Phylogenetic Relationships of Bolitoglossine Salamanders: A Demonstration of the Effects of Combining Morphological and Molecular Data Sets

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We analyzed sequence data for 555 bp of the mitochondrial gene cytochrome *b* in plethodontid salamanders, taken from 18 ingroup (tribe Bolitoglossini) and 4 outgroup (tribe Plethodontini) taxa. There were 257 variable sites, of which 219 were phylogenetically informative. Sequence differences among taxa exceeded 20%, and there were up to 15% amino acid differences among the sequences. We also analyzed 37 morphological (including karyological) characters, taken from the literature. Data were analyzed separately and then combined using parsimony and likelihood approaches. There is little conflict between the morphological and DNA data, and that which occurs is at nodes that are weakly supported by one or both of the data sets. Treated separately, the morphological and DNA data provide strong support for some nodes but not for others. The combined data act synergistically so that good support is obtained for nearly all of the nodes in the tree. Recent divergences are supported by silent transitions, and older divergences are supported by a combination of morphological, karyological, DNA transversion, and amino acid changes. Eliminating silent changes from the DNA data improves the consistency index and improves some bootstrap and decay index values for several deeper branches in the tree. However, the combined data set with all characters included provides a better supported tree overall. Maximum likelihood and parsimony with all of the data give not only the same topology but also remarkably similar branch lengths. Results of this analysis support the monophyly of the supergenera *Hydromantes* and *Batrachoseps,* and of a sister group relationship of *Batrachoseps* and the supergenus *Bolitoglossa* (represented in this study by one species of the genus *Bolitoglossa*).

Introduction

Nearly one half of the approximately 400 species of living salamanders are members of the tribe Bolitoglossini, family Plethodontidae (Frost 1985; Duellman 1993). Nine of the 10 families of salamanders are basically north temperate in distribution, whereas all salamanders of the southern hemisphere and virtually all tropical species are bolitoglossines, members of the supergenus *Bolitoglossa,* occurring from Mexico into South America. The remaining bolitoglossines comprise two other supergenera, *Hydromantes* and *Batrachoseps. Hydromantes* has an extraordinary distribution, with species in California and the Mediterranean region, and it is the only plethodontid that occurs in the Old World. *Batrachoseps* is restricted to the west coast of North America, where it ranges from Oregon to Baja California. The grouping of these three supergenera, first proposed by Wake (1966), has been widely accepted. Monophyly of the tribe and of the supergenera, and a sister-group relationship of *Bolitoglossa* and *Batrachoseps,* were postulated on the basis of morphological data (Lombard and Wake 1986), but some aspects of that analysis have been questioned (Presch 1989; Wake 1992). Our primary focus is on the relationships of the bolitoglossine supergenera, with special emphasis on *Hydromantes* and *Batrachoseps.* We present new data from a comparative study of sequences of the mitochondrial gene cytochrome *b.* These molecular data are analyzed separately, and then they are combined with morphological data from osteology, myology, karyology, and neuroanatomy. These results are used to evaluate prior hypotheses of relationship. Finally, we discuss the implications of analyzing data sets separately and combined.

Materials and Methods

Samples used for the DNA study were obtained mainly from frozen tissue stored for varying lengths of time, from a few months to more than 10 years; a few were taken from small tissue samples taken from anesthetized living specimens and preserved in 90% ethanol. Samples are listed in table 1 (voucher specimens are listed by Museum of Vertebrate Zoology accession numbers; a few specimens verified by us lack vouchers and are indicated by tissue sample numbers). The supergenus *Bolitoglossa* includes more than half of the species of the family Plethodontidae and is currently the subject of separate studies in the Wake laboratory. We selected *Bolitoglossa marmorea* to represent the supergenus. The supergenus *Batrachoseps* is taxonomically complicated (Yanev 1980), and it, too, is being studied in more detail in the Wake laboratory. We selected representatives of both of the major groups in the genus (three species, one as yet undescribed, represent the subgenus *Plethopsis*; the subgenus *Batrachoseps* is represented by three populations assigned to *B. simatus* and two subspecies of *B. pacificus*). For *Hydromantes,* we sampled all recognized American species (which represent the subgenus *Hydromantes*), a European mainland species, and three species restricted to the Italian island of Sardinia (the European species are placed in the subgenus *Speleomantes*). For outgroups, we used members of the tribe Plethodontini: two samples of the *Ensatina esch-*

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Table 1
Samples Used in this Study

Species	Museum Number	Locality
Tribe Bolitoglossini		
Genus *Hydromantes*		
Hydromantes		
1. *shastae* 1	MVZ 170730	Shasta Co., Calif.
2. *shastae* 2	MVZ 202326	Shasta Co., Calif.
3. *platycephalus* 1	MVZ 164615	Tulare Co., Calif.
4. *platycephalus* 2	MVZ 202361	Inyo Co., Calif.
5. *brunus*	S-11935	Merced Co., Calif.
Speleomantes		
6. *italicus*	MVZ 168846	Liguria Region, Italy
7. *supramontis*	S-12038	Nuoro Prov., Italy
8. *genei*	S-12039	Cagliari Prov., Italy
9. *flavus*	S-12040	Nuoro Prov., Italy
Genus *Batrachoseps*		
Batrachoseps		
10. *simatus* 1	MVZ 218033	Kern Co., Calif.
11. *simatus* 2	MVZ 158457	Kern Co., Calif.
12. *simatus* 3	MVZ 168760	Tulare Co., Calif.
13. *pacificus major*	MVZ 215862	San Diego Co., Calif.
14. *pacificus pacificus*	MVZ 172660	Ventura Co., Calif.
Plethopsis		
15. *wrighti*	MVZ 220713	Lane Co., Oreg.
16. *campi*	S-9987	Inyo Co., Calif.
17. undescribed species	MVZ 219133	Tulare Co., Calif.
Genus *Bolitoglossa*		
18. *marmorea*	MVZ 210286	Chiriqui Prov., Panama

scholtzii complex, as well as the samples of *Plethodon elongatus* and *Aneides lugubris* studied by Moritz, Schneider, and Wake (1992).

DNA prepared by boiling (for ca. 10 min) minute amounts (<5 mg) of liver or muscle in a 5% (w/v) solution of Chelex (BioRad) was amplified by the polymerase chain reaction (PCR). Primers used for amplification 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 amplify mtDNA from a wide variety of vertebrates. Primers MVZ-15, -18, and -25 were designed to match sequences from *Ensatina* and, for MVZ-18 and -25, also from *Xenopus* (Moritz, Schneider, and Wake 1992).

For double-strand reactions, template 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 typically were run for 38 cycles in a total volume of 25 μl using 0.6 units of *Taq* polymerase (Cetus). 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 and 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 reaction profiles identical to those outlined above, in 40 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. The remaining 45 μl was purified and concentrated to ca. 20 μl on disposable centrifugal filters (Millipore MC30). Dideoxy chain termination sequencing (Sanger, Nicklen, and Coulson 1977) was accomplished using U.S. Biochemical Sequenase version 2.0 kit and ^{35}S-labeled dATP. All PCR reactions included negative controls (including all reactants but DNA) to guard against contamination of reagents with DNA. The mtDNA sequences reported in this paper have been deposited in the GenBank database under accession numbers U89610–U89631. Outgroup samples of *Aneides, Ensatina,* and *Plethodon* are from those reported by Moritz, Schneider, and Wake (1992) (GenBank accession numbers L75796, L75808, L75820, L75821).

Data obtained from the DNA study (which we call the molecular data) were studied in relation to morphological (osteology, myology, nerves and sensory system) and karyological characters presented by Marlow, Brode, and Wake (1979), Lombard and Wake (1986), Wake (1989), and Sessions and Kezer (1991). We selected 37 characters, collectively termed the morphological data, all of which were phylogenetically informative. We used characters numbered 1–13, 16–27, and 29–30 in Lombard and Wake (1986), with the following modifications: We recognized an additional state for *Hydromantes* for their character 4, following data in Lombard and Wake (1977). Separate states for the genioglossal muscles, character 10, are recognized for *Ensatina*

and *Batrachoseps,* following Lombard and Wake (1977). Three states were recognized for the vomer, character 23: an ancestral condition and two derived states (short preorbital process in subgenus *Plethopsis*; no preorbital process in subgenus *Batrachoseps*). Five states were recognized for the premaxilla, character 26 (ancestral paired bones in *Plethodon* and *Hydromantes,* strengthening fusion in *Aneides,* paedomorphic fusion in *Bolitoglossa* and in subgenus *Batrachoseps,* and ontogenetic separation in subgenus *Plethopsis*; Wake 1966). Additional morphological characters (from Wake [1966, 1989] unless otherwise noted) are toe number (reduced to four in all *Batrachoseps*), dorsal fontanelle (present in *Batrachoseps*), prefrontals absent (subgenus *Batrachoseps*), lateral spur on the parietal bone (*Batrachoseps* and *Bolitoglossa*), numbers of caudosacral vertebrae (three in most, polymorphic for two or three in *Batrachoseps,* two in *Bolitoglossa*; Wake and Dresner 1967), features of chromosome 14 (unique state in subgenus *Speleomantes*; Nardi 1991), sex chromosomes (in several species of the subgenus *Speleomantes*; Nardi 1991), degree of overlap of the brainstem motor nuclei (two states, outgroups and Bolitoglossini; Roth et al. 1988), caudal musculature (differentiated in the subgenera of *Hydromantes*; Serra and Stefani 1974), transverse processes of trunk vertebrae (differentiating the subgenera of *Hydromantes*), chromosome arm ratios (differentiating the subgenera of *Hydromantes*; Sessions and Kezer 1991), and red blood cells (enucleated in subgenus *Batrachoseps*). The full morphological data matrix is available from the authors.

MtDNA sequences were read from both ends and aligned by eye with each other and, using amino acid translations, to the same region of the samples of *Ensatina, Plethodon,* and *Aneides* studied earlier (Moritz, Schneider, and Wake 1992). In some analyses, silent transitions were removed from the data set by recoding third-position transitions as purines and pyrimidines and first-position C-to-T changes as pyrimidines. In mtDNA, all third-position transitions are silent, and C-to-T changes in the first position are silent if the amino acid coded is leucine.

Phylogenetic analyses of the DNA data and of the combined data sets were conducted using PAUP (version 3.1.1; Swofford 1991*a*). For parsimony analysis, all characters were treated as unordered. A heuristic search was conducted with random taxon addition for 30 replications. We performed three analyses: (1) 37 morphological characters, all of which were informative, (2) all mtDNA characters (256 variable characters, 219 parsimony informative), and (3) all data combined (293 variable characters, 266 parsimony informative). Decay indices (Bremer 1988) were calculated using PAUP. One tree was created for every branch in the most parsimonious tree, where only the branch of interest is not collapsed to the base, using Autodecay 3 (Ericksson and Wikstrum 1995). A separate analysis was performed for each of these constraint trees. The shortest tree was then obtained for each constraint tree, and only trees that failed to satisfy the constraint that the branch of interest be monophyletic were kept. Heuristic searches were performed with 25 random taxon addition replicates for each constraint tree. The difference in length between the tree obtained and the most parsimonious tree is the decay index for that branch.

Maximum-likelihood analyses were performed using the DNAml program included in PHYLIP version 3.4 (Felsenstein 1993). Maximum-likelihood analysis was conducted using the entire sequence, employing three categories of change with a ratio of 3 first-position to 1 second-position to 20 third-position changes. Furthermore, transversions were weighted 10 times transitions. These modifications were based on empirically determined biases (Moritz, Schneider, and Wake 1992).

All bootstrap values reported are the result of 1,000 bootstrap replicates using heuristic searches and the TBR option in PAUP. The character incongruence indices IM and IMF (Mickevitch and Farris 1981; Kluge 1989; Swofford 1991*b*) were used to assess the morphological against the DNA data set. Farris et al.'s (1995) test of incongruence was applied to the data by choosing 20 random data sets from the combined data set equivalent in numbers of characters to the original morphological and molecular data sets. The number of steps for the shortest trees from the randomized subsets was calculated using PAUP. The test statistic consisted of the number of times the total number of sets from the random partitions exceeded the actual sum of the two separate data sets (Farris et al. 1995). Consistency index values reported are based solely on informative characters. We used signed-ranks tests proposed by Templeton (1983; Larson 1994) to compare the significance of trees given the data.

Results

Characteristics of the Molecular Data

We obtained a common continuous sequence of 555 bp for the cytochrome *b* gene for 18 ingroup and 4 outgroup sequences. There were 257 variable sites, 219 of which were phylogenetically informative. We found as much as 24% sequence difference (fig. 1), resulting in up to 15% inferred amino acid differences. The distribution of changes for the three base pair positions, and the proportion that are silent transitional changes, are indicated in figure 2.

Phylogenetic Analyses

Parsimony analysis of the mtDNA data with all character changes included resulted in a single most-parsimonious tree, with 870 steps and a consistency index of 0.43 (fig. 3). In this tree, European and American species of *Hydromantes* each form a monophyletic group (with decay indices of 5 and 3, respectively), and they are sister taxa, forming a monophyletic group (with a decay index of 4) that is the sister taxon of a group containing all other taxa in the study, except for two samples of *Ensatina.* Thus, two of the putative outgroup taxa cluster with *Batrachoseps* and *Bolitoglossa*; however, this group is poorly supported, with a decay index of 1. *Batrachoseps* (*Batrachoseps*) and *Batrachoseps*

Genera:	*Hydromantes*									*Batrachoseps*									*Bol.*	*Ple.*	*Ane.*	*Ensatina*
Subgenera:	*Hydromantes*					*Speleomantes*				*Batrachoseps*					*Plethopsis*							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	-	0	0.027	0.027	0.054	0.058	0.087	0.087	0.087	0.092	0.098	0.092	0.103	0.096	0.109	0.105	0.114	0.109	0.114	0.13	0.082	0.082
2	0.009	-	0.027	0.027	0.054	0.058	0.087	0.087	0.087	0.092	0.098	0.092	0.103	0.096	0.109	0.105	0.114	0.109	0.114	0.13	0.082	0.082
3	0.07	0.07	-	0	0.054	0.075	0.103	0.098	0.103	0.092	0.098	0.092	0.098	0.085	0.114	0.11	0.12	0.103	0.103	0.12	0.06	0.06
4	0.068	0.068	0.005	-	0.054	0.075	0.103	0.098	0.103	0.092	0.098	0.092	0.098	0.085	0.114	0.11	0.12	0.103	0.103	0.12	0.06	0.06
5	0.081	0.079	0.067	0.065	-	0.087	0.109	0.098	0.109	0.114	0.12	0.114	0.138	0.107	0.147	0.133	0.141	0.125	0.125	0.125	0.092	0.087
6	0.145	0.141	0.147	0.149	0.168	-	0.046	0.064	0.046	0.092	0.098	0.092	0.104	0.09	0.104	0.106	0.116	0.139	0.104	0.121	0.092	0.098
7	0.166	0.162	0.173	0.171	0.182	0.134	-	0.065	0	0.109	0.114	0.109	0.115	0.107	0.12	0.105	0.098	0.141	0.125	0.13	0.103	0.114
8	0.162	0.16	0.175	0.177	0.189	0.137	0.15	-	0.065	0.098	0.103	0.098	0.115	0.096	0.109	0.116	0.12	0.13	0.12	0.12	0.114	0.114
9	0.171	0.168	0.178	0.177	0.187	0.135	0.004	0.146	-	0.109	0.114	0.109	0.115	0.107	0.12	0.105	0.098	0.141	0.125	0.13	0.103	0.114
10	0.184	0.184	0.195	0.197	0.204	0.225	0.19	0.222	0.195	-	0.011	0	0.023	0.017	0.087	0.094	0.092	0.103	0.109	0.087	0.082	0.082
11	0.195	0.193	0.202	0.204	0.207	0.23	0.208	0.227	0.209	0.065	-	0.011	0.034	0.028	0.092	0.099	0.098	0.109	0.114	0.092	0.087	0.087
12	0.193	0.191	0.207	0.209	0.213	0.224	0.199	0.218	0.2	0.056	0.029	-	0.023	0.017	0.087	0.094	0.092	0.103	0.109	0.087	0.082	0.082
13	0.196	0.194	0.194	0.196	0.2	0.23	0.2	0.217	0.202	0.119	0.136	0.127	-	0.03	0.098	0.099	0.103	0.103	0.132	0.103	0.092	0.098
14	0.182	0.18	0.182	0.184	0.178	0.222	0.212	0.221	0.21	0.117	0.126	0.116	0.074	-	0.102	0.092	0.096	0.107	0.107	0.09	0.085	0.085
15	0.207	0.21	0.219	0.223	0.241	0.218	0.22	0.214	0.217	0.212	0.205	0.207	0.207	0.202	-	0.039	0.049	0.136	0.147	0.13	0.125	0.13
16	0.215	0.215	0.248	0.25	0.252	0.231	0.206	0.219	0.204	0.21	0.2	0.199	0.184	0.18	0.113	-	0.039	0.127	0.133	0.127	0.122	0.122
17	0.207	0.211	0.24	0.236	0.243	0.221	0.206	0.22	0.204	0.222	0.205	0.205	0.209	0.205	0.1	0.099	-	0.136	0.136	0.136	0.136	0.136
18	0.201	0.205	0.191	0.196	0.2	0.231	0.225	0.241	0.23	0.205	0.196	0.2	0.215	0.206	0.239	0.248	0.236	-	0.141	0.125	0.109	0.098
19	0.188	0.19	0.199	0.193	0.209	0.196	0.21	0.22	0.209	0.219	0.209	0.206	0.223	0.22	0.232	0.227	0.229	0.231	-	0.125	0.103	0.103
20	0.195	0.195	0.181	0.184	0.19	0.209	0.212	0.218	0.211	0.208	0.211	0.206	0.219	0.216	0.212	0.226	0.24	0.213	0.183	-	0.114	0.114
21	0.166	0.168	0.146	0.142	0.164	0.184	0.195	0.222	0.191	0.208	0.207	0.198	0.207	0.189	0.226	0.239	0.249	0.203	0.199	0.193	-	0.016
22	0.166	0.169	0.142	0.139	0.157	0.19	0.191	0.213	0.191	0.199	0.198	0.193	0.215	0.188	0.219	0.235	0.231	0.189	0.202	0.19	0.074	-

FIG. 1.—Sequence divergence between taxa. Uncorrected percent difference above and Kimura two-parameter distances below.

(*Plethopsis*) are well-supported monophyletic groups, with decay indices of 9 and 19, respectively, but support for the monophyly of *Batrachoseps* as a whole is relatively weak, with a decay index of 1. The monophyly of *Batrachoseps* + *Bolitoglossa* is also only weakly supported (decay index of 1).

The analysis of morphological data (fig. 4) produced four equally most-parsimonious trees with a length of 59 and a consistency index of 0.90. The lack of resolution seen is largely due to a lack of character variation among closely related taxa, rather than character conflict.

A combined analysis of the molecular and morphological data resulted in a single most-parsimonious tree with 932 steps and a consistency index of 0.45 (fig. 5). The tree topology is identical to one of four most-parsimonious trees (not shown) found in an analysis with the removal of silent sites. Monophyly of each of the genera *Hydromantes* and *Batrachoseps* is well supported, with bootstrap values of 91%–93% and decay indices of 6–10. Monophyly of the subgenera *Hydromantes, Speleomantes, Batrachoseps,* and *Plethopsis* is well supported, with bootstrap values of 90% or higher and decay indices of from 5 to 20. Monophyly of *Batrachoseps* + *Bolitoglossa* is supported by a bootstrap value of 82% and a decay index of 6.

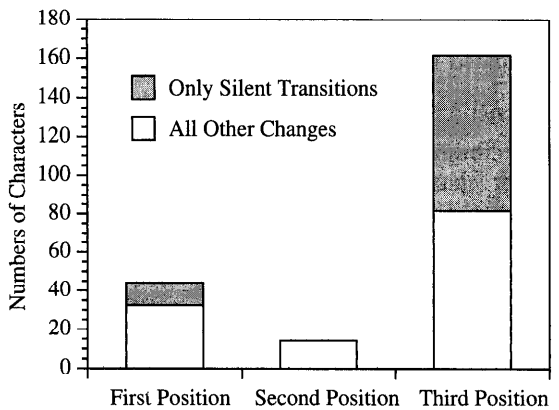

FIG. 2.—Informative sites at each codon position. The number of informative sites at each codon position is indicated, with sites characterized by only silent transition changes indicated by the shaded portion of the first and third position bars.

A maximum-likelihood tree based on the sequence data has a likelihood length of −4297.2 (not shown). The topology of this tree is identical to the topology of the combined analysis. As in the maximum-parsimony tree, both *Hydromantes* and *Batrachoseps* are monophyletic groups, each containing two monophyletic subgroups (the subgenera), and *Batrachoseps* is the sister taxon of *Bolitoglossa.*

The data sets are highly congruent as measured by two congruence statistics (Swofford 1991*b*). IMF = 0.0103 and IM = 0.05138. D_{xy}, the incongruence length difference (Farris et al. 1995), is 5. The measured incongruence is insignificant when applying Farris et al.'s (1995) test of congruence for the two data sets (P = 0.60). The results of the Templeton (1983) tests are: (1) the molecular tree is significantly less parsimonious than the morphological tree when the morphological data alone are considered (T_S, the test statistic = 10, n = 20, $P < 0.005$), (2) the tree produced from the combined data is not significantly less parsimonious than the morphological tree, (3) the morphological tree is not significantly less parsimonious than the molecular tree, and

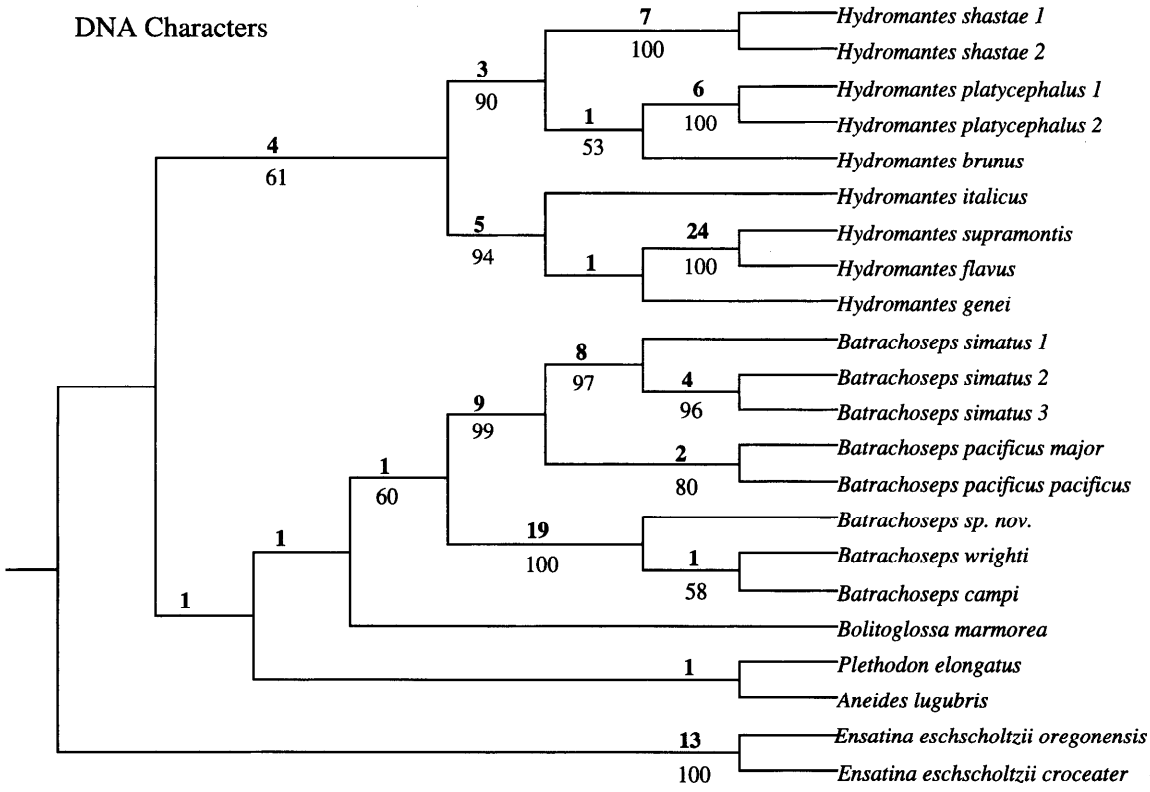

FIG. 3.—The single most-parsimonious tree with all DNA data, all characters unordered. The tree is 870 steps long and has a consistency index of 0.43. Bootstrap values (percentages) from 1,000 replicates are indicated below the branches, and decay index values are indicated above.

(4) the tree produced from the combined data is not significantly less parsimonious than the molecular tree.

Discussion

The low IM and IMF values as well as the insignificance of the results of the test of incongruence reflect the overall congruence between the morphological and molecular subsets of the total data, and the signed-ranks test reveals incongruence between the separate hypotheses based on the two data sets. As Farris et al. (1995) pointed out, any test which fails to consider the strength of a particular hypothesis may appear to reveal incongruence when it does not exist. In this case, the molecular evidence weakly supports a nonmonophyletic Bolitoglossini. This poorly supported hypothesis strongly conflicts with the morphological data; because the Templeton test does not consider the strength of this alternative hypothesis, it is rejected as a significantly less parsimonious tree. The incongruence test of Farris et al. (1995) and the IM and IMF values more accurately represent the fact that no strongly supported branch from the tree based on one subset of the total data conflicts with a well-supported branch based on the other subset of the data. Therefore, in spite of a significantly less parsimonious molecular data set hypothesis compared to the morphological hypothesis using morphological data alone, we conclude that almost no conflict exists between the two data sets.

In prior studies, support for monophyly of the Bolitoglossini was perceived to be great, but the relationships of *Hydromantes, Batrachoseps,* and *Bolitoglossa* were equivocal, with equal support for sister group relationships between (1) *Hydromantes* and *Bolitoglossa,* and (2) *Batrachoseps* and *Bolitoglossa* (Lombard and Wake 1986). The former relationship has been supported mainly by features of their far-reaching, fully projectile tongues, traits that are likely candidates for homoplasy (Lombard and Wake 1977). The latter relationship has been supported mainly by two traits uniquely derived in the family: reduction in the number of chromosomes from 14 to 13 and presence of a lateral tab on the parietal bone of the skull. There was no support for a *Hydromantes* + *Batrachoseps* sister group.

Our reanalysis of the morphological data utilizes mainly the same characters (but unordered) of Lombard and Wake (1986), plus 10 additional characters that were not relevant to the taxa in the earlier study. Once again, support for the Bolitoglossini is great, with 12 characters changing unambiguously between the Plethodontini and the Bolitoglossini. The potential sister groups *Hydromantes* + *Bolitoglossa* and *Batrachoseps* + *Bolitoglossa* are each supported by four characters (not shown).

In contrast, the DNA characters provide more support for a *Batrachoseps* + *Bolitoglossa* sister group (7) (fig. 6) than for a *Hydromantes* + *Bolitoglossa* sister group (2). Only two characters support monophyly of the Bolitoglossini.

Treated separately, the morphological and molecular data provide strong support for some nodes but not for others. The molecular data do not provide support

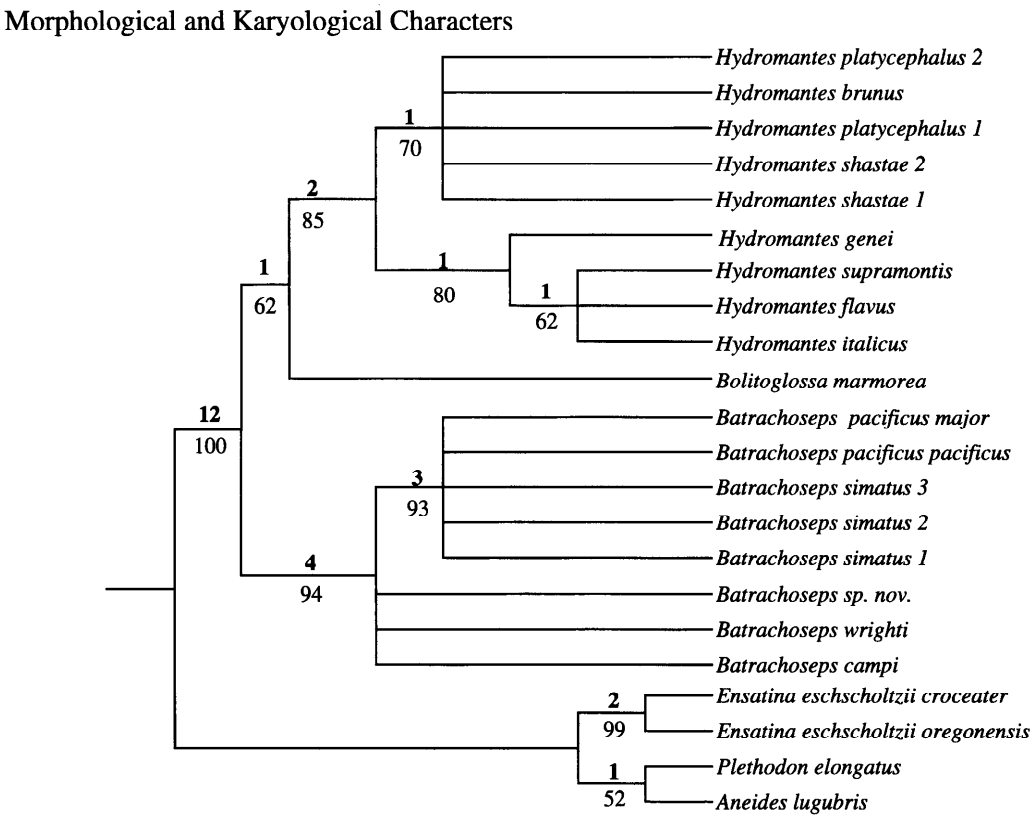

FIG. 4.—A strict consensus of the four most-parsimonious trees with morphological data analyzed unordered. The tree is 57 steps long and has a consistency index of 0.91. Bootstrap values from 1,000 replicates are indicated below the branches, and decay index values are indicated above.

for a monophyletic Bolitoglossini, but do not strongly support an alternative. However, the combined data act synergistically to give good support to all of the nodes in our favored tree (fig. 5). The morphological data weakly support a sister group relationship between *Bolitoglossa* and *Hydromantes* and strongly support a monophyletic Bolitoglossini. The molecular data strongly support *Bolitoglossa* and *Batrachoseps* as sister taxa. Both the decay index and the number of characters changing unambiguously along the branch leading to *Bolitoglossa* + *Batrachoseps* in the combined analysis are greater than the sum of the two data sets taken by themselves (figs. 5 and 6). The morphological data essentially constrain the Bolitoglossini to be monophyletic, overriding the DNA trees, in which *Plethodon* and *Aneides* are closely related to *Batrachoseps* + *Bolitoglossa,* and greatly add to DNA support for a monophyletic *Batrachoseps* + *Bolitoglossa.* Thus, the *Batrachoseps* + *Bolitoglossa* sister group is supported by 13 unambiguous character changes, 3 morphological and 10 molecular (fig. 6; seven unambiguous molecular changes occur in the molecular-only analysis). In contrast, the *Hydromantes* + *Bolitoglossa* sister group is supported by only six characters, four morphological and two molecular. Recent divergences are supported by silent transitions, and older divergences are supported by a combination of morphological, karyological, and DNA transversion characters.

A number of researchers have explored the consequences of analyzing different data sets separately or combining the data sets for analysis (reviewed by de Queiroz, Donoghue, and Kim 1995), and there have been recent theoretical (Huelsenbeck, Bull, and Cunningham 1996) as well as empirical (Sites et al. 1996) studies. In cases of conflict known to us, robustness of the alternative phylogenetic hypotheses has not been equivalent (e.g., Sites et al. 1996). Our result suggests that combined analyses will be successful in circumstances in which the separate data sets are appropriate and robust, for different phylogenetic depths.

An unanticipated outcome of these studies is the contrast in the degree of differentiation of the two clades that were our primary focus, the genera *Hydromantes* and *Batrachoseps.* The former has an extraordinary distribution, with one group of species in California and the other in extreme southeastern France, in northwestern to central Italy, and on the island of Sardinia. *Batrachoseps,* which is primarily distributed in California, with one species endemic to Oregon, is more differentiated with respect to the molecular data than is *Hydromantes,* implying that the two clades within each genus are at least equally old, with those of *Batrachoseps* probably being older. Wake, Maxson, and Wurst (1978) used a combined allozyme and immunological data set to examine relationships in *Hydromantes* and found concordant results, suggesting that the two clades within the

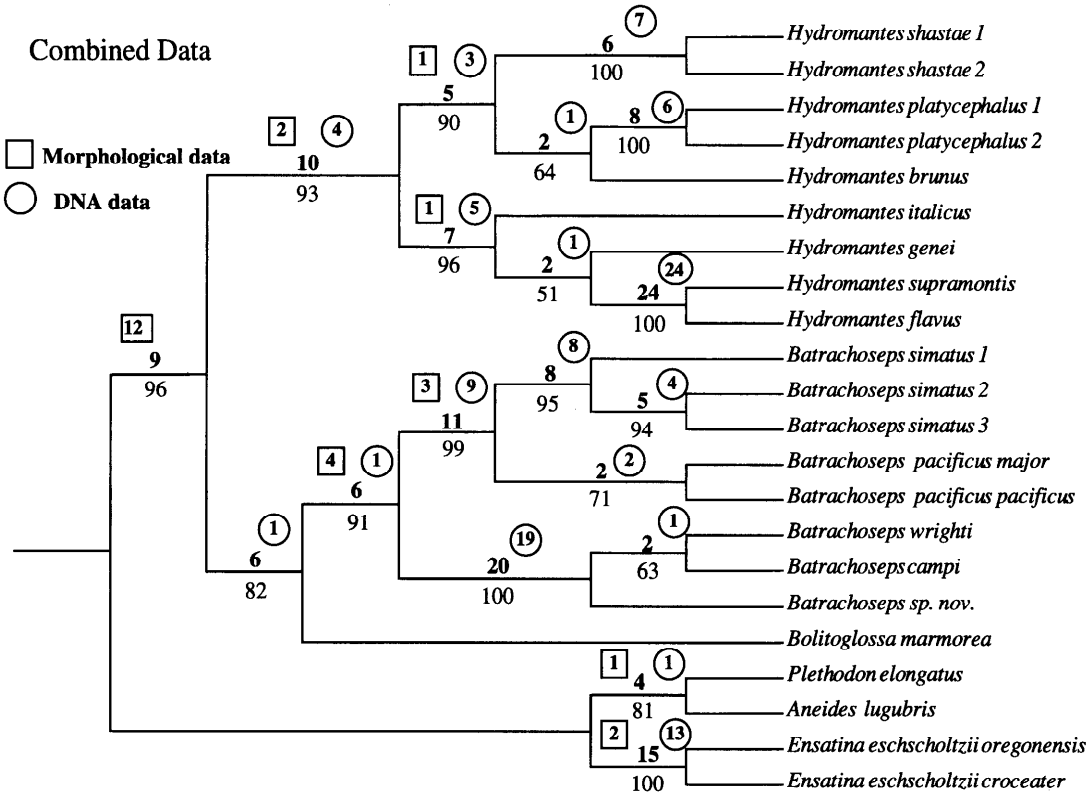

FIG. 5.—The single most-parsimonious tree using all data combined, all characters unordered. The tree is 932 steps long and has a consistency index of 0.45. Bootstrap values from 1,000 replicates are indicated below the branches and decay index values are indicated above. Numbers of morphological (box) and molecular (circle) characters for each node are indicated. The decay indices from each of the data sets by themselves are also indicated.

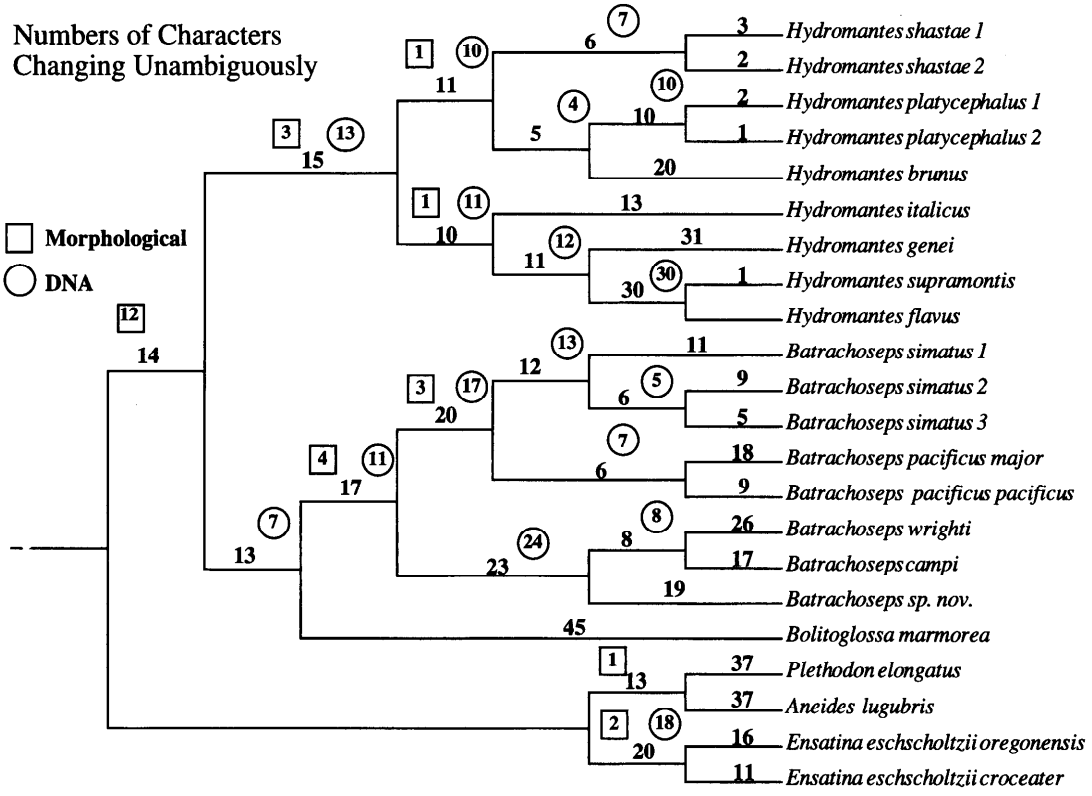

FIG. 6.—Numbers of characters changing unambiguously in the most parsimonious tree with the data combined. Changes from the separate analyses are indicated above the values from the combined analysis.

genus separated approximately 28 MYA, an awkward timing that does not correspond to possible vicariant events in earth history. The most recent land connection across the present Atlantic Ocean was on the order of 50 MYA, while the connections across the Pacific Ocean via the Bering Land Bridge occurred during the past one million years. Nascetti et al. (1996) used a larger number of proteins than did Wake, Maxson, and Wurst (1978) and measured Nei genetic distances of essentially infinity between the two subgenera; they declined to speculate on timing of the divergence of the two clades. Lanza and Vanni (1981) favored the 50-million-year date (their reasoning was questioned by Sage, Prager, and Wake 1982). In an overview and discussion of the historical biogeography of the genus *Hydromantes,* Lanza et al. (1995) favored the 50-million-year date but offered little to support that conclusion other than the date of the north Atlantic intercontinental land connection.

The two clades of *Hydromantes* share a remarkable, highly specialized, and unique tongue projection mechanism that is complex and involves many integrated parts—skeletal, muscular, and neurological. Lanza and Vanni (1981) and Lanza et al. (1995) raise what is admitted to be a remote possibility: that *Hydromantes* is polyphyletic, and the two clades are morphologically convergent. While an extremely high allozyme genetic distance might support such a suggestion, our data refute this hypothesis. Furthermore, our data suggest that if molecular evolution has proceeded at the same approximate rate in *Hydromantes* and *Batrachoseps,* the subclades of *Batrachoseps* (which have remained in western North America, presumably where they arose) are older than the subclades within *Hydromantes,* one of which has wandered to the Old World (all other plethodontids are restricted to the New World). We believe that the unique morphological features (tongue, skull, tail, feet) and unique synapomorphies that characterize all of its species argue in favor of continued recognition of a single monophyletic genus, *Hydromantes.*

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