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MITOCHONDRIAL DNA RATES AND BIOGEOGRAPHY IN EUROPEAN NEWTS (GENUS *EUPROCTUS*)

ADALGISA CACCONE,[1,2] MICHEL C. MILINKOVITCH,[2] VALERIO SBORDONI,[1] AND JEFFREY R. POWELL[2]

[1]*Dipartimento di Biologia, Università di Roma "Tor Vergata," 00133 Rome, Italy*
[2]*Department of Biology, Yale University, New Haven, Connecticut 06520, USA;*
E-mail: caccone@beagle.biology.yale.edu

Abstract.—Sequence divergence for segments of three mitochondrial DNA (mtDNA) genes encoding the 12S and 16S ribosomal RNA and cytochrome *b* was examined in newts belonging to the genus *Euproctus* (*E. asper, E. montanus, E. platycephalus*) and in three other species belonging to the same family (Salamandridae), *Triturus carnifex, T. vulgaris,* and *Pleurodeles waltl*. The three *Euproctus* species occur (one species each) in Corsica, Sardinia, and the Pyrenees. This vicariant distribution is believed to have been determined by the disjunction and rotation of the Sardinia–Corsica microplate from the Pyreneean region. Because time estimates are available for the tectonic events that led to the separation of the three landmasses, we used sequence data to estimate rates of evolution for the three gene fragments and investigated whether they conform to the rate-constancy hypothesis. By the Tajima (1993, Genetics 135:599–607) test, we could not detect rate heterogeneities for the ribosomal genes and for transversions in the cytochrome *b* gene. Assuming that these sites are evolving linearly over time and that cessation of gene flow occurred simultaneously with vicariant events, we compared the time of divergence estimated by molecular distances with the divergence times based on the geological estimates. Because we have two estimates of divergence time from the geological record, the split of Corsica/Sardinia from the Pyrenees and the split of Corsica from Sardinia, we could compare ratios of molecular divergence with the ratio of geological time divergence. The ratios are very similar, indicating that the molecular clock hypothesis cannot be rejected. These geological events also allowed us to calculate absolute rates of evolution for ribosomal and cytochrome *b* genes and compare them to rates for the same regions in other salamandrids and other vertebrates. Ribosomal mtDNA rates are comparable to those reported for other vertebrates, but cytochrome *b* rates are 3–7 times lower in salamanders than in other ectotherms. From a phylogenetic perspective, our data suggest that the cladogenic events leading to species formation in *Euproctus* and *Triturus* occurred very closely in time, indicating that the two genera may not be monophyletic. A duplication of the cytochrome *b* gene in *T. carnifex* was found, and the implications of this finding for mtDNA phylogenetic studies are discussed. [Biogeography; cytochrome *b*; *Euproctus*; mitochondrial DNA rates; mitochondrial ribosomal genes; molecular clocks; *Triturus*.]

The ability to accurately estimate the times of certain evolutionary events is crucial in many areas of evolutionary biology, from population genetics to organismal evolution. In particular, molecular datings have always been very appealing because they may be used when no other information, such as stratigraphy and paleontology, is available for time estimates. However, the use of molecules as time indicators relies on assessing whether molecular divergence is linear over time. This issue has been controversial since its introduction by Zuckerkandl and Pauling (1965) as the molecular clock or rate-constancy hypothesis.

Since its first formulation as a universal clock ticking at similar rates in all organisms, a number of studies have challenged this notion, showing that nucleotide substitution rates vary dramatically within gene regions and among divergent taxonomic groups, in both the nuclear and mitochondrial genome (Wu and Li, 1985; Britten, 1986; Powell et al., 1986; Vawter and Brown, 1986; Springer and Kirsch, 1989; Bulmer et al., 1991; Avise et al., 1992; Martin et al., 1992). For mitochondrial DNA (mtDNA), the substitution rate of 1–2%/million years, based on primate mtDNA (Brown et al., 1979, 1982) and commonly used for vertebrates, has been tested in different groups of vertebrates and different genes. Based mainly on ribosomal RNA (rRNA) and cytochrome *b* genes, the results of these studies confirm

the existence of rate heterogeneities not only among different genes but also for the same gene among different vertebrate groups (Meyer and Wilson, 1990; Mindell and Honeycutt, 1990; Hillis and Dixon, 1991; Allard et al., 1992; Martin et al., 1992; Cantatore et al., 1994; Martin 1995a, 1995b).

Although several hypotheses, such as generation time (Wu and Li, 1985; Li et al., 1996), DNA repair efficiency (Britten, 1986), nucleotide generation time (Martin et al., 1992), metabolic rates, and directed nucleotide substitutions (Martin and Palumbi, 1993; Rand, 1994; Martin, 1995b), have been used to explain rate differences among groups, identifying the factors or combination of factors that affect nucleotide substitution rates among distantly related taxa is a difficult task. Species groups that have diverged over long evolutionary periods are likely to differ for many other life history and physiological traits in addition to those under scrutiny. Because of these observations, there is a general consensus that using the molecular clock hypothesis in comparisons among distantly related taxa must be done with extreme caution and perhaps avoided altogether.

Alternatively, this concept has been generally accepted for comparisons among closely related species (but see Zhang and Ryder, 1995, for an interesting exception); because they share a recent common evolutionary history, such species are less likely to differ significantly in generation time, metabolic rate, body size, or other physiological and life history traits than are more distantly related taxa (Hillis et al., 1996).

Once the extent and reliability of the molecular clock in a particular group has been evaluated, one common, although controversial, application of the constant-rate hypothesis is its calibration against absolute time, which requires the availability of independent time estimates. Geological dating is the most common form of independent timing, with dates for the cladogenic events of interest obtained from the fossil record and/or from a well-dated biogeographic scenario. Dates based on fossils are usually considered preferable to biogeographic dates because they should provide both minimum and maximum time estimates. However, these estimates strongly depend on the resolution of the fossil record (Marshall, 1990). Datings based on biogeography must be treated cautiously as well; it is often quite difficult to demonstrate an association between a specific cladogenic event and a biogeographic scenario unless there are many representatives from taxonomically diverse groups that have been affected by the same vicariance event (e.g., Knowlton et al., 1993).

One well-dated geological event that influenced the distribution of several groups of organisms in the Mediterranean region is the disjunction and rotation of the Corsica–Sardinia microplate from the Iberian Peninsula. Based on paleomagnetic and stratigraphic data, Corsica and Sardinia split from the continent about 29 million years ago (MYA) as a single landmass. The rotation and disjunction of the two islands started 15 MYA and was completed by 9 MYA (Alvarez, 1972, 1974; Alvarez et al., 1973; Bellon et al., 1977; Bonin et al., 1979; Orsini et al., 1980; Cherchi and Montadert, 1982; Esu and Kotsakis, 1983). Presently, there are several taxonomically diverse groups with closely related species (possibly sister taxa) on each of the three landmasses (Corsica, Sardinia, Pyrenees). Most of these taxa have poor dispersal capabilities, and thus it is likely that their distributions have resulted from vicariance rather than dispersal. Obviously, it is still possible that the cladogenic events predated the geological events. However, several groups of plants and animals show concordant distributions, which suggests that a single event was responsible (Sbordoni et al., 1990).

In this study, we utilized a group of newts belonging to the genus *Euproctus*, whose distribution traditionally has been associated with the disjunction of the Sardinia–Corsica microplate from the Pyrenees. Because the timing of these microplate movements is well established, these species provide an opportunity to calibrate molecular rates against absolute time. The

three species included in the genus *Euproctus* are confined to mountain streams in the Pyreneean region (*E. asper*), in Corsica (*E. montanus*), and in Sardinia (*E. platycephalus*). To place this genus in a phylogenetic framework, we also analyzed multiple outgroups (Watrous and Wheeler, 1981; Maddison et al., 1984) that were expected to have different levels of divergence from *Euproctus*. We used two species within the genus *Triturus*, which is supposed to be the closest relative to the genus *Euproctus*, and *Pleurodeles waltl*, a species included in the same family as the other species (Salamandridae) but more distantly related to *Euproctus* than is *Triturus* (Wake and Özeti, 1969). Because the main aim of this study was to examine molecular rates and not phylogenetic relationships, we included in this study only two *Triturus* species as representative of the two main lineages of the genus, *T. vulgaris* and *T. carnifex*, a small-bodied species and a large-bodied species belonging to the subgenera *Paleotriton* and *Triturus*, respectively.

We estimated molecular rates and calibrated them against absolute time for the three most commonly studied mtDNA genes (cytochrome *b* and 12S and 16S rRNA) for a total of 1,617 bp of mtDNA. We investigated whether sequence divergence was accumulating according to the rate-constancy hypothesis along the *Euproctus* lineages by using Tajima's rate test (Tajima, 1993). The results of this test were then checked against the time estimates derived from geology. Molecular rates for the three mtDNA regions were obtained and compared with similar estimates from other vertebrates in light of the reported findings of differential rates between endotherms and ectotherms. The gene-specific rates presented here may be useful for inferring times of divergence in other salamandrids, and possibly other amphibians, where no dating from fossil and/or biogeographic sources is available. Phylogenetic analyses were also conducted to investigate the relationships among the three *Euproctus* species in light of recent molecular data that have brought into question the monophyly of this genus and of the genus *Triturus* (Caccone et al., 1994; Titus and Larson, 1995). We also present evidence of a duplication of the cytochrome *b* gene in *T. carnifex*. Although the two cytochrome *b* fragments have large sequence divergence, they differ only by a 1-bp deletion. This finding is used as an example of the potential danger of using paralogous gene sequences in mtDNA phylogenetic studies.

Material and Methods

We used three individuals each for *E. montanus* and *E. platycephalus*, two individuals for *E. asper*, and one each for *T. vulgaris*, *T. carnifex*, and *P. waltl*. The specimens of *E. montanus* were from Corsica (France), *E. platycephalus* samples were collected in Belví (Sardinia, Italy), *E. asper* samples were obtained from a breeding colony in the Laboratoire Souterrain du CNRS (Moulis, France), *T. vulgaris* and *T. carnifex* samples were from Nettuno (Latina, Latium, Italy), and the *P. waltl* sample was obtained from the Museum of Vertebrate Zoology (University of California, Berkeley; sample FC-11135).

DNA was extracted following the method of Caccone et al. (1994). Modified primer pairs L1091 + H1478 (Kocher at al., 1989) and 16Sar + 16Sbr (Palumbi et al., 1991) were used for polymerase chain reaction amplifications of 358 bp and 558 bp of the 12S and 16S rRNA genes, respectively. Modified primers cyt *b* GLU (5'-TGACATGAAAAAYCAYCGTTG, LI14724; Pääbo, 1990), cyt *b* B2 (5'-CCCTCAGAAT GATATYTGTCCTCA, H15149; Kocher et al., 1989), MVZ 15 (5'-GAACTAATGG CCCACACWWTACG; Moritz et al., 1992), MVZ 25 (5'-TAAAGAAACATGAAAYAT TGG; Moritz et al., 1992), and MVZ 18 (5'-GTCTTTGTATGAGAAGTATG; Moritz et al., 1992) were used to amplify 684 bp of the cytochrome *b* gene. Details of both amplification and sequencing protocols have been reported by Caccone et al. (1994). Sequences were determined with an automated sequencer (Applied Biosystems 373A) following the manufacturer's protocols. To ensure accuracy, strands

were sequenced in both directions for each individual.

Sequences were aligned using the program MALIGN (version 1.5, Wheeler and Gladstein, 1993). Indels (insertions/deletions) were coded as additional single characters. With one exception of a duplicate cytochrome *b* copy in *T. carnifex*, no indels were found in the cytochrome *b* gene. Few indels were present in both rRNA genes. When indels of different lengths overlapped, each size class was considered a different character state. Aligned sequences were analyzed by the maximum parsimony (Farris, 1970), maximum likelihood (Felsenstein, 1981), and neighbor joining (Saitou and Nei, 1987) methods. The robustness of the phylogenetic hypotheses was tested by 1,000 bootstrap replicates (Felsenstein, 1985). Most-parsimonious (MP) trees were derived by the branch-and-bound search (Hendy and Penny, 1982) as implemented in PAUP 3.1.1 (Swofford, 1993) with ACCTRAN optimization. The consistency index (CI; Kluge and Farris, 1969) was used to examine overall levels of homoplasy of the data. Consistency indices were calculated after the exclusion of uninformative characters (Sanderson and Donoghue, 1989). The MP tree and the shortest trees supporting competing phylogenetic hypotheses were compared using Templeton's (1983) test, as detailed by Larson (1994).

We performed a phylogenetic analysis of the 12S and 16S rRNA sequences with the following parameters: including or excluding gaps, all substitutions unweighted, transversions weighted three times more than transitions, or using only transversions. For the cytochrome *b* sequences, additional analyses excluding transitions in third positions of all codons and in first positions of leucine codons were performed (Irwin et al., 1991). A similar transversion/transition weighting scheme was used in the maximum likelihood (ML) analyses. ML distances were calculated using the program DNADIST in PHYLIP 3.5 (Felsenstein, 1993). Additionally, we estimated four-parameter (TK) distances (Takahata and Kimura, 1981). Distances based only on transversions were calculated by the Jukes–Cantor (JC) method (Jukes and Cantor, 1969). For cytochrome *b*, TK distances were calculated for all positions and for third codon positions only, using a computer program written by Etsuko Moriyama; the same program provided base compositions for all sequences.

Rate homogeneity for nucleotide substitutions among lineages was tested by the Tajima method (Tajima, 1993). Given three aligned sequences (nos. 1, 2, 3) and excluding sites with gaps, the number of sites in which nucleotides in sequence 1 differ from those in sequences 2 and 3 (m_1) is calculated. Similarly, the number of site differences between sequence 2 and sequences 1 and 3 (m_2) and between sequence 3 and sequences 1 and 2 (m_3) is obtained. In mitochondrial sequences, where transitions could occur more frequently than transversions, Tajima (1993) suggested performing the test on the two classes of changes separately. Thus, m_1 is divided into the number of transition (s_1) and transversion (v_1) differences. The null hypothesis of this test is that the expected number of substitutions is the same in sister lineages (so that the observed number of substitutions follows a Poisson distribution). When species 3 is the outgroup, this equality can be tested by using a chi-square test with two degrees of freedom: $\chi^2 = [(s_1 - s_2)^2/(s_1 + s_2)] + [(v_1 - v_2)^2/(v_1 + v_2)]$. This method is called the 2D method. The chi-square test can also be done when the outgroup among three sequences is not known (2DN method). If we consider the same three sequences (1, 2, 3) with m_1, m_2, and m_3 as the number of differences among them, we choose the two sequences that have the smallest value among m_1, m_2, and m_3 and compute a chi-square value as in the previous case. Through a series of computer simulations, Tajima (1993) showed that the above methods are appropriate over a wide range of parameters, suggesting that they are essentially similar in power to the two-parameter likelihood ratio test (Muse and Weir, 1992) and the relative rate test of Wu and Li (1985). The power of the 2D method

decreases as outgroup branch lengths increase, and it is a function of the number of nucleotides examined and the number of observed substitutions.

RESULTS

Sequence Variation

For all individuals, we sequenced 358 bp of the 12S rRNA gene (about 44% of the gene), 558 bp of the 16S rRNA gene (about 34% of the gene), and 684 bp of the cytochrome *b* gene (including 85% of the gene and a few bases of the transfer RNA for glutamic acid [tRNA GLU]). Conspecific individuals had identical sequences. The alignment is shown in Figure 1, followed by the additional indel characters used in the phylogenetic analyses.

Percentages of A + T composition, variable sites, and informative sites in the three gene fragments are shown in Table 1. As expected, third codon positions of cytochrome *b* are most variable and most A + T biased (63.4%). In 12S, 80.1% of all substitutions are transitions, and in 16S and cytochrome *b*, 67.1% and 65.3%, respectively, are transitions. TC and AG transitions occur with similar frequencies in the ribosomal genes, but TC transitions outnumber AG transitions in cytochrome *b* by between 2:1 and 3:1. AT and AC transversions account for the majority (70–94%) of all transversions. In cytochrome *b*, 76.7% of all transitions occur in third positions. About 88% of transversions are in third positions, constituting 30.5% of all changes. Transition/transversion ratios ranged from 4.8 to 1.6 in the 12S gene, from 7.0 to 1.37 in the 16S gene, and from 3.5 to 1.3 in the cytochrome *b* gene.

The number of nucleotide substitutions per site was calculated for the three gene fragments both separately and combined, using TK and ML distances. Ribosomal gene TK distances ranged from 0.07 to 0.13, and pairwise distances for the 12S gene were smaller than those for the same pairwise comparison in the 16S gene. Cytochrome *b* TK distances ranged from 0.11 to 0.26 using all positions and from 0.38 to 1.46 using only third codon positions. The TK distances for the combined data set ranged from 0.09 to 0.18 (distance matrices are available from the corresponding author upon request). The smallest distances always occurred between the two island species of *Euproctus*, *E. platycephalus* and *E. montanus*. The largest distances included the majority of the comparisons of *P. waltl* with all other taxa.

Phylogenetic Analyses

Initially, we assumed all character transformations to be equally probable and analyzed all characters with equal weighting with no phylogenetic constraints. The stability of these results was then evaluated by comparison with the results of weighted searches where we down-weighted transitions; we used a 3/1 transversion/transition cost (according to the observed transition/transversion ratios) and also used only transversions. We also analyzed the combined data set (rRNA plus cytochrome *b* genes) using equal weighting for transitions and transversions in the rRNA genes and only transversions for the cytochrome *b* gene in all codon positions or only in third codon positions.

Figure 2a summarizes the MP analyses for the combined ribosomal genes. The different weighting methods yielded a single MP tree with the same topology. For the unweighted and the transversions-only searches, trees constrained by monophyly for both *Euproctus* and *Triturus* were 14 and 4 steps longer than the MP tree, respectively. For the same two searches, trees having either *Euproctus* or *Triturus* monophyletic were 10 and 4 steps longer than the MP tree, respectively. Analyses of ribosomal genes separately yielded trees with identical topology. Figure 2b shows the MP tree for the cytochrome *b* gene. A single MP tree with the same topology was obtained using different weighting methods. This tree differed from the tree in Figure 2a in the placement of *T. vulgaris*. For the unweighted and transversions-only searches, constrained trees for which both *Euproctus* and *Triturus* were monophyletic were 11 and 15 steps longer than the MP tree, respectively. For the search including

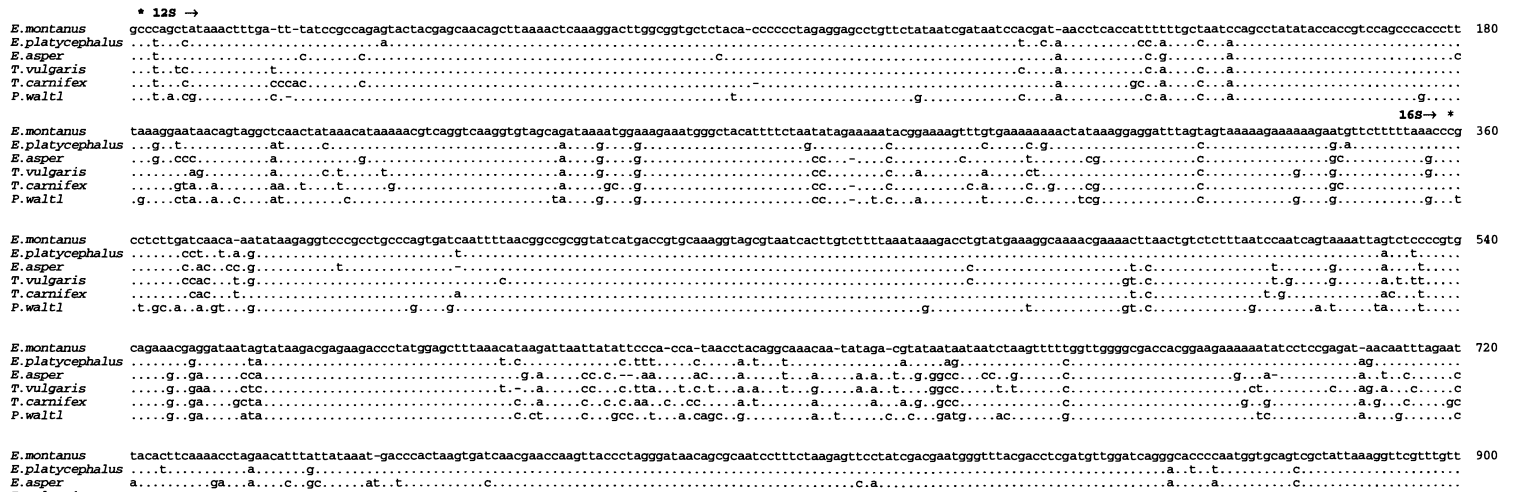

FIGURE 1. Sequence data for the 12S and 16S rRNA and cytochrome *b* genes. A complete nucleotide sequence is given for *Euproctus montanus*. Other sequences with nucleotides identical to *E. montanus* are represented by dots. Unknown characters are indicated with question marks; dashes indicate alignment gaps. Sites 1–358 correspond to the 12S rRNA gene; sites 359–916 correspond to the 16S rRNA gene; and sites 935–1,600 correspond to the cytochrome *b* sequence. The additional gap characters are shown at positions 1,601–1,617. Asterisks mark the start of each gene fragment.

TABLE 1. Percentage of variable and informative sites by gene and codon positions across the six taxa of newts studied and A + T percentages for the same regions.

Region	% variable	% informative	No. sites	% A + T
12S	20.7	8.1	358	58.9
16S	23.3	12.4	558	59.6
Cytochrome *b*				
First codon	26.1	10.8	222	56.8
Second codon	3.2	1.8	222	59.9
Third codon	79.7	60.4	222	63.4
All	33.8	24.3	666	59.8

transitions and transversions in first and second positions and only transversions in third positions, trees with either *Euproctus* or *Triturus* monophyletic were 4 and 8 steps longer, respectively. For the search using only transversions, trees with either *Euproctus* or *Triturus* monophyletic were 15 and 11 steps longer, respectively.

Figure 3 summarizes the MP analyses combining the three data sets. We obtained a single MP tree with the same topology for all the weighting schemes tested. Table 2 presents a summary of the phylogenetic results with different weighting schemes, including bootstrap values and number of additional steps needed to obtain the shortest tree having *Euproctus* and *Triturus* monophyletic. The highest bootstrap values occurred when transitions were globally down-weighted or excluded and when they were excluded from only the cytochrome *b* data set. ML and neighbor joining (NJ) trees of the combined ribosomal genes, cytochrome *b* only, and all three genes together produced trees identical to the MP trees for the same data sets (Figs. 2, 3).

We used Templeton's (1983) test to determine whether we could reject the hypotheses of monophyly for the two genera (*Euproctus* and *Triturus*). We tested the

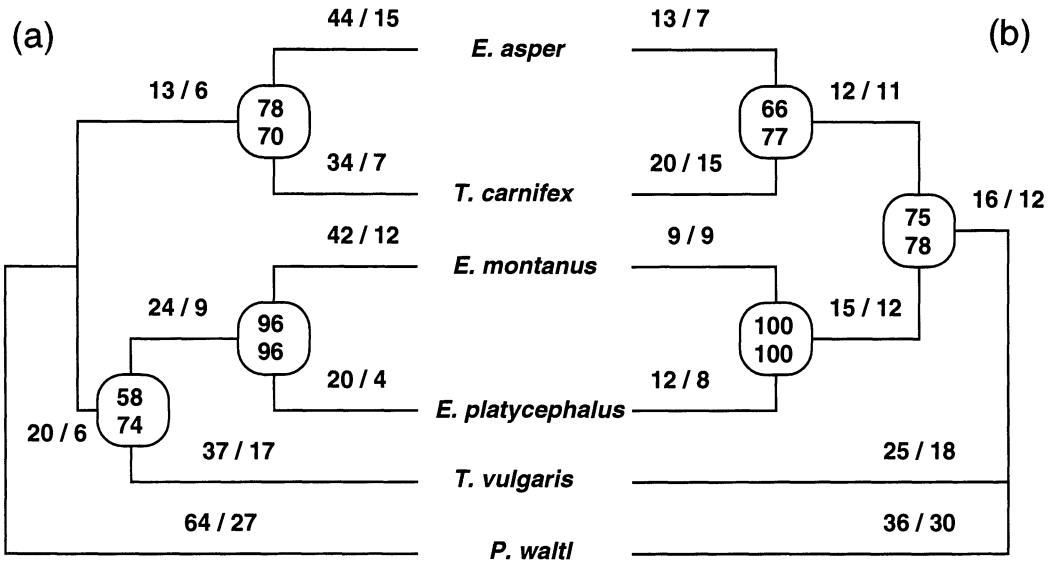

FIGURE 2. Maximum parsimony trees for species of *Euproctus*, *Triturus*, and *Pleurodeles*. (a) Tree for the rRNA sequences. The first numbers on each branch are the number of changes when all characters (length = 298, CI = 0.662) were included. The second numbers on each branch are the number of changes when only transversions (length = 103, CI = 0.600) were included. Circled nodes include bootstrap percentages for the unweighted search (top number) and for the search using only transversions (bottom number). (b) Tree for cytochrome *b* sequences. The first numbers on each branch are the branch lengths based on equal weighting of all substitutions types and using only transversions in third positions and first positions of leucine codons (length = 158, CI = 0.704); the second numbers on each branch are the branch length using only transversions (length = 122). Circled nodes include bootstrap percentages for the first (top number) and second (bottom number) searches.

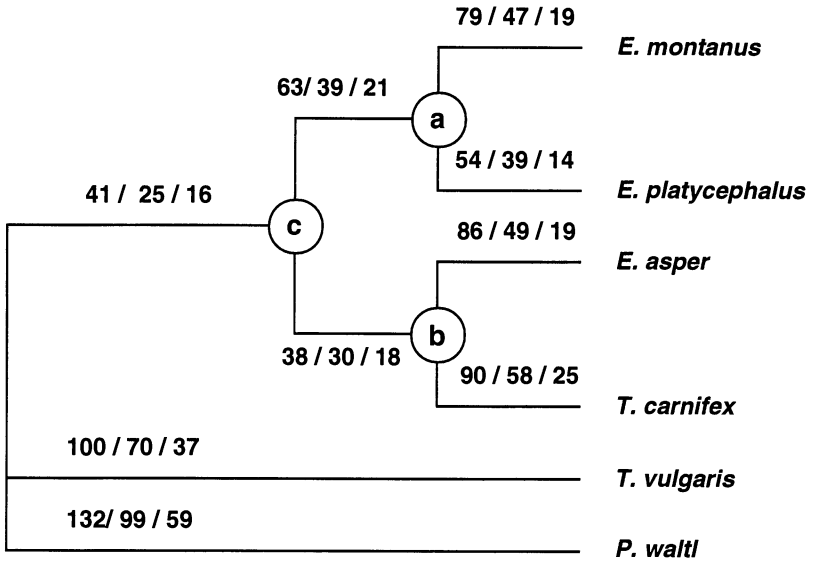

FIGURE 3. MP tree based on the combined *Euproctus, Triturus,* and *Pleurodeles* data sets. Numbers on branches are branch lengths. On each branch, the first number is based on an unweighted search (length = 683, CI = 0.651), and the second number is based on a search using all substitutions with no weights for the rRNA genes and only transversions for the cytochrome *b* gene (length = 456, CI = 0.652). The third number on each branch is based on a search using only transversions (length = 228, CI = 0.500) for all three genes. In all cases the additional gaps characters were included. Bootstrap values for the circled nodes (a, b, c) are listed in Table 2.

combined data set (ribosomal genes plus cytochrome *b*) using equal weighting for all characters, transversions only in the three genes, and equal weighting for the rRNA genes but only transversions for the cytochrome *b* gene (Table 2). Using equal weights for transitions and transversions and including gaps, the shortest tree with *Euproctus*, or *Triturus*, or both genera monophyletic required 10, 6, and 18 steps, respectively, more than the MP tree. However, these trees were not significantly different from the MP tree (significance was only borderline for the *Triturus* monophyly tree), as implied by the results of Templeton's tests. Using only transversions and including gaps, the MP tree was 6, 3, and 16 steps shorter than trees supporting either of the two genera or both genera being monophyletic. Although the shortest trees supporting monophyly for either *Euproctus* or *Triturus* were not significantly different from the MP tree (again, significance was only borderline for the *Triturus* monophyly tree), the shortest tree supporting monophyly for both genera was significantly different from the MP tree (Table 2). When we differentially weighted substitutions in the three genes (equal weights for transitions and transversions in the rRNA genes and using only transversions in cytochrome *b*), the MP trees obtained were always highly significantly different from the topologically constrained trees ($n = 17$, Wilcoxon's $Ts = 18$, $P \ll 0.009$ for monophyly of *Euproctus*; $n = 47$, $Ts = 360$, $0.05 > P > 0.02$ for monophyly of *Triturus*; $n = 39$, $Ts = 120$, $P \ll 0.009$ for monophyly of both genera, including gaps).

Extra Copy of Cytochrome b in Triturus carnifex

Figure 4 shows the sequences of two different 388-bp cytochrome *b* fragments found in the same individual of *T. carnifex*. Initial amplification and sequencing of this specimen using a conserved primer pair (cyt *b* GLU–cyt *b* B2) revealed a copy of the gene, referred to hereinafter as extra cytochrome *b*, with many substitutions and a 1-bp deletion at position 417. This

TABLE 2. Summary of phylogenetic analysis for the combined data set (rRNA genes and cytochrome *b*) using maximum parsimony (MP) and neighbor joining (NJ). For the MP searches, we used different combinations of substitutions (transitions [TS], transversions [TV]) and different substitution weights (TS/TV = 1/1, 1/3, 1/2), including or excluding gaps (in or out). For each search, we report the bootstrap percentages for each node (see Fig. 3), the total tree lengths of topologically unconstrained trees, and the number of additional steps needed to obtain alternative topologies with *Euproctus*, *Triturus*, and both genera monophyletic.

	MP								
	TS + TV								
	1/1		1/3		TV		TS + TV rRNA + TV cyt *b*[a]		
	In	Out	In	Out	In	Out	In	Out	NJ[b]
Bootstrap values									
Node a	100	100	100	100	100	100	100	100	100
Node b	61	66	80	86	70	78	82	85	78
Node c	54	53	72	79	65	73	76	80	55
Tree lengths, unconstrained	703	682	1,102	1,081	228	207	471	450	
No. extra steps, monophyly constrained[c]									
Euproctus	10	11	24	25	6	7**	13***	14***	
Triturus	6*	6*	32	32	3*	3*	17***	17***	
Both	18	19**	52	43	16***	17***	26***	27***	

[a] Search with transitions and transversions weighted equally for the rRNA genes and only transversions included for the cytochrome *b* gene.

[b] Majority consensus tree among the 100 NJ trees produced from the 100 maximum-likelihood distance matrices generated after bootstrap resampling of the original data set. Analyses were carried out with TS/TV = 1/2 and gaps excluded.

[c] Significance levels of Templeton's test. The test was not carried out on the searches with 1/3 TS/TV weights. * $P < 0.05$; ** $P < 0.025$; *** $P < 0.01$.

same primer pair amplifies only the functional mitochondrial copy in all the other species. An alternative primer (MVZ 15) with internal gene sequence amplified a fragment without the deletion. Although the cyt *b* GLU primer originates from a conserved region (the tRNA GLU), the extra cytochrome *b* was always amplified when the cyt *b* GLU primer was used in combination with either of two other primers, cyt *b* B2 or MVZ 18. Similarly, template dilutions (1/50, 1/100, and 1/1,000 from the original concentration) always produced the extra cytochrome *b* fragment when the cyt *b* GLU primer was used. Even though the extra *T. carnifex* copy showed a 1-bp deletion as compared with the functional sequence, it did not have any termination codons.

Table 3 summarizes sequence differences between the two *T. carnifex* cytochrome *b* fragments (extra cytochrome *b* and cytochrome *b*) and compares these with the cytochrome *b* fragment of *E. asper* (its closest relative according to cytochrome *b* phylogenetic analyses). The extra cytochrome *b* sequence and the functional conspecific copy differed as much as *E. asper* cytochrome *b* differed from that of *T. carnifex* (13.6% vs. 14.4%, respectively). Base composition and A + T percentages were not different in the two conspecific cytochrome *b* fragments. The sense strand of the *T. carnifex* cytochrome *b* gene is extremely biased against G at the third position, as is typical of all vertebrate mitochondrial genomes, but so is the extra cytochrome *b*. MP, ML, and NJ analyses were carried out on the complete cytochrome *b* data set including the extra cytochrome *b* fragment. The tree topology was identical to that in Figure 2b, with the extra cytochrome *b* of *T. carnifex* forming a clade either with the conspecific sequence or with *E. asper*; however, both alternative topologies were weakly supported.

Divergence Rates

Table 4 shows the results from the Tajima rate test (Tajima, 1993) for the three *Eu-*

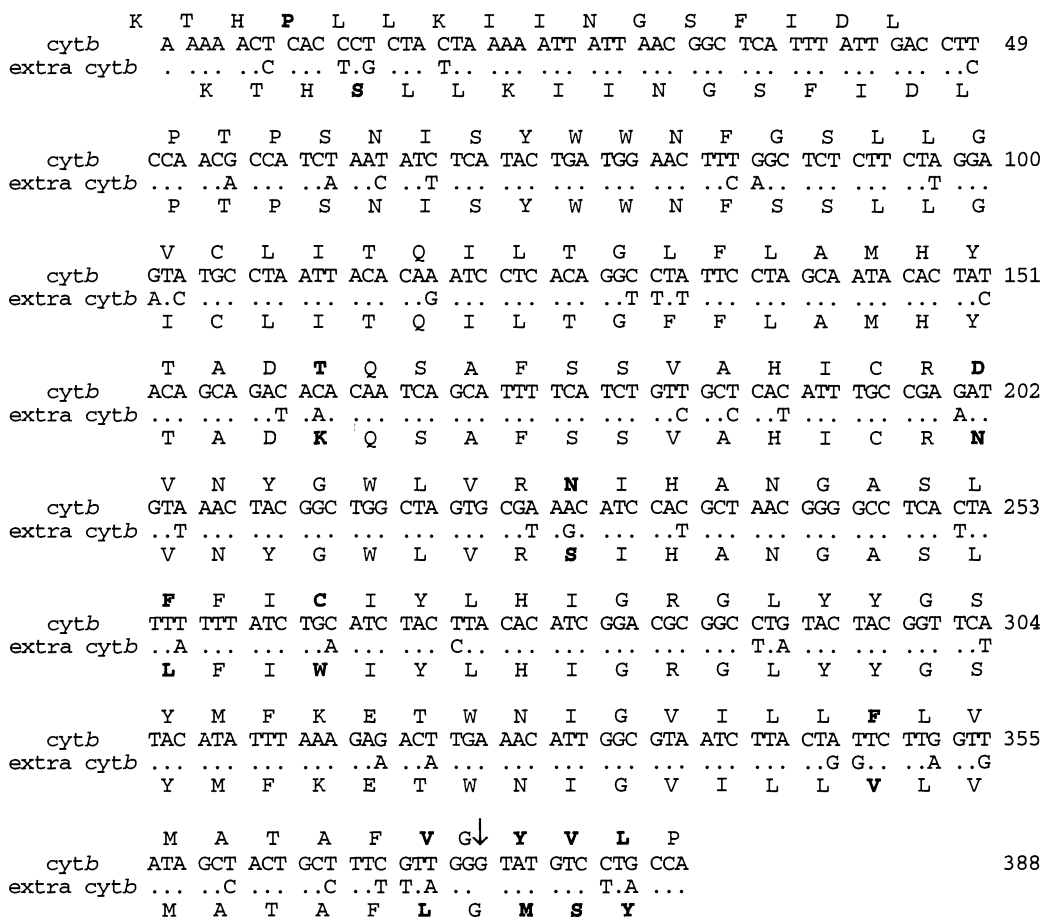

FIGURE 4. Sequence comparison of cytochrome *b* and extra cytochrome *b* in *Triturus carnifex*. A complete nucleotide sequence is given for cytochrome *b*; dots represent identical nucleotides in the extra cytochrome *b* sequence. The arrow indicates the position of the inferred deletion that causes a shift of the reading frame in the extra cytochrome *b*. The amino acid translation is shown on the top (cytochrome *b*) and on the bottom (extra cytochrome *b*) of the nucleotide sequence. Amino acid differences between the two sequences are in bold.

proctus species. Rates were tested between *E. montanus* and *E. platycephalus* (using *E. asper* as the outgroup) and along the lineage leading from *E. asper* to the *E. montanus–E. platycephalus* clade (using *T. vulgaris* and *P. waltl* as outgroups). We did not use *T. carnifex* as an outgroup because of its unclear phylogenetic position (Fig. 2). Because previous phylogenetic analyses were unable to completely resolve some of the relationships among taxa, the tests were conducted both with and without assignment of a specific outgroup. We also tested transversions alone because transitions might be approaching saturation in our data set.

We could not reject the hypothesis of constant substitution rates for the ribosomal genes; all 2D, 2DN, and V tests were not statistically significant. Using all positions for cytochrome *b*, rate constancy was rejected in almost all the comparisons between *E. montanus* and any of the other five species; rate constancy rejection among these lineages decreased when we used only third codon positions. Using transversions exclusively, we could reject the molecular clock hypothesis in only a few

TABLE 3. Sequence differences between the extra cytochrome *b* in *Triturus carnifex* (extra cyt *b* CAR) and the two mitochondrial functional copies in the same species (cyt *b* CAR) and its closest relative *Euproctus asper* (cyt *b* ASP). Data are pairwise comparisons. Values above the diagonal are numbers of transition/transversion differences; below the diagonal are the numbers of silent/replacement changes.

Gene	Extra cyt *b* CAR	Cyt *b* CAR	Cyt *b* ASP
Extra cyt *b* CAR	—	37/16	41/14
Cyt *b* CAR	41/12	—	46/10
Cyt *b* ASP	42/14	50/7	—

cases. This finding could reflect the more linear accumulation of transversions than of the other classes of changes over time. Alternatively, it could merely be a function of reduced sample size of the changes tested; the test loses strength when small numbers of substitutions are used (Tajima, 1993).

DISCUSSION

Sequence Variation and Phylogenetic Considerations

The pattern of nucleotide composition among the cytochrome *b* sequences is similar to that observed in other studies (Hedges et al., 1991; Irwin et al., 1991; Moritz et al., 1992). The highest bias against G content occurs at third codon positions, and among transitions there is a purine:pyrimidine bias (TC = 55%, AG = 23%). Cytochrome *b* has more informative sites than do the ribosomal genes, most occurring in third codon positions. The ribosomal genes were less variable, with more phylogenetically informative sites in the 16S than in the 12S (Table 1). Transition/transversion ratios range from about 7 for the most closely related species (*E. platycephalus* and *E. montanus*) to 1.3 for the most distant comparisons for the three gene fragments.

In vertebrate mtDNA, a high transition bias is well known (Brown et al., 1982; Moritz et al., 1987; reviewed by Meyer, 1993). Because of this high bias, transitional substitutions reach saturation quickly and may be of limited use in phylogenetic reconstructions. In such instances, using only transversions (or weighting them more heavily than transitions) may be preferred because with increased divergence

TABLE 4. Tajima rate test (Tajima, 1993) for three mitochondrial genes (12S + 16S and cytochrome *b*) of *Euproctus, Triturus,* and *Pleurodeles.* The observed number of transition (s_1) or transversion (v_1) differences are given between sequence 1 and both sequences 2 and 3 (s_2 and v_2, and s_3 and v_3 are similarly defined for species 2 and 3, respectively). Chi-square values are obtained using species 3 as outgroup (2D) or without the use of outgroups (2DN) and considering transversions only (V).

Gene	Species[a]			No. different sites						Chi-square[b]		
	1	2	3	s_1	v_1	s_2	v_2	s_3	v_3	2D	2DN	V
12S + 16S	M	P	A	12	4	20	3	43	11	2.14		0.20
	M	A	W	27	11	29	8	31	17	0.55		0.47
	M	A	V	34	13	24	7	25	8	2.52	0.09	0.80
	P	A	W	28	8	35	9	29	16	0.84		0.06
	P	A	V	26	9	32	7	15	18	0.87	3.01	0.25
Cytochrome *b*												
All positions	M	P	A	16	3	32	7	61	21	6.93*		1.60
	M	A	W	54	18	34	11	29	29	6.24*	8.50**	1.69
	M	A	V	30	8	62	21	39	15	6.96***	3.30	5.83**
	P	A	W	45	15	27	12	32	27	4.83	6.19*	0.33
	P	A	V	45	18	32	13	38	15	3.00	0.65	0.81
Third codon positions	M	P	A	14	2	26	7	45	20	6.38*		2.78
	M	A	W	38	18	28	9	23	23	4.52	6.62*	3.00
	M	A	V	24	7	45	21	28	11	16.89*	1.39	0.50
	P	A	W	33	14	21	10	24	22	3.34	4.70	0.67
	P	A	V	32	15	28	11	25	12	0.89	0.21	0.62

[a] M = *E. montanus*; P = *E. platycephalus*; A = *E. asper*; V = *T. vulgaris*; W = *P. waltl.*
[b] Significance levels: * $P < 0.05$; ** $P < 0.025$; *** $P < 0.01$.

transversions seem to accumulate more linearly over time (Brown et al., 1982; Hixson and Brown, 1986; Miyamoto and Boyle, 1989; Milinkovitch et al., 1995). This notion has been recently challenged by several reports cautioning against a priori assignments of weights or rejecting altogether any weighting strategy (Hedges et al., 1992; Brower and DeSalle, 1994; Reeder, 1995; Simon et al., 1996). Especially for rRNA genes, globally weighting all substitutions may not be a wise strategy because of the large rate differences existing between different regions (Vawter and Brown, 1993; Ortí et al., 1996; Simon et al., 1996) and indeed between individual nucleotide positions within stem and loop regions (Van de Peer et al., 1993; Sullivan et al., 1995; Hickson et al., 1996). For instance, some transitions might be located in a conserved stem and still be phylogenetically informative, whereas loop positions may also have both highly conserved and highly variable nucleotides. Some loops have extreme length variation, with the variable sections containing many multiple substitutions. Extreme rate variation is especially true for the 12S rRNA region used in this study, which tends to have fewer positions free to vary than do other parts of the 12S gene and can exhibit higher among-site rate variation (Simon et al., 1996). Globally weighting positions of the rRNA genes ignores the problem of among-site rate variation. However, differential weighting of substitutional changes seem less controversial for coding genes than for rRNA genes because of the well-known substitution patterns at coding positions. In cytochrome *b* in particular, transversions tend to accumulate more linearly with time than do transitions (Irwin et al., 1991; Martin et al., 1992; Moritz et al., 1992; Cantatore et al., 1994). Because of these observations, we tend to favor for this data set equal weighting of transitions and transversions for rRNA genes and the use of transversions only for the cytochrome *b* gene.

The phylogenetic relationships obtained using the ribosomal genes (Fig. 2a), the cytochrome *b* gene (Fig. 2b), and the combined data set (Fig. 3) are substantially similar. In all cases, the two island *Euproctus* species, *E. montanus* (Corsica) and *E. platycephalus* (Sardinia), are sister species. The remaining branches are less well supported when the rRNA and cytochrome *b* genes are analyzed separately and when transitions and transversions are weighted equally. However, when we applied a differential weighting scheme to the two types of genes, using transitions and transversions equally weighted for the rRNA genes and only transversions for the cytochrome *b* gene, the topology of the tree shown in Figure 3 is strongly supported in terms of bootstrap values (>75% for all the nodes), additional steps necessary to obtain alternative topologies, and the statistical significance of the Templeton (1983) tests (Table 2). The two island species of *Euproctus* appear to be almost equally distant from *E. asper* as from *T. carnifex*, implying that the two genera are not monophyletic.

These data lead us to question the monophyletic status of the genus *Euproctus*. Morphological, karyological, and ecological data suggest that *E. asper* is distinct from the other two *Euproctus* species, but these data do not provide conclusive evidence of the group's monophyly (Bolkay, 1928; Jaylet, 1966; Thorn, 1968; Wake and Özeti, 1969; Bucci-Innocenti et al., 1978; Accordi et al., 1984; Thiesmaier and Hornberg, 1990). Many characters that are phylogenetically informative for the other genera of the family are suspected to be either primitive or autapomorphies in *Euproctus* (Wake, pers. com.). Our sequence data also lead us to question the monophyly of the genus *Triturus*. Doubt concerning its taxonomic status has also been raised by a recent molecular study of the relationships within the family Salamandridae (Titus and Larson, 1995). In agreement with our findings, this study clustered a large-bodied *Triturus* species not with conspecifics but with *E. asper*, the only large-bodied *Euproctus* species. Both studies also show that the split between the two *Triturus* subgenera must be quite old, agreeing with previous immunological (Busack et al., 1988),

isozyme (Kalezic and Hedgecock, 1980; Rafinsky and Arntzen, 1987; Arntzen and Sparreboom, 1989), fossil, and biogeographic (Oosterbroek and Arntzen, 1992) evidence, which indicates a Miocene radiation for the genus (reviewed by Giacoma and Balletto, 1988; Macgregor et al., 1990; Halliday and Arano, 1991).

Certainly our data are not conclusive in establishing that *Euproctus* and/or *Triturus* are not monophyletic. However, they do support a close relationship between the two genera and are consistent with the antiquity of the cladogenic events in both genera proposed on morphological, biogeographical, and fossil grounds (30–25 MYA). Regardless of the monophyletic status of the genera, the splitting events that originated the main lineages of *Triturus* and *Euproctus* may well have occurred within a relatively short period of time, and the sequence data cannot clearly resolve these splits because they occurred so close together. Alternatively, the ambiguities of the molecular data could result from limited taxon sampling and long-branch attraction (reviewed by Smith, 1994). A more comprehensive study, which should include all the *Triturus* and *Euproctus* species plus multiple outgroups, could help clarify the issues. However, a reanalysis of the behavioral (reviewed by Halliday, 1977) and morphological characters used to propose monophyly of the genera may also be warranted. If our phylogeny is correct and the two genera radiated synchronously, this fact has important implications for the rate of acquisition of novel morphologies and behavioral traits.

Duplication of Cytochrome b in Triturus carnifex

The extra cytochrome *b* copy found in *T. carnifex* could be located in the mitochondria either as a duplication of a single mtDNA type or in the form of heteroplasmy, with two mtDNA haplotypes in the same individual. Large duplications of the mtDNA molecule and heteroplasmy have been reported in several natural populations of both *T. cristatus* and *T. carnifex* (Wallis, 1987; Wallis and Arntzen, 1989). These appear to be tandem duplications in the D-loop region. In one case, an individual of *T. carnifex* from Macedonia had an 8-kb duplication extending from the D-loop to at least the origin of the L-strand replication. Alternatively, the extra cytochrome *b* could be located in the nuclear genome; mtDNA-like sequences have been found in the nuclear DNA of several organisms (Smith et al., 1992). Although additional experimental work is necessary to clearly establish the genomic location of the duplicated sequence, the large amount of divergence between the two cytochrome *b* sequences, in association with the strong compositional bias maintained by the extra cytochrome *b* copy, suggests that the extra cytochrome *b* is still located in the mitochondria. Both the extent of sequence divergence from the other cytochrome *b* copies and the results of the phylogenetic analyses indicate that the duplication event is quite old. The presence of a deletion would argue for the extra cytochrome *b* to be a nonfunctional copy, i.e., a pseudogene. If the extra cytochrome *b* is a pseudogene, it should have an increased number of replacement substitutions and lower transition bias at silent sites (Li et al., 1985). We did observe an increased number of replacement substitutions between the cytochrome *b* of *E. asper* and the extra cytochrome *b* of *T. carnifex* (Table 3). However, no differences in transition bias at silent sites were found. Although this could imply that the extra cytochrome *b* is not a pseudogene, it is not strong evidence because the transitional mutation bias in the mtDNA could also be responsible for keeping the observed transition bias.

The finding of an anomalous copy of the cytochrome *b* gene using an extremely conserved primer (cyt *b* GLU), which is routinely used in a variety of vertebrates, highlights the need for caution in choosing cytochrome *b* sequences to be included in phylogenetic analyses. Although other examples of cytochrome *b* duplications have been reported (reviewed by Smith et al., 1992), they usually are easily recognizable by the occurrence of large deletions or the presence of termination codons in the am-

plified segment. The case described here exemplifies a particularly insidious situation. The extra cytochrome *b* sequence we found did not have any large indels or termination codons. Although this extra sequence was highly divergent from the sequences of the other taxa studied (Table 4), the only clue that alerted us to its peculiarity and prompted more experimental analyses was the presence of a single base pair deletion, which changed the reading frame to amino acids absent in any of the other taxa. Because of this observation, we returned to the same DNA preparation and extracted from the genome another cytochrome *b* sequence with no base pair deletion and 13.6% divergence from the extra cytochrome *b* copy. Without that single base pair deletion, we would have used the extra cytochrome *b* sequence in our phylogenetic analyses, erroneously comparing paralogous genes.

One has to wonder how often similar phenomena actually go undetected. Although tandem duplications of mitochondrial ribosomal DNAs have been reported (Moritz and Brown, 1986, 1987), identification of a comparable phenomenon in rRNA genes will be very difficult, because of the lack of a reading frame. By far, 12S and 16S sequences are the most commonly used mtDNA sequences in phylogenetic studies. Although there are very highly conserved motifs in these ribosomal genes (Simon et al., 1994) that should show unusually high variation rates in a pseudogene, reports of pseudogene findings are quite rare. Does this mean that they do not occur or that they have not been recognized as such?

Testing for Homogeneity of Molecular Rates

Several tests are available in the literature to evaluate the molecular clock hypothesis. The relative rate (Sarich and Wilson, 1967; Wu and Li, 1985), likelihood ratio (Muse and Weir, 1992), and Tajima (1993) tests are among the most commonly used. We chose the Tajima test because of its advantages. Like the others, it is limited to examining no more than three taxa at a time, but only the Tajima test can be used without knowing the phylogenetic relationships of the test sequences. Contrary to other types of tests, this test is not based on distance values but is applied directly to the inferred number of substitutional changes. Moreover, it is particularly attractive for mtDNA studies, where transitions and transversions occur at different rates, because transitions and transversions can be tested separately. Most importantly, it can also be used when the pattern of substitution rates is unknown and/or when different sites have different substitution rates. Notwithstanding these advantages, the results of this test must be interpreted with caution and with a clear understanding of their implications. We are testing our data against the null hypothesis that the expected number of substitutions between sister lineages is the same. However, this equality could hold even when the rate is not constant; for example, when two sister taxa have the same number of substitutions along their terminal branches, the pattern of accumulation of the substitutions may be different. Therefore, if two sequences do not pass this test, we can safely argue that they have different rates. However, we cannot argue forcefully that the molecular rates are constant along the branches leading to two sequences that passed the test. Obviously, the density of branching is important in detecting differences in dynamics, but the statistical analysis becomes more difficult, if not impossible.

Fortunately, we can test the results of the Tajima tests against the geologically derived datings. Because we have two independent estimates of divergence times within the genus *Euproctus*, we can examine the clocklike behavior of these sequences. Table 5 presents comparisons of the DNA distances schematically represented in Figure 5, i.e., the distance of *E. asper* versus *E. montanus–E. platycephalus* (a) with the distances between *E. montanus* and *E. platycephalus* (b) for different classes of substitutions. Assuming that substitutions are accumulating linearly over time, then the ratios of the molecular distances (a/b) should be similar to the ratio of the geo-

TABLE 5. Comparison of DNA distances among the three *Euproctus* species for rRNA (12S + 16S) and cytochrome *b* genes using different types of substitutions (transversions [TV], transitions [TS]). Average distances on all substitutions (TS + TV) are Takahata–Kimura distances, TV-based distances are Jukes–Cantor distances. a = sequence divergence of *E. asper* from the *E. montanus–E. platycephalus* clade; b = sequence divergence of *E. montanus* from *E. platycephalus*.

Comparisons[a]	12S + 16S		Cytochrome *b*		
	TV + TS	TV only	TV + TS	TV only	TV 3rd position
a	0.109	0.029	0.222	0.056	0.160
b	0.066	0.013	0.114	0.026	0.066

[a] See Figure 5.

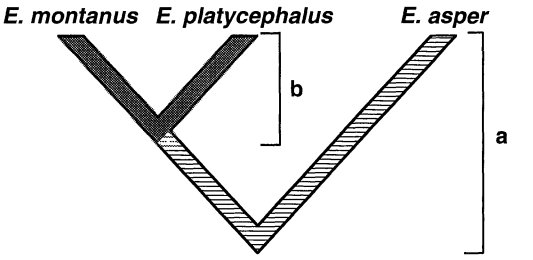

FIGURE 5. Relationships among the three *Euproctus* species. ▤ = divergence of *E. asper* from the *E. montanus–E. platycephalus* clade (a); □ = divergence of *E. montanus* and *E. platycephalus* (b).

logical estimates for the separation of the landmasses. The ratios range from 1.9 to 3.2 (29/15 to 29/9) for the geological divergences and from 1.7 to 2.4 for the molecular distances, consistent with a linear accumulation of substitutions over time for both ribosomal and cytochrome *b* genes. If *E. asper* split from the *E. montanus–E. platycephalus* clade 29 MYA, then on the basis of the distances in Table 5 we can estimate the time of divergence between *E. montanus* and *E. platycephalus* and compare it with the time estimate based on geology. Based on all substitution changes, *E. platycephalus* and *E. montanus* diverged between 15 (cytochrome *b*) and 18 (rRNA genes) MYA; time estimates based on transversions only are slightly lower (12–13.5 MYA, respectively). Both sets of time estimates suggest that the split between the two island species occurred at the beginning of the disjunction of the two islands (15 MYA) rather than at the end (9 MYA).

Evolutionary Rates for the Three mtDNA Genes and Calibrations with Absolute Time

By calibrating the estimates of *Euproctus* DNA divergence with absolute time, we obtain rates for the ribosomal genes (per million years since last common ancestor) of about 0.38% for all substitutions or 0.10% for transversions only. The cytochrome *b* gene evolves nearly twice as fast as the ribosomal genes; rates per million years for the cytochrome *b* are 0.77% for all substitutions, 0.19% for transversions only, and 0.55% for transversions in third codon positions. Both ribosomal and cytochrome *b* rates agree closely with estimates for the same genes in two other genera of salamandrids from North America, *Notophthalmus* and *Taricha*. Fossil data indicate that *Notophthalmus* and its sister taxon *Taricha* were well differentiated morphologically and biogeographically by the middle Miocene (Estes, 1981). From the published rRNA sequence data (Titus and Larson, 1995), we used TK distances to calculate the percentage of sequence divergence (11.7% for transitions + transversions; 4% for transversions only). Assuming a divergence time of 25 MYA and a molecular clock, we obtained a rate of 0.47%/million years for transitions plus transversions and 0.16%/million years for transversions only. Using the same time estimate for the divergence of *Taricha* from *Notophthalmus*, Tan and Wake (1995) computed cytochrome *b* rates for the two genera, obtaining a rate of 1–0.7% sequence divergence per million years. A comparable cytochrome *b* rate has been reported by Splolsky et al. (1992) for mole salamanders (Ambystomatidae).

Among vertebrates, evolutionary rates of mtDNA seem to have decreased in mammals as compared with poikilotherms (reviewed by Rand, 1994). But within poikilotherms, the available evidence suggests that divergence time could be assessed accurately using mtDNA rate constants (Irwin et al., 1991; Martin et al., 1992; Cantatore et al., 1994). Published evolutionary rates for mtDNA ribosomal genes in en-

dotherms and ectotherms are 0.5–1%/million years for transitions plus transversions and 0.14%/million years for transversions only (Meyer and Wilson, 1990; Mindell and Honeycutt, 1990; Hillis and Dixon, 1991; Kraus and Miyamoto, 1991; Allard et al., 1992; Meyer, 1993; Ritchie et al., 1996). These rates are comparable to the ones found in *Euproctus* and other salamandrids. Cytochrome *b* gene rates differ substantially among vertebrates. In mammals, rates for all positions are 0.6–1.4%/million years and are about 0.5%/million years for transversions in third codon positions. In sharks, fishes, and turtles, rates for the same positions are considerably lower, ranging from 0.12%/million years (all substitutions) to about 0.2%/million years (only transversions in third codon position) (Martin et al., 1992; Bowen et al., 1993; Cantatore et al., 1994; B. Shaffer, pers. comm.). Even in other amphibians, particularly bufonid frogs (Graybeal, 1993), cytochrome *b* rates are lower than those reported for warm-blooded vertebrates. This evidence has been used to suggest that cytochrome *b* rates are ancestrally slow, with mammals having increased their rates by at least 2–3-fold. This variation in rates among vertebrates has been attributed to a series of physiological and life-history variables, including differences in body size and body temperature. Endothermic animals have greater mtDNA mutation rates than do ectotherms, and within each group there is a negative correlation between body size and mtDNA rates (Avise et al., 1992; Adachi et al., 1993; Bowen et al., 1993; Martin et al., 1992; Martin and Palumbi, 1993; Rand, 1994; Martin, 1995a, 1995b). Rate estimates for *Euproctus* and other salamandrids are approximately seven (transitions + transversions) to three (transversions only) times higher than the comparable rates in other poikilotherms and are very close to the estimates for mammals. This result suggests that body temperature may not be a good predictor of evolutionary rates of the cytochrome *b* gene for all organisms. There may be no causal relationship between body temperature and molecular rates, or its effects in some organisms may be counterbalanced by other factors (e.g., generation time, body size, life span, age at first reproduction, rate of population increase, mutation rate).

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