Molecular Ecology (2005) **14**, 2475–2491 doi: 10.1111/j.1365-294X.2005.02605.x

Phylogeography of two European newt species — discordance between mtDNA and morphology

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Abstract

The newts *Triturus vulgaris* and *Triturus montandoni* are sister species that exhibit contrasting levels of intraspecific morphological variation. *Triturus vulgaris* has a broad Eurasiatic distribution encompassing both formerly glaciated and unglaciated areas and shows substantial morphological differentiation in the southern part of its range, while *T. montandoni*, confined to the Carpathians, is morphologically uniform. We analysed sequence variation of two mtDNA fragments of the total length of *c.* 1850 bp in 285 individuals of both species collected from 103 localities. Phylogenetic analysis of 200 unique haplotypes defined 12 major clades, their age estimated at *c.* 4.5–1.0 million years (Myr). Most of the older clades were found in the southern part of the range, and also in central Europe, mainly in Romania. The distribution of mtDNA clades points to the existence of several glacial refugia, located in the Caucasus region, Anatolia, the Balkan Peninsula, Italy, and more to the north in central Europe. The concordance between mtDNA based phylogeny and the distribution of *T. vulgaris* subspecies was weak. *Triturus montandoni* haplotypes did not form a monophyletic group. Instead they were found in six clades, in five of them mixed with *T. vulgaris* haplotypes, most likely as a result of past or ongoing hybridization and multiple introgression of mtDNA from *T. vulgaris* to *T. montandoni*. Patterns of sequence variation within clades suggested long-term demographic stability in the southern groups, moderate and relatively old demographic growth in the populations inhabiting central Europe, and high growth in some of the groups that colonized northern parts of Europe after the last glacial maximum.

Keywords: introgression, molecular clock, northern refugia, phylogeography, Salamandridae, *Triturus montandoni*, *Triturus vulgaris*

Received 12 January 2005; revision accepted 1 April 2005

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Introduction

The amount of intraspecific genetic variation, its geographic distribution and genealogical relationships among alleles are the consequences of the unique history of each species. Modern molecular techniques coupled with increasingly

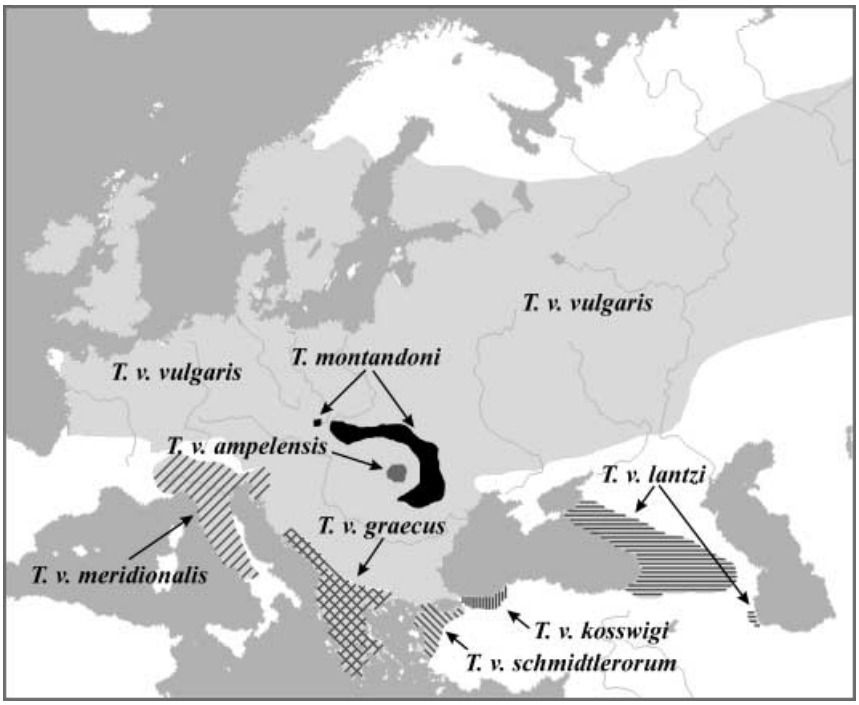

Fig. 1 Distribution of *Triturus vulgaris* and *Triturus montandoni* as well as *T. vulgaris* subspecies in Europe and western Asia (Raxworthy 1990; Zavadil *et al*. 2003; Schmidtler & Frantzen 2004).

sophisticated analytical methods effectively employ the existing patterns of genetic variation for inferring their historical causes (Avise 2000, 2004). It is widely acknowledged that Pleistocene climatic changes affected most species inhabiting temperate regions, causing range shifts, repeated bouts of secondary contact, fragmentation of species ranges, local extinctions and demographic fluctuations (Avise 2000; Hewitt 2000, 2004). These in turn promoted differentiation by increasing the effect of random genetic drift and inducing diverse selective pressures leading to local adaptations and possibly to the development of reproductive isolation between populations. However, the importance of Pleistocene glaciations for speciation in temperate regions is not well understood (Avise *et al*. 1998; Ribera & Vogler 2004; Weir & Schluter 2004; Zink *et al*. 2004).

The urodele amphibians are poikilotherm organisms with limited dispersal capabilities. Genetic differentiation in these animals is often very old, dating back to the Miocene and Oligocene (Tan & Wake 1995; Tarkhnishvili *et al*. 2000; Riberon *et al*. 2001; Weisrock *et al*. 2001), but may be also of Pleistocene origin (Church *et al*. 2003; Lecis & Norris 2004; Shaffer *et al*. 2004). In several groups a high level of genetic differentiation is not paralleled by substantial morphological differentiation (Good 1989; Good & Wake 1992; Shaffer *et al*. 2004; Veith *et al*. 2004). In this context, comparing the phylogeographic structure between two closely related newt species with diverse levels of intraspecific morphological variation is of particular interest. Extensive intraspecific morphological differentiation should be accompanied by deeper phylogeographic pattern than in morphologically uniform taxa. However, if morphological variation has been shaped mainly by selection, this simple prediction may not hold. In newts, morphological differentiation is reflected in elaborate male secondary sexual traits that are displayed during complex courtship. These traits are thought to have arisen via sexual selection, which is likely to be a major force triggering morphological differentiation in this group (Halliday 1990).

The smooth newt (*Triturus vulgaris*) and Montandon's newt (*Triturus montandoni*) are sister species, which, according to the allozyme molecular clock, diverged *c*. 6 Ma (Rafiński & Arntzen 1987; Zajc & Arntzen 1999). *Triturus vulgaris* is widely distributed in Eurasia ranging from western Europe, with the exception of Iberia, to western Siberia. A number of subspecies have been described in the southern part of its range, corresponding to the regions of glacial refugia postulated for many animal and plant species (Fig. 1). The differences between these forms pertain mainly to male secondary-sexual characters. According to recent reviews (Raxworthy 1990; Schmidtler & Frantzen 2004), seven or eight subspecies are recognized (Fig. 1). Transition zones, characterized by gradients of allozyme allele frequencies were described between several pairs of subspecies in the former Yugoslavia and Romania (Kalezić 1984; Rafiński *et al*. 2001).

In contrast to *T. vulgaris*, the range of *T. montandoni* is restricted to the eastern and western Carpathians and the

easternmost part of the Sudetes (Fig. 1; Zavadil *et al*. 2003); morphological variation in this species is small, and no subspecies has been described (Zavadil *et al*. 2003).

At lower elevations in the Carpathian Mountains where their ranges overlap, hybridization between *T. vulgaris* and *T. montandoni* is widespread; the hybrids are fertile and the structure of the hybrid zone is mosaic and bimodal (Kotlík & Zavadil 1999; Babik *et al*. 2003; Babik & Rafiński 2004). Bimodal distribution of genotypes and the patterns of morphological variation imply strong assortative mating within parental species (Babik *et al*. 2003; Babik & Rafiński 2004). The extent of mtDNA introgression exceeds that of the nuclear markers, suggesting that mtDNA introgression could be extensive during the evolutionary history of these species.

Two general issues may be addressed through examining the phylogeographic structure of *T. vulgaris* and *T. montandoni*. First, we aimed at a better understanding of the impact of the Pleistocene glaciations on the origin and structuring of mtDNA sequence variation, and consequently, on patterns of speciation in urodele amphibians. This required estimating the divergence time of the mtDNA clades, analysing their geographic distribution in the context of putative glacial refugia and assessing their demographic history. Second, we tested the hypothesis that morphological variation is explained by overall genome divergence through a comparison of the geographic distribution of mtDNA lineages and the distribution of morphologically diagnosable forms (species and subspecies). If this is not the case, mtDNA introgression or the action of selection, or both, could produce a discordance between mtDNA and morphology-based specific and subspecific boundaries. This could be partially addressed with the mtDNA sequence data presented here, that also provide a starting point for further analyses employing nuclear sequence markers.

Materials and Methods

Samples and laboratory methods

We analysed a total of 285 individuals of *Triturus vulgaris* and *Triturus montandoni* from 103 localities (Table 1, Fig. 2),

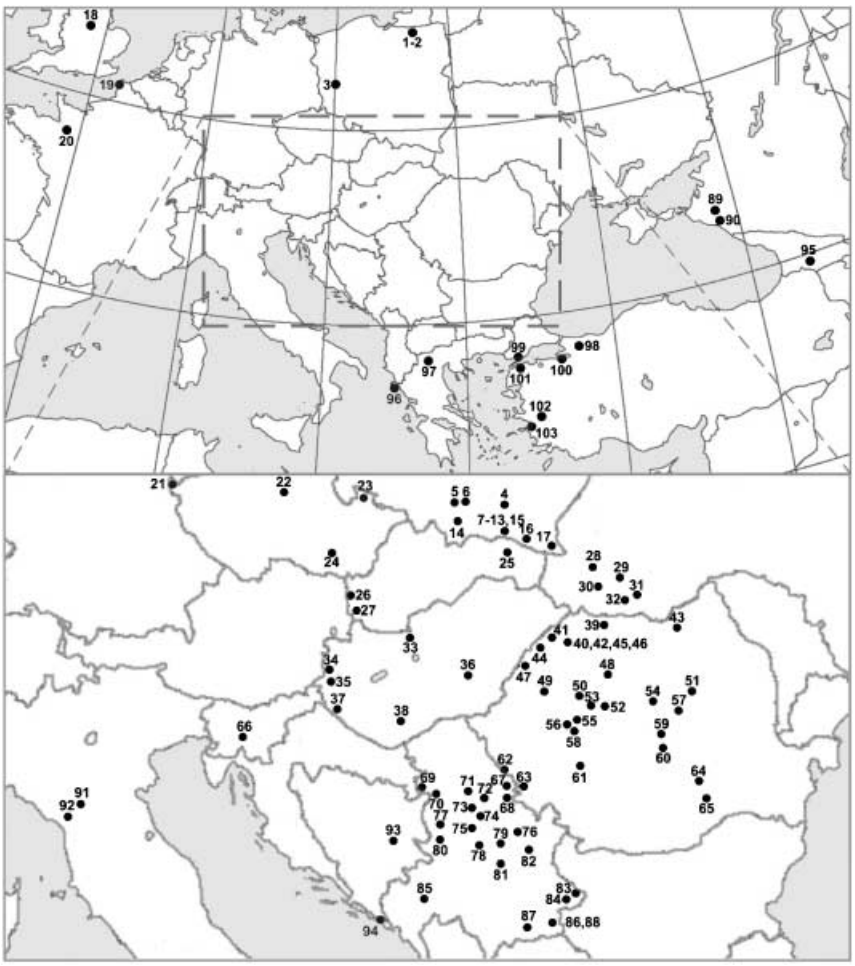

Fig. 2 Sampling localities. In a few cases a single dot represents several adjacent localities; numbers correspond to those in Table 1.

Table 1 Sample localities used in the study

No.	Locality	Coordinates	*n*	Ssp	Clades	Haplotypes	Collector
	Poland						
1	Guty	54°04′N, 21°40′E	3	*Tvv*	G	G17, G18, G19	M. Pabijan
2	Fuleda	54°06′N, 21°39′E	2	*Tvv*	G	G15, G16	M. Pabijan
3	Głogów	51°39′N, 16°05′E	3	*Tvv*	L3	L66, L67, L69	J. Rafiński
4	Cieszęciny	50°07′N, 21°23′E	1	*Tvv*	L3	L68	WB
5	Mników	50°03′N, 19°43′E	3	*Tvv*	G, L3	G14, L67, L70	WB
6	Przegorzały	50°03′N, 19°51′E	5	*Tvv*	L3	L57, L67(3), L71	WB
7	Załęże	49°40′N, 21°28′E	1	*Tvv*	L3	L62	WB
8	Bartne	49°34′N, 21°21′E	2	*Tm*, *Tvv*	I, L3	I8, L63	J. Rafiński
9	Potok Krokowy	49°32′N, 21°23′E	1	h	I	I11	WB
10	Wołowiec	49°32′N, 21°22′E	1	h	I	I6	WB
11	Kotań	49°31′N, 21°29′E	1	*Tvv*	I	I7	WB
12	Rostajne1	49°31′N, 21°26′E	4	*Tm*, *Tvv*	F, I, L3	F25, F26, I8, L65	WB
13	Rostajne2	49°30′N, 21°25′E	2	*Tm*	I, L3	I4, L67	WB
14	Łopuszna	49°29′N, 20°07′E	1	*Tm*	I	I3	A. Osikowski
15	Tysowe1	49°27′N, 21°29′E	1	*Tm*	L3	L65	WB
16	Komańcza	49°19′N, 22°03′E	2	*Tm*	I, L3	I2, L65	WB
17	Ustrzyki Górne	49°06′N, 22°38′E	5	*Tm*	I	I1, I7, I9(2), I10	WB
	UK						
18	Leicester	52°38′N, 01°07′W	2	*Tvv*	L1	L8(2)	JWA
	France						
19	Ambleteuse	50°47′N, 01°36′E	2	*Tvv*	L1	L7(2)	R. Jehle
20	Mayenne	48°18′N, 00°36′W	4	*Tvv*	L1	L7(4)	R. Jehle
	Germany						
21	Klingenthal	50°21′N, 12°28′E	4	*Tvv*	L2	L24, L28, L32(2)	R. Jehle
	Czech Republic						
22	Slavhostice	50°18′N, 15°21′E	5	*Tvv*	L2	L29(2), L33, L37(2)	M. Sandera
23	Karlova Studánka	50°04′N, 17°18′E	8	*Tm*	L2, L3	L39, L40, L60, L61, L62, L67(3)	M. Liana
24	Nosislav	49°01′N, 16°40′E	1	*Tvv*	L2	L55	M. Sandera
	Slovakia						
25	Lipníky	49°03′N, 21°23′E	4	*Tvv*	F	F22, F23, F24, F30	WB
26	Devínské Jezero	48°12′N, 16°58′E	2	*Tvv*	L2	L55(2)	P. Mikuličeк
27	Bratislava-Cunovo	48°02′N, 17°11′E	2	*Tvv*	L2	L55(2)	P. Mikuličeк
	Ukraine						
28	Maidan	48°36′N, 23°30′E	4	*Tm*	J	J11(4)	NAP
29	Ust′-Chorna	48°19′N, 23°59′E	2	*Tm*	G, J	G12, J11	NAP
30	Mala Ugolka	48°15′N, 23°36′E	3	*Tm*	F, I, J	F27, I5, J11	NAP
31	Rakhiv	48°03′N, 24°14′E	1	*Tm*	J	J9	NAP
32	Kobyla Hora	48°02′N, 24°05′E	4	*Tm*	J	J10(2), J11(2)	NAP
	Hungary						
33	Pilis, Janos Lake	47°41′N, 18°54′E	5	*Tvv*	L2	L34(2), L35, L36, L38	T. Kovacs
34	Körmend	46°60′N, 16°37′E	2	*Tvv*	L2	L29, L31	WB
35	Zalahásházgy	46°55′N, 16°37′E	2	*Tvv*	L3	L56, L58	WB
36	Öcsöd — Békésszentendrás	46°53′N, 20°24′E	4	*Tvv*	F	F12, F15, F19, F21	J. Rafiński
37	Ortilos	46°16′N, 16°55′E	5	*Tvv*	L1, L3	L9, L10, L11, L12, L57	T. Kovacs
38	Bátászek	46°12′N, 18°49′E	4	*Tvv*	L2, L3	L27, L29, L30, L50	WB
	Romania						
39	Izvoarele	47°50′N, 23°40′E	1	*Tm*	J	J8	JWA
40	Craidorolţ	47°36′N, 22°42′E	2	*Tvv*	F	F33(2)	IS
41	Cŏuaş	47°34′N, 22°32′E	2	*Tvv*	F	F29, F34	IS
42	Acâş	47°32′N, 22°45′E	2	*Tvv*	F	F31, F32	IS
43	Sadova	47°32′N, 25°29′E	1	*Tm*	G	G13	JWA
44	Andrid	47°30′N, 22°20′E	2	*Tvv*	F	F17, F18	IS
45	Săcăşeni	47°29′N, 22°41′E	2	*Tvv*	F	F13, F35	IS
46	Supuru de Sus	47°26′N, 22°45′E	2	*Tvv*	F	F28(2)	IS
47	Sălard	47°13′N, 22°01′E	2	*Tvv*	F	F16, F20	IS
48	Cluj	46°46′N, 23°36′E	5	*Tva*	J	J12, J14, J15(2), J16	DC
49	Briheni	46°30′N, 22°24′E	2	*Tva*	F	F3, F14	IS
50	Cărpiniş	46°19′N, 23°02′E	4	*Tva*	J	J2(2), J3, J17	DC
51	Crăcurele	46°16′N, 26°19′E	3	*Tvv*	G, J	G8(2), J13	DC
52	Izvoru Ampoiului	46°09′N, 23°10′E	3	*Tva*	F	F10(2), F11	DC
53	Zlatna	46°07′N, 23°13′E	4	*Tva*	J	J1(2), J20(2)	DC
54	Rupea	46°02′N, 25°12′E	5	*Tvv*	J	J22(3), J23, J24	DC
55	Săcărâmbu	45°58′N, 23°02′E	3	*Tva*	F, J	F11, J4, J21	DC

Table 1 *Continued*

No.	Locality	Coordinates	*n*	Ssp	Clades	Haplotypes	Collector
56	Deva	45°53′N, 22°54′E	4	*Tva*	F, J	F1, J5, J7, J18	DC
57	Reci	45°51′N, 25°56′E	4	*Tvv*	G, J	G10(2), G11, J19	DC
58	Călan-Bai	45°44′N, 22°59′E	2	*Tva*	F, J	F2, J6	IS
59	Predeal	45°31′N, 25°34′E	7	*Tm*	G	G7(2), G8(5)	DC
60	Baiu	45°24′N, 25°35′E	4	*Tm*	G	G1(2), G8, G9	DC
61	Câmpu lui Neag	45°18′N, 23°01′E	3	*Tva*	F	F4(2), F5	DC
62	Moraviţă	45°16′N, 21°16′E	2	*Tvv*	F	F6, F9	IS
63	Greoni	45°05′N, 21°07′E	2	*Tvv*	F, L3	F7, L41	IS
64	Scroviştea	44°40′N, 25°58′E	3	*Tvv*	G	G2, G3, G6	DC
65	Băneasa	44°29′N, 26°03′E	5	*Tvv*	G	G4(2), G5, G6(2)	DC
	Slovenia						
66	Podstrmec	45°48′N, 14°34′E	4	*Tvm*	L1	L2, L3, L4(2)	JWA
	Serbia						
67	Mesic	45°06′N, 21°23′E	1	*Tvv*	L3	L42	JCI
68	Kusić	44°53′N, 21°28′E	1	*Tvv*	F	F8	JCI
69	Jamena	44°52′N, 19°04′E	2	*Tvv*	L1	L17	JCI
70	Glušci	44°52′N, 19°33′E	1	*Tvv*	L3	L18	JCI
71	Beograd	44°50′N, 20°30′E	2	*Tvv*	L3	L48, L49	JCI
72	Ivanovo	44°44′N, 20°43′E	1	*Tvv*	L3	L47	JCI
73	Trešnja	44°36′N, 20°34′E	6	*Tvv*	L1, L3	L19, L20, L52, L53, L54(2)	JCI
74	Djurinci	44°30′N, 20°39′E	1	*Tvv*	L1	L5	JCI
75	Arandjelovac	44°17′N, 20°40′E	2	*Tvv*	L1	L21, L24	JCI
76	Milanovac	44°12′N, 21°36′E	2	*Tvv*	L3	L45, L46	JCI
77	Valjevo	44°12′N, 20°00′E	2	*Tvv*	L1	L6, L13	JCI
78	Grivac	44°00′N, 20°41′E	1	*Tvv*	L3	L43	JCI
79	Gornja Sabanta	43°57′N, 21°00′E	2	*Tvv*	L1	L25, L51	JCI
80	Karan	43°53′N, 19°56′E	2	*Tvv*	L1	L22, L23	JCI
81	Guberevac	43°51′N, 20°46′E	2	*Tvv*	L1	L15, L26	JCI
82	Rtanj	43°47′N, 21°56′E	2	*Tvv*	D, L1	D7, L1	JCI
83	Dimitrovgrad	43°03′N, 22°50′E	1	*Tvv*	D	D3	JCI
84	Vlasi	43°00′N, 22°38′E	2	*Tvv*	D	D3(2)	JCI
85	Bjelasica	42°54′N, 19°38′E	1	*Tvv*	L1	L14	JCI
86	Bosilegrad Mlekominci	42°27′N, 22°30′E	1	*Tvv*	D	D4	JCI
87	Moravica	42°26′N, 21°45′E	1	*Tvv*	D	D6	JCI
88	Bosilegrad Zli Dol	42°25′N, 22°27′E	2	*Tvv*	D	D5(2)	JCI
	Russia						
89	Kaluzhskaya	44°46′N, 38°58′E	2	*Tvl*	A	A6, A7	A. Yanchukov
90	Goryachiy Klyuch	44°37′N, 39°07′E	2	*Tvl*	A	A4, A5	A. Yanchukov
	Italy						
91	Vernio	44°02′N, 11°09′E	3	*Tvm*	H	H4(2), H5	S. Vanni
92	Pisa	43°43′N, 10°24′E	5	*Tvm*	H	H1(2), H2(2), H3	M. Ragghianti
	Bosnia and Herzegovina						
93	Podromanija	43°55′N, 18°46′E	1	*Tvv*	L1	L16	JWA
	Croatia						
94	Zljebi	42°28′N, 18°38′E	3	*Tvg*	D	D1, D2(2)	JWA
	Georgia						
95	Bakuriani	41°44′N, 43°32′E	4	*Tvl*	A	A1, A2(2), A3	D. Tarkhnishvili
	Greece						
96	Vátos	39°36′N, 19°48′E	2	*Tvg*	C	C1, C2	M. Sandera
97	Aetos	40°39′N, 21°29′E	2	*Tvg*	K	K1	B. Trapp
	Turkey						
98	Adapazari	40°47′N, 30°24′E	5	*Tvk*	B	B1, B2(2), B3, B4	KO
99	Bolayir	40°31′N, 26°46′E	5	*Tvs*	E	E3, E9(3), E10	KO
100	Gemlik	40°26′N, 29°09′E	5	*Tvs*	E	E1(2), E2(3)	KO
101	Lapseki	40°21′N, 26°41′E	5	*Tvs*	E	E8, E11, E12, E13, E14	KO
102	Bozdağ	38°20′N, 28°02′E	5	*Tvs*	E	E5(4), E6	KO
103	Efes	37°55′N, 27°20′E	5	*Tvs*	E	E4, E5(2), E7(2)	KO

Localities are arranged by countries and within countries in the north–south order; *n*, number of individuals sequenced. For each locality species and subspecies assignation (Ssp), clade associations as well as complete list of haplotypes are given; species and subspecies codes: *Tvv*, *Triturus v. vulgaris*; *Tva*, *T. v. ampelensis*; *Tvg*, *T. v. graecus*; *Tvk*, *T. v. kosswigi*; *Tvl*, *T. v. lantzi*; *Tvm*, *T. v. meridionalis*; *Tvs*, *Triturus vulgaris schmidtlerorum*; *Tm*, *Triturus montandoni*; h, hybrid; as hybrids we classified only individuals revealed as such by the genotypes at nuclear markers (Babik *et al.* 2003); in parentheses number of individuals with a given haplotype if larger than one. Names of collectors who are also authors are given as initials.

spanning the ranges of the currently recognized subspecies of *T. vulgaris* (Fig. 1; Raxworthy 1990; Schmidtler & Frantzen 2004) and the entire range of *T. montandoni*. As outgroups we used *Triturus boscai*, *Triturus helveticus* and *Triturus italicus*, the closest relatives of the *vulgaris/montandoni* clade (Rafiński & Arntzen 1987; Zajc & Arntzen 1999). The more distantly related *Triturus alpestris* was added to the divergence time analysis in order to increase genealogical depth and to strengthen the molecular dating of divergence times (see below).

DNA from frozen or alcohol-preserved tissues was extracted using a standard proteinase K–phenol–chloroform method or the DNeasy Tissue Kit (QIAGEN). Two mtDNA fragments were amplified and sequenced for all samples: a *c*. 1016-bp fragment comprising 951 bp of the ND2 gene and almost the entire tRNA-Trp gene (further referred to as 'ND2'), and *c*. 835-bp fragment covering 683 bp of the ND4 gene, the whole tRNA-His and tRNA-Ser genes and 15 bp from the 5′ end of the tRNA-Leu gene (further referred to as 'ND4'). For most ingroup individuals each fragment was amplified in one piece using primers L3870-H5018 for ND2 and ND4-Leu (Arévalo *et al*. 1994) for ND4. Several museum samples that gave poor quality DNA were amplified in two or three shorter fragments with primer pairs L3870-H4421, L3975-H4421, L4302-H5018, L4339-H4718 and L4600-H5018. For all three outgroups the ND2 fragment was amplified using primers L3780-H5018; the ND4 fragment in *T. boscai* and *T. italicus* was amplified with primers ND4-Leu, whereas in *T. helveticus* it was amplified in two pieces using primers L10377-H11513 and L11014-H11687. The sequences of all primers are given in Table 2.

Thirty-microlitre PCRs contained 3 μL of 10 × PCR buffer with $(NH_4)_2SO_4$ (Fermentas), 2.5 mM $MgCl_2$, 1 μM of the forward and reverse primers, 0.2 mM of each dNTP and 0.5 U of *Taq* polymerase (Fermentas). The cycling scheme was as follows: 94 °C for 2min, 56 °C for 45 s, 72 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 56 °C for 45 s, 72 °C for 45–90 s (depending on the expected product size) and a final extension step at 72 °C for 3 min. The PCR product was purified with the Wizard PCR Preps DNA Purification System (Promega), or Clean-Up columns (A & A Biotechnology) and sequenced using the BigDye Terminator Kit; the sequencing reaction products were purified using ExTerminator columns (A & A Biotechnology), and run on an ABI 3100 genetic analyser (Applied Biosystems). Sequences were checked by eye and aligned manually in BIOEDIT 5.0.9 (Hall 1999). Sites with indels were excluded from further analyses. To test if the amplified fragments represented mitochondrial genes and not nuclear pseudogenes, we compared sequences obtained from mtDNA extracted from mitochondria of two individuals, purified by centrifugation at the sucrose gradient, with sequences from total genomic DNA.

Table 2 Primers used for amplification and sequencing of fragments of ND2 and ND4 genes in mtDNA. Primers ND4 and Leu were from Arévalo *et al*. (1994), the remaining primes were developed for the present study

Primer	Sequence (5′–3′)
	ND2
L3780	TCGAACCTACCCTGAGGAGAT
L3870	CCCCAAATATGTTGGTGGAA
L3975	CCATCACTGGTTTTTAGCATGA
L4302	CAAAAACTAGCCCCAATAGCA
L4339	CCAGCCATCAGCTAAACACAAACC
L4600	TCATGATTAAAAACACCAACGC
H4421	AGGTGTGCAATGGATGAGTATG
H4718	GCGGATATGGCTATTAAAGCAG
H5018	TCTGGGTTGCATTCAGAAGA
	ND4
ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC
Leu	CATTACTTTTACTTGGATTTGCACCA
L10377	ACATGCTGACTGCTTCCACTAA
L11014	ACCTGAAGTCCCTAAACGCATA
H11513	CACACATGAGAATAGCCCAGAG
H11687	TTTGGTTCCTAAAACCAATGGA

Phylogenetic analyses

The phylogenetic congruence of the ND2 and ND4 data sets was tested using the partition-homogeneity test (Farris *et al*. 1994) with 100 replicates as implemented in PAUP 4.10b (Swofford 2002). As the result of the test was not significant, further analyses were performed on the combined data. Using MODELTEST 3.5 (Posada & Crandall 1998) which tests the relative fit of 56 models of sequence evolution to the data, we determined the most appropriate model of nucleotide substitution. Following both the likelihood-ratio test and Akaike information criterion, this was the Tamura–Nei model with rate heterogeneity and a nonzero proportion of invariant sites (TrN + Γ + *I*) with the following parameter settings: nucleotide frequencies 0.339 (A), 0.263 (C), 0.116 (G), 0.282 (T), gamma shape parameter α = 1.10, proportion of invariant sites *I* = 0.42. A maximum-likelihood (ML) tree with MODELTEST-derived parameters was constructed with the PHYML program using the method of Guindon & 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. Another tree was constructed using the Bayesian approach with MRBAYES 3.1 (Ronquist & Huelsenbeck 2003) under the general time reversible model of sequence evolution

(GTR + Γ + *I*), an approximation to the TrN model supported in MRBAYES; priors were set to default values. Four Metropolis coupled Monte Carlo Markov chains (three of them 'heated', temperature = 0.20) were run for 2×10^7 generations and sampled every 1000 generations. The first half of the 'forest' was discarded as burn-in, resulting in 10^4 sampled trees. This analysis was repeated five times with shorter runs over 2×10^6 generations to evaluate the congruence of the likelihood values. All analyses started with trees that were randomly generated. Log-likelihood values were plotted against generation time, which ensured that no trees were retained prior to the run reaching stationariness. To calculate the posterior probability of each bipartition, the majority-rule consensus tree was constructed over 11 000 trees, representing the two sets of trees (the long and one of short runs) that had converged best in terms of tree topology.

For several recognized clades and for the outgroup sequences we computed nucleotide diversities (π) and net sequence divergences (*Da*) using Kimura 2-parameter model with MEGA2.1 (Kumar *et al*. 2001). Standard errors were obtained through 1000 bootstrap replicates.

Dating times of divergence

We used the phylogram resulting from the maximum-likelihood analysis as the preferred tree topology for the estimation of divergence times. To scale branch lengths over the tree topology, a semiparametric method was used with the software R8S (Sanderson 2002, 2003). 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. The analyses with R8S were performed using the PL method and the truncated-Newton algorithm, for computational reasons, on a reduced data set of 115 haplotypes, representing all major clades. Calibration points were derived from a molecular phylogenetic study on the entire Salamandridae family (Steinfartz, Caccone & Arntzen, in preparation), which, on the basis of such palaeogeographic events as Europe–North America and Sardinia–Corsica splits, estimated the divergence times of several outgroup taxa relative to the ingroup at 34 Myr (point I in Fig. 3), 22 (II), 21 (III) and 20 Myr (IV) (Fig. 3). Empirical 95% confidence intervals to the temporal estimates were obtained from estimating branch lengths in 200 bootstrapped data sets, keeping 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).

Demographic analysis

The demographic history of the clades with at least 20 sequences available was assessed with two complementary approaches. ML-based estimators of theta (θ_{ML}, $\theta = 2N_f\mu$ for mitochondrial genes, where N_f is the female effective population size and μ is mutation rate) and exponential population growth parameters (g) were computed jointly with FLUCTUATE 1.4 (Kuhner *et al*. 1998). This coalescent-based method takes into account genealogical relationships among haplotypes. The estimates of θ and g are obtained by Monte Carlo Markov chain searches through the genealogy space. The transition/transversion ratio was set to 9, the rate of growth-parameter change to 0.01, the Watterson (1975) estimator was used as a starting value of θ, and an UPGMA tree constructed from Kimura 2-parameter distances as a starting genealogy. We ran FLUCTUATE several times with different numbers of short and long chains to ensure consistency of the estimates. The final estimates were based on a run of five short chains of 5000 steps each and two long chains of 50 000 steps; the sampling increment was set to 20. The estimates of g are biased upwards (Kuhner *et al*. 1998); therefore, following Lessa *et al*. (2003), a conservative approach in testing for significance was adopted, with values larger than three standard deviations (SD) of g regarded as significant.

The second approach was a mismatch-distribution analysis, following Schneider & Excoffier (1999) with ARLEQUIN 2.001 (Schneider *et al*. 2000). Mismatch analysis was shown to perform well in cases of population subdivision and when the demographic history of the populations involved is more complex than a simple model of sudden expansion (Rogers 1995). Both of these factors are likely to occur in any real data set, including ours. Goodness-of-fit to the sudden-expansion model was tested using a parametric bootstrap approach (10 000 replicates). We report the values of θ_0, θ_1 and τ. θ_0 and θ_1 are the estimators of theta before and after expansion, and τ is the time since expansion, measured in mutational time units. These values were estimated using generalized nonlinear least squares. Their approximate confidence intervals were obtained through parametric bootstrapping (10 000 replicates). It should be noted that confidence intervals for θs obtained this way are overly large and thus conservative (Schneider & Excoffier 1999).

Results

Two hundred different haplotypes were identified among 285 ingroup sequences (GenBank Accession nos for ND2: AY951337–501, for ND4: AY951508–647). Two individuals from Bosilegrad Zli Dol, Serbia (locality 88) possessed identical sequences, including a complex insertion involving 8 bp at the 3′ end of the ND2 gene that caused a frameshift. This was not entirely unexpected, as length variation is

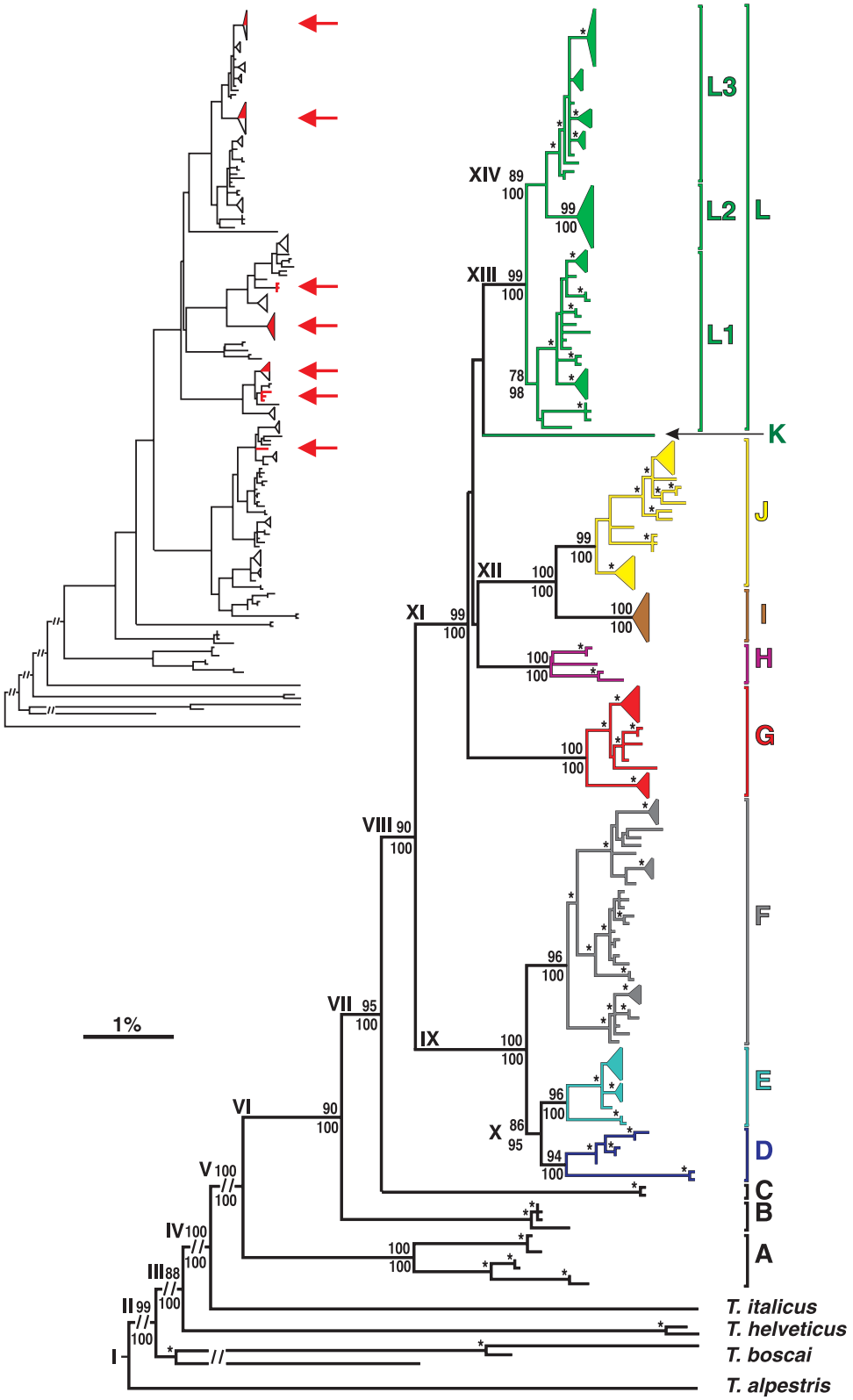

Fig. 3 Maximum-likelihood (PHYML) phylogenetic tree of *Triturus vulgaris* and *T. montandoni* haplotypes. Bootstrap values ≥ 70% for the major nodes are shown above branches, Bayesian posterior probabilities for these nodes are given below branches. The remaining nodes with bootstrap values and posterior probabilities ≥ 70% are indicated with asterisks. Several groups of closely related haplotypes are represented by triangles, their height corresponding to the number of haplotypes. The tree was rooted using *Triturus boscai*, *Triturus helveticus* and *Triturus italicus*. *Triturus alpestris* was added to the tree to show the first calibration point. Roman numbers refer to the major splits, their estimated times of divergence are given in Table 4. Inset shows the phylogenetic distribution of the haplotypes found in *T. montandoni* (red).

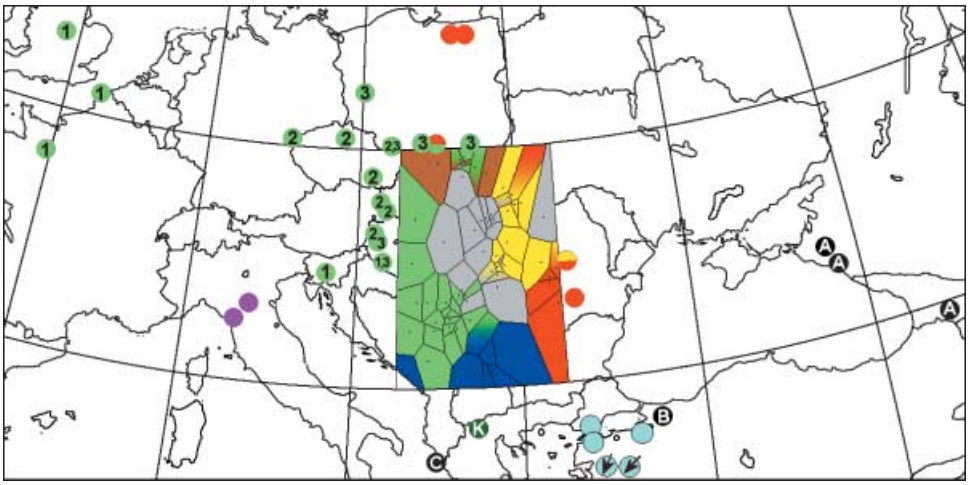

Fig. 4 Eurasian distribution of mitochondrial haplotypes observed in the newts *Triturus vulgaris* and *Triturus montandoni*. Haplotypes are organized in lineages (see Fig. 3) that are represented by the following colours: lineages A, B and C: black; lineage D: dark blue; lineage E: light blue; lineage F: grey; lineage G: red; lineage H: purple; lineage I: brown; lineage J: yellow; lineage K: dark green; lineages L1, L2 and L3: light green. In central Europe, where sampling was dense (between 42° and 50°N and 18° and 26°E) the distribution of mtDNA lineages is extrapolated through the use of Dirichlet-cells. Multicoloured cells and dots refer to localities with two or three haplotypes in the population.

regularly observed at the 3′ end of the ND2 gene in Salamandridae (Weisrock *et al*. 2001). Also, four 1-bp indels were found in tRNA-coding parts. Excluding positions with indels, 1849 bp was obtained for all ingroup individuals. Five hundred and five (27.3%) nucleotide positions were variable and 389 (21.0%) were parsimony informative. Among 1629 sites in coding regions, 481 (30.2%) were variable with 113 (23.5%) in the 1st, 46 (9.6%) in the 2nd and 322 (66.9%) in the 3rd codon position. Ninety seven of 543 (17.9%) amino acid sites were variable. Sequences of both fragments amplified from genomic DNA and from purified mtDNA were identical.

All six outgroup sequences represented unique haplotypes (GenBank Accession nos for ND2: AY951502–507, for ND4: AY951648–53). A 6-bp deletion occurred at the 3′ end of the ND2 gene in both *Triturus helveticus* sequences, whereas two deletions, of 1 and 5 bp were encountered in this region in two of three *Triturus boscai* sequences, involving the stop codon. Two more 1-bp indels occurred in the *T. helveticus* tRNA-His gene, and one 1-bp indel in the *Triturus italicus* tRNA-Trp gene. Excluding indels, the complete alignment including outgroups comprised 1836 bp; 773 (42.1%) of these were variable and 640 (35.2%) parsimony informative.

Phylogenetic analysis and geographic distribution of phylogroups

Both methods used for inferring phylogeny gave virtually identical clustering patterns with several distinct clades and partially resolved relationships among them (Fig. 3). The distribution of the clades shows a clear geographic pattern (Fig. 4). The oldest clade (A) includes all individuals of *T. v. lantzi*, a subspecies from the Caucasus region. This clade itself is deeply differentiated into two branches — north and south of the Caucasus (*Da* = 2.09%). Another ancient clade (B) represents *T. v. kosswigi*. The next successive branch (clade C) comprises *T. v. graecus* from the island of Corfu. The remaining haplotypes form two groups; one of them consists of clades D, E and F, with D and E being sister groups; the second monophyletic unit includes six clades (G–L) with poorly resolved relationships. The G clade includes both *Triturus vulgaris* and also Romanian *Triturus montandoni*. Clade I accommodates *T. montandoni* from the northern part of the species range, mainly from Poland. The distribution of its sister clade J is centred in Transylvania, but J-group haplotypes were also found in *T. montandoni* from the Ukrainian Carpathians. Clade L has by far the widest geographic distribution, encompassing a part of central and western Europe (Fig. 4), and is divided into three subclades (Figs 3 and 4).

In general, the distribution of clades is allo- or parapatric. We identified four regions where several clades meet, with haplotypes from divergent groups present in one locality (Fig. 4). These regions are (i) Serbia (ii) eastern Transylvania (iii) western Hungary and (iv) the Ukrainian Carpathians.

Haplotypes found in *T. montandoni* do not form a monophyletic unit but were found in six branches (F, G, I, J, L2 and L3) intermingled with *T. vulgaris* haplotypes. Only the northern *T. montandoni* haplotypes form a well-supported monophyletic group (clade I). The most diverse *T. montandoni* populations, in terms of mtDNA, were those in the Ukrainian Carpathians, with haplotypes belonging to

Table 3 Below diagonal: net sequence divergence (*Da*) between clades, based on Kimura 2-parameter distance; above diagonal: standard errors of the estimates (1000 bootstrap replicates). Nucleotide diversities (π) within groups on the diagonal, with standard errors (1000 bootstrap replicates) in parentheses. Codes correspond to clades identified in the phylogenetic analyses (Fig. 3). All values are expressed as percentages

	L1	L2	L3	K	J	I	H	G	F	E	D	C	B	A
L1	0.29 (0.07)	0.20	0.19	0.39	0.35	0.42	0.31	0.36	0.38	0.40	0.40	0.45	0.46	0.49
L2	0.70	0.16 (0.04)	0.20	0.38	0.23	0.42	0.33	0.40	0.40	0.42	0.42	0.50	0.49	0.47
L3	0.83	0.89	0.58 (0.09)	0.35	0.33	0.41	0.32	0.36	0.37	0.37	0.38	0.46	0.45	0.47
K	2.77	2.72	2.48	0 (0)	0.43	0.47	0.41	0.48	0.47	0.48	0.45	0.56	0.51	0.53
J	2.30	2.46	2.04	2.99	0.90 (0.13)	0.27	0.32	0.39	0.38	0.42	0.39	0.44	0.46	0.45
I	2.68	2.89	2.47	3.43	1.43	0.20 (0.05)	0.40	0.44	0.46	0.48	0.46	0.51	0.52	0.50
H	1.96	2.10	1.77	2.88	2.10	2.47	0.77 (0.14)	0.36	0.36	0.38	0.36	0.46	0.45	0.45
G	2.40	2.67	2.34	3.32	2.72	3.10	2.31	0.67 (0.11)	0.42	0.45	0.44	0.52	0.49	0.53
F	3.20	3.41	3.07	4.01	3.44	3.64	3.16	3.50	1.11 (0.13)	0.23	0.21	0.48	0.47	0.46
E	3.10	3.39	3.09	4.04	3.65	3.92	3.21	3.54	1.27	0.54 (0.10)	0.21	0.52	0.47	0.50
D	3.22	3.39	3.06	3.99	3.50	3.68	3.08	3.61	1.20	1.03	0.99 (0.15)	0.50	0.46	0.48
C	4.28	4.60	4.15	5.25	4.54	4.80	4.26	4.81	4.18	4.57	4.48	0.11 (0.09)	0.52	0.53
B	4.19	4.39	3.80	4.68	4.16	4.46	4.09	4.67	4.35	4.44	4.19	4.83	0.25 (0.07)	0.51
A	4.80	4.55	4.52	5.36	4.57	5.18	4.45	5.09	4.87	5.01	4.89	5.86	4.95	1.70 (0.21)

four clades (F, G, I, and J). The isolated *T. montandoni* population of Jeseniki Mountains at the western margin of the species range (no. 23, $n = 9$, see Table 1 and Figs 2 and 4), had only haplotypes from the *vulgaris* subclades L2 and L3.

Nucleotide diversity (π) within clades varied from 0.00 (clade K) to 1.70% (clade A). Restricted to clades with at least 10 sequences, π fell between 0.16 (clade L2) and 1.11% (clade F) (Table 3). Net sequence divergences (*Da*) ranged from 0.70 (between clades L2 and L3) to 5.86% (between clades A and C). *Da* between the outgroup species and *T. vulgaris*/*montandoni* clades ranged from 15.33% to 17.14% in comparisons with *T. italicus*, 17.45–18.33% with *T. boscai* and 20.43–21.78% with *T. helveticus*.

Dating the time of divergence

Dating of major mtDNA cladogenic events within the *T. vulgaris*/*montandoni* group is presented in Table 4 (see also Fig. 3). The divergence of clades A–C and a major split between clades D–F and G–L are of Pliocene origin, whereas most of the other splits are placed in the early or middle Pleistocene.

Demographic analyses

The results of the coalescent-based θ_{ML} and *g* analyses as well as mismatch analysis are given in Table 5. As large sample sizes for subclades L1–L3 were available, these were included as separate units. The highest value of $\theta_{ML} = 7.109$ was obtained for the L2 subclade, for which also the highest growth rate was estimated. Both values were also high for L3. Moderate or no growth was inferred in clades F, G, J and L1, with θs of the same order of magnitude except for clade F ($\theta_{ML} = 4.83$). For Turkish samples (clade E), θ_{ML} had the lowest (= 0.617) value, with no significant growth.

The results of the mismatch analysis resembled those for the coalescence-based analysis (Table 5, Fig. 5). The sudden expansion model was not rejected at $\alpha = 0.05$ in any of the clades; however, it is evident that in most clades the shapes of the mismatch distributions are far from unimodal (Fig. 5). The mismatch distribution is clearly unimodal only in the L2 subclade, implying recent ($\tau = 2.20$ mutational units) demographic growth. The θ_1 value, corresponding to theta after expansion, was much lower than θ_{ML}. In

Table 4 Dating of cladogenic events within the *Triturus vulgaris*—*T. montandoni* radiation with empirical 95% confidence intervals in parentheses

Cladogenic event (see Fig. 3)	Lineages involved	Age in Myr
I	*T. alpestris* vs. #	[34]
II	*T. boscai* vs. #	[22]
III	*T. helveticus* vs. #	[21]
IV	*T. italicus* vs. #	[20]
V	A vs. #	4.5 (3.6–5.2)
VI	B vs. #	3.4 (2.8–3.8)
VII	C vs. #	3.0 (2.4–3.3)
VIII	D–F vs. G–L	2.6 (2.1–3.0)
IX	D–E vs. F	1.3 (1.0–1.5)
X	D vs. E	1.2 (0.9–1.3)
XI multifurcation	G vs. H vs. I, J vs. K vs. L	1.9 (1.5–2.1)
XII	I vs. J	1.0 (0.7–1.2)
XIII	L1 vs. L2, L3	0.75 (0.49–0.97)
XIV	L2 vs. L3	0.50 (0.27–0.66)

Capital letters refer to clades as in Fig. 3; # represents the set of remaining taxa. Calibration points I–IV with time estimates in brackets are derived from a molecular phylogenetic study on the family Salamandridae (Steinfartz, Caccone & Arntzen, in preparation).

subclade L3, the shape of the mismatch distribution is closer to bimodal, with the higher mode centred around seven pairwise differences, suggesting an older episode of growth (τ = 7.47 mutational units). Again, for this subclade θ_1 was substantially lower than the ML estimate. For the remaining clades the results of mismatch and coalescent-based analyses are concordant, with similar θ_1 and θ_{ML} values. Shapes of the mismatch distributions are multimodal (E, G, L1), or, if closer to unimodal (F and J), the mode is shifted to the right and, accordingly, times of demographic expansions for those clades are much older than for L2 and L3. Overlapping 95% confidence intervals for θ_0 and θ_1 in clades E and G indicate the absence of demographic expansion in these groups. The *g* values were also the lowest and nonsignificant in these clades.

Formal demographic analyses were not performed for the remaining clades, but in four of these (A, B, D, and H), all inhabiting the southern part of the range of *T. vulgaris*, the divergences among haplotypes were high, even within single populations (Fig. 3 and Table 3). In contrast, clade I, grouping the haplotypes found in *T. montandoni* from the northern part of its range, shows a star-like phylogeny.

Discussion

Time of divergence of the major clades

The origin of several mtDNA *Triturus vulgaris* clades dates back to the Pliocene. This is the case for the extreme southern and eastern clades A–C which are over 3 Myr old. Also, the split between the overlapping central and south-eastern European group of clades D–F vs. the southern, central and northwestern European group of clades G–L, is placed in the Pliocene, at *c.* 2.6 Myr. Thus, the substantial

Table 5 The results of demographic analyses for clades with more than 20 sequences

Group	*n*	θ_{ML} (SD)	*g* (SD)	P_{sudden}	*N* (95% CI)	τ (95% CI)	θ_0 (95% CI)	θ_1 (95% CI)
E	25	0.617 (0.110)	85.6 (102.4)	0.160	10.353 (3.85–22.61)	3.60 (0.56–23.56)	0.540 (0.000–1.127)	0.695 (0.297–37.615)
F	40	4.828 (0.608)	434.6 (63.6)*	0.121	20.17 (17.05–25.66)	25.26 (18.36–30.02)	0.00 (0.000–0.299)	4.654 (2.838–204.006)
G	32	1.178 (0.176)	213.9 (89.6)	0.737	12.14 (6.74–18.86)	21.69 (12.05–35.63)	0.000 (0.000–0.737)	1.070 (0.507–5.527)
J	38	2.955 (0.424)	374.6 (69.2)*	0.095	16.99 (13.54–22.84)	22.865 (15.28–28.77)	0.000 (0.000–0.287)	2.970 (1.703–43.362)
L1	33	2.797 (0.435)	575.1 (103.5)*	0.302	11.13 (7.31–19.50)	8.49 (3.62–22.36)	0.299 (0.000–1.077)	2.236 (1.208–46.267)
L2	21	7.109 (3.114)	4395.6 (912.3)*	0.734	3.10 (1.49–5.33)	2.20 (0.82–6.34)	0.057 (0.000–0.175)	1.355 (0.292–380.070)
L3	46	4.773 (0.855)	2715.5 (293.9)*	0.169	5.42 (3.70–8.67)	7.47 (3.47–11.28)	0.000 (0.000–0.156)	0.949 (0.554–126.180)

n, number of sequences; θ_{ML}, the coalescent-based estimator of theta; *g*, the exponential growth rate; asterisks denote significant *g*-values; P_{sudden}, probability, based on 10 000 bootstrap replicates, that the mismatch distribution conforms to the model of sudden expansion; *N*, mean number of pairwise differences; τ, time since expansion measured in mutational time units ($\tau = \mu t$, where μ is mutation rate per locus per generation and *t* is the number of generations); θ_0 and θ_1 are theta values before and after expansion, respectively. All θ-values are given as percentages per site.

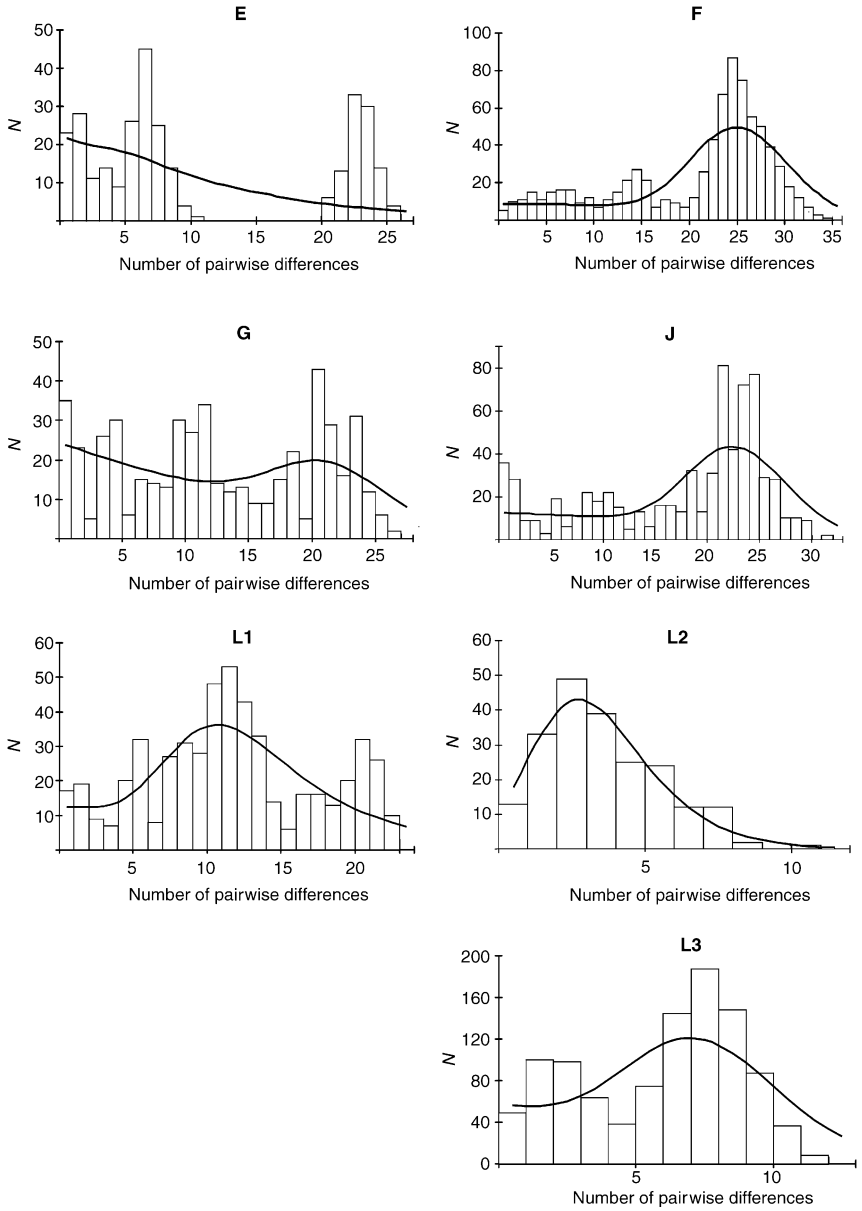

Fig. 5 Mismatch distributions in the clades with at least 20 sequences. Unimodal distributions of the pairwise differences most pronounced in the clades F, L2, and L3 indicate demographic expansion, with the location of the peak indicating the time of expansion. Multimodal, or 'ragged' distributions, clearly visible in the clades E and G suggest long-term demographic stability, Black curves show shapes of theoretical distributions according to the sudden expansion model.

fraction of intraspecific genetic differentiation predates the onset of Pleistocene glaciations. This pattern is repeatedly found in temperate urodeles (Tan & Wake 1995; Tarkhnishvili *et al*. 2000; Riberon *et al*. 2001; Weisrock *et al*. 2001) and hence may indicate that climatic changes coupled to glaciations were not a major trigger of speciation in this group. Comparative data of Avise *et al*. (1998) also show that speciation in amphibians is generally a longer process than in other vertebrate groups.

On the other hand, the origin of several younger mtDNA clades seems to be linked to the Pleistocene glaciations. The clades G–L emerged in a short time span *c*. 1.9 Ma that coincides with the early Pleistocene. The splits within the essentially northwestern European clade L into subclades L1, L2 and L3 occurred at *c*. 0.75 Ma and *c*. 0.50 Ma, respectively (Table 4), and may have been related to the changes in the climatic regime marked by a dominant role of climatic oscillations of 100 kyr periodicity and increased amplitude (Webb & Bartlein 1992; EPICA community members 2004).

Phylogeographic pattern and glacial refugia

The present distribution of mtDNA clades points to the existence of multiple glacial refugia. In the southern part of the range, seven clades (A–E, H, K) with localized distributions suggest differentiation *in situ* and long-term existence of newts in these areas. Such differentiation was

likely promoted by climatic changes during, or even predating, the Pleistocene (see previous section) and could have been facilitated by the complex geographic structure of Italy, the southern Balkans, Anatolia and the Caucasus with mountain chains acting as effective barriers to dispersal. Extensive sampling in southern areas, e.g. Greece, Albania, Bulgaria, would probably reveal additional ancient mtDNA lineages. These results agree with the view of the Mediterranean as an important centre of European biodiversity and endemism but not necessarily the main source of populations colonizing deglaciated areas after the last glacial maximum (LGM) (Bilton *et al*. 1998; Petit *et al*. 2003).

High mtDNA diversity was also found further to the north. Romania houses three major, parapatrically distributed clades (F, G, J). Of these, clade J was found almost exclusively in Transylvania, in an area broadly corresponding to the distribution of *T. v. ampelensis* (Schmidtler & Frantzen 2004). This group probably survived several glacial cycles *in situ*, which is corroborated by allozyme data confirming the distinctiveness of *T. v. ampelensis* (Rafiński *et al*. 2001). Its sister clade, I, is represented by the haplotypes found in *Triturus montandoni* from Poland and the Ukrainian Carpathians. This northern group of *T. montandoni* most likely survived glaciations in some part of its present range.

Two subclades were distinguished in the F group (Fig. 3). One of these occupies a small area around the Iron Gate, i.e. in southwestern Romania and in adjacent Serbia. The range of the second subclade is much larger and encompasses western Romania, eastern Hungary, and eastern Slovakia. The deep split between these two subclades, dated at *c*. 0.8 Myr pre-dates the last glacial cycle, suggesting separate refugia through several glacial cycles. The G group is found both in *Triturus vulgaris* south of the eastern Carpathians and in Romanian *T. montandoni*. Its haplotypes were also found in eastern Poland and in Russia (E. Karvonen, unpublished). Thus, identification of glacial refugia for the G lineage is not straightforward, although southern Romania seems a reasonable candidate. Three observations support this notion. First, the relatively high haplotype diversity found there. Second, the presence of the same haplotypes in *T. montandoni*, which most likely had a refugium in the eastern Carpathians. Third, southern Romania is also a postulated refugium for the newt *T. cristatus* (Wallis & Arntzen 1989), which has an overall distribution similar to that of *T. v. vulgaris*. We hypothesize that the introgression of the G-group haplotypes into *T. montandoni* occurred during its coexistence with *T. vulgaris* throughout the last glacial period (see below).

Our data support the emerging view of the Carpathians and Carpathian Basin as important refugial areas (Willis & van Andel 2004). This hypothesis stems from studies of fossil remains of several tree species (Willis *et al*. 2000), palaeoclimate reconstructions based on malacological data (Sümegi & Krolopp 2002), and the patterns of genetic variation in various animal and plant species (e.g. Lagercrantz & Ryman 1990; Schmitt & Seitz 2001; Stewart & Lister 2001; Jaarola & Searle 2002; Brunhoff *et al*. 2003; Babik *et al*. 2004).

The L lineage has a broad geographic distribution; it now inhabits western and most of central Europe. Its centre of differentiation was probably located in Serbia as evidenced by the high diversity in this area. This applies specifically to the L1 and L3 lineages found in western and central Europe, respectively. The L2 lineage occurs only in central Europe, and its refugium during the LGM was probably located in the southwestern part of the Carpathian Basin.

The phylogeographic pattern, considered together with our time estimates, suggests that the Pleistocene glaciations did not result into completed speciation, but played a major role in generating substantial genetic diversity and the structuring of the available variation in the newts in question, likely through the allopatric fragmentation across multiple glacial refugia.

Mitochondrial phylogeny vs. morphology-based subspecific and specific boundaries

The two oldest lineages correspond to the two southern subspecies *T. v. lantzi* and *T. v. kosswigii*. However, such agreement is the exception rather than the rule in the *T. vulgaris/montandoni* clade. In *T. v. graecus*, inhabiting the southern Balkans, we found three distantly related mtDNA lineages (Figs 3 and 4). Two of them cluster with haplotypes found in the nominotypical subspecies. Haplotypes from the J group predominate in populations from the area inhabited by *T. v. ampelensis*, although the F haplotypes may also be present in the same populations. Samples from Italy form a distinct clade representing *T. v. meridionalis*. However, in Slovenia, where on the basis of morphology and allozymes this subspecies also occurs (Schmidtler & Schmidtler 1983; Kalezić 1984; Schmidtler & Frantzen 2004), we found sequences belonging to the L1 subclade related to a haplotype from Serbia. The range of clade E coincides with the range of *T. v. schmidtlerorum* (Schmidtler & Franzen 2004). The nominotypical subspecies, *T. v. vulgaris*, represented by clades D, F, G, J, K and L, is paraphyletic with respect to *T. v. ampelensis*, *T. v. meridionalis*, *T. v. schmidtlerorum*, *T. montandoni* (see below) and partly to *T. v. graecus*.

If mtDNA phylogeny indeed correctly describes relationships within *T. vulgaris*, it has to be assumed that certain male characteristics such as the tail filament, dorsal ridges and straight (nondenticulate) dorsal crest evolved independently several times. This is not unlikely as recent studies repeatedly show that in Salamandridae, similarities in the courtship behaviour and associated morphological traits often evolve independently, most likely through the action of sexual selection and, in consequence, are poor

predictors of phylogenetic relationships (Titus & Larson 1995; Veith *et al*. 1998; see also Stuart *et al*. 2002).

Alternatively, if the morphological traits reflect true relationships, this pattern of mtDNA variation could result from extensive introgression of the mitochondrial genomes. In *T. vulgaris* from former Yugoslavia, several transition zones between subspecies were described from allozymic and morphological variation (Schmidtler & Schmidtler 1983; Kalezić 1984). A population intermediate in allozyme frequencies between *T. v. ampelensis* and the nominal subspecies was reported from Romania by Rafiński *et al*. (2001). Also, a transition zone between *T. v. kosswigi* and *T. v. vulgaris* was suggested in northwestern Turkey (Freytag 1957). We do not expect a priori that transitional zones at nuclear and cytoplasmic markers would coincide (Funk & Omland 2003; Garcia-París *et al*. 2003; Sequeira *et al*. 2005).

These two hypotheses are not mutually exclusive, and more insights into the causes of the observed discordance may be gained from nuclear genealogies. Male sexual traits may be encoded by only a small fraction of the genome (True *et al*. 1997; Orr 2001; Kopp *et al*. 2003; Presgraves *et al*. 2003). If so, and if these traits evolved independently due to the action of sexual selection, phylogenetic reconstructions based on neutrally evolving nuclear sequences should demonstrate independent evolution, although, due to inherent stochasticity in gene trees constructed from unlinked markers, not necessarily exhibiting the same relationships as cytoplasmic markers.

One of the most striking results of this study is the paraphyly of *T. vulgaris* with respect to *T. montandoni* and the polyphyly of the latter species in the mtDNA-based phylogeny. *Triturus montandoni* is distinguished from *T. vulgaris* in gross morphology (Zavadil *et al*. 2003), morphometric traits (Rafiński & Pecio 1989; Babik & Rafiński 2004), allozymes (Rafiński & Arntzen 1987), chromosomes (Zbożeń 1997) and courtship behaviour (Pecio & Rafiński 1985). Both species also show a marked although incomplete behavioural sexual isolation (Michalak *et al*. 1997; Michalak & Rafiński 1999). The majority of mtDNA haplotypes found in *T. montandoni* belong to three clades: I, present in the northern part of species range, J, detected in the Ukrainian Carpathians, and G, where the same haplotypes are present in populations of both species in Romania. All three groups meet in the Ukrainian Carpathians. Additionally, haplotypes from clades F, L2, L3 were found locally in *T. montandoni*, but these almost certainly are the result of ongoing hybridization. *Triturus* cf. *montandoni* is known from the fossil record since the Upper Miocene (Hodrová 1987) or Pliocene (Roček 1994), and on the basis of allozyme data Rafiński & Arntzen (1987) dated its split from *T. vulgaris* as 6 ± 2 Ma. Thus, if the lineage I, the only confined to *T. montandoni*, reflected the origin of this species from Transylvanian *T. vulgaris*, an exceptionally slow rate of mtDNA evolution would need to be invoked, as the applied calibration of the molecular clock imply much younger divergence of phylogroups I and J, *c*. 1.0 Myr. Moreover, an additional hypothesis would be needed to explain the pattern of mtDNA variation in Romania. Therefore, we propose that all three major lineages are the result of *T. vulgaris* mtDNA introgression into *T. montandoni* populations and replacement of its original mtDNA. This requires at least two independent introgression events. One, resulting in the emergence of clade I, would occur *c*. 1.0 Ma, and the second, much younger, dating at most to the LGM. Both species hybridize readily where their ranges meet (Fuhn *et al*. 1975; Kotlík & Zavadil 1999), and data from a Polish hybrid zone indicate that introgression of mtDNA is bidirectional and more extensive than that of the nuclear markers (Babik *et al*. 2003).

Replacement of the original *montandoni* mtDNA by *vulgaris* mtDNA could have been facilitated by reductions of effective population sizes of *T. montandoni* in refugia during glacial periods. Moreover it cannot be excluded that the original *T. montandoni* mtDNA has been replaced entirely (cf. Wilson & Bernatchez 1998) or that it remains unsampled.

Demographic history of the phylogroups

In five of seven analysed clades the estimates of θ_1 and θ_{ML} were similar. In groups L2 and L3 substantially larger θ_{ML} than θ_1 resulted most likely from the small amount of information contained in star-shaped genealogies (Kuhner *et al*. 1998).

Theta values were the smallest, and significant growth was not detected in the Turkish E clade. Relatively deep differentiation among haplotypes within clades A, B, D and H also suggests long-term demographic stability. It is not surprising as the southern populations should have been less affected by Pleistocene climatic oscillations (Hewitt 2000, 2004). Moderate and ancient population growth with relatively large effective population sizes were detected in populations represented by clades F and J. This pattern may reflect the colonization of the present-day ranges of these groups, or a demographic expansion after a bottleneck during one of the previous glacial cycles. The star-shaped phylogeny in group I probably arose from a severe bottleneck in its northern refugium and a quick recovery following climate amelioration after the LGM. The same pattern accompanied by a high growth rate was observed for the L2 group and could have had similar causes.

Three mtDNA groups now occupy large areas inhospitable for newts during the last glacial cycle. Thus we expected to find evidence for rapid population growth, connected to range expansion in these groups, but found it in the L3 subclade only. Most likely this is a consequence of our sampling scheme. Sampling in the area of putative expansion of both L1 and G groups was rather limited compared to the postulated refugia.

It could be argued that demographic analyses based on mtDNA variation are unwarranted, because some of the mtDNA clades comprised not one but two species. In the particular case this, however, does not necessarily violate assumptions of the analytical methods. Because haplotypes from clades F, L2 and L3 present in *T. montandoni* individuals are derived from local and most likely contemporary (cf. Babik *et al*. 2003) introgresion, they largely provide information on the past demographic history of *T. vulgaris* in the respective areas. On the other hand, the presence in both species of the same haplotypes from the G clade in a portion of Romania, without any species-specific clustering, raises the intriguing possibility that the ongoing female-mediated gene flow obliterates specific boundaries and produces a single, area-specific female demographic history.

Acknowledgements

The manuscript benefited from comments of Walter Durka, Maciej Pabijan and Jacek M. Szymura. Jacek M. Szymura provided purified newt mtDNA. Maciej Pabijan provided *Triturus alpestris* sequence. We would like to thank Ivan Aleksić, Severus Daniel Covaciu-Marcov, Marcin Liana and Natasa Tomasević for invaluable help in the field. The following persons provided tissue samples: Robert Jehle, Tibor Kovacs, Marcin Liana, Petr Mikuliček, Artur Osikowski, Maciej Pabijan, Jan Rafiński, Ernesto Recuero, Matilde Ragghianti, Martin Sandera, Jacek M. Szymura, David Tarkhnishvili, Benny Trapp, Stefano Vanni, Alexey Yanchukov, Annie Zuiderwijk. The work was supported by grants 6 PO4C 027 20 from the Polish State Committee for Scientific Research, BW/IZ/15/2003 from the Jagiellonian University, B1725 from the Ministry of Science, Technology & Development of Republic of Serbia, DAPTF Seed Grant 2003 and Biod-Iberia under the European Commission Human Potential Program.

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