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Molecular survey of morphological subspecies reveals new mitochondrial lineages in *Podarcis muralis* (Squamata: Lacertidae) from the Tuscan Archipelago (Italy)

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Abstract

Recent analyses of molecular markers have significantly revised the traditional taxonomy of *Podarcis* species (Squamata: Lacertidae), leading to critically reconsider the taxonomic value of several subspecies described only on morphological bases. In fact, lizards often exhibit high morphological plasticity both at the intra-specific and the intra-population level, especially on islands, where phenotypic divergences are mainly due to local adaptation, rather than to evolutionary differentiation. The Common wall lizard *Podarcis muralis* exhibits high morphological variability in biometry, pholidosis values and colour pattern. Molecular analyses have confirmed the key role played by the Italian Peninsula as a multi-glacial refuge for *P. muralis*, pointing out the lack of congruence between mitochondrial lineages and the four peninsular subspecies currently recognized. Here, we analyse a portion of the protein-encoding cytochrome *b* gene in the seven subspecies described for the Tuscan Archipelago (Italy), in order to test whether the mitochondrial haplotypes match the morphologically based taxonomy proposed for Common wall lizard. We also compare our haplotypes with all the others from the Italian Peninsula to investigate the presence of unique genetic lineages in insular populations. Our results do not agree completely with the subspecific division based on morphology. In particular, the phylogenetic analyses show that at least four subspecies are characterized by very similar haplotypes and fall into the same monophyletic clade, whereas the other three subspecies are closer to peninsular populations from central Italy. From these results, we conclude that at least some subspecies could be better regarded as simple eco-phenotypes; in addition, we provide an explanation for the distinctiveness of exclusive lineages found in the archipelago, which constituted a refuge for this species during last glacial periods.

Key words: Common wall lizard – cytochrome *b* – insular populations – Mediterranean islands – phylogeny – phylogeography – subspecies – Tuscan Archipelago

Introduction

The genus *Podarci*s Wagler, 1830 has evolved and diversified in the Mediterranean Basin (Arnold 1973; Arnold et al. 2007), where it represents the predominant reptile group (Harris and Arnold 1999). The diversification of the taxon dates from the Oligocene, and it was followed by the event of radiation from the Miocene (16–10 Ma; see Poulakakis et al. 2005). Nineteen species are recognized today (Sindaco and Jeremćenko 2008), but because of its recent spreading this genus seems to be characterized by a high incidence of cryptic lineages, leading to the occurrence of peculiar patterns of genetic variability (e.g. Carretero 2008). In this context, the recent emergence of molecular tools has significantly revised the traditional systematic of *Podarci*s species (e.g. Harris and Arnold 1999; Carranza et al. 2004), as already detected for the *Podarcis hispanica* species complex (Harris and Sá-Sousa 2002), *P. erhardii* (Poulakakis et al. 2003) and *P. tiliguerta* (Harris et al. 2005). Molecular studies have also increased the uncertainty of several described morphological subspecies, as pointed out by Podnar et al. (2004, 2005) for *P. melisellensis* and *P. sicula*, respectively. In fact, several *Podarcis* species show high plasticity at both the intra-specific and the intra-population level in morphological characters (such as body size, shape and colour patterns; see Arnold et al. 2007 for details), which greatly complicates their taxonomy. The re-evaluation of morphological subspecies recognized in small islands is particularly needed (e.g. Böhme 1986), because, though insular subspecies may sometimes deserve species status from a molecular point of view, their phenotypic divergences are mainly due to local adaptation, suggesting the occurrence of simple eco-phenotypes (e.g. Biaggini et al. 2009).

The Common wall lizard *Podarcis muralis* (Laurenti, 1768) is distributed in southern, western and central Europe, where it occupies a wide variety of habitats. Within the genus, *P. muralis* is considered the 'least Mediterranean' of all the species (Corti and Lo Cascio 2002), being present in the southern part of its range at higher altitudes (e.g. southern Italy and southern Greece). Moreover, this species is not present in most Mediterranean islands, except for some Tyrrhenian islands and the Samothrace Island. The high morphological variability in colour pattern, biometry and pholidosis values led up to a complex taxonomy in the past, allowing the proliferation of many morphological subspecies (e.g. Mertens and Wermuth 1960; Gruschwitz and Böhme 1986; Guillaume 1997). Concerning the Italian Peninsula, which represents the primary range of the species from where it spread to the rest of Europe quite recently (Harris and Arnold 1999), four morphological subspecies are currently recognized: *P. muralis muralis* (Laurenti, 1768), *P. m. maculiventris* (Werner, 1891), *P. m. nigriventris* Bonaparte, 1836 considered by Gruschwitz and Böhme (1986) to

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be synonymous with *P. m. brueggemanni* (Bedriaga, 1879) and *P. m. breviceps* (Boulenger, 1905). In addition, at least seven morphological subspecies were described during the 20th century for the Tuscan Archipelago, which includes seven main islands situated between Corsica and central Italy: *P. m. baldasseronii* (Taddei, 1949) on Palmaiola Island, *P. m. colosii* (Taddei, 1949) on Elba Island, *P. m. marcuccii* (Lanza, 1956) on Argentarola Islet and *P. m. beccarii* (Lanza, 1958) on Port'Ercole Islet (both near Monte Argentario), *P. m. vinciguerrai* (Mertens, 1932) on Gorgona Island, *P. m. insulanica* (Bedriaga, 1881) on Pianosa Island and *P. m. muellerlorenzi* (Taddei, 1949) on La Scola Islet (east of Pianosa). Noteworthy, the populations of the two paleo-islands Monte Massoncello (Livorno, Tuscany) and Monte Argentario (Grosseto, Tuscany) are currently ascribed to *P. m. nigriventris*, whereas in the past they have been respectively classified as *P. m. colosii* and *P. m. paulinii* (Taddei, 1949), because of their morphological dissimilarity from all other peninsular populations.

Although widespread at a continental scale, the Italian populations of *P. muralis* show higher genetic variability than those from the rest of Europe, as estimated both by allozyme electrophoresis (Capula 1997; Capula and Corti 2010) and molecular markers analyses (Giovannotti et al. 2010). In particular, mitochondrial analysis has recently confirmed the key role played by the Italian Peninsula as a glacial refuge for *P. muralis* during the Plio-Pleistocene climatic fluctuations. These findings support the 'Refugia-within-refugia' scenario (Gòmez and Lunt 2007) for the genetic differentiation of Italian populations and also denote a lack of congruence between mitochondrial lineages and the four peninsular subspecies described on a morphological basis (Giovannotti et al. 2010).

Recent works highlight that reptiles, and lizards in particular, can be used not only as sensitive biogeographic indicators, because of their limited dispersal capacity (Lenk et al. 1999), but also as model organisms for ecological, evolutionary and phylogeographic studies (Camargo et al. 2010). Concerning *P. muralis*, despite the recent phylogeographic and phylogenetic achievements, molecular data from insular populations are still lacking, as well as the genetic patterns of colonization of the Tuscan Archipelago. In addition, the genetic uniqueness of insular subspecies still requires confirmation by molecular analysis. Since the morphological subspecies described for the Tuscan Archipelago represent insular isolates (three of them, *P. m. muellerlorenzi*, *P. m. marcuccii* and *P. m. beccarii*, are each restricted to a single islet), we presume that most of them may be indistinguishable on a molecular basis. Anyway, due to the complex paleogeographic history of the archipelago, we hypothesized that peculiar genetic lineages could be detected in this area. Therefore, we collected representatives of all the seven morphological subspecies of *P. muralis* described for the Tuscan Archipelago, as well as specimens from the two paleo-islands of Monte Argentario and Monte Massoncello in order to: (1) test whether mitochondrial haplotypes match the morphologically based taxonomy proposed for insular subspecies during the 20th century; (2) add new molecular data that will be useful for a comprehensive analysis of *P. muralis* genetic variability; (3) investigate the role played by these islands during the last glaciations peaks, in order to understand also the biogeography of other species.

Materials and methods

As a whole, our study included the analysis of 55 *P. muralis* samples (Table S1) distributed as following: 35 specimens from the 'La Specola' Natural History Museum (Firenze, Italy), encompassing all the morphological subspecies reported for the Tuscan Archipelago and covering the entire distribution range of the species on these islands (i.e. 10 localities; Fig. 1); two museum specimens of *P. m. maculiventris* from Verona and eight specimens collected near Pavia (Lombardy, 45°11′09″N, 9°09′23″E) and Bereguardo (Lombardy, 45°15′16″N, 9°00′44″E) in order to obtain an intra-specific comparison of insular and continental subspecies genetic variability; 10 *P. m. nigriventris* collected from two peninsular populations near Calci (Tuscany, 43°43′18″N, 10°31′21″E) and Borgo Montello (Latium, 41°30′35″N, 12°46′26″E), to compare them with the populations of Monte Argentario and Monte Massoncello (Fig. 1).

Museum specimens were previously collected and classified by taxonomists, so that their subspecific assignment could be scored with certainty. Tissues samples were taken from one posterior leg or soft organs, and stored at room temperature in ethanol 96%. In the field, individuals were captured by noosing (Blomberg and Shine 1996) and quickly released after tail tips (2 mm on average) were collected. DNA from ethanol-preserved museum specimens was extracted after homogenization and rehydration, using the DNAeasy®Tissue kit (QiagenGmbH, Hilden, Germany) according to the manufacturer's instructions, while DNA from fresh tail tips was extracted with standard K-Proteinase digestion according to the Archive Pure DNA Tissue kit (Eppendorf AG, Hamburg, Germany) protocol.

We selected the 5′ region (i.e. 405 bp) of the mitochondrial protein-encoding cytochrome *b* (cyt *b*) gene to compare our sequences to those already published for populations of northern, central and southern Italy. Polymerase chain reactions (PCRs) were performed using universal primers L14725 (5′-GTGACTTGAAAAACCACCGTTG-3′, modified from Irwin et al. 1991) and H15149 (5′-GCCCCTCAGAATGATATTTGTCCTCA-3′, Kocher et al. 1989). Thermal conditions involved an initial denaturation step of 3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 47°C, 30 s at 72°C and a final extension step of 7 min at 72°C. Twenty-microlitre reactions were used for all the amplifications, containing PCR Buffer with 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.2 μM of each primer, 0.5 U of MasterTaq polymerase (Eppendorf AG), and approximately 100 ng of genomic DNA. Sequencing was carried out on an ABI 155 3730XL (Macrogen Inc., Seoul, Korea). Mitochondrial DNA sequences were corrected by eye, then alignments were made using Clustal W (Thompson et al. 1994), implemented in BioEdit 7.0 (Hall 1999). The basic sequences statistics, pairwise comparison of uncorrected sequences divergence (*p*-distance) and nucleotide composition were obtained using MEGA 4.1 (Kumar et al. 2008). We also estimated the net nucleotide divergence (*Da*) between main mtDNA clades based on cyt *b* sequences selecting K2P (Kimura-2-parameter; Kimura 1980) correction in MEGA, in order to quantify the between-group variation taking into account the within-group variation in haplotypes. Noteworthy, this metric can be used to estimate the splitting times between groups (Nei 1987). Divergent times between mtDNA lineages identified in this study were estimated using the evolutionary rates already published for *P. erhardii* cyt *b* sequence (1.45–1.59% Myr; Poulakakis et al. 2003), assuming that rates among related taxa are likely to be similar (Avise 1994).

To look for the presence of new mitochondrial lineages, we selected published haplotypes of *P. muralis* from the Italian Peninsula (i.e. 30 haplotypes, accession numbers: FJ867365–FJ867394; Giovannotti et al. 2010) corresponding to the same DNA fragment analysed in this work (i.e. 405 bp). List of peninsular haplotypes retrieved from the literature are reported in Table S1, while relative sampling localities are shown on the map (Fig. 1). Interestingly, these authors highlighted the presence through the Italian Peninsula of two main mtDNA clades: clade 1, further split in subclade 1A (central Apennine, Thyrrenian side, central and northern Adriatic coast; 16 haplotypes) and subclade 1B (western Po Plain and Alps; 5 haplotypes), and clade 2, which contains haplotypes from southern Italy (9 haplotypes). We also took into account the subdivision indicated by the literature within each subclade (see Giovannotti et al. 2010 and subdivision therein). Finally, homologous sequences of *P. erhardii* (accession number: FJ867395) and *P. sicula* (accession number: FJ867396) were retrieved from

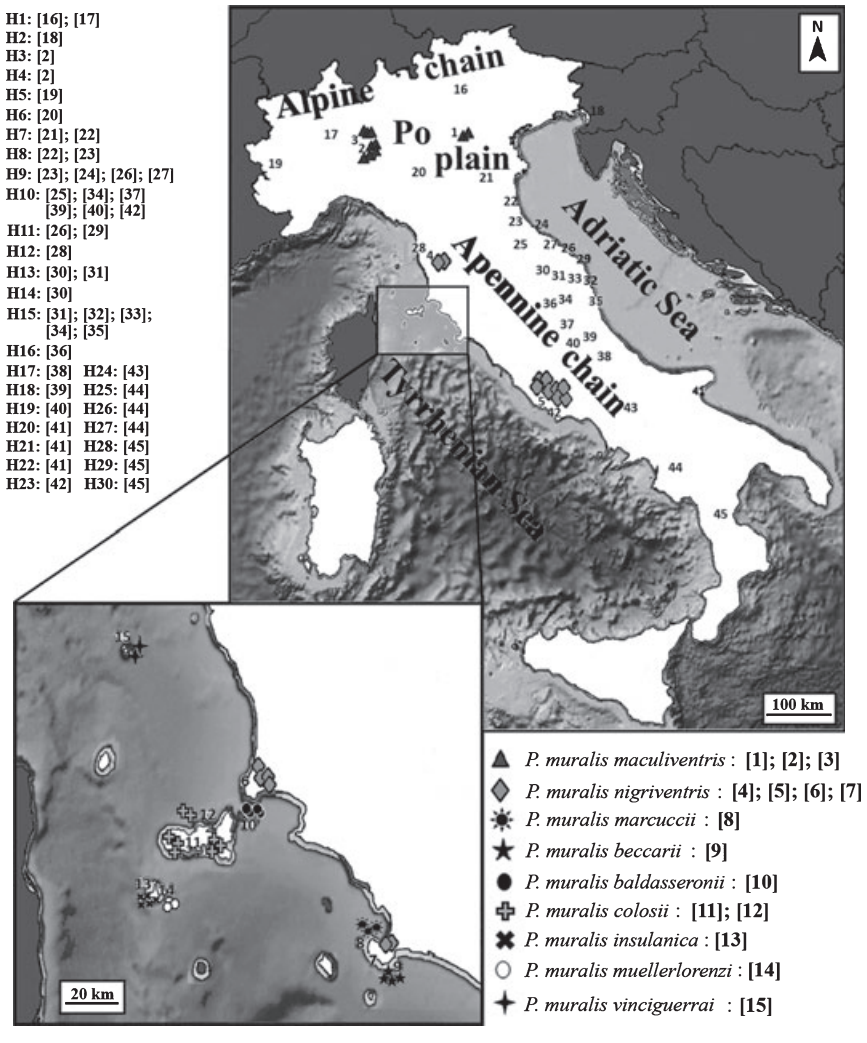

Fig. 1. Distribution of samples analysed in this study. Sampling locations of haplotypes retrieved from the literature are listed on left, while each morphological subspecies considered in this work is indicated with a different symbol. Numerical codes for all the localities are listed in Table S1

GenBank as outgroup taxa. Phylogenetic relationships were inferred with Maximum Likelihood (ML) and Bayesian Inference (BI) analysis. For the ML analysis, 88 alternative models of evolution were tested using jModelTest 0.1.1 (Posada 2008). Once the optimal model was determined according to the Akaike criterion (Akaike 1974; Sakamoto et al. 1986), it was selected to estimate ML tree in PAUP*4.0b10 (Swofford 2002), using heuristic search (TBR branch swapping, random addition sequence). The ML starting tree was obtained by stepwise addition and replicated 10 times, with each replicate starting with a random input order of sequences. Support for nodes was estimated by bootstrapping with 1000 replicates (Felsenstein 1985). We conducted BI analysis as implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), which calculates posterior probabilities using a Markov Chain Monte Carlo sampling approach (Huelsenbeck et al. 2001; Altekar et al. 2004). The best model was selected in MrModelTest 3.7, then two Markov chains were run from random trees for 4×10^6 generations and monitored to ensure that the standard deviation of split frequencies was < 0.01; the earliest 20% of the data sampled were discarded as 'burn-in'.

In order to estimate the gene genealogy of *P. muralis* cyt *b* haplotypes, we performed the haplotype network reconstruction using TCS 1.21 software (Clement et al. 2000), including also published sequences of peninsular populations as comparison. According to the statistical algorithm from Templeton et al. (1992), the number of mutational steps by which pairwise haplotypes differ was calculated, computing the probability of parsimony for pairwise differences until the probability exceeds 0.95. The minimum number of mutational steps required to connect different groups of haplotypes in a parsimony way was also determined using the fix connection limit option. Afterwards, the population genetic structure was determined by estimating the molecular variance and calculating the Φ-statistic through the Analysis of Molecular Variance (AMOVA, Weir and Cockerham 1984; Excoffier et al. 1992), as implemented in Arlequin 3.11 (Excoffier et al. 2005), starting from pairwise genetic difference calculated between cyt *b* haplotypes. Statistical support was estimated by 10 000 randomized permutations.

Results

Cyt *b* sequences were obtained for all the samples considered in this study (i.e. 55 specimens). Neither gaps nor premature stop codons were found in the final alignment. Moreover, no ambiguous positions were scored in any sequences obtained. Fifteen different cyt *b* haplotypes were identified (Accession Nos. FR821781–FR821795), eight of them related to the populations of the Tuscan Archipelago, four to *P. m. maculiventris* and three to *P. m. nigriventris* from Latium (Table S1). Of the 41 variable characters observed in our data set, 5 (12.2%) were at first, 2 (4.9%) at second and 34 (82.9%) at third codon position; 99 variable sites (50 being parsimony informative) and 11 amino acid changes were observed when the outgroup taxa were included, while excluding the outgroup 32 characters were parsimony informative and six amino acid

changes occurred. Nucleotide composition was also estimated from the alignment: frequencies of A, C and T were respectively 27.5%, 26.4% and 34.1%, with a lower value observed for G (12.1%). Observed nucleotide frequencies are consistent with those estimated by Podnar et al. (2009) in the mitochondrial genome of *P. muralis* (accession number: NC_011607) and comparable with those observed by Giovannotti et al. (2010) for Italian populations of *P. muralis*. Noteworthy, the strong bias observed against guanine is typical of mitochondrial gene, but not of nuclear ones (Giovannotti et al. 2010; see also Desjardins and Morais 1990). The transition/transversion ratio was 5.5 without the outgroup and 4.1 when the outgroup was included.

Uncorrected (*p*) sequence divergence values between populations of the archipelago range from 0.0% (between populations from Pianosa Island and La Scola Islet) to 5.4% (between Pianosa/La Scola and Monte Massoncello populations). Four haplotypes were found in populations ascribed to *P. m. colosii* from Elba Island and the facing Portoferraio Rock. Two of these haplotypes are exclusive of the morphological subspecies (i.e. haplotypes E3 and E4), while the others are shared with populations of different islands. In particular, haplotype E1ins was found also in *P. m. insulanica* and *P. m. muellerlorenzi* (from Pianosa Island and the facing La Scola Islet, respectively), while haplotype E2bal was also found in *P. m. baldasseronii* from Palmaiola Island. Interestingly, *P. m. nigriventris* populations from Monte Massoncello and Calci share the same haplotype (N1), whereas specimens from Monte Argentario share haplotype BEC with *P. m. beccarii* and haplotype MAR with *P. m. marcuccii*, which differ minimally from each other (0.2%). Finally, haplotype VIN from Gorgona Island (i.e. *P. m. vinciguerrai*) presents a high level of genetic differentiation from all the other insular populations and seems to be close to mainland populations from central Italy. Overall, haplotypes from the Tuscan Archipelago (i.e. actual and paleo-islands) differ remarkably from those ascribed to *P. m. maculiventris* analysed in this study (uncorrected *p*-distance, 5.1%), while a lower divergence exists with peninsular populations ascribed to *P. m. nigriventris* (uncorrected *p*-distance, 3.1%). Concerning the subspecific definition of our samples, divergences range from 0.0% (between *P. m. insulanica* and *P. m. muellerlorenzi*) to 5.7% (between *P. m. marcuccii*/*P. m. beccarii* and *P. m. maculiventris*). Uncorrected *p*-distances within and between morphological subspecies of the Tuscan Archipelago are summarized in Table 1. The comparison between populations specifically sequenced for this study and cyt *b* sequences retrieved from previous studies indicates that *P. m. maculiventris* haplotypes M1 and M4 correspond to published haplotypes H1 and H3 (northern Italy), respectively; moreover, within *P. m. nigriventris* haplotypes N1 and N2 match with published haplotypes H12 and H10 (central Apennine, Thyrrenian side; Giovannotti et al. 2010).

Phylogenetic analyses

We investigated the evolutionary relationships among *P. muralis* populations by comparing haplotypes from insular populations of the Tuscan Archipelago with those from the Italian Peninsula (including also published sequences retrieved from the literature, i.e. 30 haplotypes). Therefore, our phylogenetic analyses were conducted using 43 haplotype sequences (41 *P. muralis* haplotypes, 1 *P. erhardii* and 1 *P. sicula*). For the ML analysis, the TPM2uf + I + G model (freqA = 0.2687; freqC = 0.2893; freqG = 0.1131; freqT = 0.3289; Inv = 0.3690; Γ = 0.5150) was the most appropriate model of evolution for our data, according to the Akaike criterion. A similar posterior-probability tree was produced by the Bayesian Inference analysis selecting the GTR + I + G model of evolution. Both the analyses confirm the existence of a strong phylogenetic structure within *P. muralis* and reveal significant differences from previous studies (Fig. 2).

Regarding the subspecific definition of our samples, *P. m. nigriventris* populations from Monte Massoncello and the mainland (i.e. Calci and Borgo Montello), as well as *P. m. vinciguerrai* from Gorgona Island, are close to haplotypes from central Italy (ML = 75, BI = 0.97; Fig. 2) previously ascribed to subclade 1A (Giovannotti et al. 2010). Interesting enough, within this group *P. m. nigriventris* from Monte Argentario, *P. m. beccarii* and *P. m. marcuccii* group together in a monophyletic cluster (the '*Argentario*' genetic lineage) with high support values in both the analyses (ML = 99, BI = 1.00; Fig. 2). However, our phylogenetic analyses do not support the existence of subclade 1A as a well-resolved molecular lineage, because evolutionary relationships within it are poorly resolved. In fact, cluster 1Ae (from central Italy, Adriatic side; Giovannotti et al. 2010) diverges a lot from all the other haplotypes (ML = 80; BI = 1.00). Noteworthy, haplotypes referred to *P. m. baldasseronii*, *P. m. insulanica*, *P. m. muellerlorenzi* and *P. m. colosii* cluster together in a well-distinguishable clade, which clearly differs from all the other molecular lineages described till now (the '*Elba*' clade; ML = 88, BI = 0.97; Fig. 2). Finally, our specimens of *P. m. maculiventris* cluster with published haplotypes from northern Italy referred to subclade 1B by previous authors (ML = 91, BI = 1.00, Fig. 2), while no samples specifically

Table 1. Uncorrected sequence divergences (*p*-distance) within and between (along and below the diagonal, respectively) the morphological subspecies analysed in this study

Morphological subspecies	[mac]	[nig]	[bec]	[mar]	[vin]	[col]	[bal]	[ins]	[mue]
Podarcis muralis maculiventris [mac]	0.012								
Podarcis muralis nigriventris [nig]	0.052	0.014							
Podarcis muralis beccarii [bec]	0.057	0.013	n/c						
Podarcis muralis marcuccii [mar]	0.057	0.014	0.002	n/c					
Podarcis muralis vinciguerrai [vin]	0.046	0.017	0.025	0.027	n/c				
Podarcis muralis colosii [col]	0.049	0.047	0.046	0.046	0.042	0.007			
Podarcis muralis baldasseronii [bal]	0.049	0.043	0.042	0.042	0.042	0.005	n/c		
Podarcis muralis insulanica [ins]	0.049	0.050	0.049	0.049	0.044	0.006	0.007	n/c	
Podarcis muralis muellerlorenzi [mue]	0.049	0.050	0.049	0.049	0.044	0.006	0.007	0.000	n/c

n/c, non-calculated (one haplotype).

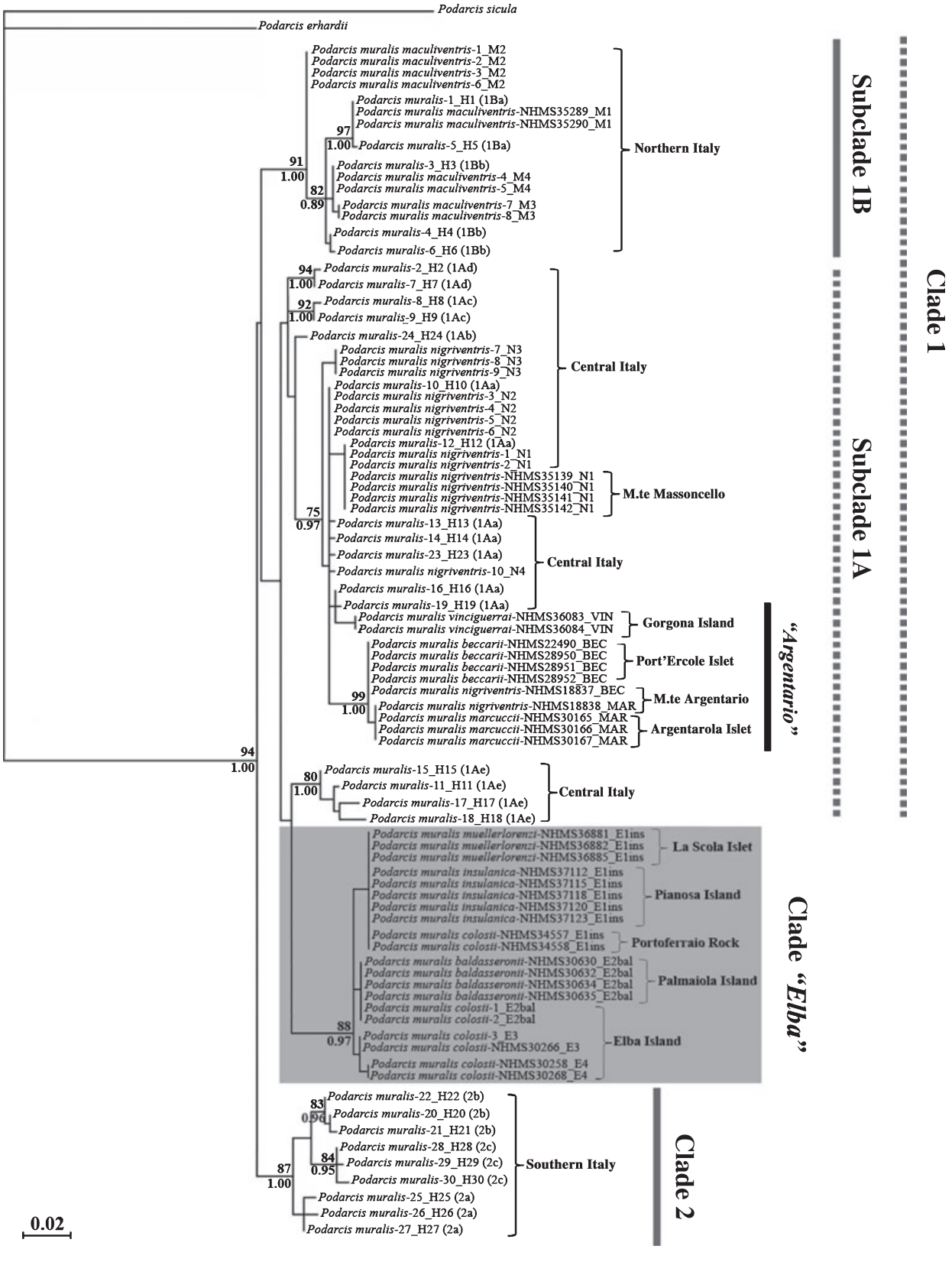

Fig. 2. Maximum Likelihood (ML) best tree obtained for data, using the model described in the text. ML bootstrap values (≥ 75%) and posterior probabilities estimated from the Bayesian analysis (≥ 0.90) are reported above and below the nodes, respectively. Morphological subspecies, haplotype codes and relative sampling locations are reported for each specimen specifically sequenced for the present work. For sequences retrieved from the literature, besides the haplotype codes, previous subdivision in clusters is reported in round brackets. Grey solid bars indicate subclades and clades proposed by previous authors that have been confirmed by ML and BI analyses, whereas molecular assemblages without support are signalled with grey dotted bars

sequenced for this study fall into the main clade 2, which includes all the peninsular haplotypes from southern Italy and show a high degree of genetic divergence from all the other Italian populations (ML = 87; BI = 1.00; Fig. 2).

Phylogeographic analyses

Considering our data set and published sequences from the Italian Peninsula, the reconstruction of the cyt *b* gene genealogy led to identification of several distinct networks when the 95% connection threshold (that was established at eight connections) was chosen (Fig. 3). Interestingly, published haplotypes referred by previous authors to subclade 1A from central Italy split into two different haplotype networks (i.e. A1 and A2): in particular, haplotypes referred to *P. m. nigriventris*, *P. m. vinciguerrai*, *P. m. marcuccii* and *P. m. beccarii* fall into the same network with those from central Italy (network A1), whereas haplotypes from the Adriatic side (i.e. cluster 1Ae) are disconnected at this point (network A2). Anyway, networks A1 and A2 join together at the 93%

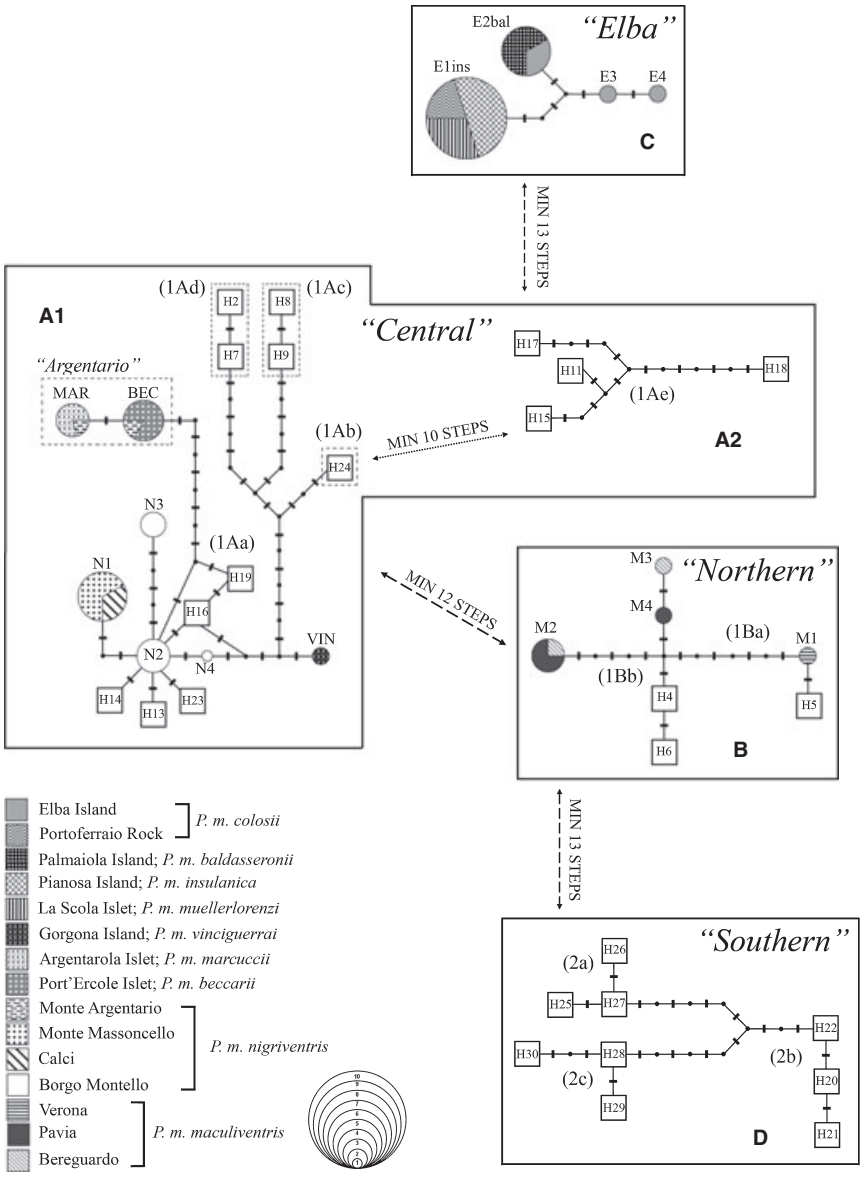

Fig. 3. Ninety-five per cent parsimony networks for *Podarcis muralis* cyt *b* haplotypes from the Italian Peninsula and the Tuscan Archipelago: (A1) haplotypes from central Italy (southern Latium, northern Tuscany, northern Marche Apennines) comprising *Podarcis muralis nigriventris*, *P. m. beccarii*, *P. m. marcuccii* and *P. m. vinciguerrai*; (A2) haplotypes from central Italy (Adriatic side); (B) haplotypes from northern Italy comprising *P. m. maculiventris*; (C) insular haplotypes referred to the '*Elba*' clade (*P. m. colosii*, *P. m. baldasseronii*, *P. m. insulanica*, *P. m. muellerlorenzi*); (D) haplotypes from southern Italy. Solid boxes indicate networks that could not be joined together in a parsimony way. Dashed boxes indicate disconnection at the 98% parsimony connection limit (within network A1). Previous subdivision is also reported in round brackets within each network. Haplotypes identified in this study are indicated with circles (different textures assigned to each population); squares indicate published haplotypes from the Italian Peninsula; morphological subspecies sampled in more than one population are also indicated with squared graphs in the legend; size of circles corresponds to haplotypes frequencies. Minimum number of substitutions required to connect networks, although in a not significant way, are indicated with dotted arrows

parsimony threshold in a '*Central*' network that includes all the haplotypes from central Italy (Fig. 3). Interestingly, network A1 shows a classical 'star-like' pattern, which is a likely consequence of populations postglacial expansion (e.g. Biaggini et al. 2009). Haplotype VIN from Gorgona Island (i.e. *P. m. vinciguerrai*) never disconnects from network A1, whereas further subdivisions were observed within this network at the 98% threshold (i.e. cluster 1Ab, 1Ac, 1Ad and the '*Argentario*' group; Fig. 3). Haplotypes of *P. m. maculiventris* join with published sequences from western Po Plain and Alps (i.e. subclade 1B) in the '*Northern*' network B (Fig. 3). Noteworthy, analysis confirms the distinctiveness of the insular haplotypes ascribed to the '*Elba*' clade by phylogenetic analysis, that group together (i.e. network C) and disconnect from all the others. Interesting enough, this network could be joined to network A2 only allowing a non-significant connection limit of 13 substitutions, the same threshold requested to connect haplotypes from network B (i.e. northern Italy) to those of the '*Southern*' network D, which contained only haplotypes from southern Italy (i.e. those from main clade 2; Fig. 3).

Uncorrected *p*-distances values between the five distinct molecular groups identified by phylogeographic analysis (i.e. the '*Northern*' group B, the '*Central*' groups A1 and A2, the '*Elba*' group C and the '*Southern*' group D) are definitely higher than those observed within each of them, as reported in Table 2. Net nucleotide divergences (*Da*) between the '*Elba*' clade and the other molecular lineages detected range from 3.0% to 4.3%, and reach higher values (5.3%) considering subdivisions proposed by previous authors (data not shown). We also estimated *Da* between the '*Elba*' clade and the '*Argentario*' genetic lineage: interestingly, the observed divergence of 4.3% is comparable with that estimated between our specimens ascribed to *P. m. maculiventris* and *P. m. nigriventris* (4.1%).

Finally, AMOVA analysis was performed on populations ascribed with certainty to different morphological subspecies (i.e. 55 samples, 15 localities) in order to investigate their genetic structure. Therefore, populations were initially split into three groups according to the evolutionary relationships observed: one for populations ascribed to *P. m. maculiventris* (localities [1] to [3]; Fig. 1), one including those ascribed to *P. m. nigriventris*, *P. m. marcuccii*, *P. m. beccarii* and *P. m. vinciguerrai* (localities [4] to [9] and [15]; Fig. 1) and the last containing populations of the '*Elba*' clade (*P. m. colosii*, *P. m. baldasseronii*, *P. m. muellerlorenzi* and *P. m. insulanica*; localities [10] to [14]). Analyses show that the majority of the total molecular variance is distributed among the groups (76.59%; $\Phi ct = 0.76591$, $p < 0.001$), while the variance among populations within each group (18.41%; $\Phi sc = 0.78660$, $p < 0.001$) and within each population (5.00%; $\Phi st = 0.95004$, $p < 0.001$) are clearly much lower. The variance among groups increased splitting apart the '*Argentario*' lineage from the other peninsular populations ascribed to *P. m. nigriventris*, including the Monte Massoncello population (83.67%; $\Phi ct = 0.83672$, $p < 0.001$). Again, the variance among populations within groups (10.89%; $\Phi sc = 0.66673$, $p < 0.001$) and within each single population (5.44%; $\Phi st = 0.945588$, $p < 0.001$) remained very low.

Table 2. Ranges of uncorrected *p*-distance (in percentages values) within and between (along and below the diagonal, respectively) mitochondrial groups identified by the haplotype network analysis (95% connection limit). Values referred to the '*Elba*' clade (network C) are indicated in bold

Groups	A1 (%)	A2 (%)	B (%)	C (%)	D (%)
'*Central*' A1	1.9				
'*Central*' A2	3.8	1.4			
'*Northern*' B	4.8	4.6	1.1		
'*Elba*' C	**4.5**	**3.9**	**5.0**	**0.7**	
'*Southern*' D	4.6	4.6	4.7	**4.8**	1.7

Discussion

Insular populations of *P. muralis* have been ascribed to different subspecies in the past, because of their extreme phenotypic variability, particularly in colour pattern. As already observed for peninsular subspecies (Giovannotti et al. 2010), our analyses do not completely agree with the subspecific division indicated for the Tuscan Archipelago by morphological taxonomy. Regarding insular populations of *P. muralis*, at least four subspecies (i.e. *P. m. colosii*, *P. m. insulanica*, *P. m. muellerlorenzi* and *P. m. baldasseronii*) are characterized by a very low genetic divergence, although they are deeply differentiated from *P. m. beccarii*, *P. m. marcuccii* and *P. m. vinciguerrai*, which seem to be closer to the peninsular subspecies *P. m. nigriventris* (Figs 2 and 3). Interestingly, some authors already suggested that at least *P. m. colosii* and *P. m. baldasseronii* should be placed in synonymy, because they largely overlapped in their morphology (Müller 1922). Although these taxonomic hypotheses date back to the beginning of the 20th century, our molecular data suggest a similar scenario, challenging the current taxonomy of the group.

Phylogenetic analyses

Our phylogenetic analyses reveal the existence of at least one new mitochondrial lineage within *P. muralis*, the '*Elba*' clade, that is exclusive of the Tuscan Archipelago and differs a lot from all the other mitochondrial lineages described for *P. muralis* till now (Figs 2 and 3). Within this clade, four insular subspecies previously described only on morphological bases were grouped together with very similar haplotypes (i.e. *P. m. colosii*, *P. m. insulanica*, *P. m. muellerlorenzi* and *P. m. baldasseronii*). Noteworthy, phylogenetic analyses do not support the hypothesis that this clade is nested within any other previously described (Fig. 2), suggesting that it may represent a third well-defined molecular clade besides previous described clade 1 and 2.

Moreover, both the ML and BI analyses show that populations ascribed to *P. m. beccarii*, *P. m. marcuccii* and *P. m. vinciguerrai*, as well as all the populations ascribed to *P. m. nigriventris* from the two paleo-islands Monte Massoncello and Monte Argentario are close to peninsular haplotypes from central Italy. Interesting enough, populations of Monte Argentario and both Port'Ercole and Argentarola islets clearly represent a single genetic stock, being characterized by low intra-clade genetic variability with very high bootstrap support (Fig. 2). Surprisingly, *P. m. vinciguerrai* from Gorgona Island seems to be very close to the peninsular populations of *P. m. nigriventris*. This finding suggests a complex phylogeographic scenario for this today isolated population, or, as we suspect, a recent anthropogenic introduction of individuals from the mainland.

Finally, our phylogenetic analyses confirm the presence of a strong phylogenetic structure within *P. muralis* through its Italian range (Fig. 2), as reported by previous studies (Harris and Arnold 1999; Capula 1997; Capula and Corti 2010; Giovannotti et al. 2010). In particular, both ML and BI analyses do not support previous recognized subclade 1A as a well-resolved molecular lineage, suggesting a high genetic complexity for peninsular populations of *P. muralis* especially concerning central Italy, probably due to the presence of multiple glacial refugia during the last glacial period (Gòmez and Lunt 2007). This result seems to diminish the distinctiveness of the whole main clade 1 and therefore we prefer to consider it as a highly heterogeneous molecular assemblage, rather than a well-defined phylogenetic clade. Interestingly, published haplotypes of *P. muralis* from Austria (accession number: AY185096, Podnar et al. 2004) and Greece (accession number: AF486232, Poulakakis et al. 2003) fall into this highly heterogeneous molecular assemblage (data not shown), whereas haplotypes from Spain (accession number: AY234155, Busack et al. 2005) and France (accession number: AF248007, Sourget-Groba et al. 2001) cluster with haplotypes from northern Italy.

Phylogeographic analyses

The 95% parsimony network analyses of cyt *b* haplotypes confirmed the existence of different molecular lineages within the Tuscan Archipelago that could not be joined together in a parsimonious way. Within network A1, the genetic stock represented by *P. m. marcuccii*, *P. m. beccarii* and *P. m. nigriventris* from Monte Argentario is well differentiated from all the other peninsular haplotypes, as well as from all the other insular populations grouped into network C (i.e. those ascribed to *P. m. colosii*, *P. m. insulanica*, *P. m. muellerlorenzi* and *P. m. baldasseronii*). Concerning *P. m. nigriventris*, molecular analyses do not show significant differences between Monte Massoncello and mainland populations, although a deep divergence characterized the population sampled in the area of Monte Argentario (previously described as a different morphological subspecies, *P. m. paulinii*). We suggest that observed divergence could be ascribed to an ancient differentiation of this isolated population, followed by a recent disconnection of the two facing islets as a result of the raising of the sea level after the last glacial period. Noteworthy, recent studies suggest that the 95% parsimony connection limit represents a useful tool for the identification of deeply divergent molecular lineages and even new cryptic species from sequence data (e.g. Hart and Sunday 2007), when applied to non-recombining loci with rapid lineage sorting (e.g. mitochondrial DNA sequences). Therefore, we can assume that haplotypes grouped into network C (i.e. the '*Elba*' clade) have been isolated for a long time from all the other insular populations of the archipelago, as well as from the mainland.

Following the evolutionary rates proposed for *P. erhardii* cyt *b* sequences (1.45–1.59% Myr; Poulakakis et al. 2003), divergence time between the '*Elba*' clade and the other main mitochondrial groups identified in this study ranged from 2.7 to 2.0 Ma, and from 3.3 to 2.0 Ma when all peninsular cluster identified in the present work, as well as in previous studies were considered. Moreover, the genetic differentiation between the '*Elba*' and the '*Argentario*' lineages within the Tuscan Archipelago populations would correspond to a divergence time of 2.8 Ma. Similarly, recent analyses of a 488-bp portion of 16S rDNA gene suggest that the '*Elba*' clade diverged about 2–3 Ma from mainland populations (data not shown), assuming the evolutionary rate estimated for *P. erhardii* (0.46% Myr; Poulakakis et al. 2005). The 'continentalization' of the Tuscan Archipelago, owing to the presence of natural bridges between the islands and the Italian Peninsula during several phases of glacial peaks, allowed many apterous groups, like Reptiles and Amphibians, to colonize the islands. In fact, climate changes occurred during geological eras have caused significant oscillations in the sea level, especially in the Tyrrhenian Sea. Information concerning the paleogeographic evolution of the Tuscan Archipelago are reported by some authors (e.g. Boccaletti et al. 1990; Dapporto et al. 2007), but, surprisingly, very few studies have attempted to assess the real similarity of insular species compared with mainland species (e.g. Dapporto and Cini 2007). As a result, paleogeography has generally been accepted as the key factor leading to the species assemblages on this archipelago. During the Lower Pliocene (4 Ma), the lowering of the sea level (over 100 m below the current level) gave rise to the Tuscan Archipelago. In this period, Elba and Pianosa islands were connected one another by the 'Pianosa' sea ridge and joined the Italian coast. Then, during the Middle Pliocene (2.8 Ma), owing to a lift of the sea level, islands were separated from the coast and in some cases (e.g. Pianosa) completely submerged. Islands emerged again in the Lower Pleistocene (125 000 ya), while Elba and Pianosa joined one more time the Italian coast during the Würm glaciation (18 000 ya). Assuming the evolutionary rate proposed for the related species *P. erhardii*, the observed divergence between different mitochondrial lineages found in the Tuscan Archipelago seems to be related to the first rising stages of the archipelago. Most probably islands were not completely isolated from the mainland, but a possible explanation for the distinctiveness of the '*Elba*' clade from the mainland populations is that during introgressive sea phases, the area surrounding the Pianosa Ridge might have been exposed to frequent sea flooding and then, during the regressive phases, water was replaced by salt swamps, which did not represent the optimal habitat for *P. muralis*. In fact, there are evidences that the peri-Thyrrenian area was affected by back and intradeep basins characterized by extended areas of autochtonous evaporites (Boccaletti et al. 1990). Moreover, it is possible that insular populations expanded their range during dry periods, but the migration fronts that eventually came into contact and exchanged genetic material became extinct during periods of flooding.

AMOVA analysis suggests that genetic exchange among the archipelago and the mainland has been reduced for a long time, confirming also the distinctiveness of *P. muralis* populations from Monte Argentario and facing islets. Therefore, our results support the hypothesis that the islands of the Tuscan Archipelago can be considered as relict islands, as their lizards populations are deeply differentiated from the mainland ones (Capula and Corti 2010). The strong genetic differentiation observed for insular populations of *P. muralis* is consistent with the high genetic polymorphism highlighted by previous authors (Capula 1997; Capula and Corti 2010) and suggests a complex phylogeographic pattern that can be ascribed to the presence of multiple glacial refugia in this area during the Pleistocene, according to the 'Refugia-within-refugia' hypothesis (Gòmez and Lunt 2007).

Further directions

Our study denotes that several morphological subspecies described during the last century for the Tuscan Archipelago are characterized by minimal or no difference in mitochondrial DNA haplotypes. Similarly, Podnar et al. (2004) assembled 20 morphological subspecies of *P. melisellensis* into three main mtDNA clades showing uncorrected *p*-distance values similar to those found in our study. According to Frost and Hillis (1990), we believe the taxonomic position of a group should be consistent with its evolutionary history, and despite our aim was not to rise more taxonomic considerations only on the basis of mtDNA data, we conclude that our results contradict the subspecific division of *P. muralis* populations of the Tuscan Archipelago. Therefore, we believe that at least in some cases the extreme phenotypic polymorphism shown by insular populations of *P. muralis* could be better explained by microevolutionary processes, rather than deep evolutionary divergence. In fact, insular systems represent discrete geographical entities where, despite the small size of the area, it is possible to find a great variety of habitats. Therefore, we suggest that further studies should be focused on the analysis of the genetic population structure of insular populations with nuclear markers, as well as on morphological traits (by using geometric morphometrics) in order to assess the actual selective pressures shaping phenotypic variability on islands. In fact, when phenotypic divergence exceeds neutral expectations, it is easy to invoke selection in driving divergence at a local scale (Camargo et al. 2010). Moreover, the evolution of different eco-phenotypes has been generally interpreted as a result of local adaptations to different environments (e.g. Vervust et al. 2007; Herrell et al. 2008).

Phylogenetic relationships of *P. muralis* insular populations would be most appropriately described by recognizing at least the '*Elba*' clade, which reach the same level of divergence generally chosen to define subspecies according to an evolutionary species concept (e.g. Podnar et al. 2004). On the contrary, genetic drift phenomena and bottleneck events could explain the deep divergence of the '*Argentario*' lineage from the mainland genetic stock. Interestingly, molecular analysis has often highlighted the presence of genetically distinct lineages in other insular populations of *Podarcis*, as observed for the critically endangered species *Podarcis raffonei* from Aeolian islands, previously considered as a subspecies of *P. wagleriana* (Capula 1994). Recently Biaggini et al. (2009) have found a high genetic similarity between *P. sicula* populations from Campanian islands and the mainland (central Italy, Thyrrenian side). In the light of our findings, we argue that different degrees of molecular divergence may result from variations in sea-depth (< 100 m for the Campanian islands) or possibly different size of the investigated islands (definitely greater for Elba Island, 223 km^2).

In conclusion, our study offers some interesting clues to go through the complex phylogeographic history of the Tyrrhenian herpetofauna. Furthermore, we provide new evidence on the genetic variability of *P. muralis*, adding more sequence data for the reconstruction of the phylogeographic patterns of this widespread species. Finally, our results will help in the understanding of the still poorly clarified genetic variation of insular populations of Mediterranean lacertid lizards.

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Riassunto

Identificazione di nuovi lignaggi mitocondriali in Podarcis muralis *(Squamata: Lacertidae) mediante analisi molecolare delle sottospecie morfologiche descritte per l'Arcipelago Toscano (Italia)*

L'avvento delle recenti tecniche di indagine bio-molecolare ha profondamente rivisto la sistematica classica delle specie appartenenti al genere *Podarcis* (Squamata: Lacertidae), portando alla ridefinizione tassonomica di numerose sottospecie descritte esclusivamente su base morfologica. Le lucertole, in effetti, esibiscono un'elevata variabilità morfologica sia a livello intra-specifico, sia all'interno della stessa popolazione. Tale plasticità è particolarmente accentuata sulle isole, dove la divergenza fenotipica che caratterizza le popolazioni è principalmente legata a fenomeni di adattamento locale, piuttosto che a una reale divergenza evolutiva. La lucertola muraiola (*Podarcis muralis*) mostra una notevole variabilità legata ad aspetti di natura biometrica, alla folidosi e al pattern cromatico. Le analisi molecolari hanno recentemente confermato il ruolo chiave giocato dalla Penisola italiana come rifugio glaciale per la specie, che da qui si sarebbe successivamente diffusa nel resto d'Europa. Inoltre, è stata evidenziata una mancanza di congruenza tra i diversi lignaggi mitocondriali e le quattro sottospecie peninsulari attualmente riconosciute su base morfologica. Nel presente studio, abbiamo analizzato un frammento del gene mitocondriale citocromo *b* nelle sette sottospecie descritte per le isole dell'Arcipelago Toscano (Italia), al fine di verificare la corrispondenza tra gli aplotipi mitocondriali e la tassonomia tradizionale proposta per la lucertola muraiola. Inoltre, gli aplotipi individuati sono stati confrontati con tutti quelli finora descritti per la Penisola italiana, al fine di evidenziare l'eventuale presenza di lignaggi genetici peculiari nelle popolazioni insulari. I risultati ottenuti non concordano del tutto con la classificazione sottospecifica effettuata su base morfologica. In particolare, le analisi filogenetiche hanno dimostrato che almeno quattro sottospecie sono caratterizzate da aplotipi molto simili tra loro e ricadono all'interno dello stesso clade monofiletico, mentre le altre tre risultano più simili alle popolazioni peninsulari del centro Italia. Sulla base di questi risultati, riteniamo che lo status tassonomico di almeno alcune delle sottospecie indagate dovrebbe essere riconsiderato, classificando le rispettive popolazioni come semplici eco-fenotipi; inoltre, in questo lavoro abbiamo fornito una possibile spiegazione per comprendere il differenziamento dei lignaggi molecolari esclusivi ritrovati nell'arcipelago. Infatti, queste isole hanno rappresentato un rifugio per la specie durante gli ultimi periodi glaciali.

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Supporting Information

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

Table S1. List of all the specimens used in this study including sequences retrieved from the literature (samples specifically sequenced for the present work are highlighted with asterisks*).

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