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Phylogenetic relationships of the European newts (genus *Triturus*) tested with mitochondrial DNA sequence data

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Abstract

European newts (genus *Triturus*) are widely studied, but their phylogeny is not yet unambiguously resolved. Fragments of mitochondrial DNA experiencing different rates of evolution (the ATPase and 12S rRNA genes) were sequenced in order to test a phylogenetic hypothesis derived from biochemical and behavioral data. Well-supported branches of the existing phylogeny gained support in our study. The monophyletic origin of the hypothesized *T. boscai* – *T. italicus* clade remained ambiguous, whereas strong support was gained for the sister-taxon relationship of *T. vulgaris* and *T. montandoni*. The position of *T. vittatus* as a sister taxon to the *T. marmoratus* species group was also supported. The phylogenetic position of *T. alpestris* could not be clarified. With an in-group taxon sampling denser than in previous molecular phylogenetic studies and under the *a priori* selection of species from the genera *Cynops*, *Neurergus* and *Paramesotriton* as out-groups, the monophyly of *Triturus* was strongly supported. It cannot be excluded, however, that the presumed out-group actually belongs to the in-group, rendering *Triturus* paraphyletic as was concluded from recently published 12S and 16S rRNA sequence data.

Contents

Introduction

Phylogenies are a cornerstone to the study of evolution: without them we are unable to reconstruct and understand pattern and process of evolutionary change. To obtain a robust phylogenetic hypothesis requires the gathering of multiple independent and complementary data sets, because usually no single data set is sufficiently powerful to simultaneously resolve older and more recent cladogenetic events.

The genus *Triturus* (European newts) has been the subject of extensive phylogenetic analysis with various data sets [osteological (Bolkay, 1928; Rafinski and Pecio, 1989), immunological (Busack et al., 1988), morphological (Giacoma and Balletto, 1988), biochemical (Rafinski and Arntzen, 1987), behavioral (Arntzen and Sparreboom, 1989), cytogenetic (Macgregor et al., 1990)], and is considered by some to be the phylogenetically best studied genus in the world (Halliday and Arano, 1991). Despite these concerted efforts, its phylogeny is not fully resolved and several competing hypotheses are available, none of them with unambiguous overall support. We take the study by Arntzen and Sparreboom (1989) as the basis of our work because the phylogeny they present is based on two independent and complementary data sets. Moreover, their hypothesis resolves the earlier as well as the later events in the *Triturus* radiation and is robust under the jack-knife test.

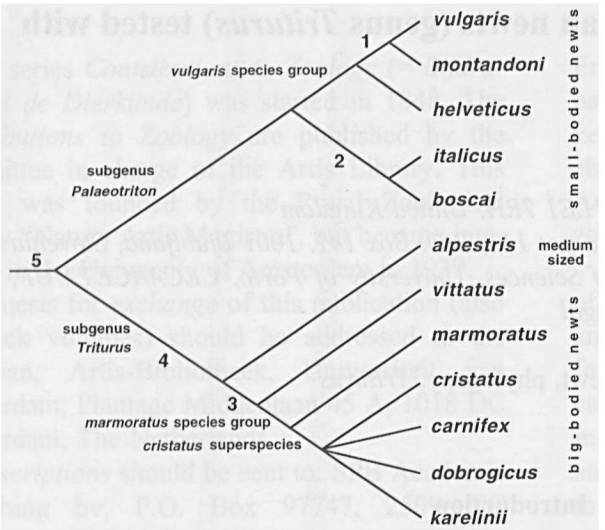

Fig. 1. Phylogeny for the genus *Triturus* based on available biochemical and behavioral characters. Numbers refer to specific questions as addressed in the text. Out-group taxa are taken from the genera *Cynops*, *Neurergus*, and *Paramesotriton*.

Nine or twelve *Triturus* species are currently recognized, depending on the criteria used for species recognition. Here we refer to twelve species, with four of them (*Triturus cristatus*, *T. carnifex*, *T. karelinii* and *T. dobrogicus*) grouped together in the *T. cristatus* superspecies (Wallis and Arntzen, 1989). The 'big'- and 'medium-sized' newts (*T. cristatus*, *T. marmoratus*, *T. vittatus* and *T. alpestris*) are organized in the subgenus *Triturus* and the 'small-bodied' newts (*T. boscai*, *T. helveticus*, *T. italicus*, *T. montandoni* and *T. vulgaris*) are placed in the subgenus *Palaeotriton*. The detailed configuration is given in Fig. 1, alongside with the nomenclature that we adopt. For the following sections of the phylogenetic tree the support is limited or contradictory: 1) the sister-taxon status of *T. vulgaris* and *T. montandoni* is supported by allozyme data only; 2) the monophyly of *T. boscai* and *T. italicus* hinges on the interpretation given to behavioral characters; 3) the status of *T. vittatus* as the sister taxon of the *T. marmoratus* species group may be called into question on the basis of its overall similarity to some small-bodied species, *T. vulgaris* in particular; 4) the support for the position of *T. alpestris* in the subgenus *Triturus* is relatively weak due to the non-independence of some behavioral synapomorphies; 5) the monophyly of *Triturus*, traditionally taken for granted, has been put in doubt by molecular data (Titus and Larson, 1995).

We tested the phylogenetic hypothesis of *Triturus* with a newly generated set of independent data. Two fragments of the mitochondrial (mt)DNA molecule experiencing different evolutionary rates were studied. The slowly evolving 12S rRNA gene (Kocher et al., 1989; Hickson et al., 1996) was partially sequenced to test some of the supposedly earlier events of the *Triturus* radiation, while the fast evolving ATPase gene (Kumar, 1996) was partially sequenced to test the branching order among some supposedly closely related species in the *T. vulgaris* species group. Sequence data for cytochrome b produced ambiguous phylogenetic results, perhaps due to the comparison of non-homologous sequences (Caccone et al., 1997; cf. Zhang & Hewitt, 1996) and were discarded.

Materials and methods

Two specimens were selected from each of nine *Triturus* species, if possible from different localities. As out-groups to the genus, representatives of the salamandrid genera *Cynops*, *Neurergus* and *Paramesotriton* were selected, following Arntzen and Sparreboom (1989). *Triturus boscai* was taken as out-group to the *T. vulgaris* species group. Specimens for which DNA was extracted were sampled as follows: *T. alpestris* from Mayenne, France and Pola de Sierra, Spain (subspecies *cyreni*); *T. boscai* from Toledo, Spain; *T. cristatus* from Limanowa, Poland and Sinaia, Romania; *T. helveticus* from Ambleteuse, France and Canterbury, U. K.; *T. italicus* from Conversano, Italy; *T. marmoratus* from El Berrueco near Madrid, Spain and Rochechouart, France; *T. montandoni* from Ustrzyki, Poland; *T. vittatus* from an unknown locality in Israel and from Adapazari, Turkey (subspecies *ophryticus*) and *T. vulgaris* from Ambleteuse, France. Out-group taxa *Cynops ensicauda*, *Neurergus strauchii* and *Paramesotriton* sp. were obtained from the pet trade with no reliable locality information.

Total DNA was extracted from approximately 50 mg of frozen or ethanol-preserved tissue (liver,

heart or tail muscle) following standard protocols (Sambrook et al., 1989). For PCR-amplification of the 12S rRNA region the forward (L2475) and reverse (H2897) 'universal' primers were used (Kocher et al., 1989). The forward and reverse ATPase primers L9858 (5'-CTCCTCCTTAATGATATGCCACA-3') and H10307 (3'-TTCACCCCAACTACCCAACTATC-5') were designed in our laboratory by G. Rowe. Double stranded DNA amplifications were performed using standard PCR protocols (Erlich, 1989) with negative controls to test for contamination. The 12S rRNA fragment was amplified under stringent conditions (annealing temperature 65°C, extension time 25 seconds, up to 28 cycles), while the ATPase primers required milder conditions (annealing temperature 45-50°C, extension time of 60 seconds, minimum of 30 cycles). The amplified products were separated on 1.5% low melting point agarose gels, cut out of the gel and purified by successive phenol, phenol-chloroform and chloroform-isoamylalcohol extractions, followed by ethanol precipitation. Both strands were directly sequenced using the dideoxy chain-termination method (Sanger et al., 1977). Multiple sequences were aligned with Clustal-V software (Higgins and Sharp, 1988) with manual adjustments. The ATPase gene sequences were translated into amino-acid sequences and the reading frame was determined by alignment to sequences of the teleost fish *Cottus kessleri* (Grachev et al., 1992) and human sequences (Arnason et al., 1996) obtained from GenBank. When the sequences for individuals of the same species were not identical, the consensus sequence was used in the phylogenetic analysis with variable sites taken as polymorphisms. The percent sequence divergence was calculated following Mindell and Honeycutt (1990). We aligned our sequences to the published salamandrid 12S sequences (Caccone et al., 1994; Hay et al., 1995), that include the in-group taxa *T. vulgaris* and *T. carnifex* and out-group taxa from the genera *Euproctus*, *Notophthalmus*, *Pleurodeles* and *Salamandra*. Furthermore, the sequences were aligned to those published by Titus & Larson (1995), that include *T. alpestris*, *T. karelinii* and representatives of the salamandrid genera *Chioglossa*, *Cynops*, *Euproctus*, *Neurergus*, *Mertensiella*, *Notophthalmus*, *Pachytriton*, *Paramesotriton*, *Pleurodeles*, *Salamandra*, *Salamandrina*, *Taricha* and *Tylototriton*.

Phylogenetic reconstruction was performed under the principle of parsimony, using the PAUP 3.1.1 software (Swofford, 1991) with the 'branch-and-bound' search option (the 'heuristic search' method was used in the bootstrap runs). Two *a priori* selected strategies were followed. First, a uniform transition – transversion ratio and uniform positional ratio was used, i.e., 'no weighting'. Second, different weights were assigned to transitions versus transversions inversely proportional to the frequency of different substitutions among in-group taxa as traced from the tree obtained with the unweighted approach, with 24 as the base number. Gap derived character-state changes were given the same weight as transversions, also on an *a priori* basis. Positional weighting factors for the first, second, and the third codon positions were determined for the protein coding DNA fragment (ATPase) in a similar way. Bootstrap scores (Felsenstein, 1985; Hillis and Bull, 1993) were determined over 5000 replications to obtain an impression of the strength of support from the data for the phylogenetic tree showing maximum parsimony. Templeton's (1983) non-parametric test was used to test one-sided for the significance of differences between documented phylogenies and the newly derived alternatives.

Results

Aligned sequences represent 212 bp of the ATPase gene and covers the short stretch of overlap between regions coding for ATPase 6 and ATPase 8 (Fig. 2). The first nucleotide corresponds to position 256 in the *Cottus kessleri* ATPase sequence (Grachev et al., 1992). Aligned sequences represent 301 bp of 12S rRNA gene. The first nucleotide corresponds to position 233 in the *Mertensiella luschani* 12S rRNA sequence (Titus and Larson, 1995). No insertions or deletions ('indels') had to be inferred to align the ATPase sequences. Alignment of the 12S sequences required two indels at one position among the *Triturus* species and two indels covering four positions in the out-group taxa. More indels on nine positions were required when

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01 CAGAATACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ACACCCCCCT AGAGGAGCCT GTTCTATAAT CGATAATCCA CGATAAACCT
02 CGGAACACTA CGAACAACAG CCTAAAACTC AAAGGACTTG GCGGTGCTCT ACACCCACCT AGAGGAGCCT GTTCTATAAT CGATAATCCA CGATAAACCT
03 CAGAACACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCC ACACCCCCCT AGAGGAGCCT GTTCTATAAT CGATATTCCT CGATAAACCT
04 CAGAAYACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ACACCCCCCT AGAGGAGCCT GTTCTATAAT CGAYAMCCCC CGATAAACCT
05 CAGAATACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ACACCCCCCT AGAGGAGCCT GTTCTATAAT CGATAACCCC CGATAAACCT
06 CAGAGTACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ACACCCACCT AGAGGAGCCT GTTCTGTAAT CGATAATCCA CGATAAACCT
07 CAGAGTACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ATACCCCCCT AGAGGAGCCT GTTCTATAAT CGATAATCCC CGATAAACCT
08 CAGAATACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ACACCCCCCT AGAGGAGCCT GTTCTATAAT CGATAATCCC CGATAAACCT
09 CAGAGTACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ACACCCCCCT AGAGGAGCCT GTTCTATAAT CGATAATCCA CGATAAACCT
10 CAGAGTACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ACACCCCCCT AGAGGAGCCT GTTCTATAAT CGATAATCCC CGATAAACCT
11 CAGAGTACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ACACCCCCCT AGAGGAGCCT GTTCTATAAT CGATAATCCA CGATAAACCT
12 CAGAGTACTA CGAGCAACAG CTTAAAACTC AAAGGACTTG GCGGTGCTCT ATACCCCCCT AGAGGAGCCT GTTCTATAAT CGATAATCCC CGATAAACCT

01 CACCATCTAT TGCCAATACA GCCTATATAC CACCGTCCAG CCCACCCTTT AAAGGGCTA- --GTAGGCAC AACTATAAAC ATAAAAACGT CAGGTCAAGG
02 CACCATCTGT TGCTAATACA GCCTATATAC CACCGTCCAG CCCACCCTCT AAGGGTATAA TAGTAGGCAC AACTACAAAC ATAAAAACGT CAGGTCAAGG
03 CACCATCTAT TGCCAATACA GCCTATATAC CACCGTCCAG CCCACCCTTC AAAGGACTAA CAGTAGGCAC AACTATAAAC ATAAAAACGT CAGGTCAAGG
04 CACCATCTAT TGCYARTACA GCCTATATAC CACCGTCCAG CCCACCCTTT AAAGGRWAAA YAGTAGGCAC AAYTATAGAC ATAAAAACGT CAGGTCAAGG
05 CACCATCTGT TGCCAATACA GCCTATATAC CACCGTCCAG CCCACCCTTT AAGGGTAAAA CAGTAGGCAC AACTATAGAC ATAAAAACGT CAGGTCAAGG
06 CACCACTTGT TGCCAATACA GCCTATATAC CACCGTCCAG CCCACCCTTC AAAGGGTAAA AAGTAGGCAA AATTATACAC ATAAGAACGT CAGGTCAAGG
07 CACCATCTAT TGCCAATACA GCCTATATAC CACCGTCCAG CCCACCCTTT AAAGGAAAGA CAGTAGGCAC AACTACACAC ATATAAACGT CAGGTCAAGG
08 CACCATCTAT TGCCAATACA GCCTATATAC CACCGTCCAG CCCACCCTTT AAAGGTAAAA CAGTAGGCAC AACTATACAC ATAAAAACGT CAGGTCAAGG
09 CACCACTTAT TGCCAATACA GCCTATATAC CACCGTCCAG CCCACCCTTC AAGGGACCAA AAGTAGGCAC AATTATACAC ATAAAAACGT CAGGTCAAGG
10 CACCATCTAT TGCCAATACA GCCTATATAC CACCGTCCAG CCCACCCTTT AAAGGAAAGA CAGTAGGCAC AACTACACAC ATATAAACGT CAGGTCAAGG
11 CACCATTTAT TGCTAATACA GCCTATATAC CACCGTCCAG CCCACCCTTT AAGGGTCAAA YAGTAGGCAT AATTATAGAC ATARAAACGT CAGGTCAAGG
12 CACCATCTAT TGCCAATACA GCCTATATAC CACCGTCCAG CCCACCCTTT AAAGGAAAGA CAGTAGGCAC AACTACATAC ATATAAACGT CAGGTCAAGG

01 TGTAGCACAT AAGATGGGAA GAAATGGGCT ACATTTTCTA GCTTAGAAAA TACGGAAAAG CTTGTGAAAC AAAACTATAA AGGAGGATTT AGCAGTAAAA A
02 TGTAGCAAAT AAGATCGGAA GAAATAGGCT AC-TTTGCTA CCTTGAAAAA TACGGAAAAG CTTGTGAAAC AAAACTATAA AGGAGGACTT AGCAGTAAAA A
03 TGTAGCATAT AAGATGGGAA GAAATGGGCT ACATTTTCTA ACTTAGAAAA TACGGAAAAG CTTGTGCAAC AAAACTATAA AGGAGGATTT AGCAGTAAGA A
04 TGTAGCGTAT GAGATGGGAA GAAATGGGCT ACATTTTCTA ACCTAGAAAA CACGGAAAAG TTTATGAAAC AAAACTATAA AGGAGGATTT AGTAGTAAAA A
05 TGTAGCAGAT AAGATGGGAA GAAATGGGCT ACATTTTCTA ACCTAGAAAA CACGGAGAAG TTTATGAAAC TAAACTATAA AGGAGGATTT AGTAGTAAAA A
06 TGTAGCAAAT AAAGCGGGAA GAAATGGGCT ACATTTTCTA ATCTAGAAAA CACGGAAAAG TCTGTGAAAT AGAACTACAA AGGAGGATTC AGAAGTAAAA A
07 TGTAGCAAAT AAGATGGGAA GAAATGGGCT ACATTTTCTA A-CTAGAAAG CACGGAAAAG TTTATGAAAC TAAGCTATGA AGGAGAATTT AGCAGTAAAA A
08 TGTAGCAGAT AAGATGGGAA GAAATGGGCT ACATTTTCTA ACCTAGAAAA CACGGAAGAG TCTATGAAAC TAAACTATGA AGGAGGATTT AGCAGTAAAA A
09 TGTAGCAAAT AAAGCGGGAA GAAATGGGCT ACATTTTCTA ACCTAGAAAA TACGGAAAAG TCTATGAAAT AAAACTACGA AGGAGGATTT AGCNGTAAAA A
10 TGTAGCAAAT AAGATGGGAA GAAATGGGCT ACATTTTCTA A-CTAGAAAA CACGGAAAAG TTTANGAAAC TAAGCTATAA AGGAGAATTT AGCAGTAAAA A
11 TGTAGCAAAT AAGATGGGAA GAAATGGGCT ACATTTTCTA AYCTAGAAAA CACGGAAAAG TCYATGAAAC AGRACTATAA AGGAGGATTT AGAAGTAAAA A
12 TGTAGCAAAT AAGATGGGAA GAAATGGGCT ACATTTTCTA ACCTAGAAAA CACGGAAAAG TTTATGAAAC TAAGCTATAA AGGAGAATTT AGCAGTAAAA A

                                  ***
05 ACACAACCCT GAAACTGACC ATGAATTTAG GCTTTTTTGA CCAATTTATA AGCCCCACCG TACTAGGCAT TCCTTTAATC GGCYTATCCT TAACACTCCC
07 ACACAACCCT GAAAYTGACC ATGAATTTAG GCTTTTTTGA CCAATTTATA AGCCCTACTA TACTAGGGGT GCCGCTAATT GGCYTAGCCC TTACACTACC
10 ACACAACCCT GAAGCTGACC ATGAACTTAG GCTTTTTTGA CCAATTTATA AGCCCCACTA TACTAGGAGT CCCATTAATT GGCYTAGCCC TTACGCTTCC
12 ACACAACCCT GAAGCTGACC ATGAACTTAG GCTTTTTTGA CCAATTTATA AGCCCTACTA TACTAGGAGT CCCATTAATT GGCYTAGCCC TTACGCTTCC

05 GTGACTAATA TTCCCTAAAA CAACTAATCA TTGACTAAAT ATCGACCTCT CAACCAAACA AACTTGATTC TTCGGACTAT TTACAAAACA ACTTATACTC
07 ATGATTACTG TTTCCTAANA CAACTAACCA TTGGTTAACC AACCGCCTAT CAACAACACA AACTTGGCTC TTTGGTATAT TTACTAAACA ACTTATACTT
10 ATGATTGCTG TTTCCAAAGA CAACAGACCA TTGGCTAAAT AACCGCCTAT CAACCACACA AACTTGGTTC TTTGGTATAT TTACTAAGCA GCTTATGCTC
12 ATGATTACTG TTTCCAAAGA CAACAGACCA TTGGCTAAAT AACCGCCTGT CAACCACACA AACTTGGTTC TTTGGTATAT TTACTAAGCA ACTTATACTT

05 CCAATTAATA TT
07 CCAATTGGCG TT
10 CCAATTGGCA TT
12 CCAATTGGCA TT

05 TQPWNWPWI* MNLGFFDQFM SPTVLGIPLI GLSLTLPWLM FPKTTNHWLN IDLSTKQTWF FGLFTKQLML PINI
07 TQPWNWPWI* MNLGFFDQFM SPTMLGVPLI GLALTLPWLL FPXTTNHWLT NRLSTTQTWL FGMFTKQLML PIGV
10 TQPWSWPWT* MNLGFFDQFM SPTMLGVPLI GLALTLPWLL FPKTTDHWLN NRLSTTQTWF FGMFTKQLML PIGI
12 TQPWSWPWT* MNLGFFDQFM SPTMLGVPLI GLALTLPWLL FPKTTDHWLN NRLSTTQTWF FGMFTKQLML PIGI
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Fig. 2. mtDNA sequence data for 12S rRNA (top panel), ATPase (middle panel) and ATPase amino acid sequences (lower panel), with overlap between ATPase genes. DNA sequences are light strands, from 5' to 3' end. The taxa studied are : 1) *Cynops ensicauda*, 2) *Neurergus strauchii*, 3) *Paramesotriton* sp., 4) *Triturus alpestris*, 5) *T. boscai*, 6) *T. cristatus*, 7) *T. helveticus*, 8) *T. italicus*, 9) *T. marmoratus*, 10) *T. montandoni*, 11) *T. vittatus,* and 12) *T. vulgaris*. Asterisks indicate the termination codon for the ATPase 8 gene.

published sequences were added to the data set, lengthening the fragment to 305 positions, with no regions of ambiguous alignment (sensu Titus and Larson, 1995).

One-hundred and sixty-one of the 212 ATPase nucleotide positions were identical across the four species studied and 38 variable positions differed by a single substitution in one taxon (Fig. 2). This left 13 (6.1%) nucleotide positions with potentially, phylogenetically relevant information. The sequence difference between *T. boscai* and in-group taxa ranged between 18.9% and 19.3%. Within

the *vulgaris* species group the distances were considerably lower, ranging from 3.3% between *T. montandoni* and *T. vulgaris* to 10.4% between *T. montandoni* and *T. helveticus*. Taking the short stretch of sequence overlap between both ATPase genes into account, the transition: transversion ratio among in-group taxa was 3.2 and the number of substitutions at the first, second, and third codon position were 5, 3, and 17, respectively. Following these relationships, the weights assigned to transitions versus transversions were 3 and 21 whereas the weights applied to the first, second and third codon positions were 8, 14, and 2. At the level of amino acid codon usage, 16 variable characters were found with nine synapomorphic character states for the *T. helveticus – T. montandoni – T. vulgaris* clade, four autapomorphic character states for *T. helveticus* and three synapomorphies were found for *T. montandoni – T. vulgaris*. The DNA sequences of *T. vulgaris* and *T. montandoni* differed only by silent substitutions.

Two-hundred and thirty-five of the 301 nucleotide positions on the 12S fragment were identical across the 12 taxa studied and 25 variable positions differed by a single substitution in one taxon, leaving 41 (13.6%) phylogenetically informative sites (Fig. 2). Half the number of variable sites were found in one-third of the 12S fragment, at the 3' end. The sequence difference between out-group and in-group taxa ranged between 6.0% and 14.0%. Within the genus *Triturus* the distances were considerably lower, ranging from 0.7% between *T. montandoni* and *T. vulgaris* [which is less than that found within *T. alpestris* (3.0%) and *T. vittatus* (1.7%)] to 10.6% between *T. cristatus* and *T. alpestris*. The average sequence difference between in-group species and an out-group taxon ranged from 7.4% (*Cynops*) to 11.2% (*Neurergus*). The transition : transversion ratio among in-group taxa was 2.8 and weights determined were 4 and 20.

Given *Cynops*, *Neurergus* and *Paramesotriton* as out-groups, the monophyly of *Triturus* appears to be strongly supported with a 89% bootstrap replication score (*P*b) (Fig. 3). Two major in-group clusters are formed. The first, with *P*b = 85%, consists of *T. vittatus* as a sister taxon to the *T. marmoratus* species group (*P*b = 98%). The second group contains *T. alpestris* and all small-bodied *Triturus* species, with a low bootstrap support (*P*b = 59%). Within this group, the monophyly of the *T. vulgaris* species group is strongly supported (*P*b = 100%), whereas the relationship of *T. alpestris*, *T. boscai,* and *T. italicus* among themselves is poorly resolved (*P*b < 55%). On the basis of 12S sequence data no firm conclusion can be drawn regarding the branching order within the *vulgaris* species group. However, the ATPase sequence data provided strong support for the sister taxon status of *T. montandoni* and *T. vulgaris* (*P*b = 96, Fig. 3) and the same bootstrap value was observed when amino-acid sequence data were analyzed instead of the DNA sequence. Under the weighting scheme topologically similar results were obtained, with three reservations: 1) with the ATPase gene fragment, a score of *P*b = 100 was observed for the *vulgaris – montandoni* clade, while not with the 12S rRNA gene fragment; 2) generally less phylogenetic resolution and lower bootstrap replication scores were observed on well-established branches; and, 3) an equally parsimonious solution was found in which the genus *Triturus* is paraphyletic. Sequences derived from the same or congeneric species by different authors (Caccone et al., 1994;

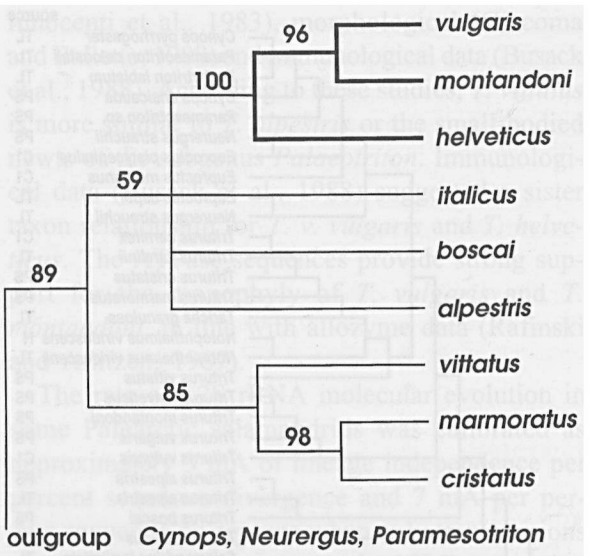

Fig. 3. Composite phylogeny for the genus *Triturus*, constructed from mitochondrial DNA sequence data. Thin and thick lines represent branching order as reconstructed from 12S rRNA and ATPase sequence data, respectively, with bootstrap replication scores indicated along branches.

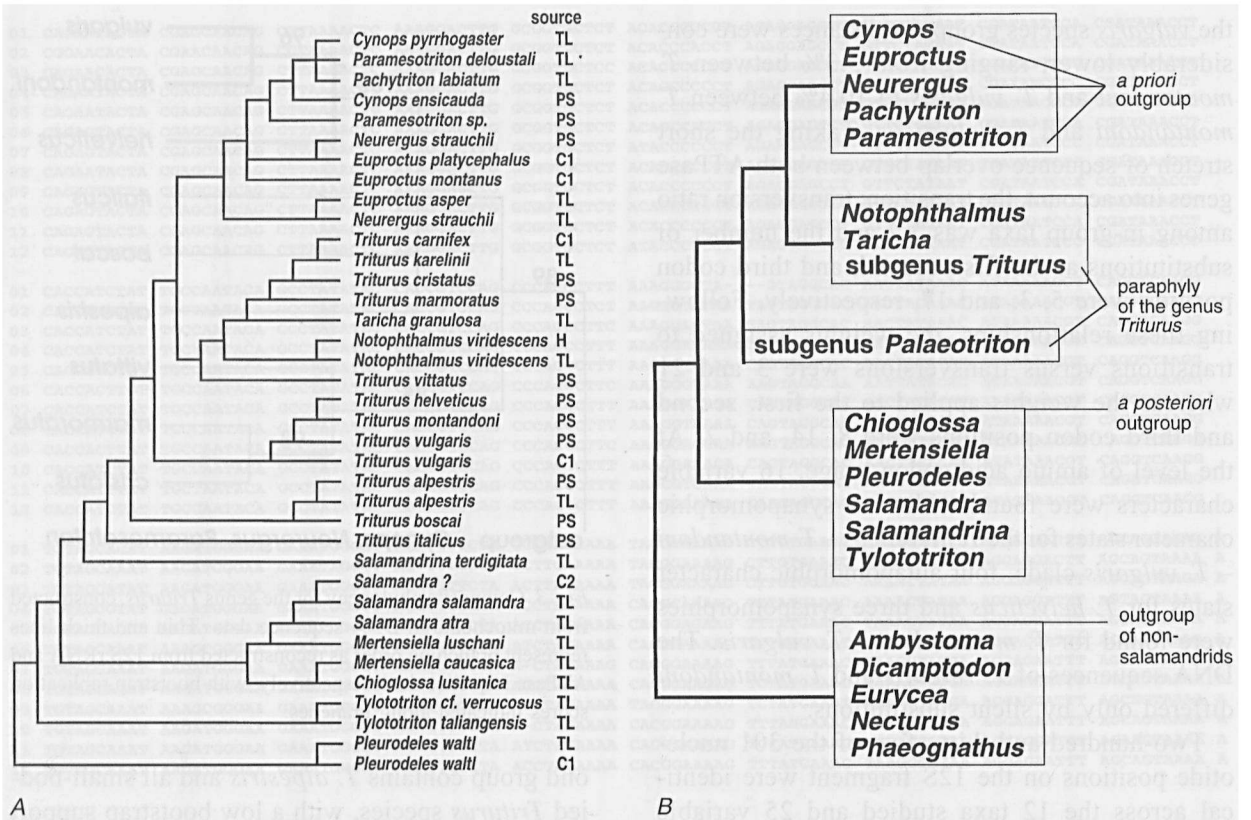

Fig. 4. (A) Molecular phylogeny for salamandrid salamanders reconstructed from 12S rRNA sequence data as published by C1, Caccone et al. (1994); C2, Caccone et al. (1994: 523); H, Hay et al. (1995); TL, Titus and Larson (1995) and PS, present study. (B) Schematic phylogeny for salamandrid genera as derived from 12S and 16S rRNA sequence data (adapted from Titus and Larson, 1995). Note the possible paraphyly of the genus *Triturus*.

Titus & Larson, 1995; Hay et al., 1995 and present study) are very similar to one another and consequently the taxa they represent are placed close together in the phylogenetic tree that has maximum parsimony (i.e., *Cynops* sp., *Neurergus strauchii*, *Notophthalmus viridescens*, *Paramesotriton* sp., *Pleurodeles waltl*, *Salamandra salamandra*, *Triturus alpestris*, *T. cristatus* superspecies and *T. vulgaris*).

With a wide range of out-groups included in the analysis, the monophyly of the genus *Triturus* was not supported. The sister clade to the subgenus *Palaeotriton* would not be the subgenus *Triturus* (i.e., *T. vittatus*, *T. marmoratus,* and the *T. cristatus* superspecies), but the subgenus *Triturus* plus *Taricha* and *Notophthalmus* and the sister clade to this group would be composed of *Cynops*, *Euproctus*, *Neurergus*, *Pachytriton,* and *Paramesotriton* (Fig. 4A). Templeton's test indicated that the difference between the shortest tree and the tree in which *Triturus* is monophyletic was significant ($P < 0.05$).

Discussion

We have tested an existing phylogeny of *Triturus* against independent molecular data. Two PCR-amplified fragments of the mtDNA molecule with relatively low (12S rRNA) and high rates of evolution (ATPase) were employed to test some of the earlier and more recent speciation events of the *Triturus* radiation, respectively. The chosen fragments showed evolutionary rates that were anticipated and that were appropriate for addressing phylogenetic questions at this taxonomic level. Faster evolving protein-coding ATPase genes can be reliable tracers of evolutionary history among

close relatives (as within the *T. vulgaris* species group), where silent, third codon position substitutions account for most variation (Zardoya and Meyer, 1996). On the other hand, if the saturation of transitions is suspected, it is imperative to reduce the emphasis on, or even eliminate, this class of substitutions from phylogenetic analysis (Disotell et al., 1992; Knight and Mindell, 1993). Although intuitively appealing, differential weighting may downweight informative transitions in conserved regions and upweight transversions primarily present in more variable regions of the molecule, thereby obscuring phylogenetic relationships (Titus and Larson, 1995; Mindell and Tacker, 1996). Given the observed transition bias, saturation was not prevalent among the compared sequences. The observation that with weighting the bootstrap support for congruent sections of competing phylogenetic solutions dropped and the phylogenetic resolution decreased, supports this view.

Our analysis presents a hypothesis that is concordant with well-supported areas in the established *Triturus* phylogeny. As to the specific questions we raised, some appear to be solved while others are not. The new data do not help to elucidate the relationship between *T. boscai* and *T. italicus*. Their monophyly was suggested by allozyme data and, ambiguously, by behavioral data (Arntzen and Sparreboom, 1989). The observation of 'flamenco' behavior in the sexual repertoire of *T. helveticus* (M. Faria, pers. comm.) and *T. marmoratus pygmaeus* (T. Halliday, pers. comm., M. Sparreboom, pers. comm.) further erodes the support for *T. boscai* – *T. italicus* monophyly. Similarly, the current study does not convincingly clarify the position of *T. alpestris*. The phylogenetic position of this species could also not be satisfactorily resolved from behavioral data (Arntzen and Sparreboom, 1989). Bolkay (1928) placed it in a third subgenus with intermediate characteristics, in between *Triturus* and *Palaeotriton*.

The 12S rRNA sequence data support the placement of *T. vittatus* as the sister taxon to the *T. marmoratus* species group in the subgenus *Triturus*, as was inferred from its breeding behavior (Arntzen and Sparreboom, 1989). This challenges the inferences from the phenetic analyses of osteological (Rafinski and Pecio, 1989), karyological (Bucci-Innocenti et al., 1983), morphological (Giacoma and Balletto, 1988) and immunological data (Busack et al., 1988). According to these studies, *T. vittatus* is more similar to *T. alpestris* or the small-bodied newts of the subgenus *Palaeotriton*. Immunological data (Busack et al., 1988) suggested a sister taxon relationship for *T. v. vulgaris* and *T. helveticus*. The ATPase sequences provide strong support for the monophyly of *T. vulgaris* and *T. montandoni*, in line with allozyme data (Rafinski and Arntzen, 1987).

The rate of 12S rRNA molecular evolution in some Palearctic salamandrids was calibrated as approximately 3 mA of lineage independence per percent sequence divergence and 7 mA per percent sequence divergence when only transversions are considered (Caccone et al., 1994). Applying these rates provides an estimate of 12-19 mA for the age of the *T. vittatus* lineage (versus the *T. marmoratus* species group) in the subgenus *Triturus* and an estimate of 14-19 mA for the *T. boscai* lineage (versus the *T. vulgaris* species group) within the subgenus *Palaeotriton*. This is in line with estimates from fossil, biochemical, molecular and biogeographical data that converge to 14-15 mA for the *T. vittatus* lineage and to 13-15 mA for the *T. boscai* lineage (reviewed in Oosterbroek and Arntzen, 1992).

The monophyly of the genus *Triturus*, although widely accepted, is defined on the basis of somewhat vague character state descriptions ('a suite of behavioral character states' and 'a high level of sexual dimorphism' – Halliday, 1977) that are not explicit synapomorphies. Moreover, a feature such as the potential for interbreeding (Wolterstorff and Herre, 1935) is explicitly plesiomorphic. The monophyly of the genus *Triturus* has recently been put into question in a molecular phylogenetic study of the family Salamandridae (Titus and Larson, 1995) and in a molecular biogeographical study of *Euproctus* (Caccone et al., 1994). In both of them only two *Triturus* species were involved. With just the representatives of the genera *Cynops*, *Neurergus* and *Paramesotriton* as out-groups, a denser in-group taxon sampling does not challenge the hypothesis of *Triturus* monophyly. However, 12S and 16S mtDNA sequence data indicate that the genera *Cynops*, *Neurergus* and *Paramesotriton*

themselves may be in-group taxa relative to *Triturus* (Fig. 4B). In the light of these results, their *a priori* choice as out-groups to the genus *Triturus* may have been unfortunate.

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