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Molecular Phylogenetics and Evolution 45 (2007) 211–226
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Phylogeny and biogeography of the alpine newt *Mesotriton alpestris* (Salamandridae, Caudata), inferred from mtDNA sequences

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Received 29 November 2006; revised 8 March 2007; accepted 8 March 2007
Available online 27 March 2007

Abstract

In this paper, we performed phylogenetic analyses of *Mesotriton alpestris* populations from the entire range of species distribution, using fragments of two mtDNA genes, cytochrome *b* (309 bp) and 16S rRNA (~500 bp). Sequence diversity patterns and phylogenetic analyses reveal the existence of a relict lineage (Clade A) of late Miocene origin, comprising populations from south-eastern Serbia. This lineage is proposed to be ancestor to a western and an eastern lineage, which diverged during the middle Pliocene. The western lineage is further divided in two clades (Clades B, C) of middle Pliocene origin that represent populations from Italy (B) and populations from central Europe and Iberia (C). Further subdivision, dated back to the middle-late Pliocene, was found within the eastern lineage, representing southern (Clade D) and central-northern (Clade E) Balkan populations, respectively. Extensive sequence divergence, implying greater isolation in multiple refugia, is found within eastern clades, while the western clades seem to have been involved in the colonization of central, western and north-eastern Europe from a hypothetical refugium in central Europe. The extent of divergence does not support the current taxonomy indicating cryptic speciation in the Balkans, while paedomorphic lineages were found to have been evolved during early-middle Pleistocene probably as a response to the ongoing dramatic climatic oscillations.

Keywords: *Mesotriton alpestris*; mtDNA; Phylogeography; Balkan Peninsula; Multiple refugia; Cryptic speciation; Facultative paedomorphosis; Cytochrome *b*; 16S rRNA

1. Introduction

The most recent taxonomic revisions of the genus *Triturus* (García-París et al., 2004; Litvinchuk et al., 2005), further supported by Steinfartz et al. (2006), place the alpine newt in the monotypic genus *Mesotriton*. *M. alpestris* constitutes a distinct monophyletic clade within European newt's phylogeny (García-París et al., 2004; Litvinchuk et al., 2005; Steinfartz et al., 2006; Weisrock et al., 2006), which has been proposed to diverge some 10–28 Mya (Macgregor et al., 1990; Zajc and Arntzen, 1999; Steinfartz et al., 2006). The species is, albeit vaguely, regarded as the most primitive species among European newts on the basis of cranial osteological characters (Bolkay, 1928) and mating behavior traits (Halliday, 1977). Its range expands from northeast France to western Ukraine and from southern Denmark to northern Italy and the Balkans, down to northern Peloponnisos. Isolated parts of its range exist in northern and central Spain, as well as in south and central Italy (Griffiths, 1996; Denoël et al., 2001) (Fig. 1A). Currently, up to nine subspecies are considered to be valid (see Griffiths, 1996; Zuiderwijk, 1997; for the most recent accounts) mainly according to external morphological features and coloration patterns. Five of them are endemics of the Balkan Peninsula. In the western and central part of the

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doi:10.1016/j.ympev.2007.03.012

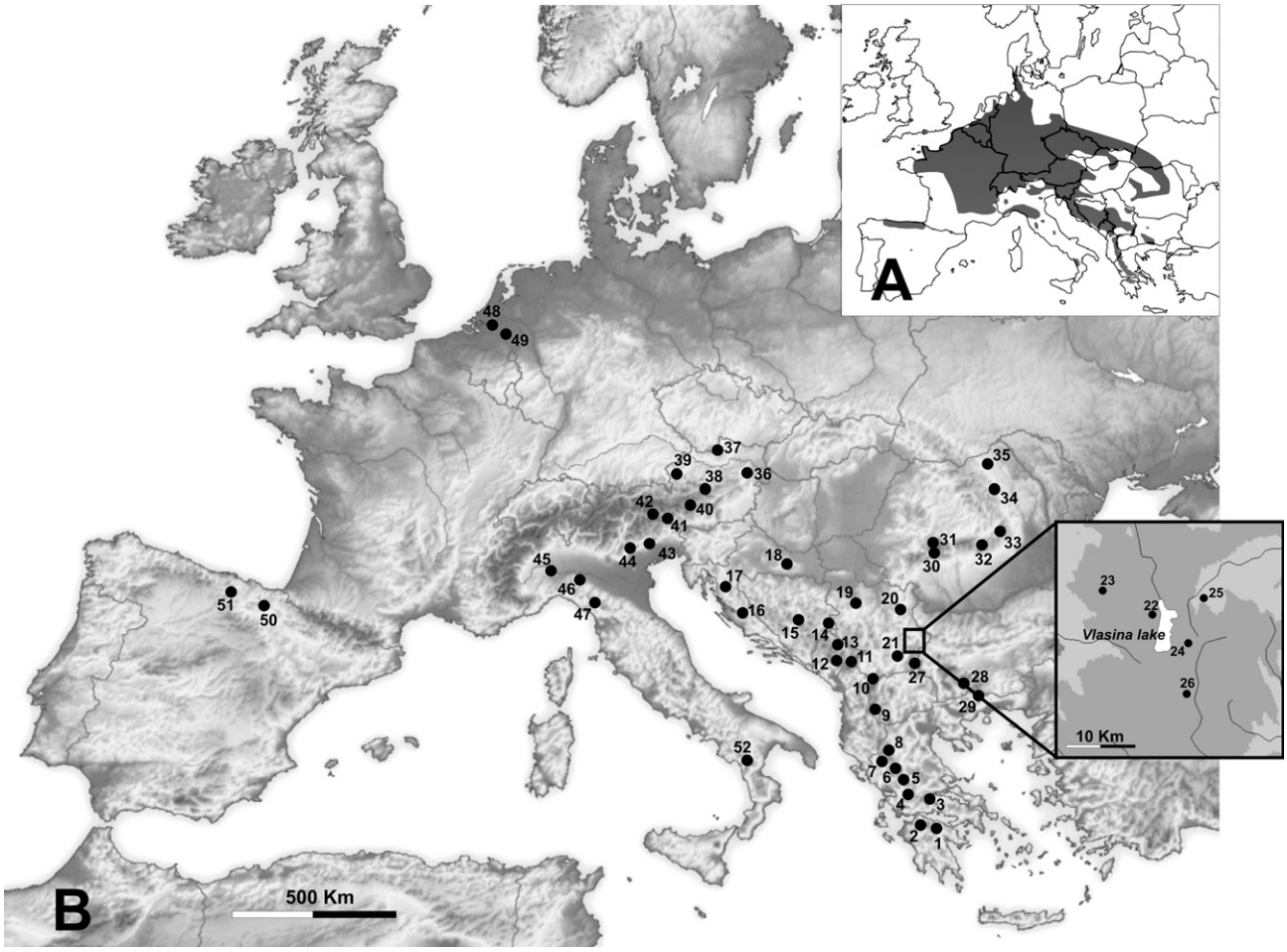

Fig. 1. (A) Distribution of *M. alpestris* in Europe (redrawn and modified from Denoël et al., 2001). (B) Sampling localities of *M. alpestris* used in the study. Population numbers correspond to those presented in Table 1.

Balkans, subspecific status has been assigned to a number of isolated populations inhabiting high-mountain glacial lakes, which exhibit facultative paedomorphosis (ssp. *montenegrinus*, *piperianus*, *serdarus*, *reiseri*), while in the southern Balkans the alpine newt is represented by ssp. *veluchiensis.* Three other subspecies have been described and are distributed in the Cantabrian Mts. (ssp. *cyreni*), in the Apennines (ssp. *apuanus*) and in Calabria (ssp. *inexpectatus*). Finally, the nominotypical subspecies (ssp. *alpestris*) expands from the Rodope Mountains through Balkans to the rest of its distribution range. Its subspecific taxonomy, especially of the Balkan taxa, has been highly controversial as there is discrepancies in results obtained by morphological (Sotiropoulos et al., 2001 and references therein), allozymic (Breuil and Guillaume, 1985; Arano and Arntzen, 1987) and cytogenetic analyses (Herrero et al., 1989). The species has been proposed to have originated and diversified in the Balkans (Arano and Arntzen, 1987; but see Herrero et al., 1989). However, a robust intraspecific phylogeny of the species is still lacking, since all the relevant molecular investigations were based on individuals from few populations of only two, out of the nine currently recognized, subspecies.

Lately, molecular phylogenetic studies have revealed considerable intraspecific pre-Pleistocene divergence within several palearctic Urodela species (*Mertensiella caucasica*, Tarkhnishvili et al., 2000; *Chioglossa lusitanica*, Alexandrino et al., 2000; *Lyciasalamandra luschani*, Weisrock et al., 2001; *Salamandra atra*, Riberon et al., 2001; *Neurergus strauchii*, Steinfartz et al., 2002; *Lissotriton vulgaris*, Babik et al., 2005; *Lissotriton boscai*, Martínez-Solano et al., 2006). Most of such intraspecific cladogenic events involve historically disjunct populations or lineages, possibly remnants of formerly widespread ancestral groups, and/or postglacial expansions from alternative refugia. In many cases, such deep phylogenetic splits are masked under a conserved or less differentiated external morphology, implying cryptic speciation processes (e.g. Tan and Wake, 1995; Highton, 2000; Shaffer et al., 2004; Martínez-Solano et al., 2006).

In Europe, the majority of speciation events have been proposed to have taken place in the three major refugial areas (Iberian, Apennine and Balkan Peninsulas) (Hewitt, 1996, 2004; Taberlet et al., 1998). Among them, the Iberian Peninsula has been proposed to consist of multiple isolated refugia, as several concordant differentiation

patterns of animal and plant species have been detected (Paulo et al., 2001; Gómez and Lunt, 2006; Martínez-Solano et al., 2006). Most of these isolated ''refugia-within-refugia'' constitute hot spots of endemism, as a result of long-term persistence of species during the repetitive Pleistocene glacial cycles. Similarly, patterns of intraspecific genetic divergence observed within the Appenine Peninsula, were attributed to isolation of populations in distinct refugia in the opposite sides of the Apennines (Raggianti and Wake, 1986; Bianco, 1990; Scillitani and Picariello, 2000).

Physiographic traits along with habitat heterogeneity have been considered as responsible for the remarkably high levels of endemism observed in the Balkans (Blondel and Aronson, 1999; Griffiths et al., 2004). The Balkan Peninsula is characterized by a long mountainous formation of northwest–southeast orientation (Dinarids, Pindic chain) that constitutes a significant west–east physical barrier, with major influence to climate conditions (Griffiths et al., 2004). The pollen record has revealed the existence of several temperate tree 'oases' situated in mid-altitudes of the Pindic chain, serving as refugia and centers of endemism for numerous—mostly mountainous—animal and plant species (Tzedakis, 1993; Tzedakis et al., 2002; Griffiths et al., 2004). However, the establishment of a similar pattern in the Balkans is still lacking.

In this study, alpine newts of all valid subspecies were collected from several localities across the entire range of species distribution, and the DNA sequences were obtained from the cytochrome *b* (cyt *b*) and 16S rRNA (16S) genes to infer the intraspecific phylogenetic relationships.

Our main goals were to (i) examine the extent of intraspecific genetic variation and produce a robust phylogeny, (ii) estimate time divergences among the different lineages identified, (iii) produce a historical interpretation of the species distribution, and (iv) examine the congruence of the emerged pattern with the current subspecific taxonomy.

2. Materials and methods

2.1. DNA extraction, amplification, and sequencing

Muscle samples or tail tips were obtained from 114 individuals of the alpine newt from 51 localities across Europe (Table 1, Fig. 1B). Tissue samples were homogenized in a digest buffer and total genomic DNA was extracted using Proteinase K, purified by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1), one with chloroform/isoamyl alcohol (24:1), and precipitated using isopropanol.

Two target genes were selected for molecular phylogenetic analysis: (1) a partial sequence (309 bp) of the mitochondrial protein-encoding cytochrome *b* gene (cyt *b*), and (2) a partial sequence of the mitochondrial gene encoding 16S rRNA (16S). The universal L14841 and H15149 primers (Kocher et al., 1989) were used to amplify the cyt *b* region of the mtDNA. The polymerase chain reaction (PCR) was performed as follows in the presence of 3 mM $MgCl_2$: 35 cycles of denaturation at 94 °C for 45 s, annealing at 47 °C for 45 s, and extension at 72 °C for 60 s. Primers 16Sar-L and 16Sbr-H (Palumbi et al., 1991) were used to amplify a segment of approximately 500 bp from the 16S rRNA region of the mtDNA, according to the following PCR conditions and in the presence of 3 mM $MgCl_2$: 35 cycles of denaturation at 94 °C for 60 s, annealing at 47 °C for 60 s, and extension at 72 °C for 60 s. The light strands were sequenced for all 114 individuals using an ABI Prism 377 DNA fragments analyzer.

Complementary sequences were obtained for one specimen of *Mesotriton alpestris inexpectatus* from Calabria (Accession numbers: DQ092232 and DQ092279 for cyt *b* and 16S, respectively; Carranza and Amat, 2005), while various salamandrid taxa were included as outgroups. Specifically, individuals from *Triturus vittatus* (cyt *b*: AY336659 Veith et al., 2004; 16S: AY336630 Veith et al., 2004), *Lissotriton vulgaris vulgaris* (cyt *b*: U55948 Caccone et al., 1997; 16S: U04705 Caccone et al., 1994), *Lissotriton vulgaris graecus* from Peloponnisos, Greece (cyt *b*: EF089337 this study, 16S: EF089303 this study), *Pleurodeles waltl* (cyt *b*: AY336654 Veith et al., 2004; 16S: AY336625 Veith et al., 2004), *Pleurodeles poireti* (cyt *b*: AY336641 Veith et al., 2004; 16S: AY336612 Veith et al., 2004) and *Salamandrina terdigidata* (cyt *b*: AY695901 Canestrelli et al., 2006; 16S: AY898735 Mattoccia et al., 2005).

2.2. Alignment and genetic divergence

The alignment of the concatenated cyt *b* and 16S rRNA sequences was performed with Clustal X (Thompson et al., 1997) and corrected by eye. Additionally, the 16S was aligned based on its secondary structure, to facilitate proper alignments. Although some alignment gaps were inserted to resolve length differences between sequences, all positions could be unambiguously aligned and were therefore included in the analyses. Cytochrome *b* sequences were translated into amino acids prior to analysis and did not show any stop codons, suggesting that probably all were functional.

For several recognized clades and subclades including multiple haplotypes, average uncorrected sequence divergences (*p*-distance) were estimated in MEGA computer package (v.3.1, Kumar et al., 2004). Additionally, haplotype and nucleotide diversity values within each clade and subclade were calculated with ARLEQUIN v. 2001 (Schneider et al., 2000).

2.3. Phylogenetic analyses

Analyses for phylogenetic inference were conducted using two methods: maximum likelihood (ML), and Bayesian inference (BI). Nucleotides were used as discrete, unordered characters. To examine whether the sequences

Table 1
Sample localities of *M. alpestris* used in the study

No.	Locality	Country	Coordinates	*n*	Subspecies	Clade	Haplotype	Acc. No. cyt *b*	Acc. No. 16S rRNA
1	Kyllini Mt.	Greece	37°56′N, 22°25′E	2	*veluchiensis*	D3	D3-1	DQ481513	DQ481488
2	Rakita (Panachaiko Mt.)	Greece	38°10′N, 21°54′E	4	*veluchiensis*	D3	D3-2	DQ481514	DQ481489
3	Katavothra (Oeta Mt.)	Greece	38°46′N, 22°19′E	3	*veluchiensis*	D4	D4-1	DQ481515	DQ481490
4	Velouchi Mt.	Greece	38°57′N, 21°49′E	3	*veluchiensis*	D4	D4-1	DQ481516	DQ481491
5	Elati (Kerketio Mt.)	Greece	39°31′N, 21°31′E	2	*veluchiensis*	D4	D4-2	DQ481517	DQ481492
6	Zygos Mt.	Greece	39°53′N, 21°17′E	2	*veluchiensis*	D4	D4-3	DQ481518	DQ481493
7	Drakolimni (Tymphi Mt.)[a]	Greece	39°58′N, 20°45′E	2	*veluchiensis*	D2	D2-1	DQ481519	DQ481494
8	Drakolimni (Smolikas Mt.)[a]	Greece	40°05′N, 20°54′E	3	*veluchiensis*	D2	D2-2	DQ481520	DQ481495
9	Podgorečko Lakre (Jablanica Mt.)	F.Y.R. Macedonia	41°15′N, 20°32′E	3	*alpestris*	D1	D1-1	DQ481521	DQ481496
10	Donje ravne mlake (Šara Mt.)	Serbia	41°54′N, 20°41′E	2	*alpestris*	D1	D1-2	DQ481522	DQ481497
11	Bukumirsko Lake (Žijovo Mt.)[a]	Montenegro	42°36′N, 19°33′E	2	*montenegrinus*	E1	E1-1	DQ481523	DQ481498
12	Kapetanovo Lake (Lukavica Mt.)[a]	Montenegro	42°48′N, 19°14′E	2	*piperianus*	E1	E1-2	DQ481524	DQ481499
13	Zminičko Lake (Sinjavina Mt.)[a]	Montenegro	42°59′N, 19°15′E	2	*serdarus*	E1	E1-3	DQ481525	DQ481500
14	Seljani (Rogatica)	Bosnia & Herzegovina	43°48′N, 19°05′E	2	*alpestris*	E2	E2-1	DQ481526	DQ481501
15	Prokoško Lake (Vranica Mt.)[a]	Bosnia & Herzegovina	43°57′N, 17°45′E	2	*reiseri*	E2	E2-2	DQ481527	DQ481502
16	Žegar (Ušljebrka)	Croatia	44°09′N, 15°51′E	1	*alpestris*	E2	E2-3	DQ481528	DQ481503
17	Kutarevo (Lika)	Croatia	44°51′N, 15°10′E	1	*alpestris*	E2	E2-4	DQ481529	DQ481504
18	Jankovac (Papuk)	Croatia	45°31′N, 17°41′E	1	*alpestris*	E2	E2-5	DQ481530	DQ481505
19	Joševa	Serbia	44°22′N, 19°50′E	2	*alpestris*	E2	E2-6	DQ481531	DQ481506
20	Ljubina bara (Južni Kučaj Mt.)	Serbia	43°56′N, 21°40′E	2	*alpestris*	E2	E2-7	DQ481532	DQ481507
21	Jezero (Sveti Ilija Mt.)	Serbia	42°36′N, 21°51′E	2	*alpestris*	E2	E2-8	DQ481533	DQ481508
22	Vlasina	Serbia	42°44′N, 22°19′E	5	*alpestris*	A	A-1	DQ481534	DQ481509
23a	Mlačiške Meane	Serbia	42°46′N, 22°14′E	4	*alpestris*	A	A-1	EF089304	EF089270
23b				1		A	A-2	EF089305	EF089271
24	Vlasina Stojkovića	Serbia	42°42′N, 22°23′E	5	*alpestris*	A	A-1	EF089306	EF089272
25a	Klisura	Serbia	42°45′N, 22°25′E	3	*alpestris*	A	A-4	EF089307	EF089273
25b				1		A	A-5	EF089308	EF089274
25c				1		A	A-3	EF089309	EF089275
26a	Bosica	Serbia	42°37′N, 22°23′E	3	*alpestris*	A	A-1	EF089310	EF089276
26b				1		A	A-3	EF089311	EF089277
26c				1		A	A-5	EF089312	EF089278
27	Zli Do (Bosilegrad)	Serbia	42°25′N, 22°27′E	2	*alpestris*	E2	E2-8	DQ481535	DQ481510
28	Satovca (W. Rodope Mts.)	Bulgaria	41°37′N, 23°58′E	2	*alpestris*	E2	E2-9	DQ481536	DQ481511
29	Elatia (C. Rodope Mts.)	Greece	41°30′N, 24°18′E	3	*alpestris*	E2	E2-9	DQ481537	DQ481512
30	Taul Secat, Retezat N.P.	Romania	c. 45°19′N, 22°42′E	2	*alpestris*	E2	E2-10	EF089313	EF089279
31	Zanoaga Lake, Retezat N.P.	Romania	c. 45°20′N, 22°43′E	3	*alpestris*	E2	E2-10	EF089314	EF089280
32	Vidrard Lake, Fagaras Mts.	Romania	c. 45°21′N, 24°38′E	3	*alpestris*	E2	E2-11	EF089315	EF089281
33	Prahova Valley	Romania	c. 45°28′N, 25°34′E	3	*alpestris*	E2	E2-11	EF089316	EF089282
34	Sihastria Monastery	Romania	c. 46°56′N, 26°11′E	2	*alpestris*	C3	C3-1	EF089317	EF089283
35	Varatec	Romania	c. 47°38′N, 26°22′E	2	*alpestris*	C3	C3-1	EF089318	EF089284
36	Gaaden	Austria	47°49′N, 16°04′E	1	*alpestris*	C3	C3-1	EF089320	EF089286

37a	Brand	Austria	c. 48°52′N, 15°01′E	3	*alpestris*	C3	C3-1	EF089325	EF089291
37b				1		C3	C3-2	EF089326	EF089292
38	Irdning	Austria	47°30′N, 14°05′E	2	*alpestris*	C3	C3-1	EF089322	EF089288
39	Obertrum	Austria	47°56′N, 13°04′E	1	*alpestris*	C3	C3-1	EF089319	EF089285
40	Mallnitz	Austria	46°59′N, 13°09′E	1	*alpestris*	C3	C3-3	EF089324	EF089290
41	Klein-Kordin-Alm	Austria	c. 46°37′N, 12°54′E	1	*alpestris*	C3	C3-1	EF089321	EF089287
42	Obertilliach	Austria	46°42′N, 12°36′E	1	*alpestris*	C3	C3-1	EF089323	EF089289
43	Venecia-Trieste	Italy	n.a.	1	*alpestris*	C2	C2-1	EF089327	EF089293
44	Monte Cesen, Valdobiadene	Italy	n.a.	1	*alpestris*	C2	C2-2	EF089328	EF089294
45	North Italy	Italy	n.a.	1	*apuanus*	B	B-1	EF089329	EF089295
46	Ceppo di Lencisa, Genova	Italy	n.a.	1	*apuanus*	B	B-2	EF089330	EF089296
47	Spezia	Italy	44°15′N, 09°32′E	1	*apuanus*	B	B-3	EF089331	EF089297
48	Eindhoven	The Netherlands	51°25′N, 05°28′E	1	*alpestris*	C3	C3-1	EF089332	EF089298
49a	Leende	The Netherlands	51°19′N, 05°32′E	1	*alpestris*	C3	C3-1	EF089333	EF089299
49b				1		C3	C3-4	EF089334	EF089300
50	Navarra	Spain	n.a.	1	*cyreni*	C1	C1-1	EF089335	EF089301
51	Zubilabala-Erreka	Spain	43°02′N, 02°41′E	2	*cyreni*	C1	C1-2	EF089336	EF089302
52	Calabria	Italy	Carranza & Amat 2005	1	*inexpectatus*	B	B-4	DQ092232	DQ092279

[a] Populations exhibiting facultative paedomorphosis.

from the two genes should be combined in a single analysis, a partition-homogeneity test was run in PAUP (v.4.0b10, Swofford, 2002), and significance was estimated by 1000 repartitions. This test, which was described as the incongruence-length difference test by Farris et al. (1995), indicated no conflicting phylogenetic signals between the datasets ($p = 0.26$) and, given that the mtDNA genes are linked, datasets from both genes were analysed together.

For maximum-likelihood (ML) analysis (Felsenstein, 1981), the best-fit model of DNA substitution and the parameter estimates used for tree construction were chosen under the Akaike Information Criterion (AIC; Akaike, 1974) in Modeltest (v.3.7, Posada and Crandall, 1998). The Hasegawa–Kishino–Yano (HKY) model with rate heterogeneity and a nonzero proportion of invariable sites had the highest likelihood score ($-\ln L = 4255.5142$) and showed a significantly better fit than the other less complicated models (model parameters: HKY + I + G, Ti/Tv ratio = 3.6636; $I = 0.5164$, $G = 0.8144$; base frequencies $A = 0.3375$, $C = 0.2334$, $G = 0.1524$, $T = 0.2766$). A maximum-likelihood (ML) tree with Modeltest-derived parameters was constructed with the PHYML program using the method of Guindon and Gascuel (2003). Because of the simultaneous adjustment of the topology and branch lengths this algorithm rapidly reaches an optimum and avoids getting trapped in local optima. It is exceptionally fast compared to other ML-based programs thus enabling the analysis of relatively large data sets and bootstrapping. We tested the robustness of the topology with 1000 bootstrap replicates.

We performed Bayesian analysis with the program MrBayes (v3.1, Huelsenbeck and Ronquist, 2001). Each gene partition received its own best-fit model of DNA substitution under the Akaike Information Criterion as estimated in Modeltest (cyt *b*: HKY + I + G; 16S: GTR + I + G). *Salamandrina terdigidata* was used as the outgroup. Analysis was run with four chains for 10^7 generations and the current tree was saved to file every 100 generations. This generated an output of 10^5 trees in each run. The $-\ln L$ stabilized after approximately 10^5 generations and the first 10^4 trees (10% ''burnin'' in Bayesian terms, chain had not become stationary) were discarded as a conservative measure to avoid the possibility of including random, sub-optimal trees. We used PAUP (v.4.0b10, Swofford, 2002) to obtain a 50% majority-rule consensus tree. The percentage of samples recovering any particular clade in a Bayesian analysis represents that clade's posterior probability (Huelsenbeck and Ronquist, 2001). We used one of the methods of Leaché and Reeder (2002) to assure that our analyses were not trapped on local optima. In particular, the posterior probabilities for individual clades obtained from separate analyses (4 runs) were compared for congruence (Huelsenbeck and Imennov, 2002), given the possibility that two analyses could appear to converge on the same ln-likelihood value while actually supporting incongruent phylogenetic trees.

2.4. Divergence time and molecular-clock testing

A molecular-clock likelihood-ratio test (LRT), $2\Delta = \ln L_{\text{non-clock}} - \ln L_{\text{clock}}$, which is distributed as χ^2 with n-2 degrees of freedom, where n is the number of sequences, was performed to determine whether there was a statistical difference in evolutionary rates among clades (Huelsenbeck and Crandall, 1997). Since the result indicated significant rate heterogeneity among clades, we used a semi-parametric method implemented in the software r8s v 1.7 (Sanderson, 2003), to scale branch lengths over the tree topology. This method allows evolutionary rates to vary between branches within certain limits using a penalized-likelihood function (PL) that includes a roughness penalty and a smoothing parameter. These control the trade-off between the smoothing of rate change across adjacent branches and the goodness-of-fit in the model. A cross-validation procedure (Sanderson, 2002) was used to find the optimal smoothing parameter value ($S = 320$). The analyses with r8s were performed using the PL method and the truncated-Newton algorithm, on a data set of 46 haplotypes, representing all major clades and the outgroups.

In order to calibrate the phylogenetic trees, we used an external calibration point based on the assumption that divergence between *Pleurodeles waltl* from Iberia and *P. poireti* from northern Africa was initiated by a vicariance event at the end of the Messinian salinity crisis, approximately 5.33 Mya, when the re-flood of the Mediterranean, after the opening of the Strait of Gibraltar, separated European and African populations of *Pleurodeles* (Carranza and Arnold, 2004; Carranza and Wade, 2004; Veith et al., 2004; Carranza and Amat, 2005).

Empirical 95% confidence intervals to the temporal estimates were obtained from estimating branch lengths in 100 bootstrapped data sets, keeping the topology and the model of evolution constant, and running r8s analysis for each branch-length set. Analyses were performed with the r8s bootstrap kit (Eriksson, 2003).

2.5. Demographic analysis

The demographic history of clades with sufficient number of sequences available was assessed by a mismatch-distribution analysis, following Schneider and Excoffier (1999) with ARLEQUIN v. 2.001 (Schneider et al., 2000). Recent growth is expected to generate a unimodal distribution of the pairwise differences between sequences (Rogers and Harpending, 1992). The distribution is compared with that expected under a model of population expansion (Rogers, 1995). We report the values of θ_0, θ_1, and τ, which are the estimators of theta before and after expansion, and the time since expansion (measured in mutational time units), respectively. These values were estimated using generalized nonlinear least squares. The goodness-of-fit to the sudden expansion model was tested using MonteCarlo simulations of 1000 random samples. The sum of squared deviations between observed and expected mismatch distributions was used as a test statistic and its P value represents the probability of obtaining a simulated sum of squared deviations larger than or equal to the observed one.

The history of effective population size was also assessed by means of other statistics including Tajima's D test (Tajima, 1989) and Fu's Fs test (Fu, 1997).

3. Results

Of the 811 sites examined, there were 75 variable cyt *b* sites, 67 of which were parsimony-informative (132 and 108, respectively, when the outgroups were included in the analysis), and 76 variable 16S rRNA sites, 71 of which were parsimony-informative (143 and 112, respectively, including outgroups). Forty different haplotypes were identified among 115 ingroup sequences (Tables 1 and 2). Twenty-nine haplotypes (72.5%) were unique and fixed in their corresponding populations (Table 1). The remaining haplotypes were distributed in more than one population, while haplotype C3-1 found to be the most widespread occurring in ten populations (Table 1).

Ingroup uncorrected sequence divergence ranged from 0 to 13.1% for the cyt *b*, while for 16S rRNA uncorrected sequence divergence ranged between 0% and 7.6% (Table 3).

Maximum-likelihood analysis under the HKY + I + G model resulted in a topology with $\ln L = -4262.6813$ (G-shape parameter with four discrete rate categories = 0.814; proportion of invariable sites = 0.516; nucleotide frequencies: $A = 0.316$, $C = 0.214$, $G = 0.192$, $T = 0.278$).

For the Bayesian inference method, identical topologies were recovered for each of the 4 runs with the full dataset, although posterior probabilities for some of the nodes differed slightly between each of the Bayesian runs (Fig. 2). The remaining, after burn-in, 9×10^4 trees, were combined as a 50% majority-rule consensus tree with a mean -ln likelihood of −4310.88 (cyt *b*: G-shape parameter with four discrete rate categories = 0.853; proportion of invariable sites = 0.3654; nucleotide frequencies: $A = 0.283$, $C = 0.276$, $G = 0.147$, $T = 0.295$; 16S: G-shape parameter with four discrete rate categories = 0.457; proportion of invariable sites = 0.467; nucleotide frequencies: $A = 0.372$, $C = 0.197$, $G = 0.168$, $T = 0.263$).

Both methods used for inferring phylogeny gave virtually identical clustering patterns with several distinct clades and partially resolved relationships among them (Fig. 2). The distribution of the clades shows a clear geographic pattern (Fig. 1). The oldest clade (A) includes samples from Vlasina Ravine (No. 22–26, Table 1) and its monophyly was supported by a high bootstrap value. This clade itself is deeply differentiated from the other major branch (Table 3). The remaining haplotypes constitute two major lineages that are in accordance with the geographical origin of the respective populations (Fig. 2): a western lineage comprising two major clades (B, populations from Italy conventionally belonging to ssp. *apuanus* and *inexpectatus*; C,

Table 2
Distribution of haplotypes and diversity measures of the various clades found in the phylogenetic analysis (see Table 1 and Fig. 2)

Haplotype	Clades										
	A	B	C1	C2	C3	D1	D2	D3	D4	E1	E2
A-1	17										
A-2	1										
A-3	2										
A-4	3										
A-5	2										
B-1		1									
B-2		1									
B-3		1									
B-4		1									
C1-1			1								
C1-2			2								
C2-1				1							
C2-2				1							
C3-1					15						
C3-2					1						
C3-3					1						
C3-4					1						
D1-1						3					
D1-2						2					
D2-1							2				
D2-2							3				
D3-1								2			
D3-2								4			
D4-1									6		
D4-2									3		
D4-3									2		
E1-1										2	
E1-2										2	
E1-3										2	
E2-1											2
E2-2											2
E2-3											1
E2-4											1
E2-5											1
E2-6											2
E2-7											2
E2-8											4
E2-9											5
E2-10											5
E2-11											6
n	25	4	3	2	18	5	5	6	11	6	31
Hd	0.530 (0.112)	1.000 (0.177)	0.667 (0.314)	1.000 (0.500)	0.314 (0.138)	0.600 (0.175)	0.600 (0.175)	0.533 (0.172)	0.655 (0.111)	0.800 (0.122)	0.903 (0.025)
Pi	0.00092 (0.00022)	0.00372 (0.00104)	0.00248 (0.00117)	0.00744 (0.00372)	0.00041 (0.00019)	0.00521 (0.00152)	0.00223 (0.00065)	0.00132 (0.00043)	0.00203 (0.00042)	0.00198 (0.00030)	0.01165 (0.00117)

n: number of sequences (sample size); Hd: haplotype diversity (±SD); Pi: nucleotide diversity (±SD).

Table 3
P-distances (%) between clades and subclades

	A	B	C1	C2	C3	D1	D2	D3	D4	E1	E2
A	(0.0/0.2)	7.1	7.4	7.2	7.6	7.3	7.5	7.6	7.3	7.2	7.1
B	12.5	(0.3/0.3)	4.0	3.9	4.3	3.7	3.6	3.4	3.6	4.2	4.6
C1	9.3	6.9	(0.1/0.4)	0.6	1.0	5.2	5.2	4.8	4.7	4.7	4.7
C2	9.9	5.5	3.3	(0.0/2.0)	0.5	4.9	4.9	4.2	4.4	4.4	4.4
C3	10.6	6.1	3.0	2.8	(0.1/0.0)	4.9	4.9	4.2	4.4	4.4	4.4
D1	12.1	8.9	9.5	8.9	8.9	(0.3/1.0)	1.2	1.1	1.3	3.6	3.8
D2	11.7	9.0	9.3	7.7	8.3	3.4	(0.1/0.4)	1.4	1.5	4.1	4.3
D3	12.9	9.7	10.6	9.6	9.9	4.7	4.4	(0.0/0.4)	0.7	3.6	4.0
D4	12.9	9.4	10.0	9.3	10.3	4.8	3.9	1.5	(0.1/0.4)	3.3	3.7
E1	12.3	7.6	7.9	8.3	7.6	7.3	6.0	8.1	7.5	(0.0/0.4)	1.8
E2	13.1	8.3	8.2	8.3	7.6	7.6	6.3	8.3	7.8	2.3	(1.0/1.6)

Codes correspond to clades and subclades identified in the phylogenetic analyses (Fig. 2). Above diagonal: 16S; below diagonal: cyt *b*; diagonal: within clades mean (%) *p*-distance (16S/cyt *b*).

populations from Iberia and central Europe) and an eastern lineage comprising two major clades (D, populations from southern Balkans; and E, populations from central and northern Balkans) supported by high bootstrap values (Fig. 2). The western clade C could be further subdivided into three subclades; C1 includes populations from Iberia conventionally representing ssp. *cyreni*, C2 includes populations from northeastern Italy, and C3 that includes populations of ssp. *alpestris* from central Europe and northern Romania. Within the eastern lineage, both clades contain all conventional Balkan subspecies. The southern clade D comprises four well-differentiated subclades (D1–D4) (Fig. 2, Table 3). Subclade D1 comprises populations from Jablanica and Sara mountains, while the second subclade (D2) contains two populations isolated in different mountain massifs of northern Pindus (Table 1, Fig. 1) exhibiting facultative paedomorphosis. The remaining subclades D3 and D4 contain populations from Peloponnisos and central Greece, respectively, conventionally belonging to ssp. *velouchiensis*. On the contrary, northern clade E comprised two divergent subclades. Three conventional subspecies (ssp. *serdarus*, *piperianus* and *montenegrinus*), that exhibit facultative paedomorphosis, form a monophyletic unit (subclade E1) with high bootstrap support (100%) and posterior probabilities (1.0) (Fig. 2). Subclade E2 contains the majority of central and northern Balkan populations, as well as populations from central and south Carpathians (Romania) (Table 1, Fig. 2), which with one exception, are conventionally classified to the nominotypical subspecies ssp. *alpestris*. Another paedomorphic population from Prokosko Lake (No. 15), conventionally belonging to ssp. *reiseri*, clusters within subclade E2 (Fig. 2).

3.1. Dating the time of divergence

Dating of major mtDNA cladogenic events within the *M. alpestris* group is presented in Fig. 2. The divergence of clade A is of pre-Pliocene origin and a major split between western (Clades B, C) and eastern (Clades D, E) lineages are of Pliocene origin. Further subdivision between B–C and D–E are of Pliocene origin, while all the remaining splits are placed in the Pleistocene.

3.2. Demographic analysis

The results of the mismatch analysis for the two widespread subclades (E2, C3) are presented in Table 4 and Fig. 3. The sudden expansion model was rejected at $\alpha = 0.05$ in subclade E2 but not in subclade C3. The mismatch distribution is clearly unimodal in the C3 subclade, implying recent ($\tau = 0.369$ mutational units) demographic growth. In subclade E2, the shape of the mismatch distribution is multimodal, with the higher modes centered around two, 12 and 14 pairwise differences, suggesting older episodes of growth ($\tau = 14.061$ mutational units). Similarly, other tests of changes in population size, Tajima's *D* and Fu's *F*s, were not significant for E2 but highly significant for C3 (Table 4). For the ancestral clade A, the sudden expansion model was not rejected at $\alpha = 0.05$ and the shape of the mismatch distribution is clearly unimodal ($\tau = 1.000$ mutational units) (Table 4, Fig. 3). However, Tajima's *D* and Fu's *F*s tests were not significant (Table 4).

4. Discussion

4.1. Evolutionary history of the alpine newt

The results of the present study revealed a well-resolved phylogeny at the species level and identified a number of haplotype clades that, based on the observed levels of sequence divergence (Table 3), represent long-separated lineages and diverse evolutionary histories within genus *Mesotriton*. All phylogenetic analyses indicated that the genus is monophyletic (bootstrap value 100% and posterior probability 1.0 for ML and BI analyses, respectively), which is in agreement with previous molecular studies (Zajc and Arntzen, 1999; Carranza and Amat, 2005; Litvinchuk et al., 2005; Steinfartz et al., 2006; Weisrock et al., 2006).

Phylogenetic analyses (both methods used: ML, and BI) indicate the existence of three major lineages of the alpine

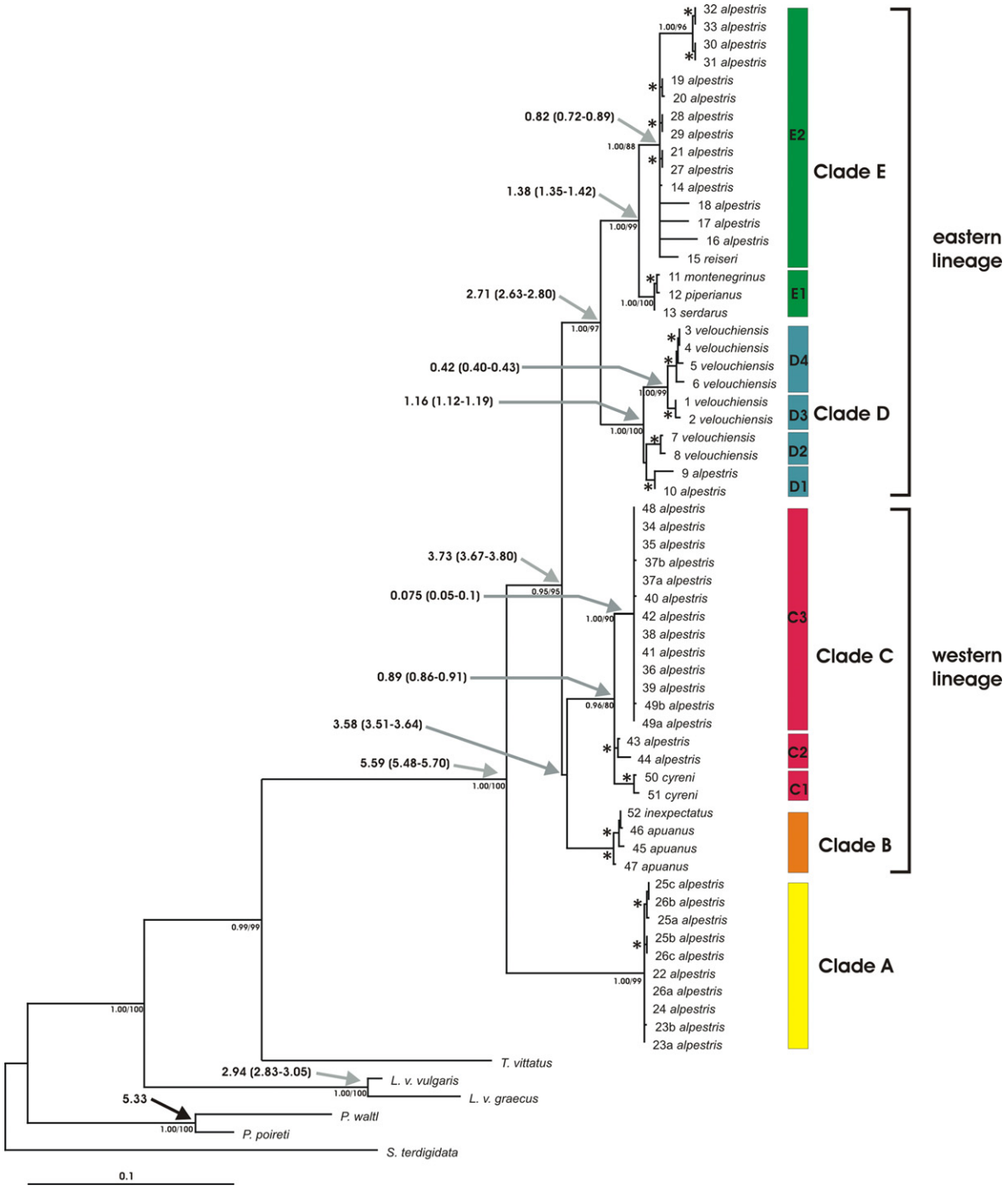

Fig. 2. Phylogenetic relationships among alpine newt populations in Europe. Phylogenetic analyses of maximum likelihood (ML) and Bayesian inference (BI) produced trees with the same topology regarding the major lineages. Only the ML tree is presented. Bayesian posterior probabilities and bootstrap values $\geqslant$80% for the major nodes are shown on branches (BI/ML). The remaining nodes with posterior probabilities and bootstrap values $\geqslant$80% are indicated with asterisks. Gray arrows present the estimated dating of cladogenic events with empirical 95% confidence intervals. The black arrow indicates the calibration point used for dating of clades and subclades.

newts with very high bootstrap values and posterior probabilities (Fig. 2).

4.1.1. A deep phylogenetic split of late Miocene origin

The phylogenetic position of Clade A represents an unexpected and interesting finding for the phylogeny of *M. alpestris*. This clade is well supported (100% bootstrap, 1.0 posterior probability), and the individuals forming it were collected from five local populations in the Vlasina Ravine (southeastern Serbia) (Table 1, Fig. 1B). This ancestral clade comprises five closely related haplotypes and exhibits an average divergence of 7.1–7.6% for the 16S and 9.3–13.1% for the cyt *b* from the remaining clades, which corresponds to a divergence time of late Miocene

Table 4
Mismatch analysis and neutrality tests for the two widespread (E2, C3) and the ancestral (A), alpine newt clades

	Balkans (E2) ($n = 31$)	Central Europe (C3) ($n = 18$)	Ancestral clade A ($n = 25$)
τ	14.061	0.369	1.000
θ_0	0.004	0.019	0.026
θ_1	17.246	∞ (99999.00)	2.386
Goodness-of-fit test			
SSD	0.07174	0.00202	0.00385
P	0.01100 (NS)	0.72000	0.68300
Tajima's *D* test	0.15262	−1.71304	−0.14952
P	0.62700 (NS)	0.02100	0.47900 (NS)
Fu's *F*s test	2.57487	−2.60267	−1.60232
P	0.85500 (NS)	0.00000	0.07400 (NS)

n: number of sequences; NS: non significant.

origin (5.5–5.7 Mya; Fig. 2). It seems that this date, and our calibration in general, is plausible since our estimation of the date of divergence between *Lissotriton vulgaris graecus* and *L. v. vulgaris* (2.8–3.1 Mya, mean 2.9 Mya; Fig. 2), is in agreement with the divergence time of the respective taxa (2.4–3.3 Mya, mean 3.0 Mya) estimated by Babik et al. (2005). The time of divergence of Clade A is surprisingly identical to the time of a deep phylogenetic break within *Lissotriton boscai* from Iberia (~5.8 Mya; Martínez-Solano et al., 2006). It is plausible that the general climatic and physiographic conditions in Europe at this time have promoted such deep cladogenic events within many urodeles. Therefore, this similarity of cladogenic events could trigger a more detailed investigation on the issue.

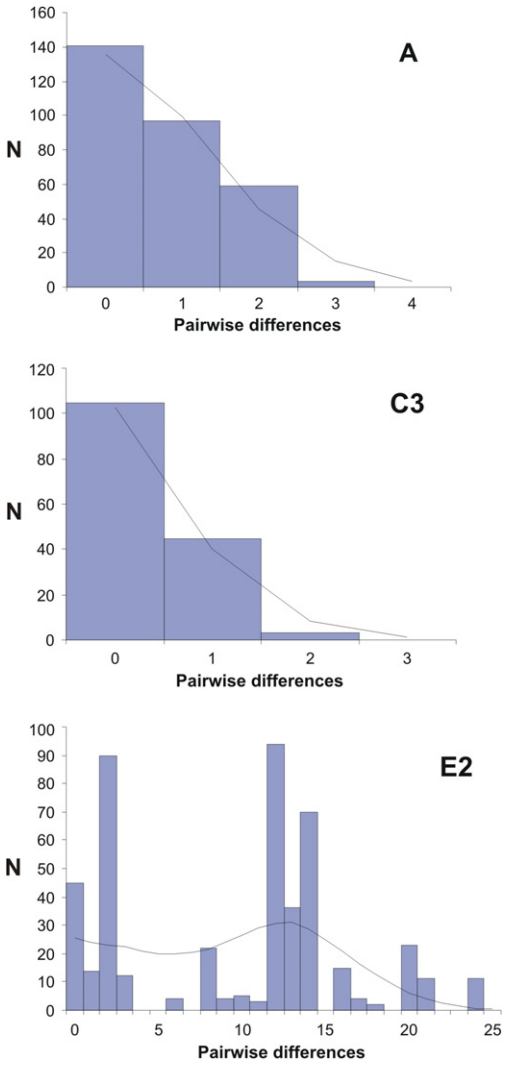

Fig. 3. Mismatch distributions in the two most widespread clades (C3, E2) and in the ancestral clade A. Unimodal distribution of the pairwise differences, pronounced in clades C3 and A, indicates demographic expansion, with the location of the peak indicating the time of expansion. The multimodal distribution in clade E2 suggests long-term demographic stability. Black curves show shapes of theoretical distributions under the sudden expansion model.

For the Vlasina Ravine population we suggest to be remnant of the oldest known stock of this newt in Europe. According to our data, it remains uncertain whether this taxon is completely confined in the Vlasina area or, less likely, expands further east to the Balkan Mountains (Stara Planina, Bulgaria). For this purpose, further sampling is required in order to discover its full distribution range. Populations of this ancestral form might have been trapped and survived up to the present in a hypothetical relict refugium of the Vlasina area giving rise to clade A. Even though deep phylogeographic breaks may occur within a continuously distributed species in the absence of any geographic barriers to gene flow (Irwin, 2002), the extent of the genetic distinctiveness of the Vlasina lineage could imply a long-term persistence in isolated pockets of relatively constant favorable microenvironments lacking any kind of gene flow with all other lineages (Fig. 4). Vlasina Ravine could offer the conditions for such a long-term isolation since it appears to be distinct in respect of geomorphological origin (Dimitrijević, 1995; Knežević-Djordjević and Krstić, 1996). It is situated at 1200 m asl., completely surrounded by several mountains up to 2000 m asl., and covers an area of approximately 50 km^2. The area appears to be of particular biogeographic interest due to other phylogeographic peculiarities (e.g. brown trout, Marić et al., 2006), and to a number of Tertiary relict populations of several plants such as *Prunus laurocerasus*, *Juglans regia* and *Taxus baccata* (Stevanović et al., 1995).

4.1.2. Pliocene: separation of eastern and western lineages

We hypothesize a Balkan origin of the alpine newt with the most recent common ancestor of Eastern and Western

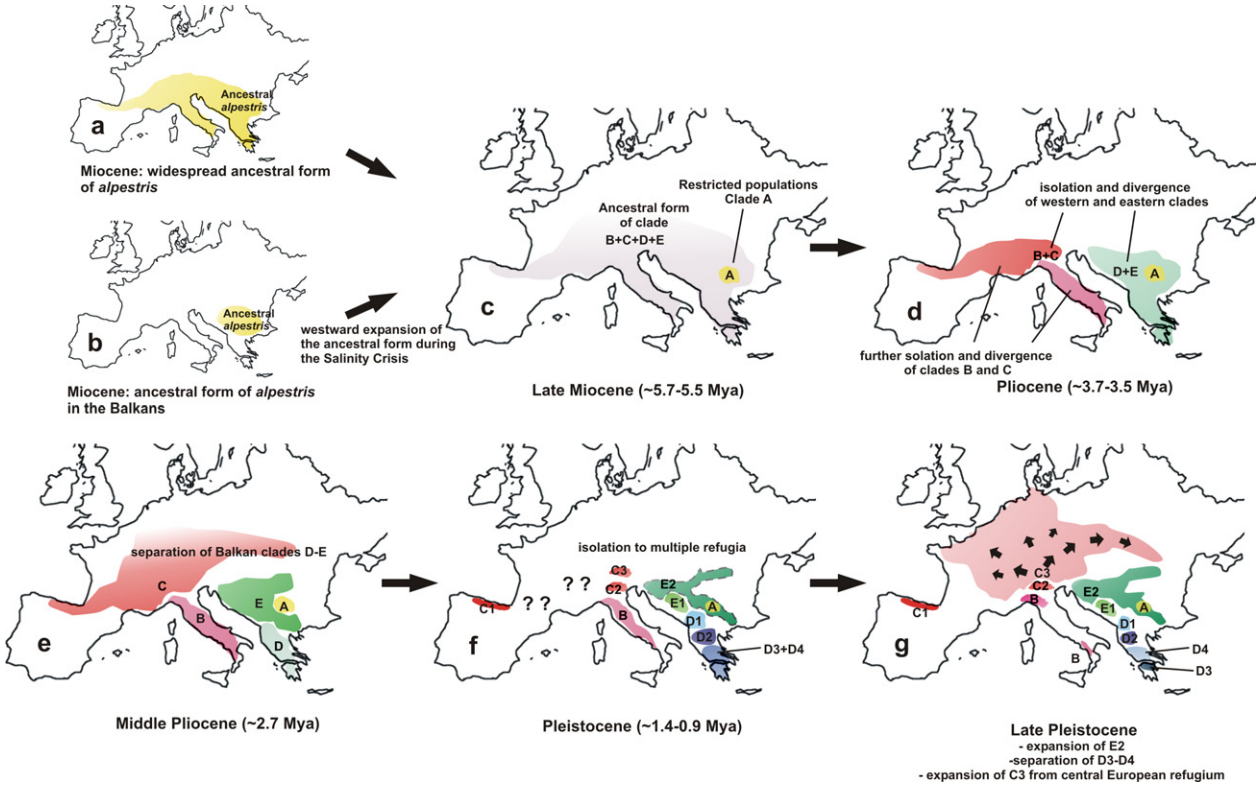

Fig. 4. Hypothetical biogeographic scenario of *M. alpestris* diversification in Europe. Capital letters correspond to clades and subclades identified in the phylogenetic analyses (Fig. 2).

lineages of Miocene origin (Figs. 2 and 4). Since the oldest known fossil of *Triturus* cf. *alpestris* comes form the upper Pliocene of Slovakia (Hodrova, 1984), two alternatives can be postulated concerning the prior history of the species in Europe: (a) either a widespread across Europe ancestral form during the late Miocene (Fig. 4a or b), a westward expansion of the ancestral form during the salinity crisis (Fig. 4b) like many other freshwater organisms (Bianco, 1990; Durand et al., 2002; Tsigenopoulos et al., 2003 and references therein).

Separation of Apennine and Balkan Peninsulas took place after the end of the salinity crisis (~5.33 Mya) as a result of the re-flooding of the Mediterranean Basin (Krijgsman et al., 1999; Duggen et al., 2003; Meijer and Krijgsman, 2005). Consequently, the Apennines emerged and were additionally isolated completely from the Alps to the north in middle Pliocene (Steininger and Rögl, 1984; Yilmaz et al., 1996). This large scale vicariant event probably caused range fragmentation of the ancestral lineage and formation of the Western (B + C) and Eastern (D + E) clades around 3.7 Mya, followed by further diversification of the western lineage to clades B and C around 3.5 Mya (Figs. 2 and 4).

Clade B expresses a high genetic divergence from all other phylogroups bearing four closely related haplotypes, and appears to be confined to the Apennine Peninsula since that period of time. Clade C contains populations from Iberia, north-eastern Italy, central Europe and northern Carpathians (Table 1, Fig. 2), displaying considerable amounts of divergence from all other lineages (Table 3). It seems that this clade was the most widespread in Europe in this period of time, a hypothesis further supported by the discovery of fossil cf. *alpestris* from the upper Pliocene of Slovakia. The eastern lineage must have followed two diversification routes during the middle-late Pliocene (around 2.7 Mya) giving rise to two geographically consistent clades: a southern clade D, comprising populations from southern Dinarids and the Pindus massifs, and a northern clade E that comprises populations from central and northern Balkans, south-central Carpathians and Rodope Mts. (Fig. 2, Table 1). This cladogenic event could possibly coincide with the physiographic characteristics of the area at this period. Extensive inflows of the sea during Pliocene at the area of Axios and Strymon river basins reached northwards almost to the central Balkans along the Vardar and perhaps Morava valleys (Meulenkamp, 1985; Yilmaz et al., 1996). This area was characterized by mostly saline soils that, being hostile to amphibians, might have act as a significant barrier to gene flow promoting the isolation and diversification of alpine newt populations.

4.1.3. Multiple pleistocene refugia within the balkan peninsula and the colonization of europe

Multiple cladogenic events seem to have taken place at the early Pleistocene, around 1.4–1.2 Mya, probably relating to the oscillating glacial cycles. Paedomorphic lineages

(subclades D2 and E1; Table 1, Fig. 2) seem to have evolved at this period of time in parallel in both major Balkan clades. The appearance of facultative paedomorphosis correlates with the ongoing Pleistocene glacial cycles probably as a response to the adverse environmental conditions (see Roček, 1995).

Apart from the hypothetical relict refugium of the ancestral clade A, at least six other distinct refugia can be allocated in the Balkan Peninsula, each one related to each of the six clades and subclades identified within the eastern alpine newt lineage (D1–D4, E1–E2). The geographic position of the involving subclades (D1–D4, E1–E2) and the timeframe of these cladogenetic events support the application of the refugia-within-refugia (Gómez and Lunt, 2006) scenario for the Balkan Peninsula. These refugia should have been located mostly on the Dinaric and Pindic Massifs (Fig. 4f). The number of the respective phylogroups increases towards the southern Balkans to the periphery of the species range indicating a greater isolation than previously thought. Given the amount and the estimated time of divergence among these subclades, it seems that the respective populations have survived the adverse climatic conditions of the Pleistocene in allopatry with limited postglacial expansion and contact. The later is supported by the almost complete fixation of the different haplotypes in many single populations within each of the phylogroups identified (no introgression has detected), as well as the limited geographical range of each of them. Exception to the rule is subclade E2 which is widespread in the central and northern Balkans up to central Carpathians, comprising 11 haplotypes distributed in 15 populations (Tables 1 and 2). However, the results of the mismatch distributions and other tests of neutrality indicate long-term demographic stability and perhaps older episodes of growth. The incidence of several groupings within E2 (Fig. 2) possibly refers to several other minor or secondary refugia in the area from which populations expanded and contracted during Pleistocene cycles. Further subdivision is indicated in the southern Balkans. Separation of subclades D3 (Peloponnisos) and D4 (central Greece) dates back to the middle-late Pleistocene (~0.4 Mya, Fig. 2, Fig. 4g). Although Peloponnisos was repeatedly connected to continental Greece over the Gulf of Corinth until the last glacial period (Würm, ~18 Kya) (Dermitzakis, 1990; Perissoratis et al., 2000; Perissoratis and Conispoliatis, 2003), the observed amount of divergence (as well as the estimated allozyme divergence of $D_{nei} = 0.321$; Sotiropoulos, unpublished) favors such an earlier separation of the respective alpine newt populations. This greater isolation in southern Balkans could be attributed to alterations of the climatic conditions that caused extensive range fragmentation of the alpine newt populations in favorable mountain islands.

Apart from a major split within the western lineage during the middle Pliocene, which led to the isolation of clade B to the Apennine Peninsula, the consecutive subdivision of western clade C into subclades C1–C3 is estimated to have taken place during Pleistocene times (Fig. 2). The geographic position of the respective subclades indicates the location of at least three distinct refugia (Fig. 4f). One should have been located in the Cantabrian Mts., in which the isolation of Iberian populations (C1) gave rise to ssp. *cyreni* since the middle Pleistocene (~0.8–1.0 Mya, Fig. 2). Two other distinct refugia should have been located on the south and north environs of the Alps corresponding to clades C2 and C3, respectively. The existence of alternative cryptic refugia in the Hungarian plains and in the river valleys of the Alps has been suggested in many studies for many animal and plant taxa (e.g. Taberlet and Bouvet, 1994; Willis et al., 2000; Deffontaine et al., 2005). The incidence of fossil cf. *alpestris* from the Pleistocene of Slovakia (Hodrova, 1984) could possibly indicate the location of the latter refugium (C3) in that area. The corresponding subclade C3 seems to have played a major role in the colonization of Europe (Fig. 4g). It is the most widespread among all the clades revealed in the analyses including populations from central, western, and eastern Europe. It contains just four closely related haplotypes (Tables 1 and 2), expresses the lowest levels of haplotype and nucleotide divergence compared to all other clades and subclades (Table 2), and found to conform significantly to a sudden expansion model (Table 4 and Fig. 3). It is difficult to put a date on spread into Europe on the basis of the molecular phylogeny, as the gene fragments used here change relatively slowly, but the estimated time of separation within C3 (Fig. 2) suggests that might have happened 50,000–100,000 years ago (Fig. 4g). If recent origin of central European alpine newts is accepted, the Pliocene and at least some of the Pleistocene fossil records of the species in central Europe (Roček, 1994) must represent earlier invasions from the source area. The descendants of these invasions became extinct subsequently, perhaps during the cold phases that accompanied Pleistocene glaciations.

This hypothesis conforms to Steinfartz et al. (2006) and Pabijan and Babik (2006) which suggest a western origin of central and eastern European populations. However the later authors failed to suspect a different origin of south-central Carpathian populations suggesting a consecutive isolation and divergence from the invading western populations. Our results indicate a different origin of southern and central Carpathian lineages which have been originated from northern Balkan lineages (Fig. 2). The results show two colonization routes, with relative different strengths and span (an extended one from the Alp region and a second, limited one, from the Balkan area), that meet in central-north Carpathians and probably form a contact zone of secondary origin (Fig. 4g). However, the last hypothesis remains to be examined in detail. Our results agree with the general colonization scenario applied for numerous animal and plant species (e.g. Hewitt, 1996, 2004; Taberlet et al., 1998), which suggests colonization of central and northern Europe from the marginal refugial populations (e.g. subclade C3) while southern populations actually imbedded and remained on site shifting their ranges upwards to higher altitudes thus preserving greater amounts of variation.

4.2. Taxonomic implications

The analyses of molecular data do not agree with the subspecific taxonomy of *M. alpestris*. To understand further the evolutionary relationships between these nine subspecies, the geographical origin (site and region) and the subspecific status of the observed haplotypes are given in Table 1 and Fig. 2.

Our data suggest that the western (clades B + C) and the eastern lineage (clades D + E) have had a long independent evolutionary history and they might constitute different cryptic species, with their respective subclades forming different subspecies. The Apennine and Calabrian taxa (ssp. *apuanus* and *inexpectatus*, respectively), form a strongly supported monophyletic unit comprising four closely related haplotypes (0.3% divergence for cyt b and 16S; Table 3) with significant amount of divergence from all other lineages and a long time in isolation. These results refute the separate subspecific taxonomy of the respective populations, at least in the mitochondrial level. An interesting case concerns ssp. *cyreni* from Iberia (subclade C1). Its clear chromosome differences (Herrero et al., 1989) gained within a relatively short time-frame (since their estimated divergence time during middle Pleistocene, ~0.9 Mya), along with its apparent geographic isolation to all other lineages, could support its separate taxonomic status. Apart from ssp. *cyreni*, two other distinct subclades are evident within the western lineage (C2, C3), containing populations conventionally assigned to the nominotypical ssp. *alpestris*. Within the eastern lineage, three conventional paedomorphic subspecies from Montenegro (ssp. *montenegrinus*, *piperianus*, *serdarus*) comprise a strongly supported monophyletic unit (E1) sharing a unique 16S haplotype and expressing a low genetic divergence in cyt *b* (0.4%; Table 3). According to allozyme (Arano and Arntzen, 1987) and cytogenetic (Herrero et al., 1989) data, only ssp. *serdarus* may warrant a separate subspecific status being distinct in regard to allele frequencies and chromosome features. However, all the above conventional subspecies fail to present any distinctiveness in body proportions and coloration patterns, from other conspecific members, expressing a conservative phenotype (Sotiropoulos et al., 2001). Another paedomorphic population from Prokosko Lake, described as ssp. *reiseri*, does not differentiate considerably either in mtDNA haplotypes or in morphology from the members of subclade E2 which in turn are all conventionally assigned to ssp. *alpestris*. Similarly, in the southern Balkans the alpine newt is represented by ssp. *veluchiensis* (distributed in central Greece and possibly Peloponnisos) and by two other well differentiated lineages (subclades D1, D2) that need further examination.

The considerable amount of genetic differentiation observed between haplotypes from the Vlasina area (Clade A) and the rest of the samples (Table 3), and its phylogenetic position outside the clade (B + C + D + E) containing all other alpine newt populations (Fig. 2), raises questions regarding the inclusion of this population within *M. alpestris*. Although significant genetic differentiation within *M. alpestris* has already been reported (3.0% sequence difference of the 12S rRNA between ssp. *alpestris* and ssp. *cyreni*; Zajc and Arntzen, 1999), the observed divergences of clade A (Table 3) are higher than the reported genetic distance between congeneric amphibian species (Johns and Avise, 1998; Veith et al., 2004; Carranza and Amat, 2005; Steinfartz et al., 2006). More interestingly, this presumably relict population expresses a conservative morphology. Morphometric and qualitative trait analyses place the Vlasina population well within the nominotypical populations' phenotype (Sotiropoulos et al., 2001). If we place the alpine newts of Vlasina in the same taxon as the populations of the surrounding Balkan areas, then Balkan clades of the alpine newt become paraphyletic. Consequently, in order to resolve the taxonomic problems of the alpine newt in this area, the population from Vlasina (Clade A) should best be considered as a separate evolutionary species. However, the recovery of this very distant clade, confined in a restricted area within a larger distribution of other *Mesotriton* lineages, raises questions concerning its true nature. Being confined to a mitochondrial gene genealogy, the instance of incomplete lineage sorting, due to retention of ancestral polymorphisms, becomes another plausible scenario having as a subsequent outcome the difficulty of inferring true species relations in space and time (Avise et al., 1983; Rosenberg, 2002, 2003; Maddison and Knowles, 2006).

Consequently, to review the specific status of the particular lineage, as well as of the whole *Mesotriton* complex, utilization of nuclear genetic variation is essential in order to confirm or rule out the possibility of incomplete lineage sorting (Patton and Smith, 1994; García-París et al., 2003), hybridization and introgression events (García-París et al., 2003), the latter being feasible considering the conservative morphology and chromosome features of the genus (Herrero et al., 1989 and references therein).

Although morphological and genetic variation within *M. alpestris* has already been studied, the recovery of many divergent lineages, which were previously largely ignored perhaps due to incomplete or limited sampling design, could further constitute the initial material for finer-scale phylogenetic and taxonomical resolution.

Acknowledgments

The present study was partially funded through the Operational Program for Education and Initial Vocational Training (O.P. "Education") in the framework of the project "Pythagoras II—Support of University Research Groups", and by the Serbian Ministry of Science and Environmental Protection ("Patterns of amphibian and reptile diversity on the Balkan Peninsula", Grant 143052). We are grateful to N. Poulakakis for helping in technical matters. Salvador Carranza generously provided key DNA samples from Spain and Italy, Suvad Lelo from Prokoško Lake, Serge Bogaerts from the Netherlands, Werner Mayer

from Austria, and Dan Cogălniceanu from Romania. A. Caccone and three anonymous reviewers provided critical comments and suggestions on an early version of the manuscript.

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