ROMA TRE
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Phylogeography of two newts sub-endemic to the Italian Peninsula: *Lissotriton vulgaris meridionalis* and *Triturus carnifex* (Amphibia, Salamandridae)

Ph.D. Student: Michela Maura

Tutor: Prof. Marco A. Bologna

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Thesis Defense on the 25th February 2013 in front of the following jury:

Prof. James D. Harris (European committee member)
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European evaluators:
Dr. Catarina Pinho
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Table of contents

PREFACE

This PhD thesis is focused on the evolutionary history of two amphibians sub-endemic of the Italian Peninsula.

The introduction section briefly addresses the issues related to the effects of Pleistocene climatic oscillations on phylogeographic patterns of temperate species across the globe, in the Western Palaearctic, and in the Italian peninsula. Finally, I enunciate the aims of the PhD research project.

The Chapters 2, 3, and 4 are structured as independent manuscripts with their own introductions, materials and methods, results and discussion sections, but with a common reference list in the end of the thesis.

Chapter 2 is conform to the paper published in *PLoS ONE* (abstract not included): *Canestrelli D, Salvi D, Maura M, Bologna MA, Nascetti G (2012). One Species, Three Pleistocene Evolutionary Histories: Phylogeography of the Italian Crested Newt, Triturus carnifex. PLoS ONE 7(7): e41754.*

Chapter 3 is based on the submitted paper (abstract not included)*: Maura M, Salvi D, Bologna MA, Nascetti G, Canestrelli D. 'Northern purity' under minor revision: the Italian Smooth newt as a case study . Biological Journal of Linnean Society (submitted).*

Chapter 4 is based on the paper in preparation: *Alluvial plain expansions counterbalanced the negative demographic effects of glacial climate in the Italian crested newt, Triturus carnifex*. In this chapter an overview of the main hypothesis tested, the experimental design, and a brief discussion of preliminary results are presented.

The conclusion section briefly synthesizes and discusses the main outcome of this thesis in the light of the aims proposed.

ABSTRACT

In the western Palaearctic region phylogeographic patterns of temperate species have been investigated intensively. Nevertheless, as more phylogeographic studies become available, a high variability of temperate species responses to Pleistocene climatic oscillations continue to emerge. In this thesis, I investigated population genetic structure, phylogeography and demographic history of two newts subendemic of the Italian Peninsula, *Triturus carnifex* and *Lissotriton vulgaris meridionalis*.

Triturus carnifex showed three main parapatric and ancient mtDNA lineages distributed in three separated geographic districts, namely the Italian Peninsula, northern Italy and western Croatia/Slovenia. Multiple evidences indicate that these three main lineages have had independent evolutionary histories trough Pleistocene and that they have showed different responses to Pleistocene climatic oscillations encompassing multiple refugia along the Apennine chain, lowland refugia in large peri-coastal plains, and a 'cryptic' northern refugium.

On the other hand, *Lissotriton vulgaris meridionalis* showed ten allo-parapatric mtDNA lineages, likely of Middle Pleistocene origin, distributed in distinct geographic compartments across the Italian Peninsula and north Italy. The phylogeographic structure observed, time estimates of common ancestors and geographic distribution of each lineage suggested that *L. v. meridionalis* survived last glacial-interglacial cycles in several refugia, many of them located in north Italy and likely in peri-Alpine and peri-northern Apennines areas. Thus, for this species, a scenario of glacial survival of northern lineages at foothills of water-donating mountains would likely explain such a pattern of 'northern richness' observed.

When comparing the evolutionary histories of these newts with those of co-distributed temperate species, while the pattern of 'northern richness' showed by *L. v. meridionalis* is unprecedented, *T. carnifex* exemplified in one species most of the responses to Pleisotcene climatic cycles previously observed in temperate species from Italy. Interestingly, both species showed glacial survival in north of Italy, and together with previous evidences from phylogeographic and palaeo-environmental studies indicated that this area allowed long-term persistence of temperate species during Pleistocene glacial-interglacial cycles.

This study gave a contribution to unravelling the complex scenario of temperate species' responses to Plio-Pleistocene climatic oscillations, in the regional context of Italy. Moreover, the north of Italy has been identified as a key area to focus future investigation and to evaluate the role of peri-mountains and coastal refugia for the evolution of the Italian biota

General background

Since late Pliocene (around 3.6 million years ago –Mya-) the Earth has underwent severe climatic oscillations (Zachos et al. 2001; Ravelo et al. 2004). Glacial and interglacial periods have cyclically been alternating with an increasing amplitude from 41 thousand years (Ky), trough Late Pliocene and the Early Pleistocene (2.6 - 0.780 My), to 100 ky, during Middle (0.780 – 0.126 My) and Late Pleistocene (0.125 - 0.0117 My; Berger & Jansen 1994; Head & Gibbard 2005). Accordingly with the increase of amplitude in climatic oscillations, the Earth experimented the more severe and also better known ice ages. The glacial periods involved the spread of considerable polar ice sheets, glaciations in the major mountain blocks (e.g. Alps, Andes etc.), much lower temperatures, and globally reduced water availability (e.g. Ray & Adams 2001). As a consequence of the large volume of accumulated ice, the sea level dramatically drop down, up to about 120 m, producing land bridges in several coastal areas and archipelagos across the world (Lambeck et al. 2002). Palaeo-environmnental changes induced by these climatic oscillations were profound and had a great impact on the distributions and assemblages of species across the globe as well as on pattern of genetic diversity, as shown by fossil and pollen data and by phylogeographic studies (Hewitt 1996, 2000, 2011a and references therein). Species went extinct over large parts of their ranges, undergoing cycles of range contraction and expansion across latitude (e.g. in the Boreal and Temperate regions), altitude (e.g. in the mountain regions across the globe) or longitude (e.g. across temporary available areas as land bridges). Generally, species' responses to Plio-Pleistocene climatic oscillations were different according to the interplay between regional physiographic features and their ecological requirements (Hewitt 1996, 2011a).

In the western-Palaearctic region, temperate species underwent to cycles of range contractions southward in the three main Mediterranean Peninsulas- Iberia, Italy and the Balkans - and of range expansions northward, following the alternation of glacial and interglacial periods (Hewitt 2011b). These historical processes have left traces in the current pattern of species' genetic diversity. While southern Mediterranean regions exhibit higher genetic diversity, due to the prolonged demographic stability

of populations in these refugial areas, a lower genetic diversity is generally observed in the northern part of species ranges, as result of the repeated bottlenecks during the expansion phases (the 'southern richness - northern purity' paradigm, Hewitt 1996). Moreover, early data have showed that temperate species have shared common routes of postglacial colonization northward which can be referable to four main paradigms as described by Hewitt (1999) and Habel et al. (2005).

However, the growing literature of the last decade has reported several exceptions to the above mentioned general paradigms. These exceptions mainly regarded structure and location of glacial refugia, distances of post glacial colonization routes, and demographic trends experimented by populations during both glacial and interglacial periods. For instance, a considerable genetic substructure within the three Mediterranean peninsulas has emerged, highlighting a multiple-refugia scenario (Hewitt 2011b, and references therein). Accordingly, while the long term demographic stability in these refugia had a major role in the maintenance of the observed 'southern richness', the combined action of microevolutionary processes such as allopatric fragmentations, divergence in isolation and, possibly secondary admixtures, would better explain the formation of the high genetic diversity found in these areas (Schmitt et al. 2006; Gomez & Lunt 2007; Krystufek et al. 2007; Canestrelli et al. 2008, 2010). As another scenario emerging, glacial refugia for temperate species have been identified also in areas north to the 'classical' Mediterranean peninsulas, so-called the northern 'cryptic' refugia (Stewart & Lister 2001, Stewart et al. 2010; but also, extra-Mediterranean refugia, Schmitt & Varga 2012). These refugia were found in areas of sheltered topography that provided suitable microclimates for temperate species survival also at northern latitudes. The occurrence of these refugia in Central and Northern Europe has challenged the perspective of long dispersal routes northward, introducing different and shorter routes of postglacial colonization. Finally, demographic stability or even traces of demographic growth during glaciations have been reported for some temperate species inhabiting lowland habitats (e.g. Canestrelli et al. 2007; Canestrelli & Nascetti 2008; Porretta et al. 2011). These unexpected demographic trends would have been favoured by the increase in the extent of lowlands during the glacial-induced marine regressions, which counterbalanced the negative effect of the glacial climate, thus prompting 'glacial stability or even expansion' rather than 'glacial contraction' of refugial populations.

Altogether these phylogeographic, palaeobotanical and palaeoclimatic studies provide evidence for a wide plethora of species' responses to Pleistocene climatic oscillations and suggest that variation in the location of

glacial refugia, postglacial colonization routes, and demographic trends of temperate species may have been wider than previously thought.

Among the southern Mediterranean peninsulas, the Italian peninsula nicely exemplifies such a high variability of temperate species' responses. Although its young age did not allowed old genetic divergences within taxa, the severe local paleoenvironmental changes induced by Plio-Pleistocene climatic oscillations had a main role in shaping the high biodiversity observed in the Italian region. The glaciation of Alps, the formation of scattered glaciers along the Apennines, and paleoenvironmental changes related to climate induced sea-level oscillations seemed to have triggered those scenarios found in other areas of Europe and described above. For instance, a multiple refugia scenario along Italy has been observed in a growing number of amphibians, reptiles and mammals as well as in plants (see Canestrelli et al. 2010, Vega et al. 2010, Hewitt 2011b, and references therein). Most of these species underwent to cycles of allopatric fragmentations in multiple refugia in southern Italy as a consequence of the paleo-insularization of this area - i.e. formation of seaways in lowland areas - during Plio-Pleistocene marine transgressions (e.g. in the Volturno River, Crati-Sibari Plain, Cantanzaro Plain and Straits of Messina; see Hewitt 2011b). Many other species showed a phylogeographic structure with a southern, central and northern genetic component, likely related to major mountain blocks, suggesting long-term persistence in multiple refugia located across the entire Italy (e.g. Podnar et al. 2005; Canestrelli et al. 2007; Canestrelli & Nascetti 2008; Barbanera et al. 2009). As some of these studies hypothesized, the glacial survival of independent lineages in north of Italy would have occurred in coastal or peri-coastal areas (e.g. the Padano-Venetian plain and the Arno river plain; Canestrelli et al. 2007; Canestrelli & Nascetti 2008), which during glacials underwent to an increase in lowland extent and, where stable and suitable conditions for the glacial persistence of thermophilic taxa occurred, as reported by paleoenvironmental data (Amorosi et al. 2004; Ricci-Lucchi 2008). However, to date few studies with a dense sampling design in the north of Italy have been accumulated (e.g. Garner et al. 2004; Podnar et al. 2005; Ursenbacher et al. 2006; Canestrelli et al. 2007; Canestrelli & Nascetti 2008; Magri 2008; Barbanera et al. 2009). Thus, while these studies start challenging the early paradigm of 'southern richness – northern purity', there is still little knowledge on the contribution of the northern part of Italy to the long-term persistence of temperate species and to the assembly of Italian and European biota.

Aims

The main aim of this work was to contribute unravelling the complex scenario of temperate species' responses to Plio-Pleistocene climatic oscillations along the Italian peninsula. In particular, by developing a dense sampling design in the northern part of Italy, we aimed to shed light on the role of this area for the evolution of the Italian biota. In order to achieve these aims we investigated population genetic structure, phylogeography and demographic history of two amphibians, the Italian crested newt, *Triturus carnifex*, and the Italian smooth newt, *Lissotriton vulgaris meridionalis*. These two newts share particularly suitable features for the study of the genetic imprints of historical processes. Indeed, within their range (mainly spanning central and northern Italy), they are common and widespread across a wide environmental and altitudinal ranges, but they also show phylopatric habits and poor dispersal abilities (Smith & Green 2005; Razzetti & Bernini 2006; Jehle et al. 2011). The results found for each species were then compared with those previously found for co-distributed temperate species, in order to identify shared patterns and major processes in the evolution of the Italian biota.

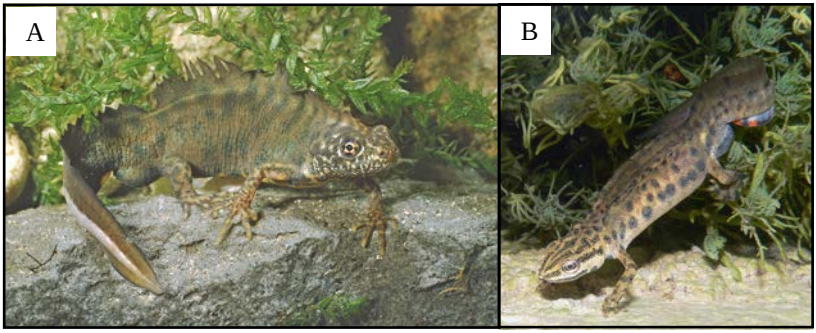


Figure 1. A: a male of the Italian crested newt, *Triturus carnifex*. B: a male of the Italian smooth newt, *Lissotriton vulgaris meridionalis*.

CHAPTER 2

One species, three Pleistocene evolutionary histories: phylogeography of the Italian crested newt, *Triturus carnifex*

INTRODUCTION

Identifying Pleistocene refugia is a central task of phylogeographical research (Waltari et al. 2007; Stewart et al. 2010; Hewitt 2011a, and references therein), and ongoing climate change has led to increased interest in the identification and characterization of these areas (Keppel et al. 2012). Glacial refugia allowed species to survive during unfavourable climatic phases of the Pleistocene and are often hotspots of current intraspecific diversity. Recent studies have shown that a plethora of microevolutionary processes encompassing demographic size variations, population fragmentations, secondary contacts, and population admixture acted in glacial refugia and contributed to shape the high refugial genetic diversity (Hewitt 2004, 2011a, 2011b; Gomez & Lunt, 2007; Byrne et al. 2008; Wang et al. 2009; Canestrelli et al. 2010, 2012a; Shafer et al. 2010). Therefore, glacial refugia are important both for long-term preservation of species and for their evolutionary potential. Moreover, evidence supporting the importance of genetic diversity with respect to community structure and ecosystem resilience is growing (Whitham et al. 2006; Hughes et al. 2008). Thus, assessing the geographic location of glacial refugia and understanding what microevolutionary processes were involved in the formation and long-term maintenance of genetic diversity hotspots in these areas have crucial relevance with respect to conservation at multiple levels of biodiversity (Hampe & Petit 2005; Vandergast et al. 2008; Keppel et al. 2012). Interestingly, in many regions worldwide, glacial refugia for temperate species have been identified in coastal lowlands (e.g. Burns et al. 2007; Marske et al. 2009; Bisconti et al. 2011; Porretta et al. 2011) and/or at the so-called ‘rear edges’, the current low-latitude margins of species’ ranges (e.g. Canestrelli et al. 2006; Larmuseau et al. 2009; Provan & Maggs, 2011; Recuero & Garcia-Paris 2011). Populations in these areas are expected to be especially threatened by climate change, rising special concerns for their long-term conservation (see Hampe & Petit 2005; Araujo et al. 2006;

Parmesan 2006; and Keppel et al. 2012, for a thorough discussion of the genetic consequences of recent climate change).

The western Mediterranean region has been the subject of intensive phylogeographical efforts (Hewitt 2004, 2011b; Petit et al. 2005; Nieto Feliner 2011). The Balkan, Iberian, and Italian peninsulas have long been identified as the most important refugial areas in the region, but as more studies were performed, increasingly complicated patterns appeared. For example, within these three peninsulas, a scenario of multiple refugia is emerging for a growing number of species (see Hewitt 2011b, and references therein). While in many cases long-term demographic stability can explain the occurrence of intraspecific diversity hotspots in these areas, in many others repeated cycles of population fragmentation and secondary admixture were involved (e.g. Gonçalves et al. 2009; Previšić et al. 2009; Canestrelli et al. 2010, 2012a). Moreover, peri-glacial areas and coastal lowlands exposed by glaciation-induced sea-level lowstands are emerging as important, previously underrated refugia, which allowed some species to overcome the negative demographic effects of glacial climate (Bisconti et al. 2011). On the whole, these studies reveal that the species' responses to Pleistocene climatic oscillations were even more diverse than previously thought (Stewart & Lister 2001; Hewitt 2004; Gomez & Lunt 2007; Schmitt 2007; Maggs et al. 2008; Rull 2009; Bisconti et al. 2011; Schneeweiss & Schonswetter 2011).

In this study, we examined the phylogeography of the Italian crested newt, *Triturus carnifex*. This amphibian belongs to the *T. cristatus* superspecies, a species group distributed in Europe and western Asia (reviewed in Jehle et al. 2011). *T. carnifex* is common and widespread from sea level to about 1000 m a.s.l. (Andreone & Marconi 2006, but see also Giacoma et al. 1988) where it breeds in a wide range of freshwater habitats (Giacoma et al. 1988; Pavignano et al. 1990; Ficetola & De Bernardi 2004). *T. carnifex* is generally considered a poor disperser with short migration distances, generally less than 1 Km / year (Arntzen & Wallis 1991; Jehle et al. 2011; but see also Schabetsberger et al. 2004). It is mainly distributed in the Italian peninsula south of the Alpine arc, reaching East Slovenia, northern Croatia and Austria (Jehle et al. 2011; Figure 1C). Interestingly, it is absent in most of the central and southern Calabria, an area that has been repeatedly indicated as the most important glacial refugium for temperate species in the Italian peninsula (see Canestrelli et al. 2010, 2012a; Vega et al. 2010, and references therein).

Previous studies based on allozyme markers (Scillitani & Picariello 2000; Arntzen 2001) suggested that the genetic diversity of *T. carnifex* populations is geographically structured into two population groups distributed north and south of the north-central Apennine chain. Thus, both

the geographic pattern of distribution and early evidence of population structure suggest that the Plio-Pleistocene evolutionary history of *T. carnifex* could be unique, not conforming to the scenario inferred for most temperate species along the Italian peninsula, i.e. a (more or less) fragmented glacial range encompassing the Calabrian region (the main refugium in southern Italy) and a postglacial northward expansion along the peninsula (e.g. Canestrelli et al. 2006, 2008). Therefore, in this study we carry out phylogenetic, molecular dating and population structure analyses in order to investigate the evolutionary history of this newt. Finally, through a comparison with previous studies of co-distributed species, we put in evidence novel patterns of phylogeographic concordance and their significance for the evolution of regional biotas.

MATERIALS AND METHODS

Ethics statement

The Italian Ministry of Environment approved all animal procedures in this study, including capture, handling, and tissue sampling (DPN/2D/2003/2267). Since the study did not involve laboratory work on living animals, authorization from the Ministry of Health was not required. Newts were captured with nets at breeding ponds. We collected tissue samples from tail tips, anaesthetizing newts by submerging them in a 0.1% solution of MS222 (3-aminobenzoic acid ethyl ester). Immediately after the completion of the procedure, tissue samples were stored in 96% ethanol and all newts were released at the collection site. No newts were brought to the laboratory and no newts were sacrificed. All sampling took place in public areas and no additional permits or approvals were required for these sites.

Sampling and laboratory procedures

A total of 231 *Triturus carnifex* individuals were sampled from 37 localities spanning the species' range. Detailed information about sampling localities and number of individuals sampled in each locality are shown in Table 1 and Figure 1C. DNA extraction was performed by following the standard cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1987). Two mitochondrial fragments were amplified and sequenced for all individuals. One fragment comprised part of the NADH dehydrogenase subunit 4 gene and the $tRNA^{His}$ gene (hereafter referred to as ND4), and the other fragment comprised the NADH dehydrogenase subunit 2 gene (hereafter referred to as ND2). Preliminary amplifications and sequencing of the ND4 fragment were performed using primers ND4 and

LEU (Arévalo et al. 1994), and then the internal primers ND4carnF1 (ACCCCATTAACAAAAGAAATAGCA) and ND4carnR2 (GTGTTTCATAACTCTTCTTGGTGTG) were designed and used to screen all individuals. Preliminary amplifications and sequencing of the ND2 fragment were performed using primers H5018 and L3780 (Babik et al. 2005), and then the internal primer TCND2F2 (TCCTTGCTTGAATAGGACTAGAAAT) was designed and used in conjunction with H5018 to screen all individuals.

Amplifications were performed in a 25 µl volume containing MgCl2 (2.5 mM), reaction buffer (5×, Promega), the four dNTPs (0.2 mM each), the two primers (0.2 µM each), the enzyme Taq polymerase (1U, Promega) and 2 µl of DNA template. Polymerase chain reaction (PCR) was performed with a step at 95 °C for 5 min followed by 30 (ND4) or 35 (ND2) cycles of: 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and a single final step at 72 °C for 10 min. Purification and sequencing of the PCR products were carried out by Macrogen Inc. (www.macrogen.com) by using an ABI PRISM 3700 sequencing system. All sequences were deposited in GenBank (accession numbers: JQ598071-JQ598166).

Data analysis

Electropherograms were visually checked using FinchTv 1.4.0 (Geospiza Inc.) and aligned using Clustal X 2.0 (Larkin et al. 2007). MEGA5 (Tamura et al. 2011) was used to analyse sequence variation.

Phylogenetic relationships among *T. carnifex* haplotypes were inferred using Maximum Likelihood (ML) and Maximum Parsimony (MP) methods. For these analyses, the closely related species *T. macedonicus* was used as outgroup (GenBank accession number NC015794).

The best-fit model of nucleotide substitution for our dataset was selected among 88 alternative models using the Akaike Information Criterion (AIC, Akaike 1973) implemented in jModelTest 0.1.1 (Posada 2008). We first analysed the ND4 and ND2 fragments separately, and then combined. TIM1+Γ (Posada 2003) was the best fit model in all cases. Consequently, the combined dataset (with the gamma distribution shape parameter = 0.10) was used in all subsequent analyses.

ML analyses were performed with PhyML 3.0 (Guindon et al. 2010). Tree topologies were estimated using the SPR&NNI option, which performs both the available methods [i.e the Nearest Neighbor Interchanges (NNI), and the Subtree Pruning and Regrafting (SPR)] and returns the best solution among the two. MP analysis was computed using PAUP (Swofford 2003), with all characters equally weighted and unordered. A heuristic search was carried out, with tree bisection and reconnection (TBR) branch swapping

Table 1. Geographic location, number of individuals (n) and haplotype composition of the 37 populations sampled of *Triturus carnifex*.

Population	Country	Locality	Latitude (N)	Longitude (E)	n	Haplotypes (n)
1	Italy	Cecita	39°22'	16°30'	**6**	**SV23 (1), SV24 (5)**
2		San Pietro in Guarano	39°20'	16°21'	**9**	**SV24 (9)**
3		Laghicello	39°25'	16° 5'	**6**	**SIV26 (6)**
4		Due Uomini	39°33'	16° 1'	**15**	**SIV25 (4), SIV26 (5), SIV27 (6)**
5		Taverna Magnano	40°03'	16° 07'	**6**	**SIII29 (1), SIII30 (5)**
6		Alberobello	40° 49'	17° 14'	**3**	**SII28 (3)**
7		Conza	40° 52'	15° 18'	**7**	**SII21 (7)**
8		Torre Palermo	41° 50'	16° 2'	**15**	**SI22 (15)**
9		Sepino	41° 23'	14° 34'	**1**	**SI32 (1)**
10		Sessano	41° 37'	14° 20'	**9**	**SI33 (4), SI34 (5)**
11		Campo di Mele	41°23'	13°31'	**7**	**SI12 (1), SI13 (6)**
12		Circeo	41° 20'	13° 2'	**5**	**SI14 (2), SI15 (3)**
13		Doganella	41°45'	12°47'	**7**	**SI17 (1), SI19 (6)**
14		Jenne	41°52'	13°11'	**7**	**SI16 (1), SI17 (4), SI18 (2)**
15		Rocca di mezzo	42° 9'	13°38'	**5**	**SI9 (2), SI10 (1), SI17 (2)**
16		San Quirico	42° 26 '	13°50'	**7**	**SI8 (1), SI22 (6)**
17		Campo Imperatore	42°25'	13°37'	**7**	**SI17 (7)**
18		Navegna	42° 9'	13° 2'	**11**	**SI11 (1), SI17 (10)**
19		Alviano	42°37'	12°14'	**7**	**SI6 (1), SI7 (2), SI19 (3), SI20 (1)**
20		Rufeno	42°46'	11°53'	**2**	**SI19 (1), SI20 (1)**

Table 1 (continued). Geographic location, number of individuals (n) and haplotype composition of the 37 populations sampled of *Triturus carnifex*.

Population	Country	Locality	Latitude (N)	Longitude (E)	n	Haplotypes (n)
21		Roccalbegna	42°44'	11°30'	**8**	**SI1 (2), SI19 (5), SI20 (1)**
22		Greve in Chianti	43°35'	11°18'	**8**	**SI3 (1), SI4 (6), SI5 (1)**
23		Firenze	43°45'	11°15'	**6**	**SI2 (3), SI3 (2), SI19 (1)**
24		San Severino Marche	43° 18'	13° 4'	**8**	**CI13 (2), SI31 (6)**
25		Senigallia	43°41'	13°12'	**5**	**CI12 (1), CI13 (4)**
26		Terra del sole	44°11'	11°57'	**5**	**CI13 (5)**
27		Mulino di Pianoro	44°21'	11°19'	**5**	**CIII10 (2), CIII11 (3)**
28		Minucciano	44°10'	10°12'	**2**	**CIII10 (2)**
29		Stagno Bargone	44°19'	9°29'	**5**	**CII5 (3), CII6 (2)**
30		Piampaludo	44°27'	8°34'	**2**	**CII9 (2)**
31		Donega	44°56'	9°14'	**5**	**CII7 (2), CII8 (3)**
32		Montevecchia	45°43'	9°23'	**5**	**CII9 (5)**
33		Porto Caleri	45° 5'	12° 19'	**4**	**CII3 (3), CII4 (1)**
34		Le Poscole	45° 36'	11° 23'	**5**	**CI2 (5)**
35		Busa de San Piero	45°48'	12°08'	**3**	**CI1 (3)**
36	Slovenia	Komen	45°49'	13° 45'	**5**	**N1 (5)**
37	Croatia	Svetvinčenat	45° 4'	13° 52'	**5**	**N2 (5)**

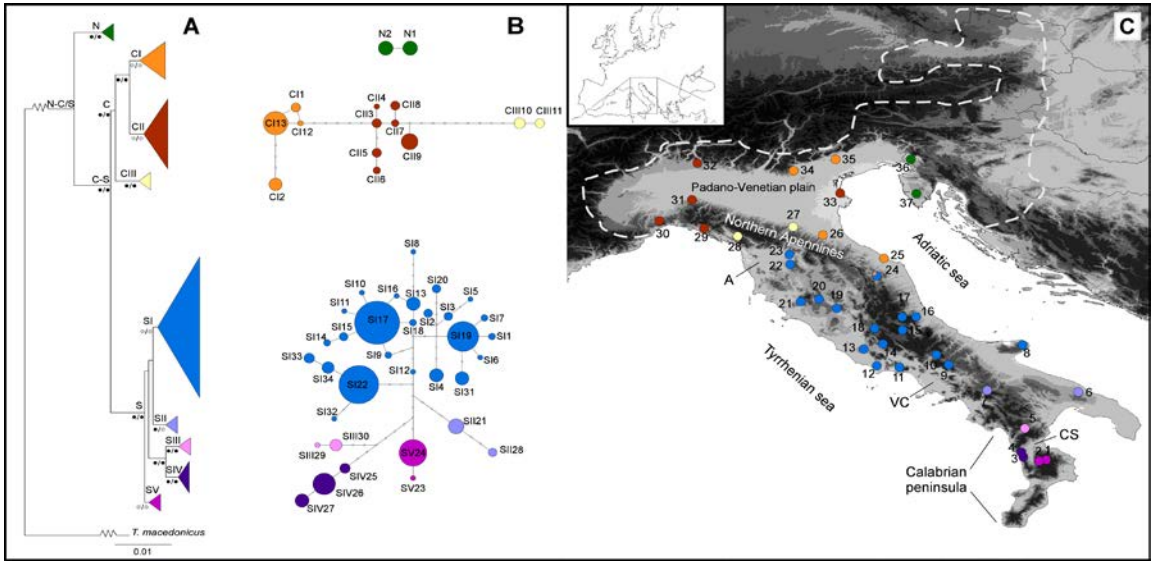


Figure 1. Geographic distribution of the 37 sampling sites and phylogenetic relationships of the 49 haplotypes found in *Triturus carnifex*. A, Maximum likelihood (ML) tree showing the phylogenetic relationships among the 49 haplotypes found in *Triturus carnifex*. Terminal haplogroups were collapsed. Clade names and bootstrap (bs) values of ML and Maximum parsimony (MP) trees (ML/MP), respectively, are shown above and below each node (grey circles: bs > 70%; black circles: bs > 85%). B, Statistical parsimony networks, with haplotypes numbered as in Table 1. Circles size is proportional to haplotype frequency; open dots represent missing intermediate haplotypes. C, Geographical distribution of the 37 populations sampled. Populations (shown as pie diagrams) are coloured according to the main haplogroups in panels A and B. White dotted line shows the northern edge of the species' distribution; A, Arno river basin, CS, Crati–Sibari plain, VC, Volturno–Calore river drainage basin. Inset: Geographical location of the study area within the western Palearctic region.

and 10 rounds of random sequence addition. The robustness of the inferred ML and MP tree topologies was assessed by the non-parametric bootstrap method with 1000 replicates.

Phylogenetic relationships among haplotypes were also inferred by the statistical parsimony procedure for phylogenetic network estimations (Templeton et al. 1992) by using the software TCS 1.2.1 (Clement et al. 2000).

Time to the most recent common ancestor (TMRCA) of the main mtDNA lineages was estimated by using the distance-based least squares (LS) methods recently described by Xia & Yang 2011 and implemented in the software DAMBE (Xia & Xie 2001). The hypothesis of clock-like evolution of our sequences was assessed by performing a likelihood ratio test in DAMBE. This test did not reject the molecular clock hypothesis for our dataset. To specify a tree topology we used the ML tree previously estimated by PhyML. The divergence between *T. carnifex* and *T. macedonicus* was used to set a calibration point. According to Arntzen et al. (2007), this divergence was estimated to date back to the end of the Messinian salinity crisis and the consequent reflooding of the Adriatic Sea [5.337 million years ago (Ma)]. Finally, to perform the LS analysis in DAMBE we set the 'softbound' option and 'MLCompositeTN93' genetic distance, as suggested by Xia & Yang (2011), along with 1000 bootstrap re-samplings to obtain standard deviations of the time estimates.

To understand how genetic variance was hierarchically distributed among groups, among populations within groups, and within populations, we performed the analysis of molecular variance (AMOVA) by using Arlequin 3.5.1.2 (Excoffier & Schneider, 2005). Groups were defined a priori, according to the main geographic discontinuities in the distribution of genetic variation, as defined by previous phylogenetic analyses. The analysis was run using the Tamura & Nei (1993) model (TrN+Γ), which is the best approximation of the TIM1+Γ model available in ARLEQUIN. The significance of the variance components and fixation indices was tested using 10100 permutations.

To assess the occurrence of a significant pattern of isolation-by-distance, the correlation between geographic and genetic distances separating populations was evaluated using Mantel tests with the software ZT (Bonnet & Van de Peer 2002). Following suggestions by Rousset (1997), geographic distances were log-transformed, and genetic distances were estimated as the mean distances among populations calculated with MEGA by using the TrN+Γ model. Mantel tests were performed for the entire data set and for each main clade defined by previous phylogenetic analyses, along 1000 bootstrap replicates.

RESULTS

For all individuals analysed the ND4 fragment was 638 bp in length, comprising 563 bp of the (3') NADH dehydrogenase subunit 4 gene and 75 bp of the $tRNA^{His}$ gene, and the ND2 fragment was 635 bp. The combined dataset (overall 1273 bp) included 128 variable positions, of which 70 were parsimony informative. We did not find indels or stop codons within the coding region of either the ND2 or the ND4 fragments. A total of 49 haplotypes were found in the combined fragment, and their geographic distribution is presented in Table 1.

The tree obtained by the ML method is shown in Figure 1A. The log-likelihood score for the ML tree was −3853.93018. MP analysis yielded 1324 most parsimonious trees of 186 steps in length (consistency index = 0.715; retention index = 0.869). Tree topologies were identical between MP and ML trees at main nodes, with minor differences at some terminal nodes. Three main clades were found, and their geographic distribution among populations is shown in Figure 1C. One clade (referred to as clade N) included only two haplotypes and was geographically restricted to north-eastern samples 36 and 37. The second clade (referred to as clade C) was found among samples from the Padano–Venetian plain and northwestern Apennines (samples 24–35), and the third clade (referred to as clade S) was widespread throughout the reminder of the species' range along the Italian peninsula (samples 1–24). Average Tamura–Nei sequence divergence among the three clades was 0.029 (standard error (SE) 0.006) for the clade pairs N–C and N–S, and 0.021 (0.004 SE) between clades C and S. Co-occurrence among these main clades was observed only in sample 24 (clades C and S). Three main subclades (referred to as CI, CII, and CIII) were observed within clade C, but they showed no clear geographic pattern of distribution (Figure 1 and Table 1). Instead, five subclades within clade S showed a clear geographic association. Subclades SIII, SIV, and SV were restricted in the Calabrian peninsula (samples 5, 3–4, and 1–2, respectively). Subclade SII (samples 6–7) was distributed north of this area to the Volturno–Calore basin. Finally, subclade SI (the most frequent in the dataset) was widespread throughout the remainder of the Italian peninsula. All of the above clades and subclades were supported by high bootstrap values (>70%).

Phylogenetic networks among the haplotypes found are shown in Figure 1B. Under the 95% criterion for a parsimonious connection, three distinct networks were generated (N, C, and S), and they corresponded to the three main clades of the phylogenetic trees. The haplotypes of networks C and S formed three and five subgroups, respectively, clearly corresponding to the subclades yielded by the tree-building methods.

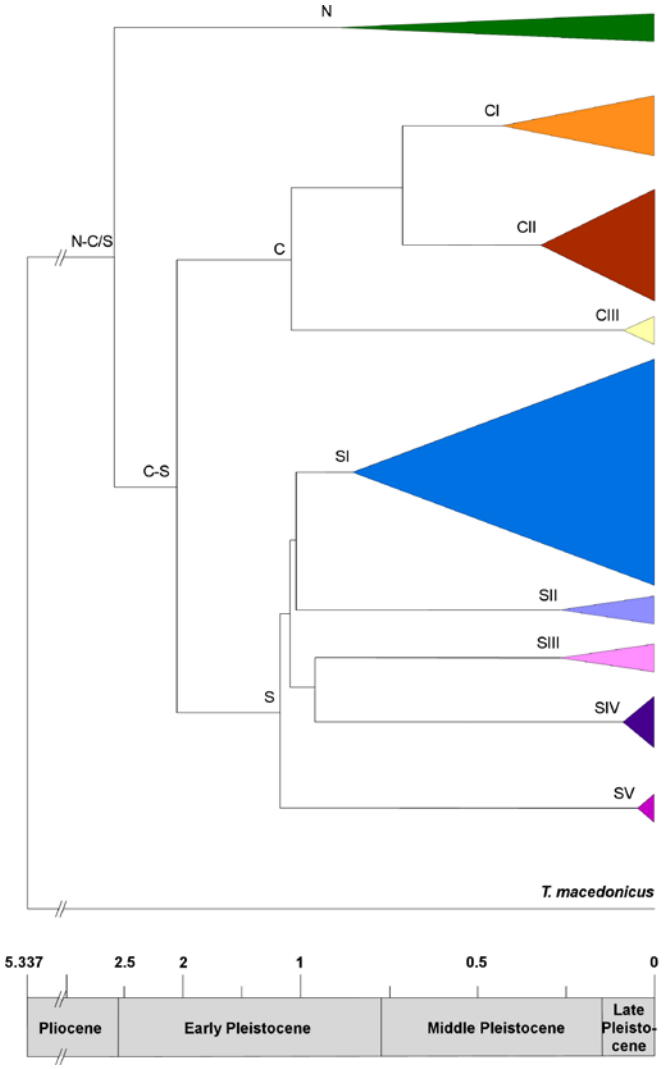


Figure 2. Chronogram of the main mtDNA lineages found in *Triturus carnifex*. Chronogram showing the estimated times to the most recent common ancestor (TMRCA) for the main mtDNA lineages of *Triturus carnifex*. The calibration point (5.337) and the ranges of the main historical epochs on the scale bar are reported in million years. Clades were named as in Figure 1A.

TMRCA estimates for the main mtDNA lineages are shown in the chronogram of Figure 2. The TMRCA for the entire ingroup was estimated to have occurred between the Late Pliocene and Early Pleistocene (2.619 ± 0.426 Ma), and the divergence between clades C and S fell well within the Early Pleistocene (2.049 ± 0.364 Ma). Finally, most of the splits within these clades likely occurred late in the Early Pleistocene.

For the AMOVA analysis the following seven groups were defined (see Figure 1): (1-2), (3-4), (5), (6-7), (8-24), (25-35), (36-37). Since the three subclades found within clade C did not show a clear geographic structure (see above), all individuals carrying haplotypes from clade C were assigned to a single group (25-35). This analysis showed that 70.59% of the overall genetic variance can be attributed to differences between groups, 24.40% to differences among populations within groups, and 5.01% to differences within populations. All the covariance components were highly significant (Table 2).

The Mantel tests performed between genetic distances and log-geographic distances suggested the occurrence of a statistically significant ($P < 0.01$) but weak pattern of isolation by distance, both within the entire dataset ($R^2 = 0.29$) and within the range of clade S ($R^2 = 0.31$). No significant pattern of isolation by distance was detected among the populations of clade C.

Table 2. Summary of the molecular variance analyses, with populations grouped according to the phylogenetic results.

Source of variation	Degree of freedom	Percentage of variation*	Fixation Indices*
Among groups	6	70.59*	F_{CT}= 0.70*
Among populations within groups	30	24.40*	F_{SC} = 0.83*
Within populations	191	5.01*	F_{ST}= 0.95*

*P < 0.001

DISCUSSION

Our results revealed an unexpected phylogeographic pattern compared with previous studies of the genetic structure among *T. carnifex* populations. Indeed, analyses of allozyme genetic variation carried out by Scillitani & Picariello (2000) and Arntzen (2001), consistently identified two main lineages, roughly distributed in northern and peninsular Italy respectively. In addition, the study of the mtDNA variation within the *T. cristatus* species group by Arntzen et al. (2007) identified two main lineages within the range of *T. carnifex*, one found among samples from peninsular Italy and the other among samples located east and north of the Alpine arc. In contrast, our results revealed the occurrence of three main mtDNA lineages (clades N, C and S; Figure 1), which apparently contrasts with the results of those previous studies. Nevertheless, when the respective sampling schemes are compared, each of those previous studies may have overlooked samples within the range of one of the three lineages. Both allozyme studies were based on samples collected south of the Alpine arc, and thus, lacked samples from the putative range of our clade N, while the study by Arntzen et al. (2007) lacked samples from the Padano–Venetian plain, i.e. the putative range of our clade C.

Divergence and secondary contact among main lineages

The divergence among the three lineages was roughly estimated to have occurred from the Plio-Pleistocene boundary to early in the Lower Pleistocene (Figure 2). Palaeoenvironmental changes were profound during this epoch and have been studied intensively in the Mediterranean region (Thompson 2005; Blondel et al. 2010; Kahlke et al. 2011). Climate became cooler and dryer, and the prevailing features of today's climate, including marked seasonality, became established at this time (Suc 1984; Bertoldi et al. 1989; Combourieu-Nebout & Vergnaud Grazzini 1991; Zachos et al. 2001; Klotz et al. 2006; Joannin et al. 2007). Accordingly, pollen spectra and fossil data, indicate substantial changes in the distributions and assemblages of temperate species (Suc 1984; Bertini 2003; Kostopoulos et al. 2007; Bertini 2010; Kahlke et al. 2011). It is likely that *T. carnifex* experienced major distributional changes during this period, including major fragmentations into three lineages, as suggested by its current genetic structure (our data; Scillitani & Picariello 2000; Arntzen 2001; Arntzen et al. 2007), at least under the assumption of niche conservatism.

The phylogeographic breaks found by us among the main lineages of *T. carnifex* closely match prominent biogeographic discontinuities along the Italian peninsula (see Hewitt 2011b).

The geographic area where lineages C and N have been found in close contiguity falls on the north-eastern side of the Padano–Venetian plain. Although outside this area we did not detect any admixed populations where the two lineages co-occur, the continuous distribution of *T. carnifex* across this phylogeographic discontinuity strongly suggests the existence of a secondary contact zone between lineages C and N. Interestingly, this geographic area not only marks the range boundaries of many species, but has also been indicated as an effective biogeographical crossroad between the Dinaric and Italian districts for many taxa and as a site of clustering of contact zones and hybrid zones between both intraspecific lineages and closely related species (this study; Amann et al. 1997; Santucci et al. 1998; Taberlet et al. 1998; Hewitt 1999, 2011a; Zeisset & Beebee 2001; Babik et al. 2005; Magri et al. 2006; Audisio et al. 2009; Verardi et al. 2009).

The southern edge of the lineage C range is delimited by a well-known biogeographic barrier, the northern Apennines. Lineage C extends narrowly to the south at both the eastern and western sides of this mountain area, and at least on the eastern side, it establishes a secondary contact zone with lineage S, as revealed by the co-occurrence of haplotypes from both lineages in our single sample 24. Clusters of species' range edges, phylogeographic breaks, and contact zones along the northern Apennines have similarly been found in several temperate species, including amphibians (Di Giovanni et al. 1998; Canestrelli et al. 2007; Canestrelli & Nascetti 2008; Stefani et al. 2004).

The clustering of phylogeographic breaks, secondary contact zones, and species' range edges identifies both the north-eastern side of the Padano–Venetian plain and the northern Apennines as suture zones (under the extended definition by Swenson & Howard 2005). This has several significant implications. First, the phylogeographic concordance between the pattern observed in *T. carnifex* and those previously found in several other species is strong evidence in favour of the historical rather than stochastic origin of the observed discontinuities (Irwin 2002; Kuo & Avise 2005; Avise 2008). Second, suture zones offer unique opportunities to compare levels and patterns of gene exchange in relation to divergence history and phenotypic evolution of different taxa (Moritz et al. 2009; Hewitt 2011a). In the western Palearctic region, such opportunities have been especially exploited for the northern and central portion of the region (e.g. Taberlet et al. 1998; Petit et al. 2003; Hewitt 2011a), whereas limited research has been devoted to cases in the Mediterranean region (but see Gomez & Lunt 2007; Canestrelli et al. 2010 and references therein). The two zones underscored here are located in key areas of the western Palearctic region and are transitional between peninsular Italy and the continent (northern Apennines), and between the Italian and the Balkan

regions (north-eastern side of the Padano–Venetian plain). Thus, future studies in these areas could help to gain deeper insights on the evolutionary history of the Mediterranean hotspot of biodiversity. Finally, because these zones are hotspots of divergence and evolutionary potential, they also merit special consideration with respect to biodiversity conservation (see Vandergast et al. 2008).

One species, three Pleistocene evolutionary histories I: Northern 'cryptic' refugia

The geographic distribution of the three lineages (N, C, and S), their estimated divergence times, and their intra-lineage phylogeographic structures suggest that they have had independent and substantially different evolutionary histories throughout most of the Pleistocene.

Lineage N was observed only at the easternmost portion of the species range (sites 36–37). This observation is also confirmed by a comparison of previously published sequence data (Espregueira Themudo et al. 2009; Wielstra et al. 2010) with our data. In fact, when our haplotypes N1 and N2 are compared with the more eastern and southern samples from these previous studies (one sequence from Sinac, Croatia and one sequence from Kramplje, Slovenia; Genbank accessions: GQ258936, GQ258952; GU982385, GU982459), average sequence divergence never exceeded 0.009, that is well below the value we found among haplotypes belonging to clades C and N (0.021, 0.004 SE; data available upon request). Although more samples east of the Alpine chain clearly will be needed before inferring the Pleistocene evolutionary history of lineage N, data currently available suggest long-term isolation of this lineage in western Croatia/Slovenia. The occurrence of lineage C in the Padano–Venetian plain, *T. macedonicus* in the Balkans, *T. cristatus* in central Europe, and *T. dobrogicus* in eastern Europe (see Jehle et al. 2011), make neighbouring areas, including the Italian and the Balkan peninsulas, less plausible as long-term refugia for this lineage. Multiple lines of evidence suggest the existence of northern (cryptic) refugia for temperate species (Stewart & Lister 2001; Stewart et al. 2010), areas of sheltered topography that provided suitable microclimates for the survival of thermophilous species outside the traditional southern peninsular refugia. This hypothesis has recently received support from several phylogeographic studies of various temperate organisms, including plants and animals (see Stewart et al. 2010, for a review). In the western Croatia/Slovenia area, the occurrence of ancient and divergent lineages of *T. carnifex* echoes previous findings from several phylogeographic, palaeobotanical and palaeoclimatic studies (Barron & Pollard 2002; Willis & Van Andel 2004; Magri et al. 2006;

Magri 2008), thus indicating the prominent contribution of this northern refugium to the present-day genetic pools of many temperate species.

One species, three Pleistocene evolutionary histories II: 'Refugia-between-refugia'

The occurrence of clade C in the Padano–Venetian plain and its deep divergence with clades N and S provide strong evidence in support of a long-term persistence of this lineage in this area and suggest that the Padano–Venetian plain could have acted as a long-term refugium for *T. carnifex* over multiple Pleistocene glaciations. The occurrence of closely related lineages around the range of the Padano–Venetian lineage would disprove the alternative hypothesis of a recent (re)colonization of this region from neighbouring areas, including north and west of the Alpine arc and the eastern coast of the Adriatic Sea, i.e. the distribution areas of the closely related species *T. cristatus* and *T. macedonicus*. Palaeogeographic, sedimentological, and palaeontological (fossil and pollen) data have shown that following the south-eastern widening of the Padano–Venetian plain due to glaciation-induced marine regressions (Corregiari et al. 1996; Amorosi et al. 1999; Garzanti et al. 2011) a vast alluvial plain environment was established in this area (Amorosi et al. 2004). This provided a paleoenvironmental scenario suitable for the survival of temperate species, particularly amphibians, even during Pleistocene glacial phases. Considering that lineage C showed a lack of geographic structure and that the highest genetic diversity of this lineage occurs along the eastern edge of its range, survival of this lineage in coastal or peri-coastal portion of the Padano–Venetian plain throughout the Pleistocene appears particularly plausible. A similar scenario was hypothesized previously on the basis of phylogeographic data for two other temperate amphibians, the Italian tree frog, *Hyla intermedia* (Canestrelli et al. 2007), and the pool frog, *Pelophylax lessonae* (Canestrelli & Nascetti 2008). Interestingly, this phylogeographic concordance, indicating long-term survival of essentially thermophilic taxa within the Padano–Venetian plain, suggests that a major glacial refugium for Mediterranean biodiversity just between the well-known Apennine and Balkan refugia could have passed mostly unseen for a long time. The post-glacial reflooding of the south-eastern portion of the Padano–Venetian plain likely erased most of the genetic imprints of such a refugial range in many species. Nevertheless, with an appropriate sampling scheme, such imprints could still be found in other species. This issue merits further research, even considering the growing interest in peri-coastal lowlands as glacial refugia and biodiversity hotspots in many regions worldwide (see Bisconti et al. 2011, and literature therein).

One species, three Pleistocene evolutionary histories III: Multiple refugia in peninsular Italy

In contrast to the northern lineages, lineage S showed a clear phylogeographic structure with five main subclades. Three of these subclades (SIII, SIV, and SV) occurred in the northern and central portions of the Calabrian peninsula, an area documented as a major hotspot of intraspecific biodiversity and as a site of multiple refugia for an increasing number of species (Santucci et al. 1996; Podnar et al. 2005; Canestrelli et al. 2006, 2007, 2008; Canestrelli & Nascetti 2008; Barbanera et al. 2009; Vega et al. 2010; Canestrelli et al. 2010, 2012a). The other two subclades (SI and SII) occurred along the remainder of the Italian peninsula. The phylogeographic discontinuities between these latter subclades are located near the Volturno–Calore river drainage basin, another area of clustering of phylogeographic breaks and secondary contact zones for several species and intraspecific lineages (e.g. Nascetti et al. 2005; Canestrelli et al. 2008; Barbanera et al. 2009). For both the Calabrian and the Volturno–Calore areas, glacio-eustatic sea-level oscillations throughout the Pleistocene and consequent insularization of southern Italy during multiple interglacial transgressions have been indicated as the most likely source of historical barriers to the dispersal of terrestrial fauna (Santucci et al. 1996; Canestrelli et al. 2006, 2007, 2010, 2012a). According to ecological and phylogenetic studies no evidence exists to support a sea crossing, even of modest distance, by this species or its close relatives. Thus, a scenario of palaeoinsularization as the source of the observed phylogeographic pattern appears plausible for *T. carnifex.*

The occurrence of five genetically divergent and geographically separated sub-clades within lineage S and the clustering of their estimated divergence times early in the Middle Pleistocene (Figure 2) suggest that the species survived most of the Pleistocene climatic oscillations within multiple separate refugia. This pattern is emerging in an increasing number of temperate species from the Italian peninsula (Santucci et al. 1996; Podnar et al. 2005; Canestrelli et al. 2006, 2007, 2008; Canestrelli & Nascetti 2008; Barbanera et al. 2009; Vega et al. 2010; Canestrelli et al. 2010, 2012a). Nevertheless, contrary to most species studied to date, *T. carnifex* is currently absent from the south-central and southern portion of the Calabrian region, the hotspot of genetic diversity and the area richest in distinct refugia and divergent lineages in most of the studied species. Furthermore, contrary to findings from these previous studies, the ancient derivation of subclade SI (about 1 My; see Figure 2) suggests a long-term refugium in the northern portion of the peninsula rather than a post-glacial recolonization of this area from the south (see also Canestrelli et al. 2007).

Interestingly, recent updates of palaeoenvironmental data for the north-western peninsula (particularly the Arno river basin) indicate the presence of areas of prolonged ecological stability along the coastal plains (Ricci-Lucchi et al. 2008), which could have acted as a glacial refugium for both plant and animal temperate species (see also Canestrelli et al. 2007; Ricci-Lucchi et al. 2008; Porretta et al. 2011).

CONCLUSIONS

The growing number of phylogeographic studies concerning the western Palaearctic region shows that the species' responses to Pleistocene climatic oscillations can be surprisingly diverse. The Italian crested newt, *Triturus carnifex*, exemplifies several of these species' responses. Multiple sources of evidence suggest that the three main lineages of this species have had substantially independent evolutionary histories in three distinct geographic districts throughout the Pleistocene, showing differential responses to Quaternary climate oscillations. Such responses encompass survival in a northern cryptic refugium (lineage N) and in peri-costal refugia (lineage C and sublineage SI), as well as in multiple refugia spanning most of the Italian peninsula (lineage S). These different regional responses also suggest diverse historical demographic trends for each lineage, which merit further investigation by using multi-locus data including information from variable regions of the nuclear genome. In addition, although different regional responses may be attributable primarily to the availability of suitable habitat through time associated with physiographic features and palaeogeographical histories, they may also be due to differences in lineage ecologies, which affect individualistic reactions to local factors (Stewart 2009; Hornsby & Matocq 2012). The development of lineage-specific ecological niche models will allow testing of whether distinct evolutionary lineages within this species have different niche associations and thus, unique responses to past and future climatic shifts (Hornsby & Matocq 2012).

The finding of three ancient and independent evolutionary units within the Italian crested newt also has important conservation implications. This species is currently listed as Least Concern on the International Union for Conservation of Nature (IUCN) Red list of Threatened Species 'in view of its wide distribution, tolerance of a broad range of habitats, presumed large population' (IUCN 2012). However, our results do not fit this assessment and suggest that the three lineages should be considered distinct

conservation units (ESUs; sensu Moritz 1994). Furthermore, lineage S appears fragmented into several population units of historical derivation that should be considered demographically independent (see Avise 2008) for management purposes also (MUs; sensu Moritz 1994).

Finally, the importance of an appropriate sampling design in phylogeographic studies has been emphasized recently, and for temperate species of the western Palearctic special attention has been given to the southern portion of the species' ranges (Taberlet et al. 1998; Hampe & Petit 2005; Gómez & Lunt 2007; Canestrelli et al. 2012a). The case of *T. carnifex* presented here reinforces this claim, and indicates substantial variation in the southern range of the species that would have passed unseen otherwise. This study also clearly shows that northern portions of peninsular species' ranges cannot be overlooked when developing sampling strategies, especially when our data are compared to those of previous assessments of genetic variation in this species.

CHAPTER 3

‘Northern purity’ under minor revision: the Italian smooth newt as a case study

INTRODUCTION

Responses to Plio-Pleistocene climatic oscillations have seen to vary among species and regions (Hewitt 1996; Hewitt 2011a). While patterns of phylogeographic concordance among co-distributed species would be expected as a consequence of similar responses to common regional paleoclimatic and environmental factors, more phylogeographic studies have been added and more species specific patterns are emerging (Stewart 2009; Hewitt 2011a). These patterns have been mainly explained by differences in species life-history (Hewitt 2000; Stewart 2009), or by the ecological interactions among species during glacial-interglacial cycles (Thompson 2005; Stewart 2009). Accumulating knowledge on phylogeographic patterns of co-distributed species may help understanding the complexity of species’ responses to past-climate changes and also how species can face to current climate change (e.g. Keppel et al. 2012).

Temperate Europe has received an intense phylogeographic research effort in comparison to other regions (Beheregaray 2008) and either unique patterns or new lines of concordance are emerging. In fact, while many species conformed to the paradigmatic scenario of glacial contraction southward in the three Mediterranean peninsulas (Italy, Iberia, and the Balkans) and postglacial colonization northwards (following the four paradigms of Hewitt 1999 and Habel et al. 2005), many other species showed deviations from these general scenarios. For instance, some species contracted their ranges in distinct southern refugia (e.g. Anatolia, Turkey, Caucaso; Babik et al. 2005; Médail & Diadema 2009) or in refugia located in Central or Northern Europe (so called northern ‘cryptic’ refugia, Stewart et al. 2010; but also, extra-Mediterranean refugia, Schmitt & Varga 2012). Moreover, a considerable genetic substructure within the three Mediterranean peninsula has emerged, highlighting a scenario of ‘refugia-within-refugia’ and differentiation of distinct lineages not only among these areas but also within them (see Hewitt 2011b, and references therein) highlighting the wide variation of . temperate species’ responses to local climatic and environmental glacial factors (Hewitt 2011b).

In the Italian peninsula, many temperate species conformed to a scenario of cycles of range contractions southward in one or more refugia,

and, postglacial colonization northwards (e.g. Canestrelli et al. 2006, 2008, 2010; Magri 2008). However, some others, showed cycles of range contractions in multiple refugia along the entire Apennine peninsula (Canestrelli et al. 2010, 2012a,b; Vega et al. 2010, Hewitt 2011b, and references therein), and among these, some encompassed also one refugium in the continental part of Italy (i.e. north Italy, below Alps; Podnar et al. 2005; Ursenbacher et al. 2006; Canestrelli & Nascetti 2008; Barbanera et al. 2009; Canestrelli et al. 2007, 2012b). The unexpected glacial survival of temperate species in northern areas has been hypothesized to be favoured by milder and stable climatic conditions (Amorosi et al. 2004; Ricci-Lucchi 2008) occurring in some coastal or peri-coastal areas during glacials (e.g. Padano-Venetian plain coast, northern Tyrrhenian coast, Canestrelli et al. 2007; 2012b; Canestrelli & Nascetti 2008). However, to date few studies with a dense sampling design in the north of Italy have been accumulated (e.g. Garner et al. 2004; Podnar et al. 2005; Ursenbacher et al. 2006; Canestrelli et al. 2007; Canestrelli & Nascetti 2008; Magri 2008; Barbanera et al. 2009) and the role of this area for the long term survival of temperate species is still little known. Moreover, the continental part of Italy is a key transitional area between the Italian peninsula and the rest of Europe, thus improving our knowledge on phylogeographic patterns encompassing this area would allow a better understanding of the evolution of both the Italian and the European biota.

In this study we analysed the genetic structure of the Italian smooth newt, *Lissotriton vulgaris meridionalis*, a temperate species mainly distributed in north and centre of Italy, in the north-western Croatia and western Slovenia, and, being absent from the south of Italy, which is inhabited by the related species, *Lissotriton italicus* (Smith & Green 2005; Razzetti & Bernini 2006, and references therein). Moreover, a previous wide-range study on the phylogeography of *Lissotriton vulgaris* showed a genetic structure highly fragmented and the survival of Pleistocene climatic oscillations in multiple refugia located not only in southern Europe and southwestern Asian (encompassing the Italian and Balkan peninsula, Turkey, Anatolia and Caucasus), but also in central Europe (e.g. close to the Carpathian mountains, Babik et al. 2005). In addition, *L. v. meridionalis* is generally considered a low dispersal and phylopatric to the freshwater habitats where it breeds (Smith & Green 2005). Thus, the current distribution of this species, its responses to past glacial stages, and its ecology make this newt particularly suitable for investigating the imprint of Pleistocene climatic oscillations on phylogeographic pattern of temperate species in the continental part of Italy. Moreover, although in the study of Babik et al. 2005 the few samples used from Italy detected an old-differentiated mitochondrial lineage (1.9 Mya) from the others, suggesting

indeed a separated Pleistocene evolutionary history of this lineage, the phylogeographic pattern of *L. v. meridionalis* is still unknown.

Using phylogeographic, molecular dating, and historical demographic analyses we investigated the evolutionary history of the Italian smooth newt, *Lissotriton vulgaris meridionalis*. In particular, by developing a dense sampling design in the northern part of Italy and through a comparison with previous studies of co-distributed species we aimed to shed light on the role of this area for the evolution of the Italian biota.

MATERIALS AND METHODS

Sampling and laboratory procedures

We sampled a total of 82 *Lissotriton vulgaris meridionalis* individuals from 24 localities throughout the species' range (see Figure 1B and Table 1for detailed information about sampling localities and number of individuals for each locality). Newts were anaesthetizing by submersion in a 0.1% solution of MS222 (3-aminobenzoic acid ethyl ester) and tissue samples from tail tips were collected and stored in 96% ethanol. After sampling, individuals were released at the collection site. DNA extraction was performed by following the standard cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1987).

Two mitochondrial fragments were amplified and sequenced for all individuals. One fragment comprised part of the NADH dehydrogenase subunit 4 gene and the $tRNA^{His}$ gene (hereafter referred to as ND4), and the other fragment comprised the NADH dehydrogenase subunit 2 gene (hereafter referred to as ND2). Preliminary amplifications and sequencing of the ND4 fragment were performed using primers ND4 and LEU (Arévalo et al 1994), and then the internal primers ND4vulgF1 (ATCCGAATTTCTATAATCMTTACCC) and ND4vulgR1 (CTTCTTGGTAGGTAGAGAGGGTTTA) were designed and used to screen all individuals. Preliminary amplifications and sequencing of the ND2 fragment were performed using primers H5018 and L3780 (Babik et al 2005), and then the internal primer LVND2F1 (AATCAGCAACAAAATACTTTTTAACG) was designed and used in conjunction with H5018 to screen all individuals.

Amplifications were performed in a 25 µl volume containing MgCl2 (2.5 mM), reaction buffer (5×, Promega), the four dNTPs (0.2 mM each), the two primers (0.2 µM each), the enzyme Taq polymerase (1U, Promega) and 2 µl of DNA template. Polymerase chain reaction (PCR) was performed

Table 1. Geographic location, number of individuals (n) and haplotype distribution for each of the 24 populations sampled of *Lissotriton vulgaris meridionalis*. * samples from Genbank (see in the text for accession numbers).

Sample	Locality	n	Latitude	Longitude	Haplotypes (n)
1	Arcade	1	45°46'	12°12'	M6b (1)
2	Bo' de Pavei	1	45°49'	12°10'	M5c (1)
3	Collesel di San Rocco	2	45°50'	12° 9'	M5a (1), M5b (1)
4	Crocetta del Montello	1	45°49'	12° 2'	M6d (1)
5	Campiglia dei Berici	1	45°19'	11°32'	M4c (1)
6	Porto Caleri	3	45° 5'	12° 19'	M4a (2), M4d (1)
7	Castelleone	4	45°16'	9°45'	M6c (1), M6e (1), M6a (1), M9a (1)
8	Cesano Maderno	3	45°36'	9° 7’	M6a (2), M2a (1)
9	Donega	8	44°56'	9°14'	M9a (2), M9b (2), M9c (2), M9d (1), M9e (1)
10	San Germiniano	3	44°43'	10°26'	M10a (1), M8f (1), M9a (1)
11	Mulino di Pianoro	7	44°21'	11°19'	M8a (3), M8b (3), M8c (1)
12	Vernio*	1	44°02'	11°09'	M8a (1)
13	Terra del sole	4	44°11'	11°57'	M8e (3), M4b (1)
14	Camaldoli	1	43°47'	11°49'	M8g (1)
15	Senigallia	2	43°41'	13°12'	M8d (1), M3h (1)
16	Pisa*	3	43°43'	10°24'	M1a (1), M1f (1), M3d (1)
17	Greve in Chianti	4	43°35'	11°18'	M1a (3), M1b (1)
18	Roccalbegna	5	42°44'	11°30'	M3a (3), M3c (1), M3f (1)
19	Orbetello	4	42°26'	11°13'	M3i (1), M3l (3)
20	Alviano	3	42°37'	12°14'	M3a (1), M3b (1), M8d (1)
21	Palo Laziale	6	41°56'	12° 5'	M3a (2), M3e (3), M3f (1)
22	Navegna	4	42° 9'	13° 2'	M1c (2), M1d (1), M1g (1),
23	Rocca di mezzo	3	42° 9'	13°38'	M1e (2), M7c (1)
24	Doganella	6	41°45'	12°47'	M7b (1), M7d (3), M7e (1), M3a (1)
25	Circeo	1	41° 20'	13° 2'	M7f (1)
26	Sepino	5	41° 23'	14° 34'	M7a (5)

with a step at 95 °C for 5 min followed by 30 (ND4) or 38 (ND2) cycles of: 94 °C for 1 min, 55°C (ND4) - 56 °C (ND2) for 1 min, 72 °C for 1 min, and a single final step at 72 °C for 10 min. Purification and sequencing of the PCR products were carried out by Macrogen Inc. (www.macrogen.com) by using an ABI PRISM 3700 sequencing system. All sequences were deposited in GenBank (accession numbers: in submission).

Nucleotide variation, phylogenetic analyses and molecular dating

Electropherograms were visually checked using FinchTv 1.4.0 (Geospiza Inc.) and aligned using Clustal X 2.0 (Larkin et al. 2007). Previously published sequences of ND4 and ND2 fragments (Babik et al. 2005, Genbank accession number: AY951639, AY951495, AY951454-56, AY951609-11) of four individuals sampled in Central Italy (localities number 12 and 16 in Tab.1) were added to our analyses.

Nucleotide variation and corrected net sequence divergence (Tamura & Nei 1993) between the main haplotype groups were estimated using the software MEGA 5 (Tamura et al. 2011).

The appropriate model of nucleotide substitution for our dataset was chosen among 88 distinct models using the Akaike Information Criterion (AIC, Akaike 1973) implemented in jModelTest 0.1.1 (Posada 2008). TIM2 + Γ with the gamma distribution shape parameter = 0.141) resulted as the best fit model for ND4 and ND2 fragments analyzed both separated and combined.

Phylogenetic relationships among haplotypes were inferred by a network-building approach. Phylogenetic networks provide a more appropriate representation of intraspecific genetic variation in comparison to tree-building approach (Posada & Crandall 2001). We constructed phylogenetic networks by using two different distance-based algorithms, the statistical parsimony implemented in TCS (Clement et al. 2000) and the median-joining procedure (Bandelt et al. 1999) implemented in NETWORK 4.6.1 (www.fluxus-engineering.com). However, because both methods recovered a topology with relatively distant haplotypes (such that some node haplotypes are missing), we consider only results from the median-joining method since in these particular cases this method has been shown to outperform the statistical parsimony method (Cassens et al. 2005).

Estimates of time to the most recent common ancestors (TMRCAs) of the mtDNA haplogroups were obtained by using the distance-based least squares (LS) method (Xia & Yang 2011) implemented in the software DAMBE (Xia & Xie 2001). A likelihood ratio test was performed with this software to test if our sequences evolved in a clock-like manner. This test did not reject the molecular clock hypothesis for our dataset. Then, to

perform the LS analysis we specified a tree topology calculated with the Maximum Likelihood method as implemented in PHYML software (Guindon et al. 2010), using the model of sequence evolution suggested by JMODELTEST, the SPR&NNI algorithm, and the related subspecies, *L. v. vulgaris* as outgroup (genbank accession: AY951562, AY951396). We set the root of the tree to 1,9 million years ago (Mya), corresponding to the divergence time between *L. v. vulgaris* and *L. v. meridionalis* as estimated by Babik et al. (2005). We used the 'softbound' option and 'MLCompositeTN93' genetic distance, as suggested by Xia & Yang (2011). Standard deviations of the time estimates were obtained by means of 1000 bootstrap re-samplings.

Population genetic structure and historical demography

We investigated the geographic structure of genetic variation following two distinct approaches. In both cases populations with one individual were excluded from the dataset. First, we searched for groups of populations that are geographically homogeneous but maximally genetically differentiated from each other by using the simulated annealing procedure of spatial analyses of molecular variance implemented in SAMOVA (Dupanloup et al. 2002). We performed this analyses setting the number of groups (*K*) from 2 to 12 *a priori* in order to test the best clustering option in terms of highest and significant value of F_{CT} (i.e. the among-group variance component). To verify the consistency of the results, we run the analyses five times for each *K* value with 1000 independent annealing processes.

Second, we tested if the geographic structure of genetic variation was mainly explained by the interaction of contemporary microevolutionary processes related to isolation by distance (i.e. genetic drift and gene flow) or by genetic differentiation among groups, by using (Partial) Mantel Tests implemented in the IBD web service 3.23 (Jensen et al. 2005). To perform these analyses we constructed three pairwise population matrix based on 1), the corrected mean genetic distance (TrN+Γ model, Γ= 0.141) calculated with MEGA, 2) the geographic distance (calculated by GEOGRAPHIC DISTANCE MATRIX GENERATOR 1.2.3., Ersts 2012) and subsequently log-transformed (following suggestions by Rousset 1997), and, 3) a binary matrix (the indicator matrix) in which the value '0' indicates that two populations are within the same group, and '1' in the opposite case. We assessed the significance of correlations and of partial correlations between these matrix controlling in one case for the geographic distance and in the

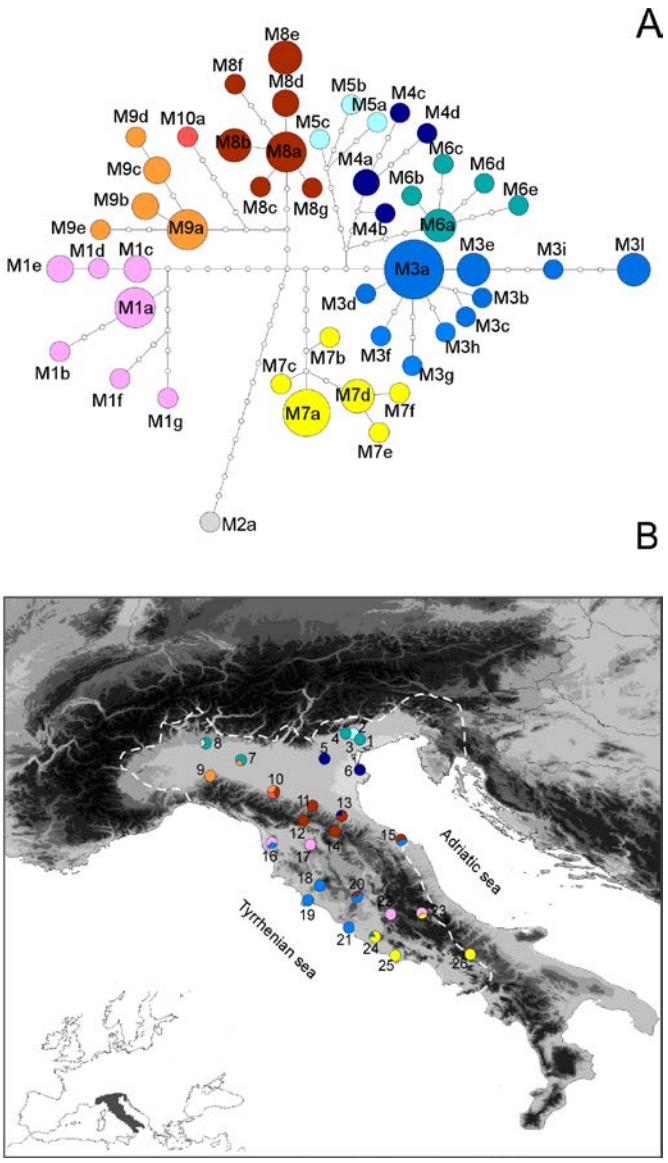


Figure 1. A: Median-Joining network showing phylogenetic relationships among the 49 haplotypes (numbered as in Table 1) found within *Lissotriton vulgaris meridionalis*. Circles size is proportional to haplotype frequency and, open dots represent missing intermediate haplotypes. B: Geographical distribution of the 10 haplogroups found across the 24 sampled populations shown as pie diagrams. White dotted lines show boundaries of the subspecies' range. Inset: geographical location of the study area within the western Palaearctic region

other one for the indicator matrix by using Mantel and Partial Mantel Tests along 1000 bootstrap replicates.

The occurrence of past demographic changes in each haplogroup found (with n > 9) for the Italian smooth newt was inferred by using two different methods. First, a mismatch distribution analyses (Rogers & Harpending 1992) was performed with the software ARLEQUIN 3.5.1.2 (Excoffier et al. 2005). In this analyses the observed distribution of nucleotide differences between haplotypes (mismatch distribution) is compared with that expected under both a demographic expansion model (Rogers & Harpending 1992) and a sudden spatial expansion model (Excoffier 2004). We used the sum of squared deviations between estimated and observed mismatch distributions as goodness-of-fit statistics and its significance was assessed along 1000 bootstrap replicates. Second, the F_S Fu (Fu 1997) and R_2 (Ramos-Onsins & Rozas 2002) statistics were also used to infer past demographic trends. Both tests have been shown to outperform most of the other test statistics commonly used with the same aim (see Ramos-Onsins & Rozas 2002), and in particular, F_S Fu to better perform with large sample size and R_2 with small sample size (Ramos-Onsins & Rozas 2002). High, negative and significance values of F_S and small, positive and significance values of R_2 give a signal of past demographic expansion. The significance of these values was assessed through 1000 coalescent simulations, carried out under the hypothesis of population equilibrium and selective neutrality. Moreover, for the estimated F_S values the 2% cut-off criterion was used to assess the 5% nominal level of significance (Fu 1997).

RESULTS

Nucleotide variation, phylogenetic analyses and molecular dating

For all individuals analysed the ND4 fragment was 644 bp in length, comprising 587 bp of the (3') NADH dehydrogenase subunit 4 gene and 57 bp of the $tRNA^{His}$ gene, and the ND2 fragment was 651 bp. The combined dataset (overall 1295 bp) included 109 variable positions, of which 61 were parsimony informative. We did not find indels or stop codons within the coding region of either the ND2 or the ND4 fragments. A total of 49 haplotypes were found in the combined fragment, and their geographic distribution is presented in Figure 1B and Table 1.

The Median-Joining network among the haplotypes showed 10 haplotype groups (M1-10; Fig.1A) occupying terminal positions in the network (such that internal positions are missing). The geographic

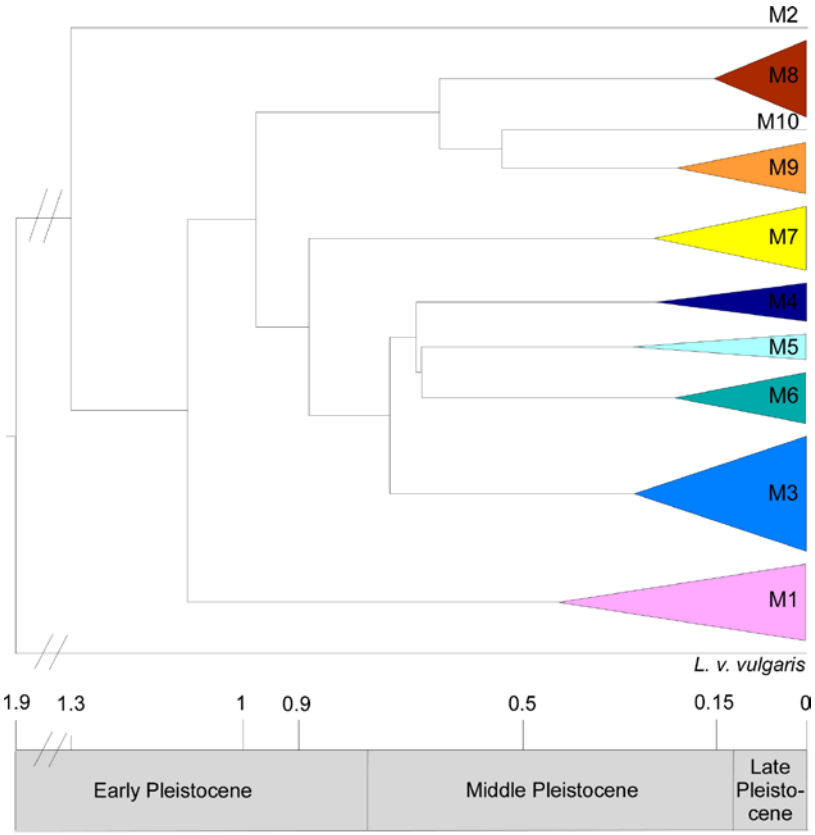


Figure 2. Chronogram showing the estimates of time to the most recent common ancestors (TMRCA) for the 10 mtDNA lineages of *Lissotriton vulgaris meridionalis*. The calibration point (1.9) and the ranges of the main historical epochs on the scale bar are reported in million years.

distribution of these haplogroups was clearly geographic associated and restricted to a few contiguous populations (Figure 1B). Six haplotype groups were distributed in northern Italy, M9 (samples 7, 9, 10), M10 (sample 10) and M2 (sample 8) mainly in the western part, M4 (sample 5, 6, 13) and M5 (samples 2 and 3) in the eastern one, and M6 (samples 1, 4, 7, 8) along the pre-Alpine area. M8 was distributed in both north (samples 10-15, 20) and south (samples 10-15, 20) sides of northern Apennines, whilst the other three haplotype groups M1, M3, M7 occurred in the remaining of the species' range along the central Italian peninsula. M3 was mostly distributed along the Tyrrhenian sea side (samples 15, 16, 18-21, 24) reaching the Adriatic side in sample 15; M7 in the south (samples 23-26); and, M1 both close to the northern (samples 16 and 17) and central Apennines (samples 22, 23), showing indeed an apparently fragmented distribution. Co-presence was observed in 9 localities (7, 8, 10, 13, 15, 16, 20, 23, 24) and occurred among most of the haplogroups.

Net average sequence divergence among the 10 haplogroups ranged from 0.004 (SE 0.002) between M3 and M4 to 0.018 (SE 0.004) between M2 and M10 (see Table 2).

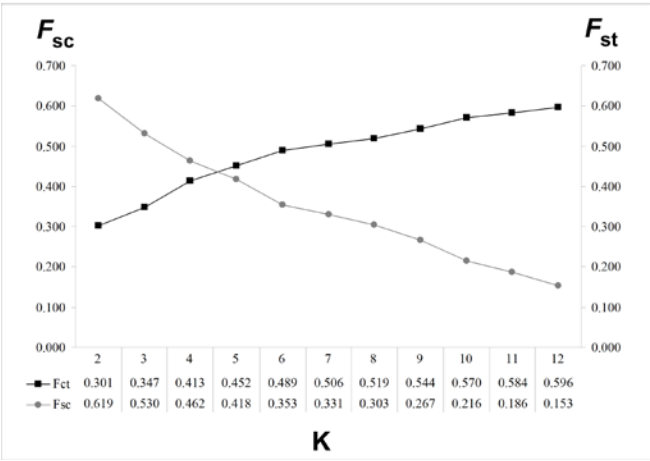
TMRCA estimates for the mtDNA haplogroups found are shown in the chronogram of Figure 2. The TMRCA for the entire ingroup was estimated to have occurred in the late Early Pleistocene (1.312 ± 0.290 Ma), and the following splits among the haplotype groups fell within the late Early Pleistocene and the Middle Pleistocene. Finally, the TMRCAs of each haplotype group ranged from the early Middle Pleistocene (i.e. 0.399±0.108 Mya of M8) to the early Late Pleistocene (i.e. 0.156±0.065 Mya of M5).

Population genetic structure and historical demography

The spatial analysis of molecular variance showed that F_{CT} values progressively increased (from 0.301 to 0.596) and F_{SC} values progressively decreased (from 0.619 to 0.153) when K increases from K=2 to 12 (see Figure 3A). After K=9 population structures were not informative anymore as one population at a time is removed from groups (see Figure 3B). At K=9 the groups recovered nearly correspond to the haplogroups identified in the phylogenetic network (M1, M3-M9) plus a group composed by samples 16-17.

The Mantel test performed between genetic and log geographic distances suggested the occurrence of a very weak pattern of isolation by distance (R^2= 0.11, P < 0.001), which is not more statistically supported when performing the partial mantel test between these two variables controlling for the long-term differentiated groups. On the other way, the

A



Number of groups (*K*)	Group composition
2	(17, 22) (3, 6, 7, 8, 9, 10, 11, 13, 15, 16, 18, 19, 20, 21, 23, 24, 26)
3	(16, 17, 22, 23) (3, 6, 7, 8, 9, 10, 11, 13, 15, 18, 19, 20, 21, 24, 26)
4	(16, 17, 22, 23) (24, 26) (9, 10, 11, 13) (3, 6, 7, 8, 15, 18, 19, 20, 21)
5	(16, 17, 22, 23) (24, 26) (9, 10) (11, 13) (3, 6, 7, 8, 15, 18, 19, 20, 21)
6	(16, 17, 22, 23) (24, 26) (9, 10) (11, 13) (3, 6, 7, 8) (15, 18, 19, 20, 21)
7	(16, 17, 22, 23) (24, 26) (9) (10, 11, 13) (3, 6) (7, 8) (15, 18, 19, 20, 21)
8	(16, 17) (22, 23) (24, 26) (9, 10) (11, 13, 15) (3, 6) (7, 8) (18, 19, 20, 21)
9	(16, 17) (22, 23) (24, 26) (9, 10) (11, 13, 15) (3) (6) (7, 8) (18, 19, 20, 21)
10	(16, 17) (22, 23) (24, 26) (9, 10) (11, 13) (3) (6) (7, 8) (19) (15, 18, 20, 21)
11	(16, 17) (22, 23) (24) (26) (9, 10) (11, 13) (3) (6) (7, 8) (19) (15, 18, 20, 21)
12	(16, 17) (22, 23) (24) (26) (9, 10) (11, 13) (3) (6) (7, 8) (19) (18, 21) (15, 20)

Figure 3. A:SAMOVA (Spatial Analysis of Molecular Variance) results. Fixation indices F_{CT} and F_{SC} ($P < 0.001$) relative for each pre-defined value of K from 2 to 12. B: Group composition for each values of *K*.

Table 2. Net average sequence divergence among haplogroups based on Tamura & Nei (1993) distances. Standard deviations (1000 bootstrap replicates) in parentheses.

	M3	M8	M6	M4	M5	M9	M7	M2	M10
M3									
M8	0.008 (0.003)								
M6	0.005 (0.002)	0.011 (0.003)							
M4	0.004 (0.002)	0.009 (0.003)	0.005 (0.002)						
M5	0.006 (0.002)	0.011 (0.003)	0.006 (0.002)	0.005 (0.002)					
M9	0.009 (0.003)	0.006 (0.002)	0.011 (0.003)	0.01 (0.003)	0.011 (0.003)				
M7	0.007 (0.002)	0.009 (0.003)	0.009 (0.003)	0.008 (0.002)	0.01 (0.003)	0.009 (0.003)			
M2	0.015 (0.003)	0.015 (0.004)	0.017 (0.004)	0.014 (0.003)	0.017 (0.004)	0.015 (0.004)	0.015 (0.003)		
M10	0.011 (0.003)	0.008 (0.002)	0.014 (0.004)	0.012 (0.003)	0.012 (0.003)	0.006 (0.002)	0.012 (0.003)	0.018 (0.004)	
M1	0.01 (0.003)	0.01 (0.003)	0.012 (0.003)	0.011 (0.003)	0.011 (0.003)	0.009 (0.002)	0.01 (0.003)	0.014 (0.003)	0.009 (0.003)

Mantel test between genetic distances and long-term differentiated groups (R^2= 0.40) as well as, the partial Mantel test between these two variables controlling for log geographic distances (R^2= 0.33) showed significant correlations ($p < 0.001$).

Results from mismatch distribution analyses and values of the demographic summary statistics for the five haplogroups tested are reported in Figure 4. The observed mismatch distributions for all the haplogroups was not significantly different from those expected from a pure demographic and spatial expansion (Figure 4). For M7, M9 and M1 the spatial expansion expected models fitted better than those ones from pure demographic expansions, whilst for M3 was the reverse. F_s and R_2 values were not statistically significant in all the haplogroups, with the only exception of M3 where a small (R_2=0.0795) and statistically significant ($p < 0.01$) R_2 value was detected (Figure 4).

DISCUSSION

The phylogeographic pattern showed by *Lissotriton vulgaris meridionalis* is highly structured in ten haplotype groups with a net genetic divergence ranging from 0.4% to 1.8%. Both the distribution of haplotypes and the spatial analyses of molecular variance showed that haplotype groups have a clear geographical association. The genetic variance observed and its geographic distribution was mainly explained by genetic differentiation among haplogroups rather than by a pattern of isolation by distance, as shown by Mantel tests. Thus, contemporary micro-evolutionary processes related to isolation by distance (i.e. genetic drift and gene flow) have likely played a minor role relative to historical divergence in shaping the genetic structure of the Italian smooth newt.

The time of divergence among the haplogroups was roughly estimated at the late Early Pleistocene (1.312 ± 0.290 Mya). Since then *L. v. meridionalis* underwent to a series of not simultaneous splits along the Middle Pleistocene. The transition between the Early and Middle Pleistocene (the interval between about 1.2 and 0.500 Mya, Head & Gibbard, 2005) also known as, the 'mid-Pleistocene revolution' (Berger & Jansen 1994) was characterized by an increase of amplitude in the rhythm of climatic oscillations (since 41ka of the Early to 100-ka of the Middle Pleistocene). The increasing severity and duration of cold stages, have had a profound effect on species and palaeo-landscapes, especially in the northern hemisphere biotas (see Head & Gibbard 2005; Hewitt 2011a, and references therein). As largely documented by fossil, pollen and phylogeographic studies in the western-Palaearctic region species underwent to cycles of severe reductions and expansions in the range distribution, and/or to extinctions over large parts of their ranges (Hewitt 2000, 2011a,b and references therein). It is likely that the high number of splits occurring in *L. v. meridionalis* during the Early-Middle Pleistocene transition was a consequence of multiple fragmentation events caused by these climate transformations.

The phylogeographic pattern showed by *L. v. meridionalis* with ten independent evolutionary lineages distributed in separated geographic compartments across the whole subspecies range, strongly conform to a scenario of multiple glacial refugia. Estimates of time to the most recent common ancestors of each haplotype group ranged from the Middle Pleistocene to the early Late Pleistocene, predating in all cases the last glacial stage, thus suggesting that these lineages would have had independent evolutionary histories across several glacial/interglacial cycles. According to the current geographic distribution of these ten lineages *L. v. meridionalis* would have survived in refugia located in the Apennine

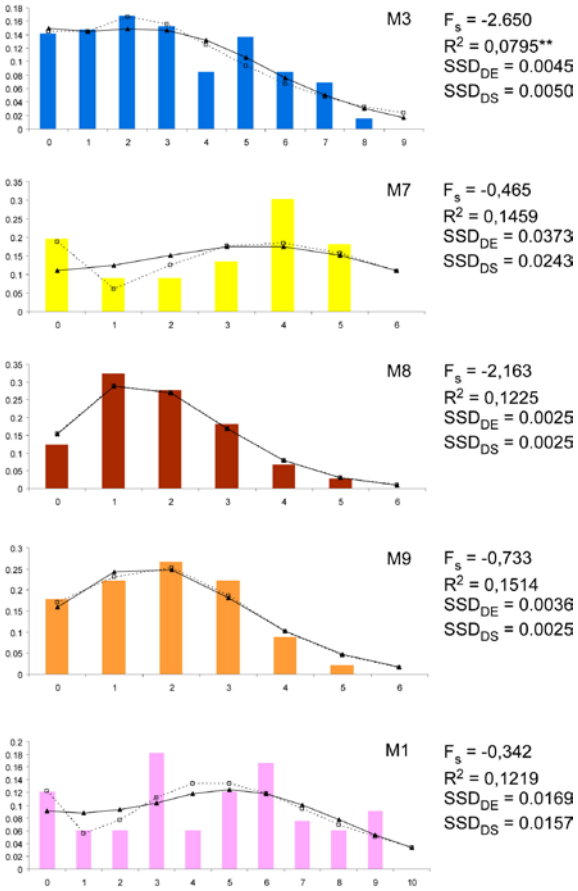


Figure 4. Mismatch distribution analyses and values of the demographic summary statistics for M1, M3, M7, M8 M9 haplogroups (n > 9) identified by phylogenetic and SAMOVA analyses. Bars (coloured according to Fig. 1a and 2) show the observed distributions of pairwise nucleotide differences; ▲ with solid lines show the expected distribution under a model of a pure demographic expansion; □ with dotted line show the expected distribution under a model of spatial expansion. SSD, sum of square deviations for the goodness-of-fit between the observed mismatch distribution and the mismatch distribution expected under both a pure demographic expansion (SSD_{DE}) and a spatial expansion model (SSD_{SE}). F_S: Fu's FS statistic (Fu, 1997). R_2: Ramos-Onsin and Roza's test (**P<0.01).

Peninsula (M1, M3 and M7), in north Italy (M2, M3, M4, M5, M6, M7, M9 and M10) and, at both sides of the northern Apennines (M8).

The current geographic distribution of haplogroups in close contiguity and the co-occurrence of different haplogroups in several localities (Fig. 1) suggest that secondary contacts after recent expansions would have occurred among these lineages. However, while mismatch analyses did not reject the hypothesis of recent demographic and spatial expansion for all the haplogroups tested, the other historical demographic analyses supported a scenario of demographic stability for all of them (see Figure 4). Thus, a higher sample size as well as the analysis of both nuclear and mitochondrial genetic diversity would be necessary to make a more robust inference on the population demographic histories of these lineages.

Multiple refugia across Italy has been observed in a growing number of species encompassing plants and vertebrates, e.g. amphibians, reptiles and mammals (Canestrelli et al. 2010, 2012a, b; Vega et al. 2010 and Hewitt, 2011 and references therein). In some of these species most of refugia have been detected in the southern portion of their ranges, from where a postglacial northward colonization has been inferred (e.g. *Bombina pachypus, Rana italica, Talpa romana, Fagus sylvatica;* Canestrelli et al. 2006, 2008, 2010; Magri 2008). However, our data can firmly discard this latter scenario of multiple southern refugia and postglacial northward colonization for *L. v. meridionalis*. The topology of the phylogenetic network, with haplogroups occupying terminal positions and intermediate nodes missing, did not support a derivation of haplogroup one from another. This result together with the estimated divergence times among haplogroups and their geographic distribution in close contiguity, definitely discard the hypothesis of a recent (re)colonization of a portion of the subspecies range from refugial areas (e.g. from southern refugia to northern areas). Moreover, the occurrence of closely related lineages around the subspecies range (i.e. the nominal subspecies *L. v. vulgaris* in the north / north-east of the Alpine arc and its close related species *L. italicus* in the south Italy) discards also the hypothesis of a recent (re)colonization from neighboring areas out of its current range.

On the other hand, the phylogeographic pattern showed by *L. v. meridionalis* fits to the scenario of glacial survival in multiple refugia across the Apennine Peninsula and north Italy, as also described for other temperate species (e.g. *Podarcis sicula*; *Vipera aspis*; *Hyla intermedia*; *Triturus carnifex*; *Pelophylax lessonae*; Podnar et al. 2005; Barbanera et al. 2009; Canestrelli et al. 2007, 2012b; Canestrelli & Nascetti 2008;). The north Italy, in particular the Padano-Venetian plain, would have allowed the glacial survival of many temperate species, and among amphibians of *Hyla intermedia*; *Triturus carnifex, Pelobates fuscus, Pelophylax lessonae*

(Canestrelli et al. 2007, 2012b; Crottini et al. 2007; Canestrelli & Nascetti 2008) and, according to this study also of *L. v. meridionalis.* However, while in north Italy all of these species showed the occurrence of one endemic lineage, thus suggesting the glacial survival in one refugium, *L. v. meridionalis* showed the occurrence of several lineages, thus suggesting the glacial survival in multiple refugia, a scenario to date unprecedented among co-distributed species. Moreover, taking into account the current geographic distribution of these northern lineages it is likely that *L. v. meridionalis* would have survived in refugia located in pre-Alpine and peri-northern Apennine areas.

A growing literature based on fossil, pollen, phylogeographic and also paleoenvironmental data advocate the glacial persistence of temperate species in European refugia located northern to the 'classical' Mediterranean ones (Stewart & Lister 2001; Stewart et al. 2010; Schmitt & Varga 2012). Evidences based on fossil and pollen data suggest the occurrence of these refugia in areas of sheltered habitats in deeply incised valleys that provided suitable microclimates for the survival of thermophilic species (Stewart & Lister 2001; Schmitt & Varga 2012). In some of these cases, refugia were apparently located at the foothills of water donating mountains systems (e.g. the glaciated Carpathians, Alps or Balkan mountains) areas where likely wetter microclimate condition persisted during glacials in contrast with more arid conditions of the adjacent lowland loess steppe areas (Schmitt & Varga 2012). A scenario of glacial survival in several refugia located in distinct humid areas at the foothills of the glaciated Alps and northern Apennines appears particularly suitable for amphibians such as *L. v. meridionalis*, strictly connected to freshwater habitats for reproduction, phylopatric and with very low dispersal capability (Smith & Green 2005, and references therein). This scenario inferred for *L. v. meridionalis* and together with the growing literature regarding the extra-Mediterranean refugia suggest that refugia located also in northern regions have acted as long-term reservoirs of genetic diversity for temperate species, thus contrasting the paradigmatic view of genetic 'northern purity'.

In conclusion, our study indicates that *L. v. meridionalis* underwent since the late Early Pleistocene to multiple fragmentation events leading to the fragmentation in ten independent evolutionary lineages distributed in separated geographic compartments across the whole subspecies range. This pattern suggests that *L. v. meridionalis* survived multiple glacial-interglacial cycles in several refugia, many of them occurring in the northern part of Italy, to date an unprecedented scenario among co-distributed species.

The high genetic fragmentation found in *L. v. meridionalis* mirrors that found in close related lineages occurring in the rest of the species range (e.g. *L. v. vulgaris* and *L. v. graecus*, Babik et al. 2005) and, also those of

other small body newts, such as *L. italicus* (Canestrelli et al. 2012a), endemic to south Italy, and *L. boscai* (Martínez-Solano et al. 2006) endemic to the Iberian peninsula, suggesting that these newts are particularly prone to retaining the genetic traces of Pleistocene climatic oscillations and, thus very useful model for phylogeographic studies.

Alluvial plain expansions counterbalanced the negative demographic effects of glacial climate in the Italian crested newt, *Triturus carnifex*

BACKGROUND

The phylogeographic pattern of *Triturus carnifex* suggests the long-term persistence of this species in the Padano–Venetian plain (clade C) and along the Italian peninsula (clade S; Canestrelli et al. 2012b). Genetic data showed the occurrence of two differentiated lineages in these areas and molecular dating suggests they have had independent evolutionary histories since the Early Pleistocene. Moreover in both clades, the genetic diversity pattern suggests that coastal or peri-coastal plains could have acted as refugial areas and allowed the persistence of this species during glaciations (Canestrelli et al. 2012b).

A vast alluvial plain environment was established in the northern Adriatic area following the south-eastern widening of the Padano–Venetian plain due to glaciation-induced marine regressions (Corregiari et al. 1996; Amorosi et al. 1999; Garzanti et al. 2011). Paleoegeographic, sedimentological, and paleoentological (fossil and pollen) data suggest that paleoenvironments in this area were suitable for the survival of temperate species, particularly amphibians, even during Pleistocene glacial phases (Amorosi et al. 2004). In particular, temperate species in these areas could have maintained large populations or even experienced range/demographic expansions during glacials as a consequence of increased availability of terrestrial habitat due to sea-regression.

This scenario was previously hypothesized on the basis of genetic data for two temperate amphibians, the Italian tree frog, *Hyla intermedia* (Canestrelli et al. 2007), and the pool frog, *Pelophylax lessonae* (Canestrelli & Nascetti 2008). In these species phylogeographic data suggest a long term persistence of populations in the northern Adriatic and historical demographic reconstructions showed in both species a trend of glacial expansion. The hypothesis of a major glacial refugium for *T. carnifex* just

between the well-known Apennine and Balkan refugia seem also plausible and merits further research.

As preliminary steps of such research we extended sampling of *T. carnifex* in the Padano-Venetian plain and we generated sequence data at two mitochondrial gene fragments for these samples and at two nuclear gene fragments for the entire set of samples available. We carried out species distribution modeling (SDM) of clade C and clade S under both Last Glacial Maximum (LGM ~ 18.000 ya) and Last Interglacial (LIG, ~130,000 -116,000 ya) and conditions and reconstructed their demographic histories. The main aim of this study will be of testing whether the glacial widening of the Padano–Venetian plain provided increased habitat suitability for lineages C and S of *T. carnifex* and allowed the maintenance of stable populations or even induced a demographic growth. Under this scenario we expect to identify suitable habitats for these lineages in the newly exposed northern Adriatic area and to detect demographic stability or growth of populations at multiple loci. Alternatively, a pattern of demographic contraction and of low suitability of this area for these lineages would disprove its role as glacial refugia for lineage C and S of *T. carnifex* .

MATERIALS AND METHODS

Sampling and laboratory procedures

A total of 265 *Triturus carnifex* individuals sampled from 43 localities spanning the entire range of clade C and S were included in the analyses. Relative to the sampling detailed in Chapter 2 (Figure 1c and Table 1), 31 individuals from seven new localities sampled in the sub-range of clade C were added in this study (see Figure 1 and Table 1). Detailed information about new sampling localities and number of individuals sampled in each locality are shown in Table 1 and Figure 1. For information regarding the remaining populations see Figure 1c and Table 1 of Chapter 2.

Two mitochondrial gene fragments, ND4 (638 bp) and ND2 (635 bp), were sequenced for all individuals following DNA extraction amplification and sequencing procedures described in Chapter 2. Two nuclear gene fragments were amplified and sequenced for selected individuals: the Beta fibrinogen, seventh intron (FIB) and the Calreticulin intron C (CALC). In particular, the FIB gene fragment (638 bp) was obtained from sequences from 74 and 92 individuals for clade C and S, respectively; and the CALC gene fragment (411 bp) was obtained from 82 and 96 individuals for clade C and S, respectively. Amplifications and sequencing were performed using primers FibF (GCAAAGAATGAGAGCATTGGC) and FibR

(GACATTGAAATTTAGCAAGCACA) (Nadachowska & Babik 2009) for the FIB fragment, and primers CalC 3F (CGTTTGCGTCCAGTGTATTG) and CalC 4R (GTCCTTGTTGATCTGCAGGTTT) (Espregueira Themudo et al. 2009) for the CALC fragment. Amplifications were performed in a 25 µl volume containing MgCl2 (2.5 mM), reaction buffer (5×, Promega), the four dNTPs (0.2 mM each), the two primers (0.2 µM each), the enzyme Taq polymerase (1U, Promega) and 2 µl of DNA template. Polymerase chain reaction (PCR) was performed for FIB with a step at 94°C for 3 min followed by 35 cycles of: 94 °C for 30 sec, 59 °C for 30 sec, 72 °C for 80 sec, and a single final step at 72 °C for 10 min for CALC with a step at 95 °C for 4 min followed by 35 cycles of: 94 °C for 30 sec, 56 °C for 30 sec, 72 °C for 1.30 min, and a single final step at 72 °C for 3 min. Purification and sequencing of the PCR products were carried out by Macrogen Inc. (www.macrogen.com) by using an ABI PRISM 3700 sequencing system.

Electropherograms were visually checked using FinchTv 1.4.0 (Geospiza Inc.) and aligned using Clustal X 2.0 (Larkin et al. 2007). For each nuclear gene, we did not find statistically significant evidence for recombination using the Pairwise Homoplasy Index (PHI) test ($p > 0.05$) (Bruen et al. 2006) implemented in Splitstree 4.11 (Huson & Bryant 2006).

Table 1. Geographic location and number of individuals (n) of the seven new localities of *Triturus carnifex* sampled.

Population	Country	Locality	Latitude (N)	Longitude (E)	n
38	Italy	Torino	44°54'	7°47'	**3**
39		Ivrea	45°29'	7°54'	**8**
40		Cesano Maderno	45°37'	9°7'	**2**
41		Castelleone	45°16'	9°45'	**6**
42		Campiglia dei Berici	45°19'	11°32'	**3**
43		Cesena	45°36'	9° 7’	**7**
44		San Romano	44° 1'	12° 4'	**2**

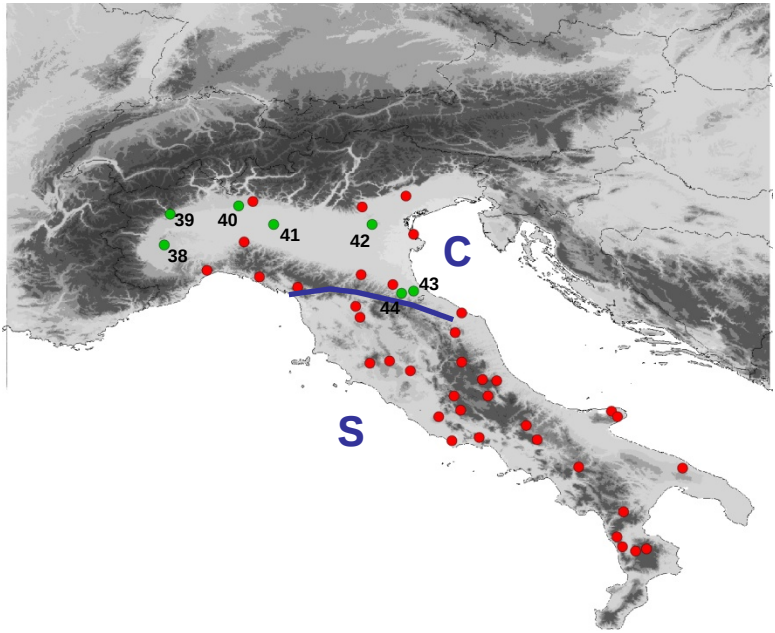


Figure 1. Geographic distribution of the 44 sampling sites of *Triturus carnifex* used for the demographic and modelling analyses. Green circles represent localities of new samples included in this study (see Table 1 of this chapter for details); red circles represent samples used in Chapter 2 (see Table 1 and Figure 1c).

Demographic analyses

The historical demography was assessed separately for the clade C and S of *Triturus carnifex* using the software DnaSP 5 (Librado & Rozas 2009). We used the mismatch distribution analysis (Rogers & Harpending 1992) and the raggedness statistic *r* (Harpending 1994) to compare the observed distribution of nucleotide differences between pairs of haplotypes with the expected distribution under demographic expansion model. Additionally, we calculated the statistics Tajima's *D* (Tajima 1989), Fu's F_S (Fu 1997), and Ramos-Onsin and Roza's R_2 (Ramos-Onsin & Roza 2002). Statistical significance was assessed through 10,000 coalescent simulations.

Specis distribution modeling

Distribution models for clade C and S of *Triturus carnifex* were generated using MAXENT3.3.3e (Phillips et al. 2006). MAXENT implements maximum entropy modelling of the geographical distributions of species; combining presence-only data with ecological-climatic layers to predict species' occurrence in areas where data points are unavailable. We used MAXENT to predict species occurrence under Current, LGM and LIG conditions. We built the models using the default parameters for convergence threshold (10^5) and number of iterations (500). To ensure the consistency of the model predictions, 75% of the localities were used to train the model and 25% were used to test it. The climatic layers (19 bioclimatic variables) for the LIG (resolution 30 arc-seconds) and LGM conditions (resolution 2.5 arc-min) were downloaded from the WorldClim database website (http://www.worldclim.org). For LGM prediction, we used data from the Model for Interdisciplinary Research on Climate (MIROC); for LIG prediction we used data from Otto-Bliesner et al. (2006).

PRELIMINARY RESULTS AND DISCUSSION

The mitochondrial haplotypes found in the new sampling localities were confirmed to belong to the clade C of *Triturus carnifex*. Thus, the current range of this lineage ranges from the Adriatic slope of northern Apennines across the Padano-Venetian plain as far west as the Italian foothills of the western Alps.

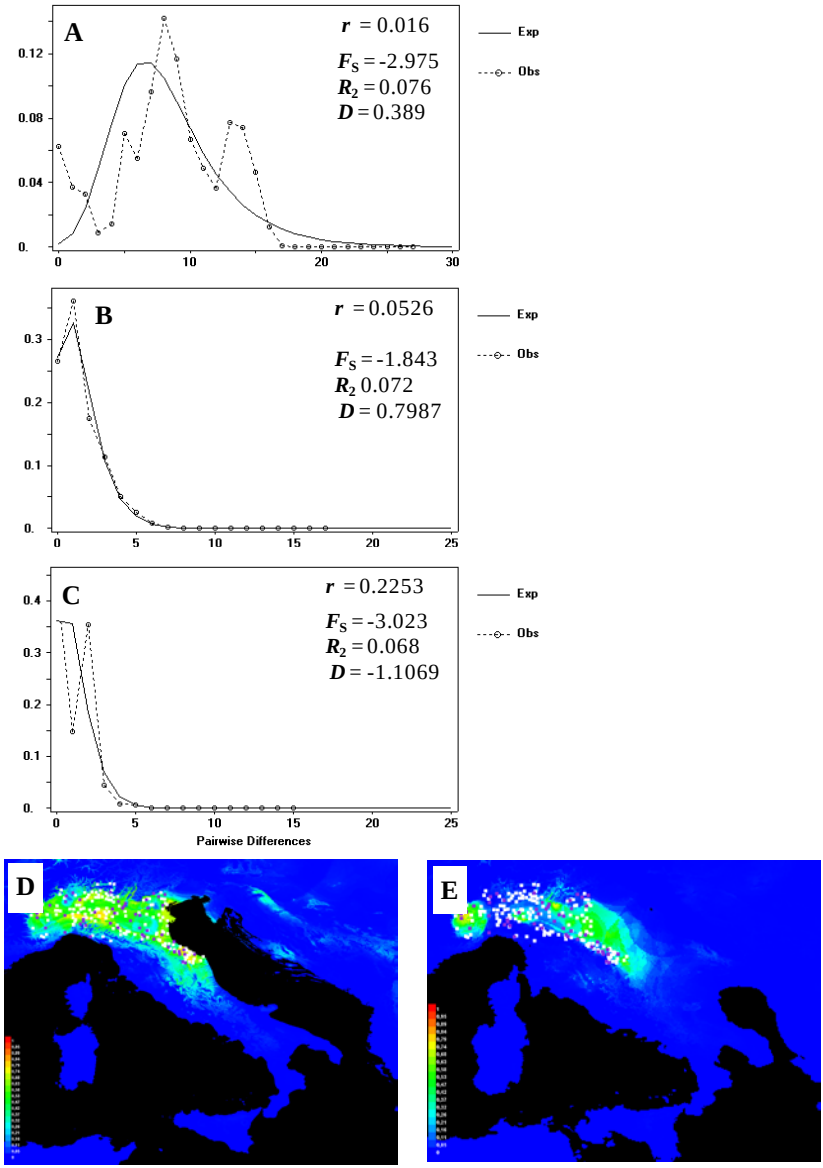
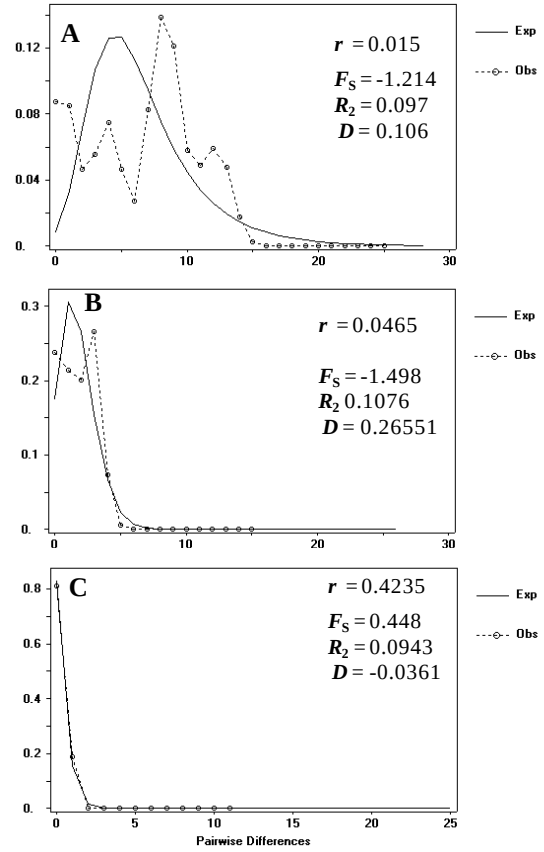
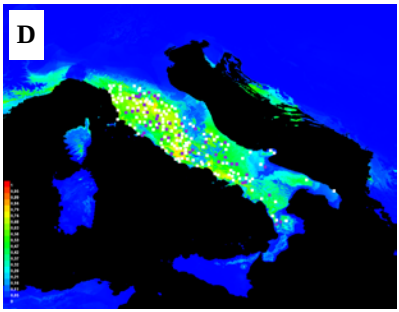


Figure 2. A-C: mismatch distribution graphs and demographic test statistics Fu's F_S, R_2, raggedness *r*, and Tajima *D* of the mitochondrial genes (A), *FIB* (B), and *CALC* (C) for the mtDNA Clade C of *Triturus carnifex*. D, E: distribution models at the Last Interglacial period (D) and at the Last Glacial Maximum (E) for the mtDNA Clade C of *T. carnifex*.





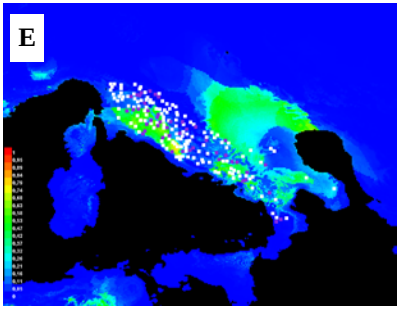


Figure 3. A-C: mismatch distribution graphs and demographic test statistics Fu's F_S, R_2, raggedness *r*, and Tajima *D* of the mitochondrial genes (A), *FIB* (B), and *CALC* (C) for the mtDNA Clade S of *Triturus carnifex*. D, E: distribution models at the Last Interglacial period (D) and at the Last Glacial Maximum (E) for the mtDNA Clade S of *T. carnifex*.

Historical demographic analyses, performed at multiple mitochondrial and nuclear loci, provided evidence of past population stability for both the clade C and clade S (Figure 2 A,B,C; Figure 3 A,B,C), while not rejecting the hypothesis of population expansion. The observed mismatch distributions based on mitochondrial and nuclear haplotypes were in all cases not significantly different from those expected under a demographic expansion model ($r = 0.005$; $P > 0.05$). Non significant values of Fu F_S and R_2 indicated past population stability for both clade C and clade S. Moreover, the non significant and in most cases positive values of Tajima *D* suggest that a demographic contraction (bottleneck) of the clade C and S can be ruled out.

Species distribution models for both clade C and S at the LIG (Figure 2D and Figure 3D) fits well with the distribution models of these clades under Current condition and reflect their current distribution in the Padano-Venetian plain and along the Apennines, respectively. These areas were overall less suitable for *T. carnifex* under LGM conditions (Figure 2E and Figure 3E). Models for this epoch show one suitable area for clade C nearby the foothills of the western Alps and a restricted area with high suitability for clade S around the Arno basin, which could have acted as refugia. The occurrence of a glacial refugia for clade S in coastal and peri-coastal areas of the Arno plain was already postulated based on phylogeographic data (see Chapter 2). An overall loss of suitable habitat was predicted in the central-eastern Padano-Venetian plain for clade C and across the Apennines for clade S. On the other hand, models under LGM conditions, identified a vast area of suitability for *T. carnifex* in the emerged lands of the northern Adriatic, which somehow compensated the lost of suitability predicted for both clade C and S elsewhere.

Overall, based on these preliminary data, both historical demographic inferences and range variations over space and time of clade C and S predicted by SDM suggest that the glacial widening of Padano-Venetian plain provided refugial areas for *T. carnifex* during the Last Glacial Maximum. An increased number of sequenced individuals, a combined demographic analysis of data from multiple loci, an increase number of SDM models and, a quantitative analysis of the area of suitable habitats under LIG and LGM conditions would further confirm this emerging pattern. However, the phylogeographic (and demographic) concordance between the amphibian taxa so far analyzed within the Padano–Venetian plain, suggests that a major glacial refugium for temperate species just between the well-known Apennine and Balkan refugia could have played an important role for the maintenance of Mediterranean biodiversity across Pleistocene ice ages. Moreover, together with other evidences from

different geographical areas (e.g. Sardinia island in the Mediterranean sea, see Bisconti et al. 2011, and references therein) these studies suggest that coastal plain increases have counterbalanced the negative effects of glacial climate in temperate species.

Conclusions

The growing number of phylogeographic, paleoenvironmental, fossil and pollen studies in the western Palaearctic region has showed that temperate species' responses to Plio-Pleistocene climatic oscillations have been widely diverse. Moreover, these studies indicated a variety of refugial structures and locations, such as the microrifuge (Rull 2009), periglacial refugia (Maggs et al. 2008) as well as the aforementioned scenarios of refugia-within-refugia, northern 'cryptic' refugia, and coastal refugia. As more studies become available, either distinctive patterns or new lines of concordance continue to emerge providing new insights on the current and historical pattern of biodiversity of the western Palaearctic region.

The evolutionary histories inferred for the Italian crested newt, *Triturus carnifex*, and the Italian smooth newt, *Lissotriton vulgaris meridionalis*, well exemplifies such a high variability of species' responses and refugial structures.

The Italian crested newt showed three main parapatric mtDNA lineages having Late Pliocene-Early Pleistocene divergence. The lineage N was observed in western Croatia/Slovenia; the lineage C, widespread throughout the Padano–Venetian plain, did not show a clear phylogeographic structure, while the lineage S, widespread south of the northern Apennine chain, was further geographically structured into five sublineages, likely of Middle Pleistocene origin. The phylogeographic structure observed within *T. carnifex* and divergence time estimates among these three lineages, suggest that they have had independent evolutionary histories since the Early Pleistocene and that responses to Pleistocene environmental changes in this single species have been as diverse as those found previously among several co-distributed temperate species combined. Consistent with the landscape heterogeneity, physiographic features, and paleoegeographical evolution of its distribution range, these responses encompass multiple refugia along the Apennine chain, lowland refugia in large peri-coastal plains, and a 'cryptic' northern refugium.

Within the Italian smooth newt, ten mtDNA lineages from distinct geographic compartments across the Apennine Peninsula and north Italy were found. The origin of these lineages ranged from Middle to Late Pleistocene suggesting that *L. v. meridionalis* survived last glacial-interglacial cycles in several refugia, many of them occurring in the northern part of Italy. While this pattern conforms to a multiple refugia scenario across the Apennine Peninsula and the north of Italy, as already

observed in many species, the high number of refugia located in north of Italy is to date an unprecedented pattern among species of the Italian temperate biota and definitely in contrast with the paradigmatic view of genetic 'northern purity'. Moreover, taking into account the current geographic distribution of these northern lineages it is likely that *L. v. meridionalis* would have survived in refugia located in pre-Alpine and peri-northern Apennines areas. Areas at the foothills of mountain systems have been proposed as glacial refugia for temperate species in other areas of the western Palaearctic region (see Schmitt & Varga, 2012; Médail & Diadema 2009 and references therein), likely favoured by more humid conditions in these areas than in the surrounding lowlands, and such scenario may well explain such a unique pattern of 'northern richness' found in *Lissotriton vulgaris meridionalis.*

When comparing the phylogeographic patterns of *T. carnifex* and *L. v. meridionalis* with those of co-distributed species, it becomes evident that temperate species survived the Pleistocene climatic oscillations in a combination of glacial refugia spanning the entire Italian peninsula. In both species as well as in other temperate species (e.g. *Hyla intermedia*, *Pelophylax lessonae*, *Podarcis sicula* and *Vipera aspis*) refugia have been located along the southern, central and northern Apennines conforming to a multiple refugia model. These glacial refugia allowed the persistence of temperate biota which was not merely the result of postglacial colonization processes from refugia located in the southern Italian peninsula as invoked by early paradigms (Hewitt 1996, 1999).

What is more, the phylogeography of these two newts showed that northern regions of Italy acted as glacial refugia as well. A distinct lineage of *T. carnifex* was found in the Padano-Venetian Plain, and together with previous studies on other species (i.e. *Hyla intermedia*, *Pelophylax lessonae*, *Pelobates fuscus*, *Podarcis sicula* and *Vipera aspis*), indicated that this area allowed long-term persistence of temperate species during Pleistocene glacial-interglacial cycles. The phylogeographic structure and distribution of genetic diversity within *L. v. meridionalis* and *T. carnifex* suggested that within north of Italy refugia were located at the foothills of mountain chains as well as in coastal or peri-coastal plains. Indeed, preliminary results from an ongoing research suggested that during the Last Glacial Maximum the widening of the Padano-Venetian plain due to sea-level regression, provided suitable habitat for the survival of both the Padano-Venetian and the Apennine lineages of *T. carnifex*. Moreover, together with the scenario of past demographic stability inferred for these lineages, all these findings indicated that lineage C and S of *T. carnifex* would have benefited from the glacial widening of lowlands areas, counterbalancing the negative effects of demographic contraction usually

expected as a consequence of glacial climate condition. Finally, the phylogeographic (and demographic) concordance between the amphibian taxa so far analysed within the Padano–Venetian plain, suggests the occurrence of a major glacial refugium for temperate species just between the well-known Apennine and Balkan refugia. Thus, this refugial area could have played an important role for the maintenance of Mediterranean biodiversity across Pleistocene ice ages.

In conclusion, this study gave a contribution to unravelling the complex scenario of temperate species' responses to Plio-Pleistocene climatic oscillations in the regional context of Italy. The evolutionary histories inferred for these two sub-endemic newts provided evidence for the occurrence of multiple refugia along the Italian peninsula and identified north Italy as a key area to focus future investigation to evaluate the role of peri-mountains and coastal refugia for the evolution of the Italian biota.

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