

Genetic differentiation and history of populations of the Italian treefrog *Hyla intermedia*: lack of concordance between mitochondrial and nuclear markers

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Abstract The genetic differentiation among 33 populations of the Italian treefrog, *Hyla intermedia* (Anura: Hylidae), was investigated using both biparentally (23 allozyme loci) and maternally (partial mitochondrial cytochrome *b* gene) inherited markers. Two main population groups were evidenced by both markers, located north and south of the northern Apennines. However, the pattern of differentiation between these two groups was much less pronounced at allozymes than at mtDNA, leading to gene flow estimates that were 25 times lower at mitochondrial than at nuclear level. Also, the mtDNA divergence between the two groups was particularly marked for two cospecific lineages of anuran amphibians (the *P*-distance being on average 9.04%), while their average genetic distance at allozymes was comparatively low ($D_{NEI} = 0.07$). This contrasting pattern of nuclear versus mitochondrial genetic variation is discussed in the context of: (1) marker specific selection, (2) secondary contact and sex-biased gene flow and (3) ancestral polymorphism and colonization from north to south. Finally we emphasize how, for population genetic studies, the use of multiple markers having distinct evolutionary properties can help unravel the existence of more complex evolutionary histories.

Keywords Allozymes · Mitochondrial DNA · Italian treefrog · Population structure · Discrepant genetic markers

Introduction

The introduction of genetic markers in the study of geographical patterns of variation between natural populations has had important consequences on the general perception of the natural history of several taxa. For instance, in amphibians which are generally conservative in their morphological evolution (Cherty et al. 1978; Richards and Moore 1996), often phylopatric and with relatively low dispersal abilities (Marsh and Trenham 2000; Jehle and Arntzen 2002), a huge amount of studies using molecular markers have provided evidence of the existence of cryptic biodiversity and complex population structures that were previously unsuspected (e.g. Veith 1996; Ron et al. 2006).

For the study of genetic differentiation among natural populations, a wide array of markers have now become available, from both nuclear and organelle genomes (Avice 2000, 2004; Zhang and Hewitt 2003; Ballard and Whitlock 2004). The relative importance of nuclear versus mitochondrial markers for the study of population genetics and evolutionary processes has long been discussed (e.g. Moore 1995; Hoelzer 1997; Avice 2000; Hare 2001), especially following evidence that distinct markers can give discrepant estimates of genetic differentiation among populations, leading to discrepant inferences regarding populations' structure and history (e.g. Palumbi and Baker 1994; Piel and Nutt 2000; Shaw 2002; Johnson et al. 2003). Some differences among mtDNA and nuclear markers are

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usually expected, because of their different effective size (typically four times lower in the former), but several other causes can contribute or even play a prevalent role in enhancing these differences. Because of the lower effective size of mtDNA, historical events implying demographic size changes, such as population bottlenecks or founder effects, are expected to affect genetic diversity at this marker more strongly than at nuclear ones (e.g. Fay and Wu 1999). This leads to mtDNA lineages becoming reciprocally monophyletic more quickly in diverging groups. Gender-biased factors also have been shown significantly to affect the magnitude of differentiation at different marker loci. For instance a male-biased sex ratio, implying a reduced female effective size, would enhance the effect of genetic drift on the (maternally inherited) mtDNA compared to the (biparentally inherited) autosomal genome (e.g. Arnaud-Haond et al. 2003). On the other hand, gender-biased patterns of dispersal or phylopatriy can produce different patterns of distribution of genetic variation at mitochondrial and nuclear genomes, because of differences in rates of gene flow (e.g. Palumbi and Baker 1994; Goudet et al. 2002). Finally, even selection can produce discrepancies among distinct kinds of markers. Indeed, fixation of new allele variants through directional selection (selective sweeps) can lead to increased estimates of differentiation among the diverging lineages, both at the selected locus and at those loci that are tightly linked to it (Maynard Smith and Haigh 1974). If acting on a mitochondrial gene, this leads to the fixation of a single mtDNA variant, because animal mitochondrial genome generally lacks recombination and therefore evolves in many ways as a single genetic locus, implying that a stronger mitochondrial differentiation will be found compared to nuclear genes, whichever mtDNA gene fragment we study (Schlotterer 2003; Ballard and Whitlock 2004). In contrast, balancing selection, promoting the maintenance of polymorphism, leads to a less pronounced differentiation at the affected loci with respect to those evolving under selective neutrality (Broughton and Harrison 2003).

In this paper, we present a new case of remarkable discrepancy between nuclear (allozymes) and mitochondrial markers in depicting the pattern and depth of differentiation among populations of the Italian treefrog, and discuss the possible contribution of different evolutionary processes in generating the observed pattern.

The Italian treefrog *Hyla intermedia* is a small, cryptically coloured amphibian which breeds in ponds, pools, temporary waters and various other freshwater habitats (Lanza 1983). It is a relatively thermophilic species, with

the majority of populations (>90%) being distributed below 500 m a.s.l. (e.g. Mazzotti et al. 1999). Because of the scarcity of morphological and chromatic differentiation, the populations of this species were long attributed to the European species *Hyla arborea* (Linnaeus 1758), together with all other Palaearctic populations of treefrog. Some authors (e.g. Tscudi; cited in Schreiber 1875) even believed the species to be cosmopolitan. Later studies based on genetic and bioacoustic markers revealed the existence, within the Palearctic region, of several distinct species within the former *H. arborea* (e.g. Paillette 1967; Schneider 1974; Kawamura et al. 1977; Nascetti et al. 1985). Based on an allozyme survey, Nascetti et al. (1995) attributed the treefrog populations from peninsular Italy and Sicily to the new species *Hyla italica*, which showed a genetic divergence (Nei 1972) of $D_{NEI} = 0.47$ with respect to the other European populations regarded as *H. arborea*. Subsequently, a synonymizing of *H. italica* (Nascetti et al. 1995) with *H. intermedia* (Boulenger 1882), and the consequent replacing of the latter name with the former, was proposed (Dubois 1995).

Here we investigate the pattern of population differentiation within the Italian treefrog, through a further analysis of the genetic variation in this species, assessed at both nuclear (allozymes) and cytoplasmic level (PCR-RFLP and sequence analyses of a cytochrome *b* gene fragment). Our aims are both to assess the present geographical pattern of differentiation among populations of the Italian treefrog and to elucidate the evolutionary processes involved in generating this pattern.

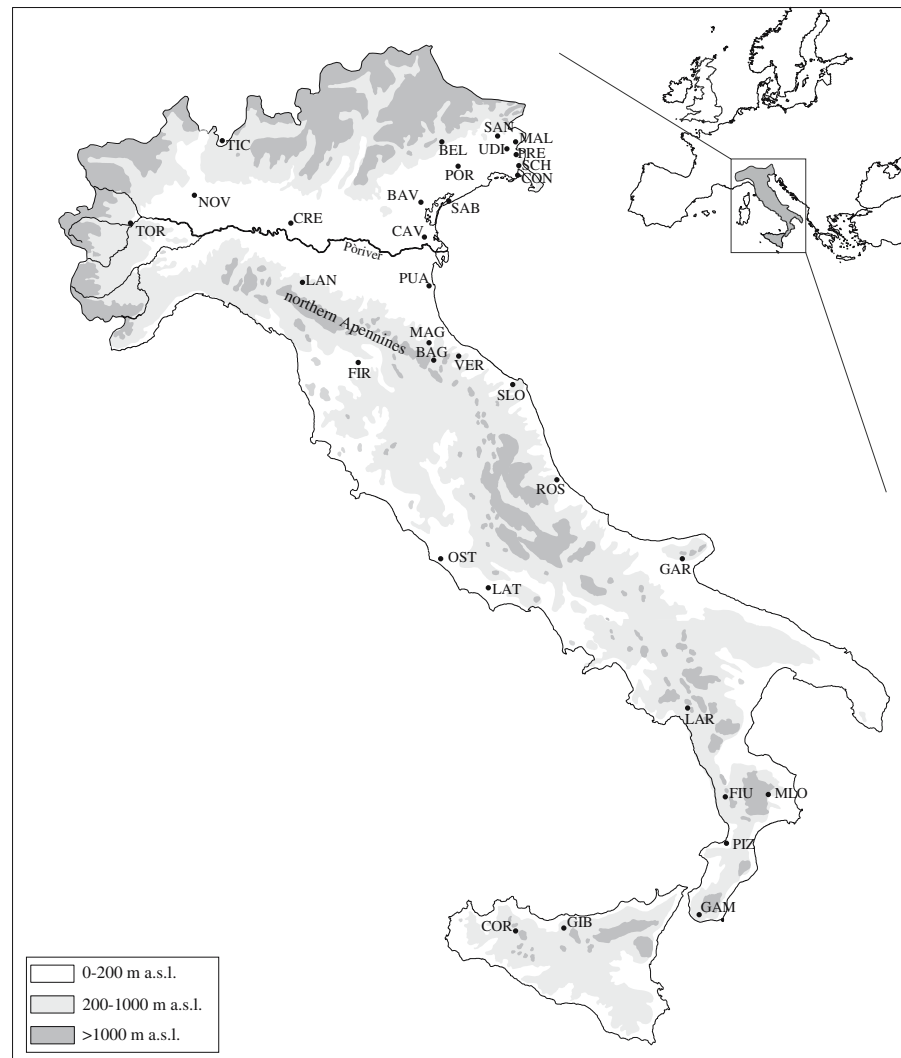
Materials and methods

Altogether 497 specimens of *H. intermedia* were collected from 33 populations, covering the entire species range (Fig. 1). The geographic origin of samples studied and sample sizes are given in Table 1. Tissue samples were obtained through a toe-clipping procedure. Each sampled individual was then released in the same collection place. Collected samples were carried to the laboratory in liquid nitrogen containers, then stored at -80°C until further analysis could be carried out. Samples from Verucchio, San Daniele and Langhirano were provided by the Department of Animal and Human Biology of Turin University and had been stored in 70% ethanol.

Allozymes

Horizontal electrophoresis was carried out using 10% starch gel. Enzyme systems analysed and their putative encoding loci are listed in Table 2 together with the

Fig. 1 Geographic range of *Hyla intermedia* and geographic location of the 33 samples studied. Samples are encoded as in Table 1



electrophoretic techniques utilized. Isozymes were numbered in order of decreasing mobility, from the most anodal one. Alleles at each locus were designated by their mobility (in mm, standardized conditions) relative to the most common one (100) in a reference population (Corleone, Sicily).

Estimates of allele frequencies and population genetic variability (as mean observed heterozygosity, Nei's (1978) unbiased estimate of expected heterozygosity, percentage of polymorphic loci and mean number of alleles per locus) were computed with the software BIOSYS-2 (Swofford and Selander 1999). Exact significance probabilities for Hardy–Weinberg equilibrium (HW) were assessed for each locus in each sample, then the Bonferroni correction for multiple tests was applied (Rice 1989).

Genetic distances between populations were assessed by calculating Nei's (1972) standard genetic distances, which were then used to build an UPGMA

phenogram. 1,000 bootstrap pseudoreplicates over loci were run to test the reliability of the UPGMA phenogram with the BOOTDIST option in BIOSYS-2. The consensus UPGMA was then obtained using the subroutines NEIGHBOR and CONSENSE in the software PHYLIP 3.5c (Felsenstein 1993). Because UPGMA analysis forces populations into a dichotomous branching pattern, we also carried out a Principal Component Analysis (PCA) with the software PCAGEN 1.2 (Goudet 1999), in order to detect potentially intergraded populations relative to the main lineages detected. The statistical significance of each axis was evaluated over 10,000 randomizations.

mtDNA

DNA was extracted from frozen or alcohol-preserved tissues following the standard Sambrook et al. (1989) extraction protocol. A fragment of 332 bp of the

Table 1 Geographic origin and sample size (*n*) of the 33 populations of the Italian treefrog studied

Sampling location	Location reference code	Latitude N	Longitude E	<i>n</i>	
				Allozymes	mtDNA
Corleone	COR	37°49'	13°18'	25	11
Gibilmanna	GIB	37°55'	14°01'	35	12
Gambarie	GAM	38°09'	15°41'	28	8
Pizzo	PIZ	38°44'	16°10'	23	9
Fiumefreddo	FIU	39°20'	16°07'	8	4
Macchia Longa	MLO	39°15'	16°46'	15	–
Lago Remmo	LAR	40°11'	15°52'	21	–
S. Giovanni Rotondo	GAR	41°43'	15°43'	20	4
Lido di Ostia	OST	41°45'	12°20'	11	7
Latina	LAT	41°28'	12°56'	10	–
Roseto	ROS	42°40'	13°59'	–	6
San Lorenzo	SLO	43°34'	13°26'	19	19
Firenze	FIR	43°49'	11°28'	16	6
Bagno di Romagna	BAG	43°50'	11°57'	9	9
Magliano	MAG	44°60'	12°5'	24	24
Punta Alberete	PUA	44°30'	12°16'	17	17
Verucchio	VER	43°59'	12°25'	–	7
Langhirano	LAN	44°37'	10°16'	–	5
Cremona	CRE	45°09'	10°01'	15	15
Novara	NOV	45°29'	8°39'	22	25
Torino	TOR	45°07'	7°34'	8	18
C. Ticino	TIC	46°01'	8°54'	–	6
Belluno	BEL	46°08'	12°12'	–	7
Cavarzere	CAV	45°08'	12°04'	7	4
Bavaria	BAV	45°34'	12°05'	17	11
San Daniele	SAN	46°10'	13°05'	–	12
Cordenons	POR	45°57'	12°38'	16	9
Punta Sabbioni	SAB	45°30'	12°34'	6	–
Brazzacco	UDI	46°05'	13°10'	10	–
Malina	MAL	46°05'	13°19'	11	6
Schiavetti	SCH	45°49'	13°33'	21	7
Cona	CON	45°54'	13°32'	12	5
Preval	PRE	45°56'	13°33'	15	8

mitochondrial gene encoding for the cytochrome *b* was amplified by means of the polymerase chain reaction (hereon, PCR) using primers MVZ15 (5'-GA-ACTAATGGCCCACACWWTACGNAA-3'; Moritz et al. 1992) and H15149 (5'-AAACTGCAGCCCCT-CAGAATGATATTTGTCCTCA-3'; Kocher et al. 1989). PCR cycling protocol was: 95°C for 5 min followed by 39 cycles of 93°C for 1 min, 45°C for 45 s, 72°C for 1 min 30 s and a single final step at 72°C for 10 min.

Preliminary tests of restriction endonucleases were carried out for 30 individuals drawn from six populations (PUA, TOR, MAL, FIR, OST and PIZ). Of the ten restriction enzymes surveyed (*HaeIII*, *RsaI*, *Sau3a*, *DraI*, *EcoRI*, *MaeII*, *AluI*, *SspI*, *StuI* and *XhoI*), the six, which showed restriction site polymorphisms (*HaeIII*, *RsaI*, *Sau3a*, *DraI*, *MaeII* and *AluI*) were retained and used to screen all remaining samples.

A volume of 17.3 µl of PCR product was digested overnight with five units of enzyme following manufacturer's instructions. Restriction fragments were

separated on 3% agarose gel, stained with 0.5 µg/ml Ethidium Bromide and visualized under UV light.

Haplotype sequences were obtained using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems, Foster City, USA) following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. The sequencing chromatograms were analysed with the program CHROMAS (Technelysium Pty Ltd., Australia). Alignments were carried out using the software CLUSTALX 1.81 (Thompson et al. 1997).

The null hypothesis of constant substitution rate was tested by means of Tajima's relative rate test (1993) as implemented in MEGA 2.1. Pairwise sequence divergence (*P*-distance) and phylogenetic analyses were computed using the software PAUP* 4.0b10 (Swofford 2003). Phylogenetic trees were inferred using Maximum Likelihood (ML), Neighbour-Joining (NJ) and Maximum Parsimony (MP) analyses. Unweighted MP trees were obtained using the exhaustive search. The optimal model of sequence evolution for the ML and NJ analyses was assessed using the hierarchical

Table 2 Enzymes studied, their code number, encoding loci, buffer systems and staining technique reference

Enzyme	EEC	Encoding loci	Buffer systems	Staining reference
Malate dehydrogenase	1.1.1.37	<i>Mdh-1</i>	5	b
		<i>Mdh-2</i>	5	b
Malate dehydrogenase (NADP ⁺)	1.1.1.40	<i>Mdhp</i>	2	d
Isocitrate dehydrogenase	1.1.1.42	<i>Idh-1</i>	2	b
		<i>Idh-2</i>	2	b
6-PhosPhogluconate dehydrogenase	1.1.1.44	<i>6Pgdh</i>	5	b
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	<i>Gapdh</i>	2	d
Superoxide dismutase	1.15.1.1	<i>Sod-1</i>	1, 2	d
		<i>Sod-2</i>	1, 2	d
Aspartate aminotransferase	2.6.1.1	<i>Aat-1</i>	5	c
		<i>Aat-2</i>	5	c
Creatine kinase	2.7.3.2	<i>Ck</i>	2	b
Adenylate kinase	2.7.4.3	<i>Adk</i>	2	d
Aminopeptidase (Leu-Ala)	3.4.1.1	<i>Pep-C1</i>	2	e
		<i>Pep-C2</i>	2	e
		<i>Pep-C4</i>	2	e
Adenosine deaminase	3.5.4.4	<i>Ada</i>	2	e
Carbonic anhydrase	4.2.1.1	<i>Ca</i>	3	e
Mannose phosphate isomerase	5.3.1.8	<i>Mpi</i>	3	e
Glucose phosphate isomerase	5.3.1.9	<i>Gpi</i>	5	c
Phosphoglucomutase	5.4.2.2	<i>Pgm-2</i>	4	a
Unidentified proteins		<i>Pt-2</i>	1	b
		<i>Pt-3</i>	1	b

Buffer systems 1) Discontinuous Tris/Citrate (Poulik 1957); 2) Continuous Tris/Citrate (Selander et al. 1971); 3) Tris/Versene/Borate (Brewer and Sing 1970); 4) Tris/Versene/Maleate (Brewer and Sing 1970); 5) Phosphate-Cytrate (Harris 1966). Staining technique reference; a) Brewer and Sing (1970); b) Shaw and Prasad (1970); c) Selander et al. (1971); d) Ayala et al. (1972); e) Harris and Hopkinson (1976)

likelihood-ratio test as implemented by MODELTEST 3.6 (Posada and Crandall 1998) associated with PAUP*. This analysis supported the Kimura 2-parameter model (K-2-P; Kimura 1980) as the best fit substitution model for the data. ML analysis was carried out using heuristic search with tree-bisection–reconnection (TBR) branch swapping. Nodal support for the inferred trees was evaluated by the bootstrapping method with 1,000 pseudoreplicates. *Pseudacris regilla* was used as the outgroup (GenBank Accession Number: AY363197; Ripplinger and Wagner 2004).

Estimating F_{ST} and gene flow for comparative purposes

Population genetic structure was investigated using F_{ST} among pairs of samples as estimated by the parameter θ of Weir and Cockerham (1984) using the software FSTAT 2.9.3 (Goudet 2001). The significance of the θ estimates was assessed by 1,000 permutations, and the Bonferroni correction for multiple simultaneous tests (Rice 1989) was applied.

Indirect estimates of gene flow (Nm) among populations were obtained from F -statistics (Wright 1951) as $Nm = (1 - F_{ST}) / (4 F_{ST})$. This estimate assumes the infinite-island model of population structure and gene

flow (Wright 1951). Although the majority of real populations probably violate these assumptions (Whitlock et al. 1999), this estimate is still considered a useful tool for comparative purposes (e.g. Neigel 2002; Mosen et al. 2003) and will therefore be used for this aim.

Results

Allozymes

Eight of 23 loci surveyed (*6Pgdh*, *Gapdh*, *Sod-2*, *Ck*, *Adk*, *Gpi*, *Pt-2* and *Pt-3*) were monomorphic for the same allele in all the 27 populations surveyed at allozymes (the table of allele frequencies at polymorphic loci is available from the authors upon request).

Eight of 174 tests for HW equilibrium were significant ($P < 0.05$). However, they did not concern specific loci or geographic locations, and were not significant after the Bonferroni correction.

Populations of the Italian treefrog close to the area of parapatry with the European species *H. arborea* (UDI, MAL, SCH, CON and PRE) showed alleles of this species (*Idh-1*⁹³, *Sod-1*⁸⁴, *Aat-2*⁹⁰, *Pep-C1*¹⁰⁸, *Pep-C2*⁹⁵, *Pep-C4*¹¹⁰ and *Ada*⁹³; Nascetti et al. 1995)

introgressed with frequencies varying across loci and populations. The contact zone between these sister species and patterns of introgression are being analysed in another paper, and will therefore not be further considered here.

Five of 15 polymorphic loci (*Mdhp*, *Idh-2*, *Aat-2*, *PepC-2* and *Ca3*) showed marked differences in allele frequencies among populations located either south or north (hereon group-S and group-N, respectively) of the northern Apennine (Figs. 1, 2). The allele *Mdhp*¹⁰⁴, the most common allele at locus *Mdhp* in almost all populations of the group-N, is only present in four populations of the group-S with frequencies never above 0.09, whereas the loci *Idh-2*, *Aat-2*, *PepC-2* and *Ca3* showed alleles found in almost all northern populations that are observed only in one (*Aat-2*⁸⁵ and *PepC-2*⁹⁰) or two (*Idh-2*¹¹⁰ and *Ca3*¹¹⁰) southern populations (see Fig. 2). The same geographical pattern of variation was not apparent at the other ten polymorphic loci (the complete data set is available upon request).

Nei's (1972) standard genetic distance (D_{NEI}) between populations ranged from 0.00 and 0.11, with the highest value observed between SCH (group-N) and LAR (group-S) and between TOR (group-N) and several populations from group-S. Populations close to the northern side of the northern Apennines (MAG, BAG and SLO) showed lower values of D_{NEI} if compared with populations from group-S (D_{NEI} [\pm SD]

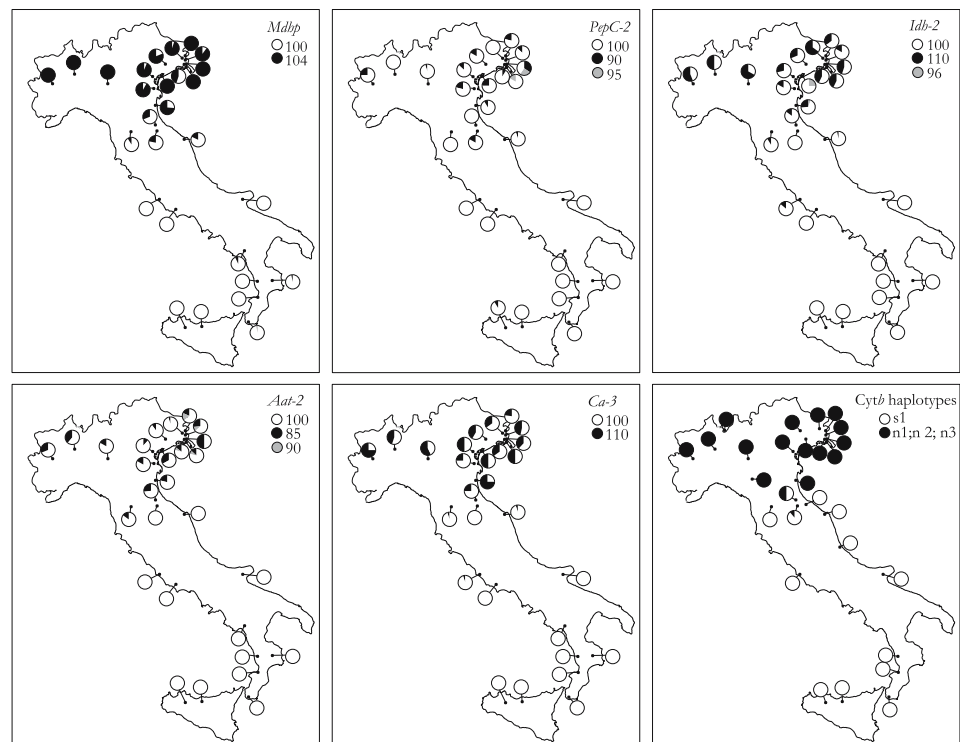
being on average 0.02 [\pm 0.01], 0.03 [\pm 0.01] and 0.02 [\pm 0.01], respectively) than if compared with populations from group-N (D_{NEI} being on average 0.04 [\pm 0.01], 0.07 [\pm 0.02] and 0.06 [\pm 0.02], respectively). Between group-N and group-S mean D_{NEI} was 0.07 (\pm 0.02), whereas within each group it was 0.02 (\pm 0.01) and 0.01 (\pm 0.01), respectively.

A phenogram based on a UPGMA (Fig. 3) grouped samples into two main clusters corresponding to group-N and group-S as defined above. The only exception to this geographical pattern are the three samples close to the northern side of the Apennines (MAG, BAG and SLO) which are instead included in the southern cluster. Since all other nodes in the UPGMA phenogram receive little bootstrap support (<70%), additional inferences cannot be made with accuracy from the UPGMA phenogram.

The first two principal components resulting from PCA (scatterplot shown in Fig. 4) cumulatively accounted for 80.3% of the total genetic variance, although only the first PC was significant ($P < 0.05$). The first PC explained 72.8% of the total variance and discriminated between the same two groups of samples as observed in the UPGMA tree. Nevertheless, along this PC axis, the sample from MAG was intermediate among these two groups.

The level of differentiation among all populations was strong and highly significant ($\theta = 0.311$; $P < 0.001$).

Fig. 2 Pie-diagrams showing the geographic variation of allele frequencies at loci *Mdhp*, *PepC-2*, *Idh-2*, *Aat-2*, *Ca-3* and of haplotype frequencies at the mitochondrial gene encoding for the cytochrome *b*



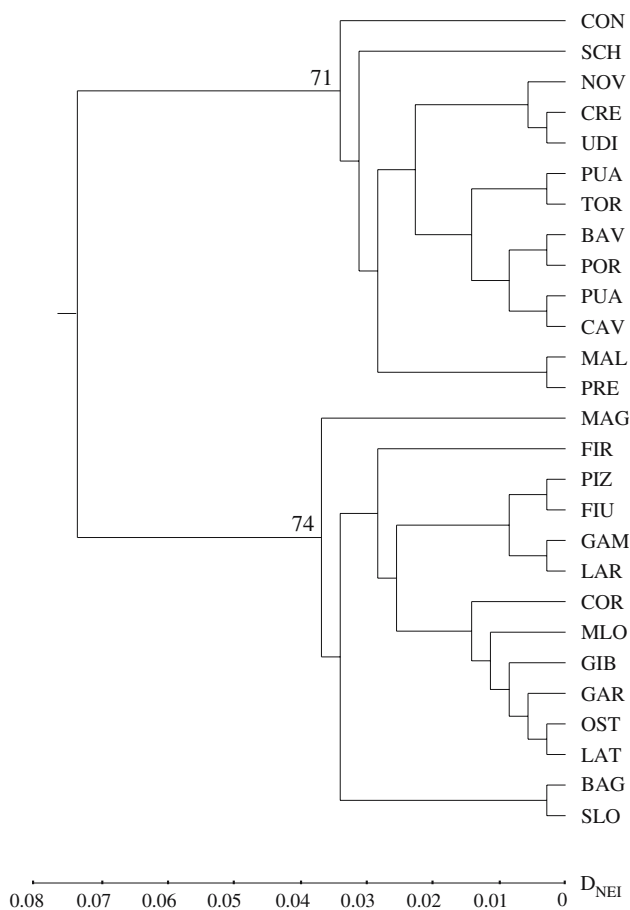
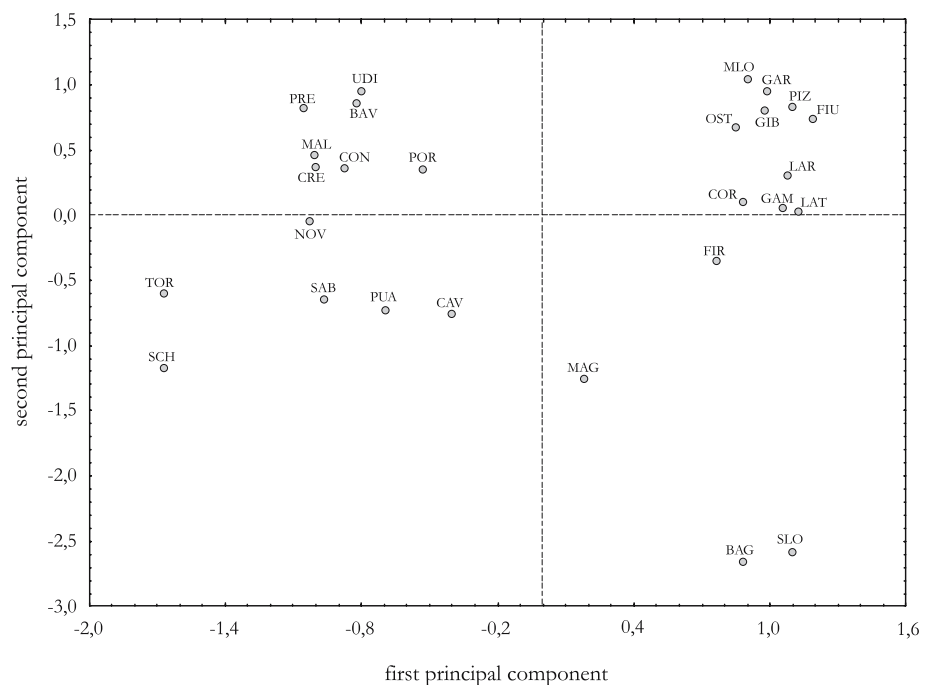


Fig. 3 UPGMA phenogram of the populations sampled of the Italian treefrog, based on Nei's (1972) standard genetic distance (D_{NEI}). Bootstrap values >70% after 1,000 pseudoreplicates are shown

Fig. 4 Principal component analysis of allele frequencies among the studied samples of the Italian treefrog. The horizontal axis is significant ($P < 0.01$), whereas the vertical is not. Samples are encoded as in Table 1



Values of pairwise θ among samples for which mitochondrial data were also collected are given in Table 3. Observed values of θ within each group suggested a higher level of population differentiation within group-S than within group-N (average θ was 0.126 [± 0.080] and 0.046 [± 0.038], respectively). The sample from MAG shows lower values of θ with respect to samples from group-S than when compared to samples from group-N. However, it is the “southern” sample that shows the lowest values of θ in comparisons with samples from group-N (never exceeding 0.295). The sample from BAG (group-S) shows the highest values of θ of the intra-group comparisons. Indeed, in three cases (when compared to GIB, GAR and OST) it shows values above 0.420, which are of the same degree as the values observed in the inter-group comparisons. When excluding these two populations, average θ between groups was 0.439 (± 0.087), a value that under an infinite islands model (Wright 1951) leads to an estimate of the average between groups gene flow of $Nm = 0.319$.

Estimates of population genetic variability are given in Table 4. The unbiased estimate of expected heterozygosity (H_E) varied from 0.016, observed at samples from GAR and LAT, to 0.140 observed at SCH. Mean values of H_E for group-N was 0.112 (± 0.020), whereas for group-S it was 0.049 (± 0.022), values which differ significantly (Mann–Whitney tests: $U_{(n=27; df=1)} = 3$; $P < 0.01$). The highest values for H_E were observed in a group of populations close to Eastern Prealps (populations MAL, SCH, CON and PRE) which, as

Table 3 Pairwise values of θ (Weir and Cockerham 1984) for the 22 samples of *Hyla intermedia* for which both mitochondrial and allozyme data were collected

	COR	GIB	GAM	PIZ	FIU	GAR	OST	SLO	FIR	BAG	MAG	PUA	CRE	NOV	TOR	CAV	BAV	POR	MAL	SCH	CON	PRE
COR	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	0.156**	0.230*	0.816**	1.000**	1.000**	1.000**	1.000**	0.827**	1.000**	0.884**	1.000**	1.000**	1.000**
GIB	0.056*	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	0.170**	0.239*	0.821**	1.000**	1.000**	1.000**	1.000**	0.834**	1.000**	0.891**	1.000**	1.000**	1.000**
GAM	0.010	0.181**	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	0.107	0.199*	0.795**	1.000**	1.000**	1.000**	1.000**	0.802**	1.000**	0.858**	1.000**	1.000**	1.000**
PIZ	0.054*	0.084*	0.074*	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	0.125**	0.211*	0.803**	1.000**	1.000**	1.000**	1.000**	0.811**	1.000**	0.868**	1.000**	1.000**	1.000**
FIU	0.092*	0.223*	0.120*	0.027	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	0.125	0.758**	1.000**	1.000**	1.000**	1.000**	0.750**	1.000**	0.797**	1.000**	1.000**	1.000**
GAR	0.077*	≤0.000	0.209**	0.108*	0.279**	x	≤0.000	≤0.000	≤0.000	≤0.000	0.125	0.758**	1.000**	1.000**	1.000**	1.000**	0.750**	1.000**	0.797**	1.000**	1.000**	1.000**
OST	0.124*	0.037	0.208**	0.092*	0.232**	0.051	x	≤0.000	≤0.000	0.087	0.186*	0.787**	1.000**	1.000**	1.000**	1.000**	0.791**	1.000**	0.847**	1.000**	1.000**	1.000**
SLO	0.146**	0.263**	0.117*	0.165**	0.137*	0.137*	0.262**	x	≤0.000	0.249**	0.289**	0.853**	1.000**	1.000**	1.000**	1.000**	0.871**	1.000**	0.922**	1.000**	1.000**	1.000**
FIR	0.045*	0.155**	0.085**	0.088*	0.068*	0.175**	0.153*	0.030	x	0.063	0.171*	0.779**	1.000**	1.000**	1.000**	1.000**	0.779**	1.000**	0.833**	1.000**	1.000**	1.000**
BAG	0.251*	0.420**	0.112	0.245*	0.185*	0.486**	0.442**	0.033	0.098*	x	0.016	0.584**	0.807**	0.860**	0.827**	0.662**	0.553*	0.750**	0.552*	0.722*	0.685**	0.737**
MAG	0.143**	0.191	0.159**	0.136**	0.111*	0.188**	0.187**	0.071**	0.027	0.107*	x	0.347**	0.486**	0.552**	0.508**	0.358*	0.302**	0.432**	0.297*	0.409*	0.379**	0.421**
PUA	0.424**	0.438**	0.428**	0.347*	0.320**	0.433**	0.353**	0.351**	0.311**	0.380**	0.177**	x	0.112	0.169**	0.131**	≤0.000	≤0.000	0.062*	≤0.000	0.036	≤0.000	0.050
CRE	0.588**	0.615**	0.566**	0.537**	0.514**	0.619**	0.529**	0.493**	0.454**	0.523**	0.295**	0.087*	x	≤0.000	≤0.000	≤0.000	0.085**	≤0.000	0.167**	≤0.000	≤0.000	≤0.000
NOV	0.494**	0.573**	0.521**	0.492**	0.453**	0.566**	0.481**	0.425**	0.392**	0.398**	0.232**	0.063*	0.007	x	≤0.000	≤0.000	0.1152**	≤0.000	0.275**	≤0.000	≤0.000	≤0.000
TOR	0.595**	0.624**	0.506**	0.530**	0.462**	0.640**	0.484**	0.479**	0.425**	0.443**	0.277**	0.026	0.001	≤0.000	x	≤0.000	0.1108**	≤0.000	0.204**	≤0.000	≤0.000	≤0.000
CAV	0.524**	0.590**	0.431**	0.455**	0.396**	0.626**	0.480**	0.376**	0.333**	0.347**	0.168*	0.018	0.091*	0.017	0.013	x	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000	≤0.000
BAV	0.449**	0.487**	0.427**	0.410**	0.363**	0.486**	0.381**	0.358**	0.315**	0.364**	0.188**	0.024	0.044	0.023	0.048*	≤0.000	x	0.027	≤0.000	≤0.000	≤0.000	0.014
POR	0.419**	0.449**	0.357**	0.350**	0.374*	0.478**	0.309**	0.368**	0.320**	0.387**	0.181**	0.026	0.065*	0.030	0.036	0.018	≤0.000	x	0.072**	≤0.000	≤0.000	≤0.000
MAL	0.474**	0.398**	0.322**	0.350*	0.331**	0.491**	0.302*	0.377**	0.320**	0.302**	0.142*	0.024	0.004	≤0.000	0.085*	0.060	0.056*	0.027	x	0.028**	≤0.000	0.051**
SCH	0.489**	0.518**	0.434**	0.454**	0.385**	0.518**	0.417**	0.415**	0.357**	0.379**	0.264**	0.148**	0.150**	0.040	0.090*	0.101*	0.126*	0.107*	0.056*	x	≤0.000	≤0.000
CON	0.531**	0.528**	0.432**	0.447**	0.388**	0.570**	0.424**	0.421**	0.378**	0.376**	0.211**	0.049	0.042	0.006	0.068*	0.040	0.069*	0.080*	0.044	0.083*	x	≤0.000
PRE	0.417**	0.407**	0.382**	0.342**	0.317**	0.427**	0.311**	0.342**	0.293**	0.333**	0.148**	≤0.000	0.073*	0.015	0.067*	0.017	0.012	0.012	0.008	0.092**	0.060*	x

Allozyme data are below the diagonal; mitochondrial data are above the diagonal

* $P < 0.05$ after 1,000 permutations

**Significant after Bonferroni correction

Table 4 Estimates of genetic variability for sampled populations of the Italian treefrog (SD in parentheses). Samples are encoded as in Table 1

Sample	A	P_{95}	H_O	H_E
COR	1.2 (0.1)	8.7	0.026 (0.023)	0.034 (0.023)
GIB	1.3 (0.1)	8.7	0.026 (0.013)	0.027 (0.013)
GAM	1.2 (0.1)	13	0.056 (0.036)	0.048 (0.032)
PIZ	1.3 (0.1)	13	0.045 (0.023)	0.056 (0.030)
FIU	1.2 (0.1)	13	0.071 (0.042)	0.059 (0.036)
MLO	1.3 (0.1)	21.7	0.074 (0.036)	0.065 (0.030)
LAR	1.3 (0.2)	13	0.050 (0.030)	0.045 (0.028)
GAR	1.1 (0.1)	8.7	0.011 (0.009)	0.016 (0.010)
OST	1.2 (0.1)	13	0.027 (0.014)	0.037 (0.017)
LAT	1.1 (0.1)	8.7	0.017 (0.014)	0.016 (0.012)
SLO	1.3 (0.1)	13	0.067 (0.032)	0.061 (0.029)
FIR	1.3 (0.1)	26.1	0.070 (0.028)	0.067 (0.027)
BAG	1.2 (0.1)	17.4	0.048 (0.023)	0.052 (0.025)
MAG	1.3 (0.1)	26.1	0.089 (0.038)	0.096 (0.036)
PUA	1.4 (0.1)	30.4	0.113 (0.039)	0.108 (0.036)
CRE	1.2 (0.1)	17.4	0.049 (0.025)	0.077 (0.035)
NOV	1.2 (0.1)	17.4	0.091 (0.041)	0.090 (0.040)
TOR	1.4 (0.1)	39.1	0.121 (0.039)	0.129 (0.039)
CAV	1.3 (0.1)	30.4	0.119 (0.042)	0.110 (0.038)
BAV	1.5 (0.1)	34.8	0.113 (0.041)	0.106 (0.034)
POR	1.3 (0.1)	26.1	0.088 (0.034)	0.092 (0.035)
SAB	1.2 (0.1)	21.7	0.087 (0.044)	0.101 (0.041)
UDI	1.4 (0.1)	39.1	0.072 (0.026)	0.099 (0.033)
MAL	1.6 (0.1)	34.8	0.134 (0.041)	0.138 (0.038)
SCH	1.4 (0.2)	30.4	0.132 (0.049)	0.140 (0.049)
CON	1.4 (0.1)	34.8	0.125 (0.046)	0.134 (0.043)
PRE	1.6 (0.1)	47.8	0.129 (0.031)	0.131 (0.035)

A mean number of alleles per locus, P_{95} percentage of polymorphic loci (95% criterion), H_O observed heterozygosity, H_E expected heterozygosity (Nei's 1978 unbiased estimate)

stated above, also presented introgressed alleles of the sister species *H. arborea*. Removing these samples (plus UDI, also introgressed) from the comparisons of the level of genetic variability between the two groups of populations did not significantly affect our results (average H_E for group-N was 0.102 [± 0.016]; Mann–Whitney tests: $U_{(n=22; df=1)} = 3$; $P < 0.01$). Other measures of genetic variability show the same geographical pattern as H_E .

mtDNA

Five composite RFLP-haplotypes were found over all samples. One of these (a1) was observed only in one individual from MAL, a population close to the hybrid zone between *H. intermedia* and *H. arborea*. It was also observed in all samples studied of *H. arborea* from Germany (Verardi, unpublished data). In all samples located south of the northern Apennines the only haplotype observed was s1 (see Fig. 2). It was also the only haplotype found at VER and SLO, while in samples from BAG and MAG it was observed at frequencies of 0.8 and 0.5, respectively. In all the other northern populations n1 was the most common haplotype, with frequencies varying from 0.73 (at BAV) to 1.0 (the majority of samples). Two other haplotypes were found among samples of the group-N, never

exceeding 0.22: n2, which was observed at BAV and POR, and n3 at MAG, PUA and BAV.

Sequences of 332 bp of the mitochondrial cytochrome *b* gene, corresponding to the positions 16,347 to 16,678 of the *Xenopus laevis* mitochondrial genome (Roe et al. 1985), were obtained from two individuals for each RFLP-haplotype (GenBank accession numbers: AY093690, AY093692–AY093695).

The NJ tree illustrating relationships among the haplotypes found is given in Fig. 5 (the MP and ML trees have an identical topology, hence are not shown). Average sequence divergence (P -distance) among *H. arborea* haplotype (a1) and *H. intermedia* haplotypes (n1, n2, n3 and s1) was 14.23%. Among *H. intermedia* haplotypes 32 variable sites were observed: 31 in third position and one in second position. No amino acid replacements were found. Sequence divergences between haplotypes n1, n2 and n3 ranges from one to three base pairs, all transitions in the third position. The most differentiated haplotype was s1, with an average 9.04% of sequence divergence with respect to the other *H. intermedia* haplotypes.

To assess the linearity of accumulation of substitutions over time we carried out a total of ten Tajima (1993) tests among all possible pairs of haplotypes, using a previously published sequence of *P. regilla* as an outgroup. The null hypothesis of constant

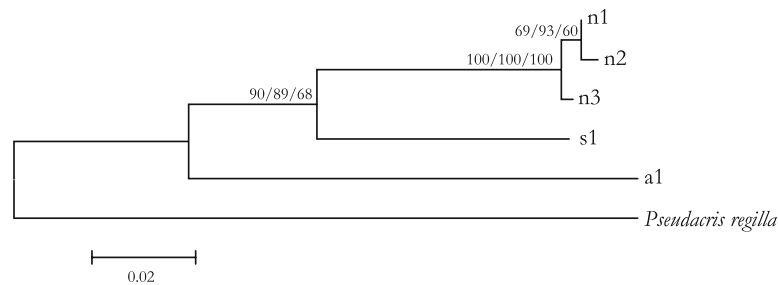


Fig. 5 Neighbour-Joining tree based on K-2-P genetic distances among the cytochrome *b* haplotypes found within the studied samples of *Hyla intermedia* (a1 is a typical *Hyla arborea* haplotype, found introgressed into *H. intermedia*). Maximum Parsimony and Maximum Likelihood trees presented identical

topologies. Bootstrap supports over 1,000 replicates are shown at the nodes (Maximum Likelihood/Neighbour-Joining/Maximum Parsimony). *Pseudacris regilla* (GenBank accession number: AY363197; Ripplinger and Wagner 2004) was used as outgroup

substitution rates could not be rejected based on those tests (all $P > 0.05$).

The pattern of population differentiation was very strong and significant, with a value of θ overall samples being 0.777 ($P < 0.01$). Pairwise θ estimates between population pairs (Table 3) showed the existence of two groups of populations identical to those evidenced with allozymes. When excluding the samples from BAG and MAG (see below), very weak population differentiation was found within each group, with average θ as low as 0.000 (± 0.000) and 0.032 (± 0.061) for group-S and group-N, respectively. On the other hand, average θ between the two groups was as high as 0.950 (± 0.084), which under the infinite islands model (Wright 1951) gives an estimate of the between groups gene flow of $Nm = 0.013$. Populations from BAG and particularly MAG (i.e. the only two where both southern and northern haplotypes were found to be co-present) showed intermediate values of θ when compared either to populations from group-S or to those from group-N.

Discussion

The analysis of genetic variation at 23 nuclear loci and a fragment of the mitochondrial cytochrome *b* gene has clearly shown the existence of a remarkable genetic structuring within the Italian treefrog. This pattern appeared mainly attributable to differences between two geographically coherent groups of populations, one comprising those located north of the northern Apennines, and the other those located south. Populations closest to the northern side of this mountain chain were genetically intermediate between the two groups.

Although substantially concordant in depicting spatial pattern of genetic variation, allozymes and mtDNA led to markedly discrepant estimates of the

pattern of genetic divergence between the two groups, as well as of the graduality of the intergradation among them. At the mitochondrial level, a sharp phylogeographic break was found, with only two close samples (MAG and BAG) showing the two haploforms co-present. Furthermore, the divergence between the two haploforms was particularly high ($P = 9.04\%$), resembling that found between several congeneric amphibian species. For instance, Lee et al. (1999) observed a sequence divergence of 10.74–10.96% at cytochrome *b* between the Korean *Hyla japonica* and *Hyla suweonensis*, whereas values of 6.30–10.03% were found by Martinez-Solano et al. (2004) among midwife toads of the species *Alytes dickhilleni*, *Alytes maurus*, *Alytes muletensis* and *Alytes obstetricans*. By contrast, genetic divergence at allozymes was weaker ($D_{NEI} = 0.07$), and well below the levels usually observed among congeneric amphibian species (e.g. Avise and Aquadro 1982; see also Weigt et al. 2005). Also the intergradation between the two groups of populations appeared less sharp at allozyme than at mitochondrial level. Samples from SLO and FIR, both clustered within the southern group and sharing the single “southern” haplotype s1, showed “northern” nuclear alleles, with low frequencies, at all but one of the differentiated loci. These are also the samples from the southern group that show the lowest genetic differentiation compared to the northern Apennine samples MAG and BAG. Finally, the discrepancy between the two kinds of markers is also evident from pairwise estimates of population subdivision, as measured by conventional F -statistics and, consequently, by the F_{ST} -based gene flow estimates. Given that the mitochondrial genome is haploid and maternally inherited, estimates of gene flow are expected to be fourfold lower at mtDNA than at nuclear markers, whereas our results indicated a Nm estimate 25 times lower at the mitochondrial than at the nuclear level.

At least three distinct hypotheses can be made to explain such discrepancies: (1) deviations from expectations under neutral evolution because of forces acting on individual markers, such as balancing selection on allozymes and/or selective sweeps or purifying selection against deleterious mutations on mtDNA; (2) past fragmentation followed by secondary contact through sex-biased gene flow; (3) origin of the southern group through colonization from northern areas, associated with a founder effect, and retention of ancient polymorphisms at allozymes within the northern group.

Balancing selection favours the maintenance of similar allele frequencies, leading to patterns of population subdivision, as measured by conventional F -statistics, which should be much less apparent (Schierup et al. 2000). Nevertheless the footprint of natural selection on multiple loci depends on the intensity of selection and the recombination rate among loci (Broughton and Harrison 2003). Therefore, to play a role in our case study, either this phenomenon would have to be applied simultaneously to several protein loci, or the recombination rate among them would have to be very low, two highly unlikely occurrences (but see Hare and Avise 1998). On the other hand, a selective sweep resulting from directional selection on mtDNA could have led to fixation of distinct new haplotypes within one or both of the two groups of populations, leading to higher θ -values at the mtDNA level (e.g. Slatkin and Wiehe 1998; Andolfatto 2001). Inflated estimates of population differentiation at the mtDNA could also be the result of background selection (Charlesworth et al. 1993; Charlesworth 1998). However, both these kinds of selection would not alter the substitution rate at neutral nucleotide sites (Birky and Walsh 1988; Charlesworth 1998). So, while they could account for the large difference in N_m estimates, they appear not suitable to account for the large discrepancy in the estimates of divergence between the two groups. Therefore, scenario (1) of differential constraints on marker evolution, leading to deviations from expectations under neutral evolution, appears on the whole unlikely.

The influence of the northern Apennines in shaping patterns of distribution and differentiation among related species or groups of population within them, has been pointed out for several taxa (e.g. Di Giovanni et al. 1998; Stefani et al. 2004 and references therein). Since the Italian treefrog is a relatively thermophilic species, with more than 90% of its populations located under 500 m a.s.l. (e.g. Mazzotti et al. 1999), it appears very likely that this mountain chain has played the role of a barrier to dispersal in this species along the north–

south axis. Following scenario (2), this mountain chain could have acted as an extrinsic barrier to gene flow, by separating the two population groups, most probably during glacial maxima, when scattered glaciers were present at high altitude (Cremaschi 2003a, b; Giraudi 2004). This phase would have been followed by secondary contact, during subsequent interglacials, that re-established gene flow. However, to generate the observed patterns and discrepancies, this gene flow would need to be strongly male-biased and directional (mainly from south to north, see Fig. 2). Unlike mammals and birds, for which sex-biased gene flow has been largely documented (e.g. Greenwood 1980; Baker et al. 1998; Helbig et al. 2001; Crochet et al. 2003; Johnson et al. 2003), much less is known about amphibians (Goudet et al. 2002). Because it is polygynous, the male is expected to be the dispersing sex in frogs (Lampert et al. 2003). To our knowledge, case studies concerning anuran amphibians in which male-biased dispersal was invoked to account for genetic data have been reported for only three species: *Rana cascade* (Monsen and Blouin 2003), *Physalaemus pustulosus* (Lampert et al. 2003) and *Bombina orientalis* (Szymura et al. 1985). In the case of the Italian treefrog, a strong male-biased gene flow from southern to northern areas could have led to extensive introgression at differentiated nuclear loci, giving rise to the general geographic pattern of variation at allele frequencies (see Fig. 2), whereas inter-locus variance (Lewontin and Krakauer 1973; Baer 1999 and reference therein) could easily account for differences among them. However, although not completely implausible, this hypothesis alone appears not appropriate to explain the observed geographic pattern of distribution of the genetic variability. In fact, it would imply such a geographical asymmetry in gene flow that it appears unlikely, also considering the lack of ecological or biogeographic features that could underlie this pattern.

The alternative hypothesis (3), of persistent ancient polymorphisms at allozymes within the northern group and the origin of the southern group through colonization from northern areas, could also account for the observed pattern of geographical distribution of genetic variation. Because ancestral polymorphisms can be retained, a large historical population size should be assumed (e.g. Ting et al. 2000). Present knowledge of palaeoenvironments in northern Italy allows this assumption (e.g. Montuire and Marcolini 2002; Cattani 2003). In fact, during glacial periods, marine regressions led to a southward migration of the coastal line, till 300 km south-east of its present location (Cremaschi 2003b), allowing the formation of freshwater

environments where large populations could have persisted (e.g. Miola et al. 2003). By contrast, during interglacials, when the coastal line migrated northward following marine transgression, large populations could have persisted in the entire Pò river plain (e.g. Ravazzi and Strick 1995; Amorosi et al. 1999; Cattani 2003). The colonization of southern habitats may have occurred during these latter periods, with the establishment of more favourable climatic conditions that allowed treefrogs to cross the northern Apennines. Moreover, the discovery of treefrog fossils from the Early Pleistocene (late Villafranchian) of Apricena (Apulia, southern Italy; Delfino and Bailon 2000) suggests that such colonization would have pre-dated this period, an occurrence also compatible with the high degree of divergence observed at the mtDNA. A founder effect accompanying this event could explain the observed reduced genetic variability of southern populations with respect to populations of the northern group (on average less than half). A reduced historical population size in peninsular Italy may also have been favoured by the more fragmented distribution of lowland habitats in this area. This scenario could also easily account for the observed discrepancy in the pattern of differentiation as depicted by mitochondrial and nuclear markers (and thus in the F_{ST} -based N_m estimates), whereby shared alleles between the two groups are only observed at nuclear loci whereas at mtDNA reciprocal monophyly has been achieved. As mentioned above, varying effective population size can have deep and diversified effects on population genetic patterns at nuclear and mitochondrial genomes (e.g. Birky et al. 1983; Avise et al. 1984; Hoelzer 1997 and references therein). In particular, the achievement of the reciprocal monophyly is a process primarily influenced by the effective population size (e.g. Neigel and Avise 1986) and it has been shown to be much faster in mtDNA than even in a single nuclear locus (and far much faster than in a sample of several nuclear loci; Hudson and Coyne 2002). Thus it can have major effects on frequency-based estimates of population differentiation, as are those considered here (see also Canestrelli et al. 2006). However, it is worth noting that other forces could also have favoured the achievement of the reciprocal monophyly at mitochondrial level. These might include the above-mentioned selective forces, or the fixation of new mutations arisen in the wave front of the expanding population (during the colonization of southern habitats), which is also a process whose probability has been shown to be largely influenced by the effective population size (Edmonds et al. 2004). More data about both species' ecology and genetic variation

(including extensive mitochondrial and nuclear DNA sequencing) will help to clarify this point.

The two hypotheses (2) and (3) are not completely mutually exclusive. Furthermore, each one gives a more plausible explanation for particular aspects of the dataset: the geographical distribution of genetic variability is best explained by hypothesis (3), well supported by paleoecological and biogeographic information, whereas the patterns of variation among populations closest to the northern Apennines may well have been generated by secondary contact between previously isolated lineages.

Therefore, the most likely scenario appear the following: within the areas located north of the northern Apennines, the maintenance of a large historical population size would have allowed for the retention of ancestral polymorphisms, which may be responsible for much of the genetic variability found in this area. The areas south of the northern Apennines would have been colonized by the northern populations before the Early Pleistocene, an event accompanied by a founder effect leading to the observed reduced genetic variability in populations from peninsular Italy. The intervening of unsuitable conditions at medium to high altitude in the northern Apennines during subsequent pleniglacials would have made this mountain chain an efficient barrier to dispersal, leading to prolonged isolation between the two population groups. More recently, the intervening of climatic conditions favourable to crossing this geographic barrier would have led to a secondary contact allowing the re-establishment of some gene flow between them.

Conclusions

In this paper, we have presented a case of remarkable discrepancy between nuclear and mitochondrial markers in depicting the pattern of population differentiation between two groups of populations within the Italian treefrog. The most plausible scenario accounting for the observed pattern of differentiation and the discrepancy among markers appears to imply several historical events: (a) the retention of ancestral polymorphisms within the northern group, favoured by a large historical population size; (b) the origin of the southern group through colonization from northern areas before the late Villafranchian; (c) the isolation between the two population groups during subsequent pleniglacials, with the northern Apennine acting as a barrier to dispersal; (d) a subsequent secondary contact between them.

Based on the results of the mtDNA analysis alone, and with a sparser sampling scheme, one could have

supposed the existence of two distinct species within the Italian treefrog, with the northern Apennine acting as an extrinsic barrier to gene flow. Nevertheless, allozyme data and the study of contact populations do not support this hypothesis. On the other hand, allozyme data alone would have suggested the existence of two moderately differentiated groups of populations, whose divergence could be viewed as of recent origin. Only the joint study of both nuclear and mitochondrial markers, has made it possible to put in evidence the existence of a more complex evolutionary history. In the face of the extensive use of a single kind of marker in studies of molecular taxonomy and systematics, the case study we have presented here constitutes a serious cautionary note, and a strong argument against the use of single markers and/or genetic distances for identifying species or investigating evolutionary processes at levels other than the single marker studied (see also Ferguson 2002; Hudson and Coyne 2002; Lee 2003). This latter point appears of particular relevance for taxa such as amphibians, whose taxonomy and systematics have been so deeply revised in the light of genetic studies (Veith 1996; Borkin 1999).

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