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Citation
Wielstra, B., Themudo, G. E., Guclu, O., Olgun, K., Poyarkov, N. A., & Arntzen, J. W. (2010). Cryptic crested newt diversity at the Eurasian transition: t. *Molecular Phylogenetics And Evolution*, *56*(3), 888-896. doi:10.1016/j.ympev.2010.04.030

Version:	Publisher's Version
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Downloaded from:	https://hdl.handle.net/1887/3281014

Note: To cite this publication please use the final published version (if applicable).

Molecular Phylogenetics and Evolution 56 (2010) 888–896

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Cryptic crested newt diversity at the Eurasian transition: The mitochondrial DNA phylogeography of Near Eastern *Triturus* newts

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ARTICLE INFO

Article history:
Received 21 September 2009
Revised 15 April 2010
Accepted 23 April 2010
Available online 7 May 2010

Keywords:
Anatolia
Balkan Peninsula
Caucasia
Historical biogeography
Iran
Mediterranean
Phylogeny
Triturus arntzeni
Triturus cristatus superspecies
Triturus karelinii

ABSTRACT

Crested newts of the *Triturus karelinii* group occur in a phylogeographically understudied region: the Near East. Controversy surrounds the systematic position of these newts within the complete crested newt assemblage (the *Triturus cristatus* superspecies). We explore the situation using mitochondrial sequence data (ND2 and ND4, ≈1.7 kb) and employing different methods of phylogenetic inference (Bayesian inference and Maximum Likelihood using mixed models) and molecular dating (r8s and BEAST). The *T. karelinii* group is monophyletic and constitutes one of four main lineages in the *T. cristatus* superspecies. The separation of the *T. karelinii* group from the remaining crested newts around 9 Ma is related to the formation of the Mid-Aegean Trench, which separated the Balkan and Anatolian landmasses. The *T. karelinii* group comprises three geographically structured clades (eastern, central and western). The genetic divergence shown by these clades is comparable to that among recognized crested newt species. We suggest the uplift of the Armenian Plateau to be responsible for the separation of the eastern clade around 7 Ma, and the re-establishment of a marine connection between the Black Sea and the Mediterranean at the end of the Messinian Salinity Crisis to have caused the split between the central and western clade around 5.5 Ma. Genetic structuring within the three clades dates to the Quaternary Ice Age (<2.59 Ma) and is associated with alternating periods of isolation and reconnection caused by periodic changes in sea level and surface runoff.

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1. Introduction

Historical biogeography seeks to understand the processes governing the spatio-temporal distribution of biodiversity. Patterns in biodiversity can be established objectively by exploring the genotype (Avise, 2004; Riddle et al., 2008). Phylogeography refers to the section of historical biogeography that aims to uncover the geographical distribution of genealogical lineages within (groups of related) species (Avise, 2000). Present-day distribution patterns are the result of past processes and, aided by gene geography, factors responsible for former vicariance and dispersal can be inferred.

Although each species will have had unique evolutionary responses, similarities in genetic patterns are expected to be present among the components of a region's contemporary biodiversity (Arbogast and Kenagy, 2001; Avise, 2004). The phylogeographic approach provides the foundation for formulating biogeographical hypotheses, initially for individual model species and ultimately for entire communities. It can be seen as a tool for reconstructing paleogeological scenario's, analogous to, for example, paleontology and palynology (Riddle et al., 2008).

The Mediterranean region serves as a natural laboratory for phylogeographically oriented studies. The area has experienced a turbulent geological and climatological history, resulting from the continental collision of Eurasia and Africa–Arabia (e.g. Meulenkamp and Sissingh, 2003; Popov et al., 2004; Rögl, 1998). This dynamic past is reflected by the rich biodiversity characterizing the region today (Mittermeier et al., 2005; Myers et al., 2000). The accumulation of phylogeographical information enables the extraction of prevailing biogeographical patterns in the Mediterranean region. However, phylogeographic research has been biased towards the southern European peninsulas (e.g. Beheregaray, 2008).

Amphibians provide an excellent model system for phylogeographical studies (Avise, 2004; Zeisset and Beebee, 2008). The crested newt *Triturus cristatus* superspecies is distributed in a large

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doi:10.1016/j.ympev.2010.04.030

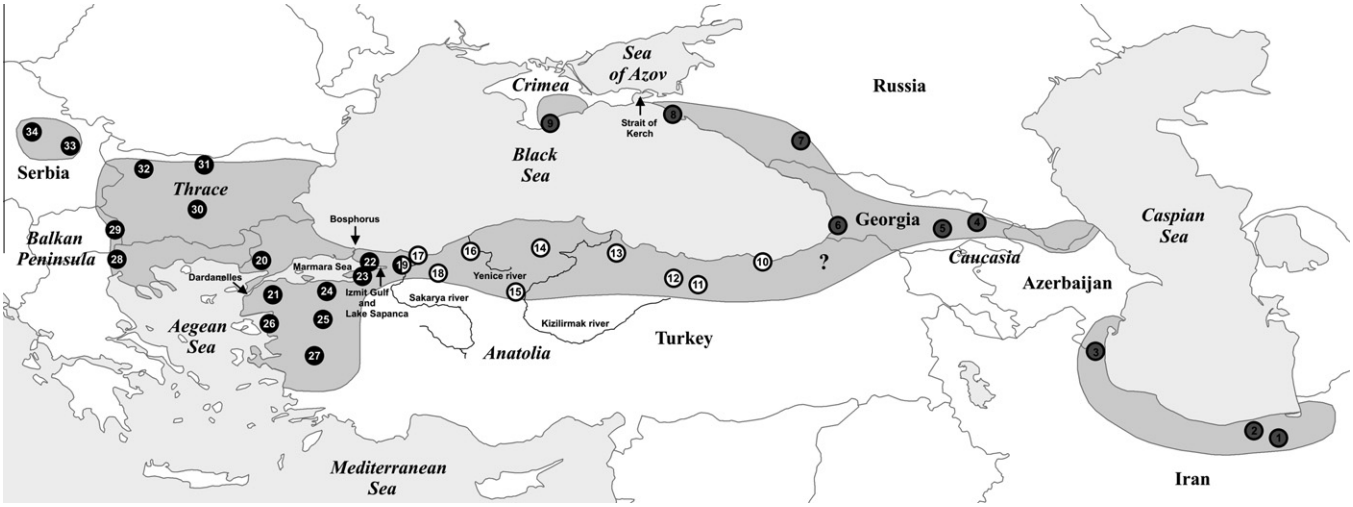

Fig. 1. Approximate distribution of crested newts belonging to the *T. karelinii* group (modified from Arntzen (2003)). Sampled populations are numbered and correspond to Table 1. Localities belonging to the eastern, central or western clade are shown in gray, white or black (cf. Fig. 2). Crested newt presence could not be established in the area indicated with a question mark; distribution could be discontinuous here (see Section 4).

segment of the Mediterranean region (e.g. Arntzen, 2003). Crested newts have been subjected to previous phylogeographic analyses, but the emphasis lay on the Balkan Peninsula (e.g. Wallis and Arntzen, 1989; Arntzen et al., 2007). The range of the crested newts traditionally referred to as '*T. karelinii*' encompasses, next to an isolated Serbian enclave and Thrace on the Balkan Peninsula, the regions Anatolia, Caucasia, Crimea and the southern shore of the Caspian Sea (Fig. 1).

Wallis and Arntzen (1989), based on limited sampling, already hinted at the presence of substantial genetic variation in '*T. karelinii*'. Subsequently, Steinfartz et al. (2007) suggested that '*T. karelinii*' actually constitutes a paraphyletic grouping. Recently, Espregueira Themudo et al. (2009) uncovered two distinct clades in '*T. karelinii*', which were postulated to represent two distinct species (elevating the subspecies *arntzeni* to species level). The phylogenetic scope of these studies – reflected in geographically restricted, low density sampling – hinders the translation of taxonomical interpretation to geographical population; firstly, it is not settled how many distinct forms are included, and secondly, it is not clear how these forms would be distributed (cf. Arntzen and Wielstra, in press). For ease of communication, we refer to the '*T. karelinii*' crested newts as the *T. karelinii* group throughout this paper.

We present a range-wide phylogeographic analysis for the *T. karelinii* group, based on mitochondrial DNA sequence data. We (1) investigate the phylogenetic position of the *T. karelinii* group in the genus *Triturus*, (2) explore the distribution and structuring of genetic variation within the *T. karelinii* group and (3) formulate a hypothesis on the biogeographical history of the *T. karelinii* group, based on a qualitative comparison of gene geography and paleo-reconstructions.

2. Materials and methods

2.1. Sampling strategy

Sampling covers the entire distribution range of the *T. karelinii* group and includes 144 individuals from 34 georeferenced localities (Table 1, Fig. 1 and Supplementary data Appendix 1). Representatives of the other *Triturus* species – the four remaining crested newt species (*T. carnifex*, *T. cristatus*, *T. dobrogicus* and *T. macedonicus*) and the two marbled newt species (*T. marmoratus* and *T. pygmaeus*) – are included (Supplementary data Appendix 1). The Pyrenean brook newt *Calotriton asper*, being the closest living relative of *Triturus* (e.g. Zhang et al., 2008), serves as an outgroup.

2.2. Laboratory methods

Total genomic DNA was extracted from a small amount of tissue, using the DNeasy Tissue Kit (Qiagen). Two mitochondrial protein coding genes were amplified by PCR: the complete subunit 2 (ND2) and a segment of subunit 4 (ND4) of the NADH dehydrogenase gene complex. For details on primers see Table 2. Reaction conditions were initial denaturation for 180 s at 94 °C, 35 cycles composed of 30 s denaturation at 94 °C, 30 s annealing at 58 °C and 60 s extension at 72 °C, and a 240 s final extension step at 72 °C. PCR products were purified with the Wizard SV Gel and PCR Clean-up System (Promega). Cycle sequencing of both forward and reverse strands was done commercially through Macrogen, Inc.

2.3. Phylogenetic analysis

The forward and reverse sequences were checked by eye and a consensus sequence was compiled with Sequencher 4.5 (Gene Codes Corporation). Sequences were aligned manually in MacClade 4.08 (Maddison and Maddison, 2005). The two mitochondrial fragments were collated and identical sequences were merged into haplotypes.

We tested four data partitioning strategies: a single partition (all data), two partitions (per gene), three partitions (per codon position) and six partitions (per gene and codon position). For each data partition, the most appropriate model of sequence evolution was determined with MrModeltest 2.2 (Nylander, 2004), based on the Akaike Information Criterion.

The data was analyzed under Bayesian inference with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) for each partitioning strategy. Four Metropolis Coupled Monte Carlo Markov Chains were ran, one cold and three incrementally heated, starting from a random topology. The heating parameter was set to 0.01, to facilitate mixing between chains. Two separate runs of twenty million generations were conducted simultaneously and for each run the cold chain was sampled every 1000 generations. When using more than

Table 1
Populations of the *T. karelinii* group sampled in this study, with details on the distribution of haplotypes (if more than one copy is present in a population the frequency is stated in parentheses). *N* is the sample size per population and population numbers correspond to Fig. 1. See Supplementary data Appendix 1 for the division of individuals in haplotypes.

No.	Country and locality	Coordinates		*N*	Haplotypes
		Latitude	Longitude		
1	Iran: Alandan	36°14′N	53°28′E	3	TkarA05 (3)
2	Iran: Qu'Am Shahr	36°26′N	52°48′E	2	TkarA03, TkarA04
3	Azerbaijan: Avyarud	38°30′N	48°36′E	5	TkarA01 (2), TkarA02, TkarA06, TkarA07
4	Georgia: Telavi	41°54′N	45°29′E	5	TkarA08 (2), TkarA09 (3)
5	Georgia: Tsodoreti	41°44′N	44°34′E	4	TkarA09 (4)
6	Georgia: Kobuleti	41°49′N	41°49′E	5	TkarA10 (5)
7	Russia: Psebay	44°04′N	40°51′E	5	TkarA11 (3), TkarA12, TkarA13
8	Russia: Cape Malyi Utrish	44°43′N	37°28′E	5	TkarA14 (4), TkarA15
9	Ukraine: Nikita	44°32′N	34°15′E	5	TkarA16 (4), TkarA17
10	Turkey: Yomra	40°52′N	39°52′E	1	TkarB01
11	Turkey: Şebinkarahisar	40°17′N	38°08′E	5	TkarB02 (4), TkarB03
12	Turkey: Reşadiye	40°27′N	37°29′E	5	TkarB04 (3), TkarB05, TkarB06
13	Turkey: Kavak	41°07′N	36°01′E	5	TkarB07, TkarB08 (3), TkarB09
14	Turkey: Cebeci	41°12′N	34°02′E	5	TkarB10 (5)
15	Turkey: Kalecik	40°05′N	33°21′E	5	TkarB11 (5)
16	Turkey: Bartın	41°29′N	32°09′E	5	TkarB12 (3), TkarB13 (2)
17	Turkey: Karasu	41°05′N	30°46′E	2	TkarB14 (2)
18	Turkey: Abanta Gölu	40°37′N	31°17′E	2	TkarB15 (2)
19	Turkey: Adapazarı	40°45′N	30°23′E	6	TkarB16 (2), TkarC02, TkarC05 (3)
20	Turkey: Keşan	40°55′N	26°38′E	5	TkarC01 (4), TkarC19
21	Turkey: Çan	40°01′N	26°56′E	5	TkarC03 (2), TkarC04, TkarC12, TkarC13
22	Turkey: Gebze	40°54′N	29°30′E	5	TkarC08 (2), TkarC09, TkarC10, TkarC11
23	Turkey: Orhangazi	40°33′N	29°20′E	3	TkarC06 (2), TkarC07
24	Turkey: Mustafa Kemalpaşa	40°04′N	28°21′E	5	TkarC14 (3), TkarC15 (2)
25	Turkey: Bigadiç	39°21′N	28°13′E	3	TkarC18 (3)
26	Turkey: Dikili	39°11′N	26°50′E	5	TkarC16 (4), TkarC17
27	Turkey: Bozdağ	38°22′N	28°06′E	5	TkarC18 (5)
28	Greece: Dafnochori	40°57′N	22°48′E	3	TkarC19 (3)
29	Macedonia: Mitrašinci	41°45′N	22°46′E	5	TkarC19 (3), TkarC20 (2)
30	Bulgaria: Rakovski	42°16′N	24°58′E	4	TkarC20 (4)
31	Bulgaria: Levski	43°25′N	25°08′E	5	TkarC20 (2), TkarC21 (3)
32	Bulgaria: Lilyache	43°19′N	23°32′E	2	TkarC20 (2)
33	Serbia: Sicevac	43°56′N	21°37′E	4	TkarC22, TkarC23, TkarC24, TkarC25
34	Serbia: Arandelovac	44°19′N	20°35′E	5	TkarC20 (2), TkarC26, TkarC27, TkarC28

Table 2
Primers used for PCR amplification and sequencing of the ND2 and ND4 mitochondrial protein coding genes.

Primer	Sequence (5′–3′)	Reference
	ND2 fragment	
L3780	TCGAACCTACCCTGAGGAGAT	Babik et al. (2005)
H5018	TCTGGGTTGCATTCAGAAGA	Babik et al. (2005)
	ND4 fragment	
	Ingroup:	
KARF4	AGCGCCTGTCGCCGGGTCAATA	Arntzen et al. (2007)
KARR1	AACTCTTCTTGGTGCGTAG	Arntzen et al. (2007)
	Outgroup:	
ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC	Arévalo et al. (1994)
Leu	CATTACTTTTACTTGGATTTGCACCA	Arévalo et al. (1994)

one data partition, parameters for each were unlinked and rates were allowed to vary independently. Tracer 1.5 (Rambaut and Drummond, 2007) was used to check for stabilization of overall likelihood within and convergence between runs. The first quarter of sampled trees was discarded as burn-in and the inference was drawn from the remaining 'forest'.

The optimal partitioning scheme was selected based on the differences in the harmonic mean of the −ln likelihood scores resulting from the Bayesian inferences for the four partitioning schemes (excluding the burn-in). By subtracting the score of the next simpler model and multiplying the outcome by −2, the 2 ln Bayes factor was calculated for each partitioning scheme (Brandley et al., 2005; Nylander et al., 2004). A value for 2 ln Bayes factor exceeding 10 was used as a threshold for preferring the more complex model (Kass and Raftery, 1995).

We expanded upon the phylogenetic analyses by conducting a partitioned Maximum Likelihood analysis with RAxML 7.0.3 (Stamatakis, 2006). We conducted this analysis for the sixfold (per gene and codon position) data partitioning scheme only, applying independent GTR + G + I substitution models for each partition. Clade robustness was assessed by 1000 rapid bootstrap replicates.

2.4. Molecular dating

Divergence times were estimated with the programs BEAST 1.5.3 (Drummond and Rambaut, 2007) and r8s 1.71 (Sanderson, 2003).

In BEAST, we applied the uncorrelated lognormal relaxed clock model and a coalescent model assuming constant size. The data was analyzed under the sixfold partitioning scheme (per gene and codon position) and each partition was allowed its own model of sequence evolution (as previously determined with MrModeltest). The tree resulting from the Bayesian analysis (based on the sixfold partitioning scheme) was used as starting topology. Divergence times were estimated based on two independent 100 million generation runs, sampled every 1000 generations, after discarding the first quarter of generations as burn-in.

In r8s we used the penalized-likelihood approach in combination with the truncated-Newton algorithm. The outgroup *C. asper* was pruned from the dataset, while keeping the root position, to avoid performing the time estimation on a basal polytomy. The optimal smoothing parameter ($S = 1$) was first determined by a

cross-validation procedure, using the Bayesian consensus tree as input. Mean temporal estimates and 95% confidence intervals were subsequently determined by profiling the last thousand sampled Bayesian topologies for each of the two runs resulting from the inference using the sixfold partitioning scheme.

We used two independent calibration points, one fossil-based and one geology-based. A *Triturus* fossil dated at 24 Ma was interpreted as approximating the crown of the genus *Triturus*. This treatment is derived from a comprehensive study on divergence times within the family Salamandridae, using multiple fossils and applying cross-validation with paleogeological data (Steinfartz et al., 2007). The origin of the Adriatic Sea at 5.33 Ma, at the end of the Messinian Salinity Crisis, was interpreted as the vicariant event which separated the *T. carnifex*–*T. macedonicus* species pair (sensu Arntzen et al., 2007).

In r8s the two calibration points used were set as fixed. In BEAST the calibration points were effectively fixed by appointing them a normally distributed prior with a small (0.001) standard deviation. We used the calibration points both separately and in conjunction. This means we conducted a total of six dating analyses: two different dating methods with three calibration strategies each.

3. Results

The 144 *T. karelinii* group individuals comprise 61 haplotypes, the other *Triturus* samples and the *C. asper* outgroup each represent unique haplotypes (for details and GenBank Accession Numbers see Supplementary data Appendices 1 and 2). Sequences could be unambiguously aligned; the only length variation is observed at the 3′ end of ND2, with a one or two triplet deletion in the crested newts, relative to the marbled newts and the outgroup *C. asper*. The total alignment comprises 1699 bp (1035–1041 for ND2 and 658 for ND4). The corresponding data matrix has been submitted to TreeBASE (submission ID 10307).

Table 3
List of the partitioning schemes tested, with the number of characters present in each partition and the models selected per partition based on MrModeltest.

Partitioning scheme	No. of characters	Model
All data		
ND2 + ND4	1699	GTR + I + G
Per gene		
ND2	1041	GTR + G
ND4	658	GTR + I + G
Per codon position		
ND2 + ND4 1st pos	567	GTR + I + G
ND2 + ND4 2nd pos	566	HKY + I + G
ND2 + ND4 3rd pos	566	GTR + G
Per gene and codon position		
ND2 1st pos	347	GTR + G
ND2 2nd pos	347	HKY + I + G
ND2 3rd pos	347	GTR + G
ND4 1st pos	220	GTR + G
ND4 2nd pos	219	HKY + I + G
ND4 3rd pos	219	GTR + I + G

Table 4
Evaluation of the optimal partitioning scheme for the mitochondrial sequence data based on Bayes factor analysis. N.a., not applicable; i.e. a simpler model for Bayes factor comparison is unavailable.

Partition scheme	Harmonic mean (−ln)	2 ln Bayes factor
All data	8657.29	N.a.
Per gene	8851.68	−388.78
Per codon position	8308.15	1087.05
Per gene and codon position	8270.46	75.37

Models of sequence evolution for each tested partition are noted in Table 3. The Bayes factor analysis, comparing the results of the Bayesian inference under different partitioning schemes, suggests that treating each codon position for each mitochondrial fragment separately is preferred over the three tested alternatives (Table 4). We only present the Bayesian phylogenetic tree obtained using this data partitioning model (Fig. 2). The outcome of the partitioned Maximum Likelihood analysis is congruent with the Bayesian inference (Fig. 2).

The *T. karelinii* group forms a monophyletic assemblage, separated from the other crested newts with statistically significant support. Three unambiguously supported, geographically coherent clades are present in the group, which are from hereon designated as 'eastern', 'central' and 'western', in line with their distributions. The eastern clade encompasses Caucasia, Crimea, and the southern Caspian Seashore, the central clade is distributed in northern Turkey, along the southern shore of the Black Sea, and the western clade comprises western Asiatic Turkey and the Balkan Peninsula. The eastern clade is the first to split off and the central and western clade are sister groups. The central and western clades are parapatrically distributed (occurring in syntopy at locality 19), whereas no geographical overlap was found for the eastern and central clades.

The eastern, central and western clades show substantial genetic substructuring. We divided each clade into three (not necessarily monophyletic) groups of haplotypes (Fig. 2). The relatively homogenous groups from Caucasia (eastern II) and Crimea (eastern III) are nested within the genetically diverse southern Caspian Seashore group (eastern I). The central clade shows three reciprocally monophyletic groups (central I–III), with representatives of central II and central III observed in syntopy at locality 16. In the western clade, a basal haplotype is found in European Turkey (western I), whereas haplotypes from Asiatic Turkey (western II) are paraphyletic with respect to the remaining European ones (western III).

Temporal estimates for significantly supported splits among *Triturus* species and within the *T. karelinii* group are provided in Table 5. Dates are similar across methods and using different calibration strategies.

4. Discussion

4.1. Systematic position

The *T. karelinii* group composes one of four mitochondrial DNA lineages (the others being *T. carnifex* plus *T. macedonicus*, *T. cristatus* and *T. dobrogicus*) that form a basal polytomy in the *T. cristatus* superspecies (Fig. 2; cf. Arntzen et al., 2007). This means that either the mitochondrial data analyzed here contain too little information to resolve the order of speciation events (a soft polytomy), or that the four lineages truly split simultaneously (a hard polytomy). Espregueira Themudo et al. (2009), using a suite of nuclear markers, managed to recover more detailed crested newt relationships (clustering *T. dobrogicus* with the *T. carnifex* and *T. macedonicus* lineage) despite cladogenesis having occurred in close temporal proximity. This implies that the basal polytomy we found is, at least in part, a soft one.

The initial split of the ancestor of the *T. karelinii* group from the remaining crested newts is estimated to have occurred around 9 Ma (Fig. 2 and Table 5). In the Early through Middle Miocene (23.03–11.61 Ma), the ancestral crested newt distribution presumably encompassed the continuous Balkan–Anatolian landmass (Fig. 3A; Steininger and Rögl, 1984). During the late Middle and early Late Miocene (ca. 12–9 Ma), the formation of the Mid-Aegean

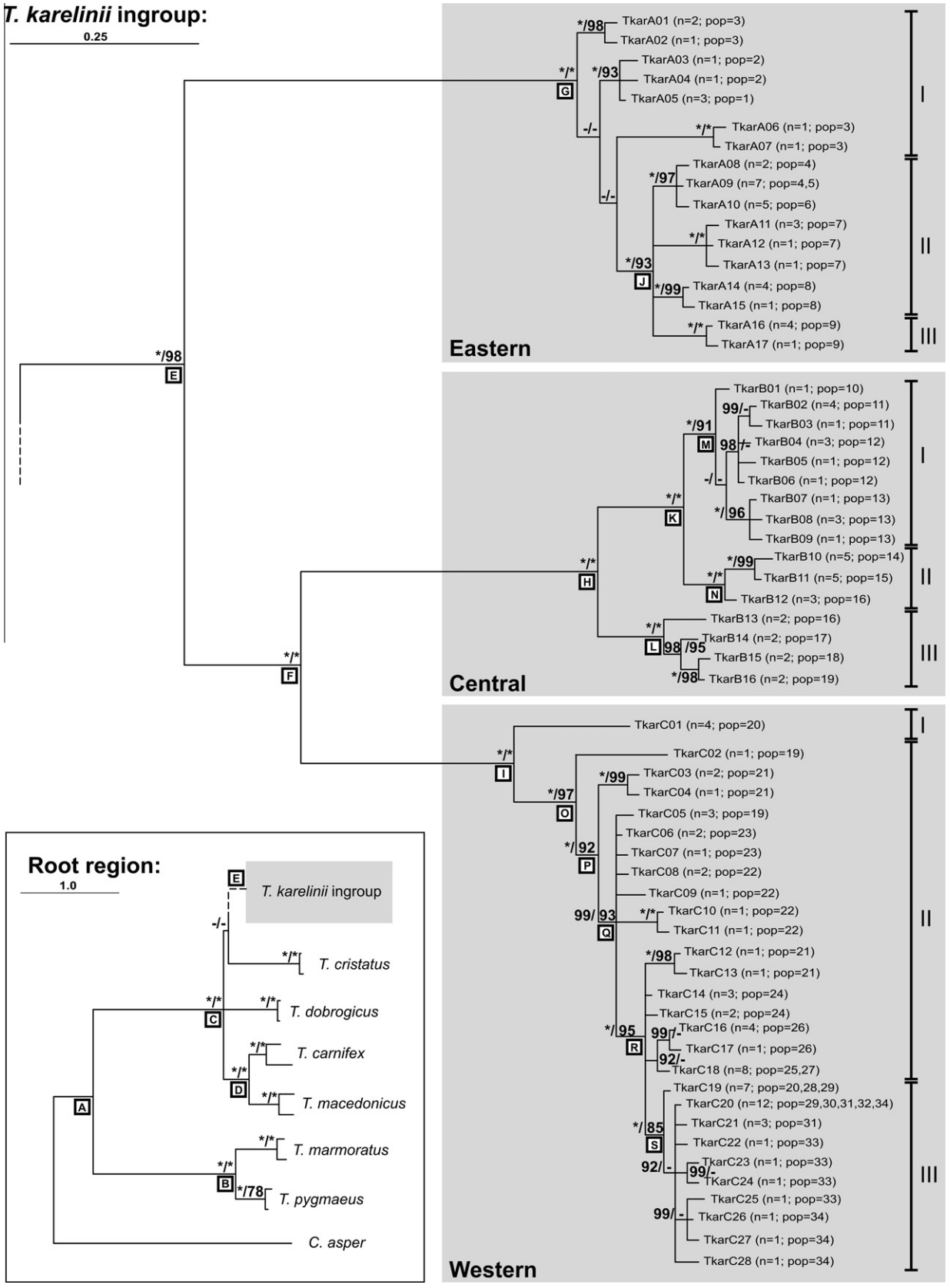

Fig. 2. Majority rule consensus phylogenetic tree resulting from the Bayesian inference. Focus lays on the *T. karelinii* group; the inset shows the root region of the tree on a different scale. Three major clades identified in the *T. karelinii* group are labeled eastern, central and western, and subdivided into three groups each, labeled I, II and III (see text for details). Tips are labeled with haplotype identifiers, for which the frequency (*n*) and the populations (pop) where they are found are stated in parentheses (cf. Fig. 1, Table 1 and Supplementary data Appendices 1 and 2). Scale bars denote expected changes per site. Node support is indicated as Bayesian posterior probability before and Maximum Likelihood bootstrap after the slash. Both are presented as percentages, with a 100% denoted with an asterisk and 75% or less with a vertical bar. For those nodes accompanied by a boxed letter, temporal estimates are presented in Table 5.

Table 5
Divergence time estimates among *Triturus* species and within the *T. karelinii* group (in Ma). Nodes are coded according to Fig. 2. For each of the two programs used (r8s and BEAST), three calibration strategies are applied: 1, the *Triturus* crown fixed at 24 Ma; 2, the *T. carnifex–T. macedonicus* split fixed at 5.33 Ma; and 3, both calibration points together (see text for rationale). When nodes were used as calibration point they are marked with an asterisk. 95% confidence intervals are stated in parentheses.

Node	r8s			BEAST		
	1	2	3	1	2	3
A	24*	25.7 (19.4–32.1)	24*	24*	29.0 (20.8–37.7)	24*
B	5.3 (4.1–6.5)	5.7 (3.8–7.6)	5.3 (4.1–6.5)	4.5 (3.1–5.9)	5.4 (3.5–7.3)	4.8 (3.5–6.3)
C	9.3 (8.0–10.7)	9.9 (7.7–12)	9.4 (8.2–10.6)	8.6 (6.8–10.5)	10.4 (8.1–12.8)	9.4 (7.9–11.1)
D	5.2 (4.1–6.4)	5.33*	5.33*	4.4 (3.2–5.7)	5.33*	5.33*
E	6.8 (5.4–8.2)	7.2 (5.2–9.1)	6.8 (5.5–8.2)	6.7 (5.1–8.4)	8.1 (5.8–10.5)	7.2 (5.6–8.9)
F	5.4 (4.2–6.5)	5.7 (4.1–7.2)	5.4 (4.3–6.5)	5.1 (3.8–6.5)	6.2 (4.4–8.1)	5.5 (4.2–6.9)
G	1.3 (1.0–1.7)	1.4 (1.0–1.9)	1.3 (1.0–1.7)	1.1 (0.7–1.5)	1.3 (0.9–1.8)	1.2 (0.8–1.6)
H	1.8 (1.3–2.3)	1.9 (1.3–2.6)	1.8 (1.3–2.3)	1.6 (1.1–2.2)	1.9 (1.3–2.6)	1.7 (1.1–2.3)
I	2.1 (1.5–2.7)	2.2 (1.5–2.9)	2.1 (1.5–2.7)	1.8 (1.2–2.5)	2.2 (1.4–3.0)	2.0 (1.3–2.6)
J	0.8 (0.5–1.0)	0.8 (0.5–1.1)	0.8 (0.5–1.0)	0.6 (0.4–0.9)	0.7 (0.4–1.1)	0.7 (0.4–1.1)
K	0.9 (0.6–1.2)	0.9 (0.6–1.3)	0.9 (0.6–1.2)	0.7 (0.4–1.0)	0.9 (0.5–1.3)	0.9 (0.5–1.3)
L	0.7 (0.3–1.0)	0.7 (0.3–1.0)	0.7 (0.3–1.0)	0.5 (0.2–0.7)	0.6 (0.3–0.9)	0.6 (0.3–0.9)
M	0.5 (0.3–0.7)	0.5 (0.3–0.8)	0.5 (0.3–0.7)	0.3 (0.2–0.5)	0.4 (0.2–0.6)	0.4 (0.2–0.6)
N	0.4 (0.2–0.6)	0.4 (0.2–0.7)	0.4 (0.2–0.6)	0.3 (0.1–0.5)	0.3 (0.1–0.6)	0.3 (0.1–0.6)
O	1.3 (0.9–1.6)	1.3 (0.8–1.8)	1.3 (0.9–1.6)	1.0 (0.7–1.4)	1.2 (0.8–1.7)	1.2 (0.8–1.7)
P	0.9 (0.6–1.2)	1.0 (0.6–1.3)	0.9 (0.6–1.2)	0.7 (0.4–1.0)	0.8 (0.5–1.2)	0.8 (0.5–1.2)
Q	0.7 (0.5–0.9)	0.7 (0.5–1.0)	0.7 (0.5–0.9)	0.5 (0.4–0.8)	0.7 (0.4–0.9)	0.7 (0.4–0.9)
R	0.4 (0.3–0.6)	0.5 (0.3–0.7)	0.5 (0.3–0.6)	0.3 (0.2–0.5)	0.4 (0.2–0.6)	0.4 (0.2–0.6)
S	0.3 (0.2–0.4)	0.3 (0.2–0.5)	0.3 (0.2–0.4)	0.2 (0.1–0.3)	0.2 (0.1–0.4)	0.2 (0.1–0.4)

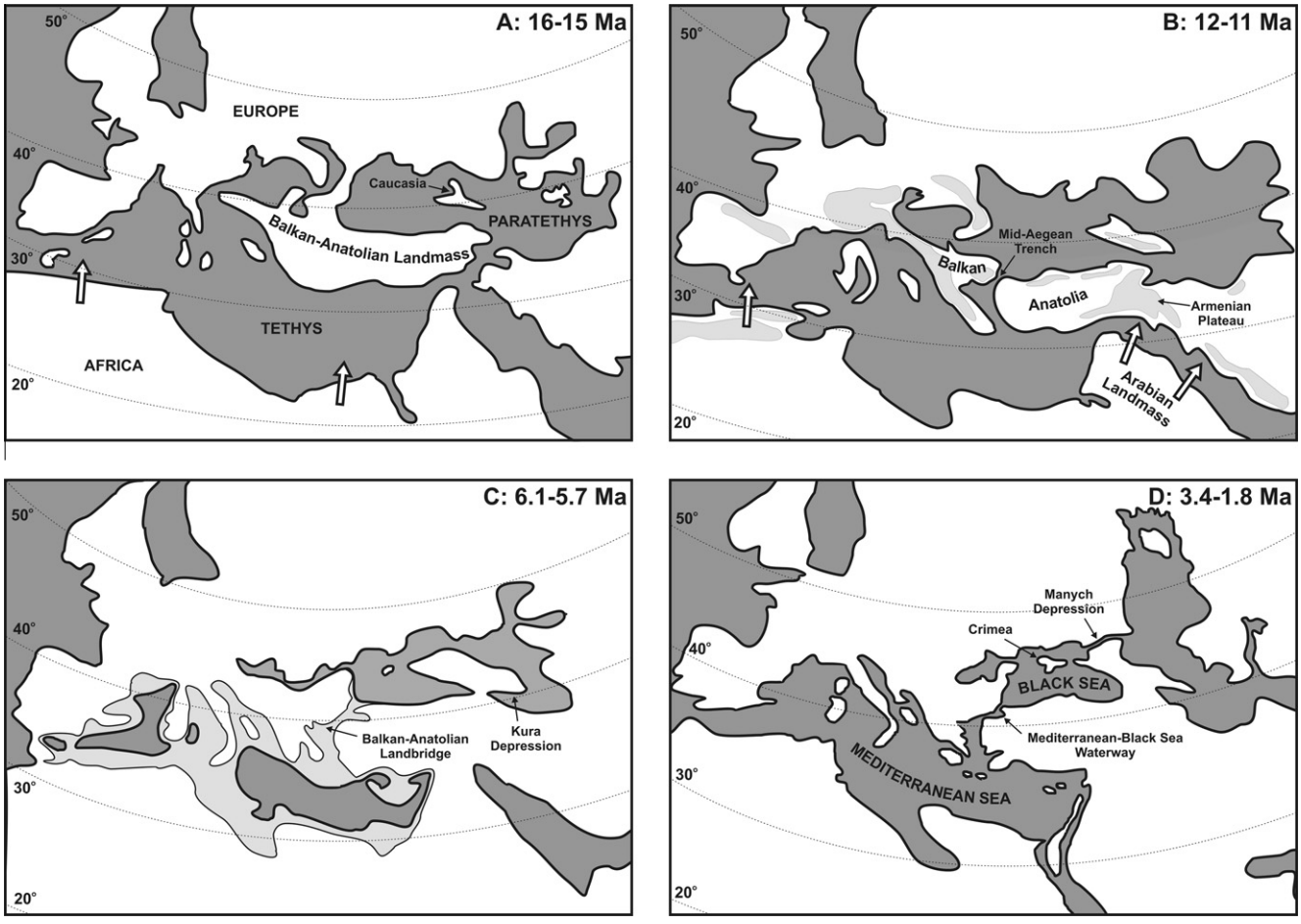

Fig. 3. Paleogeological reconstructions for the Mediterranean region, showing stages relevant for the *T. karelinii* group (adapted from Popov et al. (2004)). (A) A continuous Balkan–Anatolian landmass allows a continuous distribution for a proto crested newt. (B) The origin of the Mid-Aegean Trench splits of the *T. karelinii* group in Anatolia from the Balkan crested newts. Extensive orogenesis (shaded light gray) due to the movement of Africa towards Europe leads to the uplift of the Armenian Plateau, which isolates the eastern clade. (C) The desiccation of the Mediterranean during the Mesinnian Salinity Crisis exposes part of the sea bed (shaded light gray), including a land bridge between the Balkans and Anatolia, allowing crested newts to colonize Europe from Asia. Caucasia gradually becomes connected to the Asian mainland, but there is still marine embayment via the Kura Depression. (D) After the conclusion of the Mediterranean Salinity Crisis, a marine connection between the Mediterranean and the Black Sea is re-established, causing the split between the central and western clades. The Manych Depression starts to close, making Caucasia a land bridge between Asia and Europe. Crimea is gradually moving towards the mainland. Periodical drops in global sea level during the Pleistocene cause occasional terrestrial connections between the Balkans and Anatolian and the Russian mainland and Crimea.

Trench caused a divide between the Balkan and Anatolian landmasses and initiated a long-lived marine communication between the Mediterranean and the Paratethys (Fig. 3B; Dermitzakis and Papanikolaou, 1981; Rögl, 1998). We propose that the formation of this barrier severed the ancestor of the *T. karelinii* group from the remaining crested newts. For a reconstruction of the historical biogeography of the other crested newt species, see Arntzen et al. (2007).

4.2. The origin of the eastern, central and western clades

We uncovered three distinct, geographically structured mitochondrial DNA clades in the *T. karelinii* group (Fig. 2). The basal split that gave rise to the eastern clade is placed around 7 Ma (Fig. 2 and Table 5). Temporally and geographically, this split is congruent with the uplift of the Armenian Plateau (10–5 Ma), caused by the Arabia–Eurasia collision (Fig. 3B; e.g. Meulenkamp and Sissingh, 2003; Popov et al., 2006). We propose this orogeny isolated ancestral eastern clade crested newts in what is now Iran. This vicariance event might have been reinforced by episodic marine connections between the Mediterranean and the Paratethys via the Turkish–Iranian region (Chepalyga, 1995; Popov et al., 2006). Remaining land currently inhabited by the eastern clade was probably not yet accessible; Caucasia, which would eventually divide the Paratethys into the current Black and Caspian Seas and constitute a terrestrial passageway to southern Russia, was still an archipelago at the time (Rögl, 1998).

The split of the central and western clades in the *T. karelinii* group is dated around 5.5 Ma (Fig. 2 and Table 5). This coincides with the conclusion of the Messinian Salinity Crisis, an event which comprised the temporary desiccation of the Mediterranean. Isolation of the Mediterranean from the Atlantic was established at 5.59 Ma by the tectonically induced closing of the Betic and Rifian corridors (Krijgsman et al., 1999). Subsequent evaporation resulted in a dramatic drop in sea level and created opportunity for crested newts to move between Anatolia and the Balkan Peninsula via terrestrial connections (Fig. 3C; Çağatay et al., 2006; Sakınç and Yaltırak, 2005). At 5.33 Ma, the barrier cutting of the Mediterranean from the Atlantic gave way as the Strait of Gibraltar was formed (Krijgsman et al., 1999). During the resulting 'Zanclean flood', the Mediterranean abruptly re-filled and sea water from the Atlantic penetrated, via the Aegean Sea, into the Black Sea (e.g. Clauzon et al., 2005; Elmas, 2003; Sakınç and Yaltırak, 2005). We propose the resulting Mediterranean–Black Sea Waterway bisected a continuously distributed crested newt stock into an Anatolian and a Balkan component, corresponding to the central and western clades (Fig. 3D).

4.3. Structuring within the three clades

The phylogeographical pattern of the eastern clade suggests a center of origin along the southern shore of the Caspian Sea and colonization of the Caucasus and Crimea at a later point. During the Messinian (7.25–5.33 Ma), soon after the initial split of the eastern clade from the remaining members of the *T. karelinii* group, the first land based connection with the Greater Caucasus landmass would appear (Fig. 3C; e.g. Popov et al., 2006). However, an extensive marine embayment by the Black and Caspian Seas and recurring communication between the two via the Kura and Manych Depressions (situated south and north of the Greater Caucasus ridge) will have seriously hampered northward expansion (Fig. 3C and D; e.g. Popov et al., 2006; Ryan et al., 2003). On the other hand, the periodical drop in global sea level during the Quaternary Ice Age may have reduced the impact of this dispersal barrier (Lambeck et al., 2002). The distribution of the eastern I and eastern II groups is presently discontinuous; central Azerbaijan appears to be devoid of crested newts (Fig. 1; Arntzen, 2003; personal observation). Crimea originated as an island in the Eastern Paratethys and became peninsular at the Plio-Pleistocene boundary at 2.59 Ma (Fig. 3D; e.g. Popov et al., 2006; Tugolesov et al., 1985). The Strait of Kerch, which connects the Sea of Azov to the Black Sea (Fig. 1), currently separates the Crimean newts (eastern III) from the remainder of the range (eastern I–II). During Quaternary glaciations, however, lowering of the sea level caused land-based connections to emerge (Ryan et al., 2003).

The central clade shows three distinct groups of haplotypes. A basal split separates samples from either side of the Yenice river valley, with the exception of Bartin (locality 16), where they co-occur (Figs. 1 and 2). A subsequent bifurcation separates samples from either side of the river Kızılırmak, with no syntopy detected. Rivers are sometimes considered a driving force for generating phylogeographical structuring in amphibians, but spatial coincidences may also be formed at a later point (Lemmon et al., 2007; Pauly et al., 2007). Denser sampling is required to distinguish between these competing hypotheses.

The phylogeographical pattern observed in the western clade suggests a Balkan origin, subsequent expansion into Anatolia, and eventual secondary colonization of the Balkan Peninsula, where new colonizers came into contact with newts already present. Currently, the Bosphorus, the Marmara Sea and the Dardanelles separate the Balkan Peninsula and Anatolia (Fig. 1). The Dardanelles already came in place during the Pliocene (5.33–2.59 Ma) (Çağatay et al., 2006). On the other hand, the outflow of the Marmara Sea into the Black Sea has since been re-ordered due to tectonic movements. The Bosphorus originated during the Holocene (11.7 Ka to present); before that time, the Istanbul Peninsula was directly accessible from the Balkan Peninsula via a terrestrial route (Kerey et al., 2004). Throughout the Pleistocene (2.59 Ma–11.7 Ka), the Marmara and Black Seas were connected via the İzmit Gulf–Lake Sapanca–Sakarya Valley waterway (Fig. 1; Elmas, 2003). This geological history explains the presence of the western clade on either side of the Bosphorus, but not the long term presence in western Anatolia or the recently derived haplotypes in the Balkan Peninsula. However, global sea level fluctuations corresponding to the Quaternary climatic oscillations likely caused periodical emergence of the shallow sea straits separating Europe and Asia (Lambeck et al., 2002).

4.4. Taxonomic considerations and perspectives for further research

We applied a dense intraspecific sampling regime and found that the *T. karelinii* group, from a mitochondrial perspective, constitutes a monophyletic assemblage. This contradicts the notion of paraphyly proposed by Steinfartz et al. (2007). We identified three mitochondrial DNA clades within the *T. karelinii* group, which show genetic differentiation comparable to that among recognized *Triturus* species (Fig. 2). Espregueira Themudo et al. (2009) suggest that the *T. karelinii* group comprises two species: *T. karelinii sensu stricto* and *T. arntzeni*. The former name would apply to the eastern clade and the latter to the western clade. We identified a third assemblage, the central clade, for which no name appears to be available. Whereas the European *Triturus* species can be distinguished on morphological grounds (Arntzen and Wallis, 1999), a range-wide study on the morphology of the *T. karelinii* group is as yet lacking.

Mitochondrial DNA divergence in itself does not provide conclusive evidence for evolutionary independence (e.g. Ballard and Rand, 2005). Permeability of mitochondrial DNA phylogeographic breaks for nuclear DNA indicates ongoing gene flow. Congruent patterns between both genomes could suggest either true genetic incompatibility or long term spatial isolation of clades. We intend to assess the structuring of nuclear DNA by exploring nuclear DNA markers. Contact zones are suitable places to study the presence

and extent of gene flow in the *T. karelinii* group. The central and western clades occur in syntopy at the Sakarya Valley (locality 19 in Fig. 1), suggesting secondary contact after the recent closing (11.7 Ka) of the İzmit Gulf–Lake Sapanca–Sakarya Valley waterway. Whether the eastern and central clades are in contact is unclear; historical records (reviewed in Arntzen, 2003) exist from the intervening area between Kobuleti in Georgia (locality 6) and Yomra in Turkey (locality 10), but no recent observations could be made (personal observation; M. Sparreboom, in lit.).

The Mediterranean region is a biodiversity hotspot but is also experiencing intense anthropogenic pressure (Mittermeier et al., 2005; Myers et al., 2000). We hope our case study contributes to a better understanding of the biogeographical history of the region. This is not as straightforward as it may seem. The ranges of *Ommatotriton* and the *Lissotriton vulgaris* group largely overlap with that of the *T. karelinii* group and representatives of these three newt genera can often be found occurring in syntopy. Yet intriguing differences remain. The range occupied by the central *T. karelinii* group clade is practically devoid of *Lissotriton*, leaving a Caucasian clade isolated from the rest of the *Lissotriton* range. *Ommatotriton* is mostly absent from the range of the western *T. karelinii* group clade and does not share the distribution of the eastern clade in Iran. Its distribution does however encompass part of the Middle East. The evolutionary history of the *L. vulgaris* group appears to have unfolded itself over a more recent time span than that of the *T. karelinii* group (Babik et al., 2005). For *Ommatotriton* a detailed phylogeographic study is as yet lacking. Despite their ecological similarities, these three groups of newts already show incongruent scenarios. To distil a prevailing biogeographical pattern for the Near East, a wider array of species should be investigated and compared. Knowledge on Near Eastern phylogeographical patterns is rapidly accumulating and the time is ripe for a thorough review. The data available so far suggest the Near East to be a cradle for biodiversity, on a par with the southern European peninsulas.

Acknowledgments

B.W. thanks the 'J.J. ter Pelkwijk fund' for support. G.E.T. was financed through a Ph.D. grant from the Fundação para a Ciência e a Tecnologia, Lisbon, Portugal (SFRH/BD/16894/2004). N.A.P. was supported by a Naturalis 'Martin fellowship' and by a grant from the 'Pieter Langerhuizen Lambertuszoon Fonds', allocated by the 'Koninklijke Hollandsche Maatschappij der Wetenschappen'. Permits for Turkey were made available by TÜBITAK. The authors thank W. Babik, J. Badridze, S. Carranza, D. Cogălniceanu, E. Galoyan, H. Gholi Kami, A. Kidov, N. Moin, I. Serbinova, D. Tarkhnishvili and N. Üzüm for help with collecting. A. Skidmore and G. Wallis provided comments on an earlier version of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.04.030.

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