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THE POLYTYPIC SPECIES REVISITED: GENETIC DIFFERENTIATION AND MOLECULAR PHYLOGENETICS OF THE TIGER SALAMANDER *AMBYSTOMA TIGRINUM* (AMPHIBIA: CAUDATA) COMPLEX

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Abstract.—We present a phylogenetic analysis of the *Ambystoma tigrinum* complex, based on approximately 840 base pairs of mitochondrial-DNA sequence from the rapidly evolving D-loop and an adjacent intron. Our samples include populations of the continentally distributed species, *A. tigrinum,* plus all described species of Mexican ambystomatids. Sequence divergence is low, ranging from 0–8.5%, and most phylogenetic groupings are weakly supported statistically. We identified eight reasonably well-defined clades from the United States and Mexico, with the geographically isolated *A. californiense* from California as the probable sister group to the remaining taxa. Our sequence data are not capable of resolving the relationships among these clades, although the pattern of transitional-site evolution suggests that these eight lineages diverged during a period of rapid cladogenesis. We roughly calibrate a molecular clock and identify a few lineages that significantly deviate from the slow, baseline rate of 0.5–0.75% per million years. Our data also suggest that species boundaries for several U.S. and Mexican species need to be altered and that the concept of a continentally distributed, polytypic tiger salamander is not valid.

Key words.—*Ambystoma,* axolotl, biogeography, D-loop, mitochondrial DNA, phylogeny, tiger salamander.

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As evolutionary biologists continue to recognize the importance of comparative and phylogenetic approaches to the study of speciation, there has been a virtual explosion of research using DNA sequences to reconstruct intra- and interspecific phylogenies. The rationale for such work is obvious enough: collect data from neutral or nearly neutral molecular markers, and use this information to reconstruct robust, well-supported phylogenies of the populations, species, or higher groups of interest. Armed with these historical hypotheses of relationships, one is often in a reasonable position to draw inferences about topics ranging from biogeography and patterns of colonization, to rates of gene flow, to speciation, and subsequent character evolution (Avise 1994).

Perhaps nowhere in evolutionary biology is this general phylogenetic framework for studying evolutionary mechanisms more important than in analyses at the interface between intra- and interspecific differentiation (Coyne and Orr 1989; Lynch 1989; Tilley et al. 1990). Here, the questions are often formulated in terms of mechanisms of speciation: Are sister taxa sympatric or allopatric? What are the rates of accumulation of pre- and postmating isolating mechanisms? How precise is the correlation of known events from the geological past with lineage divergences? Answering these and related questions depends on well-resolved phylogenetic hypotheses. And yet, these are often situations where gene flow may still be occurring, levels of divergence are slight, and character-based confidence levels (either from likelihood or bootstrap methods) are insignificant.

How should one proceed in such cases? Here we explore these issues in the context of a large analysis of mitochondrial DNA (mtDNA) D-loop sequence divergence within and among members of the North American tiger salamander (*Ambystoma tigrinum*) complex. Among the Amphibia, the tiger salamander and its close relatives comprise one of the most widely distributed, polytypic species complexes known. Across their continental range, these animals are found in virtually all habitats except the extreme deserts, the Sierra Nevada, and the Appalachian Mountains. Because of their tremendous variation in color pattern, life history, and ecology, tiger salamanders and their close relatives (including the laboratory axolotl, *A. mexicanum*) are commonly exploited model systems in developmental, genetic, and evolutionary studies (Gehlbach 1967; Collins et al. 1980; Shaffer 1983, 1984a,b, 1993; Jones and Collins 1992; Routman 1993). Yet, without a synthetic analysis of the entire complex, it is difficult to place this work into a more general evolutionary framework. For example, *A. tigrinum* is currently recognized as the only continentally distributed, polytypic amphibian species in North America (Stebbins 1985; Conant and Collins 1991). However, its boundaries and monophyly have never been rigorously examined, and several recent authors have questioned the interpretation of a single, continentally distributed species of tiger salamander (Collins et al. 1980; Shaffer 1983, 1993). Similarly, several of the Mexican members of the complex are well-known examples of heterochrony via incomplete metamorphosis (Gould 1977; Shaffer 1984a,b, 1993), but their relationships to the transforming tiger salamanders of Mexico and the United States remain incompletely understood.

We have two goals here. First, we report the results of a survey of mitochondrial DNA (mtDNA) sequences from the entire tiger salamander complex, including virtually all of the named species and subspecies. Our analysis includes sequence data for 77 populations for about 600 base pairs (bp) of D-loop and 240 bp of an intronlike mtDNA insert that is apparently unique to ambystomatid salamanders (McKnight and Shaffer, unpubl. data). We chose the D-loop because it evolves rapidly, providing insights into recent evolutionary events (Moritz et al. 1987, Hoelzel et al. 1991). Second, we

examine our results in light of what we can, and cannot, say from a molecular analysis where shallow divergences and resultingly weak character support lead to low bootstrap *P*-values and relatively incomplete phylogenetic resolution of the included populations and species. Our goal is to attempt to extract useful evolutionary information from such a data set, and explore potential lines of interpretation that might apply to other, phylogenetically "uninformative" studies.

Current Taxonomic Status of the *Ambystoma tigrinum* Complex

The tiger salamander complex, as recognized here, includes two components. The tiger salamander, *Ambystoma tigrinum,* comprises a single species with 5–7 parapatrically or allopatrically distributed subspecies (Gehlbach 1967; Jones et al. 1988; Shaffer 1993). It is broadly distributed from southern Canada, throughout the continental United States (Gehlbach 1967; Stebbins 1985; Conant and Collins 1991). The remainder of the *A. tigrinum* complex consists of about 13 species from Mexico (Shaffer 1984a,b, 1993; Brandon 1989). These species have previously been placed in four genera, although current evidence indicates that they should all be contained within *Ambystoma* (Brandon 1989; Shaffer 1993; Reilly and Brandon 1994).

The monophyly of the tiger salamander complex has been supported by studies of both morphological (Tihen 1958; Krogh and Tanner 1972; Kraus 1988) and allozyme (Shaffer et al. 1991) characters. These analyses identify either the California tiger salamander, *A. californiense* (Kraus 1988; Shaffer et al. 1991), or one of the high-altitude populations from northwestern or central Mexico (Shaffer 1984a, 1993) as the probable sister group to the remaining members of the tiger salamander complex.

The recognition of *A. tigrinum* as a single species by most authors implies that it is monophyletic relative to the other forms, although this has never been explicitly tested. Since Dunn's (1940) overview, all authors have recognized at least five subspecies within *Ambystoma tigrinum* (Fig. 1). *Ambystoma t. tigrinum* is widely, but unevenly, distributed in the eastern United States. In the grasslands of the central United States, three subspecies are parapatrically distributed, with *A. t. melanostictum* in the northern plains, *A. t. mavortium* in the south, and *A. t. diaboli* in an narrow band of North Dakota and southern Manitoba. *Ambystoma t. nebulosum* is distributed throughout the Rocky Mountains, from southeastern Idaho to Arizona. *Ambystoma t. stebbinsi* from the San Rafael Valley in southern Arizona is sometimes recognized as a distinct taxon (Jones et al. 1988) or as a local population of *A. t. nebulosum* (Gehlbach 1965, 1967). Finally, *A. californiense* (found in California's Central Valley and inner Coast Range, Barry and Shaffer 1994) and *A. velasci* (found throughout the highlands of northern and central Mexico, Shaffer 1984a,b; Brandon 1989) is recognized either as a separate species or as a subspecies of *A. tigrinum,* depending on the authority.

The remaining 15 species of ambystomatid salamanders from Mexico are considered "closely allied" with the tiger salamander (Tihen 1958; Shaffer 1984a, 1993; Brandon 1989; Routman 1993). Shaffer (1984a,b) analyzed allozyme and morphological patterns of diversity in the Mexican taxa, and hypothesized that speciation was primarily a function of allopatric differentiation determined by the geology and hydrology of central Mexico. Both molecular clock calibrations (based on Nei's D) and geological-age estimates of the Mexican Neovolcanic Axis are consistent with an initial divergence of no more than 10 million years for the Mexican taxa, and many species are apparently much more recently derived (Shaffer 1984a).

Materials and Methods

Specimens

Because mtDNA variation is often distributed primarily among, rather than within, populations (Avise et al. 1987; Moritz et al. 1992; Routman 1993; McKnight 1995), we generally sampled one (occasionally two) individuals per population. This allowed us to maximize the number of populations we could analyze (Fig. 1; Appendix). We sampled all named Mexican ambystomatids except for *Ambystoma* (*Rhyacosiredon*) *zempoalensis* and *leorae.* Most of the Mexican taxa are known only from their type localities, and all specimens were collected from those localities. For the two widely distributed Mexican taxa (*A. velasci, A. rosaceum*), we included several samples representing their geographic range, plus divergent lineages based on previous allozyme studies (Shaffer 1983, 1984a). We also included two individuals each of *A. andersoni, A. mexicanum, A. taylori,* and *A. velasci* (from El Vergel, Chihuahua) to study within-locality variation. We included multiple populations of all subspecies that occur in the United States, except *A. t. stebbinsi* (which was unavailable and thus excluded from this study). A list of all localities is presented in the Appendix.

Sequencing

The SDS-Proteinase K/Phenol-Chloroform extraction procedure (Kocher et al. 1989), was used to extract total genomic DNA from frozen tissue samples. We constructed amplification and sequencing primers (Fig. 2) from the DNA sequence of a mtDNA clone of *A. t. tigrinum* provided to us by E. Routman. We performed symmetric and asymmetric Taq DNA polymerase mediated amplifications in 50-μl volumes, following standard procedures (Kocher et al. 1989), except we gel purified the symmetric products on 1% standard high-melting-point agarose gels, and used a 50:1 excess-to-limiting primer ratio for the asymmetric amplifications. Sequencing of the single-stranded DNA resulting from asymmetric amplifications followed instructions from a commercial kit (Sequenase, United States Biochemical). We sequenced both strands within the D-loop to confirm the sequence from the complementary strand; sequence from the 240 bp insert was not confirmed in this way. DNA sequences were recorded, inverted, and tested for conformity using the GeneJockey sequence processor (Biosoft, Cambridge, England), and aligned by eye.

Analysis

The large size of our data set placed constraints on our possible analyses. The neighbor-joining algorithm (Saitou

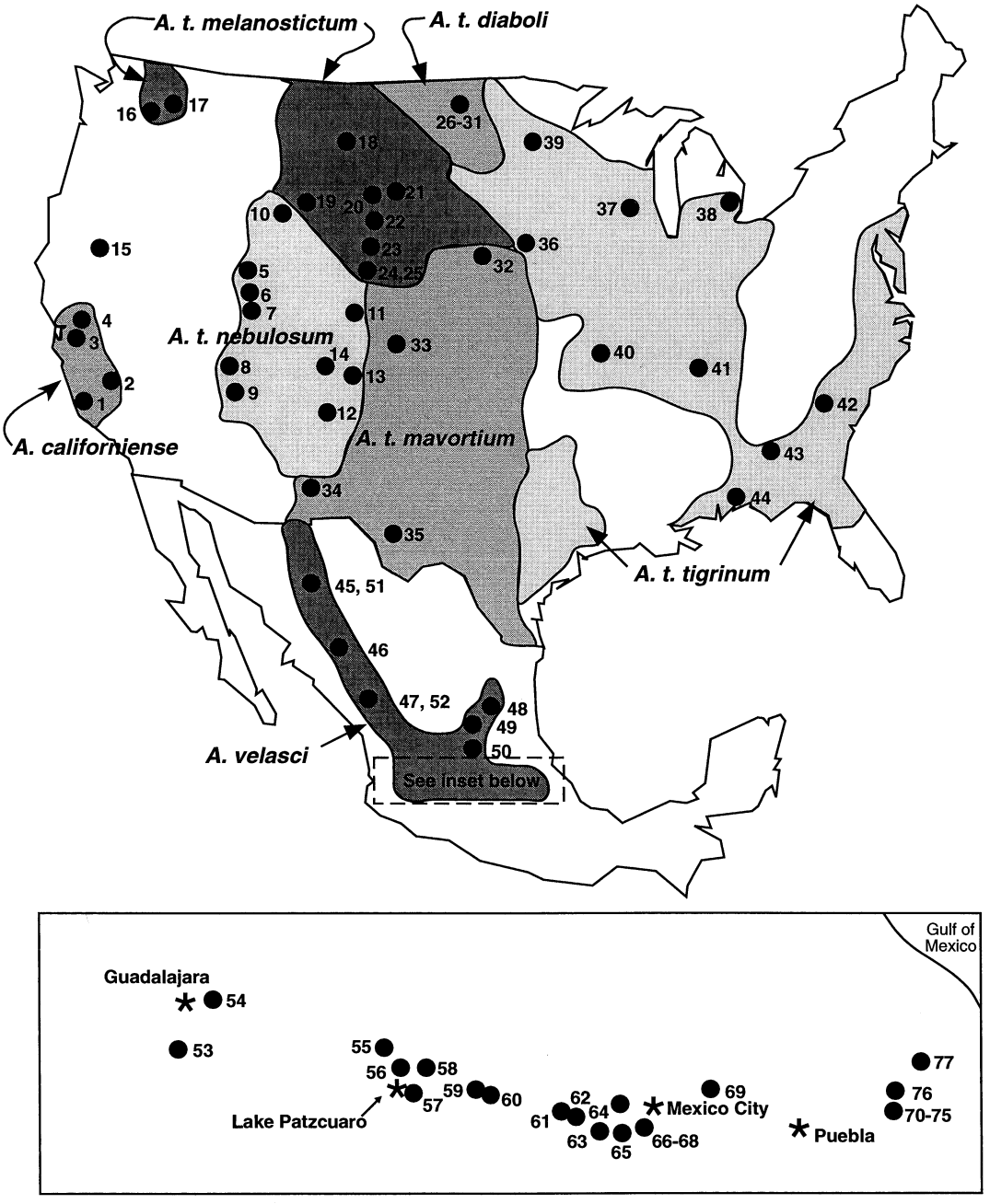
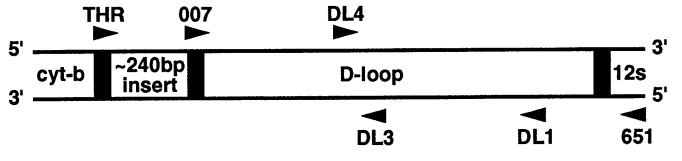


FIG. 1. Collecting localities for *Ambystoma tigrinum* complex samples from the United States and Mexico. Shaded regions show the approximate distributions of the five subspecies in the United States, plus *A. californiense* and *A. velasci.* Stars (insert map) show approximate locations of major cities and Lake Patzcuaro in central Mexico. Sample numbers correspond to those in the Appendix.

and Nei 1987) allowed us to analyze our complete data set and search for major groupings. For parsimony analyses of the entire data set (using *A. californiense* as an outgroup), we used PAUP (version 3.1.1; Swofford 1993) but were restricted to heuristic search strategies. To help identify the most parsimonious set of trees, we used 300 replicates of the random-taxon-addition prodecure, retaining the first 50 trees from each replicate and examined the consensus of the resulting 1450 equally parsimonious trees. To test for statistical significance of the accuracy of our trees (sensu Hillis and Bull 1993), we used bootstrap *P*-values (Felsenstein 1985; Felsenstein and Kishino 1993). We also examined tree-length distributions (and associated g_1 skewness statistics; Hillis 1991) to quantify the phylogenetic information of various subsets of our data.

In evaluating these analyses, we take the consensus of parsimony and neighbor joining as an indication that a grouping is not critically dependent on the model of phylogeny reconstruction; in that sense, it provides support for a group even if bootstrap values are low. Many groups are present in 100% of the equally parsimonious trees in our analysis, yet have low bootstrap support, indicating that a few characters



THR = 5' A A A C A T C G A T C T T G T A A G T C 3'

007 = 5' G C A C C C A A A G C C A A A A T T C T T G 3'

DL4 = 5' G C C A C T G G T T A A A A T C T A T G 3'

DL3 = 5' T T C G A T C C A A T T G A T G A A T G 3'

DL1 = 5' A A T A T T G A T A A T T C A A G C T C C G 3'

651 = 5' G T A A G A T T A G G A C C A A A T C T 3'

FIG. 2. Relative positions and sequences of amplification and sequencing primers. Black bands in the figure represent the transfer-RNA coding regions for the amino acids Threonine, Proline, and Phenylalanine (left to right). Primers THR, 007, and 651 were modified from the primers L15026, L16007, and H00651 respectively, of Kocher et al. (1989), using sequence data from a cloned segment of mtDNA from *Ambystoma tigrinum tigrinum*. The other primers were designed from sequences of several individual *Ambystoma*. Symmetric amplifications utilized primers THR and 651. Single-stranded DNA was produced for both the "heavy" and "light" strands, using the primers 007 and DL1. Only the light strand of the insert region was examined by asymmetric amplifications with THR and DL3.

unambiguously support a node. When the two analyses differ in group recognition (as is the case in many of the basal groupings), there were invariably low bootstrap values in the areas of conflict.

RESULTS

Patterns of mtDNA Sequence Divergence

We obtained approximately 842 bp of sequence from 83 individual salamanders. Overall levels of variation are low to moderate (Fig. 3), ranging from about 8.5% to 0 (Table 1). The greatest sequence divergence (5–8.5%) is between *Ambystoma californiense* and all other taxa, reflecting its probable sister-group relationship to the rest of the *A. tigrinum* complex. Among the remaining members of the complex, pairwise sequence divergences vary from over 7% to 0. The greatest divergences involve *A. rosaceum, A. velasci* (El Vergel), *A. t. tigrinum* (eastern United States), and *A. t. nebulosum* from south-central Colorado. In northwestern Mexico, two *A. rosaceum* samples (populations 51, 52) differ by 5.76% from each other, and by 3.61–7.13% from all others (excluding *A. californiense*). In this same geographic region, the two *A. velasci* sequences from El Vergel, Chihuahua (population 46) differ from all others by 2.30–5.76% divergence (excluding *A. californiense* and *A. rosaceum*). These large values result in a basal placement of both *A. rosaceum* and El Vergel *A. velasci* in the neighbor-joining analysis (Fig. 4). A previously unrecognized lineage of *A. t. nebulosum* from south-central Colorado (populations 13, 14) differs from all remaining samples (excluding the previous three) by 3.55–4.95%. Sequence divergences are also relatively large among certain components of the populations from the Mexican Plateau (populations 53–77) and between the eastern U.S. *A. t. tigrinum* (populations 37–44) and all others. The Mexican sequences apparently represent a complex group of populations in which geographically nearby populations may be very divergent (Figs. 4–5). For example, two *A. ordinarium* (populations 59, 60) from the Transverse Volcanic Range in the state of Michoacan are separated by about 20 km of continuous habitat, yet differ from each other by 3.61% sequence divergence. The non-transforming paedomorph *A. dumerilii* (population 56) differs from a transforming *A. velasci* population (population 57) a few kilometers away by 2.43%, whereas two transforming samples from the same region, and also separated by a few kilometers, are virtually identical (populations 57, 58; 0.13% divergent).

Although none of these divergence levels are great, they are relatively large compared with the low levels of differentiation found over much of the central United States, Rocky Mountains, and central Mexican Plateau (Table 1). In both the Rocky Mountains (populations 5–12) and Great Plains (populations 15–36), sequence divergence is very low, rarely exceeding 1%, even over hundreds of kilometers of habitat.

Monophyletic Groups

We summarize phylogenetic relationships and major monophyletic groupings using both distance (Fig. 4) and parsimony (Figs. 5–6) approaches. As is often the case for noncoding DNA sequence data, the two analyses provide similar insights to well-supported groups (Cracraft and Helm-Bychowski 1991), although they differ with respect to the interrelationships among these units. Bootstrap *P*-values were often low for both methods, even though many groups were present in 100% of the 1450 trees retained in our parsimony searching strategy (Fig. 5), and tree-skewness analyses (Hillis 1991) revealed significant phylogenetic signal at both deep and shallow levels. Even considering a liberal interpretation of 70% bootstrap *P*-values as representing a "real" alpha level of 0.05 (Hillis and Bull 1993), our results contain little unambiguous phylogenetic information. However, all nodes supported by 70% or greater bootstrap values in the neighbor-joining analysis are similarly well-supported under parsimony (the single exception is for populations 57, 58, with 78% under neighbor joining and 57% under parsimony). Thus, when a grouping is well-supported, the method of analysis appears relatively unimportant.

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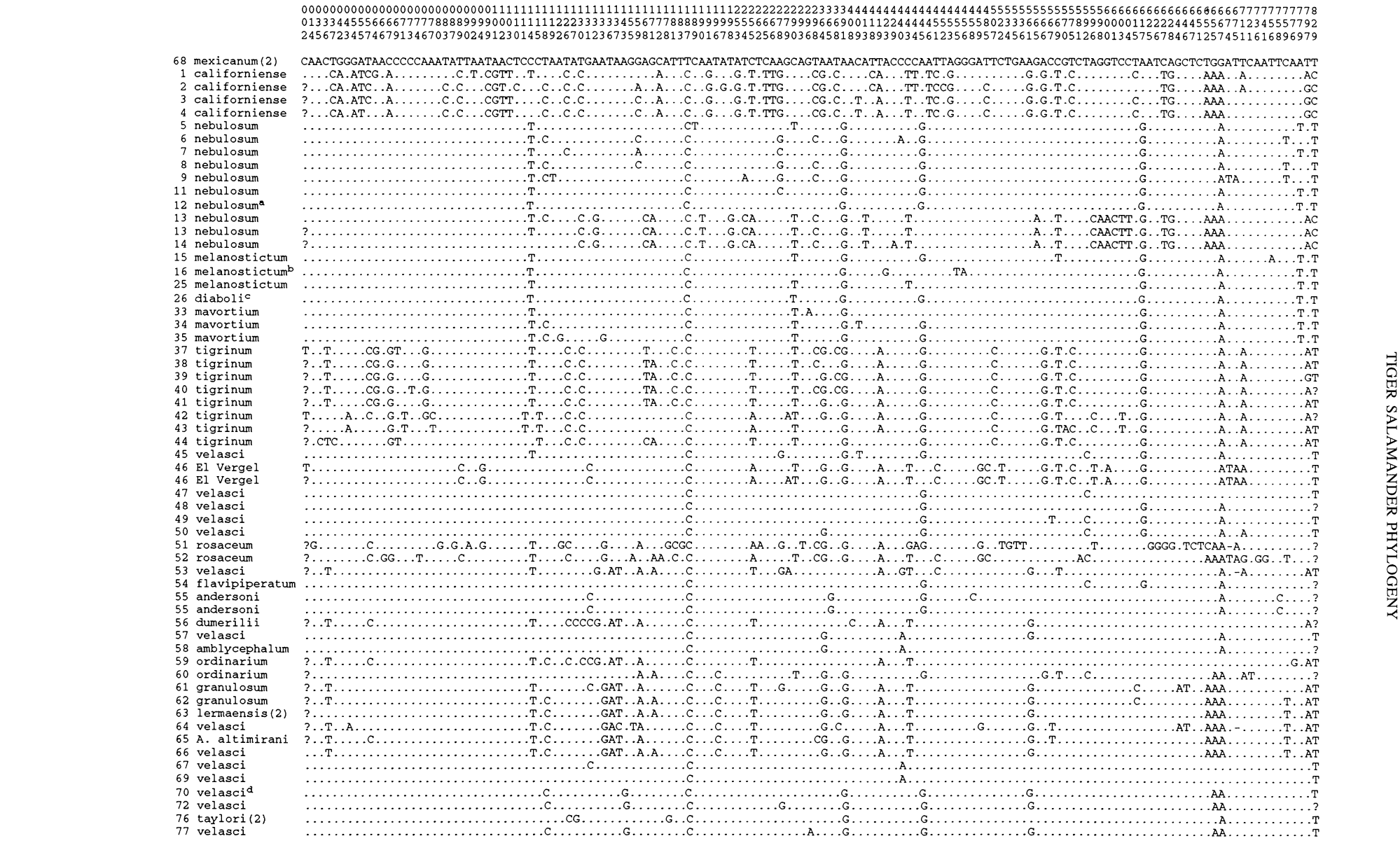
FIG. 3. Mitochondrial-DNA sequence variation from the last 26 bp of the Threonine tRNA through the 240 bp insert, the entire Proline tRNA and 503 bp of the D-loop. All 60 different sequences are compared to a wild caught *Ambystoma mexicanum*. Identical duplicate samples from the same population are identified by (2); more complex identities are described with footnotes. Sequence locations of variable sites are shown in the columns; all sites not shown are invariant. Population numbers (beginning of each row) are mapped in figure 1 and described in the Appendix.



[a] this sequence was also found in melanostictum from populations 23 and 24, diaboli from populations 30 and 31, mavortium from population 32, and tigrinum from population 36.

[b] this sequence was also found in the other sample of melanostictum from Washington, population 17.

[c] this sequence was also found in nebulosum from population 10, melanostictum from populations 18-22, and diaboli from populations 27-29.

[d] this sequence was also found in velasci from populations 71, 73-75.

TABLE 1. Matrix of percentage of sequence divergence calculated by the method of Jukes and Cantor (1969). Population numbers are the same as in figs. 1 and 2.

	A. californiense				*A. t. nebulosum*										*A. t. melanostictum*			*dia.*
	1	2	3	4	5	6	7	8	9	11	12	13a	13b	14	15	16	25	26
2	1.66																	
3	1.53	1.27																
4	1.66	1.40	0.13															
5	6.44	6.99	6.58	6.44														
6	6.30	6.44	5.89	5.76	1.14													
7	6.17	6.44	6.17	6.03	0.63	1.01												
8	6.17	6.30	5.76	5.62	1.01	0.13	0.89											
9	6.30	6.58	6.17	6.03	1.14	0.51	1.14	0.38										
11	6.17	6.72	6.30	6.17	0.38	0.89	0.25	0.76	0.89									
12	6.17	6.72	6.30	6.17	0.25	0.89	0.38	0.76	0.89	0.13								
13a	6.85	7.27	6.85	6.72	3.61	3.74	4.01	3.61	3.61	3.74	3.61							
13b	6.72	7.41	6.99	6.85	3.48	3.88	3.88	3.74	3.74	3.61	3.48	0.13						
14	6.99	7.41	6.99	6.85	3.74	4.14	4.14	4.01	4.01	3.88	3.74	0.38	0.25					
15	6.30	6.85	6.44	6.30	0.38	1.27	0.76	1.14	1.27	0.51	0.38	3.48	3.35	3.61				
16	6.44	6.99	6.58	6.44	0.76	1.40	0.89	1.27	1.40	0.63	0.51	3.88	3.74	4.01	0.89			
25	6.03	6.58	6.17	6.03	0.38	1.27	0.76	1.14	1.27	0.51	0.38	3.22	3.09	3.35	0.51	0.63		
26	6.30	6.85	6.44	6.30	0.13	1.01	0.51	0.89	1.01	0.25	0.13	3.48	3.35	3.61	0.25	0.63	0.25	
33	6.30	6.85	6.44	6.30	0.38	1.27	0.76	1.14	1.27	0.51	0.38	3.48	3.35	3.61	0.51	0.63	0.25	0.25
34	6.58	6.85	6.17	6.03	0.38	1.01	0.76	0.89	1.01	0.51	0.38	3.48	3.61	3.88	0.51	0.89	0.51	0.25
35	6.72	6.99	6.58	6.44	0.51	1.14	0.89	1.01	1.14	0.63	0.51	3.61	3.74	4.01	0.63	1.01	0.63	0.38
37	5.08	6.03	5.89	6.03	2.95	3.35	3.09	3.22	3.35	3.09	2.95	4.81	4.68	4.95	2.82	3.48	3.09	2.82
38	5.35	6.30	6.17	6.30	2.69	3.09	2.82	2.95	3.09	2.82	2.69	4.28	4.14	4.41	2.56	3.22	2.82	2.56
39	5.35	6.03	5.89	6.03	2.82	3.48	2.95	3.35	3.48	2.95	2.82	4.81	4.68	4.95	2.69	3.35	2.95	2.69
40	5.21	6.17	6.03	6.17	3.09	3.48	3.22	3.35	3.48	3.22	3.09	4.68	4.54	4.81	2.95	3.61	3.22	2.95
41	5.35	6.30	6.17	6.30	2.69	3.35	2.82	3.22	3.35	2.82	2.69	4.54	4.41	4.68	2.56	3.22	2.82	2.56
42	6.44	6.85	6.72	6.85	3.09	3.74	3.22	3.61	3.74	3.22	3.09	4.68	4.54	4.54	2.95	3.61	3.22	2.95
43	6.44	6.85	6.72	6.58	2.82	3.48	2.95	3.35	3.48	2.95	2.82	4.41	4.28	4.28	2.69	3.35	2.95	2.69
44	6.03	6.44	6.30	6.17	2.56	3.22	2.69	3.09	3.22	2.69	2.56	4.28	4.14	4.14	2.43	3.09	2.69	2.43
45	6.17	6.72	6.03	5.89	0.76	0.89	0.76	0.76	0.89	0.51	0.51	3.88	3.74	4.01	0.89	1.01	0.89	0.63
46a	5.89	5.89	5.76	5.62	2.69	3.35	3.09	3.22	3.35	2.82	2.69	4.41	4.28	4.28	2.56	2.95	2.30	2.56
46b	6.03	6.03	5.89	5.76	2.82	3.48	3.22	3.35	3.48	2.95	2.82	4.54	4.41	4.41	2.69	3.09	2.43	2.69
47	6.17	6.44	6.03	5.89	1.01	1.40	1.14	1.27	1.40	0.89	0.76	4.14	4.01	4.01	1.14	1.27	1.14	0.89
48	6.03	6.30	5.89	5.76	0.63	1.01	0.76	0.89	1.01	0.51	0.38	3.74	3.61	3.61	0.76	0.89	0.76	0.51
49	6.30	6.58	6.17	6.03	0.89	1.27	1.01	1.14	1.27	0.76	0.63	4.01	3.88	3.88	1.01	1.14	1.01	0.76
50	5.89	6.17	6.03	5.89	1.01	1.40	1.14	1.27	1.40	0.89	0.76	4.14	4.01	4.01	1.14	1.27	1.14	0.89
51	8.11	8.39	8.25	8.39	5.35	5.08	4.81	4.95	5.21	5.08	5.08	6.99	6.85	7.13	5.49	5.62	5.35	5.21
52	6.17	6.44	6.17	6.30	4.01	4.14	4.14	4.01	4.14	4.14	4.01	5.35	5.21	5.49	4.14	4.28	3.61	3.88
53	5.49	5.89	5.89	5.76	2.95	2.82	2.69	2.82	3.09	2.69	2.69	4.41	4.28	4.54	2.82	2.95	2.56	2.82
54	6.17	6.44	6.03	5.89	0.76	1.14	0.89	1.01	1.14	0.63	0.51	3.88	3.74	3.74	0.89	1.01	0.89	0.63
55a	6.30	6.58	6.17	6.03	1.14	1.53	1.27	1.40	1.53	1.01	0.89	4.28	4.14	4.14	1.27	1.40	1.27	1.01
55b	6.44	6.72	6.30	6.17	1.27	1.66	1.40	1.53	1.66	1.14	1.01	4.41	4.28	4.28	1.40	1.53	1.40	1.14
56	5.76	6.17	5.89	6.03	2.95	3.22	2.56	3.09	3.35	2.82	2.69	4.68	4.54	4.81	3.09	2.95	2.56	2.82
57	5.62	5.89	5.49	5.35	1.27	1.40	1.40	1.53	1.66	1.14	1.01	4.14	4.01	4.01	1.40	1.27	1.14	1.14
58	5.76	6.03	5.62	5.49	1.14	1.27	1.27	1.40	1.53	1.01	0.89	4.01	3.88	3.88	1.27	1.14	1.01	1.01
59	5.89	6.03	5.76	5.89	2.82	2.82	2.43	2.69	2.95	2.69	2.56	4.28	4.41	4.68	2.95	2.82	2.43	2.69
60	6.17	6.17	6.17	6.03	1.78	2.30	1.91	2.17	2.43	1.91	1.78	4.14	4.01	4.01	1.66	2.30	1.91	1.66
61	5.62	6.30	5.76	5.62	3.09	3.09	2.82	2.95	3.22	2.82	2.82	4.81	4.68	4.95	3.22	3.09	2.69	2.95
62	5.76	6.17	5.62	5.49	2.95	2.69	2.82	2.56	2.82	2.82	2.69	4.14	4.28	4.54	3.09	2.95	2.56	2.82
63	5.76	5.89	5.62	5.49	2.69	2.43	2.56	2.30	2.56	2.56	2.43	3.88	4.01	4.28	2.82	2.69	2.30	2.56
64	6.30	6.44	6.17	6.03	3.74	3.48	3.61	3.35	3.61	3.61	3.48	5.21	5.35	5.62	3.88	3.74	3.35	3.61
65	5.62	5.76	5.49	5.62	3.09	2.56	2.95	2.43	2.69	2.95	2.82	4.28	4.41	4.68	3.22	3.09	2.69	2.95
66	5.89	6.03	5.76	5.62	2.82	2.56	2.69	2.43	2.69	2.69	2.56	4.01	4.14	4.41	2.95	2.82	2.43	2.69
67	6.17	6.44	6.03	5.89	1.27	1.40	1.40	1.53	1.66	1.14	1.01	4.14	4.01	4.01	1.40	1.27	1.14	1.14
68	6.03	6.30	5.89	5.76	1.14	1.53	1.27	1.40	1.53	1.01	0.89	4.01	3.88	3.88	1.27	1.14	1.01	1.01
69	6.03	6.30	5.89	5.76	1.14	1.27	1.27	1.40	1.53	1.01	0.89	4.01	3.88	3.88	1.27	1.14	1.01	1.01
70	6.03	6.03	5.62	5.49	1.14	1.27	1.27	1.14	1.27	1.01	0.89	3.74	3.88	3.88	1.27	1.40	1.27	1.01
72	5.89	5.89	5.49	5.35	1.27	1.14	1.27	1.01	1.14	1.01	1.01	3.88	4.01	4.01	1.40	1.53	1.40	1.14
76	6.17	6.44	6.03	5.89	1.01	1.40	0.89	1.27	1.40	0.89	0.76	4.14	4.01	4.01	1.14	1.27	1.14	0.89
77	6.17	6.17	5.76	5.62	1.27	1.40	1.40	1.27	1.40	1.14	1.01	3.88	4.01	4.01	1.40	1.53	1.40	1.14

We tentatively recognize eight groups, at least seven of which are monophyletic. Based on their center of geographic distribution, we identify these seven as *A. californiense,* south-central Colorado, eastern United States, two lineages of *A. rosaceum, A. velasci* (El Vergel), and central and western Mexican Plateau. The large, remaining group, including the Rocky Mountains and Great Plains, Sierra Madre Oriental and Mexican Plateau, and eastern Mexican Plateau is more

TABLE 1. Extended.

A. t. mavortium			*A. t. tigrinum*								Mexican taxa									
33	34	35	37	38	39	40	41	42	43	44	45	46a	46b	47	48	49	50	51	52	53
0.51																				
0.63	0.38																			
3.09	3.09	3.22																		
2.82	2.82	2.95	0.51																	
2.95	2.95	3.09	0.51	0.51																
3.22	3.22	3.35	0.38	0.38	0.38															
2.82	2.82	2.95	0.51	0.25	0.25	0.38														
3.22	3.22	3.35	2.30	2.30	2.30	2.43	2.04													
2.95	2.95	3.09	2.56	2.30	2.56	2.69	2.30	0.89												
2.69	2.69	2.82	1.78	1.53	1.78	1.91	1.53	2.43	1.91											
0.89	0.63	1.01	3.22	2.95	3.09	3.35	2.95	3.35	3.09	2.82										
2.56	2.82	2.95	3.48	3.48	3.35	3.61	3.22	3.35	3.09	3.35	2.95									
2.69	2.95	3.09	3.61	3.61	3.48	3.74	3.35	3.22	3.22	3.48	3.09	0.13								
1.14	1.14	1.27	3.48	3.22	3.35	3.61	3.22	3.35	3.09	2.82	0.76	2.95	3.09							
0.76	0.76	0.89	3.09	2.82	2.95	3.22	2.82	2.95	2.69	2.43	0.63	2.56	2.69	0.38						
1.01	1.01	1.14	3.35	3.09	3.22	3.48	3.09	3.22	2.95	2.69	0.63	2.82	2.95	0.38	0.25					
1.14	1.14	1.27	2.95	2.95	2.82	3.09	2.69	2.82	2.82	2.56	0.76	2.43	2.56	0.51	0.38	0.38				
5.49	5.49	5.08	6.17	6.17	6.30	6.30	6.17	6.58	6.85	7.13	5.08	5.62	5.76	5.35	5.21	5.49	5.35			
3.88	4.14	4.01	4.41	4.14	4.28	4.28	4.14	4.95	5.21	5.08	4.01	3.74	3.88	4.01	4.14	4.14	3.88	5.76		
2.82	3.09	3.22	3.88	3.35	3.61	3.74	3.35	4.14	4.14	3.48	2.69	3.48	3.35	2.69	2.56	2.82	2.69	6.30	4.68	
0.89	0.89	1.01	3.22	2.95	3.09	3.35	2.95	3.09	2.82	2.56	0.51	2.69	2.82	0.25	0.13	0.13	0.25	5.35	4.01	2.69
1.27	1.27	1.40	3.61	3.35	3.48	3.74	3.35	3.48	3.22	2.95	1.14	2.82	2.95	0.63	0.51	0.76	0.89	5.49	4.41	2.82
1.40	1.40	1.53	3.74	3.48	3.61	3.88	3.48	3.61	3.35	3.09	1.27	2.95	3.09	0.76	0.63	0.89	1.01	5.62	4.54	2.95
2.82	3.09	3.22	3.61	3.35	3.61	3.74	3.35	4.14	4.14	3.74	2.95	4.01	4.14	2.43	2.56	2.82	2.95	6.03	4.81	1.78
1.14	1.40	1.53	3.48	3.48	3.35	3.61	3.22	3.35	3.35	3.09	1.27	2.69	2.82	0.76	0.63	0.89	0.76	5.35	4.01	2.30
1.01	1.27	1.40	3.35	3.35	3.22	3.48	3.09	3.22	3.22	2.95	1.14	2.56	2.69	0.63	0.51	0.76	0.63	5.21	3.88	2.43
2.69	2.69	2.82	3.48	3.22	3.48	3.61	3.22	4.01	4.01	3.61	2.82	3.88	4.01	2.30	2.43	2.69	2.82	5.89	4.68	1.91
1.91	1.91	2.04	3.22	2.95	2.82	3.09	2.69	3.09	3.09	2.56	1.78	2.69	2.82	1.53	1.66	1.66	1.27	5.62	3.88	2.95
2.95	3.22	3.09	4.28	4.28	4.28	4.41	4.01	4.81	4.81	4.41	2.82	3.88	4.01	3.09	2.95	3.22	3.09	5.21	4.41	2.17
2.82	2.82	2.69	4.14	3.88	3.88	4.01	3.61	4.68	4.68	4.01	2.95	4.01	4.14	2.95	2.82	3.09	2.95	5.62	3.74	2.04
2.56	2.56	2.43	4.14	3.88	3.88	4.01	3.61	4.41	4.41	4.01	2.69	3.74	3.88	2.69	2.56	2.82	2.69	5.35	3.48	2.04
3.61	3.61	3.48	4.68	4.95	4.68	4.81	4.68	5.21	5.21	5.08	3.74	4.54	4.68	3.48	3.35	3.35	3.48	5.89	4.41	2.95
2.95	2.95	2.82	3.74	3.74	4.01	3.88	3.74	4.54	4.81	4.41	3.09	4.14	4.28	3.09	2.95	2.95	3.09	5.21	3.61	2.43
2.69	2.69	2.56	4.01	3.74	3.74	3.88	3.48	4.54	4.54	3.88	2.82	3.88	4.01	2.82	2.69	2.95	2.82	5.49	3.61	1.91
1.14	1.40	1.53	3.74	3.48	3.61	3.88	3.48	3.61	3.35	3.09	1.27	2.69	2.82	0.51	0.63	0.89	1.01	5.62	4.28	2.56
1.01	1.27	1.40	3.61	3.35	3.48	3.74	3.35	3.48	3.22	2.95	1.14	2.82	2.95	0.38	0.51	0.76	0.89	5.49	4.14	2.56
1.01	1.27	1.40	3.61	3.35	3.48	3.74	3.35	3.48	3.22	2.95	1.14	2.82	2.95	0.38	0.51	0.76	0.89	5.49	4.14	2.43
1.27	1.01	1.14	3.61	3.35	3.48	3.74	3.35	3.48	3.22	2.95	1.14	3.09	3.22	0.89	0.76	1.01	1.14	5.21	4.14	2.82
1.40	1.14	1.27	3.74	3.48	3.61	3.88	3.48	3.61	3.35	3.09	1.01	3.22	3.35	1.01	0.89	1.14	1.27	5.08	4.28	2.69
1.14	1.14	1.27	3.22	2.95	3.09	3.35	2.95	3.09	2.82	2.56	1.01	2.95	3.09	0.76	0.63	0.89	1.01	4.81	4.01	2.95
1.14	1.14	1.27	3.74	3.48	3.61	3.88	3.48	3.61	3.35	3.09	1.27	3.22	3.35	1.01	0.89	1.14	1.27	5.35	4.28	2.95

problematic, because it is variously interpreted as a shallowly differentiated monophyletic (Fig. 4) or paraphyletic (Fig. 5) group. In addition to these well-supported nodes, both parsimony and neighbor-joining methods suggest several other large, often weakly differentiated sets of populations that may form monophyletic groups. In particular, both analyses indicate that the set of sequences from the Rocky Mountains and Great Plains (exclusive of populations 13 and 14 from

TABLE 1. Extended.

	Mexican taxa																			
	54	55a	55b	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	72	76
2																				
3																				
4																				
5																				
6																				
7																				
8																				
9																				
11																				
12																				
13a																				
13b																				
14																				
15																				
16																				
25																				
26																				
33																				
34																				
35																				
37																				
38																				
39																				
40																				
41																				
42																				
43																				
44																				
45																				
46a																				
46b																				
47																				
48																				
49																				
50																				
51																				
52																				
53																				
54																				
55a	0.63																			
55b	0.76	0.13																		
56	2.69	2.56	2.69																	
57	0.76	0.89	1.01	2.43																
58	0.63	0.76	0.89	2.56	0.13															
59	2.56	2.43	2.56	0.63	2.56	2.43														
60	1.53	1.91	2.04	3.74	1.78	1.66	3.61													
61	3.09	2.95	3.09	2.17	2.56	2.69	2.30	3.09												
62	2.95	3.09	3.22	2.30	2.43	2.56	2.17	2.69	0.89											
63	2.69	2.82	2.95	2.30	2.17	2.30	2.17	2.43	1.14	0.25										
64	3.48	3.61	3.74	2.95	2.95	3.09	2.82	3.74	1.53	1.40	1.40									
65	3.09	3.22	3.35	2.17	2.56	2.69	2.04	3.09	1.27	0.63	0.63	1.27								
66	2.82	2.95	3.09	2.17	2.30	2.43	2.04	2.56	1.01	0.13	0.13	1.27	0.51							
67	0.76	0.63	0.76	2.17	0.51	0.38	2.04	2.04	2.82	2.95	2.69	3.48	3.09	2.82						
68	0.63	0.76	0.89	2.30	0.63	0.51	2.17	1.91	2.95	2.82	2.56	3.35	2.95	2.69	0.38					
69	0.63	0.76	0.89	2.30	0.38	0.25	2.17	1.91	2.95	2.82	2.56	3.35	2.95	2.69	0.13	0.25				
70	0.89	1.01	1.14	2.82	0.89	1.01	2.69	1.66	2.69	2.30	2.04	3.09	2.43	2.17	1.14	1.01	1.01			
72	1.01	1.14	1.27	2.95	1.01	1.14	2.82	1.78	2.56	2.43	2.17	3.22	2.56	2.30	1.27	1.14	1.14	0.13		
76	0.76	0.89	1.01	2.56	1.01	0.89	2.56	1.78	3.09	2.95	2.69	3.74	3.09	2.82	1.01	0.89	0.89	0.89	1.01	
77	1.01	1.14	1.27	2.95	1.01	1.14	2.82	1.78	2.82	2.43	2.17	3.22	2.56	2.30	1.27	1.14	1.14	0.13	0.25	1.01

south-central Colorado), a group from northern and central Mexico (populations 47, 55, 57, 58, 67–69), and those from the eastern Mexican Plateau (populations 70–77, with 76 questionably included) are monophyletic groups, although bootstrap *P*-values are below 50% (Figs. 4–6).

Phylogenetic Relationships among Major Lineages

We tentatively recognize *A. californiense* as the sister group to the remaining members of the tiger salamander complex. This interpretation has previously been supported by allo-

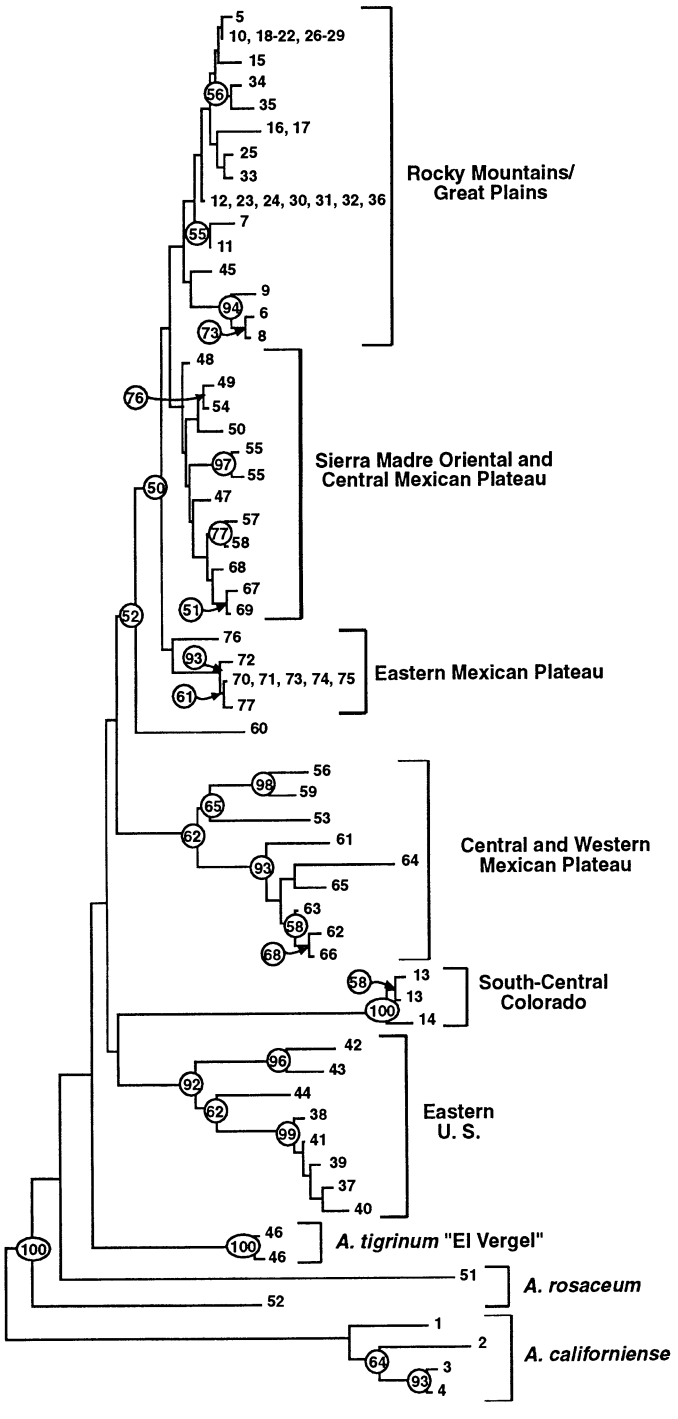


FIG. 4. Neighbor-joining tree (Saitou and Nei 1987) for all members of the *Ambystoma tigrinum* complex. Population numbers correspond to the appendix. Numbers in circles are bootstrap probability values (2000 replicates; Hedges 1992); unnumbered nodes were found in less than 50% of the bootstrap samples.

zyme (Shaffer et al. 1991), morphological (Kraus 1988), and cytochrome-*b* and 12s ribosomal-DNA sequence analyses (Shaffer and McKnight, unpubl. data) of the U.S. tiger salamanders, using the remaining North American members of the family Ambystomatidae as outgroups. The large levels of sequence divergence between *A. californiense* and all others are consistent with this interpretation (Fig. 3; Table 1), and the neighbor-joining tree (Fig. 4) recognizes this relationship, albeit with a very short, nonsignificant basal branch.

Relationships among the remaining seven major lineages are difficult to establish with this data set. Even using a 50% probability value, no resolution among most lineages is possible in either the neighbor joining or parsimony analyses. Both the neighbor joining (Fig. 4) and majority-rule parsimony (Fig. 5) analyses place the central and western Mexican Plateau, south-central Colorado, and *A. rosaceum* clades basal to the large group from the Rocky Mountains-Great Plains and the remaining Mexican populations. However, the two analyses differ drastically in the placement of the eastern United States and *A. velasci* El Vergel samples (compare Figs. 4 and 5).

Although we cannot accept with confidence any single hypothesis of relationship, it appears unlikely that the North American and Mexican lineages are each monophyletic. No analysis indicates that the North American tiger salamanders, which are generally placed in a single species, are each other's closest relatives. When the U.S. populations are constrained to be monophyletic, 11 characters support our most parsimonious tree over the hypothesis of U.S. monophyly, whereas only five characters preferentially support the alternative. Using a modification of the winning sites test (Prager and Wilson 1988; Edwards et al. 1991), we cannot statistically reject the monophyly of the U.S. *A. tigrinum* ($G = 2.306$, df = 1, $P > 0.05$; Zar 1974), although the strongest character support favors a polyphyletic interpretation of the U.S. assemblage. We tested a number of other alternative trees using the winning sites approach and could not reject any of the alternatives with these data.

DISCUSSION

In many ways, the strongest conclusion to come from this survey is the striking *lack* of differentiation among the 14 species of the tiger salamander complex. Even with the rapid rate of evolution of the D-loop, the very shallow levels of molecular divergence over vast geographical regions and across recognized species boundaries suggests that this is a recently derived complex. In comparison, the plethodontid salamander *Ensatina eschscholtzii* has recently been interpreted as a single, polytypic species with several morphologically divergent subspecies (Stebbins 1949; Wake and Yanev 1986). A survey of the protein-encoding cytochrome-*b* divergence among major *Ensatina* lineages (Moritz et al. 1992) found up to 15% divergence among populations, in contrast to 0–8.5% divergence in the more rapidly evolving D-loop across the entire tiger salamander complex. In this sense, the tiger salamanders appear more comparable to song sparrows (Zink and Dittmann 1993), freshwater turtles (Avise et al. 1992), and many other taxa that have been primarily influenced by the dynamic Pleistocene history of North America (Pielou 1991).

We now consider three areas of primary interest in this and many other analyses: phylogenetic and biogeographic patterns, rates and constancy of molecular evolution, and systematic implications. For each, we suggest interpretations

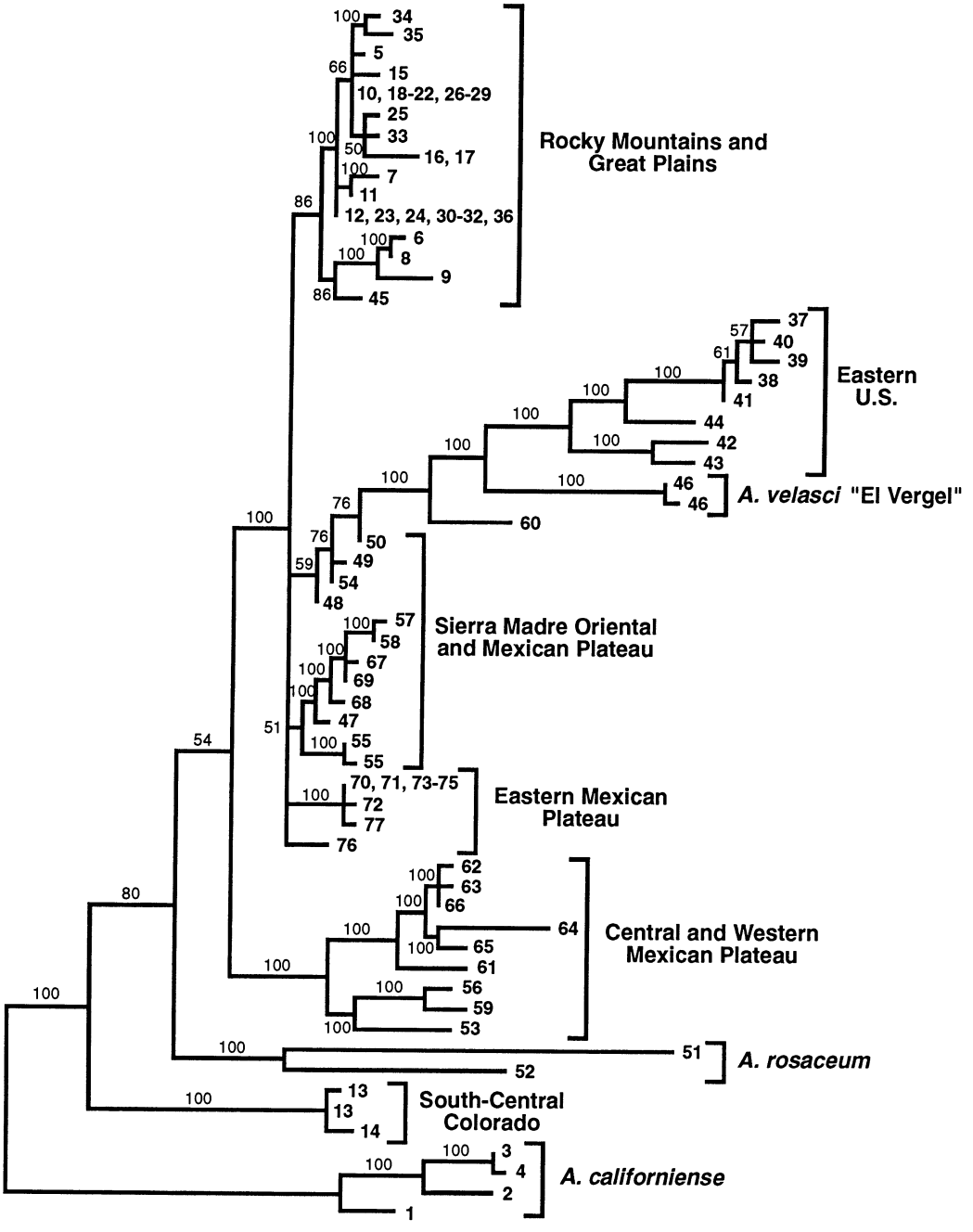


FIG. 5. Majority-rule consensus tree of 1450 equally parsimonious trees for all members of the *Ambystoma tigrinum* complex. Consistency index, 0.50; rescaled CI, 0.39; retention index, 0.79. Branch lengths as drawn are proportional to the accumulated number of evolutionary changes; numbers along branches show the percentage of the 1450 trees in which each group appeared. Population numbers correspond to the Appendix.

of the evolutionary history of the tiger salamander complex that are consistent with our data, although we recognize the limitations imposed by our frequently unresolved phylogenetic conclusions.

Phylogeny and Biogeography

Within its relatively shallow (and presumably recent) history, the tiger salamander complex shows a combination of divergent and very shallowly differentiated lineages. Even with the generally nonsignificant bootstrap *P*-values, the presence and distribution of these groups provides insights into the historical biogeography of the entire complex.

At the deepest levels of differentiation, we found eight primary lineages: *Ambystoma californiense,* south-central Colorado, eastern United States, two lineages of *A. rosaceum, A. velasci* (El Vergel), central and western Mexican Plateau, and the large group including the Rocky Mountains and Great Plains, Sierra Madre Oriental and Mexican Plateau, and east-

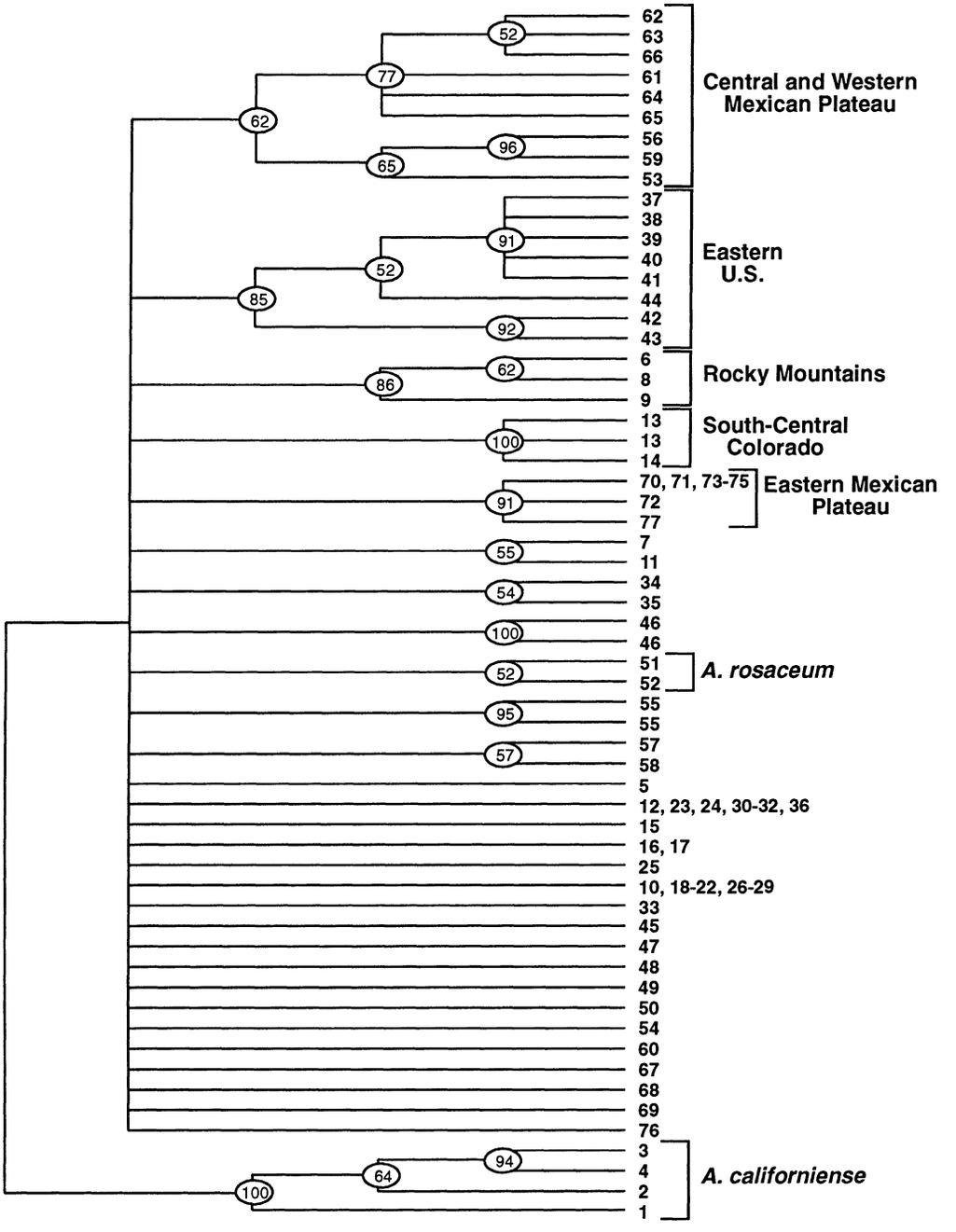


FIG. 6. PAUP majority-rule consensus tree, based on 1000 bootstrap replicates. The number within the circle at a node refers to the bootstrap *P*-value. Nodes for which the *P* -value was less than 50% are collapsed. Population numbers correspond to the Appendix.

ern Mexican Plateau (Figs. 4–5). To examine biogeographic hypotheses concerning the primary diversification of the entire complex requires an understanding of how these major lineages are related. To pursue this in greater detail, we chose a single representative of each group and analyzed this very reduced data set under parsimony, using two different strategies. First, we examined the full sequence, including all variable sites (Fig. 7a). Second, we considered transversions only, because they evolve more slowly than transitions, and might be more informative at relatively deep nodes (Fig. 7b). In both cases, we found a striking lack of resolution: multiple maximum parsimony trees, virtually no consensus among them, and an overall consensus of a single-star phylogeny with no resolution.

A key question remains: Does the "starburst" phylogeny, as reconstructed here, reflect a real pattern, or is it simply an artifact of insufficient data or a DNA sequence that evolves at an inappropriate rate? If the real pattern was a nearly simultaneous set of speciation events and geographic expansion of some lineages, then an unresolved (and probably unresolvable) series of nearly equally divergent lineages with short internal nodes and no phylogenetic signal should result

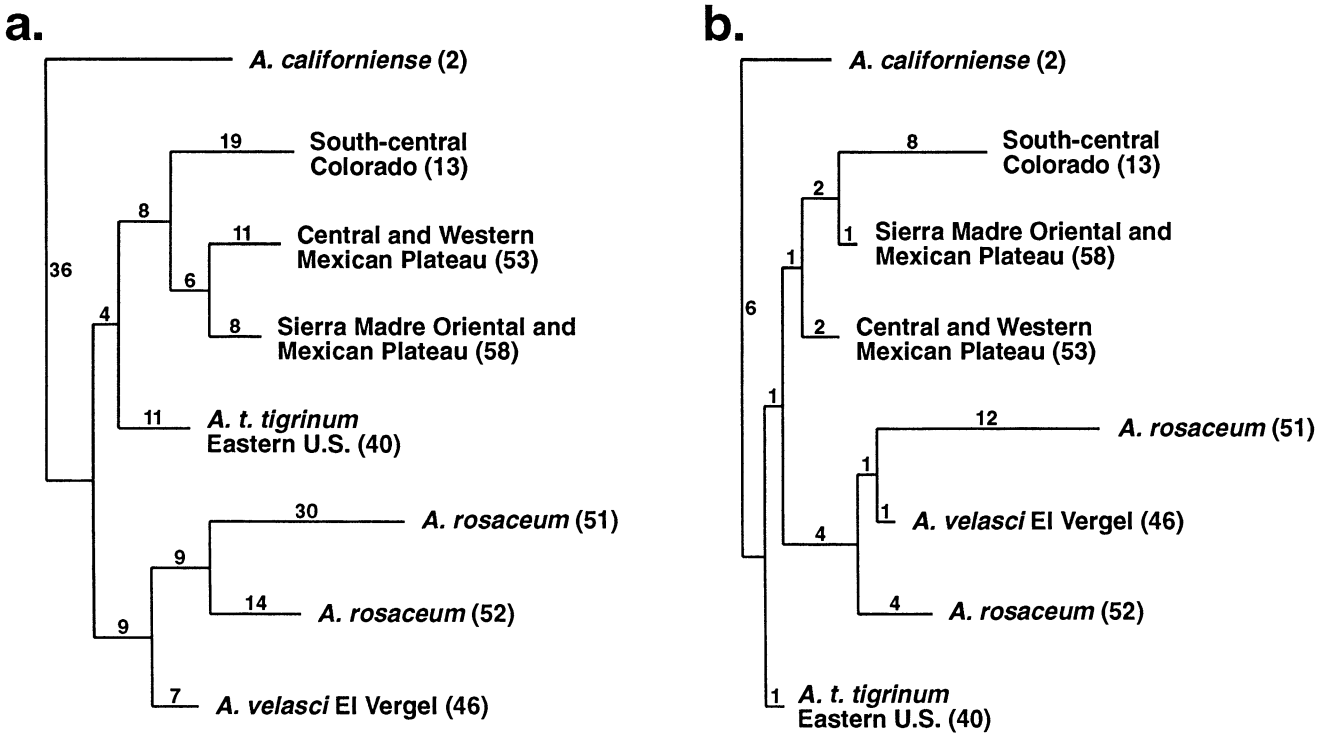


FIG. 7. PAUP trees for representatives of the eight major lineages of the tiger salamander complex (population 58 represents the Sierra Madre Oriental-eastern Mexican Plateau and Rocky Mountain-Great Plains group). (a.) Tree based on all data. (b.) Tree based on transversions only. Numbers along branches are the inferred number of nucleotide substitutions.

(Hillis 1991). When all data are considered, there is no phylogenetic signal in the resultant tree ($g_1 = -0.0004$). Although many characters appear to support each node (Fig. 7a), pairwise homoplasy values between lineages are uniformly large (ranging from 0–26 sites per comparison, depending on the tree and taxa). One possible interpretation of this pattern is that the inferred character support for internal branches is a function of long branches attracting, rather than real character support (Swofford and Olsen 1990), and that the internal branches are much shorter than the reconstructions shown in Figure 7a. When only transversional changes are considered, the data set contains marginally significant phylogenetic information ($g_1 = -0.36$; critical P-value at the 0.05 level without corrections for multiple comparisons is -0.34; Hillis 1991), homoplasy levels are essentially zero, and the number of characters supporting each internal node reduces to a very few (Fig. 7b). This pattern is consistent with a rapid initial diversification of these eight lineages, with low bootstrap values reflecting the short internal branches. Thus, a reasonable interpretation is that the near-simultaneous diversification of these lineages, with a series of synchronous speciation events, is the true historical pattern.

Within the larger groups, we generally found significantly skewed tree-length distributions (analyzed for transitions and transversions together), with sister groups separating along biogeographic boundaries. This phylogenetic signal indicates that many groups have remained stable long enough for mitochondrial lineages to sort into phylogeographically meaningful clades. For example, when analyzed alone, the eight sequences of *A. t. tigrinum* from the eastern United States had a highly significant tree skewness ($g_1 = -1.53$, $P \ll 0.01$), resulting in a single tree identical to that in Fig. 5. A similar result was found for the central and western Mexican Plateau clade ($g_1 = -1.06$, $P \ll 0.01$, based on all populations except no. 62). In the Rocky Mountain-Great Plains group, we also found significantly skewed tree-length distributions ($g_1 = -1.18$, $P \ll 0.01$ for a selection of eight relatively divergent lineages), with the structure resulting from the divergence of the southern Rocky Mountain members (populations 8, 9, 45) from those in the Great Plains and northern Rockies. However, among the Great Plains and northern Rocky Mountain lineages, there is extremely little divergence and no indication that the four recognized subspecies represent monophyletic entities.

Thus, our results are consistent with an interpretation of an initial, relatively rapid diversification of the tiger salamander complex across North America, with at least eight lineages initially involved. Most of these lineages contain phylogenetically informative tree-length distributions, with narrow-to-broad hybrid zones between parapatrically distributed groups. Whether these zones developed in primary or secondary contact is not possible to determine, in part because we have no clear data on the relationships among the major groups. Detailed analyses of hybrid-zone dynamics between *A. t. tigrinum* and *A. t. mavortium* based on allozymes (Kocher 1986; Routman 1993; Shaffer and Irschick, unpubl. data) and mtDNA restriction sites (Routman 1993) have generally been interpreted as resulting from secondary contact following isolation of *A. t. tigrinum* and *A. t. mavortium,* and our results are consistent with this interpretation.

The vast region encompassing the Great Plains, much of the Rocky Mountains, the Sierra Madre Oriental, and some Mexican Plateau populations demonstrates a fundamentally different pattern, with extraordinarily little differentiation. Regardless of what the precise history of the salamanders in this region has been, both our mitochondrial and allozyme (Shaffer, unpubl. data) results indicate that much of this region has been recently colonized, resulting in low genetic diversity (Wade et al. 1994). In the northern prairies, the distinctively patterned, dark spotted *A. t. diaboli* occupies an area that was covered in glacial ice 18,000 years ago (Andrews 1987). Assuming that it evolved de novo since the glacial retreat, this suggests that taxonomically significant color-pattern variation may evolve within a few thousand generations, outpacing the rate at which mitochondrial lineages sort out into monophyletic taxa. It also suggests that the postmetamorphic color patterns that characterize the named subspecies throughout this region may be subject to relatively strong natural selection.

Molecular Clocks and Rates of Evolution

Although the plio-Pleistocene history of North America and Mexico is reasonably well understood, our lack of phylogenetic resolution frustrates most attempts to place absolute dates, and thus rates, on sequence divergences. However, two lines of evidence provide insights into the rate and constancy of mtDNA evolution in the tiger salamander complex.

Based on previous allozyme and morphological evidence and on the neighbor-joining analysis (Fig. 4), one of the initial speciation events within the complex was between *A. californiense* and all others. If the Sierran uplift and the subsequent drying of the North American deserts isolated *A. californiense,* (and we recognize that this is an educated guess at best), then this relatively basal split can be dated at the beginning of the Sierran uplift about 5 mya (Axelrod 1980; Unruh 1991). The pairwise sequence divergences between *A. californiense* and all other tiger salamanders is about 5–8% (Table 1), suggesting a very approximate rate for mitochondrial D-loop sequence evolution of about 1–1.5% per million years, or 0.5–0.75% per million years per lineage. This rate is considerably slower than the overall mtDNA rate commonly claimed for mammals of 2% per million years (Brown et al. 1979) and is consistent with recent observations of a slow rate of mtDNA evolution in poikilothermic vertebrates in other mitochondrial genes (Martin et al. 1992; Rand 1994; Shaffer et al. submitted). This also suggests that the Mexican taxa are more recently derived than the 10 million year estimate that was previously suggested based on allozyme and geological evidence (Shaffer 1984a).

A visual inspection of the phylogram in Figure 5 implies that several lineages show a pronounced speedup in the rate of mtDNA evolution. Such heterogeneity, if real, is somewhat surprising in untranslated sequences like those presented here and would invalidate the use of a single rate to date divergences within the complex. To test for clock constancy, we applied Tajima's method (eq. 4, Tajima 1993) to several three-lineage sets of comparisons. Tajima's method is particularly appropriate for our data, because the statistical validity of the test applies even if the outgroup is not known among the three lineages (however, in this case, one cannot say which lineage has experienced the rate change).

We conducted six sets of comparisons for the most obvious candidates for rate heterogeneity in our data. (We identify populations by number, with inferred sister taxa nested in the inner pair of parentheses.) For three cases [1(33, 46)], [1(40, 46)], and [1(11, 13)] we could not reject the hypothesis of a molecular clock among the derived pair of sister sequences. In three others, we could reject a strict molecular clock [1(13, 40), $\chi^2 = 4.23$, $P < 0.05$; 1(51, 52), $\chi^2 = 4.33$, $P < 0.05$; 61(33, 40), $\chi^2 = 4.84$, $P < 0.05$], suggesting that there has been a rate speedup in the south-central Colorado lineage (population 13) relative to eastern *A. t. tigrinum,* in *A. t. tigrinum* relative to *A. t. mavortium* from the Great Plains (population 33), and between the two lineages of *A. rosaceum* (populations 51, 52). Thus, even for the D-loop, heterogeneity in the rate of mtDNA evolution leads to difficulties in a universal application of clock constants (Tajima 1993 found a similar result for protein-coding genes in hominoid mtDNA). However, in the tiger salamander complex, instances of heterogeneity appear uncommon. In addition, the two most extreme cases of rate heterogeneity found [1(51, 52) and 1(13, 40)] differed by about a factor of 2; if this is the worst case, then the "clock" still functions reasonably well, provided it is not applied too precisely.

Gene Trees, Organism Trees, and Species Boundaries

As the dust begins to settle over current debates concerning biological (Templeton 1989), phylogenetic (Cracraft 1989), or combined (Avise 1994) species concepts, two conclusions appear to be emerging. First, at very recent levels of divergence, we should anticipate that problems will emerge, because reproductive isolating mechanisms have not yet fully evolved, and gene trees have not had time to sort out into monophyletic groups (Patton and Smith 1994). The problem of gene tree sorting is particularly vexing for mtDNA, because ancestral polymorphisms may cause a true disparity between mitochondrial and organismal genealogical histories (Harrison 1989). Second, at deeper levels of divergence, the biological and phylogenetic concepts often converge on the same species boundaries.

Within the tiger salamander complex, the most deeply differentiated taxa are consistently recognized with both allozyme, mtDNA, and morphological characters, suggesting that mtDNA provides insights into the limits of these relatively differentiated species. For example, all available data (Kraus 1988; Shaffer et al. 1991; Shaffer 1993) demonstrate that *A. californiense* is the sister group to all or most of the remaining complex members. It is a monophyletic entity, and there seems little reason to include it within *A. tigrinum.* We therefore concur with Storer (1925) in supporting Gray's (1853) original decision of full species recognition. The same arguments can be made for *A. rosaceum,* although both allozyme (Shaffer 1983) and mtDNA suggest that Taylor's (1941) original conception of two species, rather than the single taxon currently recognized, may better reflect true species boundaries.

Based on similarly divergent, apparently monophyletic groups, we feel that a reasonable case can be made for the

recognition of at least eight differentiated, monophyletic species in the tiger salamander complex. In addition to *A. californiense* and the two lineages of *A. "rosaceum," A. velasci* from El Vergel, south-central Colorado, eastern United States, and the central and western Mexican Plateau all stand out as recognizable monophyletic units distinct from each other and from the largely undifferentiated samples in the Rocky Mountains, Great Plains, and the remaining regions of Mexico. In recognizing these groups, we advocate the primacy of differentiation and monophyly over the importance of hybridization; in the best studied case to date, *A. t. tigrinum* and *A. t. mavortium* clearly hybridize over a relatively broad zone of 50–100 km (Kocher 1986; Routman 1993; Shaffer and Irschick, unpubl. data). However, away from the hybrid zone, each maintains its historical integrity, and we have no reason to suspect that they will fail to do so in the future. Thus, we support the phylogenetic view (Cracraft 1989) that these are best considered separate species even though they can hybridize.

The most problematic, and most challenging, speciation issues in this complex involve the nontransforming paedomorphs or axolotls from Mexico. These populations are morphologically and ecologically distinct, and often appear to be differentiated monophyletic entities that never metamorphose in nature (for example, *A. dumerilii, A. andersoni,* and, as far as we know, *A. velasci* from El Vergel and *A. mexicanum*). However, in other cases, they exist as polymorphic populations, with both transforming and nontransforming individuals (Shaffer 1984a, 1986). Several of the obligate paedomorphs are currently recognized as species, leaving a paraphyletic remainder of transforming populations. Our feeling is that paedomorphosis should be treated as an important character in designating species boundaries if it has gone to fixation in a population. In this case, a paedomorphic lineage is ecologically and reproductively isolated from the transforming salamanders in the surrounding habitat and is on a separate, unique evolutionary trajectory. This may result in transforming species that are recognized as paraphyletic, particularly when paedomorphosis is recently derived and the time to monophyly among transforming populations is relatively great. In adopting this view of species boundaries, we are calling for the recognition of several species that are extremely similar in their mtDNA, especially in the eastern Mexican Plateau (populations 70–77). In this area, several obligate paedomorphs are morphologically distinct (Shaffer, unpubl. data) but have sequence identity (populations 70, 71, 73) or near identity (population 72), implying that these populations are among the most recently derived vertebrate species known. Although this may lead to a paraphyletic "remainder" species, it emphasizes the importance of species as independent evolutionary lineages which is, in our view, the critical point common to most current species concepts.

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Appendix

Specimens Examined

Specimen numbers in parentheses are H. B. Shaffer field numbers. All voucher specimens are currently housed in the University of California, Davis Vertebrate Collections.

1. *Ambystoma californiense* (HBS 6437): Careaga divide, 6.0 mi northwest of Los Alamos, Santa Barbara County, CA.
2. *A. californiense* (HBS 6533): Rd. 204, 0.2 mi south of junction with Rd. 205, approximtely 2.0 mi west-northwest of Friant, Madera County, CA.
3. *A. californiense* (HBS 6680): 1.2 mi north of intersection of Vasco Rd. with Scenic Avenue, along Vasco Rd., Livermore, Alameda County, CA.
4. *A. californiense* (HBS 6697): Olcott Pond, in the Jepson Prairie, approximately 9 mi south of Dixon along Highway 113, Solano County, CA.
5. *A. tigrinum nebulosum* (HTP52): Hardware Turnoff Pond, 100 yd northwest of intersection of Utah Hwy. 39 and road to Hardware Ranch, Weber County, UT (G. Wurst field number)
6. *A. t. nebulosum* (HBS 7712): Lake Salamander, at end of Lambs Canyon Rd., 8.0 mi south of Lambs Canyon exit from I-80, Salt Lake County, UT; el. 2500 m.
7. *A. t. nebulosum* (HBS 7763): drainage pond in town of Thistle, along Rt. 89, which is 15.6 mi north of the Sanpete-Utah County line, Utah County, UT; el. 1540 m.
8. *A. t. nebulosum* (HBS 7877): stock tank at "Fly in Lucky D" ranch, 1.1 mi (along dirt road) north of Rt. 9, 1.8 mi east of Zion National Park Boundary, Washington County, UT; el. 1810 m.
9. *A. t. nebulosum* (HBS 7832): Fracas Lake, 3.5 mi west of Hwy. 67, which is 9.0 miles south of Jacob Lake, Coconino County, AZ; el. 2400 m.
10. *A. t. nebulosum* (HBS 7584): pond, 17.6 mi east of continental divide at Togwatee Pass, along Rt. 26, Fremont County, WY.; el. 2170 m.
11. *A. t. nebulosum* (HBS 7163): beaver pond, 3.0 mi north of Dillon-Silverthorne ramp of I-70, along Ryan Gulch Rd., Summit County, CO; el. 2880 m.
12. *A. t. nebulosum* (HBS 7101): 31.6 mi north of Thoreau on Rt. 57, in stock tank, McKinley County, NM.; el. 2000 m.
13. *A. t. nebulosum* (HBS 5993, HBS 5994): Platoro, approximately 50 km west of Alamosa, Conejos County, CO (gift of W. Van Devender).
14. *A. t. nebulosum* (HBS 7124): beaver pond along Hwy. 550, 15.4 mi north of San Juan-La Plata County line, at Molas Pass, San Juan County, CO; el. 3220 m.
15. *A. t. melanostictum* (HBS 8974): Grass Lake, Siskiyou County, CA.
16. *A. t. melanostictum* (HBS 6792): Seep Lakes region, 2 miles south of paved road from Hwy. 17, just south of Potholes Reservoir, Columbia National Wildlife Refuge, Grant County, WA.
17. *A. t. melanostictum* (HBS 6883): small pond, approximately 1 mi northeast of Fourth of July Lake, 1 mi southeast of Sprague, Lincoln County, WA.
18. *A. t. melanostictum* (HBS 7398): 13.2–17.0 miles north of Angela, on Hwy. 22, Garfield County, MT; el. 950 m.
19. *A. t. melanostictum* (HBS 7530): 6.7 mi east of Hwy. 20, along Hwy. 172, Hot Springs County, WY; el. 1350 m.
20. *A. t. melanostictum* (HBS 7327): 12.0 mi northwest of Wyoming–South Dakota border, along Hwy. 212, in stock pond, Crook County, WY; el. 1140 m.
21. *A. t. melanostictum* (HBS 7304): small cattle tank, 15.6 mi south east of the Wyoming–South Dakota border along Hwy. 212, Butte County, SD; el. 1040 m.
22. *A. t. melanostictum* (HBS 7281): stock tank, 24.3 mi south of Hwy. 18 along Hwy. 85, Niobrara County, WY; el. 1330 m.
23. *A. t. melanostictum* (HBS 7247): stock tank 1.4 mi south of intersection of Hwy. 85 and Hwy. 313, along Hwy. 85, Goshen County, WY; el. 1400 m.
24. *A. t. melanostictum* (HBS 7206): stock tank along Rt. 143, 1.7 mi south of Midway (on Rt. 85), Laramie County, WY; el. 1720 m.
25. *A. t. melanostictum* (HBS 7169): stock tank, 3.4 mi east of Hwy. 85 along Eagle Camp Rd., which is 17.4 mi southwest of Midway, Laramie County, WY; el. 1800 m.
26. *A. t. diaboli* (HBS 5424): 1.5 mi north, 4.5 mi west of Webster, Ramsey County, ND.
27. *A. t. diaboli* (HBS 5465): 1.3–5.0 mi south of Rt. 30-Rt. 2 intersection, along Rt. 30, Benson County, ND.
28. *A. t. diaboli* (HBS 5498): 16.6–18.0 mi south of Rt. 30-Rt. 2 intersection, along Rt. 30, Benson County, ND.
29. *A. t. diaboli* (HBS 5512): 1.2–2.7 mi south of Wells County-Benson County line, along Hwy. 30, Wells County, ND.
30. *A. t. diaboli* (HBS 5522): from Hurdsford (at intersection of Hwys. 3 and 200) to 1.5 mi west of Hurdsford, along Hwy. 200, Wells County, ND.
31. *A. t. diaboli* (HBS 5535): vicinity of Goodrich, along Hwy. 200, Sheridan County, ND.
32. *A. t. mavortium* (HBS 5861): 1.7 mi east of intersection with State Hwy. 14, along road to Oakdale (approximately 3 mi west of Oakdale), Antelope County, NE.
33. *A. t. mavortium* (HBS 6593): pond, 10 mi north of Limon, Lincoln County, CO.
34. *A. t. mavortium* (HBS 6916): stock tank at Corners Ranch, 4 mi north of Red Rock, Grant County, NM; el. 1170 m.
35. *A. t. mavortium* (HBS 7927): cattle tank along State Rd. 166, approximately 35.5 mi southwest of Fort Davis, Jeff Davis County, TX (F. Kraus specimen).
36. *A. t. tigrinum* (HBS 5960): cattle tank of Compton residence, 2.2 mi west of Lawton, along Hwy. 20, Woodbury County, IA; el. 380 m.
37. *A. t. tigrinum* (HBS 5154): along Hwy. 12, approximately 2 mi north (by road), of Oregon, Dane County, WI.
38. *A. t. tigrinum* (HBS 5766): Goss Pond, between Goss Rd. and U.S. 23, Washtenaw County, MI (F. Kraus specimen).
39. *A. t. tigrinum* (HBS 5587): Along Hwy. 64, 6.7 mi north of intersection of Hwy. 64 and Hwy. 210, Cass County, MN.
40. *A. t. tigrinum* (HBS 6074): 4 mi south of Cabool, along Hwy. 63, Texas County, MO (R. Altig specimen).
41. *A. t. tigrinum* (HBS 5615): cattle tank of Mr. Culpepper, 1/4 mi south of junction of old Hwy. 149 at Oak Ridge Rd., Montgomery County, TN.
42. *A. t. tigrinum* (HBS 6652): Ellenton Bay, Savannah River Ecology Lab, Aiken County, SC (R. Semlitsch specimen).
43. *A. t. tigrinum* (HBS 6645): approximately 7.5 mi south of Box Springs, on Marion-Chatahoochee County border, GA.
44. *A. t. tigrinum* (HBS 6187): approximately 4 mi east of Jay, Santa Rosa County, FL (P. Moler specimen).
45. *A. velasci* (HBS 3846): 15.5 mi north of Temosachic, approximately 1/4 mi east of highway from Gomez Farias to Temosachic, Chihuahua, Mexico; el. 2080 m.
46. *A. velasci* (HBS 1702, HBS 1708): pond in center of El Vergel, approximately 200 m east of road, Chihuahua, Mexico; el. 2660 m.
47. *A. velasci* (HBS 2731): 1.2 mi east (by Durango-Mazatlan Hwy.) of bridge at Mimbres, Durango, Mexico; el. 2350 m.
48. *A. velasci* (HBS 5787): roadside pond, 5 mi north of turnoff to Tanquecillo, which is 27 mi north of Dr. Arroyo, Nuevo Leon, Mexico; el. 1520 m.
49. *A. velasci* (HBS 4450): cattle tank, 1.5 mi north (via Hwy. 57) of Villa Hidalgo, San Luis Potosi, Mexico; el. 1618 m.
50. *A. velasci* (HBS 4407): 1.3 mi east of Mex. Hwy. 57, along road to San Jose Iturbide, Guanajuato, Mexico; el. 2023 m.
51. *A. rosaceum* (HBS 2834): 1.3 mi east (by Durango-Mazatlan Rd.) of La Cuidad, Durango, Mexico; el. 2500 m.
52. *A. rosaceum* (HBS 3969): 14.3 mi west (by road) of Tomachic, along road from La Junta, at stream crossing under road, Chihuahua, Mexico; el. 2000 m.
53. *A. velasci* (HBS 2950): 2.3 mi east of Tapalpa, along Tapalpa-Cuidad Guzman Rd., Jalisco, Mexico; el. 2110 m.
54. *A. flavipiperatum* (HBS 2892): 5.7 mi west of turnoff to Morelia (via Mex. Hwy. 15), which is 10 mi west of Guadalajara, Jalisco, Mexico; el. 1550 m.
55. *A. andersoni* (HBS 1782, HBS 1790): creek draining east side of Lake Zacapu, Michoacan, Mexico; el. 1820 m.
56. *A. dumerilii* (HBS 1834): Lake Patzcuaro (purchased in Patzcuaro town market), Michoacan, Mexico; el. 1920 m.
57. *A. velasci* (HBS 3155): 3.2 mi (by road) west of traffic circle, east of town of Patzcuaro, along Patzcuaro-Uruapan Rd., Michoacan, Mexico; el. 1970 m.

58. *A. amblycephalum* (HBS 3055): creek crossing Hwy. 15 at town of Iratzio, 11.2 mi (by road) east of Quiroga, Michoacan, Mexico; el. 2130 m.

59. *A. ordinarium* (HBS 4191): 10.5 mi southeast San Gregorio, along Patzcuaro-Tacambaro Rd., Michoacan, Mexico; el. 2100 m.

60. *A. ordinarium* (HBS 1865): 0.4 mi west of San Jose Lagunillas, between Morelia and Hidalgo, in creek south of Hwy. 15, Michoacan, Mexico; el. 2490 m.

61. *A. granulosum* (HBS 2571): Mex. Hwy. 15, 18 km (by road) southwest of Toluca, Mexico, Mexico; el. 2500 m.

62. *A. granulosum* (HBS 3219): Mex. Hwy. 15, approximately 12 mi west of Toluca, Mexico, Mexico; el. 2500 m.

63. *A. lermaensis* (HBS 1929): Lake Lerma, 1/2 mile west of intersection road to Lerma and San Mateo Tasaquillo, Mexico, Mexico; el. 2400 m.

63. *A. lermaensis* (HBS 1950): Lake Almoloya, southwest of of Almoloya, in small lake, Mexico, Mexico.

64. *A. (Rhyacosiredon) altimirani* (HBS 8166): aqueduct stream, just south of highway from Ixtlahuaca de Rayon to Mexico City, 22.5 km west of turnoff to Villa del Carbon, Mexico, Mexico; el. 3300 m.

65. *A. (Rhyacosiredon) altimirani* (HBS 8112): artificial pond, approximately 500 mi south of Tres Marias-Santa Marta Rd., 21 km east of Santa Marta, Morelos, Mexico; el. 2810 m.

66. *A. velasci* (HBS 4263): 42.5 mi east of Valle de Bravo, along Rt. 1 (near San Bertola del Llano), Mexico, Mexico; el. 2600 m.

67. *A. velasci* (HBS 5047): Laguna de Tecocomulco, Mexico, Mexico. (H. Drummond specimen)

68. *A. mexicanum* (HBS 3182, HBS 3184): canal, 100 m south of town of Mixquic, 5.5 mi southwest of Chalco, Distrito Federal, Mexico; el. 2200 m.

69. *A. velasci* (HBS 1987): 5.4 mi north of Hidalgo-Mexico border, in Ranchero Pond along Hwy. 130 (132), Hidalgo, Mexico; el 2320 m.

70. *A. velasci* (HBS 3248): Caldera Lake, at north end of Tecuitlapa, in eastern end of crater, Puebla, Mexico; el. 2270.

71. *A. velasci* (HBS 4967): Laguna de Quechulac, 3 km south of Laguna La Preciosa, Puebla, Mexico; el. 2275.

72. *A. velasci* (HBS 4914): Laguna de San Luis Atexcac, 10 km west and 3 km south of Laguna de Alchichica, Puebla, Mexico; el. 2330 m.

73. *A. velasci* (HBS 4880): Laguna la Preciosa, Puebla, Mexico; el. 2310 m.

74. *A. velasci* (HBS 4903): well on the property of Sr. Limon, approximately 3 km south of Laguna Alchichica. Puebla, Mexico; el. 2290 m.

75. *A. velasci* (HBS 4901): trapped in a well, approximately 20 m from the shore of Laguna de Alchichica, Puebla, Mexico; el. 2290 m.

76. *A. taylori* (HBS 2321, HBS 4892): shores of Laguna de Alchichica, Puebla, Mexico; el. 2290 m (2321: sexually mature larval form; 4892: naturally transformed juvenile).

77. *A. velasci* (HBS 4367): 6.9 miles south of Las Vigas, along Microondas Rd., Veracruz, Mexico; el. 2817 m.