

# The dissection of a Pleistocene refugium: phylogeography of the smooth newt, *Lissotriton vulgaris*, in the Balkans

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### ABSTRACT

**Aim** To assess the role of the Balkan Peninsula as a Pleistocene refugium for the smooth newt, *Lissotriton vulgaris*, and to test whether its genetic differentiation is temporally compatible with the southern refugia model.

Location The Balkan Peninsula.

**Methods** Phylogeographical analyses were conducted using mitochondrial DNA (mtDNA) sequences sampled from 49 populations of *L. vulgaris*. A fossil-calibrated estimate of divergence times among major mtDNA clades was obtained.

**Results** We detected seven parapatrically distributed mtDNA clades with very little admixture among populations. Whereas most clades diverged in the Pleistocene, the earliest splits between Caucasian, Anatolian and Balkan clades occurred in the Plocene. Clades C, D, K and M have local distributions within the Balkans and have evolved in isolation from other groups. Clade L originated in the Pannonian Basin and northern margin of the Balkan Peninsula and recently expanded across central and western Europe. Clades H and E have recently arrived in the Balkans from source populations in the Apennine Peninsula and Anatolia, respectively.

**Main conclusions** The history of *L. vulgaris* involves multiple, independent refugial populations in the Balkans. Only one of them, located at the northern periphery of the peninsula, showed evidence of post-glacial expansion into western and northern Europe. The Balkans have therefore served as a reservoir of old diversity for *L. vulgaris*. By contrast, populations at the northern and eastern frontiers of the peninsula have experienced non-equilibrium dynamics. Our dating revealed that very little, if any, pre-Pliocene genetic diversity has survived in Europe, despite an extensive fossil record for this species in the Miocene and Pliocene. Differentiation of the European mtDNA clades thus seems to have been primarily moulded by Pleistocene climate change. None of the currently recognized subspecies present in the Balkans are reciprocally monophyletic in their mtDNA. We hypothesize that incomplete lineage sorting and mtDNA introgression account for the observed discrepancies.

### Keywords

Amphibia, divergence dating, fossil calibration, introgression, *Lissotriton vulgaris*, mtDNA, phylogeography, Pleistocene refugium, refugia within refugia.

## INTRODUCTION

Climate change fosters shifts in the distributions of plants and animals. During periods of global cooling, the ranges of many temperate species contracted to pockets of hospitable habitat at low latitudes or elevations. At present, such refugia can be distinguished because they maintain distinct genetic lineage(s) that have presumably persisted *in situ* during episodes of past

http://wileyonlinelibrary.com/journal/jbi doi:10.1111/jbi.12449 climate change (Médail & Diadema, 2009). At a larger scale, areas that support refugia often reveal a patchwork of parapatrically or allopatrically distributed, genetically divergent lineages (Petit *et al.*, 2003; Gómez & Lunt, 2007). Usually only a small fraction of the genetic variation present in refugia is represented in areas recolonized following climatic amelioration (Hampe & Petit, 2005; Babik *et al.*, 2009).

The traditional biogeographical model for western Eurasia states that temperate species survived unfavourable climatic periods in southern refugia located in the Iberian, Apennine and Balkan peninsulas, from which their populations expanded and colonized central and northern Europe during interglacials (Hewitt, 2000; Tzedakis et al., 2013). This model has been challenged by the recognition of actual or hypothetical cryptic northern or extra-Mediterranean refugia for certain species (Provan & Bennett, 2008; Juřičková et al., 2014). Among the European southern refugial areas, the Balkan Peninsula has received the least attention (Crnobrnja-Isailović, 2007), even though it is perhaps the most important contributor to the post-glacial recolonization of central and northern Europe in terms of number of species (Hewitt, 2011). The Balkan Peninsula is located at the junction between central Europe and Asia Minor and is characterized by extreme topographic and climatic diversity. These features may underlie the high species and subspecies diversity (Džukić & Kalezić, 2004; Kryštufek & Reed, 2004). Another factor that potentially augmented the biodiversity of the Balkan Peninsula is its capacity to hold cold stage refugia for forest communities and their associated fauna (Tzedakis, 2004; Tzedakis et al., 2013). In this study, we provide an analysis of a species of newt to contribute to our understanding of the role of the Balkan Peninsula in providing Pleistocene refugia for temperate species.

Although amphibians may be more mobile than commonly thought (Smith & Green, 2005; Schmidt et al., 2007), they nonetheless show relatively strong philopatry and a limited ability to cope with extreme temperatures and desiccation, making them susceptible to local or regional extinction due to climatic fluctuations (Araújo et al., 2006). Indeed, the deep phylogeographical structure typically revealed across the ranges of amphibians (Vences & Wake, 2007) may be a response to historical climate oscillations that extirpated local and regional populations, thus generating highly fragmented and genetically divergent populations. In Europe, present-day distributions of amphibians and reptiles are determined more by their proximity to the three glacial refugial areas than by current climate gradients (Araújo & Pearson, 2005), suggesting that the ranges of these taxa have not yet 'recovered' from the most recent major climatic switch from glacial to interglacial conditions. The Balkan Peninsula is thought to have acted as a refugial area for several amphibian species (Sotiropoulos et al., 2007; Fijarczyk et al., 2011; Vences et al., 2013; Wielstra et al., 2013). Moreover, the presence of endemic amphibian species (e.g. Proteus anguinus, Rana graeca, Pelophylax epeiroticus, Pelophylax shqiperica) suggests long-term population persistence of a range of different types of amphibians in this area.

The Balkan Peninsula also played a crucial role in the Pleistocene history of the most widely distributed European newt: the smooth newt, Lissotriton vulgaris (Linnaeus, 1758). The species currently ranges from north-western Europe to Siberia (Fig. 1a). The northern part of this distribution is inhabited by the wide-ranging nominative subspecies. In the south-western part of its distribution, L. vulgaris is differentiated into a number of morphologically distinct subspecies (Raxworthy, 1990; Schmidtler & Franzen, 2004). The Apennine Peninsula and adjacent territories are inhabited by L. v. meridionalis. The Transylvanian plateau is home to L. v. ampelensis. Lissotriton v. lantzi (sometimes considered a separate species) inhabits the northern Caucasus and parts of Transcaucasia. Western Anatolia contains two subspecies: L. v. kosswigi inhabits a small area along the Black Sea coast east of Istanbul, while L. v. schmidtlerorum (sometimes synonymized with the nominative subspecies) inhabits western Anatolia and the coast of the Marmara Sea. The southern Balkan Peninsula is inhabited by the morphologically well-defined L. v. graecus. A number of other subspecies and forms have been described, particularly along the eastern coast of the Adriatic, but their validity has been questioned (Schmidtler & Schmidtler, 1983; Kalezić et al., 1987).

In this contribution, we assess the importance of the Balkans in generating the genetic diversity seen in the smooth newt. We infer the number and distribution of refugia and determine the role of refugial populations in the evolutionary history of this species. We examine the mitochondrial DNA (mtDNA) variation in the context of morphologically distinguishable subspecies and suggest testable hypotheses that have led to their current distributions and patterns of genetic variation. By using a fossil-calibrated time tree for *Lissotriton*, we test whether the observed genetic and subspecific diversity in Balkan smooth newts is a consequence of Plio-Pleistocene climatic oscillations or processes pre-dating this period.

## MATERIALS AND METHODS

## Sampling and laboratory methods

Tissues were sampled from 1–5 individuals from 49 localities (Table 1, Fig. 1) for a total of 173 adult newts. We refer to a locality as a single newt breeding site (e.g. pond). We amplified and sequenced two mtDNA fragments: a 1014 bp fragment of NADH dehydrogenase subunit 2 (ND2) and flanking tRNA genes; and, for a subset of samples (14 individuals), a 835 bp fragment of NADH dehydrogenase subunit 4 (ND4) with associated tRNAs. See Appendix S1 in Supporting Information for details of primer sequences, PCRs and sequencing.

## Data analysis

Sequences were checked and edited using SEQSCAPE 2.5 (Applied Biosystems, Foster City, CA, USA). We added 28 *ND2* and *ND4* sequences from Babik *et al.* (2005), representing



**Figure 1** (a) The distributions of *Lissotriton vulgaris* subspecies, *L. montandoni*, and mtDNA clades present in sampled localities from this and previous studies (Babik *et al.*, 2005; Nadachowska & Babik, 2009; Zieliński *et al.*, 2013). Black circles within tokens indicate localities used for PHYLOMAPPER analyses. (b) The sampling localities in the Balkans; clades are colour-coded according to the key. Pie chart diameter is proportional to sample size. Numbers refer to populations sampled in this study (Table 1); non-numbered pie charts with filled circles in their centres represent previously studied populations.

all major mtDNA lineages within *Lissotriton* and outgroups (see Appendix S1 for GenBank accession numbers). We then constructed two datasets: (1) a 1013 bp *ND2* alignment

encompassing all sampled Balkan newts; and (2) a 1849 bp alignment consisting of concatenated ND2, ND4 and four tRNA genes (further referred to as ND2 + ND4) encompassing

<b>Table 1</b> Localities in which smooth newts ( <i>Lissotriton vulgaris</i> ) were sampled for this study ( <i>n</i> refers to the number of individuals
sampled from each locality). Haplotypes and major clade(s) found in each locality are also shown. Subspecies assignment was based on
morphology assessed in the field. Subspecies abbreviations: Lvm, L. v. meridionalis; Lvv, L. v. vulgaris; Lvg, L. v. graecus.

Number	Locality	Country	п	Clade(s)	Haplotype(s)	Subspecies	Latitude (N)	Longitude (E)
1	Barban	Croatia	5	Н	H6 (3), H7(2)	Lvm	45.060	14.044
2	Barban1		3	H,L	H6 (2), L79	Lvm	45.063	14.010
3	Salakovac		4	L	L80 (4)	Lvm	45.052	14.087
4	Srsici		3	L	L77, L78 (2)	Lvm	45.145	14.548
5	Krk		5	L	L76	Lvm	44.984	14.652
6	Donja Ploča		5	L	L74 (4), L75	Lvv	44.456	15.655
7	Donji Zemunik		5	L	L84	Lvv	44.114	15.363
8	Benkovac		5	D,L	D1 (3), D10, L84	Lvv	44.023	15.627
9	Domanovići	Bosnia and Hercegovina	5	D	D1	Lvg	43.134	17.803
10	Moševići		4	D	D1	Lvg	42.933	17.684
11	Tivat	Montenegro	5	D	D1 (4), D10	Lvg	42.397	18.739
12	Kostanjica		5	L	L83	Lvg	42.089	19.263
13	Donji Stoj		5	Κ	K11, K16 (3), K17	Lvg	41.878	19.367
14	Xërze	Kosovo	5	D	D8	Undetermined	42.341	20.573
15	Gur i Zi	Albania	4	Κ	K11 (2), K12, K13	Lvg	42.284	19.569
16	Fushë-Krujë		1	Κ	K14	Lvg	41.529	19.682
17	Kavajë		4	Κ	К3	Lvg	41.168	19.556
18	Gracen		3	Κ	K2	Lvg	41.157	19.949
19	Orik		5	Κ	K9, K10 (4)	Lvg	40.310	19.458
20	Borsh		2	С	C4,C5	Lvg	40.050	19.850
21	Gjirokastër		1	Κ	K15	Lvg	40.099	20.124
22	Sopik		1	С	C3	Lvg	39.782	20.070
23	R'zanicino	FYR Macedonia	5	Κ	K1 (4), K5	Undetermined	41.914	21.643
24	Debreste		1	Κ	K1	Lvg	41.502	21.293
25	Izvor		4	Κ	K1 (2), K4 (2)	Lvg	41.521	21.710
26	Leskoec		4	Κ	K2	Lvg	40.962	20.885
27	Garnikovo1		2	Κ	K1, K6	Lvg	41.277	22.060
28	Asprangeloi	Greece	4	Κ	K1, K18 (3)	Lvg	39.830	20.730
29	Kleisoura		5	С	C6, C7 (2), C8 (2)	Lvg	39.362	20.887
30	Archontochori		3	С	С9	Lvg	38.697	21.039
31	Lapas		4	С	C10, C11 (2), C13	Lvg	38.111	21.410
32	Sagaiika		5	С	C11 (4), C12	Lvg	38.104	21.469
33	Zevgolateio		2	М	M1	Lvg	37.253	21.978
34	Milia		3	М	M3	Lvg	37.596	22.409
35	Kalavryta		5	М	M1, M2 (3), M3	Lvg	38.014	22.024
36	Thavmako		3	Κ	K1, K8 (2)	Lvg	39.145	22.276
37	Pythio		1	Κ	K1	Lvg	40.089	22.208
38	Trilofos		1	Κ	K1	Lvg	40.358	22.477
39	Seli		5	Κ	K7	Lvg	40.567	22.022
40	Ano Kalliniki		4	Κ	K1 (3), K2	Lvg	40.854	21.451
41	Archangelos		1	Κ	K1	Lvg	41.087	22.300
42	Pefkodasos		5	Κ	K1 (4), K7	Lvg	41.030	22.580
43	Kentriko		5	Κ	K1, K7 (4)	Lvg	41.160	22.892
44	Gravuna		2	L	L81, L82	Lvv	41.008	24.678
45	Evros-Feres		1	Е	E19	Lvv	40.859	26.184
46	Evros-Lira		1	Е	E16	Lvv	41.040	26.156
47	Ostar Kamak	Bulgaria	5	Е	E15(4), E18	Lvv	41.879	25.853
48	Aleksandrovo	č	3	Е	E17	Lvv	42.602	25.099
49	Klisura		4	D	D9	Lvv	42.334	23.380

42 individuals representing all mtDNA lineages in the Balkans and outgroups (Appendix S1). The *ND2* dataset was used for network-based and demographic analyses, and for illustrating the geographical distribution of the major mtDNA lineages, while the *ND2* + *ND4* dataset was used to establish relationships and estimate divergence times among major mtDNA lineages.

# **Phylogenetic analyses**

We defined subsets of the *ND2* dataset that encompassed 1st, 2nd and 3rd codon positions of *ND2*. We used PARTITIONFINDER 1.1.0 (Lanfear *et al.*, 2012) for selecting the optimum partitioning and evolutionary models. According to the Bayesian information criterion (BIC), the best scheme

involved a single partition under a GTR+I+G substitution model. This model was implemented in a maximum likelihood (ML) analysis with rapid bootstrap (1000 replicates) in RAXML 7.4.2 (Stamatakis, 2006). Networks for the main mitochondrial clades present in the Balkans were computed with a median-joining algorithm implemented in the software NETWORK 4.6. (Bandelt *et al.*, 1999).

The relationships among major mtDNA lineages were investigated using the ND2 + ND4 dataset. We designated subsets according to codon positions and grouped all tRNA genes in a single partition. The best partitioning scheme under BIC involved the grouping of 1st codon positions of ND2 and ND4 and all tRNAs into a single partition (HKY+I+G), 2nd codon positions of both genes (HKY+I+G), and 3rd codon positions of ND2 and ND4 (GTR+G). Bayesian inference (BI) was conducted on this dataset in MRBAYES 3.2 (Ronquist et al., 2012) for 10 million generations sampled every 1000 generations. The Markov chain Monte Carlo (MCMC) runs converged before the first 1 million generations, which were discarded as burn-in (10%). We also conducted an ML analysis in RAxML using the partitioned ND2 + ND4 dataset. We implemented 100 alternative runs on distinct parsimony starting trees with thorough bootstrap analysis (1000 replicates).

#### **Demographic analyses**

Newts and other amphibians form discrete demes corresponding to breeding ponds that undergo extinction and recolonization dynamics such that the regional population can be regarded as a metapopulation (e.g. Marsh & Trenham, 2001). For demographic analyses, we treated major mtDNA clades as metapopulations and localities (i.e. ponds) as demes. It has been shown (Wakeley, 2004) that if one gene copy per locus is sampled per deme, the ancestral process producing the sample is identical to the unstructured coalescent. Therefore, for demographic analyses we randomly selected a single mtDNA haplotype from each population in which a given clade was present using sequences available from this study and Babik *et al.* (2005). For lineage E, widespread in Asiatic Turkey, we used only populations available from Thrace.

We used three statistical procedures to detect departures from the standard neutral model within the haplogroups: Tajima's *D*, Fu's  $F_S$  and Ramos-Onsins & Rozas  $R_2$ . These complementary statistics take advantage of different aspects of the site frequency spectrum (Tajima's *D* and  $R_2$ ) or are based on the distribution of haplotypes in a sample (Fu's  $F_S$ ), and are most powerful for detecting departures from neutrality assuming no recombination (Ramírez-Soriano *et al.*, 2008). We used DNASP 5 (Librado & Rozas, 2009) to calculate these statistics and their confidence intervals (1000 coalescent simulations conditioned on the number of segregating sites). We did a mismatch analysis in ARLEQUIN 3.5 (Excoffier & Lischer, 2010). Generalized nonlinear least squares were used to calculate  $\Theta_0$ ,  $\Theta_1$  and  $\tau$ , the time since expansion measured in mutational time units. A parametric bootstrap (1000 replicates) was used to test the fit to a sudden expansion model and to obtain confidence intervals on demographic statistics.

#### **Divergence dating**

Divergence times of the major mtDNA lineages within Lissotriton were estimated in a Bayesian framework using BEAST 1.8.0 (Drummond et al., 2012). Detailed BEAST settings are given in Appendix S2. We placed a calibration prior on the time to the most recent common ancestor (TMRCA) of Lissotriton and Mesotriton. We used mtDNA sequences from Mesotriton alpestris, the putative sister group to Lissotriton (Halliday & Arano, 1991; Zhang et al., 2008). Based on fossil data, we assume that the TMRCA for both genera must date to between 56.0 and 16.2 million years ago (Ma). The maximum time constraint of 56 Ma rests on the oldest European salamandrids from the Upper Palaeocene (Estes et al., 1967). The minimum time constraint is based on the oldest fossils reliably attributable to both genera (see Appendix S2 for an overview of the fossil record of L. vulgaris). Lissotriton has a rich fossil record going back perhaps to the Acquitanian (23 Ma; Böhme, 2003). However, most Lissotriton fossils have been reported without description or depiction (e.g. Böhme, 2003; Böhme & Ilg, 2003) precluding an assessment of their reliability. The first well-documented finds of Lissotriton include L. aff. helveticus from Béon, France (17.3 Ma; Rage & Bailon, 2005) and Sandelhauzen, Germany (16.2 Ma; Böhme, 2010). Few fossils are known for Mesotriton; however, an exceptional fossil of M. randeckensis (Schoch & Rasser, 2013) from the early Miocene of Germany and slightly older but more fragmentary material (M. wintershofi; Lunau, 1950) place the origin of the genus at a minimum of 16.4 Ma. Thus the time window encompassing the first well-documented representatives of Lissotriton and Mesotriton is relatively narrow (17.3-16.2 Ma) and we use the lower value as a minimum time constraint. We set a prior on the TMRCA of both genera using a normal distribution with a mean of 36 Ma and standard deviation (SD) of 10 Myr. This distribution yielded 95% quantiles between 16.4 and 55.6 Ma, fitting our expectations from the fossil data.

### Distribution of ancestral haplotypes

We used PHYLOMAPPER 1b1 (Lemmon & Lemmon, 2008) to estimate the geographical locations of ancestors of the sampled individuals. The input mtDNA genealogy consisted of BI of all non-redundant haplotypes in our *ND2* dataset, including sequences and localities from Babik *et al.* (2005), under the GTR+I+G substitution model in MRBAYES (parameter settings were consistent with BI described above). PHYLOMAPPER requires that geographical coordinates are assigned to each haplotype. If several localities shared a haplotype, then we randomly selected one of these localities for analysis. If more than one haplotype occurred in a locality, then we slightly shifted the geographical coordinates of the coincident haplotypes in order to avoid redundancy in the georeferencing. Separate analyses were conducted for clades D, H, K, L and C+M (these clades are monophyletic and were combined). Clade E, distributed predominantly in western Anatolia (Nadachowska & Babik, 2009), was omitted from this analysis. Divergence times of basal nodes for each focal clade were taken from the BEAST analysis and then nonparametric rate-smoothing was applied to obtain an ultrametric genealogy used for optimizing the dispersal parameters and locations of ancestral nodes (1000 replicates). The ancestral locations and estimated point ages were imported into ARCMAP 10.2.1 (ESRI, Redlands, CA, USA) and interpolated into a continuous map using the inverse distance weighted technique (after Chan *et al.*, 2011).

### RESULTS

# Variation in *ND2* and the geographical distribution of haplotypes

We obtained ND2 sequences (969-1013 bp) for 173 smooth newts and ND4 sequences for 14 individuals (GenBank accession numbers for ND2: KM987645-KM987696; for ND4: KP008135-KP008148). The ND2 alignment contained 198 variable positions and 185 parsimony informative sites within the ingroup (L. vulgaris). We found 54 haplotypes, 52 of which have not been previously reported (hereafter called 'new' haplotypes). The ML phylogram for the ND2 haplotypes is shown in Appendix S1. The ND2 haplotypes fall into seven major clades distributed allopatrically or parapatrically (Figs 1b & 2). Clade K (17 new haplotypes/18 total) occurs in northern Greece, Macedonia, Albania and southern Croatia. Clade D (3 new/9 total) occurs immediately northwards in Montenegro, central Croatia, Bosnia and Hercegovina, Kosovo and eastern Bulgaria. Clade L (11 new/84 total) occurs in the northern Balkans and is also widespread in central and western Europe (Babik et al., 2005); however, two haplotypes (L81 and L82) were found in north-eastern Greece. Previously reported subclades L1-L3 (Fig. 2, Appendix S1) constitute a subset of the variation in the L clade observed in the Balkans. Two major mtDNA clades occur almost exclusively in Greece: the newly recognized clade M (three new haplotypes) is limited to the Peloponnese Peninsula, while clade C (11 new/13 total) occurs in the Peloponnese, the island of Corfu and the south-eastern Greek mainland. Clade E (5 new /8 total) is limited to Thrace and southern Bulgaria in Europe but is widespread and structured in Asiatic Turkey (> 35 haplotypes; Nadachowska & Babik, 2009). Clade H (2 new/7 total) occurs in northern Croatia and northern Italy. We detected only two cases of syntopy of major mtDNA clades, both along the Adriatic coast of north-western Croatia, between clades H and L in the Istrian locality of Barban 1 (locality 2 in Fig. 1b), and also between clades D and L in the Dalmatian locality of Benkovac (locality 8 in Fig. 1b).

We were unable to reject the sudden expansion model in any of the clades (Table 2). However, we rejected demographic stability in favour of demographic expansion in all three neutrality tests for clades K and L, and in one test for clade E (Table 2). Ancestral distributions (Fig. 3) are found in southwestern mainland Greece (clades C+M), southern Albania (clade K), southern Serbia (clade D) and the north-eastern corner of the Apennine Peninsula (clade H). The ancestral distribution of clade L (with the largest number of sampled populations) is centred in the western part of the Pannonian Basin and the northern part of the Balkan Peninsula.

# Phylogeny and divergence times of major mtDNA lineages

There were 782 variable positions in the concatenated ND2 + ND4 alignment; 607 of these were parsimony informative. The topologies of ML (Fig. 2) and BI (not shown) genealogies were identical. The presence of well-differentiated clades A-L was consistent with the mtDNA phylogeny of Babik et al. (2005). Support was high at most nodes (although BI posterior probabilities were notably higher than bootstrap support in the ML analysis) with the exceptions of some branches within the L clade. The concatenated mtDNA phylogenv resolved some of the relationships at the base of the tree (particularly the positions of clades B, C and M) that were unresolved in the ND2 analysis (cf. Fig. S1 in Appendix S1). Our extended sampling in the Balkan Peninsula revealed 11 new branches (shown in red in Fig. 2), most of which fell into formerly defined lineages (C, E, D, H, K). Clade M, a previously unsampled, strongly divergent mtDNA lineage (> 4%) was discovered on the Peloponnese Peninsula. Moreover, several divergent subclades of the widespread L lineage (L4-L6) were discovered in Croatia, Montenegro and Greece.

In the four BEAST analyses effective sample sizes for all parameters exceeded 3000 (most exceeded 20,000). The average mtDNA substitution rate, measured through the neutrally evolving 3rd codon positions of ND2 and ND4, amounted to 0.0354 substitutions per site per million years. The average substitution rates for the remaining partitions (1st codon positions + tRNAs, 2nd codon positions) were 0.00691 and 0.00209 substitutions per site per million years, respectively. Averaged over all partitions, we obtained an mtDNA substitution rate of 1.48% per site per lineage per million years. A time frame for the history of Lissotriton is presented in Fig. 4 and in Appendix S2. The oldest splits among extant Lissotriton, leading to the western European L. boscai, L. helveticus and L. italicus, occurred firmly in the Miocene. Deep intraspecific splits of Miocene or Pliocene age are also apparent in Mesotriton and L. boscai. In contrast, the majority of intraspecific divergences in L. vulgaris are relatively recent. The oldest intraspecific split leading to the easternmost lineage A occurred during the Pliocene or early Pleistocene. This event was followed by several nearly



Lissotriton vulgaris mtDNA clades based on a maximum likelihood (ML) analysis of concatenated ND2 and ND4 genes. Numbers at nodes represent Bayesian posterior probabilities (PP, top) and bootstrap support in the ML analysis (BS, bottom); asterisks (\*) denote full support in both analyses; values < 0.7 PP or < 70 BS are not shown. The scale bar shows the maximum likelihood estimate of the number of nucleotide substitutions per site. Red branches represent lineages detected in this study. The names of clades follow Babik et al. (2005) and are assigned to morphologically distinguished subspecies; filled stars represent the nominative subspecies L. v. vulgaris. Mesotriton alpestris was used as an outgroup. Median-joining networks for ND2 sequences are shown for the mtDNA haplotypes occurring in the Balkans.

Figure 2 Relationships among major

synchronous splits between the late Pliocene and middle Pleistocene that included lineages B, C and M and a monophyletic group composed of clades D, E and F. All other mtDNA lineages diverged in the Pleistocene.

### DISCUSSION

### Dissection of a refugium

Our extended sampling of Balkan smooth newts revealed strong genetic subdivision and the existence of seven well-differentiated mtDNA clades. A highly divergent clade (M) restricted to the Peloponnese, and a number of subclades previously unreported by Babik *et al.* (2005) were detected. All clades are parapatrically distributed in the Balkans with only two cases of syntopy (clades H and L in Istria and clades D and L on the Dalmatian coast; 4% of populations sampled), which contrasts with the situation in central and northern Europe where syntopy was detected in 20% of localities (Babik *et al.*, 2005). Genetically diverse populations are expected if post-glacial colonization waves involve individuals from more than one refugial source population (Petit *et al.*, 2003). Expansion waves from cryptic northern refugia (Babik *et al.*, 2005; Zieliński *et al.*, 2013) and the Balkan refugium (see below) may have mixed across large parts of Europe north of the Balkans. The redistribution of genetic variation from two or more refugia could have produced the current diversity in central European populations, while refugial populations in the Balkans seem to have maintained their integrity over the course of at least the most recent Pleistocene climate shifts.

The mtDNA lineages of smooth newts in the Balkans depict the complex historical and spatial dynamics of this Pleistocene refugium. A salient result is the nearly complete lack of mtDNA admixture among populations, suggesting evolution in spatially distinct regional populations (i.e. 'refugia within refugia'; Gómez & Lunt, 2007). Our analysis supports the presence of refugia for at least four mtDNA clades (C, D, K, M) that have survived in the Balkans *in situ*. A further refugium for clade L probably occurred at the northern fringe of the peninsula and in the Pannonian Basin. A similar 'refugia within refugia' pattern was recently found for *L. v. meridionalis* in the Italian peninsula (Maura *et al.*, 2014). Moreover, we inferred idiosyncratic histories for each Balkan mtDNA clade. For example, a recent increase in population size took place in clade K. Although the spatial extent

**Table 2** Neutrality tests and demographic analyses for mtDNA clades of smooth newts (*Lissotriton vulgaris*) in the Balkans. N denotes the number of sampled populations. The empirical distributions of Tajima's D, Fu's  $F_s$  and Ramos-Onsins and Rozas  $R_2$  were drawn from coalescent simulations conditioned on the number of segregating sites (S). Asterisks mark significant values at P < 0.05. Demographic analyses were conducted under the assumptions of a sudden expansion model, confidence intervals were obtained by bootstrapping (1000 replicates): k, mean number of pairwise differences;  $\tau$ , time since expansion measured in mutational time units;  $\Theta_0$  and  $\Theta_1$  are theta values before and after expansion;  $P_{sudden}$ , probability that the mismatch distribution conforms to the model of sudden expansion; 95% CI, 95% confidence interval.

Clade	Ν	S	Tajima's D	Fu's F <sub>S</sub>	$R_2$	k (95% CI)	τ (95% CI)	Θ <sub>0</sub> (95% CI)	Θ <sub>1</sub> (95% CI)	P <sub>sudden</sub>
С	7	36	-0.196	0.752	0.139	16.21 (9.95–22.23)	17.5 (10.56–21.82)	0.00 (0.00-6.89)	167.85 (63.63-99999.00)	0.054
D	13	31	0.951	0.663	0.181	14.14 (5.64–21.42)	21.57 (1.15-29.62)	0.00 (0.00-9.93)	25.55 (12.75-999.00)	0.184
Е	5	8	-0.807	-2.004	0.107*	4.18 (1.6-7.6)	6.17 (0.01-89.13)	0.00 (0.00-9.46)	99999.00 (7.96–99999.00)	0.053
Н	4	17	0.250	2.538	0.225	9.5 (5.50-18.33)	12.68 (0.08-97.68)	0.00 (0.00-65.84)	129.43 (20.22–999.00)	0.106
L	47	92	$-1.55^{*}$	-26.489*	0.056*	12.58 (9.43-15.75)	14.71 (9.36-18.57)	0.00 (0.00-4.08)	52.81 (33.40-1957.81)	0.058
Κ	22	20	$-1.99^{*}$	-5.594*	0.081*	2.54 (0.87-4.86)	2.25 (0.04-7.76)	0.98 (0.00-1.43)	5.85 (2.87-999.00)	0.745



of this expansion is rather local, it covers most of the present range of L. v. graecus (Fig. 1). The northern part of the Balkans acted as a refugium and source population for lineage L: a lineage which colonized a large swathe of central and western Europe, a pattern consistent with leading-edge expansion in which marginal refugial populations colonize formerly inhospitable habitat after climate amelioration (Hewitt, 2000). We speculate that the more southern mtDNA lineages may not have had access to the dispersal routes towards central Europe. A distinct subclade (L6) with a disjunct distribution in Montenegro and eastern Greece is evocative of population dynamics preceding the current interglacial. The presence of haplotypes from clades H and E in the north-west and south-east of the peninsula reveals the influx of phylogeographical lineages from the Apennine Peninsula and Anatolia, respectively.

The Balkan Peninsula is an important centre of biodiversity (Kryštufek & Reed, 2004), part of which is thought to have evolved as a result of isolation in Tertiary/Quaternary **Figure 3** Ancestral distributions of major *Lissotriton vulgaris* clades in the Balkans. The coloured overlay represents ancestral distributions over time, with warm colours delimiting the oldest (ancestral) haplotypes for each clade calculated using PhyloMAPPER (only the top 60% of values are shown). An estimate is not provided for clade E. Mismatch distributions for each clade are shown next to the ancestral ranges; red curves show the expected distribution for a demographically stable population.

refugia. At the intraspecific level, the presence of several distinct genetic lineages, indicative of multiple refugia, has been inferred for amphibians (Sotiropoulos *et al.*, 2007; Wielstra *et al.*, 2013), reptiles (Böhme *et al.*, 2007; Ursenbacher *et al.*, 2008), mammals (Kryštufek *et al.*, 2007; Alexandri *et al.*, 2012) and insects (Gratton *et al.*, 2008; Previšić *et al.*, 2009). From an analysis of phylogeographical patterns of 82 plant species, Médail & Diadema (2009) pinpoint eight potential Tertiary/Quaternary refugia. Four of these are located along the Adriatic coast, two in central Greece, one in the Peloponnese and one in the Chalkidiki Peninsula. Our results suggest that the origin of mtDNA clades K, C and M corresponds with the general area of the southernmost plant refugia.

## Historical time frame

The mtDNA phylogeny of smooth newts shows a nested pattern with the oldest lineages, A (L. v. lantzi) and B (L. v. kosswigi), found in the Caucasus and Anatolia, respectively,



**Figure 4** Chronogram for smooth newts (*Lissotriton vulgaris* subspecies) and related species. Bars represent 95% highest posterior density (HPD) intervals. Closed circles at nodes denote posterior probabilities > 0.9. The chronogram was calibrated at the most recent ancestor of *Mesotriton* and *Lissotriton*; the 95% HPD interval at this node (16.8–55.9 Ma) was omitted from the figure.

followed by two sister clades (C, M) confined to L. v. graecus in southern Greece. A subsequent, relatively deep split occurs between eastern European newts, including L. v. graecus (clades D, E, F) and a geographically widespread group containing populations from eastern, western, central and northern Europe (clades G-L). This geographical and temporal pattern implies that all extant mtDNA lineages within the smooth newt and the Carpathian newt, Lissotriton montandoni (Babik et al., 2005; Zieliński et al., 2013), are descendants of Anatolian/Caucasian stock. Moreover, the divergence between Anatolian and European newts is relatively recent, dated between the middle Pliocene (Zanclean) to early Pleistocene. It follows that smooth newts were either absent from Europe before the Plio-Pleistocene, or that earlier European populations of smooth newts (or their mtDNA) went extinct and the region was then recolonized during or slightly before the Pliocene, probably from an Anatolian source population. Fossil evidence refutes the first hypothesis. Fossils similar to or even attributable to L. vulgaris are numerous in Miocene and Pliocene sediments from central, western and south-eastern Europe and Turkey (Appendix S2). The pectinate shape of the chronogram for L. vulgaris is different from what we would expect if lineages present in the Miocene survived the Pliocene and Pleistocene climatic vagaries within Europe: we would anticipate a pattern with at least some deep mtDNA clades showing divergences preceding the Pliocene, as in L. boscai and M. alpestris (Fig. 4; Martínez-Solano et al., 2006; Sotiropoulos et al., 2007) and certain other amphibians (Wielstra & Arntzen, 2011; Pabijan et al., 2013). Instead, we found that all mtDNA clades confined to Europe diverged in the late Pliocene or Pleistocene. Thus we infer that climate change during the Pliocene, possibly related to the onset of Northern Hemisphere glaciations at c. 2.75 Ma (Ravelo et al., 2004; Bartoli et al., 2005), effectively extirpated all ancestral mtDNA lineages present in Europe at that time. The oldest, nearly synchronous, cladogenetic events within the L. vulgaris mtDNA genealogy (clades B, C, M and the ancestor of D–F) were probably the result of allopatric fragmentation of the ancestral stock that survived or colonized the southern Balkans in the late Pliocene.

# Concordance between mtDNA variation and taxonomy

Our sampling overlapped the ranges of at least three subspecies (Fig. 1a). The nominative subspecies (including the morphologically similar L. v. schmidtlerorum) encompassed individuals with haplotypes from clades L, D and E (Fig. 2), but none of the oldest mtDNA clades. Thus, the current distribution of mtDNA diversity may reflect the history of fragmentation and range changes experienced by this, presumably derived, morphological form. The transfer of mtDNA between species has been well documented for L. vulgaris and L. montandoni (Babik et al., 2005; Zieliński et al., 2013). Here we demonstrate recurrent mtDNA introgression among subspecies of L. vulgaris inhabiting the Balkan Peninsula. The predominantly Italian L. v. meridionalis harbours mtDNA clade H, specific to this subspecies, but several individuals in northern Croatia carried haplotypes from clade L. These haplotypes are grouped in subclade L4, suggesting a single, not very recent mtDNA introgression event.

More extensive introgression is inferred for *L. v. graecus*. Of five mtDNA clades, C, M and K are unique to this subspecies, while clades L and D are shared with *L. v. vulgaris* (indicated by filled stars in Fig. 2). Haplotypes from the D and L clades present on the fringes of the *L. v. graecus* range are very similar or even identical to haplotypes found in the nominative subspecies in neighbouring areas; therefore they are likely to have derived from recent introgression. Introgression from *L. v. vulgaris* into *L. v. graecus* is also a probable explanation for the distribution of clade K; however, other alternatives have to be considered as well. Clade K

diverged > 1 Ma from its sister clade L. Potentially, its occurrence in L. v. graecus may be explained by incomplete sorting of ancestral polymorphism. This would imply that the divergence of L. v. vulgaris and L. v. graecus is not older than the split between K and L lineages and would require long term retention of divergent mtDNA (C, K and M) in L. v. graecus. A more likely explanation is introgression of K mtDNA from L. v. vulgaris. In this case lineages C and M would represent the ancestral mtDNA of L. v. graecus and mtDNA of the K clade would have been acquired during a range expansion by a process similar to that modelled by Currat et al. (2008) and demonstrated for Triturus newts in the Balkans (Wielstra & Arntzen, 2012). The K lineage may have been acquired recently and the donor L. v. vulgaris populations could be extinct, but it is also likely that introgression is older and the recent demographic expansion occurred in introgressed L. v. graecus populations. A third hypothesis is that genes underlying the L. v. graecus morphology adaptively introgressed into L. v. vulgaris populations harbouring K mtDNA. Under this scenario such populations would retain the mtDNA and most of the nuclear genome of L. v. vulgaris. The three hypotheses generate different predictions that may be tested with available nuclear markers (Zieliński et al., 2014). Under incomplete lineage sorting, large effective population size of L. v. graecus and recent divergence from L. v. vulgaris would be expected. If the origin of mtDNA clade K in L. v. graecus is the result of introgression, then nuclear genes would support older nuclear divergence and possibly some post-divergence gene flow. Finally, under the hypothesis of adaptive introgression, two major nuclear groups would occur in L. v. graecus, one of which would be closely related to L. v. vulgaris populations in the Balkans.

### CONCLUSIONS

The refugia within refugia hypothesis (Gómez & Lunt, 2007) holds for smooth newts in the Balkans. We found evidence for isolated populations in spatially distinct refugia in the southern and central parts of the peninsula. Consistent with the leading edge mechanism of post-glacial dispersal, we demonstrate that populations inhabiting the northern fringe of the Balkans took part in the most recent colonization of central and western Europe. Moreover, the inferred incursion of mtDNA clades associated with neighbouring subspecies at the north-western and south-eastern borders of the Peninsula is compatible with the idea of the Balkans as a crossroads for organisms inhabiting central Europe and Asia Minor, e.g. as suggested for mammals (e.g. Dubey et al., 2007). Our results suggest that the European clades originated in the Pliocene at the earliest, while climate change in the Pleistocene was probably the main trigger for subsequent mtDNA differentiation. The morphological differentiation observed in the four diagnosable subspecies in Europe (graecus, vulgaris, meridionalis and ampelenesis) is not consistent with mtDNA differentiation. None of these subspecies are reciprocally monophyletic in their mtDNA. Although this may represent incomplete lineage sorting, we found evidence for recent introgression of *L. v. vulgaris* mtDNA into *L .v. graecus*, and possible instances of past introgression events with *L. v. vulgaris* as the donor of mtDNA and *L .v. meridionalis* and *L. v. graecus* as recipients.

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### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Extended methods, primer sequences, Gen-Bank accessions and maximum likelihood tree for *ND2* dataset. **Appendix S2** Overview of *Lissotriton* fossils, BEAST settings and divergence times for mtDNA clades.

### BIOSKETCH

**Maciej Pabijan** is interested in the biology of amphibians and reptiles including their phylogeny, phylogeography and life history. The research team led by Wiesław Babik uses information from contemporary DNA sequences to infer the history of populations and closely related species.

Author contributions: W.B. and M.P. conceived the ideas; W.B., P.Z., K.D., M.C., K.S., M.L. and M.P. collected the data; M.P. and W.B. analysed the data; and M.P. and W.B. led the writing.

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