Evolutionary history of *Lissotriton helveticus*: Multilocus assessment of ancestral vs. recent colonization of the Iberian Peninsula

Ernesto Recuero *, Mario García-París

Museo Nacional de Ciencias Naturales (MNCN-CSIC), C/ José Gutiérrez Abascal, 2, 28006 Madrid, Spain

**Article Info**

Article history:
Received 13 December 2010
Revised 4 March 2011
Accepted 12 April 2011
Available online 22 April 2011

**Keywords:**
*Lissotriton helveticus*
Phylogeography
Glaciations
Refugia
Allopatric fragmentation
Range expansion

**Abstract**

The Pleistocene was characterized by climatic changes that greatly altered the distribution of organisms. Population extinctions, bottlenecks, isolation, range expansions and contractions were often associated with glaciations, leaving signatures in the spatial patterns of genetic diversity across species. *Lissotriton helveticus* belongs to a Pan-European lineage of newts that were strongly affected by glaciations and represent an excellent model to analyse the effect of generalized climatic changes in phylogeographic patterns. We studied the genetic diversity of the species using data from two mitochondrial and three nuclear genes analyzed in a Bayesian phylogenetic framework to investigate the historical processes shaping spatial patterns of genetic diversity. Mitochondrial haplotypes cluster in four different groups present in the Iberian Peninsula and of Pleistocene origin, probably by allopatric fragmentation. Nuclear genes present no obvious geographic structure patterns, suggesting gene flow and generalized incomplete lineage sorting. Populations north of the Pyrenees are closely related to those from northeastern Iberia, suggesting recent range expansion from this region. Historical demographic analyses indicate a demographic expansion starting about 100,000 years ago and more recent population declines. Compared to other *Lissotriton* species, *L. helveticus* includes only relatively young genetic lineages, suggesting a Central European pre-Pleistocene distribution followed by complete extirpation of the species during glaciations in that area. Historical demographic trends in the Iberian Peninsula are reversed with respect to the more Mediterranean species *Lissotriton boscai*, indicating different responses of both species to climate changes. Diversity patterns among *Lissotriton* species seem to be defined by four main factors: ancestral distributions, colonization capabilities, interactions with other species and effective population sizes. Differences in these factors define two types of species, referred to as “R” (refugia) and “S” (sanctuaries) that explain part of the diversity in patterns of genetic diversity created by glaciations in Western Europe.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

Climatic oscillations during the Quaternary have been crucial in the determination of present patterns of biological diversity, especially in the Holarctic region (Hewitt, 1996, 1999, 2000, 2001, 2004; Taberlet et al., 1998). On one hand they produced successive contractions and expansions of populations of different organisms, causing extinctions across broad parts of their ranges (Hewitt, 2000, 2004). Since the origins of many groups and species are much older, predating the Pleistocene (Klicka and Zink, 1997; Martínez-Solano et al., 2006; Ribera and Vogler, 2004; Zink and Slowinski, 1995), glacial periods often acted as an impoverishing force that reduced intraspecific diversity by population extinction and range reduction. On the other hand, glaciations favoured geographical isolation of populations in refugia, prompting lineage differentiation by allopatric fragmentation. During interglacial periods, more favourable environmental conditions allowed the expansion of quartered populations from their refugia into new territories, originating areas of secondary contact (e.g. Babik et al., 2005; Martínez-Solano et al., 2006; Sequeira et al., 2005). Although some generalized patterns have been depicted, including identification of common recolonization routes and clustering of secondary contact zones shared across studied taxa (Hewitt, 2004; Taberlet et al., 1998), some species present specific responses to climate oscillations that are usually reflected in peculiarities either in the recolonization processes or in the genetic and demographic structure within the main refugial areas.

The accumulation and comparison of numerous phylogeographic studies in a given area provides a valuable framework to infer common historical patterns, but also the processes and events that give rise to patterns of intraspecific genetic diversity (Buckley, 2009; Gómez and Lundt, 2006). In this context, the compilation of
data from closely related species that, sharing a common temporal evolutionary timescale, have been historically constrained by the same global events, is critical to determine the relative importance of species-specific responses in shaping patterns of intraspecific genetic structure.

The salamandrid genus *Lissotriton* is a group of five species of small newts distributed in the western Palearctic, from the Iberian Peninsula and the British Islands to western Siberia. Species in this genus are old, dating back to at least the Miocene (Babik et al., 2005; Rafinski and Arntzen, 1987; Steinfartz et al., 2007), so all of them have been under the effect of Quaternary climate changes. To date, and based on different molecular markers, there is detailed information about the evolutionary history of each of these species (Babik et al., 2005; Martínez-Solano et al., 2006; Ragghianti and Wake, 1986) except *Lissotriton helveticus*.

*L. helveticus* (Razoumovski, 1789) is a western European newt present from the northern half of the Iberian Peninsula to the West of the Czech Republic and also in most part of Great Britain (Raffaelli, 2007). It presents sympatric populations with other *Lissotriton* species: with *L. boscai* in northwestern Iberia and, more widely, with *Lissotriton vulgaris* in Great Britain and Central Europe. Hybrids with the latter species have been found sporadically but widely, with morphological surveys from populations in northern Iberia rejecting populations (García-París et al., 2004). However, more recent genetic structure. By a single sample, whereas for the rest we sampled 2–7 individuals, trying to represent most of the mitochondrial DNA (mtDNA) haplotype variation on a broad geographical scale (Fig. 1; Table 1). Our sampling is biased towards Iberian populations but is complemented by the data presented in Johanet et al. (in press), which includes multiple populations from France and England. A subset of this sampling representing all the mitochondrial groups and covering the general distribution of the species was used to obtain nuclear sequences from three different genes to compare with mtDNA genealogies (Table 1).

Total genomic DNA was extracted from ethanol preserved and frozen tissues (tail tips and liver) using a phenol–chloroform protocol (Sambrook and Russell, 2001), preceded by a digestion with proteinase K. Part of the samples corresponded to long time frozen (−80 °C), homogenized tissues used in a previous allozyme electrophoresis study (Montori, Llorente and Arano, unpublished data) that were extracted under the same protocol. Polymerase chain reaction (PCR) was used to amplify a total of 1355 base pairs (bp) of mtDNA corresponding to 644 bp of the mitochondrial Cytochrome Oxidase I gene (COX1), using the primers LCO1490 (Folmer et al., 1994) and PRO and PHE (Martínez-Solano et al., 2006). With respect to nuclear markers, we amplified 453 bp of the seventh intron of the beta fibrinogen gene (Fib), using the primers Fib-F and Fib-R (Nadachowska and Babik, 2009), 493 bp of the Chemokine receptor 4 (Cxcr4) gene using the primers Cxcr4-R and Cxcr4-F (Nadachowska and Babik, 2009) and 742 bp of an anonymous noncoding genomic DNA fragment (Tva4) using primers Tva4-F and Tva4-R (Nadachowska and Babik, 2009). For some samples we used internal primers to amplify 554 bp of the latter fragment. These primers are Tva4F-7 (5′-CCAGTCAGCAAGAG CTTAT-3′) and Tva4R-5 (5′-TGGTGTGCTCAGTCTTCTCT-3′).

PCR reactions were performed in a total volume of 25 μl, including one unit of Taq polymerase (Biotechnol., 5 U/ml), 2.5 μM of each primer, 0.4 mM of dNTPs, 17.5 mM of MgCl2, and 67 mM of a reaction buffer (Tris–HCl, pH = 8.3, Biotechnol). PCR reactions consisted of 35 cycles with a denaturing temperature of 94 °C (45 s), annealing at 56 °C (D-loop), 56 °C (COX1), 56 °C (D-loop), 56 °C (Tva4), 67 °C (Cxcr4) or 60 °C (Fib) (45 s), and extension at 72 °C (45 s). Double strand templates were cleaned using sodium acetate and ethanol to precipitate the PCR products and then re-suspended in 22 μl of ddH2O. Sequencing reactions were performed for both strands and sequenced on an ABI PRISM 3700 DNA sequencer (Applied Biosystems) following the manufacturer’s instructions and the conditions described in Martínez-Solano et al. (2006).

2. Materials and methods

2.1. Sampling and sequencing

We obtained tissue samples from a total of 100 individuals from 35 populations along the species’ range with special focus on the Iberian populations. Eight of these populations are represented by a single sample, whereas for the rest we sampled 2–7 individuals, trying to represent most of the mitochondrial DNA (mtDNA) haplotype variation on a broad geographical scale (Fig. 1; Table 1). Our sampling is biased towards Iberian populations but is complemented by the data presented in Johanet et al. (in press), which includes multiple populations from France and England. A subset of this sampling representing all the mitochondrial groups and covering the general distribution of the species was used to obtain nuclear sequences from three different genes to compare with mtDNA genealogies (Table 1).

Total genomic DNA was extracted from ethanol preserved and frozen tissues (tail tips and liver) using a phenol–chloroform protocol (Sambrook and Russell, 2001), preceded by a digestion with proteinase K. Part of the samples corresponded to long time frozen (−80 °C), homogenized tissues used in a previous allozyme electrophoresis study (Montori, Llorente and Arano, unpublished data) that were extracted under the same protocol. Polymerase chain reaction (PCR) was used to amplify a total of 1355 base pairs (bp) of mtDNA corresponding to 644 bp of the mitochondrial Cytochrome Oxidase I gene (COX1), using the primers LCO1490 (Folmer et al., 1994) and PRO and PHE (Martínez-Solano et al., 2006). With respect to nuclear markers, we amplified 453 bp of the seventh intron of the beta fibrinogen gene (Fib), using the primers Fib-F and Fib-R (Nadachowska and Babik, 2009), 493 bp of the Chemokine receptor 4 (Cxcr4) gene using the primers Cxcr4-R and Cxcr4-F (Nadachowska and Babik, 2009) and 742 bp of an anonymous noncoding genomic DNA fragment (Tva4) using primers Tva4-F and Tva4-R (Nadachowska and Babik, 2009). For some samples we used internal primers to amplify 554 bp of the latter fragment. These primers are Tva4F-7 (5′-CCAGTCAGCAAGAG CTTAT-3′) and Tva4R-5 (5′-TGGTGTGCTCAGTCTTCTCT-3′).

PCR reactions were performed in a total volume of 25 μl, including one unit of Taq polymerase (Biotechnol., 5 U/ml), 2.5 μM of each primer, 0.4 mM of dNTPs, 1.5 mM of MgCl2, and 67 mM of a reaction buffer (Tris–HCl, pH = 8.3, Biotechnol). PCR reactions consisted of 35 cycles with a denaturing temperature of 94 °C (45 s), annealing at 56 °C (D-loop), 56 °C (COX1), 56 °C (D-loop), 56 °C (Tva4), 67 °C (Cxcr4) or 60 °C (Fib) (45 s), and extension at 72 °C (45 s). Double strand templates were cleaned using sodium acetate and ethanol to precipitate the PCR products and then re-suspended in 22 μl of ddH2O. Sequencing reactions were performed for both strands and sequenced on an ABI PRISM 3700 DNA sequencer (Applied Biosystems) following the manufacturer’s instructions and the conditions described in Martínez-Solano et al. (2006).

2.2. Sequence alignment and phylogenetic analyses

Two closely related species, *L. vulgaris* and *L. boscai* were chosen as outgroups. Sequences of *L. vulgaris* were obtained in the
laboratory while those of L. boscai were obtained from GenBank [accession numbers DQ491890 (Martínez-Solano et al., 2006) and EF525956 (Smith et al., 2008)]. Sequences were compiled and revised using Sequence Navigator™ version 1.0.1 (Applied Biosystems) and aligned manually except the D-loop sequences that were aligned using the online version of MAFFT v.6 (Katoh and Kuma, 2002). Several nuclear sequences resulted in two alleles that differed at one or more sites. In these cases, we used the software Phase v2.1 (Stephens et al., 2001) to infer haplotype phase. All haplotypes were included in the alignments.

We performed Bayesian phylogenetic analyses with MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The optimal substitution models for each gene and partition were selected with ModelTest v3.7 (Posada and Crandall, 1998). Mitochondrial genes were analyzed together considering three different partition designs. First with two partitions using the best models for both genes. Second with three partitions using the best model estimated for D-loop, the best model for first and second codon positions of COX1 and the best model for the third positions of COX1. Third with four partitions using the best models estimated for D-loop and each codon positions of COX1. Nuclear genes were analyzed independently considering the best model estimated for each one. We initiated the analysis with random starting trees and ran four Metropolis coupled Monte Carlo Markov chains (three heated, one cold) for 20 millions of generations, sampling every 1000 generations. We checked for stationarity and convergence of the chains with TRACER 1.5 (Rambaut and Drummond, 2007) and discarded 2000 trees as burn-in. Posterior clade probabilities were used to assess nodal support. Additionally we constructed a haplotype network using the software TCS 1.2.1 (Clement et al., 2000), which follows the statistical parsimony algorithm described in Templeton et al. (1992). Ambiguities in the network were resolved after the recommendations in Pfenninger and Posada (2002).

We used BEAST v1.5.4 (Drummond and Rambaut, 2007) to estimate divergence times among clades. We used sequences of L. vulgaris, L. boscai and Mertensiella caucasica [GenBank accession number EU880321 (Zhang et al., 2008)] to calibrate the molecular clock. The age of the Lissotriton clade was set in 21 Mya with normal distribution and 1.0 as standard deviation (Martínez-Solano et al., 2006). The age of the root of the tree was set in 48 Mya with lognormal distribution and 1.0 as standard deviation (Martínez-Solano et al., 2006). For each time estimate, we show the mean and the 95% high posterior density interval. The analyses were run under an uncorrelated lognormal relaxed clock model and the Birth and Death process speciation tree prior with a length of

Fig. 1. (A) Geographic distribution of Lissotriton helveticus and localities sampled for this study North of the Pyrenees. (B) Localities sampled in the Iberian Peninsula. Populations are identified with numbers (see Table 1). Colours represent the four main mtDNA groups identified (blue: Northwestern; green: Asturian; yellow: East Cantabrian; red–orange: Northeastern).
chain of 250 millions sampled every 1000 generations. The analysis was performed twice and the results combined in tracer with a burn-in for each run of 25,000 trees.

2.3. Phylogeographic and historical demographic analyses

We tested the possible existence of “isolation by distance” (IBD) processes in the different mitochondrial lineages. For this we performed Mantel’s tests (Mantel, 1967) to analyse the relationships between genetic and geographical distances considering samples and groups of samples, in our case the main clades recovered in the phylogenetic and phylogeographic analyses. The matrices of geographical (km) and genetic (ML-corrected) distances were generated with Geodis v2.4 and PAUP v4.0b10 respectively (Posada et al., 2000; Swofford, 2002). To perform Mantel’s tests we used GENALEX version 6 (Peakall and Smouse, 2006), with 999 permutations to estimate the 95% upper tail probability of the matrix correlation coefficients. In the cases we found significant associations between genetic and geographical distances, the corresponding scatter plots were visually analysed, to untangle the effects of random genetic drift and gene flow in the geographical genetic structure (Hutchinson and Templeton, 1999).

We used analysis of molecular variance (AMOVA) (Excoffier et al., 1992) to characterize patterns of genetic variation at different hierarchical levels (individuals, populations, and the main groups characterized by their general geographic distribution and mtDNA lineages identified by phylogenetic analyses) as implemented by Arlequin v. 3.11 (Excoffier et al., 2005). Levels of significance of statistics characterizing variation at different hierarchical levels were assessed through 10,000 permutations.

The demographic history of the different L. helveticus lineages was inferred using different approaches. For a Bayesian coalescent approach (Drummond et al., 2005) we chose a Bayesian Skyline Plot (BSP) as our initial demographic model. BSP estimates a posterior distribution of effective population size through time using standard Markov chain Monte Carlo (MCMC) sampling procedures, calculating directly from the sequence data rather than from a predetermined genealogy, and offering credibility intervals for the given estimates (Drummond et al., 2005). This method was performed as implemented in BEAST v1.5, running 50 millions of generations. Burn-in and final BSP was determined with TRACER v1.5. The second approach were mismatch-distribution analyses of pairwise mtDNA differences (Slatkin and Hudson, 1991), comparing the observed distributions with that expected under a model of populations expansion, which are usually unimodal compared with the multimodal distributions of populations in equilibrium or that are subdivided (Martínez-Solano et al., 2007). The analyses were performed with Arlequin v3.11 (Excoffier et al., 2005) and the goodness-of-fit of the compared models was tested with 1000 bootstrap replicates. Finally, we also used Fu’s test of neutrality (Fu, 1997), searching for signals of population expansions in the analysed lineages, as implemented in DNASP v4.20.2 (Rozas et al., 2003).
3. Results

3.1. Phylogenetic analyses

A total of 100 specimens of _L. helveticus_ were studied through DNA sequencing. Details on the used mitochondrial and nuclear genes are given in Table 2. The combination of mtDNA sequences of _COX1_ (644 bp) and _D-loop_ (711 bp) data yielded 47 haplotypes. Some insertions and deletions were observed between _L. helveticus_ and the outgroups in the noncoding _D-loop_ sequences. For _COX1_ sequences we estimated the best substitution models for the different codon positions: TrN for the first positions, F81 for the second positions, TrN + I for first and second positions together and TrN for third positions. For nuclear genes we analyzed sequences from a subset of 39 individuals, with 453 bp for _Fib_, 493 bp for _Cxcr4_ and 742 bp for _Tva4_. All three genes are characterized by reduced variation in _L. helveticus_ except for a few divergent haplotypes. Sequences were deposited in GenBank and accession numbers are given in Table 1.

The different partition schemes tested in the phylogenetic analyses of the mtDNA data (see Section 2) did not show differences in topology and branch lengths. The observed structure in our phylogenetic reconstructions presents a peculiar distribution of haplotypes. On one hand we obtained three well-supported clades with in general, a well-defined geographic structure (Figs. 1 and 2; Supplementary material Fig. S1). On the other hand we find a group of haplotypes, all coming from populations from Asturias and northern León, that are not clustered together, but are distributed along the basal structure of the whole _L. helveticus_ clade (Figs. 1 and 2) and are central in the haplotype network (Fig. S1). Based on these results we defined four different geographic groups: the “Northwestern” group (blue in Figs. 1–3; S1), which includes samples from the north of Portugal and Galicia (populations 11, 12, 20); the “Asturian” group (green), formed by haplotypes found in Asturias and northern León (populations 7–10, 29); the “East Cantabrian” group (yellow), distributed mainly in Cantabria, Palencia and Burgos, but also in La Rioja and Soria (populations 13–17, 19, 26, 28, 34); and finally, the “Northeastern” group (red), found in the Northeast of the Iberian Peninsula and north of the Pyrenees to Great Britain and Germany (populations 1–6, 18, 21–27, 30–33, 35). The “Northwestern”, “East Cantabrian” and “Northeastern” clades were well supported by posterior probability values (Fig. 2). The divergence time estimates for mtDNA clades suggest a Pleistocene origin of the four main groups (all of them are recovered as well supported clades in BEAST analyses). The earliest split probably occurred around 1.28 (0.24–2.76) million years ago (Mya) and most of the nodes originated in the lower-middle Pleistocene. The age of the most recent common ancestor for the “Northwestern” clade dates back to about 0.42 (0.04–1.01) Mya, 0.55 (0.11–1.16) Mya for the “Cantabrian” group, 0.48 (0.1–1.05) Mya for the “Northeastern” and 1.0 (0.19–2.14) Mya for the “Asturian” group.

The topologies obtained with the three nuclear markers are conditioned by their low intraspecific variation and are characterized by a generalized pattern of incomplete lineage sorting. The _Tva4_ topology presents three main lineages (Fig. 3). One of them is formed by a single haplotype present in two not very distant populations, one form the “Asturian” group and other from the “Eastern Cantabrian” group. This lineage is the sister of a three haplotype group where the rest of the “Asturian” populations cluster together with the Portuguese ones and with some of the “Northeastern” populations. The third lineage is formed by haplotypes present both in the “East Cantabrian” and the “Northeastern” groups, although the most common haplotype is also present in a very distant population from Galicia.

The _Cxcr4_ topology presents three main lineages (Fig. 3) with no evident geographic pattern. The most common haplotype is present across the whole geographic range of the species, from Portugal to Germany.

The _Fib_ topology presents three main lineages (Fig. 3). One of them is formed by low frequency haplotypes present in the “Northeastern” populations, while the other two lineages present a mixed geographical composition. There are two predominant haplotypes. One is characteristic of “Asturian” populations, but is also present in Galicia and Cantabria. The other is widely distributed, from Portugal to southern Great Britain.

Although there is no evident geographic structure observed in nuclear sequences, there seems to be a weak pattern of incipient sorting of haplotypes in some of the “Northeastern” populations (Fig. 3).

3.2. Phylogeographic and historical demographic analyses

Except for the “East Cantabrian” group, we found significant relationships between geographical and genetic distances in all Mantel tests performed, both for all individuals of _L. helveticus_ and for the three remaining major lineages separately (Table 3). However, in all cases correlations are weak with coefficients ranging from 0.03 to 0.40, being highest in the “Northwestern” group that, with few samples included in the analysis, probably offers an incomplete picture of the population variation and structure in that region. Additionally, the scatter plots (Fig. 4) reflect a generalized lack of regional equilibrium, with drift apparently more influential than gene flow, similar to pattern III in _Hutchison and Templeton_ (1999), suggesting that other processes apart from isolation by distance have affected the genetic structure of the populations.

Results from AMOVA indicate that most of the observed variation among mtDNA lineages is related to differences between groups (76.16% of the total variance observed). Lower values were observed for variance related to differences among populations within groups (12.69%) and within populations (11.15%). All hierarchical components of genetic variation were highly significant (p < 0.0001).

Mismatch distributions were multimodal for the total data set and the “Northwestern” group, suggesting a history of population stability or subdivision, whereas they were clearly unimodal for the “Northeastern”, “Asturian” and “East Cantabrian” groups, which is indicative of population expansion (Fig. S2). However, in all cases, raggedness indices (r) were not significant, thus not rejecting the null hypothesis of stationarity. Fu’s Fs test was significant, again indicating population expansion, for the total data set and all groups except the “Northwestern” (Table 3). The result of the historical demographic reconstruction under the BSP model

<table>
<thead>
<tr>
<th>Gene</th>
<th>Substitution model</th>
<th>Variable/constant sites</th>
<th>Nucleotide frequencies A/C/G/T (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX1</td>
<td>TrN + I</td>
<td>258/386</td>
<td>24.93/26.88/18.86/29.33</td>
</tr>
<tr>
<td>D-loop</td>
<td>TrN + G</td>
<td>536/237</td>
<td>29.18/21.28/15.88/33.66</td>
</tr>
<tr>
<td>Fib</td>
<td>F81</td>
<td>38/415</td>
<td>35.53/20.63/19.74/24.1</td>
</tr>
<tr>
<td>Cxcr4</td>
<td>K81</td>
<td>20/473</td>
<td>Equal</td>
</tr>
<tr>
<td>Tva4</td>
<td>HKY</td>
<td>150/592</td>
<td>35.53/20.63/19.74/24.1</td>
</tr>
</tbody>
</table>
for the total sample dataset is shown in Fig. 5. When all individuals are analyzed together, we see that for the last million years the population size remained constant until about 100,000 years ago, when the populations started to experience a considerable demographic expansion that lasted for several thousands of years until it started to decrease in recent times. When we analyze the different groups separately, we find similar patterns of demographic expansions in all groups but the “Northwestern” one, which presents a relatively small but constant population decrease, although the small sample size in this particular group makes the results unreliable.

4. Discussion

4.1. Evolutionary history of L. helveticus

The phylogeographic structure of L. helveticus in the Iberian Peninsula presents a pattern that is superficially similar to the recurrent model of “refugia within refugia” (Gómez and Lundt, 2006) often found in the Mediterranean peninsulas (see for example Gonzalves et al. (2009) and references therein). Climatic fluctuations during the Quaternary probably played a key role in the origin of the main lineages in L. helveticus by favouring isolation and subsequent allopatric differentiation, as our results suggest. Based on our divergence time estimates, the original fragmentation of the main mtDNA clades in L. helveticus occurred around 400,000 to 1 million years before the present, from Lower to Middle Pleistocene. These are relatively recent events if we consider that the age of this species was estimated in about 20 millions of years (Babik et al., 2005) and that other Lissotriton species such as L. boscai and L. vulgaris present much older lineages that originated during the Pliocene and even the Miocene (Babik et al., 2005; Martínez-Solano et al., 2006), as is also the case in several other taxa with comparable distributions, e.g. Chioglossa lusitanica (Alexandrin et al., 2000, 2002), Salamandra salamandra (García-París et al., 1998, 2003), Alytes obstetricans (Gonçalves et al., 2006; Martínez-Solano et al., 2004), and Lacerta schreiberi (Paulo et al., 2001). This relatively shallow differentiation has relevant implications for the evolutionary history of L. helveticus, and represents
the basic difference with the mentioned “refugia within refugia” model.

One possible hypothesis to explain this pattern is that ancient lineages in the Iberian Peninsula have become extinct. Alternatively, ancient lineages could have never existed in this region. *L. helveticus* would have speciated during the Lower Miocene somewhere in Central or Western Europe outside the Iberian Peninsula. This situation is compatible with the fossil record, which includes remains assigned to the species in Middle and Upper Pleistocene deposits from Central Europe and Great Britain (Ashton et al., 1994; Green et al., 2006; Holman, 1998, 2000; Ivanov, 2007) and even Miocene remains found in sites north of the Pyrenees that have been identified as, or at least *affinis* to, *L. helveticus* (Rage and Bailon, 2005).

Following this scenario, the Iberian Peninsula would have been colonized, at the same time as other species like *Mesotriton alpestris* (Sotiropoulos et al., 2007) or *Rana temporaria* (Veith et al., 2003), not before the generalized cooling of the weather that started in the Pleistocene. The oldest fossil remains in this region come from Middle Pleistocene deposits (Sanchiz, 1987). The colonization of the Iberian Peninsula probably was facilitated not only by the changes in environmental conditions but also by the retreat of a competing species such as *L. boscai* into glacial refugia (Martínez-Solano et al., 2006). The phylogeographic pattern found in *L. helveticus* could therefore be explained assuming that one or several extinction events took place, probably associated with glacial maximum times, which eliminated most of the species’ populations except those present in refugial areas in the northern third

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Fs</th>
<th>$p$</th>
<th>$r$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>-11.1743</td>
<td>&lt;0.05</td>
<td>0.170</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Northeastern</td>
<td>-9.0064</td>
<td>&lt;0.0005</td>
<td>0.335</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>East Cantabrian</td>
<td>-4.2135</td>
<td>&lt;0.05</td>
<td>0.094</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Austrian</td>
<td>-3.2640</td>
<td>&lt;0.05</td>
<td>0.316</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Northwestern</td>
<td>2.1678</td>
<td>&gt;0.05</td>
<td>0.634</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

---

**Fig. 3.** Bayesian phylogenetic reconstructions of the three nuclear genealogies in *Lissotriton helveticus*. Posterior probability values are given for main clades. All sequenced individuals are represented to highlight allele frequency and the correspondence to mtDNA groups.

**Fig. 4.** Isolation by distance (IBD) plots (genetic distances vs. geographical distances, in kilometres) for the total sample dataset and including regression equations and correlation coefficients. Main groups show similar patterns when analyzed independently except the “East Cantabrian” group, with non-significant results (see Table 3).
of the Iberian Peninsula. The approximated location of these areas is coincident with refugia proposed for other species (Gómez and Lundt, 2006). One has been proposed along the Atlantic coast of the Peninsula, most likely in the northern half of Portugal, where refugia for species such as Chioglossa lusitana longipes (Alexandri-no et al., 2000, 2002), Lissotriton boscai (Martínez-Solano et al., 2006), Lacerta schreiberi (Paulo et al., 2001) or Podarcis bocagei (Pinho et al., 2007) existed. Another one would be located in the western Cantabrian mountains, as hypothesized also for other taxa like Salamandra salamandra bernardezi (García-París et al., 2003), Mesotriton alpestris cyreni (Sotiropoulos et al., 2007) or Lepus castroviejoi (Pérez-Suárez et al., 1994). A third refugia would be in the northeastern part of the Iberian Peninsula, maybe along the Ebro basin and the Mediterranean coast, or maybe associated to the complex orography of the Pre-Pyrenean ranges, as it is the case for species of the genus Calotriton (Carranza and Amat, 2005) and Pyrenean Iberolacerta (Carranza et al., 2004). A fourth one could have existed in the Sistema Ibérico Mountains, again a very complex area that served as a refugium for species like Pinus pinaster (Salvador et al., 2000) or Nebrioporus croceus (Ribera, 2003).

Molecular data allow us to determine the amount of genetic diversity that has persisted along a period of extinctions and, together with comparable data from closely related taxa, give us an idea of how much has been lost through the Pleistocene climatic change cycles. However it gives us little hints of the evolutionary history of the species in areas where it completely disappeared at a given time, as seems to be the case for L. helveticus. As it has been recently proposed (Araújo et al., 2008), species richness among amphibians and reptiles in Europe is strongly influenced by historic climate changes, which have affected specially narrow-ranging species probably because of their reduced dispersal capacity that prevents them to track climate changes, favouring endemcity in areas with more stable conditions (Jansson, 2003). The southward expansion of Polar conditions, thus, could have erased an amount of diversity at least equal to the diversity present today in the southern European peninsula.

Given our limited sampling outside the Iberian Peninsula we cannot reject the hypothesis of the persistence of some northern refugia for the species. However our data seems to indicate that populations of L. helveticus north of the Pyrenees originated from a rapid range expansion from the Iberian Northeast, most probably during the Holocene as a consequence of the amelioration of climatic conditions. This is in accordance with the genetic data presented by Johanet et al. (in press) and with previously proposed biogeographic hypotheses for this species (Zuidewijk, 1980). It also fits the general model proposed for postglacial recolonization of Western Europe (Schmitt, 2007; Taberlet et al., 1998). If we consider the limited dispersal ability of the species, this process probably took place as a contiguous range expansion. Again considering the poor capabilities of amphibians in general for oversea dispersal, the most likely way in which Great Britain was colonized was before the land bridge with the continent totally disappeared under the English Channel, 7500 years ago at the very latest (Lambeck, 1997; Martínková et al., 2007; Sanchiz, 2002), which is completely compatible with the Holocene expansion hypothesis. A full characterization, using fast evolving markers such as microsatellites, of the genetic diversity of L. helveticus outside the Iberian Peninsula is needed to complete our knowledge on the colonization routes and mechanisms of expansion of the species north of the Pyrenees.

4.2. Phylogeographic implications

The possibility that L. helveticus has expanded its distribution range in thousands of square kilometres in about 10,000 years raises several questions: why didn’t they expand to the south as well? Why is there so little introgression/admixture among mtDNA lineages? The answer to the first question may be related to the ecological requirements of the species, which include the cool and humid conditions typical of the Euro-Siberian phyto-climatic region and mountain habitats. Only a few populations of L. helveti-cus are known in Mediterranean habitats. These are often isolated and in precarious state of conservation and many populations from the Northern Plateau in central Spain have recently disappeared (Barbadillo, 2002). However in areas of favourable habitat, such as most of Galicia and northern Portugal, the species is not as common as expected. Here there is sympathy with the other Iberian species of the genus, L. boscai, which usually presents higher densities and is ubiquitously found. Apparently there is a progressive replacement of both species: as we move westwards and southwards L. helveticus is rarer than L. boscai, while L. helveticus...
becomes the dominant species as we move eastwards along the Cantabrian region. A similar pattern has been described between *L. helveticus* and *L. vulgaris* in Central Europe (Zuiderwijk, 1980), which could indicate there is some kind of competitive interaction among these species that prevents further expansions of their distributions, or at least not as fast as if there were no other competing species. The existence of wide areas of overlap between *L. helveticus* and *L. vulgaris* (Johannet et al., in press) could be associated with the timing of the range expansion process. If Central Europe and Great Britain were colonized by these two species at similar times, low densities in the newly formed populations probably allowed the simultaneous colonization and largely symmetrical distribution of both species in these regions.

The observed lack of admixture between mtDNA lineages is probably associated to the peculiarities of this molecular marker rather than to actual restrictions to gene flow among the populations. At present there is a continuous area with propitious habitat from the Pyrenees to northern Portugal and in these conditions moderately high levels of gene flow among populations is expected. A similar pattern has been described for *S. salamandra* in the same geographical area (García-París et al., 2003). According to mtDNA data, *S. salamandra* populations are strongly structured along the Cantabrian region, but this structure is lost when analyzed with nuclear markers. The persistence of ancestral mtDNA lineages in these situations of high nuclear admixture is usually explained by differences in the evolutionary dynamics between maternally inherited molecular markers such as mtDNA genes from those in the nuclear genome (Birky et al., 1989; Irwin, 2002). The effective population size for mtDNA is about four times smaller than for nuclear DNA when sex ratio is 1:1 and if the sex ratio is skewed towards males the mtDNA effective population size is progressively reduced (Birky et al., 1983). As a consequence, processes such as genetic drift are more marked at the mitochondrial level. The migration of mtDNA haplotypes could then be affected by the existence of large and already settled populations, especially if colonization of new areas is the result of contiguous range expansions, as our results suggest. In our case, the only population with mtDNA haplotypes from two different lineages is Camprovín, in the medium course of the Ebro River, giving additional support to the important role of this river as a dispersal vector for this species, allowing relatively long distance colonization and the admixture of ancestral lineages. Our nuclear results show a generalized lack of geographic structure among the observed variation. This is probably explained by incomplete lineage sorting in these markers but also by the existence of considerable gene flow among populations.

There is a shallow signal of lineage sorting in some populations of *L. helveticus* and *L. vulgaris* (Johannet et al., in press) could be associated with the timing of the range expansion process. If Central Europe and Great Britain were colonized by these two species at similar times, low densities in the newly formed populations probably allowed the simultaneous colonization and largely symmetrical distribution of both species in these regions.

The observed lack of admixture between mtDNA lineages is probably associated to the peculiarities of this molecular marker rather than to actual restrictions to gene flow among the populations. At present there is a continuous area with propitious habitat from the Pyrenees to northern Portugal and in these conditions moderately high levels of gene flow among populations is expected. A similar pattern has been described for *S. salamandra* in the same geographical area (García-París et al., 2003). According to mtDNA data, *S. salamandra* populations are strongly structured along the Cantabrian region, but this structure is lost when analyzed with nuclear markers. The persistence of ancestral mtDNA lineages in these situations of high nuclear admixture is usually explained by differences in the evolutionary dynamics between maternally inherited molecular markers such as mtDNA genes from those in the nuclear genome (Birky et al., 1989; Irwin, 2002). The effective population size for mtDNA is about four times smaller than for nuclear DNA when sex ratio is 1:1 and if the sex ratio is skewed towards males the mtDNA effective population size is progressively reduced (Birky et al., 1983). As a consequence, processes such as genetic drift are more marked at the mitochondrial level. The migration of mtDNA haplotypes could then be affected by the existence of large and already settled populations, especially if colonization of new areas is the result of contiguous range expansions, as our results suggest. In our case, the only population with mtDNA haplotypes from two different lineages is Camprovín, in the medium course of the Ebro River, giving additional support to the important role of this river as a dispersal vector for this species, allowing relatively long distance colonization and the admixture of ancestral lineages. Our nuclear results show a generalized lack of geographic structure among the observed variation. This is probably explained by incomplete lineage sorting in these markers but also by the existence of considerable gene flow among populations. There is a shallow signal of lineage sorting in some populations of the “Northeastern” group, but with differences among the three studied genes. Gene flow reduction, small population size, bottleneck, founder effects, could be some of the triggers for this incipient sorting. The results found in *L. helveticus* and other species (Irwin, 2002; Martínez-Solano et al., 2007), with differentiated mitochondrial lineages along continuously populated areas argues against the often urgent necessity to invoke the presence of physical barriers to explain the persistence in time of such patterns.

Within *L. helveticus*, estimated population sizes seem to have remained constant for most part of the last million years (Fig. 5). The absence of dramatic population size reductions could have also favoured the integrity of mtDNA lineages. Our results from historical demographic analyses suggest the existence of a population size expansion that started about 100,000 years ago, roughly corresponding with the last glacial age. This could be related, as suggested for other amphibians such as *Hyla intermedia* (Canestrelli et al., 2007), to an increase of favourable habitat for *L. helveticus*, but also with a retreat of potentially competing species like *L. boscai*, which, compared with *L. helveticus*, presents a reversed historical demographic model (as represented in Fig. 3 in Martínez-Solano et al. (2006)). The only group not showing expansion, but rather a progressive population size reduction is the Northwestern lineage, which supposedly shared refugia with *L. boscai*. The end of the last glacial period apparently affected population sizes in *L. helveticus* negatively in the Iberian Peninsula but favoured *L. boscai* (Martínez-Solano et al., 2006), which is probably associated to an expansion of Mediterranean conditions in the region and indicates the importance of autoecological traits in demographic responses to climate changes.

### 4.3. Diversity patterns in *Lissotriton*

Among the five species of *Lissotriton* we find three that present a restricted distribution, a priori reduced to the areas that served as refugia during the Quaternary: *L. montandoni* in the Carpathian Mountains, *Lissotriton italicus* in the southern half of the Iberian Peninsula and *L. boscai* in the western half of the Iberian Peninsula (Raffælli, 2007). A common pattern among these species is the lack of intraspecific morphological differentiation in their populations, which, together with their reduced distributions, could also imply a homogeneous genetic structure (Karron, 1987). However, *L. italicus* and specially *L. boscai* present deep patterns of genetic variation associated to past fragmentation of their ranges (Martínez-Solano et al., 2006; Raggiani and Wake, 1986). *L. montandoni*, in contrast, is characterized based on mtDNA (Babik et al., 2005) by extensive introgressive hybridization with its sister species, *L. vulgaris* (Babik and Rafinski, 2004; Babik et al., 2003; Kotlik and Zavadil, 1999; Litvinchuk et al., 2003; Mikulíček and Zavadil, 2008), although they are well differentiated when analyzed with nuclear markers, morphology and sexual behaviour (Babik et al. (2005) and references therein).

The other two species of the genus present much wider geographical distributions. *Lissotriton vulgaris* (Linnaeus, 1758) ranges from western Siberia and the Caucasus to Western Europe, including the British Islands but excluding the Iberian Peninsula and southern France (Raffælli, 2007). Through its range, high levels of morphological diversity have been described, concentrated mainly in the southern part of its distribution, a region where several glacial refugia could have existed, and seven or eight subspecies are usually recognized (Raffælli, 2007; Raxworthy, 1990; Schmidtler and Franzen, 2004). The characters used to describe this intraspecific diversity correspond basically to secondary-sexual characters, which are supposed to be directly influenced by sexual selection (Halliday, 1990). The existence of different phenotypic groups goes along with the existence of a high genetic diversity and a relatively deep phylogeographic structure, although it is not always concordant with the morphological variation, which can be explained by the existence of local selective processes (Babik et al., 2005). In contrast, *L. helveticus* concentrates most of its intraspecific variation in a small part of its range corresponding to the Iberian Peninsula populations.

Allopatric seems to be the principal force in the genesis of species within *Lissotriton*, as in most amphibians (Vences and Wake, 2007). Given the observed patterns of diversity, the current distributions, and the fossil record, the most likely origin of *L. boscai*, *L. italicus* and *L. vulgaris* is located in the Mediterranean peninsulas, while *L. helveticus* and *L. montandoni* originated most probably in Central Europe. Allopatric processes are also the main factor in generating the observed intraspecific diversity patterns (Babik et al., 2005; Martínez-Solano et al., 2006; Raggiani and Wake, 1986). Patterns of diversity, however, are also governed by additional factors:

The persistence of ancestral populations: diversity, both at specific and intraspecific levels, is concentrated in particular areas that represent a small part of the entire range of the genus, where long term stability of population distribution and size allow the mainte-
The capability of colonization: after Pleistocene glaciations much of Europe became a territory with optimal ecological conditions for *Lissotriton* species and virtually free of competitors. Under this situation both *L. helveticus* and *L. vulgaris* were able to expand their respective geographic ranges. In the case of *L. helveticus* this was probably a recolonization of ancestral territories for the species, while *L. vulgaris* probably profited from the absence of other *Lissotriton* species to expand outside the Balkans (Babik et al., 2005).

Interactions between species: the presence of a species in a given area seems to prevent the expansion of other species into that region. The presence of *L. vulgaris meridionalis* in the northern half of Italy might prevent the expansion of *L. italicus* from the southern half. *L. montandoni* is probably confined to its small range because its populations are surrounded by large populations of *L. vulgaris*. In the case of *L. helveticus*, further expansion both to the south and to the east might have been prevented by the presence of *L. boscai* and *L. vulgaris* respectively.

Effective population size: high population sizes will facilitate the persistence of populations and recolonization processes and will prevent possible replacement by other species. For example, a strong reduction of population size in *L. montandoni* could be related to the deep introgression with *L. vulgaris*, including the almost complete replacement of its mitochondrial genome (Babik et al., 2005). In this case, the evolution of reproductive isolation mechanisms could have prevented the complete extirpation of *L. montandoni*. On the contrary, large population sizes would have helped the persistence of old lineages in *L. boscai* (Martínez-Solano et al., 2006).

### 4.4. Biogeographic implications: sanctuaries vs. refugia

The Iberian Peninsula is the tip of the large European Peninsula and, following the principles of the Peninsular Effect (Simpson, 1964), should present a reduced diversity compared to areas closer to the "main continent" (Baquero and Tellería, 2001). However Iberia presents a high degree of endemicity and important diversity hotspots when compared with most parts of Europe, which contradicts this idea. The arrival of African taxa alone could hardly explain these differences so the question is why are endemicity and diversity higher in the Southern Peninsulas?

The phylogeographic patterns observed in the two Iberian *Lissotriton* species have been shaped by processes associated to climate changes during the Pleistocene. The resulting specific patterns conform to two different models (Fig. 6) that can be roughly applicable to many other European species and that are identifiable by their genetic signatures in mitochondrial lineages.

---

**Fig. 6.** Graphical representation of the phylogeographic models described in the text, from time 1, before climate changes, to time 4, when phylogeographic studies take place. Model “S” conforms to persistence of ancestral populations in areas within the original (ancestral) territory (represented by a grey-black zone). In this case, reduction of the distribution area and posterior range expansion is not necessarily mandatory. Model “R” conforms to species that suffered extinction in all ancestral territories. Past contraction of their distribution range is mandatory but not the posterior range expansion. Genetic diversity, larger in older populations, is represented by the intensity of dark colour. Arrows indicate direction of range expansion and white areas denote population extirpation due to climate change. High biodiversity levels in the European southern Peninsulas are promoted by the persistence of “S” and “R” species together, while only “R” species are present in central-northern Europe.
Species such as *Bufo calamita*, represent old inhabitants of the Peninsulas that persistently occupied at least parts of their ancestral ranges through the glacial cycles. In these cases, the existing populations would present traces of their long, independent evolutionary histories in that area, with deep phylogenetic lineages and genetic variation markedly structured geographically. Under this model, Pleistocene glaciations probably caused fragmentations and favoured lineage sorting, but allowed preservation of at least part of the accumulated ancestral diversity, creating, rather than refugia, sanctuaries of biological diversity. We refer to taxa under this model as type “S” (sanctuary) species. Other well-studied cases that fit this model include both geographically restricted species like *Chioglossa lusitanica* (Alexandrino et al., 2003; Steinfartz et al., 2000) or *Alytes obstetricians* (Gonçalves et al., 2006; Martínez-Solano et al., 2004). For most cases in model “S” species there are evidences of recent, big or small, range expansions after the last glacial maximum, but there are no evidences for range contractions during the Pleistocene, suggesting a certain stability of populations with migrations and range expansions limited geographically, maybe to altitudinal movements.

Species such as *L. helveticus*, represent taxa that colonized Iberia during the Quaternary in times of generalized climate changes. The cooler conditions characterizing the Pleistocene triggered southward range expansions in many Central and Northern European species. The newly colonized areas resulted in the precursors of truly glacial refugia, where only a small part of the populations could survive to generalized extinctions and most of the ancestral intraspecific diversity was lost under the ice, with the current diversification resulting from Pleistocene fragmentation and isolation in the refugia. We call them type “R” (refugia) species. In these cases a much shallower mitochondrial diversification is expected. Lineages would not probably be older than one million years and, in the case of widely distributed species, with large areas of genetically homogeneous populations. Several European taxa can be ascribed to this model, for example *Bufo calamita* (Rowe et al., 2006) or *Apodemus sylvaticus* (Michaux et al., 2003).

The complexity generated by the presence of sets of species representing each model in Iberia and other Mediterranean peninsulas results in a markedly higher diversity when compared to other regions in Central and northern Europe and gave rise to the "refugia within refugia theory" which, in fact, can be misleading. The relative contribution to the diversity of a region of “R” and “S” species can be a more useful indicator of the incidence of past glaciations and of the levels of biodiversity lost by climate changes.

It has already been suggested that neutral genetic diversity in populations is more affected by its position relative to historical refugia than to the core of the current range (Garner et al., 2004) and our data support this idea. Populations from recently colonized areas are often considered marginal and are neglected in conservation efforts because of their reduced genetic variation (Lesica and Allendorf, 1995). Nevertheless these populations can be precursors of future refugia under future global changes. Current trends in climate change threaten the persistence of many species in the Mediterranean region (Araújo et al., 2006) and the species that managed to expand their ranges further North may have more chances of survival in the near future. These “S” and “R” models can be applied not only to the Iberian but also to other Mediterranean peninsulas, suggesting that these areas acted not only as glacial refugia but as sanctuaries for diversity whose conservation is more affected by the recent human activity than by millions of years of ecological changes.

5. Conclusions

Almost all of the genetic diversity in *L. helveticus* is concentrated in the Iberian Peninsula. The analysis of mtDNA sequences reflects a geographically structured pattern that is maintained in spite of their continuous distribution in northern Iberia and the inferred existence of gene flow among the four main groups as indicated by nuclear markers. In accordance with previous morphological work (Galán Regalado, 1985) our molecular data do not support the subspecific division within *L. helveticus*.

All lineages in *L. helveticus* originated during the Pleistocene, probably during the last million years. These lineages are relatively recent if we consider that the origin of the species dates about 20 Mya and that within other Mediterranean species of *Lissotriton*, lineages originated during the Pliocene or even the Miocene. Our interpretation is that *L. helveticus* was originally a Central European species that colonized the Iberian Peninsula in the Middle Pleistocene, probably favoured by the generalized climate cooling. Glaciations eventually eradicated all ancestral lineages from its original geographical distribution but persistence of populations in southern refugia allowed the survival of the species with recent range expansions into favourable areas. The current distribution of the species in areas north of the Pyrenees is probably a consequence of a rapid Holocene expansion from the Iberian Peninsula.

*Lissotriton helveticus* belongs to a group of taxa that we denominate type “R” species, which endured changes during glacial periods sheltered in peripheral or even marginal populations in meridional areas and, consequently, lost a big part of their ancestral intraspecific diversity. In contrast, we also define a group of type “S” species, as *L. boscai*, whose ancestral distribution areas acted as sanctuaries and were not as severely affected by climatic changes as in type “R” species and that could thus retain most of their long time accumulated diversity. These types are defined by four main factors that govern the patterns of diversity observed among *Lissotriton* species: persistence of ancestral populations, capability of colonization, interspecific interactions and effective population sizes. These factors, and hence their associated species types can be generalized to many organisms in the Western Palearctic.

Acknowledgments

We thank friends and colleagues that provided samples or helped during the field work: R. Alonso, C. Martín, M. Moute Faria, X. Rubio. Special thanks to: W. Babik for outgroup taxa DNA; C. Pérez Collados, J. Sánchez Videgain and the rest of the people from the Asociación Naturalista de Aragón (ANSAR) for samples from the Zaragoza province; A. Montori and G. Llorente for their allozyme electrophoresis material; J.W. Armten, S. Caranza, M. Stöck and U. Scheidt for the crucial samples from outside the Iberian Peninsula. Thanks to I. Martinez-Solano for his help with sampling and for reviewing the manuscript. Thanks to D. Buckley for unpublished primer sequences, for his help with the analyses and for his comments on the manuscript. Thanks also to E. Albert and M. Alcobendas for their assistance in laboratory work. This work has been supported by Grants CGL2007-64621 and CGL2010-15786 from the Spanish “Ministerio de Ciencia e Innovación”.

Appendix A. Supplementary material

References

*Cochlia insignis* (Amphibia: Urodela). Heredity 88, 66–
74.

glacial refugia and postglacial recolonization in the golden-striped salamander, 


Babik, W., Rafinski, J., 2004. Relationship between morphometric and genetic variation in pure and hybrid populations of the smooth and Montandon’s newt (*Triturus vulgaris* and *T. Montandoni*). J. Zool. 262, 135–143.


Babik, W., Rafinski, J., 2004. Relationship between morphometric and genetic variation in pure and hybrid populations of the smooth and Montandon’s newt (*Triturus vulgaris* and *T. Montandoni*). J. Zool. 262, 135–143.


