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Molecular biogeography: using the Corsica-Sardinia microplate disjunction to calibrate mitochondrial rDNA evolutionary rates in mountain newts (*Euproctus*)

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Abstract

Mitochondrial DNA (mtDNA) sequence variation was examined in the three species belonging to the newt genus *Euproctus*: *E. asper*, *E. montanus*, and *E. platycephalus*, and in three other species belonging to the same family: *Triturus carnifex*, *T. vulgaris* and *Pleurodeles waltl*. The *Euproctus* species inhabit mountain streams in the Pyrenean region, Corsica, and Sardinia, respectively. This vicariant distribution is believed to be a result of the disjunction and rotation of the Sardinia-Corsica microplate from the Pyrenean region and suggested dates for each cladogenetic event are available. A total of 915 bp from 12S and 16S ribosomal rRNA genes were compared for each taxon. These are the first mt-rDNA sequence data for salamanders. Sequences were used to reconstruct phylogenetic trees, investigate evolutionary rates for these genes, calibrate them with absolute time since divergence, and compare rates with published ones.

Using *P. waltl* as the outgroup, all phylogenetic methods used (parsimony, maximum likelihood, and Neighbor Joining) produced trees with identical topologies and similar bootstrap values associated with each node. These sequence data cannot unambiguously resolve the splitting events leading to the main radiation of the genus *Triturus* and the origin of the genus *Euproctus*. These events may well have occurred very close in time, consistent with other sorts of data. Although it is unlikely strict linearity holds for all kinds of substitutions, relative rate tests of the molecular clock hypothesis could not reject clock-like behavior of sequence changes along *Euproctus* lineages. Estimates of absolute rates of base changes are 0.35% per Myr since divergence for all substitutions and 0.14% per Myr for transversions;

these estimates are similar to other vertebrate estimates. A comparison with distance measures from allozyme studies agrees quite well with regard to relative divergences of the three *Euproctus* species.

Introduction

A basic tenet of molecular systematics and evolution is that isolated populations genetically diverge monotonically with time. That this is true is hardly doubted, but what is more uncertain is whether the rate of genetic divergence is linear with time, such that the degree of genetic difference can be used as a measure of time since divergence, the issue of the molecular clock. If molecular clocks are valid, then molecular studies become very valuable in timing evolutionary events (Zuckerkandl and Pauling, 1965).

Rate heterogeneities clearly exist at several genomic levels: variable substitution rates are obvious across nucleotide sites, genes, and genomes within a phylogenetic lineage. What is controversial is whether rates of change between homologous sequences across different lineages proceed in a clock-like fashion. Relative rate tests (Wilson et al., 1977) have been used to check for the existence of universal or local clocks. While the majority of evidence tends to reject the possibility of a universal clock across very different lineages, the notion of local clock with lineage-specific rate differences seems to be more viable (see reviews in Nei, 1987; Wilson et al., 1987; Li and Graur, 1991). However, even if one is willing to accept the clock hypothesis, one problem still exists: the calibration of the clock with absolute time. Calibration of a molecular clock with time requires an independent event, most often a geological one. Geological dates for the cladogenetic events of interest may be obtained if the taxa studied have a good fossil record and/or if cladogenetic events can be associated with specific, well-dated biogeographic scenarios (Knowlton et al., 1993).

One well-studied geological event with biogeographic consequences is the disjunction and rotation of the Corsica-Sardinia microplate from the Iberian Peninsula. Both the mode and the timing of this event have been thoroughly investigated. According to paleomagnetic, stratigraphic and geomorphological data from the Tyrrenian area, Corsica and Sardinia separated as a single landmass from the continent in the Miocene about 29 Mya. After the initial disjunction, a rotation took place which brought the two islands to the approximate present position. The pattern and timing of the separation of the two islands is more complex. Even though episodic contacts between southwestern Corsica and Northern Sardinia may have persisted until very recently, the separation of these islands may have begun as long as 15 Mya and was certainly complete by 9 Myr (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).

The particularly attractive aspect of this biogeographic situation is that several taxonomically diverse groups of organisms with closely related species (likely sister taxa) exist on each of the three land masses. For many of these groups the potential

for active or passive dispersal is virtually nil, and thus the time of the cladogenetic events in each of these lineages can reasonably be assumed to coincide with the tectonic events involving the three land masses. This feature, plus the availability of two time estimates, make this situation unusually favorable for calibrating clocks for a time period (10–30 Myr) which has been rarely addressed, and for testing the validity of the clocklike nature of genetic divergence. Of course, any such dates for cladogenetic events must be considered to be minimum estimates, as biological differentiation may have preceded geological separation.

We have begun to investigate several organisms with the same distribution with respect to this biogeographic event. This paper reports on a groups of newts, belonging to the genus *Euproctus*, which are specialized to cold running fresh-water. *Euproctus* is a genus belonging to the Salamandridae family; it consists of only three species. *E. asper* is entirely restricted to the Pyrenees at high altitude; *E. montanus* occurs in the mountain streams of Corsica, and *E. platycephalus* in those of Sardinia. To obtain an accurate representation of the phylogenetic relationships among the species we planned to use multiple outgroups (Watrous and Wheeler, 1981; Maddison et al., 1984), with different expected levels of divergence from *Euproctus*. We used two species of *Triturus*, *T. vulgaris* and *T. carnifex*, as representatives of lineages closely related to the *Euproctus*, and *Pleurodeles waltl*, which is more distant from *Euproctus* than *Triturus*, but still belongs to the same family (Wake and Ozeti, 1969).

We have sequenced segments of the 12S and 16S mitochondrial ribosomal genes (mt-DNA) to (a) infer the phylogenetic relationships among these species, (b) investigate evolutionary rates for these genes, and (c) calibrate them with absolute time since divergence.

Material and Methods

Material

Three individuals for each of the three *Euproctus* species, one individual each for the two *Triturus* species and for *Pleurodeles waltl* were analyzed. DNA was extracted from liver and muscle tissues of frozen ($-80°$ C) or ethanol-preserved specimens according to the protocol of Caccone et al. (1987). High molecular weight DNA was recovered from both frozen and alcohol-preserved samples.

DNA amplification, and sequencing

Modified conserved primer pairs L1091 + H1478 (Kocher et al., 1989) and 16Sar + 16Sbr (Palumbi et al., 1990) were used for PCR amplifications of portions of the 12S and 16S genes, respectively. Double stranded amplifications were performed with a Perkin-Elmer-Cetus thermal cycler in 100 μl of a solution containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, each dNTP at

2.5 mM, each primer at 1 μM, genomic DNA (10–100 ng), and 2 units of Amplitaq (Perkin-Elmer Cetus). After a 1 min. denaturation step (94° C), each cycle of the polymerase chain reaction consisted of denaturation for 45 sec. at 94° C, annealing for 1 min. and 30 sec. at 50° C, and extension for 1 min. at 72° C. This cycle was repeated 30 times and followed by a 5 min. incubation step at 72° C. Double stranded amplified products were separated by electrophoresis in 1% agarose gels, the DNA fragments were visualized by fluorescence under UV light after ethidium bromide staining (Maniatis et al., 1982). Three to four aliquots ($\simeq 1$ μl) of the gel fragment containing the amplified product were excised from the gel and stored (-70° C). These aliquots were used directly as template in the second chain of reactions to generate single-stranded DNA for direct sequencing by the unbalanced-primer procedure of Gyllensten and Erlich (1988). In this reaction the concentration of one or the other primer is reduced 100-fold. The cycling was similar to the one described before, except for an increase in annealing temperature (58° C) and number of cycles (38). Single-stranded amplifications were purified by two phenol-chloroform extractions and two ethanol precipitations. To check for DNA contamination for both double and single-stranded PCR reactions negative controls were also assembled by omitting the DNA from the reaction mixture and including an aliquot of the gel outside the band. Sequences were determined on both strands with an automated sequencer (Applied Biosystems Model 373A) following the manufacturer protocols.

Data analysis

Sequences were aligned using the MALIGN progam (version 1.5) for multiple sequence alignment (written and distributed by W. Wheeler and D. Gladstein, American Museum of National History, New York). *All individuals sampled from the same species yielded identical sequences*, therefore one haplotype per species was used. Each species-specific sequence will be denoted with a three letter symbol as follows: ASP = *E. asper*; PLA = *E. platycephalus*; MON = *E. montanus*; CAR = *T. carnifex*; VUL = *T. vulgaris*; and PLE = *P. waltl.*

Aligned sequences were analyzed by the maximum parsimony procedures available in PAUP 3.0s (Swofford, 1991). By parsimony, the branching order(s) requiring the least number of substitutions is (are) considered most likely to reflect the actual evolutionary relationships among taxa. Positions at which only one species differs (autoapomorphies) have been included because such positions provide information about the amount of change that its sequence has undergone since it shared a common ancestor with other members of the group, and thus they provide information about rates of change. For each data set we weighted transitions (TI) and transversions (TV) equally, weighted TV/TI 3/1, and considered only TV. On the basis of known cow 12S (Gutell and Fox, 1988) and *Xenopus* 16S (Gutell et al., 1985) secondary structures, we estimated a secondary structure for our sequences. Stem regions and loop regions of mt-rDNA are subject to different selective and structural constraints; in stem regions we weighted compensatory changes half of

the weight of noncompensatory changes (Wheeler and Honeycutt, 1988). Most parsimonious (MP) trees were calculated using an exact search with branch and bounding settings. Bootstrap resampling procedures (Felsenstein, 1985) were performed with a minimum of 100 to a maximum of 5000 replicates to produce a majority consensus tree and to investigate its roubstness. Tree length distributions were also examined using the g1 statistic of Sokal and Rohlf (1981). Examining the shape of the distribution of the lengths of all trees provides an effective means of discriminating phylogenetic signal from noise in systematic data sets (Hillis, 1991; Hillis and Huelsenbeck, 1992).

The program DNAML in PHYLIP version 3.4 (Felsenstein, 1991) was used to obtain maximum likelihood (ML) trees. In addition we generated 100 ML distance matrices by bootstrap resampling of the original data set. A majority rule consensus tree was obtained among the 100 neighbor joining (NJ, Saitou and Nei, 1987) trees calculated from each of the ML data matrices. We used the Kishino and Hasegawa (1989) statistical test (as implemented in PHYLIP) to determine if the topology of the tree with the highest likelihood was significantly different from other topologies with high likelihood. Distance matrices based on Kimura (1980), maximum likelihood, and Jukes and Cantor (1969) distances (program DNADIST) were obtained for the combined data set. NJ trees were drawn from these matrices using the program NEIGHBOR in PHYLIP. Nodes were tested for robustness by bootstrapping.

Rate homogeneity was evaluated by two tests. We calculated the index of disperion [$R(t)$ = (variance)/mean of the branch lengths for sister lineages] by using Kimura distances to calculate mean divergence values across a node. If a Poisson process with a constant rate is a good model for the molecular clock, this ratio should be 1; values >2.5 are taken as evidence of extensive rate heterogeneity. For protein-coding sequences used in clock calculations, $R(t)$ is usually <2–3 (Gillespie, 1986; Allard et al., 1992). To test if the nucleotide substitution rates are the same in different lineages we used the "relative rate test" (Wilson et al., 1977), which does not require knowledge of divergence times between species. The number of transitional substitutions per site, transversional substitutions per site, and the total number of substitutions per site were estimated by Kimura's formulas (Kimura, 1980). Variances and covariances of these values were calculated following Wu and Li (1985). Levels of significance were determined according to the procedure for the standardized normal test, following the assumptions specified in Wu and Li (1985).

Results

Sequence variation, divergence, and TI/TV ratios

On the basis of the most probable multiple alignment, 15 small "indels" (insertions and deletions) occur among the six species studied. Indels were coded as single characters, irrespective of their length. When indels of different lengths overlapped,

each size class was assigned a particular character state. Analyses were repeated including and excluding gap coding. However, because of the difficulties in coding and in determining homology among inferred gap events, we will present only the results obtained excluding gaps. Although some useful information may be discarded, excluding gaps from the analysis prevents the inclusion of spurious information. In fact, the analyses with or without considering gap coding were very similar. Sequences are available in GenBank.

Approximately 380 bp of the 12S gene and 580 bp of the 16S gene were sequenced for each sample. Our analyses are based on a 358 bp segment of the 12S gene and a 557 bp segment of the 16S gene common to all the taxa studied. 730 bp (79.8%) of the 915 nucleotide positions were identical in all taxa and 95 (10%) variable positions differed by a single substitution in one taxon. This left 90 (9.8%) nucleotide positions with the potential to contribute phylogenetic information. 21% sites varied in the 16S gene and 19% in the 12S gene. 26 changes in the 12S gene and 50 changes in the 16S gene are transversions. Overall 41% of the variable nucleotide positions were characterized by transversions.

Percent divergence for all characters and only for transversions were obtained for the 12S and 16S gene fragments separately and combined for all pairwise comparisons. Table 1 (above diagonal) presents these data for all differences for both gene fragments (915 bp). When both TI's and TV's are considered, divergence ranges from a minimum value of 6.4% for the comparison between MON and PLA, to an average value of 11% for the comparisons of all the taxa with PLE. The two island *Euproctus* species (MON and PLA) show an average 10.2% divergence from the continental *Euproctus* (ASP). The two *Triturus* (CAR and VUL) are 9.6% divergent from each other. Distance matrices using three algorithms (Kimura, Jukes and Cantor and Maximum likelihood, program DNADIST in PHYLIP), which make different assumptions as to how to estimate multiple substitutions at a site, were calculated. Results were similar for all matrices. Table 1 (below diagonal) shows the Kimura distances.

Table 1. Sequence divergence between pairs of species (above diagonal) and Kimura (1980) distances (below diagonal).

	MON	PLA	ASP	VUL	CAR	PLE
MON	-	0.064	0.108	0.101	0.101	0.117
PLA	0.067	-	0.096	0.079	0.093	0.108
ASP	0.120	0.106	-	0.098	0.105	0.109
VUL	0.111	0.086	0.108	-	0.096	0.106
CAR	0.110	0.101	0.116	0.105	-	0.110
PLE	0.132	0.120	0.121	0.118	0.122	-

In vertebrate mtDNA (Hixson and Brown, 1986; Miyamoto and Boyle, 1989) as well as in nuclear rDNA (Larson and Wilson, 1989; Hedges et al., 1990) TV's tend to occur much less frequently than TI's. This TI-bias is known to decrease over time, with increasing sequence divergence, until an asymptotic value is reached. Theoretically, if all base changes are equally likely, at equilibrium the ratio of TI/TV should be 0.5. In fact, saturation for TI's has been shown to occur in 12S and 16S mtDNA at a ratio of about 1, which occurs at about 50 Myr (Mindell and Honeycutt, 1990). TI/TV ratios for the 12S gene were 2.25 and 3.4 for the closest species pairs (MON-PLA and VUL-CAR, respectively), and ranged from 2.6 to a minimum of 1.3 for all the intergeneric comparisons. For the 16S gene TI/TV ratios were as high as 7 for the MON-PLA pair, and ranged from 2.7 to 1.2 for all the other comparisons. Thus, in our data the presumed saturation ratio of 1 was approached, but not reached, only for the comparisons with PLE.

Phylogenetic analysis

While initially we anticipated using both *Triturus* and *Pleurodeles* as multiple outgroups for defining relationships within *Euproctus*, as the analysis proceeded it became clear that only *Pleurodeles* could be considered an unambiguous outgroup. Therefore all analyses were conducted using PLE as the outgroup. We first analyzed all characters weighting them equally with no phylogenetic constraint. The result of an equally weighted search for hierarchical structure in the data set may be used as the basis for comparisons with other analyses which do not make such simplified assumptions. A single MP tree was obtained using all characters unordered and excluding indel coding (Fig. 1A). Only the node linking MON and PLA (node **a**) had an associated bootstrap probablility over 90% after 5000 replicates. All the other nodes had moderate bootstrap values (38–61%). This tree is 294 steps long with a consistency index (CI) of 0.806 (CI excluding uninformative characters = 0.650) and a retention index (RI) of 0.394. We also generated MP trees by assigning different TV/TI weights to explore the stability of the tree in Fig. 1A. Usually by empirically deriving the TI bias from the percent sequence divergence values, one can weight TV and TI accordingly. In our case there were not enough low divergence points to perform a regression analysis and empirically determine the appropriate TV/TI weight. Thus, we used a 3/1 TV/TI cost, which seemed a reasonable approximation given our actual TI/TV ratios. Other variations of analysis included considering only transversions and weighting compensatory changes due to secondary structure by one half (Wheeler and Honeycutt, 1988). Three compensatory changes were found in the 12S gene fragment sequenced, while no compensatory changes were found in the 16S fragment studied. *The same parsimonious tree was obtained in all these searches, identical in topology to the one in Fig. 1A, with similar associated bootstrap values.* In all cases, frequency distributions of all tree lengths were significantly more skewed than expected by random. Asymmetrical tree distributions of this nature have been proposed to be representa-

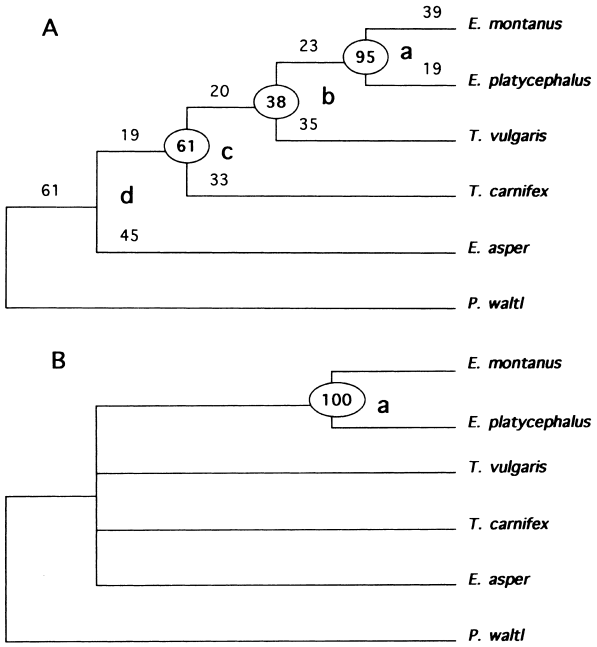

Fig. 1. (A) Single most parsimonious tree obtained by using all substitutions (TI's and TV's equally weighted). The tree is 294 steps long, the CI is 0.806 (CI excluding uninformative characters = 0.650), and the RI = 0.394. Letters indicate each node, numbers of nucleotide substitutions are shown above the branches (ACCTRAN option of PAUP). Numbers at the circled nodes represent bootstrap values based on 5000 replications using exact branch and bound search from PAUP. (B) 50% majority rule consensus tree of the first 15 most parsimonious trees, ranging in tree lengths from 294 to 301 steps.

tive of data where the ratio of random noise to phylogenetic signal is quite low (Hillis and Huelsenback, 1992).

To assess the compatibility of our data with previous phylogenetic hypotheses which argue for monophyly of the *Triturus* and *Euproctus* genera, we enforced topological constraints that preserved these monophyletic groupings. When all characters were considered, the first tree supporting *Triturus* monophyly was 296 steps long, the first one with the *Euproctus* monophyletic was 298 steps long, and the first tree supporting both *Triturus* and *Euproctus* monophyly was 298 steps long. These trees were only two and four steps longer than the most parsimonious tree (TL = 294). Figure 1B shows the 50% majority rule consensus tree of the 15 shortest trees (up to 301 steps), which represent the leftmost tail of the tree length frequency distribution. Only node **a** is always supported, while all the other nodes collapse in an unresolved polytomy. Similar patterns were obtained when only TV's were considered: the shortest tree was 96 steps long. Only one additional step away

there are two trees, one supporting *Triturus* monophyly, the other supporting *Euproctus* monophyly. A tree with both genera monophyletic is only 99 steps long.

Figure 2 shows the maximum likelihood (ML) tree obtained by weighting TV's and TIs equally. TV/TI of 3:1, 5:1, and 10:1 produced trees with the same topology and similar bootstrap probabilities as for the MP tree in Fig. 1A. For all four different TV/TI weights, ML trees obtained by enforcing monophyly for *Triturus* and *Euproctus* were tested against the unconstrained ML tree using the Kishino and Hasegawa test (1989). None of the trees tested were significantly different from any other. The neihbor-joining trees based on Kinura and Jukes and Cantor distances were identical in topology to the MP and ML trees with similar bootstrap values associated to each node.

Divergence rates

The index of dispersion, $R(t)$, the ratio of the variance to the mean distance from PLE to all the other taxa, was equal to 2.3. Relative to other DNA-sequence studies (Gillespie, 1986; Allard et al., 1992) this indicates a reasonably uniform rate of change.

To test whether the nucleotide substitution rates are identical in two different lineages, we performed three separate sets of relative rate tests (Wu and Li, 1985). In such tests, we compared the evolutionary distance between species 1 and a reference species (outgroup) with that between species 2 and the reference species. We used the three schemes presented in Fig. 3 to check rates at different hierarchical levels. Table 2 shows the differences in substitution numbers between node 0 and 1 and between node 0 to 2 with 3 as the reference. Tests were performed for

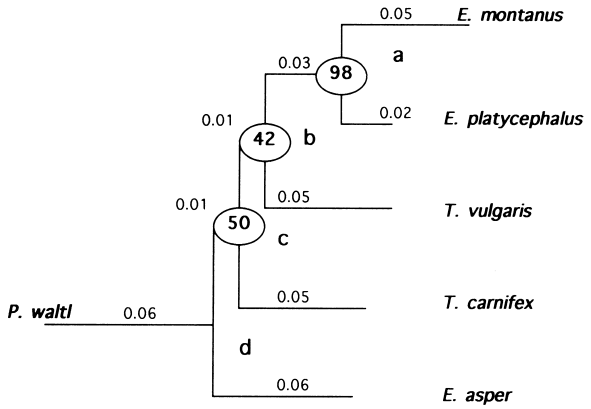

Fig. 2. Maximum likelihood tree obtained by using all substitutions (TI's and TV's weighted equally). Branch lengths are shown above branches. Nodes are indicated with small letters. Numbers at circled nodes indicate bootstrap values. See text for details of bootstrapping procedure.

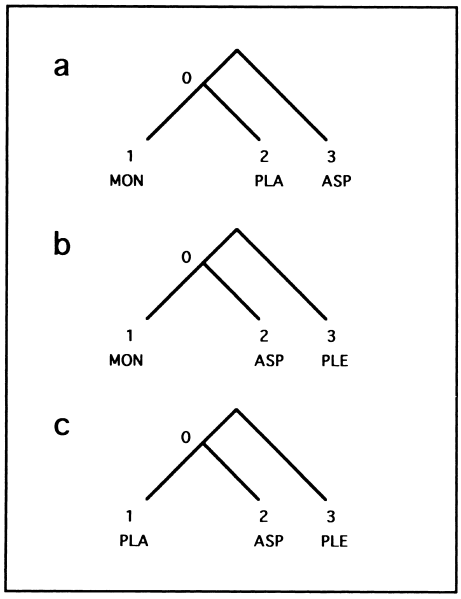

Fig. 3. Schematic representation of the three species trees used in three relative rate tests. In test **a**, ASP is used as reference species to check for homogeneity of rates between MON and PLA. In tests **b** and **c**, PLE is the reference species to check rates between MON and ASP, and PLA and ASP, respectively.

Table 2. Results of the relative rate tests on the trees diagrammed in Fig. 3 referred to in the first column below. Differences in numbers of nucleotide substitutions between node 0 to 1 and 0 to 2 for all trees were calculated for TI's, TV's and TI's + TV's, using species 3 as reference. Differences [dist.(1,3) − dist.(2,3)] were calculated by correcting for multiple hits using Kimura's (1980) formulas. Standard errors (S.E.) were computed by calculating variances and covariances following Wu and Li (1985) equations.

Tree	TI's		TV's		TI's + TV's	
	Difference	S.E.	Difference	S.E.	Difference	S.E.
a	0.0075	± 0.0084	0.0054	± 0.0041	0.0129	± 0.0100
b	0.0121	± 0.0100	0.0011	± 0.0074	0.0110	± 0.0128
c	0.0115	± 0.0098	0.0109	± 0.0068	0.0006	± 0.0124

transitions and transversions only and for the total number of substitutions. In no case was there a significant difference from zero, indicating that we cannot reject the molecular clock hypothesis.

Discussion

Phylogenetic considerations

All analyses produce trees with identical topology with *Triturus* paraphyletic (Figs. 1, 2). However, several lines of evidence indicate that this topology is not strongly supported. In the MP, ML, and NJ trees the only node with high bootstrap values is node **a**. In addition, the MP tree shown in Fig. 1A is only few steps shorter (out of nearly 300) than alternative trees supporting *Euproctus* and *Triturus* monophyly, and the consensus tree on the first 15 MP trees (Fig. 1B) is unable to resolve the cladogenetic events leading to the two *Triturus* and *E. asper*. Similarly, the ML tree (Fig. 2) is not statistically different from trees with imposed *Triturus* and *Euproctus* monophyly, as indicated by the Kishino and Hasegawa test (1989). Thus, the topology depicted in Fig. 1B is a reasonable summary of the phylogenetic content of the 12S and 16S mt-rDNA sequences, not only for the parsimony analysis but also for the other methodological approaches. *Euproctus platycephalus* and *E. montanus* are closely related and represent a distinct cladogenetic event. The lineages leading to the two *Triturus* and *E. asper* are quite old and probably separated by a relatively short time span.

The antiquity of the *Triturus* lineages indicated by our data is consistent with paleontological, morphological, behavioral, karyological, immunological, allozyme, and other DNA data from the genus. Integrative analyses of this genus (Giacoma and Balletto, 1988; Macgregor et al., 1990; Halliday and Arano, 1992) recognize twelve species on the basis of karyological and hybridization studies (Mancino et al., 1982; Bucci-Innocenti et al., 1983; Frost, 1985). Independent electrophoretic surveys (Kalezic and Hedgecock, 1980; Rafinski and Arntzen, 1987; Arntzen and Sparreboom, 1989), a micro-complement fixation study (Busack et al., 1988) and RFLP (Wallis and Arntzen, 1989) and sequence data (cytochrome b) on mtDNA (Thomaz, 1990) have contributed phylogenetic information on this genus. These studies are in broad agreement with each other and with Bolkay's (1928) morphological analysis, dividing *Triturus* into two major species groups: the *vulgaris* and the *cristatus* species groups. One representative of each were included in our study, *T. vulgaris* and *T. carnifex*, respectively. Recently these two species groups have been elevated to subgeneric status by Macgregor et al. (1990), *Paleotriton* and *Triturus*.

While different data sets produce conflicting relative positions of species within subgenera, they all agree on the existence of a deep split dividing the two subgenera within this genus. Immunological (Busack et al., 1988), isozyme (Rafinsky and Arntzen, 1987; Kalezic and Hedgecock, 1980), and cytochrome b sequence (Thomaz, 1990) data place this split at 18–21 Myr ago. This timing is concordant

with both fossil and biogeographic evidence, which indicates a Miocene radiation for the genus (Macgregor et al., 1990; Oosterbroek and Arntzen, 1992).

Morphological, ecological, karyological and behavioral studies of the three *Euproctus* species all indicate that *E. platycephalus* and *E. montanus* are the closest pair (Jaylet, 1966; Thorn, 1968; Bucci-Innocenti et al., 1978; Accordi et al., 1984; Thiesmeier and Hornberg, 1990). These data are in agreement with the molecular data available for this genus based on allozymes (Sbordoni et al., 1982, 1985; Sbordoni et al., 1990) and single-copy DNA hybridization (Sbordoni et al., 1990). Unfortunately, only limited data directly compare *Triturus* and *Euproctus*, either with each other or with the other members of the family. Morphological, karyological, and behavioral data all indicate a close relationship between the two genera. However, robust phylogenetic conclusions are hampered by the occurrence in *Euproctus* of suites of primitive and autapomorphic morphological and behavioral characters, and by difficulties in identifying homologous chromosomes (Wake and Ozeti, 1969; Giacoma and Balletto, 1988; Thiesmeier and Hornberg, 1990).

The mt-rDNA sequence data presented here are consistent with antiquity of the cladogenetic events in both *Triturus* and *Euproctus* and support a close phylogenetic relationship between the two genera. Moreover, these data suggest that the splitting events which determined the main radiation of the genus *Triturus* and the origin of the genus *Euproctus* may well have occurred in a relatively short period of time. The sequence data cannot unambiguously resolve these splits, probably not because of saturation of informative changes in the region sequenced, but because the splits actually occurred close together. This finding is important because it indicates that *E. asper* and *Triturus* spp. are almost equally distant relatives of the *E. montanus*/*E. platycephalus* clade despite numerous shared morphological and behavioural characters occurring in the three species of *Euproctus*.

Molecular rates: calibration with absolute time and comparison with other vertebrate data

Because we could not detect heterogeneity of rates of change among the lineages of *Euproctus* for the 12S and 16S rDNA sequence data (see last part of results section), and because geological evidence provides two independent dates for vicariant events, we can use the data to examine the clock-like behavior of these sequences. The corrected DNA differences between species through the earlier node is about 11% and for the most recent split is 6.7% (Table 1). Assuming these splits coincided with the geological events separating the land masses, the respective times of the phylogenetic splits are estimated at about 29 Mya and 15–9 Mya respectively (see Introduction). If we use only TV's, since they seem to evolve linearly with time (Miyamoto and Boyle, 1989; Mindell and Honeycutt, 1990), the DNA divergence through the earlier node of *Euproctus* is about 3.95% and for the most recent split is 1.30%. If we assume that the 3.95% divergence is achieved after 29 Myr of separation, then we reach an estimate of about 9.5 Myr for the split of *E. montanus* and *E. platycephalus*. This is consistent with the time estimate (9 Myr) for the completion of the separation of the two islands. On the other hand, we could not

reject a clock for the combined TI + TV data which give different estimates of relative times of divergence. Again, assuming the origin of ASP at about 29 Mya, TI + TV data imply PLA and MON split at about half that time, consistent with the older estimate of 15 Mya for the geologic separation of Corsica and Sardinia. Because the TI's are almost certainly beginning to reach saturation, we do not put much faith in TI's + TV's as a linear clock for these taxa. Therefore, on balance, we tentatively conclude that these molecular data support the younger estimate of the geological split, at least with respect to how this split affected the isolation between these newts.

Extensive allozyme data exist for these species, and we can examine whether these different molecular data yield similar estimates of divergence times for the taxa. However, in any comparison of such different kinds of data, direct comparisons are virtually impossible. We therefore examined the following relative estimates: the ratio of divergence of the *E. asper* and *E. montanus/E. platycephalus* clade compared to the divergence of *E. montanus* and *E. platycephalus* (the *a/b* ratio in Fig. 4). Another problem in comparing relative molecular divergence estimates is that diverse assumptions are used to "correct" the data. In Fig. 4 we show the relative divergence for allozymes and 12S + 16S rDNA sequences, TV's only and TI's + TV's. The allozymes ratio is based on Nei's distances corrected and weighted for fast and slow evolving loci (Sbordoni et al., 1990). Remarkably, the allozyme and TV's sequence divergence give nearly identical ratios, which coincide with the ratio of the timing of the geological events (29 Myr/9 Myr = 3.2). When TI's are included in the mt-rDNA the *a/b* ratio becomes much smaller than the other three estimates. We can thus conclude that the relative molecular divergence as measured from TV's data for 12S and 16S mtDNA and allozymes are similar to one another and consistent, assuming a molecular clock, with the geological record.

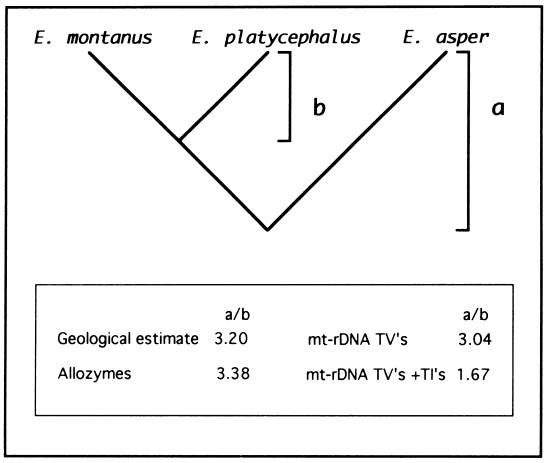

Fig. 4. Ratios of geological estimates of times of land mass separation compared to the ratios of molecular divergence estimates for the three *Euproctus* species.

We could not reject the "molecular clock" hypothesis for either TI's or TV's alone or combined (Tab. 2). Yet clearly both TI's and TV's cannot be evolving in the same clock-like fashion, e.g. linearly. This is because the $a:b$ ratio from Fig. 4 is very different when TI's are included (i.e. TI's + TV's, $a/b = 1.67$) compared to when only TV's ($a/b = 3.04$) are considered. It may be that TV's are linear while TI's are aymptotic (i.e. reaching a saturation plateau). Presumably a more densely branching tree could detect variations from linearity, if such tests were available. The precautionary note is that just because tests such as Wu and Li's (1985) cannot reject the clock for simple trees (three taxa), this is not a strong justification for assuming *linearity* of molecular divergence with time.

Even though ribosomal nuclear and mitochondrial RNAs are among the best known groups of genes, very limited data are available for amphibian mt-rDNA. Most molecular phylogenetic studies in this group have been directed at the study of evolutionary events occurring 50–200 Mya, which are best addressed by studying nuclear ribosomal genes (Hillis and Davis, 1986; Larson and Wilson, 1989; Larson, 1991). In particular, no salamander mt-rDNA sequence data had been published before this report. The published data are either restriction-data (Wallis, 1987; Wallis and Arntzen, 1989; Kraus and Miyamoto, 1990; Arntzen and Wallis, 1991; Spolsky et al., 1992) or protein-coding sequences (Thomaz, 1990; Hedges et al., 1992; Moritz et al., 1993). Fortunately, data on other vertebrates are not so limited. Analyses of 12S and 16S in Primates (Hixon and Brown, 1986), Artiodactyla (Miyamoto and Boyle, 1989; Miyamoto et al., 1990; Kraus and Miyamoto, 1991; Allard et al., 1992; Gatesy et al., 1992), Rodentia (Allard and Honeycutt, 1992), Cetacea (Milinkovitch et al., 1993), and fishes (reviewed in Meyer, 1993) have provided information on patterns and rates of nucleotide substitutions in these groups. The overall mt-DNA substitution rates are about half of those calculated for the whole mtDNA molecule. Assuming overall mtDNA rates between 1 and 2%/Myr since divergence, then mt-rDNA rates range between 0.5–1%/Myr (Brown et al., 1982; Hixon and Brown, 1986; Meyer and Wilson, 1990; Mindell and Honeycutt, 1990; Hillis and Dixon, 1991). Based on fossil data a rate of 0.14%/Myr has been obtained for Artiodactyla for transversions only (Allard et al., 1992).

By calibrating the estimates of *Euproctus* DNA divergence with absolute time, we obtain an mt-rDNA rate of 0.35%/Myr if we consider all nucleotide changes, and a rate of 0.14%/Myr if we only consider transversions. The TV's + TI's substitution rate obtained for *Euproctus* seems to be somewhat lower than the overall rate in vertebrates indicated above. However, as we noted earlier, we have little evidence of the relative rate of TI's and TV's for closely related species so our estimates of TI's may be too low. Nonetheless, the transversion rate for this amphibian mt-rDNA is remarkably similar to that calibrated for bovids.

Conclusions

This is the first use of the biogeographic events involving the movement of Corsica and Sardinia away from the continent to test for clocklike behavior of

DNA-sequence data. It is also the first study to calibrate the rate of amphibian mt-rDNA evolution. By all tests available we could not reject the clock-like behavior of mt-rDNA evolution in these species and the estimated absolute rates are consistent with those from other vertebrate species, especially for rates of transversions. However, transitions and transversions are evidently evolving differently as indicated by the different ratios of branch lengths (Fig. 4), yet the relative rate test could not reject that both were behaving as clocks. What we should state, in fact, is that we cannot reject the hypothesis that the *same number of substitutions* occurred along some pairs of branches, but this *does not prove the same dynamics of change* occurred. Obviously the density of branching is important to detect differences in dynamics, but at this point statistical analysis becomes more difficult if not impossible.

As for the phylogeny deduced for these salamanders, it was a surprise to see the possibility of paraphyly. However, we should emphasize that while paraphyly is the most parsimonious and most "likely" hypothesis, trees supporting monophyly of the genera were not rejected with any statistical certainty. If paraphyly is the true state of these genera, this has some very interesting implications with respect to the morphology and behavior interpreted to support monophyly of *Euproctus* and *Triturus*. A re-examination of these characters in the light of the present results may prove useful in evaluating the relative merits of molecular and morphological/behavioural data in phylogenetic studies, as well as providing insights into mechanisms or dynamics of change.

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References

Accordi, F., E. Grassi Milano and V. P. Gallo. 1984. The adrenal gland of *Euproctus* (Urodela, Salamandridae): Comparison of the three species and phylogenetic inferences. J. Anat. 139: 209–214.

Allard, M. W. and R. L. Honeycutt. 1992. Nucleotide sequence variation in the mitochondrial 12S rRNA gene and the phylogeny of African mole-rats (Rodentia–Bathyergidae). Mol. Biol. Evol. 9: 27–40.

Allard, M. W., M. M. Miyamoto, L. Jarecki, F. Kraus and M. Tennant. 1992. DNA systematics and evolution of the artiodactyl family Bovidae. Proc. Natl. Acad. Sci. USA 89: 3972–3976.

Alvarez, W. 1972. Rotation of the Corsica-Sardinia microplate. Nature 235: 103–105.

Alvarez, W. 1974. Sardinia and Corsica one microplate or two? Rend. Sem. Fac. Sc. Univ. Cagliari.

Alvarez, W., F. G. Franks and A. E. M. Nairn. 1973. Paleomagnetism of Plio-Pleistocene basalts from N. W. Sardinia. Nature 243: 10–11.

Arntzen, J. W. and Sparreboom. 1989. A phylogeny of the old world newts, genus *Triturus*: biochemical and behavioural data. J. Zool. (Lond.) 219: 645–664.

Arntzen, J. W. and G. P. Wallis. 1991. Restricted gene flow in a moving hybrid zone of the newts *Triturus cristatus* and *T. marmoratus* in western France. Evolution 45: 805–826.

Bellon, H., C. Coulon and J. B. Edel. 1977. Le déplacement de la Sardaigne synthèse des donnees géochronologiques, magmatiques et paléomagnétiques. Bull. Soc. Geol. France 7: 825–831.

Bolkay, St. J. 1928. Die Schadel der Salamandriden mit besonderer Rucksicht auf ihre systematische Bedeutung. Z. Anat. Entw. 86: 259–319.

Bonin, B., P. Chotin, A. Girte and J.-B. Orsini. 1979. Étude du bloc corso-sarde sur documents satellites: le problème des mouvements différentiels entre les deux iles. Rev. Geog. Phys. Geol. Dynam. 21: 147–154.

Brown, W. M., E. M. Prager, A. Wang and A. C. Wilson. 1982. Mitochondrial DNA sequences of primates: tempo and mode of evolution. J. Mol. Evol. 18: 225–239.

Busack, S. D., B. G. Jericho, L. R. Maxson and T. Uzzell. 1988. Evolutionary relationships of Salamanders in the genus *Triturus*. The view from immunology. Herpetologica 44: 307–316.

Bucci-Innocenti, S., D. Lacialfari, M. Ragghianti and G. Mancino. 1978. The karyotype of the Corsican and Sardinian mountain salamanders, *Euproctus montanus* and *E. platycephalus* (Urodela, Salamandridae). Experientia 34: 1001–1004.

Bucci-Innocenti, S., M. Ragghianti and G. Mancino. 1983. Investigation of karyology and hybrids in *Triturus boscai* and *Triturus vittatus* with a reinterpretation of species groups within *Triturus*. Copeia 1983: 662–672.

Caccone, A., G. D. Amato and J. R. Powell. 1987. Intraspecific DNA divergence in *Drosophila*: A study of parthenogenetic *D. mercatorum*. Mol. Biol. Evol. 4: 343–350.

Cherchi, A. and L. Montadert. 1982. Oligo-Miocene rift of Sardinia and the early history of the western Mediterranean basin. Nature 298: 736–736.

Esu, D. and T. Kotsakis. 1983. Les vertebres et les mollusques continentaux du Tertiarire de la Sardaigne: paleobiogeographie et biostratigraphie. Geologica Rom. 22: 177–206.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Felsenstein, J. 1991. PHYLIP: phylogenetic inference package, version 3.4: program and documentation. Department of Genetics, University of Washington, Seattle.

Frost, D. R. 1985. Amphibian species of the world. A taxonomic and geographic reference. Allen Press Inc. and Ass. Syst. Coll., Lawrence, Kans., USA.

Gatesy, J., D. Yelon, R. DeSalle and E. Vrba. 1992. Phylogeny of the Bovidae (Artiodactyla, Mammalia), based on mitochondrial Ribosomal DNA sequences. Mol. Biol. Evol. 9: 433–446.

Giacoma, C. and E. Balletto. 1988. Phylogeny of the salamandrid genus *Triturus*. Boll. Zool. 55: 337–360.

Gillespie, J. H. 1986. Rates of molecular evolution. Ann. Rev. Ecol. Syst. 17: 637–665.

Gutell, R. R. and G. E. Fox. 1988. A compilation of large subunit RNA sequences presented in a structural form. Nucleic Acids Res. 16 [Suppl.]: R175–R313.

Gutell, R. R., B. Weiser, C. Woese and H. F. Noller. 1985. Comparative anatomy of 16-S-like ribosomal RNA. Prog. Nucleic Acids Res. 32: 155–215.

Gyllensten, U. B. and H. A. Erlich. 1988. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc. Natl. Acad. Sci. USA 85: 7652–7656.

Halliday, T. and B. Arano. 1992. Resolving the phylogeny of the European Newts. TREE 6: 113–117.

Hedges, S. B., K. D. Moberg and L. R. Maxson. 1990: Tetrapod phylogeny inferred from 18S and 28S ribosomal RNA sequences and a review of the evidence for amniote relationships. Mol. Biol. Evol. 7: 607–633.

Hedges, S. B., J. P. Bogart and L. R. Maxson. 1992. Ancestry of unisexual salamanders. Nature 356: 708–710.

Hillis, D. M. 1991. Discriminating between phylogenetic signal and random noise in DNA sequences, pp. 278–294. *In* M. M. Miyamoto and J. Cracraft (eds.) Phylogenetic analysis of DNA sequences. Oxford University Press, New York, USA.

Hillis, D. M. and S. K. Davis. 1986. Evolution of ribosomal DNA: fifty years of recorded history in the frog genus *Rana*. Evolution 40: 1275–1288.

Hillis, D. M. and M. T. Dixon. 1991. Ribsomoal DNA: molecular evolution and phylogenetic inference. Quart. Rev. Biol. 66: 411–453.

Hillis, D. M. and J. P. Huelsenbeck. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. Journal of Heredity 83: 189–195.

Hixson, J. E. and W. M. Brown. 1986. A comparison of the small ribosomal RNA genes from the mitochondrial DNA of the great apes and humans: sequence, structure, evolution, and phylogenetic information. Mol. Biol. Evol. 3: 1–18.

Jaylet, A. 1966. Le caryotype de l'amphibien urodèle *Euproctus asper* (Duges). Chromosoma 18: 79–87.

Jukes, T. H. and C. Cantor. 1969: Evolution of protein molecules, pp. 21–123. *In* H. N. Munro (ed.) Mammalian protein metabolism. Academic Press, New York.

Kalezic M. L. and D. Hedgecock. 1980. Genetic variation and differentiation of three common European newts (*Triturus*) in Yugoslavia. Br. J. Herpetol. 6: 49–57.

Kimura, M. 1980. A simple method of estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111–120.

Kishino, H. and M. Hasegawa. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J. Mol. Evol. 29: 170–179.

Knowlton, N., L. A. Weigt, L. A. Solorzano, D. K. Mills and E. Bermingham. 1993. Divergence in proteins, mitochondrial DNA, and reproductive compatibility across the Isthmus of Panama. Science 260: 1629–1632.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Pääbo, F. X. Villablanca and A. C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86: 6196–6200.

Kraus, F. and M. M. Miyamoto. 1990. Mitochondrial genotype of a unisexual salamander of hybrid origin is unrelated to either of its nuclear haplotype. Proc. Natl. Acad. Sci. USA 87: 2235–2238.

Kraus, F. and M. M. Miyamoto. 1991. Rapid cladogenesis among the Pecoran ruminants: evidence from mitochondrial DNA sequences. Syst. Zool. 117–130.

Larson, A. 1991. A molecular perspective on the evolutionary relationships of the salamander families. Evol. Biol. 25: 211–278.

Larson, A. and A. C. Wilson. 1989. Patterns of ribosomal RNA evolution in salamanders. Mol. Biol. Evol. 6: 131–154.

Li, W.-H. and D. Graur. 1991. Molecular evolution. Sinauer, Sunderland, Massachusetts, USA.

Maniatis, T., E. F. Fritsch and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York.

Mancino, G., M. Raggianti and S. Bucci-Innocenti. 1982. The species groups of the genus *Triturus* (Amphibia, Caudata). Boll. Zool. 49: 118–119.

Macgregor, H. C., S. K. Sessions and J. W. Arntzen. 1990. An integrative analysis of phylogenetic relationships among newts of the genus *Triturus* (family Salamandridae), using comparative biochemistry, cytogenetics and reproductive interactions. J. Evol. Biol. 3: 329–373.

Maddison, W. P., M. J. Donoghue and D. R. Maddison. 1984. Outgroup analysis and parsimony. Syst Zool. 33: 83–103.

Meyer, A. 1993. Evolution of mitochondrial DNA in fishes, pp. 1–38. *In* P. W. Hochachka and T. P. Mommsen (eds.) The biochemistry and molecular biology of fishes, vol. 2, Elsevier Press.

Meyer, A. and A. C. Wilson. 1990. Origin of tetrapods inferred from their mitochondrial DNA affiliation to lungfish. J. Mol. Evol. 31: 359–364.

Milinkovitch, M. C., G. Orti and A. Meyer. 1993. Revised phylogeny of whales suggested by mitochondrial ribosomal DNA sequences. Nature 361: 346–348.

Mindell, D. and R. L. Honeycutt. 1990. Ribosomal RNA: evolution and phylogenetic applications. Annu. Rev. Ecol. Syst. 21: 541–566.

Miyamoto, M. M. and S. M. Boyle. 1989. The potential importance of mitochondrial DNA sequence data to eutherian mammal phylogeny, pp. 437–450. *In* B. Fernholm, K. Bremer and H. Jornvall (eds.) The hierarchy of life. Elsevier Science, Amsterdam.

Miyamoto, M. M., F. Kraus and O. Ryder. 1990. Phylogeny and evolution of antlered deer determined from mitochondrial DNA sequences. Proc. Natl. Acad. Sci. USA 87: 6127–6131.

Miyamoto, M. M., S. M. Tanhauser and P. J. Laipis. 1989. Systematic relationships in the artiodactyl tribe Bovini (family Bovidae), as determined from mitochondrial DNA sequences. Syst. Zool. 38: 342–349.

Moritz, C., C. J. Schneider and D. B. Wake. 1993. Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. Syst. Biol. 41: 273–291.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York, USA.

Oosterbroek, P. and J. W. Arntzen. 1992. Arca-cladograms of Circum-Mediterranean taxa in relation to Mediterranean paleogeography. Journal of Biogeography 19: 3–20.

Orsini, J. B., C. Coulon and T. Cocozza. 1980. Le dérive cénozoique de la Corse et de la Sardaigne. Géologie Alpine 56: 169–202.

Palumbi, S., A. Martin, S. Romano, W. O. McMillan, L. Stice and G. Grabowski. 1990. The simple fool's guide to PCR (Department of Zoology, University of Hawaii, Honolulu, 1991).

Rafinski, J. and J. W. Arntzen. 1987. Biochemical systematics of the old world newts *Triturus*: allozyme data. Herpetologica 43: 446–457.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Sbordoni, V., M. Cobolli Sbordoni, E. De Matthaeis, G. Allegrucci, D. Cesaroni, A. Caccone and G. M. Carpaneto. 1982. Orologi molecolari e paleogeografia: congruenza tra stime geocronologiche e datazioni elettroforetiche della divergenza nelle specie del genere *Euproctus* (Caudata, Salamandridae). Boll. Zool. (Suppl.) 49: 170.

Sbordoni, V., A. Caccone, G. Allegrucci, D. Cesaroni, M. Cobolli Sbordoni and E. De Matthaeis. 1985. Molecular clocks and paleogeography: dating the divergence time between *Euproctus* species (Caudata, Salamandridae). ICSEB III, 3rd International Congress of Systematics and Evolutionary Biology, Sussex, Brighton, England, 4–10 July. Abstracts, 166.

Sbordoni, V., A. Caccone, G. Allegrucci, D. Cesaroni. 1990. Molecular island biogeography, pp. 55–83. *In* Azzaroli (ed) Biogeographical aspects of insularity, Atti dei Convegni Lincei, vol. 85. Accademia Nazionale dei Lincei, Roma, Italy.

Sokal, R. R. and F. J. Rohlf. 1981. Biometry, 2nd ed. W. H. Freeman, San Francisco, USA.

Spolsky, C. M., C. A. Phillips and T. Uzzell. 1992. Antiquity of clonal salamander lineages revealed by mitochondrial DNA. Nature 356: 706–708.

Swofford, D. 1991. PAUP: phylogenetic analysis using parsimony, version 3.0s: program and documentation. Illinois Natural History Survey, Urbana.

Thiesmeier, B. and C. Hornberg. 1990. Zur Fortpflanzung sowie zum Paarungsverhalten der Gebirgsmolche, Gattung *Euproctus* (Gene'), im Terrarium, unter besonderer Berucksichtigung von *Euproctus asper* (Duges, 1852). Salamandra 26: 63–82.

Thomaz, D. M. 1990. DNA phylogeny of the European newts (genus *Triturus*) using a PCR amplified fragment of mitochondrial DNA. Thesis for the M.Sc. Dept. of Genetics, The University of Leicester, UK.

Thorn, R. 1968. Les salamandres d'Europe, d'Asie et d'Afrique du Nord. Lechevalier, Paris, France.

Wake, D. B. and N. Ozeti. 1969. Evolutionary relationships in the family Salamandridae. Copeia 1969: 124–137.

Wallis, G. P. 1987. Mitochondrial DNA insertion polymorphism and germ line heteroplasmy in the *Triturus cristatus* complex. Heredity 58: 229–238.

Wallis, G. P. and J. W. Arntzen. 1989. Mitochondrial DNA variation in the crested newt superspecies: limited cytoplasmic gene flow among species. Evolution 43: 88–104.

Watrous, L. E. and Q. D. Wheeler. 1981. The out-group comparison method of character analysis. Syst. Zool. 30: 1–11.

Wheeler, W. C. and R. L. Honeycutt. 1988. Paired sequence difference in ribosomal RNAs: evolutionary and phylogenetic implications. Mol. Biol. Evol. 5: 90–96.

Wilson, A. C., S. S. Carlson and T. J. White. 1977. Biochemical evolution. Annu. Rev. Biochem. 46: 573–639.

Wilson, A. C., H. Ochman and E. M. Prager. 1987. Molecular time scale for evolution. Trends Genet. 3: 241–247.

Wu, C.-I. and W.-H. Li. 1987. Evidence for higher rates of nucleotide substitution in rodents than in men. Proc. Natl. Acad. Sci. USA 82: 1741–1745.

Zuckerkandl, E. and L. Pauling. 1965. Evolutionary divergence and convergence in proteins, pp. 97–166. *In* V. Bryson and H. J. Vogel (eds.) Evolving genes and proteins. Academic Press, New York, New York, USA

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