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EVOLUTIONARY RELATIONSHIPS WITHIN THE *ENSATINA ESCHSCHOLTZII* COMPLEX CONFIRM THE RING SPECIES INTERPRETATION

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Abstract.—Sequences (644–681 bp) from the mitochondrial cytochrome *b* gene were obtained for 24 individuals representing the geographic range and morphological diversity of the polytypic salamander ring species *Ensatina eschscholtzii.* These data were used to estimate the phylogeny of components of the ring to test the biogeographic scenario underlying current interpretations of speciation in this complex. The analysis revealed high levels of nucleotide variation among subspecies. Strong subdivision was evident within the subspecies *platensis* and *oregonensis.* The phylogenetic hypothesis of minimum length that is best supported by the data contains one monophyletic group that includes populations from the southern Sierra Nevada and mountains of southern California (*croceater, klauberi,* and southern *platensis*) and another that includes populations of the southern and central coastal regions (*xanthoptica* and *eschscholtzii*). Samples of *oregonensis* were typically basal, but their precise branching order was unstable. Both *oregonensis* and *platensis* were paraphyletic, with several disparate lineages in *oregonensis* and a strong north–south dichotomy in *platensis.* The data were incompatible with a biogeographic model that required all subspecies to be monophyletic but were compatible with slightly modified predictions of a model assuming stepwise colonizations from north to south down the Sierra Nevada and independently down the coastal ranges. These features provide strong support for the biogeographic scenario central to the interpretation of *Ensatina eschscholtzii* as a ring species. Division of this complex into separate species on the basis of the observed patterns of monophyly for mitochondrial DNA (mtDNA) is unwarranted because further sampling could reveal additional instances of paraphyly across subspecies and, more generally, because mtDNA alone should not be used to infer species boundaries. [DNA sequences; mitochondrial DNA; cytochrome *b*; ring species; phylogenetics; geographic variation; speciation; Caudata.]

Salamanders of the *Ensatina eschscholtzii* complex provide a particularly clear example of a ring species (Stebbins, 1949). Ring species are circularly arranged polytypic taxa with gradual transitions between adjacent components but abrupt changes where the terminal races come into contact. They are supposed to exemplify speciation through gradual divergence among large population units (Rensch, 1929; Mayr, 1963) and, thus, the linkage between microevolutionary processes and speciation. This view contrasts with recent models of speciation that emphasize genetic changes in small peripheral populations (Mayr, 1982; White, 1982; Carson and Templeton, 1984). It is also contrary to the opinion that the process of speciation is qualitatively distinct from intraspecific microevolution (e.g., Goldschmidt, 1933; Gould and Eldredge, 1977).

Stebbins (1949) recognized seven subspecies distributed in the mountains encircling the central valley of California (Fig. 1). Unblotched subspecies occur on the coastal margins and replace each other from southern (*eschscholtzii*), through central (*xanthoptica*), to northern California (*oregonensis* and *picta*) into Oregon and Washington. Blotched subspecies are distributed down the inland side of the valley on the mesic western slopes of the Sierra Nevada. The subspecies *platensis* ranges widely through the Sierra but is replaced at the southern end by populations of *croceater.* The third blotched form, *klauberi,* is disjunct and occurs as isolated populations in the mountains of southern California. The blotched and unblotched forms are in contact in three places: across the top of the ring, in the central Sierran foothills where a disjunct series of populations of *xanthop-*

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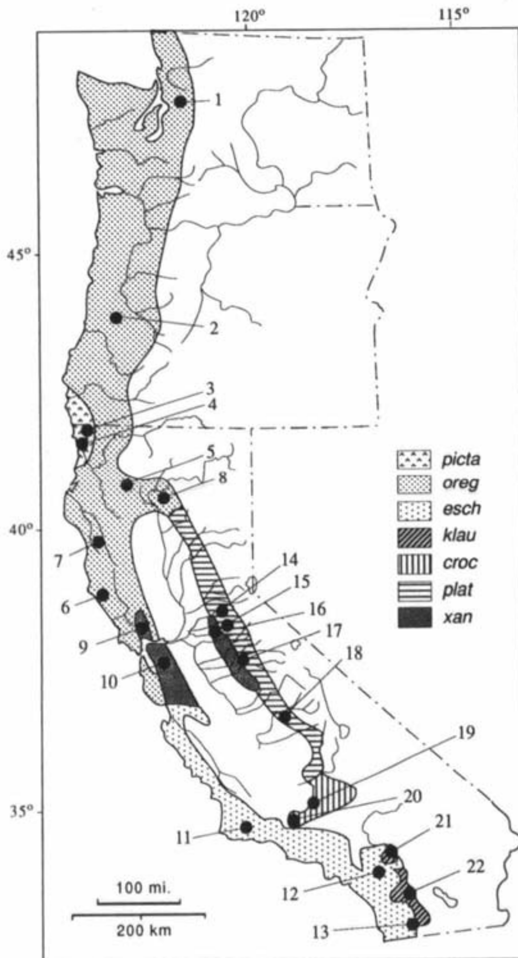

FIGURE 1. Map of western USA showing the distribution of localities from which *Ensatina* mtDNAs were sequenced. The ranges of the subspecies are defined on the basis of information in Stebbins (1949) and extensive recent collections (Wake, unpubl. data). Abbreviations, localities, and MVZ catalog numbers are in the Appendix.

tica is in contact with *platensis*, and in the mountains of southern California where *klauberi* and *eschscholtzii* meet at four disjunct sites.

Stebbins (1949) proposed that the species originally occurred in the Douglas fir and redwood forests of southern Oregon and northern California and that during periods of greater humidity, salamanders dispersed down the coastal and inland ranges to meet again in southern California. With increasing aridity, the populations became less continuous, particularly in the southern part of the range. The Sierran populations of *xanthoptica* were thought to be recently derived from the coastal populations via a transvalley leak during the more mesic periods of the Pleistocene.

Based on this model of historical biogeography, Stebbins proposed that the southward colonizations were each accompanied by differentiation such that interbreeding was precluded when the two lineages came into contact again in the mountains of southern California. The key observation (Stebbins, 1949:506) that suggested the gradual accumulation of reproductive isolation was that

> progressive genetical divergence with progressively increasing reproductive incompatibility is observed from north to south between the coastal and interior series of races—from free interbreeding in northern California, through occasional hybridization in the Sierra in central California, to sympatry with reproductive isolation in southern California.

Additional collecting and analyses (Stebbins, 1957; Brown and Stebbins, 1964; Brown, 1974; Wake et al., 1986, 1989) have supported the basic interpretation.

An analysis of allozyme variation (Wake and Yanev, 1986) revealed strong differentiation throughout the complex. Some subspecies were remarkably diverse for allozymes (e.g., *platensis, oregonensis*), whereas others (e.g., *xanthoptica, eschscholtzii*) showed little differentiation. The presence of fixed differences between local populations of the same subspecies was contrary to the view (Dobzhansky, 1958) that the continuity of the ring was the result of ongoing gene flow. Given such strong geographic structuring, the initial allozyme study did not sample sufficient populations to determine the discreteness of subspecies or their relationships. Wake and Yanev (1986:713), however, suggested that the blotched and unblotched forms "might represent separate adaptive responses to provincial selection pressures rather than discrete historical entities or incipient species."

In this paper, we used sequences from mitochondrial DNA (mtDNA) to estimate

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phylogenetic relationships among components of the ring and to evaluate the historical biogeographic scenario basic to Stebbins's (1949) evolutionary model. Specifically, we evaluated the phylogenetic predictions from two alternative scenarios: (1) Stebbins's model, which suggests independent stepwise colonizations down the coastal and inland ranges from northern Californian populations, with a recent transvalley leak of *xanthoptica*, and (2) a model based on the premise that the current distribution of subspecies is the result of secondary contacts between subspecies that had diverged considerably in geographic isolation (cf. Stebbins, 1949:503).

We compared mtDNAs to infer population history. The mtDNA "gene tree" should reasonably reflect population history where local populations are reciprocally monophyletic for their mtDNAs and diversity within populations is much less than that among populations (Avise, 1989). These conditions should be met where local populations have been small and have had restricted gene flow over long periods (ca. $4N_f$ generations; Neigel and Avise, 1986; Birky et al., 1989). These conditions are likely to be met by *Ensatina eschscholtzii* subspecies, given their extreme subdivision for allozyme alleles (Wake and Yanev, 1986) and presumed low vagility. Prompted by controversy over the taxonomy of *Ensatina* (Frost and Hillis, 1990), we point out the limitations of mtDNA for defining species boundaries.

Materials and Methods

Sampling Design

The analysis covers multiple, geographically separated representatives of each named subspecies, concentrating on morphologically "pure" populations as defined by Stebbins (1949) or our own observations. Sampling and analysis was done in three phases (see Baverstock and Moritz, 1990). The first was a pilot study, based on 378 bp of mitochondrial cytochrome *b* sequence from 15 samples of *Ensatina* and two outgroup samples, to determine the suitability of the gene. *Aneides lugubris* and *Plethodon elongatus* were selected as outgroups on the basis of other studies (Wake, 1963; Larson et al., 1981) that suggested these species as members of the sister clade of *Ensatina*, itself a monotypic genus. The pilot study revealed appropriate levels of variation (up to 12% observed sequence divergence within *Ensatina*), although phylogenetic analysis failed to resolve the branching order at the base of the tree.

In the second phase, we added nine more samples of *Ensatina* to replicate representation of some subspecies and to attempt to split long branches, a potential cause of instability or error in parsimony analysis (Felsenstein, 1978; Swofford and Olsen, 1990). We also extended the sequences by 303 bp in an attempt to increase resolution at the base of the phylogeny. The final data set included 24 individuals from 22 localities (Fig. 1) and one each from the outgroups, *Aneides* and *Plethodon*. In the third phase of sampling, we screened multiple samples from three populations for diagnostic restriction fragment length polymorphisms to assess the distribution of identified mtDNA lineages within and among localities.

DNA Preparation, Amplification, and Sequencing

DNA was prepared by phenol-chloroform extraction (Maniatis et al., 1982) or by boiling minute amounts (<5 mg) of frozen tissue in a 5% (w/v) solution of Chelex (BioRad). Both approaches yielded DNA suitable for amplification by the polymerase chain reaction (PCR).

PCR amplifications were done using primers (Fig. 2) that spanned approximately two-thirds of the cytochrome *b* gene and yielded a sequence homologous to codons 7-234 in the published sequence of *Xenopus* (Roe et al., 1985). Primers cyt-b2 (Kocher et al., 1989) and MVZ 16 (C. Orrego and D. Irwin, pers. comm.) amplify mtDNA from a wide variety of vertebrates. Primers MVZ 15, 18, and 25 were designed to match sequences from *Ensatina* and, for 18 and 25, also from *Xenopus*.

For double-strand reactions, template

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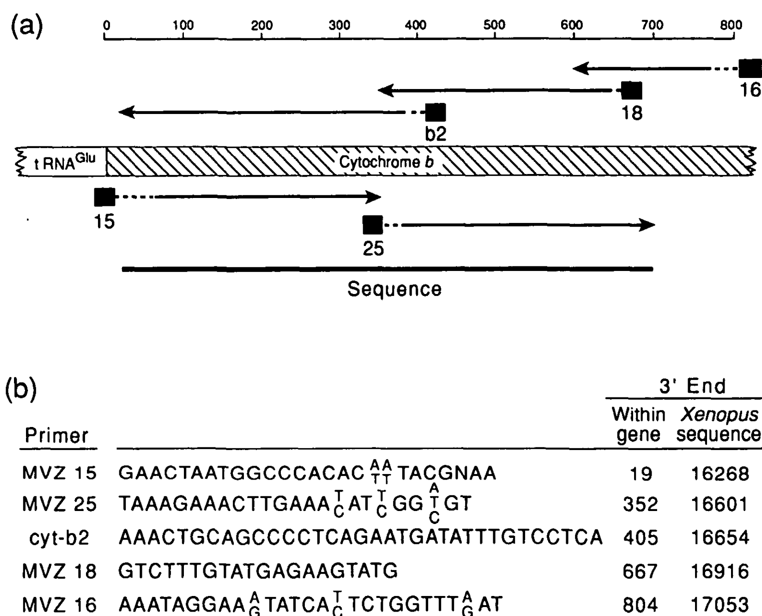

Primer		3' End: Within gene	3' End: Xenopus sequence
MVZ 15	GAACTAATGGCCCACAC[A/T][A/T]TACGNAA	19	16268
MVZ 25	TAAAGAAACTTGAAA[T/C]AT[T/C]GG[A/T/C]GT	352	16601
cyt-b2	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	405	16654
MVZ 18	GTCTTTGTATGAGAAGTATG	667	16916
MVZ 16	AAATAGGAA[A/G]TATCA[T/C]TCTGGTTT[A/G]AT	804	17053

FIGURE 2. Location (a) and definition (b) of oligonucleotide primers used for amplification and direct sequencing of cytochrome *b*. The scale represents the distance from the start of the gene in base pairs. The location of the 3′ end of the primers is defined relative to the 3′ end of the gene and to the published sequence of *Xenopus* mtDNA (Roe et al., 1985). Arrows indicate the region sequenced from each primer. Primers 15 and 25 generated light strand (antisense) sequence.

and primers were annealed at 45–50°C, using 0.5 pmol of each primer, 0.75 mM dNTPs, and 1.5 mM $MgCl_2$ in a pH 8.4 buffer with 50 mM KCl and 10 mM Tris-HCl (final concentrations). Reactions were typically run for 35 cycles in a total volume of 12.5 or 25 μl, using 0.3 or 0.6 units of *Taq* polymerase (Cetus), respectively. Aliquots of 5 μl were run on 2–4% low-melting-point agarose gels from which a small plug was taken and diluted 1:100 in 10 mM Tris–0.1 mM EDTA to provide template for single-strand reactions.

Single-strand template was prepared by asymmetric PCR (Gyllensten and Erlich, 1988) with 1:50 primer ratios and the same reaction profiles as above in 50-μl reactions with 1.2 units of *Taq* polymerase. The yield and purity of single-strand products were assessed by electrophoresis of 5-μl aliquots through 4% agarose (1× TAE) gels. For successful reactions, the remaining 45 μl was purified and concentrated to ca. 20 μl on disposable centrifugal filters (Millipore MC30). Dideoxy chain termination sequencing (Sanger et al., 1977) was done with the U.S. Biochemicals Sequenase version 2.0 kit and ^{35}S-labeled dATP. All double- and single-strand PCR reactions included negative controls to guard against contamination of reagents with DNA. No such contamination was detected.

Data Analysis

Sequences were read from both strands with >90% overlap and aligned by eye to each other and, using amino acid translations, to the same region of *Xenopus* mtDNA. The distribution and amount of variation among the sequences were analyzed with the MOSY program written in C for DOS PCs by Chris Meacham (Univ. California, Berkeley). Measures of actual divergence (cf. observed difference) were estimated using the correction of Brown et al. (1982).

For phylogenetic analysis, the general strategy was to minimize the noise gen-

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```
               K  I  H  P  L  L  K  I  I  N  N  S  F  I  D  L  P  T  P  S  N  L  S  Y  L  W  N
xan SR      AAAATTCACCCACTACTAAAAATCATTAATAATTCCTTCATTGACTTACCTACTCCATCAAACTTATCCTACCTATGAAA
xan Av      ................................................................................
xan Or      ??????????.C....................C...............................................
oreg WA     ...........TT..T................C...........T.....C...........T.....T..TT.......
oreg In     ...........C...T.......T........C..A........T..G..C...........T........TT.......
oreg OR     ??????.....TT..T............................T.....C...........T.....T..TT.......
oreg Br     ??????.....TT..G.......T........C...........T..............T..T.........T.......
oreg Me     ????CC.....TT..A.......T........C...........T..............T..T.........T.......
oreg He     ??????????????????????.......C..C...........T.....C..C........T.....T..TT.......
plat Bl     ........T..TT..T................C.................CG..........T.....T..TT.......
plat Ar     ........T..T...T..................................C...........T.....T..TT.......
plat Ha     ....C......T...T.............C..C...........T......G..........T.........T.......
plat Ma     ??????.....T...T.......T.....C..C...........T......G....................T.......
croc Te     ....C......TT..T.......T.....C..............TC..........C.....T........TT.......
croc Ve     ????C......TT..T.......T.....C..............T...........C.....T........TT.......
klau Cr     ....C......TT..T.............C....................C...........TC.G......T.......
klau CW1    ....C......TT..T.............C....................C...........TC........T.......
klau CW2    ??..C......TT..T......???....C....................C...........TC........T.......
klau SJS    ???????????.???T?............C........T...........C...........TC.G......T.......
esch SBa    ??????.....T...........T........C...........T.....A.............................
esch SBe    ??????????.T....................C...........T.....C....................T........
esch CW     ???????????T....................C...........T.....C...........T.................
picta       ??????????.T...T................C...........T.....C...........T........TT.......
Plethodon   ?????????????....T.....T........C..T..T.....TC.C..A..C....GT..TC....A..TG.......
Aneides     ????????????????????????G.C...........TG.....C.T..CG.C........T.....A..TT.......

               F  G  S  L  L  G  I  C  L  I  S  Q  I  M  T  G  L  F  L  A  M  H  Y  T  A  D
xan SR      CTTTGGGTCACTACTAGGAATCTGTTTAATTTCACAAATTATAACTGGCCTCTTTCTAGCAATACATTATACAGCAGACA
xan Av      ................................................................................
xan Or      ........................................C.T.TC.......................C..........
oreg WA     ......A.....................G...........C.T........T..........................T.
oreg In     ......A..GT.................G...........C.T..C.....T...T........................
oreg OR     ......A............G.T......G...........C.T........T............................
oreg Br     ......A......T...........C..............C.T..C.....T...T........................
oreg Me     ......A......T.....G.....C..............C.T..C.....T...T........................
oreg He     ......A............G........G...........C.T..C.....T.................C..........
plat Bl     ......A...T.............................C.T........T.................C..........
plat Ar     ......A..CT.............................C.T..C.....T.................C..........
plat Ha     ......A......T....C............A........C.G..C.....T...T......................T.
plat Ma     ......A......T.................A........C.G........T...T......................T.
croc Te     ......A......T..............G...........C.G..C.....A...T........................
croc Ve     ......A.....GT..............G...........C.G..C.....A...T........................
klau Cr     T.....A.................................C.T........T...T........................
klau CW1    T.....A..T...T..........................C.T.....T..T...T.............C..........
klau CW2    T.....A......T..........................C.T.....T..T...T.............C..........
klau SJS    T.....A.................................C.T........T...T.............C..........
esch SBa    ........................................C.C..C..................................
esch SBe    ......A.................................C.C..C..................................
esch CW     ......A.................................C.C..C..................................
picta       ......A.................C...G...........C.T........T...T........................
Plethodon   ...C..A...T..T....T.....CC....AAT......C..T..A.....G..............C...........T.
Aneides     ......T......G......AT..C.......T.........T..A..T..A.....G.....G..C........T....
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FIGURE 3. Sequences from codon 7 to codon 234 of cytochrome *b* from 23 individuals of *Ensatina* and representatives of the outgroups (*Aneides lugubris* and *Plethodon elongatus*). A complete nucleotide sequence and its amino acid translation is given for a *xanthoptica* from Santa Rosa. Other sequences with nucleotides identical to the *xan* SR sequence are represented by periods (.). Some sequences could not be reliably read close to the primers; ambiguities are represented by question marks (?). The sequence found in both individuals of *picta* is represented once. Abbreviations are defined in the Appendix.

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            T  T  S  A  F  S  S  V  V  H  I  C  R  D  V  N  Y  G  W  I  M  A  N  I  H  A  N
xan SR      CCACTTCCGCATTCTCATCAGTTGTACATATCTGCCGCGATGTAAATTACGGATGAATTATACGAAATATTCACGCCAAC
xan Av      ..........................................................C.....................
xan Or      ................................................................................
oreg WA     ....C..T.......................T...........G........G...G..T.............T......
oreg In     .......T.....T......A..........T..............C.........G................T......
oreg OR     ....C..T.......................T....................G...G..T.............T......
oreg Br     .............T......A.......C..............T.....T..C......T.............T......
oreg Me     .......T.....T......A............................T..C...G..T.............T......
oreg He     ....C..T............A..........T.................T..G...G................T......
plat Bl     ....C...............A..........T........C........T......G................T......
plat Ar     ..T.C..T............A..........T........C..G.....T......G................T......
plat Ha     ....C...............A..........T.................T.......................T......
plat Ma     ....C...............A..........T.................T.......................T......
croc Te     ....C...............A..........T.....G...................................T......
croc Ve     ....C...............A..........T.....G...........T.......................T......
klau Cr     ....C...............A.C.C......T........C.....C..T..C....................T......
klau CW1    ....C...............A...C......T........C.....C..T..C....................T......
klau CW2    ....C...............A...C......T........C.....C..T..C....................T......
klau SJS    ....C...............A.C.C......T........C.....C..T..C....................T......
esch SBa    .................................................T..G...........................
esch SBe    ....C...............A...........................................................
esch CW     ....C...............A...........................................................
picta       ....C........T......A..........T..T...........C..T......G................T.....T
Plethodon   .............T........C..............?..C.....C..T......T.A........C............
Aneides     .T.T.........T........CA.............A..C........T..T...T.A.......G......T..T..T

             G  A  S  F  F  F  I  C  I  Y  M  H  I  G  R  G  I  Y  Y  G  S  Y  M  F  K  K  T
xan SR      GGAGCCTCATTTTTCTTTATCTGCATCTACATACATATTGGACGTGGTATTTATTACGGATCCTATATATTTAAAAAAAC
xan Av      ............................................G...........T........C.........G....
xan Or      ............................................G...............................G....
oreg WA     ..............T........T..T..TT.............A.....A.....T...................G....
oreg In     .................C..T..T..T........C.....A.....G............................G....
oreg OR     ..............T........T..T..TT.............A.....A.....T...................G....
oreg Br     ...........A........T..T...........C.....G..A..C..............T..C..........G....
oreg Me     ...........A.......................C........A.....C.....T.....T..C..........G....
oreg He     ..............T.....T.....T..TT....C........A.....A.....T.....A.....G.......G....
plat Bl     ..........................T.................A.....A.....T.....T..C..........G....
plat Ar     ..........................T..T..............A.....A.....T.....T..C..........G....
plat Ha     .....T.......................T........C.....A..A..A.....T.......TC..G.......G....
plat Ma     ...........C........T........T........C.....A..A..A.....T.......T...........G....
croc Te     .............................T..............A.....A.....T...................G.G..
croc Ve     .............................T..............A.....A.....T...................G.G..
klau Cr     ...........C..T.....T..T..T..T..............A.....A.....T..G................G.G..
klau CW1    ...........C..T.....T..T..T..T.....C........A.....A.....T...................G....
klau CW2    ...........C..T.....T..T..T..T.....C........A.....A.....T...................G....
klau SJS    ...........C..T.....T..T..T..T..............A.....A.....T...................G.G..
esch SBa    ..........................T..T..............A...........T........C..........G....
esch SBe    ..........................T..T........C.....A...........T........C.....C....G....
esch CW     ..........................T..T..............A...........T........C.....C....G....
picta       .............................TT.............A..C..A.....T.....T........C....G....
Plethodon   .....T.....C..T........T..T..TC.T...........A..AGCA..C..T..........A..A.....G....
Aneides     ..T........C..T...........T..T.....C........A..CT.A.....T.....A.......A...GG....
```

FIGURE 3. Continued.

erated by homoplasy through a priori identification of classes of variation expected to be saturated (F. Villablanca, W. K. Thomas, and A. C. Wilson, submitted) and the use of appropriate weighting measures and constraints. Phylogenies were estimated using PAUP (version 3.01; Swofford, 1991). Unless otherwise noted, all analyses used a 10:1 step matrix to weight transversions over transitions. Bootstrap analyses with the step matrix applied were done using PAUP 3.0s.

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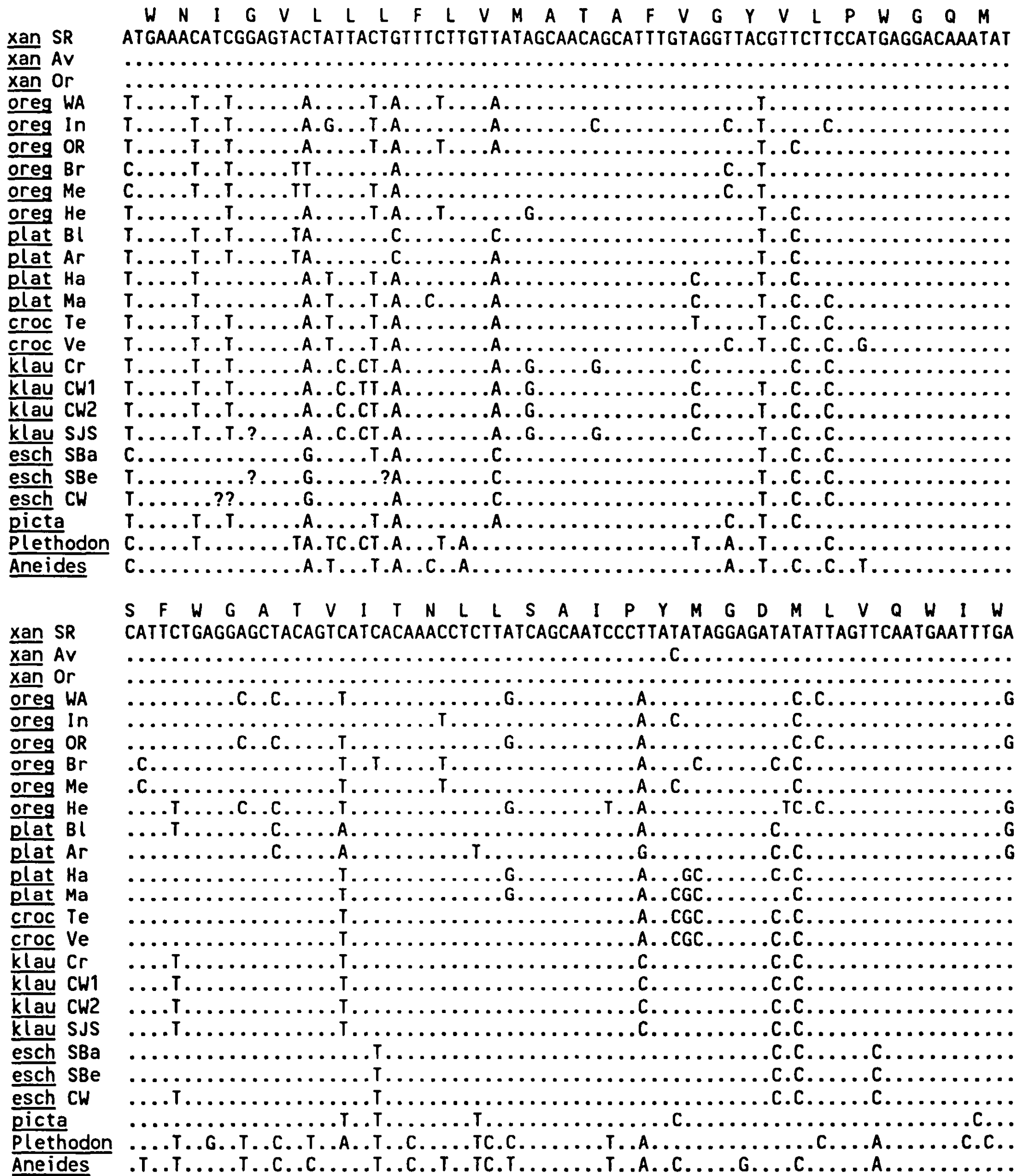

FIGURE 3. Continued.

RESULTS

Sequence Variation

Sequences 644–681 bases in length were obtained from 24 individuals of *Ensatina* and one individual each of *Plethodon elongatus* and *Aneides lugubris* (Fig. 3). The minor differences in length are due to variable sequence readability close to the primers. No substitutions causing frameshift or nonsense mutations were present. The mean base content of the light strand across all taxa (0.13 G, 0.31 A, 0.35 T, 0.20 C) is biased toward A and T, with a marked

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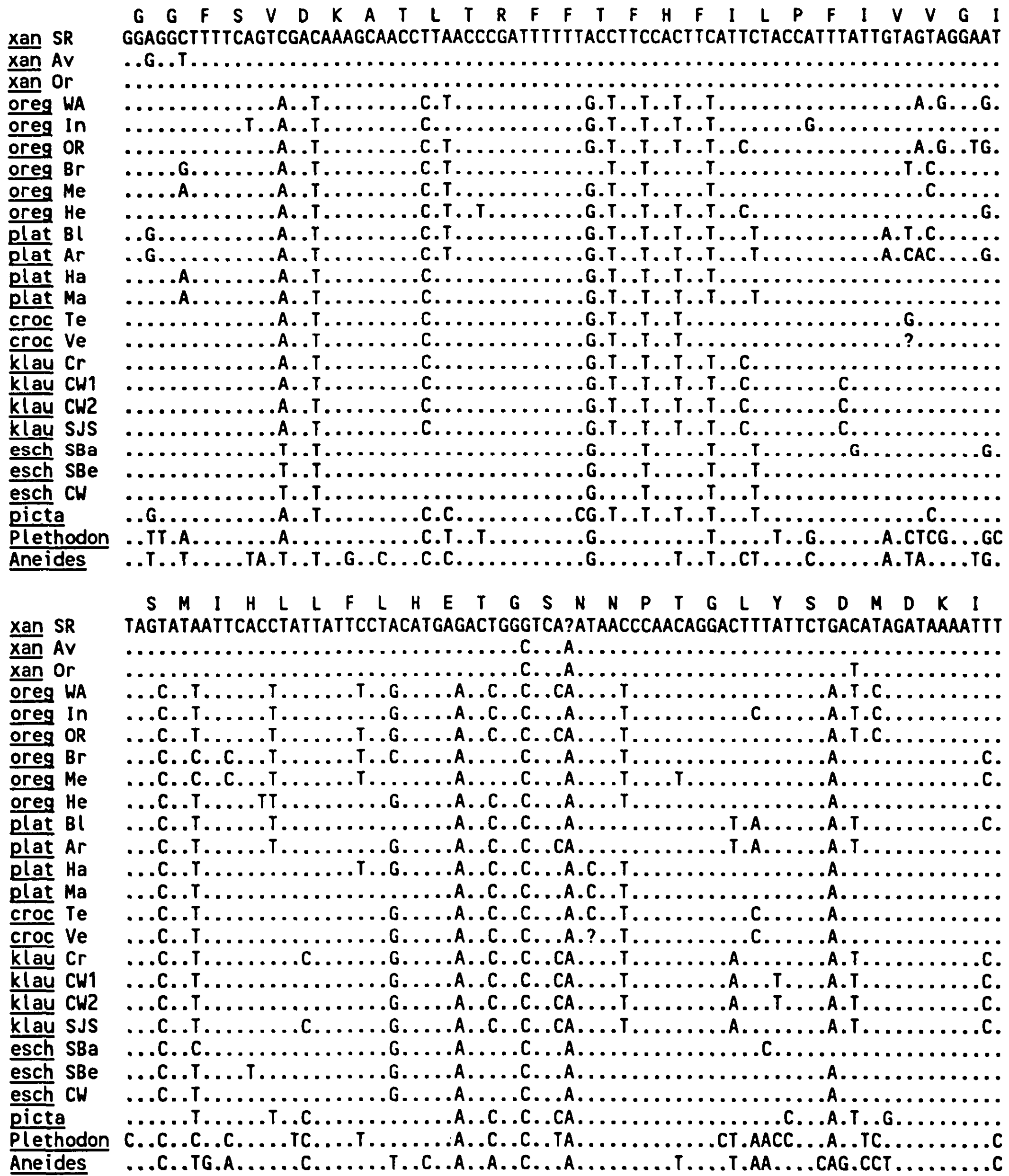

FIGURE 3. Continued.

deficit of G's. This bias in nucleotide composition is typical of vertebrate mtDNA genes (Brown, 1985).

The magnitude of variation is high. Including both *Ensatina* and the outgroups, 250 (37%) of 681 nucleotides and 51 of 227 (22%) amino acids vary (Fig. 3). Observed sequence differences are between 0.3 and 19.2%. Corrected for multiple hits (Brown et al., 1982), the values range from 0.8 to 39.7% (Table 1). Within *Ensatina*, observed sequence difference ranges from 0.3 to 12.1%, and corrected values are between 0.8 and 16.2%.

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```
            S  F  H  P  Y  F  S  Y  K  D  L  F  G  F
xan SR      CATTCCACCCATACTTCTCATACAAAGACTTATTTGGATTT
xan Av      .........................................
xan Or      .............................?....A......
oreg WA     ......................T..................
oreg In     ....T.................T..................
oreg OR     ......................T..............G...
oreg Br     ......................T.........C........
oreg Me     ......................T.........C........
oreg He     ..............?.......T..................
plat Bl     ......................T..................
plat Ar     ......................T..................
plat Ha     ................T....TT..................
plat Ma     ................T....TT........G.........
croc Te     .G...........T.......TT..................
croc Ve     .............T.......TT..................
klau Cr     ......................T..................
klau CW1    ......................T..................
klau CW2    ......................T..................
klau SJS    ......................T..................
esch SBa    .........................................
esch SBe    .........................................
esch CW     .........................................
picta       ....T.................T..........A.......
Plethodon   ....T........T..T............C..C.G..C...
Aneides     ....T?..........TCT.........?????????????
```

FIGURE 3. Continued.

Estimates of corrected DNA sequence difference are relatively low within five subspecies: *picta* (0.0%, $n = 2$), *xanthoptica* (1.6–2.5%, $n = 3$), *eschscholtzii* (1.0–2.7%, $n = 3$), *klauberi* (0.6–2.4%, $n = 4$), and *croceater* (1.2%, $n = 2$). The other two subspecies are strongly differentiated; sequence differences within *oregonensis* range from 1.3 to 14.5% ($n = 5$) and those within *platensis* are 2.4–14.0% ($n = 4$). The variation in sequence differences within subspecies does not appear to be due to different scales of sampling. Some widely separated samples within *oregonensis* and within *eschscholtzii* have similar sequences, whereas some geographically close samples within *oregonensis* and within *platensis* have very different sequences (cf. Table 1 and Fig. 1).

Phylogenetic Analysis: Character Evaluation

For phylogenetic analysis, each nucleotide was treated as a character with up to four unordered states. Phylogenetically informative variation is present at 184 of the 250 variable positions (Fig. 3). For the 250 variable positions, 52 changes (21%) are in the first codon position, 25 (10%) in the second, and 173 (69%) in the third. Transitions, especially between C and T, outweigh transversions; biases ≥10:1 are common among the more similar genomes.

To test for saturation of base substitutions (i.e., multiple hits), transitions and transversions at each codon position were plotted against maximum-likelihood estimates of relative divergence (Fig. 4; F. Villablanca, W. K. Thomas, and A. C. Wilson, submitted). These divergence estimates are analogous but not equivalent to sequence divergence and take into account deviations from even base composition, differences in substitution rate among codon positions, and any bias toward transitions (Felsenstein, 1990). Values for these parameters derived from the data are 2:1:7 changes at the 1st:2nd:3rd codon positions and a 10:1 transition/tranversion ratio among similar sequences. Nonlinearity in the plots indicates increasing saturation of substitutions for that class of characters.

The plots (Fig. 4) indicate that increases in first and third codon position transitions are nonlinear in comparison to relative divergence estimates. This nonlinearity, indicative of saturation effects, is particularly obvious for comparisons between *Ensatina*

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TABLE 1. Corrected (Brown et al., 1982) estimates of sequence divergence among samples of *Ensatina* and the outgroups. The two samples of *picta* are identical over the 681 bp analyzed and are represented once here. Localities are shown in Figure 1 and defined in the Appendix.

	xanthoptica			*oregonensis*						*platensis*			
	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *xan* SR	—												
2. *xan* Av	1.8	—											
3. *xan* Or	2.6	1.6	—										
4. *oreg* OR	13.9	13.0	12.0	—									
5. *oreg* In	12.9	12.2	11.1	8.5	—								
6. *oreg* WA	14.7	13.8	12.9	1.3	9.4	—							
7. *oreg* Br	16.2	15.2	14.4	13.5	14.3	14.5	—						
8. *oreg* Me	14.3	12.8	12.6	11.0	11.9	11.8	3.6	—					
9. *oreg* He	14.3	13.6	12.4	5.5	10.4	5.9	14.4	11.5	—				
10. *plat* Bl	12.9	11.6	10.7	8.9	10.4	9.7	12.1	10.5	8.8	—			
11. *plat* Ar	15.6	14.2	13.3	8.4	10.9	9.2	14.8	13.2	11.1	3.8	—		
12. *plat* Ha	14.5	13.5	12.8	11.5	11.0	12.4	16.0	13.7	12.6	12.6	14.0	—	
13. *plat* Ma	14.0	12.8	12.4	11.5	10.3	12.5	15.6	13.2	12.5	12.2	14.0	2.4	—
14. *croc* Te	13.2	12.4	12.0	10.6	9.2	11.1	16.2	14.0	11.8	11.6	12.8	7.1	6.8
15. *croc* Ve	12.8	12.0	11.6	10.1	8.5	10.6	14.7	13.1	11.1	10.9	12.1	7.7	7.3
16. *klau* Cr	13.7	13.2	12.3	9.7	10.3	9.9	15.7	13.9	11.8	10.7	11.2	11.1	10.7
17. *klau* CW1	15.1	14.4	13.2	10.7	11.2	11.0	16.5	14.6	12.6	11.6	10.8	12.1	11.6
18. *klau* CW2	14.8	14.1	12.8	10.3	10.7	10.5	15.9	13.9	11.9	11.1	11.5	11.6	10.9
19. *klau* SJS	13.6	13.2	12.3	10.0	10.4	10.3	15.5	13.8	11.8	10.8	11.1	11.5	11.0
20. *esch* SBa	7.3	6.5	5.7	10.8	10.2	11.4	13.7	12.0	10.9	10.4	12.0	11.4	10.9
21. *esch* SBe	6.9	6.2	5.6	10.5	9.2	11.1	14.2	12.5	10.3	8.8	10.8	10.9	10.5
22. *esch* CW	6.5	5.8	5.3	9.9	8.9	10.5	13.2	11.7	9.6	8.4	10.7	10.3	10.2
23. *picta*	12.8	11.5	11.4	7.7	9.0	8.3	15.0	12.5	10.1	9.2	9.4	12.4	11.6
24. *Plethodon*	39.7	37.8	38.0	33.4	38.5	34.4	39.2	37.4	34.2	32.7	34.4	34.3	35.2
25. *Aneides*	36.9	34.9	35.3	34.0	34.7	32.9	35.4	34.9	31.5	33.7	37.1	40.1	38.9

and the outgroups. The high level of first-base transitions appears to be due to the large proportion (11.2%) of leucines in the sequence. For this base, C ↔ T transitions at the first position are silent. The other classes of characters (first-base transversions, second-base transitions and transversions, and third-base tranversions) appear to increase linearly with the maximum-likelihood distance estimate.

Phylogenetic Analysis: Estimation of Relationships

Because of the evidence for saturation of first and third codon position transitions among the more divergent sequences, we estimated the most-parsimonious tree in two steps. First, the obviously saturated classes of information were excluded to estimate the root and the branching pattern at the base of the tree. To do this, we used a 0:1 step matrix to discount transitions at the first and third codon positions (Swofford and Olsen, 1990). Second, the shortest topology obtained was used to constrain the basal branching order in an analysis using all classes of characters, but with a 10:1 step matrix to weight transversions (the more "conservative" substitutions) over transitions. This two-step approach minimizes noise at the base of the tree due to character homoplasy while using all of the information to resolve relationships among more similar sequences (Swofford and Olsen, 1990).

The analysis excluding first- and third-base transitions produced a single shortest tree of 163 steps (Fig. 5b). Sequences from the northern populations of *platensis* are basal, followed by southwestern *oregonensis* (Branscomb/Navarro), then northern *oregonensis* and *picta*, and then a group consisting of one *oregonensis* (Ingot) and the other subspecies. The number of unambiguous changes defining the basal branches is small, particularly for those involving

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TABLE 1. Extended.

croceater		*klauberi*				*eschscholtzii*			*picta*	*Plethodon*	*Aneides*
14	15	16	17	18	19	20	21	22	23	24	25
—											
1.2	—										
9.7	10.2	—									
10.7	11.4	2.4	—								
10.2	10.7	1.9	0.8	—							
10.1	10.5	0.6	2.2	1.5	—						
10.7	10.0	10.9	11.8	11.3	11.1	—					
10.0	9.5	10.6	11.4	11.0	10.6	2.7	—				
9.3	8.9	9.5	10.3	9.9	9.5	2.4	1.0	—			
11.3	10.3	10.0	10.9	10.5	10.2	10.7	9.6	9.0	—		
36.3	37.5	35.4	35.5	34.9	34.8	35.8	38.0	36.9	36.1	—	
34.8	34.3	36.5	38.1	37.4	36.7	34.4	34.7	33.6	35.2	32.0	—

oregonensis (Fig. 5b). Exchanging positions among basal taxa (*oregonensis* and northern *platensis*) results in trees one to three steps longer.

In the second step of the analysis, which uses all classes of characters, the first three branches of the ingroup were constrained as described above. The single shortest tree (Fig. 5a) had 1,839 steps, counting each transversion as 10 steps. The only substantial difference between this and the "basal" tree (Fig. 5b) is in the location of *oregonensis* from Ingot. Relationships within clades are more clearly resolved, with evidence for monophyly of *eschscholtzii*, clearer resolution within *klauberi*, and the exclusion of *picta* from the clade consisting of north-coastal *oregonensis*. The same topology is obtained using the same constraint without differential weighting of transversions and transitions.

Using no constraints results in two shortest trees of 1,839 steps. One is the same as the single shortest tree above (Fig. 5a). The alternative topology (Fig. 5c) defines the same major clades, except that components of *oregonensis* (and *picta*) are differently located. We regard the topology in Figure 5a as a better estimate of phylogeny because the branching order of basal mtDNAs is not compromised by classes of information that can have high homoplasy (saturation) among such highly divergent sequences.

Aside from instability in the branching order of the *oregonensis* groups, most clades are strongly supported by a large number of unambiguous character states, have high repeatability in bootstrap replications, and are consistent among analyses (Fig. 5).

The major features of the "best-estimate" tree (Fig. 5a) are (1) monophyly of mtDNAs from each of the subspecies *xanthoptica*, *eschscholtzii*, *klauberi*, *croceater*, and *picta*, (2) identification of a monophyletic southern coastal clade (*xanthoptica* and *eschscholtzii*)

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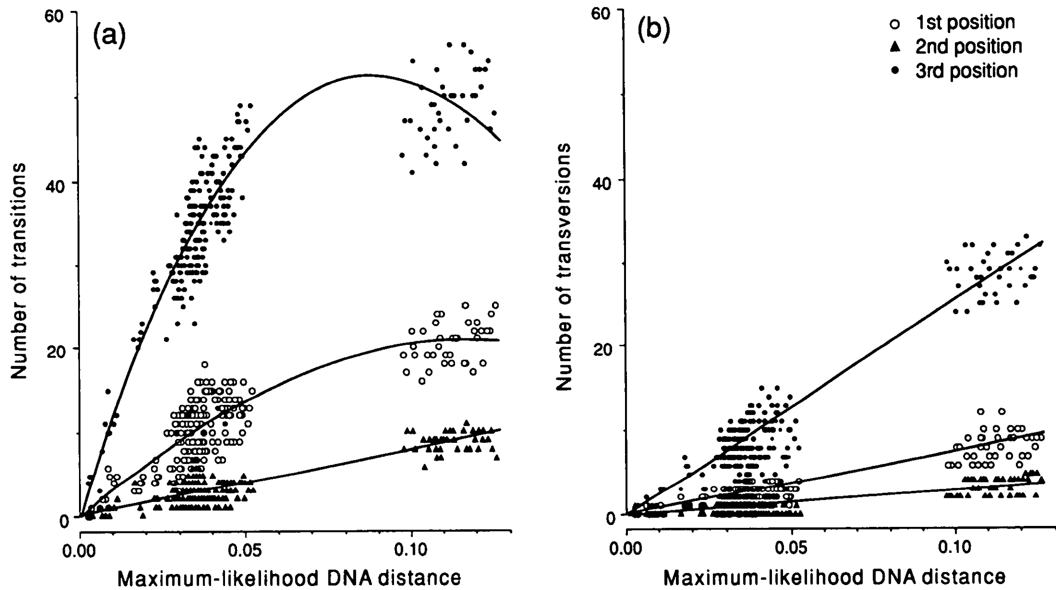

FIGURE 4. Plots of the numbers of transitions (a) and transversions (b) at the first, second, and third codon positions against maximum-likelihood indices of corrected DNA divergence (ML option in DNADIST, PHYLIP). Linear regressions are shown for all but first- and third-base transitions. For these, second-order polynomial regressions are shown. Partial F statistics for second-order polynomials (with degrees of freedom corrected for nonindependence) are $F_{20} = 4.12$, $0.1 > P > 0.05$, for first-position transitions and $F_{20} = 26.914$, $P < 0.001$, for third-position transitions. All other P values are >0.1, indicating that a linear model is more appropriate for transitions at the second codon position and transversions at all positions. The separate clouds of points for large divergences are the comparisons between *Ensatina* and the outgroups.

and a southern Sierran clade (*klauberi*, *croceater*, and southern *platensis*), and (3) paraphyly of mtDNAs from both *platensis* and *oregonensis* (see Fig. 6).

Phylogenetic Analysis: Tests of A Priori Models

An alternative way to use the data is to test whether they are consistent with a priori predictions based on competing evolutionary hypotheses. If the ring is actually a montage of secondary contacts among strongly differentiated subspecies, then each of the subspecies should, barring introgression and assuming isolation longer than $4N_f$ generations, be monophyletic for mtDNA. To test this prediction, we searched for the shortest trees satisfying the constraint that the mtDNAs from each subspecies are monophyletic. The relationships among subspecies are free to vary. The single shortest tree produced under this constraint (Fig. 7a) has 1,918 steps, which is 79 steps longer than the best-estimate phylogeny (Fig. 5a). The best-estimate phylogeny requires fewer steps for 33 characters, whereas the monophyly model is more parsimonious for 10 characters. Using a winning sites test (Prager and Wilson, 1988), we can reject the monophyly model ($\chi^2 = 12.3$, $P < 0.001$).

To test the scenario proposed by Stebbins (1949), a simple model based on independent and stepwise colonization of the Sierra Nevada and the southern Coastal ranges from appropriately located ancestral populations of *oregonensis* was constructed (Fig. 7b). This model assumes that populations were established in a gradual stepping-stone manner, with each propagule bearing derived states from its source population. This model requires 1,913 steps, 74 steps more than the minimum-length trees. Whereas the shorter tree is more parsimonious for 24 characters, the colonization model provides a better explanation for 14 characters. On this basis the model cannot be rejected ($\chi^2 = 2.63$, $0.10 > P >$

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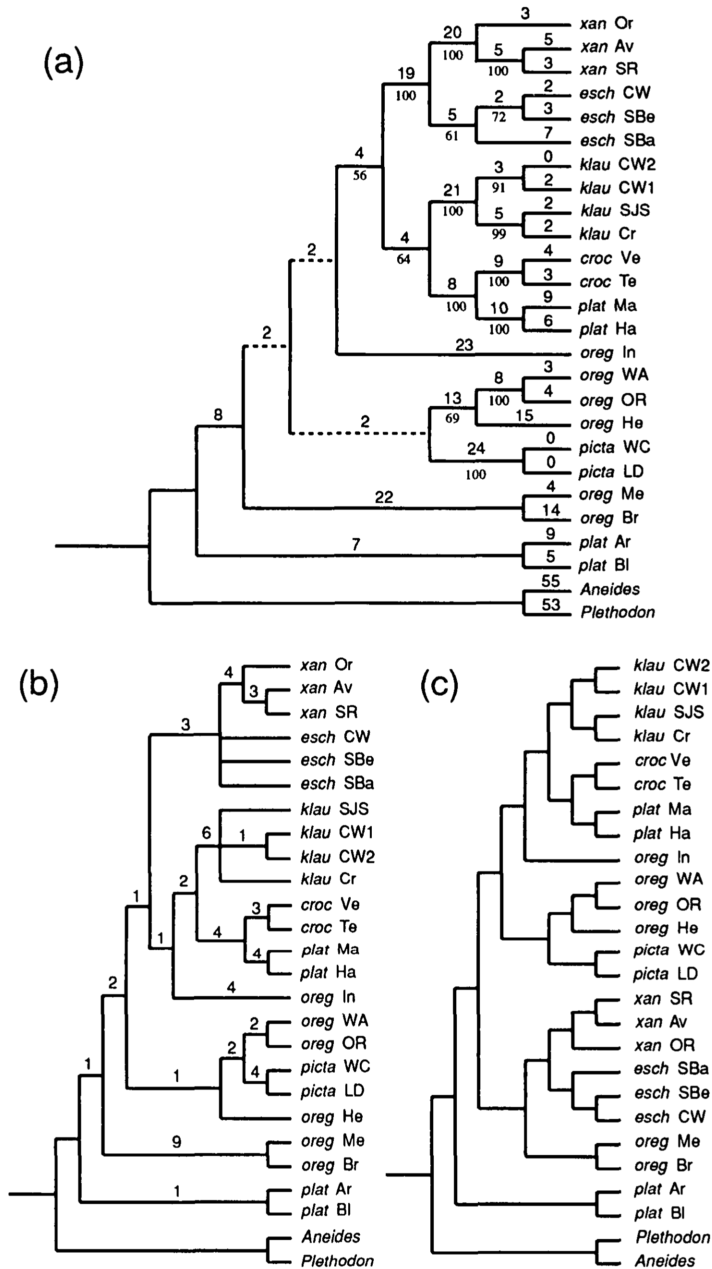

FIGURE 5. Minimum-length phylogenetic trees for mtDNAs from *Ensatina*. Abbreviations follow the Appendix. (a) "Best-estimate" tree based on all classes of characters, with the three most basal branches of the ingroup constrained and a 10:1 step matrix weighting transversions over transitions. The number above each branch is the number of unambiguous characters defining the branch. The number below is the proportion of 100 bootstrap replicates (with 10:1 step matrix applied) in which the clade to the right was present. Dashed lines indicate poorly resolved branches. (b) Shortest tree obtained using step matrices to exclude first- and third-base transitions to resolve the branching pattern at the base of the tree. The number above each branch is the number of unambiguous characters defining the branch. (c) Alternative equally parsimonious topology obtained from an analysis of all classes of characters with a 10:1 step matrix but no constraint on the order of basal branches.

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0.05), although the difference approaches statistical significance.

A closer fit between the data and the colonization model proposed by Stebbins is achieved by allowing mtDNAs from southern *platensis* and *croceater* to be monophyletic, leaving the other details the same (Fig. 7c). This model requires 1,879 steps, 40 steps more than the shortest tree, and is reasonably consistent with the data (16 vs. 13 sites; $\chi^2 = 0.31$, $P > 0.10$).

Phylogenetic Analysis: Evaluation of Sampling Effects

Except for two individuals of *klauberi* from Camp Wolahi, the above analyses are based on one individual per locality. To test whether a single individual is representative or whether sampling of multiple individuals is likely to reveal divergent mtDNAs from the same locality, restriction enzymes were used to screen multiple samples of amplified DNA (using primers 15–18; Fig. 2) from three populations of *oregonensis* from northern California, the geographic region with the highest mtDNA diversity. Scanning the available sequences (Fig. 3) revealed that digestion with *Hin*fI and *Msp*I can diagnose each of the major mtDNA lineages identified in this region (Table 2). Each of the six restriction sites screened included one or more phylogenetically informative nucleotide positions. These sites are uniform for each of the within-population samples (In, Me, Salyer [Trinity Co., CA]; Table 2), suggesting that the bulk of the variation is distributed among rather than within populations. This hypothesis is consistent with similarity of sequences from some geographically proximal populations (cf. Table 1 and Fig. 1). Thus, a sequence from a single individual is apparently representative for a population of these salamanders, at least for the purposes of broad-scale biogeographic and phylogenetic comparisons.

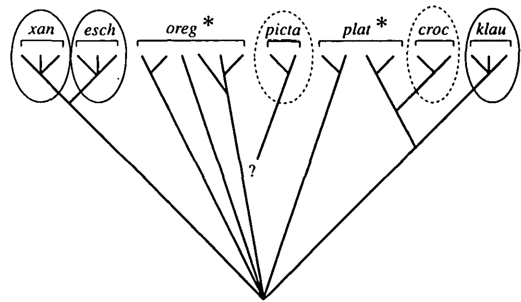

FIGURE 6. Simplified interpretation of the mtDNA phylogeny illustrating the subspecies with monophyletic mtDNAs (circled) and those with potentially or demonstrably paraphyletic mtDNA (*). mtDNAs from *croceater* and *picta* are enclosed by dashed lines to indicate the samples were taken from small geographic areas.

DISCUSSION

Biogeographic Implications

Stebbins (1949) proposed that Sierran and south-coastal populations of *Ensatina* were separately derived from northern populations, perhaps from those in the redwood and Douglas fir forests of northern California and southwestern Oregon. This prediction was largely based on the observations that these populations have the most generalized color pattern and the highest population densities. Stebbins regarded *picta* as a close approximation of the ancestral state.

The evidence from mtDNA sequences substantiates three critical components of Stebbins's (1949) biogeographic model and, therefore, of his evolutionary scenario for *Ensatina*. The first component is that populations from northern California were ancestral to the Sierran and south-coastal radiations. Populations from the top of the ring show the highest regional diversity in mtDNAs; these sequences are consistently basal in the phylogenetic analyses. In the best-estimate phylogeny (Fig. 5a), mtDNAs from *oregonensis* are paraphyletic with respect to other *Ensatina*. The very instability of the mtDNA relationships among the three groups of *oregonensis* and other *Ensatina* is to be expected if *oregonensis* was ancestral, with extant lineages having mainly primitive or uniquely derived character states. These mtDNAs are each separated from others by long branches that are expected to complicate phylogenetic analyses (Felsenstein, 1978; Swofford and Olsen, 1990). Increasing the number of

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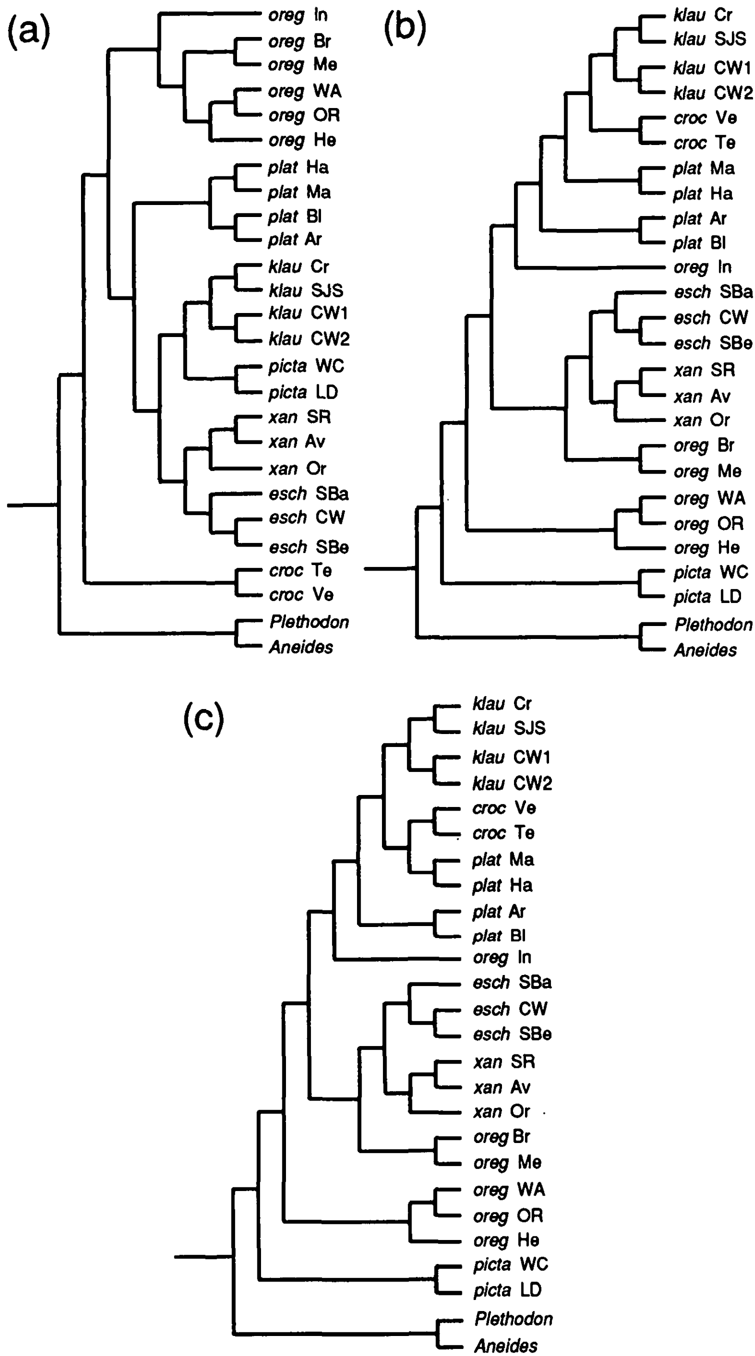

FIGURE 7. Shortest trees consistent with (a) all subspecies being monophyletic, (b) a simple model of stepwise colonization down each side of the central valley of California, and (c) a modification of the colonization model in which southern *platensis* populations (*plat* Ma, *plat* Ha) were the sister group to samples of *croceater*. The phylogeny shown in (c) was the most consistent with the data. Abbreviations follow the Appendix.

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TABLE 2. Distribution of restriction sites for *HinfI* and *MspI* within the 681-bp segment of cytochrome *b* from northern Californian *Ensatina*. Localities are shown in Figure 1 and defined in the Appendix.

		Restriction site[b]					
		HinfI				*MspI*	
Sample	Type[a]	98	457	473	614	125	593
Among populations							
picta	A	1	0	1	0	0	0
oreg He	B	1	1	0	0	1	1
oreg Br/Me	C	1	0	0	0	1	0
oreg In	D	1	0	0	1	1	1
plat Bl	E	1	0	0	0	0	1
plat Ar	F	1	0	0	0	1	1
Within populations							
oreg In ($n = 4$)	D	1	0	0	1	1	1
oreg Me ($n = 12$)	C	1	0	0	0	1	0
oreg Salyer ($n = 12$)	F	1	0	0	0	1	1

[a] Scanning the sequences (Fig. 3) for *HinfI* and *MspI* sites revealed that each of the major lineages could be diagnosed by digestion with these two enzymes (types A–F). Digestion profiles of multiple individuals from each of three localities revealed one type per locality.

[b] Restriction sites are numbered from the first base of the sequences (Fig. 3).

samples and the length of each sequence failed to improve resolution at the base of the tree. However, the mtDNA from *picta*, thought to be ancestral by Stebbins (1949), is unremarkable. It simply appears as one among many basal and strongly differentiated lineages in the area.

The second component supported by the mtDNA evidence is that the blotched salamanders of the inland ranges and the unblotched coastal forms represent independent radiations. As expected, mtDNAs from the coastal subspecies, *xanthoptica* and *eschscholtzii*, form a strongly supported monophyletic group; *croceater*, *klauberi*, and southern *platensis* also form a monophyletic group, but it is less well supported. This observation precludes two alternative biogeographic scenarios that are not compatible with the ring-species concept. One is that *xanthoptica* could be derived from the inland races, having spread from east to west to make recent secondary contact with coastal forms of *Ensatina*. The other is that *klauberi* could be derived from southern *eschscholtzii*.

However, the simplest biogeographic scenario, which assumes stepwise colonization down each side of the valley (Fig. 7b), is not well supported (although a minor modification of it [Fig. 7c] is). This discrepancy is largely due to the diversity of mtDNA within *platensis*. Two very distinct mtDNA lineages were found: one in northern *platensis* and the other in the southern samples. The former was consistently basal in the phylogenetic analyses, whereas the latter was highly derived and more closely related to *croceater* than to the northern clade.

One hypothesis to explain the dichotomy of mtDNA within *platensis* is that the northern and southern groups stem from two divergent lineages of mtDNA independently derived from southern *oregonensis*. One lineage has persisted and diversified during the colonization of the southern Sierras. The other is (as yet) found only as far south as the central Sierra Nevada. This interpretation requires lineage sorting (Avise et al., 1984; Neigel and Avise, 1986) but with unusually high levels of mtDNA differentiation within the ancestor. This is precisely the situation in extant populations of *oregonensis* from northern California. This hypothesis predicts that further sampling of *oregonensis* will reveal mtDNAs that are closely related to the divergent lineages found within *platensis*.

The third essential component of Stebbin's (1949) model was that diversification

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within *Ensatina* was gradual, as suggested by the increasing levels of isolation between blotched and unblotched salamanders from north to south, but there was no indication of the time involved. The large divergences among *Ensatina* mtDNAs, as well as the large intraspecific genetic distances for allozymes (Wake and Yanev, 1986), are consistent with a long evolutionary history. The great diversity of mtDNA within *oregonensis* (estimated divergences up to 14.5%; Table 1) suggests that this is an ancient group. The levels of mtDNA differentiation of *croceater* + southern *platensis* from *klauberi* (10.2–11.6%; Table 1) and of *eschscholtzii* from *xanthoptica* (5.8–7.3%; Table 1) suggest that each of the southern radiations has been diverging over a long period. However, the low divergence of both mtDNA and allozymes (Wake and Yanev, 1986) among geographically distant samples of *eschscholtzii* and between coastal and inland populations of *xanthoptica* suggests that the spread of these subspecies has been relatively recent. Thus, both of the well-documented secondary contacts within *Ensatina*, i.e., between *xanthoptica* and *platensis* and between *eschscholtzii* and *klauberi* (reviewed in Wake et al., 1989), may be relatively recent.

The general support from the mtDNA evidence for the biogeographic scenario proposed by Stebbins (1949) substantiates *Ensatina* as a ring species and thus as an exemplar of gradual microevolutionary divergence leading toward speciation. The mtDNA evidence confirms that *klauberi* and *eschscholtzii* are the end points of two independently evolving lineages that are now genetically and in some places (Brown, 1974; Wake et al., 1986) reproductively isolated.

Implications for Systematics: mtDNA and Species Boundaries

The number and distribution of species in *Ensatina* has long been contentious (Stebbins, 1949); recent discussion has focused on the implications of different species concepts (e.g., Frost and Hillis, 1990). The observed monophyly of mtDNAs from some subspecies, notably *klauberi*, could be taken as support for the recognition as independent evolutionary lineages, i.e., species, under some definitions. However, because of differences in the behavior of organellar and nuclear genes, the use of organellar genes alone to identify species boundaries can lead to errors.

Because of its clonal and maternal inheritance, mtDNA has a lower effective population size and can be strongly differentiated geographically, whereas nuclear genes cannot (Birky et al., 1983, 1989). This discrepancy will be exaggerated where females disperse less often than males, as appears to be the case in *Ensatina* (Stebbins, 1954; Wake, unpubl. data). Thus, in old, geographically subdivided taxa, phylogenetic analysis could reveal geographically discrete monophyletic groups of mtDNAs (i.e., phylogeographic category 1 of Avise et al., 1987). However, such an analysis cannot identify species borders because putative taxa may be united by nuclear gene flow. We have uncovered such cases in *Ensatina*, e.g., among populations of *oregonensis* along a transect in northern California and within *platensis* in the central Sierra Nevada (Jackman and Wake, in prep.). The opposite situation, where otherwise discrete taxa are paraphyletic for mtDNA, is well known (e.g., Ferris et al., 1983; Whittemore and Schall, 1991) and appears to be the case for *platensis* and *oregonensis*, which were regarded by Stebbins (1954) as morphologically separable but are shown here to be paraphyletic for mtDNA. The use of mtDNA patterns without corroboration from other evidence would lead to incorrect conclusions about both genetic isolation and cohesion within *Ensatina*. Generally, we caution against using mtDNA patterns as the sole criterion for determining species boundaries.

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APPENDIX

Abbreviations, collecting localities, and MVZ catalog numbers for subspecies of *Ensatina eschscholtzii* from which mtDNA was sequenced (see Fig. 1): **1:** *oreg* WA: Granite Falls, Snohomish Co., Washington, MVZ 168659; **2:** *oreg* OR, Wolf Creek Rd., Lane Co., Oregon, MVZ 168668; **3:** *picta* LD: Low Divide Rd., Del Norte Co., California, MVZ 172513; **4:** *picta* WC: Wilson Creek Rd., Del Norte Co., California, MVZ 168709; **5:** *oreg* He: Helena, Trinity Co., California, MVZ (S)10938; **6:** *oreg* Me: Navarro, Mendocino Co., California, MVZ 194896; **7:** *oreg* Br: Branscomb Rd., Mendocino Co., California, MVZ 194708; **8:** *oreg* In: Ingot, Shasta Co., California, MVZ 182000; **9:** *xan* SR: Santa Rosa, Sonoma Co., California, MVZ 205681; **10:** *xan* Or: Orinda, Contra Costa Co., California, MVZ 163850; **11:** *esch* SBa: Zaca Creek, Santa Barbara Co., California, MVZ 167654; **12:** *esch* SBe: Millard Canyon, San Bernardino Mtns., Riverside Co., California, MVZ 181460; **13:** *esch* CW: Alpine (MVZ 178729) and *klau* CW1 and *klau* CW2: Camp Wolahi (MVZ 191684, MVZ 194908), San Diego Co., California; **14:** *plat* Bl: Blodgett, El Dorado Co., California, MVZ 172459; **15:** *plat* Ar: Arnold, Calaveras Co., California, MVZ 158006; **16:** *xan* Av: Avery, Calaveras Co., California, MVZ 202316; **17:** *plat* Ma: Gooseberry Flat Rd., Madera Co., California, MVZ 169033; **18:** *plat* Ha: Hartland, Tulare Co., California, MVZ 169165; **19:** *croc* Te: Tehachapi Mtn. Park, Kern Co., California, MVZ 202330; **20:** *croc* Ve: Alamo-Little Mutau Creek, Ventura Co., California, MVZ 195607; **21:** *klau* Cr: Crystal Creek, San Bernardino Co., California, MVZ 185823; **22:** *klau* SJS: Santa Rosa Mountain Rd., Riverside Co., California, MVZ 185844.

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