciation during the Pleistocene, when ice sheets extended as low as about 1500 m and the regional snow line (the lower limit of perennial snow) was about 2000 m lower than at present (about 4200 m; Kane, 1982; Rogers *et al.,* 1991). Much of the usable habitat for *C. bottae* would have been eliminated during these periods, which would have prevented contact between rubber boas dispersing from the south and those migrating from the east, north, or west. Alternatively, *C. bottae* could have occurred throughout much of present-day Lassen Volcanic National Park area at one time in the past and have been extirpated by the recent geological activity in this area. It is unclear what barriers, if any, presently separate the Sierra Nevada and the Northwestern subclades of *C. bottae.*

Nowadays, the Central Valley of California represents unsuitable habitat for rubber boas, as populations belonging to the Sierra Nevada and Northwestern subclades are found around it, but do not occur in it. The ranges of various species of amphibians (e.g., *E. eschscholtzii; Taricha torosa* (California newt); *Rana boylii* (foothill yellow-legged frog)) and reptiles (e.g., *Sceloporus graciosus* (sagebrush lizard); *Elgaria coerulea* (northern alligator lizard); *Contia tenuis* (sharp-tailed snake); *Masticophis lateralis* (California whipsnake)) are also interrupted by the Central Valley (Jennings, 1983; Leonard and Ovaska, 1998; Stebbins, 1985; Tan and Wake, 1995). Genetic studies have shown that gene flow has occurred between populations of *E. eschscholtzii* (Moritz *et al.,* 1992; Wake and Yanev, 1986; D. B. Wake *et al.,* unpublished data) and of *C. tenuis* (Feldman, 2000) on the eastern and western sides of the valley (a "transvalley leak"). A more extensive sampling of *C. bottae* populations is necessary to determine whether the Central Valley has served as an effective barrier against eastward and westward dispersal of rubber boas, as initially suggested by our findings.

The phylogeographic structure of *C. bottae* reflects both deeper and more shallow divergences. The reality of the three rubber boa clades indicates the existence of older divergences among those population assemblages. On the other hand, the very short branches in the ML tree within the *umbratica* clade and the Sierra Nevada subclade are suggestive of shallow differentiation of mtDNA haplotypes, possibly as a result of recent population expansion or of ongoing (or recent) relatively high levels of gene flow. There is more geographic structuring in the Northwestern subclade, but the populations comprising this cluster span a much larger geographic area than those forming the *umbratica* and Sierra Nevada groupings, and a more thorough sampling might alter the observed phylogeographic pattern. As perhaps true for many terrestrial vertebrates, the spatial genetic architecture of *C. bottae* is a complex outcome of contemporary demographic and ecological forces acting upon a preexisting population structure that was molded by biogeographic factors operative throughout the evolutionary history of

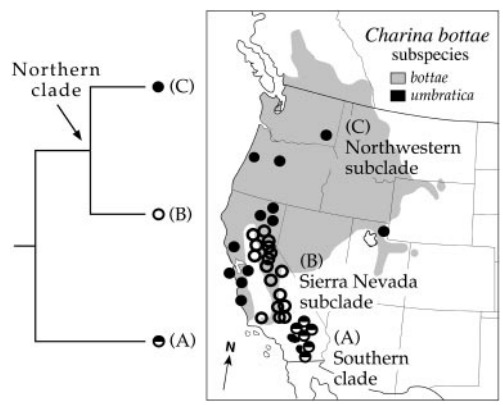

FIG. 5. Simplified interpretation of geographic structuring of mtDNA haplotypes of *Charina bottae.* A phylogenetic hypothesis indicates the relationship among the three major clades of this species complex.

the species (cf. Avise, 2000), and consequently, it reflects both shallower and deeper genetic divergences.

Taxonomic Implications of MtDNA Variation

Based on its geographic distribution and morphological differentiation, Erwin (1974) stated that *C. b. umbratica* should be elevated to full species status, but his recommendation was not followed by other authors. Our analyses show that the mtDNA haplotypes of *umbratica* form a clade separate from those of all other rubber boas. Whereas polyphyletic or paraphyletic relationships of haplotypes among descendant taxa are expected immediately following reproductive isolation, reciprocal monophyly in gene trees will usually be obtained with time (Avise, 2000; Moore, 1995, 1997). Observations of reciprocal monophyly in gene trees among related taxa thus imply a substantial time depth to their divergences. Estimates of mtDNA sequence divergence for reptile species for which branching events have been confidently dated using the fossil record or geological events range from 0.47 to 1.32% per million years (Zamudio and Greene, 1997). The smallest, uncorrected percentage sequence divergence between the *umbratica* and northern clades of *C. bottae* is 5.77%, which suggests that their separation occurred 12.3–4.4 mya. Although this estimate is for the divergence of the mtDNA lineages, not necessarily of the populations (and therefore it only indicates a maximum age for the split of the southern and northern rubber boa clusters), it still suggests that the *umbratica* clade has been an independent unit for a considerable period of time. We do not advocate making taxonomic changes solely on the basis of mtDNA data, but we recognize that assessments of a species' genetic structure can alert us to documented and/or overlooked levels of morphological differentiation among allopatric populations, and sometimes the taxonomic implications of this variation have not been fully considered (e.g., García-Moreno and Fjeldså, 1999; Parkinson *et al.*, 2000; Zink *et al.*, 1997).

Despite noticeable variation and overlap in the characters that define *C. b. bottae* and *C. b. umbratica*, the two forms still can be separated in most cases using a suite of morphological traits. Based on published data (Hoyer, 1974; Hoyer and Stewart, 2000; Nussbaum and Hoyer, 1974; Stewart, 1977, 1988; Weisman, 1988) and on our examination of additional material, we constructed the following taxonomic key for rubber boas. Agreement with any two of these three characters is likely to render a correct identification:

bottae. Frontal scale is usually subtriangular with a distinctly convex or angular posterior margin (Fig. 6A); generally there are 42 or more middorsal scale rows; there are usually 197 or more full-sized (cf. Dowling, 1951) ventral scales.

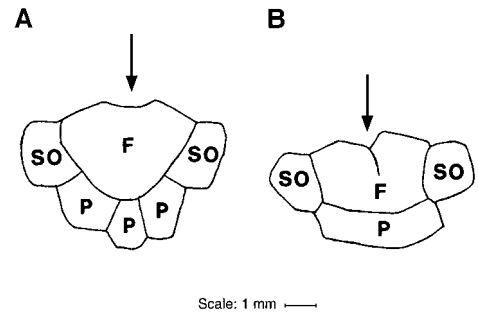

FIG. 6. Differences in general shape of head scales of (A) *Charina b. bottae* and (B) *C. b. umbratica*, with some common scale anomalies that occur in either form, such as a partially divided frontal scale and a completely divided parietal scale. These anomalies cannot be used to diagnose the two forms. Arrows indicate anterior margin. F, frontal scale; P, parietal scales; SO, supraocular scales.

umbratica. Frontal scale is usually subrectangular with a straight or only slightly convex or angular posterior margin (Fig. 6B); generally there are 41 or fewer middorsal scale rows; there are usually 196 or fewer full-sized ventral scales.

Rubber boas from Mount Pinos (locality of sample 30) and the Tehachapi Mountains (locality of samples 31 and 32), at the southern extreme of the range of the *bottae* clade, resemble specimens of *umbratica* in having low middorsal and ventral scale counts, although they tend to be more like *bottae* from more northern localities in the frontal scale character (Weisman, 1988). Additionally, alleles of two (lactate dehydrogenase, 6-phosphogluconate dehydrogenase) of the seven allozyme loci surveyed in an earlier genetic study (Weisman, 1988) were found in populations of *bottae* and *umbratica* from these two localities. On the other hand, the mtDNA sequences showed that *umbratica* possesses unique haplotypes, and Weisman's (1988) study also documented that *bottae* and *umbratica* differ completely for four (esterase, glucose 6-phosphate dehydrogenase, malate dehydrogenase, octanol dehydrogenase) of the proteins assayed (the seventh allozyme, peptidase, showed no variation across taxa). We interpret these findings as indicative that, as expected from their geographic distribution, gene flow between the northern and southern rubber boa clades last occurred between *bottae* from the Tehachapi Mountains and Mount Pinos area and *umbratica* and that shared, ancestral polymorphisms persist in the more slowly evolving nuclear genome of *bottae* and *umbratica*. In summary then, *C. bottae umbratica* is a genetically cohesive, allopatric taxon that is morphologically diagnosable, and we conclude that it is an independent evolutionary unit (cf. Frost and Hillis, 1990; Frost *et al.*, 1992; Wiley and Mayden, 2000) that should be recognized as a distinct species, *C. umbratica* Klauber, 1943.

CONCLUDING REMARKS

Phylogeography has significantly improved the description of the geographic distribution, phylogenetic relationships, and genetic distances among animal and plant populations. Although single-species studies have clearly been extremely valuable in this regard, comparative (i.e., multispecies) genetic assessments of taxa with similar distributions enable us to draw broader conclusions about regional biogeography. Recent (Barrowclough *et al.*, 1999; Cicero, 1996; Moritz *et al.*, 1992; Patton and Smith, 1990; Rodríguez-Robles *et al.*, 1999; Tan and Wake, 1995; Wake, 1997) and ongoing genetic studies of various species of amphibians, mammals, reptiles, and birds from California will allow us to assess how the complicated geomorphological history of this region has interacted with demographic processes to shape the modern genetic population structure of independent lineages of terrestrial vertebrates.

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