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ORIGINAL ARTICLE

Phylogeography of the pool frog *Rana* (*Pelophylax*) *lessonae* in the Italian peninsula and Sicily: multiple refugia, glacial expansions and nuclear–mitochondrial discordance

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ABSTRACT

Aim To infer the evolutionary history of *Rana* (*Pelophylax*) *lessonae* Camerano within its inferred Quaternary refugial range, and to shed light on the processes that have contributed to shaping the patterns of diversity within the southern European peninsulas.

Location The Italian peninsula south of the Alps and Sicily.

Methods Sequence analysis of a mitochondrial cytochrome *b* gene fragment in 149 individuals sampled from 25 localities.

Results Three mitochondrial DNA (mtDNA) phylogroups were identified, distributed in northern Italy, the whole Italian peninsula south of the northern Apennines, and Sicily. Syntopy between the northern and peninsular lineages was observed close to the northern Apennines. The northern lineage was the most differentiated, showing a net sequence divergence of 4.8 ± 0.8% with respect to the two others, whereas the net divergence between peninsular and Sicilian lineages was 2.6 ± 0.6%. Analysis of molecular variance (AMOVA) revealed that 93% of the overall variation occurred between these three groups. Historical demographic statistics support a recent expansion for both the northern and peninsular groups, but not for the Sicilian group. In both northern and peninsular Italy, such an expansion was likely to have occurred during the last glaciation.

Main conclusions Our results suggest that a number of microevolutionary processes were involved in shaping the present genetic structure of *R. lessonae* in Italy. These encompass allopatric differentiations in three distinct Pleistocene refugia, recent population expansions and secondary contacts. Our results, together with some previous work, support (1) the existence of a suture zone in the northern Apennines, and (2) the possibility of population expansions during the last glacial phase, when a vast widening of the lowland floodplain habitats followed sea-level fall, particularly in northern Italy. When compared with previous analyses of allozyme data, it appears that the peninsular mtDNA lineage has recently replaced the Sicilian one in southern Calabria, and we suggest that this event occurred due to selective introgression. The implications of such an occurrence for the study of factors underlying the patterns of diversity within this southern European biodiversity hotspot are discussed. Taxonomic implications of the results are also evaluated.

Keywords

Amphibians, historical demography, Italy, mtDNA, multiple refugia, phylogeography, *Rana lessonae,* secondary contact.

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INTRODUCTION

Quaternary climatic oscillations have been identified as major historical factors in shaping the present geographical distribution of species and their genetic diversity. Both fossil remains and genetic data suggest that species were forced by such oscillations to repeated cycles of retreat within refugial ranges during glacial phases, followed by range expansions in the subsequent interglacials (reviews in Hewitt, 2004a,b). For the Western Palaearctic region, important glacial refugia have been identified in the southern Mediterranean peninsulas of Iberia, Italy and the Balkans (Hewitt, 1996, 1999, 2000; Taberlet *et al.*, 1998), although more recently the occurrence of additional refugia in central Europe has also been suggested (Stewart & Lister, 2001; Deffontaine *et al.*, 2005, and references therein). One of the most pervasive patterns of distribution of genetic diversity, often observed among temperate species from this region, is the so called 'southern richness, northern purity' pattern (Hewitt, 1996, 2000; but cf. Bilton *et al.*, 1998; Petit *et al.*, 2003), whereby populations from putative refugial ranges harbour the largest fraction of genetic diversity of the species. The higher genetic diversity observed in populations from the putative southern refugia have been explained mainly as a function of the prolonged demographic stability that characterized these populations, with respect to those that arose from the northward range expansion, a process involving serial genetic bottlenecks (Hewitt, 1996). Based on case studies from the Iberian peninsula, more recently an alternative hypothesis has been invoked to explain such a southern richness pattern (Gómez & Lunt, 2006, and references therein). According to this hypothesis, multiple glacial refugia would have existed within the southern refugia, allowing distinct population groups to differentiate in allopatry. A major consequence of this 'refugia-within-refugia' scenario (Gómez & Lunt, 2006) is that the higher genetic diversity found within the southern ranges could have arisen not only from the prolonged stability of populations in these areas, but also from the existence of a more complex series of microevolutionary processes, encompassing allopatric differentiation, post-glacial range expansion, secondary contacts and admixture among previously differentiated lineages. Moreover, the process of genetic diversity loss during northward range expansion could also have been strengthened by the fact that not all the southern genetic diversity was represented in the populations at the leading edge (those involved in the northward expansion event). The strong phylogeographical structure observed for a growing number of taxa, and the extensive phylogeographical concordance among them, strongly support this scenario for the Iberian peninsula (Gómez & Lunt, 2006; Martínez-Solano *et al.*, 2006; Bella *et al.*, 2007).

Although the possible contribution of a multiple refugia scenario to the present patterns of genetic diversity has not been so extensively investigated for the other putative refugial ranges across Europe, several case studies concerning the Italian refugium have documented a decrease in the genetic variability of populations with increasing latitude, as well as a strong pattern of genetic differentiation among populations from the putative refugial range (Santucci *et al.*, 1996; Podnar *et al.*, 2005; Canestrelli *et al.*, 2006, 2007a,b; Ursenbacher *et al.*, 2006; Böhme *et al.*, 2007; for a brief review see Canestrelli, 2006). This suggests a multiple refugia scenario. In this respect, the pool frog *Rana* (*Pelophylax*) *lessonae* Camerano constitutes an interesting case study. It is a species distributed throughout central and eastern Europe, south to the Italian peninsula, Corsica and Sicily, where it inhabits a wide range of freshwater habitats from sea level to > 1500 m a.s.l. (e.g. at Laga Mountains, central Italy), although it is mainly distributed below 600 m a.s.l. (Günther, 1997). The patterns of geographical variation within *R. lessonae* have been studied intensively throughout its range using genetic, morphometric, immunological and bioacoustic markers (Uzzell, 1979; Uzzell & Hotz, 1979; Günther & Plötner, 1994; Santucci *et al.*, 1996, 2000; Sinsch & Schneider, 1996; Zeisset & Beebee, 2001; Snell *et al.*, 2005), and several lines of evidence have indicated the Italian peninsula as the refugial range for this species (e.g. Uzzell & Hotz, 1979; Zeisset & Beebee, 2001). Two main population groups were identified within Italy south of the Alps, located at alternative sides of the northern Apennines (Uzzell, 1979; Uzzell & Hotz, 1979). This geographical pattern was also confirmed by later studies, although the different markers gave an apparently contraditory picture of the degree of differentiation between the two groups, also leading to some taxonomical controversies (see Crochet & Dubois, 2004). Finally, a survey of the allozyme variation throughout the southern peninsula and Sicily also identified two population groups (Santucci *et al.*, 1996), one located in Sicily and southern Calabria, the other ranging from central Calabria to the rest of the peninsula. Furthermore, a wide hybrid zone between these two groups was observed in central Calabria.

Here we investigate the pattern of genetic variation of the pool frog in Italy and Sicily through sequence analysis of a partial mitochondrial cytochrome *b* gene fragment. We are interested in studying the factors underlying the high diversity that characterizes the southern European peninsulas. Therefore, in this paper our aims are: (1) to gain further insight into the evolutionary and historical demographic processes that have shaped the present geographical distribution of genetic diversity of *R. lessonae* in Italy, and (2) to assess whether, and to what extent, our results, obtained using a uniparenthally (mitochondrial) inherited marker, are concordant with those previously achieved by studying biparentally (nuclear) inherited markers. Finally, we briefly discuss the implications of our results for the taxonomy of the Italian pool frog populations.

MATERIALS AND METHODS

Samples and laboratory procedures

We collected tissue samples from 149 individuals sampled in 25 localities spanning from northern Italy to the tip of Calabria and Sicily. Sampling localities are listed in Table 1 along with

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Table 1 Geographical location, sample size (n) and estimate of genetic diversity (where $n > 4$) for the 25 populations of *Rana lessonae* studied.

Population	Locality	Latitude (N)	Longitude (E)	n	Haplotypes (n)	h	π [10^2]
1	Noto	36°54′	15°05′	5	S1(5)	0.000	0.000
2	Ficuzza	37°49′	13°24′	6	S1(6)	0.000	0.000
3	Piana degli Albanesi	37°58′	13°18′	4	S1(4)	–	–
4	Randazzo	37°52′	14°58′	6	S1(1), S2(5)	0.333	0.054
5	Riberoia	37°29′	13°15′	8	S1(7), S3(1)	0.250	0.040
6	La Verde	38°06′	16°11′	5	C1(2), C6(1), C7(2)	0.800	0.162
7	Platì	38°13′	16°03′	5	C1(1), C7(4)	0.400	0.065
8	Gioia Tauro	38°24′	15°56′	10	C1(1), C6(7), C7(2)	0.511	0.133
9	Angitola	38°46′	16°14′	5	C1(2), C11(2), C14(1)	0.800	0.259
10	Taverna	39°01′	16°35′	4	C1(1), C11(3)	–	–
11	Macchia Longa	39°26′	16°38′	5	C1(1), C11(4)	0.400	0.129
12	Due Uomini	39°34′	16°06′	6	C1(4), C2(1), C11(1)	0.600	0.162
13	Sala Consilina	40°24′	15°40′	9	C1(4), C10(2), C12(2), C13(1)	0.779	0.162
14	Vieste	41°53′	16°11′	3	C1(1), C3(2)	–	–
15	Fondi	41°23′	13°18′	3	C1(2), C8(1)	–	–
16	Castelluccio	42°50′	13°13′	4	C1(4)	–	–
17	Macerata	43°17′	13°30′	6	C1(5), C9(1)	0.333	0.054
18	Firenze	43°48′	13°10′	5	C1(2), C4(2), C5(1)	0.800	0.162
19	Garfagnana	44°06′	10°26′	2	C1(2)	–	–
20	San Marino	43°56′	12°27′	10	C1 (8), N1(2)	0.356	1.75
21	Torino	45°00′	7°40′	14	N1(6), N8(2), N9(6)	0.659	0.171
22	Dosolo	44°57′	10°38′	8	N1(5), N3(1), N4(1), N5(1)	0.643	0.179
23	Punta Alberete	44°31′	12°17′	9	N1(8), N2(1)	0.222	0.072
24	Monfalcone	45°46′	13°22′	5	N1(3), N6(1), N7(1)	0.700	0.194
25	Bologna	44°30′	11°21′	2	N1(2)	–	–

h, haplotype diversity; π, nucleotide diversity.

sample sizes, and are shown in (Fig. 1c). Tissue samples were collected through a toe-clipping procedure after anaesthesia in the field in a 0.1% solution of 3-aminobenzoic acid ethyl ester (MS) 222. *Rana lessonae* and the hybridogenetic hybrid species *Rana esculenta* are often syntopic and are hardly distinguishable on the basis of their morphological characters. Therefore, for the proper species recognition of each individual we used diagnostic characters at three allozyme loci (αGpdh, *Ldh-1*, *Gapdh*), visualized using a standard allozyme starch gel electrophoresis procedure, following Santucci *et al.* (2000). Subsequently, only *R. lessonae* individuals were used in the study.

Total genomic DNA was extracted following the standard cetyl trimethyl ammonium bromide (CTAB) protocol of Doyle & Doyle (1987). Partial sequences of the mitochondrial cytochrome *b* gene were obtained through amplification by polymerase chain reaction (PCR). After preliminary amplifications and sequencing using primers MVZ15 and MVZ16 (Moritz *et al.*, 1992), the specific primers 494Lmod (5′-CGGGTCTTTYATTGACCTCC-3′) and CYTbLmod (5′-ATTAGCTGGTGTGAARTTGTCTGG-3′) were designed and used to screen all individuals analysed. Amplifications were performed in a 50 μL volume containing $MgCl_2$ (2.5 mM), the reaction buffer (1x, Promega, Madison, WI, USA), the four dNTPs (0.2 mM each), the two primers (0.2 μM each), the enzyme *Taq* polymerase (2 U, Promega) and 2 μL DNA template. PCR cycling started with a step at 95°C for 5 min followed by 35 cycles of 93°C for 1 min, 52°C for 45 s, 72°C for 1 min 30 s, and a single final step at 72°C for 10 min. Sequencing was carried out using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems, Foster City, CA, USA) following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. Both strands were sequenced for all the specimens analysed.

Data analysis

Sequence alignment and editing were undertaken using the software ClustalX (Thompson *et al.*, 1997). Nucleotide and amino acid composition was determined using the software mega ver. 3.1 (Kumar *et al.*, 2004).

Phylogenetic relationships among haplotypes were inferred using neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods. Pairwise sequence divergence, NJ, MP and ML trees were computed using the software paup* 4.0b10 (Swofford, 2003). For MP analysis, characters were unordered and equally weighted and, for both this and ML methods, heuristic searches were conducted with 10 rounds of random sequence addition and tree bisection–reconnection branch swapping. The best-fit model of sequence evolution for the data was assessed from among 56 possible alternative models, using the Akaike information criterion as implemented in the software modeltest ver. 3.6 (Posada &

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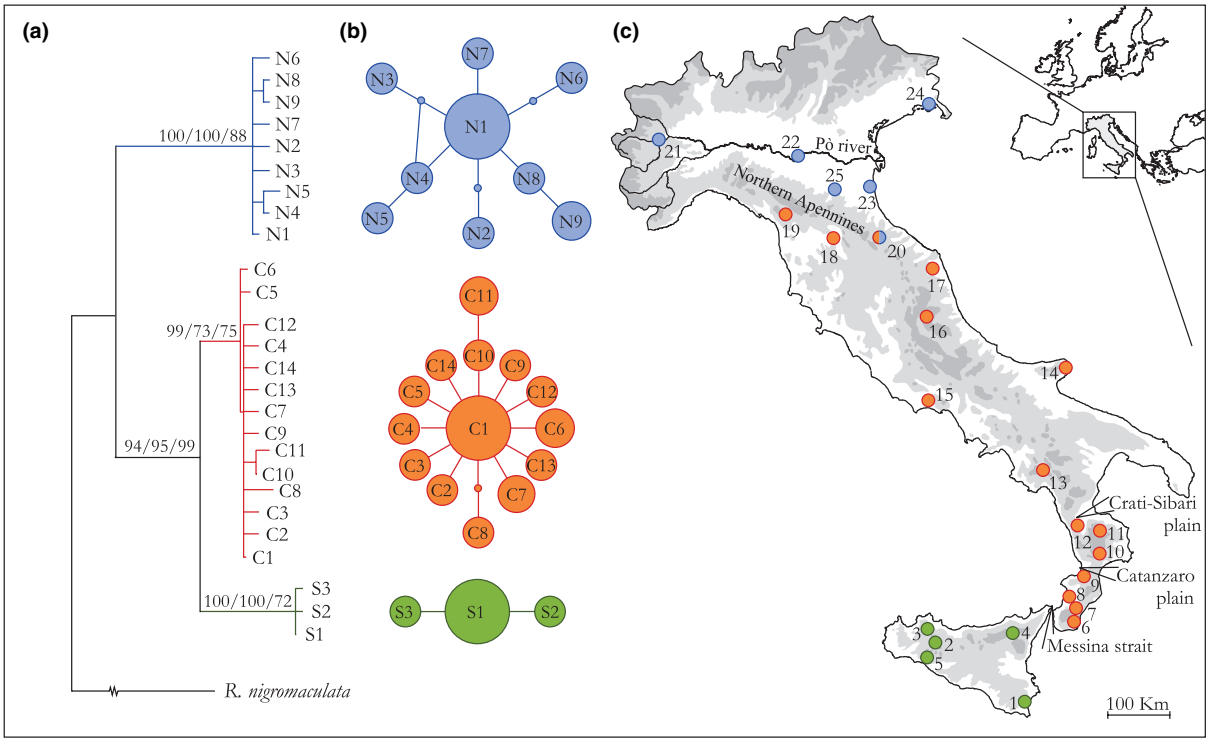

Figure 1 Phylogeography of *Rana lessonae* in the Italian peninsula and Sicily. (a) Neighbour-joining phylogram based on TN93 + I genetic distance among the 26 cytochrome *b* haplotypes found among the 149 individuals of *Rana lessonae* analysed. Bootstrap support values > 70% over 1000 pseudoreplicates are given at nodes for neighbour-joining, maximum parsimony and maximum likelihood trees (NJ/MP/ML). A previously published sequence of *Rana nigromaculata* was used as outgroup (GenBank accession number AB043889; Sumida *et al.*, 2001). (b) Minimum spanning networks obtained for the same data set. The size of circles is roughly proportional to haplotype frequency; open dots represent missing intermediate haplotypes. (c) Geographical location of the 25 populations sampled. Populations are coloured according to the presence/absence of major clades as determined by phylogenetic analyses, and are numbered as in Table 1.

Crandall, 1998). This analysis supported the model of Tamura & Nei (1993) as the best-fit substitution model for the data (TN93 + I), with the proportion of invariable sites being 0.7131 and nucleotide frequencies $A = 0.245$, $C = 0.315$, $G = 0.143$ and $T = 0.297$. This model was then used with both NJ and ML analyses. For all methods, the reliability of the inferred relationships was assessed by the nonparametric bootstrap method (Felsenstein, 1985), using a 70% threshold over 1000 pseudoreplicates (Hillis & Bull, 1993). In addition to the above tree-building methods, we also constructed a statistical parsimony network (Templeton *et al.*, 1992) using the software TCS ver. 1.21 (Clement *et al.*, 2000).

To estimate the divergence time between major lineages as they emerged from phylogenetic analyses, we first carried out a likelihood-ratio test according to Huelsenbeck & Crandall (1997) in order to test for the homogeneity of the substitution rates across all clades. The software MEGA ver. 3.1 was used to compute net sequence divergence between major lineages (TN93-corrected estimate) to correct for average divergence within lineages (Nei, 1987). Subsequently, a cytochrome *b* specific pairwise sequence divergence of 3.6% Myr^{-1} was assumed, as suggested by Babik *et al.* (2004) for European frog species, based on previous work by Beerli *et al.* (1996) and Veith *et al.* (2003).

Haplotype (h) and nucleotide (π) diversity (Nei, 1987) were estimated for each population in which sampled individuals were ≥ 5 and for the higher level clades, as inferred by phylogenetic analyses, using the software DNASP ver. 4.0 (Rozas *et al.*, 2003). The pattern of genetic differentiation among populations was investigated by estimating pairwise values of F_{ST} using the software ARLEQUIN (Schneider *et al.*, 2000). The statistical significance of the estimates was assessed based on 10,000 permutations. This software was also used to carry out an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) in order to partition the overall genetic variance into its hierarchical components among groups, among populations within groups, and within populations (significances assessed by 1023 permutations). Both the pairwise F_{ST} and AMOVA were performed incorporating the genetic distance of Tamura & Nei (1993), the best-fit model of sequence evolution for our data.

The relative roles of gene flow and genetic drift in population genetic structure were investigated by comparing geographical and genetic distance between populations with those expected under a stepping-stone model of population structure (Hutchinson & Templeton, 1999). Spearman's rank correlation coefficient was used to evaluate the strength of the correlation between geographical (km) and genetic

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$[F_{ST}/(1 - F_{ST})]$ distances, and a Mantel test (with 10,000 permutations) was used to assess the statistical significance of the correlation. These analyses were conducted with the software GENEPOP ver. 3.1 (Raymond & Rousset, 1995).

To investigate the possible occurrence of past population demographic changes, we computed the statistics F_S (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) using the software DNASP ver. 4.0 (Rozas *et al.*, 2003), and evaluated their significance by coalescent simulations (10,000 replicates). These neutrality test statistics have recently been shown to be the most powerful for the detection of past population growth (Ramos-Onsins & Rozas, 2002). Furthermore, a mismatch distribution analysis was also carried out to investigate the fit of the data to a sudden expansion model (Rogers & Harpending, 1992; Rogers, 1995) and to estimate the parameter τ, the mutational time since the expansion ($\tau = 2ut$ where u is the mutation rate per sequence and per generation, and t is time in generations), using the generalized least-square procedure proposed by Schneider & Excoffier (1999) and implemented in ARLEQUIN (Schneider *et al.*, 2000). The raggedness index (r; Harpending, 1994) was used as goodness-of-fit statistic for the observed and expected mismatch distributions, with the significance assessed using the parametric bootstrapping procedure (10,000 replicates) implemented in ARLEQUIN.

RESULTS

We sequenced a fragment of 620 bp of the mitochondrial DNA (mtDNA) cytochrome *b* gene for the 149 individuals sampled, corresponding to the positions 16,749–17,368 of the previously published *Rana nigromaculata* complete mtDNA genome (GenBank accession number AB043889). Sixty-three variable sites were observed (45 parsimony-informative), identifying 26 distinct haplotypes (GenBank accession numbers EU047772–EU047797). Fifty-six variable sites were in third codon position, six in first and one in second position, and three amino-acid substitutions were observed.

Phylogenetic analyses

The trees obtained with the different reconstruction methods (NJ, MP, ML) showed similar topologies. The MP analysis identified 12 equally parsimonious trees, 161 steps in length (consistency index excluding uninformative sites = 0.813; retention index = 0.953). The log-likelihood of the best ML tree obtained was loglk = −1586.76. The NJ phylogram based on the TN93 + I genetic distance is presented in Fig. 1a. Two major groups of haplotypes can be identified, showing a net sequence divergence of 4.8% (0.8% SD). One group comprised nine haplotypes (N1–N9) and was geographically restricted to northern Italy (samples 20–24). The second group was distributed from the northern Apennines to the rest of peninsular Italy and Sicily (samples 1–20). The two groups were found together only at the geographically intermediate sample 20 (Table 1). Within the latter group, two subclades were identified, one distributed in peninsular Italy (haplotypes C1–C14 observed in samples 6–20), the other restricted to Sicily (haplotypes S1–S3 observed in samples 1–5). These subclades presented a net pairwise sequence divergence of 2.6% (0.6% SD), and were never found at the same site.

Based on the statistical parsimony procedure implemented by the TCS software, the maximum number of mutational steps allowing for a 95% parsimonious connection between haplotypes was estimated at 10. Accordingly, it was not possible to connect all the haplotypes into a single network. Three distinct haplotype networks were generated (Fig. 1b), each corresponding to one of the three major clades as identified by the NJ, MP and ML analyses. The haplotype network connecting the haplotypes found in northern Italy (N1–N9) showed a star-like shape centred on haplotype N1, which was by far the most common haplotype of its clade (65% of occurrences). A star-like shape was also apparent for the network connecting the haplotypes of the peninsular subclade (C1–C14). Among these haplotypes, C1 was the most common (50% of occurrences), besides having been observed in all samples studied from peninsular Italy (samples 6–20).

The null hypothesis of constancy of the rate of molecular evolution was not rejected by the likelihood-ratio test ($-2 \log \Delta = 25.34$; $P > 0.05$). Applying the calibration suggested by Babik *et al.* (2004), the divergence time between the two major clades would be estimated at 1.33 (±0.22) Myr, whereas between the peninsular and Sicilian subclades it would be estimated at 0.72 (±0.16) Myr.

Genetic diversity and population structure

Estimates of population genetic diversity are presented in Table 1. In general, the populations sampled from Sicily showed little variation, with three out of five samples from this island (samples 1–5) showing the fixed haplotype S1. By contrast, among populations sampled in both peninsular and northern Italy, a wide and comparable range of diversity values was observed. At sample 20, the only one showing haplotypes from both northern and peninsular clades, a particularly high nucleotide diversity was found, about one order of magnitude greater than elsewhere. When evaluated separately for the three main clades identified by the previous phylogenetic analyses, genetic diversity estimates appeared to be of the same magnitude for both peninsular [$\pi = 1.88 \times 10^3$ (1.35×10^3 SD); $h = 0.72$ (0.05 SD)] and northern clades [$\pi = 1.75 \times 10^3$ (1.31×10^3 SD); $h = 0.56$ (0.08 SD)], and much lower for the Sicilian clade [$\pi = 0.59 \times 10^3$ (0.65×10^3 SD); $h = 0.35$ (0.09 SD)].

The overall pattern of population differentiation appeared to be strong and highly significant with $F_{ST} = 0.92$ ($P < 0.001$). Pairwise estimates of F_{ST} among populations are presented in Table 2. Values of $F_{ST} \geq 0.95$ were observed in almost all pairwise comparisons among samples from distinct groups of populations as defined by phylogenetic analyses (Sicily, 1–5; peninsular Italy, 6–20; northern Italy, 21–25), with the single exception of sample 20. However, this sample showed F_{ST} values no lower than 0.62 with respect to samples from northern Italy, but no higher than 0.13 with respect to

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Table 2 Pairwise values of F_{ST} among the 25 populations of *Rana lessonae* surveyed.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	–																								
2	0.00	–																							
3	0.00	0.00	–																						
4	0.78*	0.80*	0.76*	–																					
5	0.00	0.00	0.00	0.70*	–																				
6	0.97*	0.97*	0.97*	0.96*	0.97*	–																			
7	0.99*	0.99*	0.99*	0.98*	0.98*	0.08	–																		
8	0.97*	0.97*	0.96*	0.96*	0.97*	0.15	0.52*	–																	
9	0.95*	0.96*	0.95*	0.95*	0.96*	0.19	0.44*	0.41*	–																
10	0.98*	0.98*	0.97*	0.97*	0.97*	0.52*	0.71*	0.64*	0.00	–															
11	0.98*	0.98*	0.97*	0.97*	0.98*	0.59*	0.75*	0.68*	0.09	0.00	–														
12	0.97*	0.97*	0.96*	0.96*	0.97*	0.09*	0.45*	0.36*	0.00	0.33	0.42	–													
13	0.96*	0.96*	0.96*	0.96*	0.96*	0.13*	0.44*	0.38*	0.08	0.42*	0.49*	0.00	–												
14	0.99*	0.99*	0.98*	0.98*	0.98*	0.31	0.66*	0.50*	0.26	0.60*	0.67*	0.25	0.27	–											
15	0.97*	0.98*	0.97*	0.97*	0.97*	0.10	0.49*	0.38*	0.11	0.47	0.56*	0.03	0.08	0.25	–										
16	1.00*	1.00*	1.00*	0.99*	0.99*	0.11	0.72*	0.41*	0.14	0.67	0.72*	0.00	0.00	0.58	0.11	–									
17	0.99*	0.99*	0.99*	0.98*	0.98*	0.15*	0.62*	0.42*	0.20	0.64*	0.69*	0.00	0.05	0.46*	0.13	0.00	–								
18	0.97*	0.97*	0.97*	0.96*	0.97*	0.17*	0.50*	0.41*	0.19*	0.52*	0.59*	0.09	0.13	0.31*	0.10	0.11	0.15	–							
19	1.00*	1.00*	1.00*	0.98*	0.99*	0.00	0.62	0.29	0.00	0.53	0.62	0.00	0.00	0.37	0.00	0.00	0.00	0.00	–						
20	0.67*	0.69*	0.64*	0.69*	0.71*	0.02	0.09	0.13*	0.03	0.07	0.12	0.03	0.10	0.00	0.00	0.00	0.04	0.03	0.00	–					
21	0.98*	0.98*	0.98*	0.98*	0.98*	0.97*	0.97*	0.97*	0.96*	0.97*	0.97*	0.97*	0.97*	0.97*	0.97*	0.97*	0.97*	0.97*	0.97*	0.80*	–				
22	0.98*	0.98*	0.98*	0.98*	0.98*	0.97*	0.97*	0.97*	0.96*	0.97*	0.97*	0.97*	0.97*	0.97*	0.96*	0.98*	0.97*	0.97*	0.97*	0.74*	0.34	–			
23	0.99*	0.99*	0.99*	0.99*	0.99*	0.98*	0.99*	0.98*	0.97*	0.98*	0.98*	0.98*	0.98*	0.98*	0.98*	0.99*	0.99*	0.98*	0.99*	0.76*	0.36	0.09	–		
24	0.98*	0.99*	0.98*	0.98*	0.99*	0.96*	0.97*	0.97*	0.96*	0.96*	0.97*	0.97*	0.97*	0.97*	0.96*	0.98*	0.98*	0.97*	0.97	0.70*	0.31	0.06	0.05	–	
25	1.00*	1.00*	1.00	0.99*	0.99*	0.97	0.99	0.98*	0.96	0.98*	0.98	0.97	0.97*	0.99	0.97*	1.00	0.99	0.97	1.00	0.62	0.19	0.00	0.00	0.00	–

Populations are numbered as in Table 1. Values ≤ 0 were in all cases set to 0.
*$P < 0.05$ after 10,000 permutations.

the other samples from peninsular Italy. Among the Sicilian samples, high and significant F_{ST} values were observed only in comparisons involving sample 4, and the overall pattern of genetic differentiation appeared not to be significantly correlated to geographical distances separating populations (Spearman's $r = -0.05$; $P > 0.05$). Among samples from peninsular Italy (samples 6–20), a wide range of variation in F_{ST} values was observed (from 0.00, observed in several comparisons, to 0.75 among samples 7 and 11). However, high and significant F_{ST} values were observed mainly among samples from central and southern Calabria (samples 6–11) and among these samples and those located from northern Calabria to the northern Apennines (samples 12–20). By contrast, most pairwise comparisons within the latter geographical area were lower than average and not significant. No correlation was observed between genetic and geographical distances along the entire Italian peninsula (Spearman's $r = 0.11$; $P > 0.05$). However, a significant correlation was observed among samples located north of the northern Apennines (Spearman's $r = 0.77$; $P < 0.01$).

An AMOVA (Table 3), conducted by grouping samples according to their location with respect to the northern Apennine chain and the Messina Strait (with the three groups defined as above) showed that *c.* 93% of the overall genetic variance can be attributed to differences between the three population groups.

Table 3 Summary of the hierarchical analysis of molecular variance (AMOVA), with samples grouped as follows: Sicily (samples 1–5), peninsular Italy (6–20), northern Italy (samples 21–25).

Level of variation	d.f.	Percentage of variation	Φ statistic
Among groups	2	93.36*	$\Phi_{CT} = 0.933$*
Within groups	22	1.46*	$\Phi_{SC} = 0.219$*
Within populations	124	5.19*	$\Phi_{ST} = 0.948$*

*$P < 0.001$.

Historical demography

Historical demographic analyses were carried out separately for the three main clades as defined by phylogenetic analyses, and the results are summarized in Fig. 2. For the Sicilian clade, the small and not significant value of Fu's (1997) F_S statistic and the large and not significant value of the R_2 statistic (Ramos-Onsins & Rozas, 2002) both suggest a history of prolonged demographic stability, although a good fitting of the observed and expected mismatch distributions and a non-significant value of the r statistic (Harpending, 1994) would not allow us to reject the past occurrence of a sudden expansion of this clade. By contrast, for both northern and peninsular clades, large negative and significant values of the

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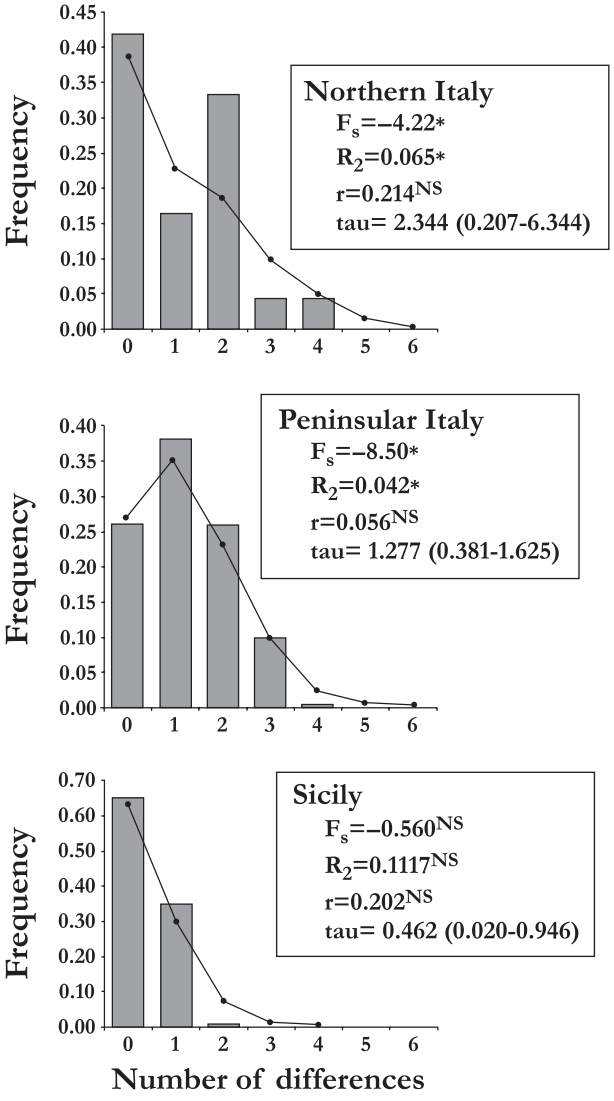

Figure 2 Mismatch distribution analysis and values of F_S (Fu, 1997), R_2 (Ramos-Onsins & Rozas, 2002) and r (Harpending, 1994) statistics for the three main mitochondrial lineages detected within *Rana lessonae* in Italy. Bars, observed pairwise differences; solid lines, expected distribution under a sudden expansion model. *$P < 0.05$; n.s., not significant.

F_S statistic and small and significant values of the R_2 statistic suggested the occurrence of a demographic expansion. This hypothesis was also supported by non-significant values of the r statistic (Harpending, 1994), although for the northern clade only a poor fitting of the expected and observed mismatch distributions was apparent. By the estimated values of parameter τ (Fig. 2), and the mutation rate per sequence per generation derived from the above-mentioned calibration suggested by Babik *et al.* (2004), time since the expansion could be dated at 105,000 yr BP (95% CI 9000–284,000) for the northern clade; 57,000 yr BP (95% CI 17,000–73,000) for the central clade; and 20,000 yr BP (95% CI 900–42,000) for the Sicilian clade.

DISCUSSION

Divergence between lineages

The analysis of mtDNA variation among the pool frog *R. lessonae* populations in peninsular Italy and Sicily revealed the occurrence of three main mtDNA lineages. The deepest genealogical divergence was observed among the pool frogs sampled north of the northern Apennines, and those further south. The existence of these two main phylogroups is consistent with previous findings based on analysis of variation at nuclear allozymes, morphological, immunological and bioacoustic characters (Uzzell, 1979; Uzzell & Hotz, 1979; Günther & Plötner, 1994; Sinsch & Schneider, 1996; Santucci *et al.*, 2000). The two groups showed a net sequence divergence from one another of 4.8 (±0.8)%. Using the calibration proposed by Babik *et al.* (2004), this indicates a split time (1.33 ± 0.22 Myr) dating back to the Early Pleistocene. Due to the intrinsic inaccuracies of the molecular clock (Ayala, 1997, 1999; Gibbons, 1998; Welch & Bromham, 2005; Ho & Larson, 2006), caution is needed when using molecular data to date historical processes. We thus regard this estimate, as well as the following ones, as indicative of approximate time windows for the inferred events, amenable to future adjustments.

In agreement with the hypothesis of Uzzell & Hotz (1979), the above-mentioned estimate suggests that the Italian pool frogs have survived much of the Pleistocene in at least two distinct peninsular refugia. According to the present distribution of the two clades, a plausible scenario to explain their divergence would involve a role for the northern Apennine chain as a historical–geographical barrier to dispersal along the north–south axis of the peninsula. This mountain chain was occupied by scattered glaciers during Quaternary glaciations (Giraudi, 2004), and its role as a historical barrier to dispersal has been suggested for several temperate species, including amphibians (Di Giovanni *et al.*, 1998; Stefani *et al.*, 2004; Canestrelli *et al.*, 2007a). In particular, an almost complete phylogeographical concordance was observed with respect to the Italian tree frog *Hyla intermedia* (Canestrelli *et al.*, 2007a, b), an amphibian species that shares several features of its natural history with *R. lessonae*, such as preferences for pools and ponds in lowland habitats. In *H. intermedia*, the deepest phylogeographical discontinuity was observed among two phylogroups located at alternative sides of the northern Apennines, and the only populations where these phylogroups were found together were located close to the northern side of the northern Apennines. This suggests that there are shared features between the evolutionary histories of the two species, encompassing a prolonged allopatry between the two main population groups located north and south of the northern Apennines, and a secondary contact through recent dispersal from south to north (see following sections). These findings, together with the observation that many species have their northern/southern range edge in proximity to the northern Apennines (Canestrelli, 2006), suggest the possible occurrence

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in this geographical area of a suture zone, at least under the expanded definition of Swenson & Howard (2005). More genetic data on multiple codistributed taxa will be necessary to strengthen this hypothesis.

The second genealogical divergence is found between clades located across the Messina Strait (the net sequence divergence being 2.6 ± 0.6%), thus separating pool frog populations sampled in Sicily from those sampled in the rest of the peninsula. The split between the two lineages was estimated to date back to the beginning of the Middle Pleistocene (0.72 ± 0.16 Myr). The existence of two main lineages of pool frog in peninsular Italy and Sicily was also evidenced by Santucci *et al.* (1996) based on allozyme data, although the allozyme differentiation between them (average $D_{NEI} = 0.4$) suggests an older separation (2–3 Myr). As the possible source of the observed divergence, Santucci *et al.* (1996) suggested a complex Plio-Pleistocene scenario of palaeogeographical evolution of the southern peninsula, a scenario that appears reasonable in the light of the mtDNA data presented here.

Genetic diversity within lineages and nuclear–mitochondrial discordance

Among populations sampled north of the northern Apennines, no phylogeographical discontinuities were found, and the overall pattern of genetic differentiation observed appeared to suggest a substantially homogeneous group of samples. Furthermore, a significant pattern of isolation by distance was also observed, suggesting that the pool frog populations from the Padanovenezian plain are currently in a migration–drift equilibrium (Hutchinson & Templeton, 1999). The estimated values of both the F_S and R_2 neutrality statistics suggested a past demographic expansion for the pool frogs in this geographical area. Although a bimodal mismatch distribution would make this scenario less straightforward, several factors have been shown to affect the general shape of this distribution (Slatkin & Hudson, 1991; Marjoram & Donnelly, 1994; Volckaert *et al.*, 2002; Paulus *et al.*, 2006; Krystufek *et al.*, 2007), and the non-significant value of the r statistic does not allow us to reject the hypothesis of past population expansion based on this analysis. Interestingly, the estimated time since the expansion (105,000 yr BP; 95% CI 9000–284,000) probably pre-dates the ending of the Last Glacial Maximum and falls within the last glacial phase. During this phase, the entire Padanovenezian plain underwent an impressive expansion due to a southward shift of the coastline by several hundred kilometres, which followed the glacio-eustatic-driven sea-level drop (Corregiari *et al.*, 1996). This phase also led to the ultimate establishment of a large Pleistocene alluvial plain environment in the area (Amorosi *et al.*, 2004). As previously suggested for the Italian tree frog *H. intermedia* (Canestrelli *et al.*, 2007a,b), it appears plausible that the pool frog populations have also benefited from such an expansion of lowland alluvial habitat in this geographical area. Therefore, independent evidence from two distinct species indicates demographic expansions, rather than reductions during the last glacial phase in the Padanovenezian plain, suggesting that the classical paradigm of glacial-reduction interglacial-expansion for temperate Mediterranean species (Hewitt, 2004a,b and references therein) may not be as general as previously thought, at least for this geographical area.

In their study of the phylogeography of *R. lessonae* in central and northern Europe, Zeisset & Beebee (2001) drew two hypotheses about the recent recolonization routes of these geographical areas from an ancestral population in northern Italy based on variation at microsatellite loci: (1) a single route out of Italy through the eastern Alpine–Carpathian gap; or (2) two separate migration routes out of Italy, one to the east and one to the west. Although we did not sequence samples from central and northern Europe, it is interesting to note that a comparison of the haplotypes we found in northern Italy with the two previously deposited in GenBank by the senior author (T. J. Beebee) could support the latter hypothesis. One was sampled in France (accession number AY057100) and presents no nucleotide differences with respect to our haplotype N8; the other was sampled in Poland (accession number AY057099) and was found identical to our haplotype N6. Haplotypes N6 and N8 were found geographically restricted to populations 24 (the easternmost one we sampled) and 21 (the westernmost one), respectively (Table 1; Fig. 1). Furthermore, from the haplotype network (Fig. 1b), these two haplotypes appear to be independent derivates from N1, the most common and widespread of the northern clade, supporting two distinct routes for the recolonization of the northern range, one to the east and one to the west of the Alpine massif.

Sicilian populations appeared among the least variable in the data set and, overall, only three haplotypes were found among them. This is in agreement with previous findings based on allozymes, showing the Sicilian samples as almost invariable (mean observed heterozygosity = 0.03, mean number of alleles per locus = 1.1; Santucci *et al.*, 1996). Both historical and contemporary processes are expected to affect the genetic diversity of populations and disentangling the two can be hard, particularly when no clear geographical pattern is expected to be associated with the hypothesized history of populations (Ficetola *et al.*, 2007). This is also the case for the pool frogs from Sicily, where both the prolonged insularity and the more recent human-driven alterations of habitats could account for the observed paucity of genetic diversity. Both processes are likely to have played a role.

The phylogeographical pattern observed among populations from the northern Apennines to the tip of Calabria (6–20) strictly match the category V pattern as described by Avise (2000), whereby a common haplotype that is geographically widespread is observed (in the present case, C1), together with several rare, closely related and geographically restricted haplotypes. This pattern has been explained mainly as the outcome of contemporary restricted gene flow among populations that were previously closely connected (Avise, 2000). Besides the geographical distribution of haplotypes, in this case the inference of presently restricted gene flow is also supported by the strong and significant pattern of population differentiation, particularly

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among Calabrian populations (6–11), and among these and the populations located further north (12–20). The overall shallow divergence between haplotypes of clade C, the star-like shape of the haplotype network centred on haplotype C1 (the ancestral and the most common and widespread haplotype of its clade), the estimated values of the statistics F_S and R_2, as well as the mismatch distribution analysis, all indicate that the pool frog of the central and southern peninsula has recently undergone a demographic expansion from a single ancestral population.

The pattern of geographical distribution of both C1 and its recent and low-frequency derived mutations (Table 1) also suggest that this expansion has involved the whole range of clade C (Fu, 1997; Avise, 2000). Furthermore, as for the northern group, from the estimated value of the expansion parameter τ, the demographic expansion appears to pre-date the ending of the last pleniglacial, and to fall within the last glacial phase (53,000 yr BP, 95% CI 17,000–73,000).

The general picture of an ancestral pool frog population in peninsular Italy, which, like the northern group, would have expanded demographically during the last glacial phase, appears well supported by the mitochondrial data at hand, and plausible even in the light of both palaeoenvironmental reconstructions (Tortora *et al.*, 2001; Lambeck *et al.*, 2004; Ferranti *et al.*, 2006; A. Amorosi, personal communication) and comparisons with other taxa (Canestrelli *et al.*, 2007b). Nevertheless, this historical scenario presents at least one major discordance with respect to what was previously observed based on allozymes (Santucci *et al.*, 1996). Based on these markers, the peninsular form of *R. lessonae* appears to be distributed from the northern Apennine chain to northern Calabria, whereas the southern form is distributed in Sicily and southern Calabria. The central portion of Calabria, particularly the area between the Catanzaro and the Crati-Sibari plains, was identified as a wide hybrid zone between the two forms. Seven unlinked diagnostic allozyme loci support this scenario (six are shown in Fig. 3). By contrast, as stated above, at mitochondrial level we observed two forms distributed at the opposite sides of the Messina Strait (Fig. 1). As we see no reason to doubt that the two lineages identified by Santucci *et al.* (1996) based on allozyme markers and those observed here are the same, the most plausible explanation for such a discordance appears to be a higher mitochondrial introgression compared with nuclear markers, which would have followed the secondary contact between the two forms in central Calabria. This strong introgression would finally have led to the complete mitochondrial substitution in southern Calabria. Two possible alternative hypotheses could be: (1) introgression of Sicilian nuclear alleles into the Calabrian mtDNA clade; and (2) incomplete lineage sorting in southern Calabria from a polymorphic ancestor. The first hypothesis appears dismissible on the basis of at least two arguments. First, and most convincingly, it appears highly unparsimonious on the basis that it would imply that the independent signals at seven unlinked nuclear loci are less representative of the population history than is that of the single mtDNA genome (Funk & Omland, 2003; Avise, 2004; Ballard & Whitlock, 2004; but cf. García-París *et al.*, 2003). Also, it has been shown that mtDNA could be particularly prone to introgress with respect to nuclear loci, even among well differentiated lineages or distinct species (Avise, 2004; Weisrock *et al.*, 2005; McGuire *et al.*, 2007). Second, southern Calabrian populations (those where introgressed northern alleles were not found at nuclear loci) share with the Sicilian ones the allele frequencies at those loci that are diagnostic with respect to peninsular populations, but also present differences with respect to Sicilian populations, leading to a value for Nei's (1972) genetic distance of 0.04 (Santucci *et al.*, 1996). This, in turn, suggests that the Messina Strait was still active as a barrier to dispersal when the recent secondary contact between the two lineages occurred, and that this event probably took place among the peninsular group and the southern Calabrian (sub)group. Finally, the hypothesis of incomplete lineage sorting also appears highly implausible. In the case of multiple haploforms in a common ancestor retained in descendent lineages, these are expected to be distributed randomly in the descendant populations, and not to be geographically restricted to the proximity of the inferred secondary contact zone (McGuire *et al.*, 2007). In the present case, instead, haplotypes from clade C were found only in southern Calabria (slightly south of the inferred secondary contact zone), not in Sicily.

As mentioned above, a deeper mitochondrial rather than nuclear introgression has been observed frequently, and several explanations have been proposed to account for such a pattern (reviewed by Ballard & Whitlock, 2004). These encompass: (1) stochastic processes due to the smaller effective population size of mtDNA with respect to nDNA; (2) selective advantages because of local mtDNA adaptations; and (3) selective introgression following mutational meltdown in small populations. In the light of present knowledge of the palaeogeographical evolution of southern Calabria, the latter explanation appears to be especially plausible. Due to glacio-eustatic sea-level oscillations and an intense tectonic activity throughout the Plio-Pleistocene, this geographical area repeatedly underwent fragmentation by means of sea sounds until the Late Pleistocene, also leading to phases of insularization of its major reliefs (Aspromonte, Serre Calabre, Capo Vaticano and Sila massifs). As has emerged from both palaeontological evidence (Bonfiglio *et al.*, 2002) and genetic data (Canestrelli *et al.*, 2006), these historical events have left deep traces in the structure of both the flora and fauna of this geographical area (cf. Pignatti, 1984; Caloi *et al.*, 1989), and were previously indicated by Santucci *et al.* (1996) as the possible source for the observed divergence between the two pool frog lineages in southern Italy. Thus it appears plausible that, during the insularization phases, a strong fragmentation of the pool frog populations in the area could have occurred, engendering the palaeodemographic context for the successful selective introgression to take place following secondary contact.

The observed discordance between mitochondrial and nuclear markers has some highly relevant implications for

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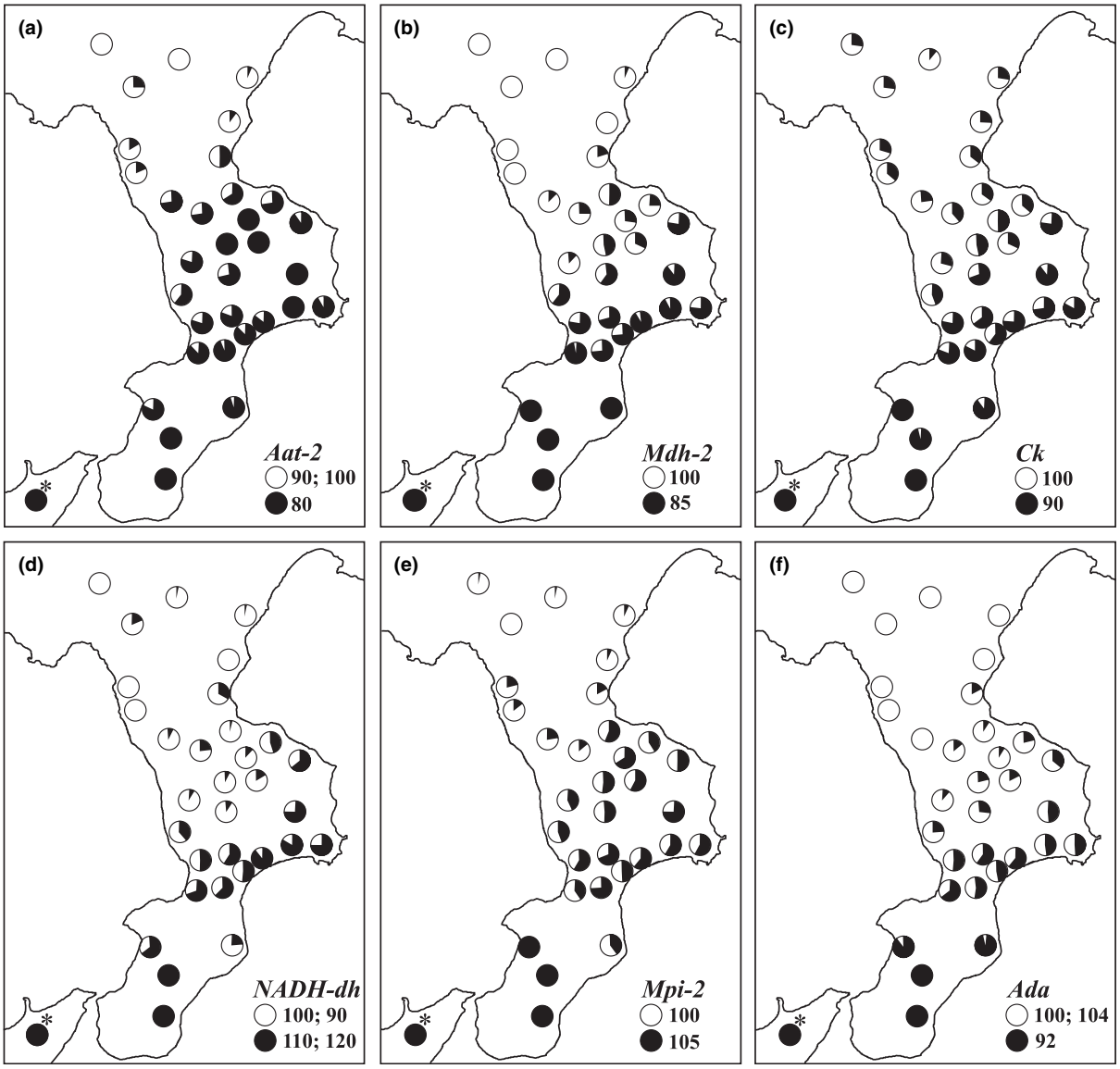

Figure 3 Pie diagrams showing allele frequency variation at loci (a) *Aat-2*, (b) *Mdh-2*, (c) *Ck*, (d) *NADH-dh*, (e) *Mpi-2* and (f) *Ada* among southern Italian samples of *Rana lessonae*. Open sections, northern alleles; filled sections, southern alleles. All samples from Sicily were found to be fixed for the same allele at each locus, and are therefore cumulatively represented by a single pie diagram (*). Modified from Santucci *et al.* (1996).

phylogeographical inferences. The need to use genetic markers from both nuclear and mitochondrial genomes to draw reliable inferences of the evolutionary history of both species and populations, although long acknowledged (Ballard & Whitlock, 2004; Canestrelli *et al.*, 2007a), appears still to be widely underrated in phylogeographical practice. In this context, the case of the pool frogs from southern Italy appears especially instructive. Although several major events in the evolutionary history of the pool frog populations south of the Alps could not have been appreciated without the mtDNA data presented here, if we had only had the mtDNA data at our disposal, we would have been able to infer the recent demographic expansion of clade C, but not to appreciate all its consequences. In fact, the supposed selective introgression of the peninsular form into southern Calabria would have completely erased the mtDNA signals of previous historical events in the area, as well as the traces of a secondary contact between the two vicariant lineages in central Calabria. Most importantly, the relative contribution of the 'multiple refugia' vs. 'prolonged stability' scenarios (see Introduction) in the southern peninsula could not have been appropriately addressed.

Finally, it is worth pointing out that the palaeogeographical context that would have led to the observed discordance between different kinds of marker for the pool frogs in southern Italy could also have affected other organisms in a similar way (but cf. Canestrelli *et al.*, 2006). Thus, our analysis shows that there is a strong need to use genetic markers from multiple source genomes in studies concerning

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phylogeography, population structure and conservation genetics of taxa from this major biodiversity hotspot.

Taxonomic implications

The most appropriate taxonomic arrangement of the Italian non-hybrid pool frog populations has been a matter of considerable uncertainty (Crochet & Dubois, 2004). Based on the existence of differences of diagnostic value in morphology, immunology, bioacoustic and mtDNA, some authors recognized populations from Italy north of the northern Apennines as belonging to the European species *R. lessonae*, while assigning populations located further south to the new species *Rana bergeri* Günther, 1985 (Günther, 1997). By contrast, based on the low level of genetic divergence observed for allozymes (Uzzell & Hotz, 1979; Santucci *et al.*, 2000), other authors consider all the Italian populations as being conspecific (Santucci *et al.*, 1996; Crochet & Dubois, 2004; Lanza *et al.*, 2006), recognizing the two lineages as separate subspecies under the names *Rana lessonae lessonae* and *Rana lessonae bergeri*. Our data support the latter arrangement. The level of genetic divergence we observed using mtDNA, although not preventing their designation (García-París *et al.*, 2001), cannot be taken as evidence that the two lineages should be assigned to separate species (Johns & Avise, 1998; Ferguson, 2002). For instance, in the case of the Italian tree frog *H. intermedia*, the average uncorrected sequence divergence between the mtDNA lineages found on alternative sides of the northern Apennines is almost twice that observed here for the pool frog, but based on the overall genetic pattern observed (at both mtDNA and nuclear markers) they are reliably regarded as conspecific lineages (Canestrelli *et al.*, 2007a,b). Previous claims in favour of the species status of the two pool frog lineages in peninsular Italy south of the Alps on the basis of mtDNA (Plötner, 1998) focused on the existence of fixed differences between fully allopatric and distantly located samples. Nevertheless, the utilization of such kinds of difference for purposes of species status recognition has recently been discouraged (Hudson & Coyne, 2002). Furthermore, we found the two lineages to be co-occurring in the geographically intermediate population located on the northern side of the northern Apennines. Although a detailed genetic analysis (with both nuclear and mitochondrial markers) of the secondary contact zone is still in progress and will be presented elsewhere, we have yet to find any evidence of genetic disequilibria, as would be expected in cases of reduced fitness in individuals of mixed ancestry, or the existence of assortative mating. The data at hand thus do not support the assignment of the pool frogs from northern Italy and those from the rest of the peninsula and Sicily to two separate species. Therefore, according to their mostly allopatric distribution, and in line with what was recently suggested by Crochet & Dubois (2004) and Lanza *et al.* (2006), we conclude that they should be regarded as distinct subspecies of *R. lessonae*, namely *R. lessonae lessonae* and *R. lessonae bergeri*.

Finally, as also suggested by Crochet & Dubois (2004) and Lanza *et al.* (2006), based on the work of Santucci *et al.* (1996), we propose also to assign the status of separate subspecies (to be described formally elsewhere) to the Sicilian pool frogs. At the mtDNA level, this appears as a monophyletic, allopatric and highly differentiated lineage of pool frogs. The overall genetic pattern observed in central and southern Calabria (Santucci *et al.*, 1996; this study) identifies this geographical area as a wide hybrid zone between the peninsular and Sicilian forms of *R. lessonae*.

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