On glacial refugia, genetic diversity, and microevolutionary processes: deep phylogeographical structure in the endemic newt *Lissotriton italicus*¹

DANIELE CANESTRELLI,* FLORINDA SACCO and GIUSEPPE NASCETTI

Dipartimento di Ecologia e Sviluppo Economico Sostenibile, Università della Tuscia, Viale dell’Università s.n.c., I-01100 Viterbo, Italy

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We investigated the phylogeographical patterns of *Lissotriton italicus*, a newt endemic to the Italian peninsula, aiming to determine why hotspots of intraspecific diversity so ‘hot’. We found two main mitochondrial DNA lineages (net sequence divergence of 6.8% at two fragments of total length of 1897 bp): one restricted to part of the Calabrian peninsula (i.e. the southernmost portion of the species range) and the other widespread throughout the rest of the species range. Both lineages, which had a parapatric distribution, showed evidence of further subdivisions, with an overall number of eight terminal haplogroups, most of whose times to the most recent common ancestors were estimated at the Late Pleistocene. Analysis of molecular variance suggested that partitioning populations according to the geographical distribution of these haplogroups can explain 97% of the observed genetic variation. These results suggest that *L. italicus* underwent repeated cycles of allopatric fragmentation throughout the Pleistocene, and that it likely survived the Late Pleistocene paleoenvironmental changes within eight separate refugia. Thus, the current hotspot of intraspecific diversity of *L. italicus* (within the Calabrian peninsula) has not been moulded by long-term stability of large populations but rather by multiple events of allopatric fragmentation and divergence. When compared with the patterns recently identified in other species, these results suggest that the occurrence of phases of allopatric divergence (eventually followed by secondary admixture) could be a common, albeit probably underrated feature in the history of formation of hotspots of intraspecific diversity. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, ••–••.


INTRODUCTION

Southern peninsulas of Europe have long been acknowledged as major hotspots of biodiversity at both species and intraspecific level, and as important centres of endemicity (Thompson, 2005; Blondel et al., 2010; Hewitt, 2011). Studies using pollen records, macrofossil remains, genetic markers, and, more recently, ecological niche modelling, have ascertained the role of these peninsulas as major glacial refugia throughout the Plio-Pleistocene (Hewitt, 2004; Schmitt, 2007; Svenning, Normand & Kageyama, 2008; Fløjgaard et al., 2009). Thus, to explain the occurrence of these southern hotspots of diversity, particularly at intraspecific level, two main scenarios have been proposed (Hewitt, 1996, 2000). According to the first scenario, within these refugial ranges, the species would have persisted as large and stable populations, avoiding the loss of diversity that characterizes small, structured or fluctuating populations. In this scenario, refugia are mostly seen as long-term repositories of intraspecific genetic diversity. According to the second scenario, within the refugial ranges, the species repeatedly underwent climate-linked cycles of fragmentation and allopatric divergence,
eventually followed by secondary admixtures. Under this scenario, refugia are instead seen as forges, melting pots of intraspecific diversity (Canestrelli et al., 2010). The first scenario has often been taken for granted and, in many cases, this assumption has also influenced the sampling schemes to a degree that did not allow the use of data to choose between alternative hypotheses (for a longer discussion on this issue see, Gómez & Lunt, 2007; Canestrelli, Cimmaruta & Nascetti, 2008). However, recent evidence for species from all three southern Mediterranean peninsulas suggests that the second scenario may have been much more pervasive than previously suspected (Gonçalves et al., 2009; Previšić et al., 2009; Canestrelli et al., 2010).

Although mostly discussed in the context of the formation of intraspecific patterns of diversity, ascertaining the relative contribution of these two historical scenarios is of major relevance beyond this level of diversity. Indeed, it could help shed more light on the close link between microevolutionary processes and patterns of biodiversity at species level, including the latitudinal gradients of species diversity (Chek, Austin & Lougheed, 2003; Hillebrand, 2004; Martin & McKay, 2004; Mittelbach et al., 2007). Similarly, it could help explain why species endemic to putative refugia often do not conform to the expected positive correlation between range size and genetic diversity, particularly compared to widespread congeners (Hamrick & Godt, 1989; Lewis & Crawford, 1995; Williams, 2007; Derieg, Sangamphai & Bruederle, 2008). Furthermore, the above mentioned microevolutionary processes are known to strongly affect co-evolutionary interaction patterns (e.g. the geographical mosaic theory of coevolution; Thompson, 2005). Finally, populations within the southern hotspots of genetic diversity are often of disproportionate relevance for the conservation of species and biota (Hampe & Petit, 2005).

In the present study, we investigate the contribution of the above-mentioned scenarios to the current population genetic structure of an Italian endemic, the small-bodied newt Lissotriton italicus (Peracca, 1898). This species was one of the first endemics for which a multiple refugia scenario in peninsular Italy was hypothesized (Ragghianti & Wake, 1986). This newt, breeding in lentic or slow-flowing water, has its range limited to the central and southern portions of the Italian peninsula (Fig. 1), mostly at low and intermediate altitudes. Within its range, it is widespread and mostly continuously distributed. The study of geographical variation in this species, carried out with allozymes (Ragghianti & Wake, 1986), revealed that the sampled populations clustered in two main groups of supposed Pleistocene origin: one comprising northern and western populations and the other comprising southern and eastern populations. Nevertheless, no studies have been conducted on a range-wide scale. In particular, the allozyme study did not cover most of the Calabrian peninsula (i.e. the southern part of the species range), whereas this has proved to be an important region, being the most refugia-rich of the Italian peninsula (Podnar, Mayer & Tvrkovic, 2005; Canestrelli et al., 2006a, 2008, 2010; Canestrelli & Nascetti, 2008; Vega et al., 2010). Accordingly, long chapters of the evolutionary history of this species may have passed unread.

Based on a sampling scheme covering the whole species range, we present phylogeographical and historical demographic analyses of the mitochondrial (mt)DNA variation of L. italicus, carried out with the aim of shedding more light on the history of its populations and the evolutionary processes that drove it. We found an unexpectedly deep phylogeographical structure, which significantly complicates the scenario suggested by previous studies. We therefore discuss the palaeoenvironmental context of this scenario, lines of concordance and discordance with observations from other species, and their implications for the evolutionary history of intraspecific hotspots of diversity, as well as for phylogeographical inferences. Finally, we also briefly evaluate the possible taxonomic implications of our findings.

MATERIAL AND METHODS

DATA GATHERING

A total of 201 Italian newts were sampled from 23 populations spanning the entire species range. The geographical location of each sampled population is shown in Figure 1 and Table 1, where the size of each sample is also reported. Tail-tips (approximately 0.5 cm) were removed from every collected individual, carried to the laboratory in liquid nitrogen, and stored at −80 ºC until subsequent analyses. All individuals were released at the point of collection.

Genomic DNA was extracted following the standard phenol–chloroform protocol (Sambrook, Fritsch & Maniatis, 1989) from all sampled Italian newts and from two further newts, one Lissotriton vulgaris and one Mesotriton alpestris, that were used as outgroups in the phylogenetic analyses. We amplified and sequenced two mtDNA fragments. The first fragment (referred to as ND2) comprised the 3′ portion of the tRNA-Met gene (60 bp), the entire gene for the NADH dehydrogenase subunit 2 (1039 bp), and the 5′ portion of the tRNA-Trp gene (38 bp). The second fragment (referred to as ND4) comprised the 3′ portion of the gene encoding for the NADH dehydrogenase subunit 4 (648 bp), the entire tRNA-His gene (70 bp), and part of the tRNA-Ser gene (42 bp). The ND2 fragment was
Figure 1. A, maximum likelihood (ML) tree showing the phylogenetic relationships among the 43 haplotypes found in *Lissotriton italicus*. Bootstrap supports (bs) for both ML and maximum parsimony (MP) trees (ML/MP) are shown at the nodes. *bs > 70%; #bs > 90%. B, geographical distribution of the 23 sampled populations, and distribution among them of main mitochondrial DNA lineages, shown as pie diagrams. The dashed line shows the northern edge of the species’ range. CA, Catanzaro plain; CS, Crati-Sibari plain. Inset: geographical location of the study area within the Western Palearctic region. C, statistical parsimony networks. The size of circles is proportional to haplotype frequency; filled dots represent missing intermediate haplotypes. Populations and haplotypes are numbered as in Table 1.
amplified and sequenced using the polymerase chain reaction (PCR) primers H5018 and L3780, whereas, for the ND4 fragment, the primers ND4 and LEU were used (for the primer sequences, see Babik et al., 2005). For both fragments, PCR reactions were carried out in a 25-mL volume, in accordance with the PCR protocol and cycling conditions described by Babik et al. (2005). Sequencing was carried out by Macrogen Inc. (http://www.macrogen.com), using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems) in accordance with the ABI PRISM BigDye Terminator Cycle Sequencing protocol.

**DATA ANALYSIS**

Sequences were checked and edited using CHROMASPRO, version 1.42 (Technelysium Pty Ltd), and were aligned using CLUSTALX (Thompson et al., 1997). For all subsequent analyses, gaps were treated as missing data. Sequences of the ND2 and ND4 fragments were concatenated using CONCATENATOR, version 1.1.0 (Pina-Martins & Paulo, 2008).

The software MEGA4 (Tamura et al., 2007) was used to determine nucleotide variation and net sequence divergence (Nei, 1987) among major groups of haplotypes, whereas haplotype (h) and nucleotide (π) diversity (Nei, 1987) were estimated using DNASP, version 5 (Librado & Rozas, 2009).

The phylogenetic relationships among haplotypes were inferred using maximum parsimony (MP) and maximum likelihood (ML) methods. MP analysis was conducted in PAUP* 4.0B10 (Swofford, 2003). It was run with the characters unordered and equally weighted, and a heuristic search was carried out, with ten rounds of random sequence addition and tree bisection–reconnection branch swapping. The optimal model of sequence evolution for our data was chosen from 88 distinct models using the Bayesian information criterion as implemented in JMODELTEST, version 0.1.1 (Posada, 2008). The method suggested TrN+Γ (Tamura & Nei, 1993) as the best-fit model for the data, with gamma distribution shape parameter = 0.142. ML analysis was carried out with the PHYML (Guindon et al., 2005), with the starting tree determined by a BIONJ analysis (default setting). For both the MP and ML analyses, the reliability of the inferred tree topologies was assessed by carrying out 1000 bootstrap pseudoreplicates. Nodes with a bootstrap support lower than 70% were considered as unresolved (Hillis & Bull, 1993). Finally, the genealogical relationships among the haplotypes found were also inferred by means of phylogenetic networks,

Table 1. Geographical location and sample size (N) of the 23 sampled populations of *Lissotriton italicus*, and geographical distribution among them of the 43 mtDNA haplotypes found

<table>
<thead>
<tr>
<th>Population</th>
<th>Latitude N</th>
<th>Longitude E</th>
<th>N</th>
<th>Haplotypes (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Serra San Quirico</td>
<td>43°26′</td>
<td>13°01′</td>
<td>2</td>
<td>DIII1(2)</td>
</tr>
<tr>
<td>2 Cascata Vitello D’oro</td>
<td>42°26′</td>
<td>13°49′</td>
<td>1</td>
<td>DIII1(1)</td>
</tr>
<tr>
<td>3 M. Redentore</td>
<td>41°18′</td>
<td>13°37′</td>
<td>14</td>
<td>DIII1(1), DIII3(13)</td>
</tr>
<tr>
<td>4 Pescolanciano</td>
<td>41°40′</td>
<td>14°20′</td>
<td>16</td>
<td>DIII1(13), DIII2(3)</td>
</tr>
<tr>
<td>5 Foresta Umbra</td>
<td>41°51′</td>
<td>16°01′</td>
<td>4</td>
<td>DIII1(4)</td>
</tr>
<tr>
<td>6 Roseto Valfortore</td>
<td>41°22′</td>
<td>15°05′</td>
<td>12</td>
<td>DIII1(8), DIII4(3), DIII5(1)</td>
</tr>
<tr>
<td>7 Conza</td>
<td>40°51′</td>
<td>15°20′</td>
<td>4</td>
<td>DIII6(3), DIII7(1)</td>
</tr>
<tr>
<td>8 Ottati</td>
<td>40°27′</td>
<td>15°19′</td>
<td>10</td>
<td>DIII1(4), DIII4(4), DIII3(1), DIII4(1)</td>
</tr>
<tr>
<td>9 Stio</td>
<td>40°18′</td>
<td>15°15′</td>
<td>7</td>
<td>DIII8(1), DIII1(2), DIII5(4)</td>
</tr>
<tr>
<td>10 Lago Castiglione</td>
<td>40°57′</td>
<td>17°07′</td>
<td>12</td>
<td>CIII1(12)</td>
</tr>
<tr>
<td>11 Alberobello</td>
<td>40°47′</td>
<td>17°14′</td>
<td>6</td>
<td>CIII3(5), CIII4(1)</td>
</tr>
<tr>
<td>12 Policoro</td>
<td>40°12′</td>
<td>16°40′</td>
<td>9</td>
<td>CIII1(1), CIII2(3), CIII5(5)</td>
</tr>
<tr>
<td>13 Lecce</td>
<td>40°21′</td>
<td>18°10′</td>
<td>7</td>
<td>CIII1(7)</td>
</tr>
<tr>
<td>14 Tarsia</td>
<td>39°37′</td>
<td>16°16′</td>
<td>9</td>
<td>CII9(9)</td>
</tr>
<tr>
<td>15 Lago dei Due Uomini</td>
<td>39°33′</td>
<td>16°01′</td>
<td>8</td>
<td>CIII2(2), CIII2(1), CIII4(1), CIII5(2)</td>
</tr>
<tr>
<td>16 San Lucido</td>
<td>39°17′</td>
<td>16°05′</td>
<td>6</td>
<td>CII6(1), CII7(3), CII8(2)</td>
</tr>
<tr>
<td>17 Crosia</td>
<td>39°34′</td>
<td>16°46′</td>
<td>8</td>
<td>BII(8)</td>
</tr>
<tr>
<td>18 Verzino</td>
<td>39°18′</td>
<td>16°51′</td>
<td>8</td>
<td>BII2(2), BII3(5), BII4(1)</td>
</tr>
<tr>
<td>19 Nocera Terinese</td>
<td>39°02′</td>
<td>16°09′</td>
<td>15</td>
<td>AIII1(1), AIII1(4), AIII2(10)</td>
</tr>
<tr>
<td>20 Polia</td>
<td>38°44′</td>
<td>16°20′</td>
<td>12</td>
<td>AIII3(12)</td>
</tr>
<tr>
<td>21 Zungri</td>
<td>38°40′</td>
<td>15°59′</td>
<td>6</td>
<td>AIII2(2), AIII4(3), AIII5(1)</td>
</tr>
<tr>
<td>22 S. Ilario dello Jonio</td>
<td>38°13′</td>
<td>16°11′</td>
<td>13</td>
<td>AIII5(5), AIII2(4), AIII3(1), AIII4(3)</td>
</tr>
<tr>
<td>23 Madonna dell’Oleandro</td>
<td>38°00′</td>
<td>15°43′</td>
<td>12</td>
<td>AIII5(12)</td>
</tr>
</tbody>
</table>
The historical demographic trends were investigated for the main mtDNA lineages through an analysis of the distribution of pairwise nucleotide differences between haplotypes (i.e. the mismatch distribution). The observed distribution was compared to those expected under both a pure demographic expansion model (Rogers & Harpending, 1992) and a sudden spatial expansion model (Excoffier, 2004). We used the sum of square deviations (SSD) between the estimated and observed mismatch distributions as a goodness-of-fit statistic, of which the significance was assessed by means of 1000 bootstrap replicates. Finally, for each lineage, we also computed the raggedness index $r$ (Harpending, 1994; significance assessed similarly to SSD) and the statistic $F_S$ (Fu, 1997; significance assessed by means of 1000 coalescent simulations). These analyses were carried out using ARLEQUIN.

RESULTS

For the 201 Italian newts analyzed, the final data matrix comprised 1897 characters. Among these, 183 were variable positions, of which 153 were parsimony informative, and a total of three indels were observed (all 1 bp long). Forty-three composite haplotypes were found, whose distribution among populations is given in Table 1 (Genbank accessions: JN788173-JN788258).

The ML tree showing the phylogenetic relationships between haplotypes is shown in Figure 1A. The log-likelihood score for the single ML tree was $-5951.96$. MP analysis recovered eighteen most parsimonious trees, 768 steps in length (consistency index $= 0.870$; retention index $= 0.945$). An essentially identical topology was revealed by both the ML and MP analysis (with a few minor differences involving terminal nodes). Two main clades were found, showing a net ML-corrected sequence divergence of 0.068 (ND2: 0.071; ND4: 0.063). The first clade (referred to as clade N) was geographically distributed in the northern and central portions of the species range, south as far as the central-western portion of the Calabrian peninsula (populations 1–16; Fig. 1B). The second clade (referred to as clade S) was distributed in the central-eastern and southern portions of the Calabrian peninsula (populations 17–23). Haplotypes of these two clades were never found in sympatry and the shortest geographical distance observed between populations belonging to these two clades (populations 16 and 19) was approximately 28 km. Within clade N, two main subclades were observed (Fig. 1), showing a net divergence of 0.019 (ND2: 0.015; ND4: 0.024): one with a more eastern and southern distribution (clade C; populations 10–13) and the other with a more northern and western distribution (clade D; populations 1–9). A
further subdivision into two haplogroups, approximately distributed along the north–south axis was observed within both clades C and D (see haplogroups CI–CII, with the haplogroup CI being paraphyletic, and DI–DII of Fig. 1). Also within the southern clade S, two main subclades were observed (A and B of Fig. 1): one found only in the two populations close to the Sila massif (clade B; populations 17 and 18) and the other found further south (clade A; populations 19–23). These two subclades showed a net divergence of 0.013 (ND2: 0.010; ND4: 0.016). Finally, within subclade A, a further subdivision into three haplogroups (AI, AII, and AIII) was observed: one observed only slightly north of the Catanzaro plain (haplogroup AIII; population 19) and the others occurring south of this plain (haplogroups AI and AII; populations 20–23). Interestingly, the co-occurrence among clades was minimal among the studied populations, only being observed among the haplogroups DI and DII at population 9 and among the haplogroups AII and AIII at population 19.

The phylogenetic networks are shown in Figure 1C. Under the 95% criterion for a parsimonious connection, the method does not allow the joining of all haplotypes found into a single network. Instead, five separate networks were obtained. All the clades identified by the phylogenetic tree analyses were also clearly apparent in the network analysis, either as separate networks or groups of more closely-related haplotypes within them.

Bayesian analysis carried out in BEAST showed effective sample sizes largely exceeding 500 for all the parameters of interest, and a full convergence of posterior likelihoods between runs. Therefore, the results of the five independent runs were combined. Estimates of the time to the most recent common ancestor for the main mtDNA clades are shown in Table 2. According to Calibration I, for all the terminal haplogroups (i.e. AI-AIII, B, CI, CII, DI, and DII), the TMRCA estimates fell well within the Late Pleistocene (approximately 10 000–125 000 years ago), whereas other TMRCA estimates within both clades N and S (i.e. for clades A, C, D, N, and S) suggested divergences of Middle Pleistocene origin (approximately 780 000–125 000 years ago). Finally, the divergence between the two main mtDNA lineages observed (i.e. N and S) was estimated to have occurred approximately 1.7 Myr ago, within the Early Pleistocene (approximately 780 000–2 600 000 years ago). Considering Calibration II, TMRCA estimates were approximately twice as ancient as those obtained with Calibration I.

AMOVA showed that most of the genetic variation (97%; $F_{CT} = 0.97$) was attributable to the differences between the eight population groups (see haplogroups AI, AII, AIII, B, CI, CII, DI, and DII of Fig. 1), whereas a minor portion of this variation was explained by either the among-populations within-groups (1.7%; $F_{SC} = 0.63$) or the within-populations (1%; $F_{ST} = 0.99$) levels of variation. However, all the variance components and fixation indices were highly significant (all $P < 0.001$).

Estimates of the haplotype and nucleotide diversity for the eight terminal haplogroups are given in Table 2. The nucleotide diversity varied from 0.00023 of clade AIII to 0.00216 of clade AI. Clade AIII also

<table>
<thead>
<tr>
<th>Clade</th>
<th>$N$</th>
<th>TMRCA</th>
<th>Genetic diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calibration I</td>
<td>Calibration II</td>
</tr>
<tr>
<td>AI</td>
<td>25</td>
<td>84 (26–156)</td>
<td>181 (54–339)</td>
</tr>
<tr>
<td>AII</td>
<td>19</td>
<td>72 (23–136)</td>
<td>156 (45–293)</td>
</tr>
<tr>
<td>AIII</td>
<td>14</td>
<td>28 (2–53)</td>
<td>61 (3–119)</td>
</tr>
<tr>
<td>B</td>
<td>16</td>
<td>72 (23–136)</td>
<td>115 (32–237)</td>
</tr>
<tr>
<td>CI</td>
<td>23</td>
<td>54 (18–101)</td>
<td>118 (36–220)</td>
</tr>
<tr>
<td>CII</td>
<td>34</td>
<td>39 (15–70)</td>
<td>84 (30–154)</td>
</tr>
<tr>
<td>DI</td>
<td>16</td>
<td>44 (18–81)</td>
<td>94 (31–179)</td>
</tr>
<tr>
<td>DII</td>
<td>54</td>
<td>93 (33–167)</td>
<td>202 (66–372)</td>
</tr>
</tbody>
</table>
showed the lowest level of haplotype diversity (0.44), whereas the highest haplotype diversity was estimated for clade CI (0.83).

The mantel test carried out between the genetic (linearized $F_{ST}$) and log-geographical distance matrices suggested the occurrence of a weak ($R^2 = 0.08$) albeit significant ($P < 0.01$) pattern of isolation-by-distance among the studied populations.

The historical demographic analyses for all the terminal haplogroups are shown in Figure 2. For the four haplogroups AI, AII, B, and CI, the observed mismatch distribution showed a closer fit with the one expected under a spatial rather than under a pure demographic expansion. Furthermore, for the haplogroups AI and CI, both the SSD statistic and the $r$ index allowed us to reject the hypothesis of a pure demographic expansion but not that of a spatial expansion. For the other four haplogroups (AIII, CII, DI, and DII), both the spatial and the pure demographic expansions models fitted equally the observed mismatch distributions, and could not be rejected on the basis of either the SSD or the $r$ statistics. Finally, no significant values of the Fu’s (Fu, 1997) $F_s$ statistic were observed.

**DISCUSSION**

The phylogeographical pattern observed in *L. italicus* is of unprecedented complexity among the species from the Italian peninsula studied to date (Canestrelli et al., 2010; Vega et al., 2010). Indeed, our data provided evidence of two divergent lineages: one restricted to the Calabrian peninsula and the other widespread throughout the rest of the species’ range, with further phylogeographical subdivisions within them, suggesting that the species could have been fragmented into eight separate refugia during its recent evolutionary history.

To set an approximate time scale to the inferred events in the evolutionary history of the Italian newt, we used two alternative calibrations sensu Zhang et al. (2008) and based on an estimated time of divergence between *M. alpestris* and *L. vulgaris* of 20.6 Myr (Calibration I) and 45.2 Myr (Calibration II) respectively. However, two arguments lead us to deserve TMRCA estimates derived from Calibration II as implausible. First, Zhang et al. (2008) suggested Calibration I to be more reliable, showing a best fit to independent biogeographical inferences and being based on more calibration points and whole mitochondrial genomes. Second, and most importantly, the time of divergence between the two main lineages of *L. italicus* estimated based on Calibration II (3.654 Myr) does not fit well-established events in the paleogeographical history of the study area. Indeed, the Calabrian arc reached its current position (i.e. the geographical continuity with the rest of the Italian peninsula) after a south-eastward migration, which started approximately 7.6 Myr from east of the Sardinia island (Duermeyer et al., 1998) and ended not before the Late Pliocene–Early Pleistocene (Rosenbaum, Lister & Duboz, 2002). Therefore, the colonization of the Calabrian peninsula by *L. italicus* (and consequently the subsequent divergence between clade N and clade S) could not have occurred before this epoch, as Calibration II would suggest. Interestingly, the same argument was also recently invoked to explain why many taxa from southern Italy show shallower divergences than that usually observed in related taxa from the Iberian peninsula (Hewitt, 2011). On the other hand, the divergence time estimate derived from Calibration I are fully consistent with this paleogeographical scenario. Thus, in the subsequent discussion, we will only rely on TMRCA estimates derived from Calibration I.

The sharp phylogeographical discontinuity between clade N and clade S was approximately coincident with the Crati-Sibari plain (Fig. 1). This plain was affected by intense tectonic activity, and was repeatedly marine-flooded during the Plio-Pleistocene marine transgressions, separating the Italian peninsula from the Calabrian and Sicilian palaeoarchipelago (Martini, Sagri & Colella, 2001; Cucci, 2004). Palaeobiogeographic, palaeontological, and, more recently, phylogeographical data have shown that the Crati-Sibari plain has repeatedly acted as an effective barrier to dispersal of terrestrial fauna and flora along the north–south axis (Pignatti, 1984; Santucci, Nascetti & Bullini, 1996; Bernasconi, Corselli & Carobene, 1997; Canestrelli et al., 2006a, 2010; Canestrelli, Cimmaruta & Nascetti, 2007), and have identified this area as a possible suture zone (sensu Swenson & Howard, 2005).

The occurrence of these two deeply divergent lineages within *L. italicus* was unexpected, according to a previous allozyme survey of variation (Ragghianti & Wake, 1986). Indeed, in that study, the authors identified ‘two weakly differentiated units’. However, the geographical distribution of these units largely resemble the one of the mtDNA subclades C and D. Furthermore, for these subclades, we estimated Middle Pleistocene divergence (Table 2), which agrees with that inferred by Ragghianti & Wake (1986). Finally, the southernmost population sampled by these authors (Grisolia) was approximately 25 km north-west of our population 15 (i.e. well within the range of clade N). Therefore, we argue that the differences in the pattern of divergence between our data and those of Ragghianti & Wake (1986) are a result of discordances between kinds of markers but, instead, to the lack of populations in the former study from the geographical range of clade S, which thus remained undetected.
Both clades N and S were further subdivided (Fig. 1), with eight terminal subclades showing restricted geographical distributions. All the TMRCAs of the eight terminal subclades (CI–CII, DI–DII for clade N and AI, AII, AIII, and B for clade S) were estimated at approximately the Late Pleistocene, whereas divergence between them was estimated at the Middle Pleistocene (Table 2). Furthermore, within both clades N and S, at least two series of fragmentation events could be inferred. Indeed, the divergence between subclade pairs C–D (clade N) and A–B (clade S) would have occurred early in the Middle Pleistocene, whereas the divergence between subclade groups CI–CII, DI–DII, and AI–AII–AIII would have occurred more recently (late Middle Pleistocene; Table 2). This overall pattern suggests that the species have undergone several rounds of fragmentation throughout the Pleistocene, with the last occurring during the Late Pleistocene glacial cycle and leading to a fragmentation into eight population units.

Historical demographic analyses and neutrality test statistics do not support recent population expansions for all the subclades (Fig. 2). However, this does not allow us to reject the hypothesis of postglacial demographic recoveries. Indeed, a strong population differentiation was observed even within the eight groups ($F_{SC} = 0.63$), with most haplotypes being private to one or few populations (Table 1). This strong pattern of population fragmentation can affect coalescence-based demographic inference, leading to a semblance of population stability (i.e. a ragged mismatch distribution and low and not significant values of the neutrality test statistics; Hein, Schierup & Wiuf, 2005). On the other hand, the lack of co-presence among several haplogroups, despite close contiguity (see, for example, the reciprocal distribution of the main clades N and S in central western Calabria), suggests that, if expansions and secondary contacts occurred between them, these would not have led to extensive admixture. However, the data in our hands does not allow us to say much more in this respect, and a closer sampling scheme, as well as analysis of both nuclear and mitochondrial genetic diversity, will be necessary to address what microevolutionary processes have shaped variation in the transition zones between the main clades.

As stated above, the phylogeographical pattern observed in *L. italicus* is particularly complex among species endemic to the Italian peninsula, in that no case studied to date has shown evidence for eight distinct refugia in southern Italy. This phylogeographical pattern shows at least two main lines of concordance with previously studied cases from the south of the Italian peninsula. The first involves the Catanzaro plain (Fig. 1), an area that, as was the Crati-Sibari plain, was repeatedly marine flooded until the last interglacial as a result of glaciation-induced marine transgressions (Ghisetti, 1979; Caloi, Malatesta & Palombo, 1989; Bonfiglio et al., 2002), and which has emerged as a source of important biogeographical and phylogeographical discontinuities (Caloi et al., 1989; Santucci et al., 1996; Podnar et al., 2005; Canestrelli et al., 2006a, 2008, 2010). The geographical distribution of clades A and B on one hand, and clades AI and AII–AIII on the other, suggests that the Catanzaro plain could have acted as a barrier to newt dispersal at least twice: the first one in the Middle Pleistocene, leading to the divergence between clades A and B, and the second one during the last interglacial, when, after a northward expansion of clade A, it could have led to the divergence between clade AIII and clades AI and AII. Although this scenario would need the support of further data (involving a closer sampling in the area), it appears plausible based on the palaeogeographical history of the area, and it has recently been invoked to explain the genetic patterns in another species with restricted dispersal abilities, the Roman mole *Talpa romana* (Canestrelli et al., 2010). The second line of concordance involves the phylogeographical discontinuity between clades C and D, located in central-southern Italy. Indeed, this discontinuity has already been noted in other species (Lenk & Wüster, 1999; Steinfartz, Veith & Tautz, 2000; Nascetti, Zangari & Canestrelli, 2005; Canestrelli, Zangari & Nascetti, 2006b; Barbanera et al., 2009). Unlike the Crati-Sibari and Catanzaro plains, this area did not undergo marine submersion during the Middle and the Late Pleistocene. Nonetheless, the observed pattern could be explained by considering: (1) the topographical structure of the area, with the south-east to north-west orientation of the Apennine chain; (2) the scattered but diffused occurrence of glaciers.
around its mountain peaks during Pleistocene glaciations (Acquafrredda & Palmentola, 1986); and (3) the essentially thermophilic habits of the Italian newt, whose population sizes are currently more abundant at low altitudes (below 600 m a.s.l.; Sindaco et al., 2006).

Finally, the complex phylogeographical structure that we observed in *L. italicus* parallels that previously observed in two closely-related species, *Lissotriton boscai* (Martínez-Solano et al., 2006) from the Iberian peninsula and *L. vulgaris* (Babik et al., 2005) from the Balkans and adjacent areas. Also in these species, complex phylogeographical structures were found, with deep divergences among several lineages and evidence for a scenario involving multiple Plio-Pleistocene refugia. When compared with that observed in other co-distributed species, including but not limited to amphibians (Gómez & Lunt, 2007; Canestrelli et al., 2008, 2010), this concordant complexity of the respective phylogeographical patterns suggests that small-bodied newts are particularly prone to retaining (and thus particularly appealing to investigate) the genetic imprints of the palaeoecological evolution of their habitats and the microevolutionary processes that it triggered.

**Main Implications and Conclusions**

The geographical coincidence of hotspots of intraspecific diversity and putative glacial refugia has long led us to assume a causative relation between the two, and thus to see these areas as long-term repositories of intraspecific variation for many temperate species, owing to the hypothesized occurrence of persistently large and demographically stable populations. Furthermore, this view has been seen as especially plausible for species endemic to putative refugia (Lewis & Crawford, 1995; Derieg et al., 2008). Our data clearly do not support this scenario for the Italian newt. Both the main lineages and the majority of haplotypes (58%) and haplogroups (63%) were found within the Calabrian peninsula, which can therefore be seen as a hotspot of intraspecific diversity for *L. italicus*. Nevertheless, although long-term persistence of the species in this area can be inferred from our data, the long-term stability of a single large population cannot. The most plausible scenario instead appears to involve multiple cycles of allopatric fragmentation as a result of the repeated formation of seaways during Plio-Pleistocene marine transgressions. Although the complexity of the genetic pattern observed in *L. italicus* is somewhat extreme in this respect, similar patterns, possibly involving evidence for secondary admixture between lineages (Canestrelli et al., 2010), have recently been documented in species or differentiated lineages endemic to various putative refugia, including southern Italy (Gómez & Lunt, 2007; Previšić et al., 2009; Canestrelli et al., 2010). This congruence among different species and areas suggests that this pattern could be more widespread than previously appreciated. Interestingly, similar patterns are emerging in areas that were historically affected by the formation of seaways during Plio-Pleistocene marine transgressions (e.g. Italy, the Aegean, Baja California, the Philippines) (Riddle et al., 2000; Papadopoulou et al., 2009; Canestrelli et al., 2010; Ravago-Gotanco & Junio-Meñez, 2010), as well as in areas where other processes were involved, such as the interplay between paleoclimatic oscillations and several physiographical features (e.g. Iberia, Australia, North America, subtropical China) (Gómez & Lunt, 2007; Byrne et al., 2008; Wang et al., 2009; Shafer et al., 2010). This concordance suggests that, beyond the diversity of physiographical features, palaeoecological histories, and the consequent phylogeographical patterns, the occurrence of phases of allopatric divergence (sometimes followed by secondary admixture) could be a common feature in the history of the formation of intraspecific hotspots of diversity.

Another issue exemplified particularly well by the case study presented here is the importance of a thorough sampling throughout a species range. The allozyme study of Ragghianti & Wake (1986) was probably the best-sampled survey of genetic variation among Italian amphibians at the time. Nevertheless, owing to the lack of populations from the south of the species range, it left unread the oldest chapter of the species’ evolutionary history (i.e. the existence of the two divergent lineages N and S). Despite early claims (Taberlet, 1998) and the above mentioned emerging patterns, many studies continue to adopt sampling schemes that are not appropriate for recovering the possible occurrence of the genetic patterns described above (Nagy et al., 2002; Böhme et al., 2007; Giovannotti et al., 2007; Rato et al., 2009; Giovannotti, Nisi-Cerioni & Caputo, 2010). Such sampling schemes are likely to fail to unravel the relative contribution of distinct microevolutionary processes to current patterns of diversity. In particular, they are likely to underrate the contribution of allopatric divergence and gene exchange, with far-reaching implications under evolutionary, ecological and conservation perspectives (Hampe & Petit, 2005; Thompson, 2005; Arnold, 2006; Gómez & Lunt, 2007; Canestrelli et al., 2008).

Finally, the occurrence of two deeply divergent lineages (sequence divergence 0.068) that are in close contiguity but not sympatry in northern Calabria could suggest the occurrence of two cryptic species within *L. italicus*, particularly compared to that previously observed in other newts. For example, at the ND4 gene, a sequence divergence of 0.050 and 0.051 was observed between *Triturus marmoratus*/*Triturus
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