Ann. Zool. Fennici 46: 247–258 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 31 August 2009

Nuclear and mitochondrial patterns of introgression between the parapatric European treefrogs *Hyla arborea* and *H. intermedia*

Andrea Verardi[1], Daniele Canestrelli[2,*] & Giuseppe Nascetti[2]

[1)] *Dipartimento di Genetica e Biologia Molecolare "Charles Darwin", Università degli Studi di Roma "La Sapienza", Via dei Sardi 70, I-00185 Roma, Italy*
[2)] *Dipartimento di Ecologia e Sviluppo Economico Sostenibile, Università della Tuscia, Largo dell'Università s.n.c., I-01100 Viterbo, Italy (*corresponding author's e-mail: canestrelli@unitus.it)*

Received 12 July 2008, revised version received 11 Nov. 2008, accepted 11 Dec. 2008

Verardi, A., Canestrelli, D. & Nascetti, G. 2009: Nuclear and mitochondrial patterns of introgression between the parapatric European treefrogs *Hyla arborea* and *H. intermedia*. — *Ann. Zool. Fennici* 46: 247–258.

Postglacial northward expansions from distinct southern European refugia have lead to the formation of several secondary contact zones between species. These zones are providing growing insights into the relevance of introgressive hybridization in shaping patterns of genetic diversity, and into the evolutionary processes involved in the completion of reproductive isolation barriers. Using both nuclear (9 allozyme loci) and mitochondrial (PCR-RFLP of a cytochrome *b* gene fragment) diagnostic markers we investigated the genetic patterns of variation across a zone of parapatry between the two European treefrogs *Hyla arborea* and *H. intermedia*. Neither F_1 and F_2 hybrids nor backcrosses were identified, indicating the lack of current gene exchange between the two species. However, introgressed alleles were observed in both species and in all markers analysed, which testifies to the occurrence of past events of introgressive hybridization. A wide variation in the frequencies of introgressed alleles was observed between loci and between the two species, suggesting the action of differential selective filtering. The historical context for the occurrence of secondary contact between the two species and its evolutionary implications are discussed, together with the possible role of human disturbance and/or reinforcing selection in leading to the observed absence of ongoing gene flow between them.

Introduction

Secondary contact zones have usually been found in areas where populations that diverged allopatrically during Pleistocenic glaciations met after the glaciers' retreat, often forming hybrid zones. As a consequence of the climatic oscillations of the Quaternary and of the presence of major geographical barriers to dispersal, hybrid zones often show a clustered geographic distribution (Hewitt 1996, 2000). In Europe these areas of hybridisation or "suture zones" (Remington 1968) have been observed in the Pyrenees, the Alps, around the border between France and Germany, and in Scandinavia (e.g. Taberlet *et al*. 1998). In many of these contact

zones hybridisation has led to introgressive phenomena, i.e. the spread of genes from one species into the other species' genome through successive backcrossing of hybrids to one or both of the parental species. A most debated topic is the impact of introgressive hybridisation on the species genome. Introgression has been considered an important and pervasive phenomenon; it can be a source of genetic novelties, which can sometimes lead to novel adaptations (Grant 1963, Arnold 1992, Mallet 2005), and may have played a major role in rapid adaptive radiations (*see* Seehausen 2004). If introgressing genes are selectively advantageous they can spread rapidly, whereas when they are disadvantageous or maladapted to the new genetic environment, they can be eliminated by selection (e.g. Harrison 1993, Martinsen *et al*. 2001). Foreign alleles can also be selectively neutral, in which case they are subjected to the laws of random evolution (Barton 2001). On the other hand, some authors argue that, as a consequence of endogenous selective pressures acting against hybrids, parental species are able to keep their genetic integrity, despite forming stable hybrid zones for thousands of generations (Barton 1979, Barton & Hewitt 1985, Jiggins *et al*. 1997). Dobzhansky (1940) suggested that when selection against hybrids is strong enough, it may promote the formation of prezygotic barriers to gene exchange in hybridising taxa. This mechanism, known as reinforcement, would prevent a disadvantageous waste of reproductive energy on hybrid offspring that are unfit or unviable. Since its conception, this theory has been heavily criticised on theoretical bases (e.g. Moore 1957, Bigelow 1965, Paterson 1978, Barton & Hewitt 1981) as well as for lack of experimental evidence (Howard 1993, Littlejohn 1981, Phelan & Baker 1987). However, in recent decades both theoretical and empirical studies have provided strong evidence supporting the reinforcement hypothesis (*see* Servedio 2004 and references therein).

The *Hyla arborea* species group comprises about 15 species distributed across the Palearctic region (*see* Faivovich *et al*. 2005). Most of these were once attributed to the single species *H. arborea*, and have been described as separate species only recently, mainly on the basis of bioacoustic and genetic studies (e.g. Paillette 1967, Schneider 1974, Kawamura *et al*. 1977, Nascetti *et al*. 1985, 1995). Among these, the Italian treefrog *H. intermedia* has been recognized as a separate taxon, endemic to the Italian peninsula and Sicily, on the basis of a survey of variation at 28 enzymatic loci and morphometric analysis (Nascetti *et al*. 1995). A putative zone of secondary contact between this species and its sister taxon *H. arborea* was identified in northeastern Italy, where evidence for introgressive hybridization between the two species were also observed. In this study, we investigate further the pattern and extent of hybridization and introgression between *H. intermedia* and *H. arborea* by means of both nuclear (allozyme electrophoresis) and mitochondrial (PCR-RFLP) markers, in order to gain insight into the historical processes most likely to have been involved in shaping the geographical patterns of genetic variation across the zone of parapatry between these species, and to evaluate the evolutionary implications.

Material and methods

Sampling and laboratory procedures

We analysed genetic variation in a total of 282 individuals belonging to the two treefrog species *H. arborea* and *H. intermedia*, from 16 sampling sites located east and west of the putative zone of secondary contact. This geographic area ranges from northeastern Italy to western Slovenia and Istria, and is bounded by the Adriatic sea and the eastern Prealps at its southern and northern portions, respectively. Sampling sites were initially identified by following males' mating calls. Therefore, all individuals were sampled from breeding ponds during the breeding seasons, which in the study area are concurrent for both species (from March to May). With the exception of those from Noghere and Cepic, all other samples were also used in previous studies (Nascetti *et al*. 1995, Canestrelli *et al*. 2007a). Geographic location of samples studied and their sizes are presented in Fig. 1 and Table 1.

Horizontal starch gel electrophoresis was carried out in order to analyze the pattern of variation at the nine protein loci previously identified as being diagnostic between the two

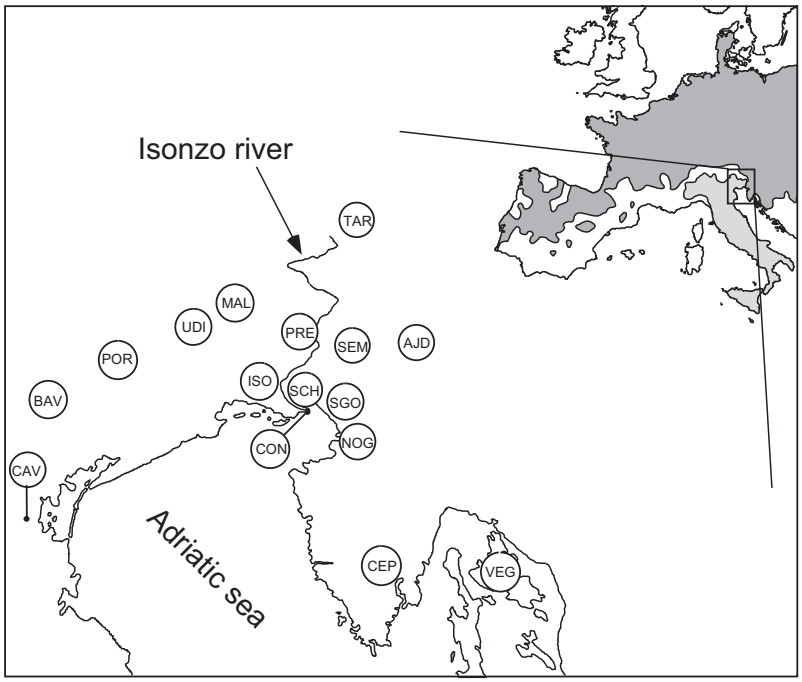

Fig. 1. Geographic distribution of the two treefrog species *Hyla arborea* (dark grey) and *H. intermedia* (light grey) in western and central Europe, and geographic location of the 16 sampled populations. Sample codes are as in Table 1.

treefrog species *H. arborea* and *H. intermedia* (Nascetti *et al*. 1995): *Icdh-1* (EC 1.1.1.42); *Xdh* (EC 1.2.1.37.); *Sod-1* (EC 1.15.1.1); *Aat-2* (EC 2.6.1.1); *Est-2* (EC 3.1.1.1); *Pep-1*, *Pep-2* and *Pep-4* (EC 3.4.13); *Ada* (EC 3.5.4.4). Running buffers, staining systems and technical details followed Nascetti *et al*. (1995).

Total genomic DNA was extracted following conventional phenol-chloroform methodology (Sambrook *et al*. 1989). A fragment of 330 bp of the mitochondrial cytochrome *b* gene was amplified in polymerase chain reaction (PCR) using the primers L14841 and H15149 described in Kocher *et al*. (1989). Reactions were carried

Table 1. Geographic locations, sample codes, sample sizes and estimates of populations' genetic variabilities for the 16 populations sampled of the European treefrog species *Hyla arborea* and *H. intermedia*. *A* = average allelic richness (El Mousadik & Petit 1996), *H* = average gene diversity (Nei 1987).

Species	Locality	Code	Sample size	Lat. N	Long. E	*A*	*H*
H. intermedia	Cavarzere	CAV	7	45°08′	12°04′	1.2	0.08
	Bavaria	BAV	17	45°34′	12°05′	1.2	0.05
	Cordenons	POR	16	45°57′	12°38′	1.1	0.07
	Brazzacco	UDI	10	46°05	13°10′	1.3	0.07
	Malina	MAL	11	46°05′	13°19′	1.6	0.21
	Isonzo	ISO	41	45°48′	13°23′	1.7	0.14
	Preval	PRE	15	45°56′	13°33′	1.7	0.21
	Cona	CON	12	45°54′	13°32′	1.6	0.21
	Schiavetti	SCH	21	45°49′	13°33′	1.6	0.22
H. arborea	Tarvisio	TAR	19	46°30′	13°34′	1.0	0.01
	Sempas	SEM	34	45°55′	13°44′	1.1	0.02
	Sgonico	SGO	37	45°44′	13°45′	1.2	0.05
	Ajdovscina	AJD	12	45°53′	13°54′	1.1	0.03
	Noghere	NOG	8	45°34′	13°48′	1.1	0.03
	Cepic	CEP	10	45°05′	14°01′	1.1	0.02
	Veglia	VEG	12	45°08′	14°40′	1.0	0.00

out in a final volume of 100 μl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, 2.5 units of Taq Polymerase and 1 μl of genomic DNA. The cycling conditions were as follows: 5 min of denaturation step at 95 °C followed by 39 cycles of 1 min at 93 °C, 1 min at 50 °C and 1 min at 72 °C, with a final extension step of 10 min at 72 °C. Negative controls were used for all sets of reactions, and 5 μl of each PCR product were run in a 1.8% agarose gel and visualised under UV light with ethiudium bromide to check for proper amplification. PCR products were then digested with four restriction enzymes (*Rsa*I, *Alu*I, *Hae*III and *Taq*I) following the manufacturer's instructions and using 10 to 15 μl of the amplification product for each reaction. In order to assess the Restriction Fragment Length Polymorphisms (RFLP), restriction fragments were separated in 6% polyacrylamide gels and visualised with ethidium bromide under UV light. Each of the RFLP composite haplotypes found was sequenced using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems), following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. Both strands were sequenced for each of the individuals analysed.

Data analysis

Allozymes

Deviations from Hardy-Weinberg equilibrium (exact tests for each locus in each population) and genotypic linkage disequilibria (exact tests for each pair of loci in each population) were assessed with GENEPOP 3.3 (Raymond & Rousset 1995). Mean allelic richness (El Mousadik & Petit 1996), average gene diversity (Nei 1987) and allelic frequencies were estimated using FSTAT 2.9.3.2 (Goudet 2001).

The patterns of genetic differentiation between populations was investigated by means of a Factorial Correspondence Analysis (FCA) as implemented by the program GENETIX 4.05 (Belkhir *et al*. 1996–2004). The analysis uses individual multilocus genotypes to estimate the proportion of the overall inertia among populations. The 'centre of gravity' for each population was plotted in a 2D space of the first two factors, which together explain the majority of variation in the data.

The assignment of individuals to population/species and admixture analyses on the basis of multilocus genotype data were carried out using two different methods, both based on a Bayesian approach: the method proposed by Pritchard *et al*. (2000) and implemented in the program STRUCTURE 2.1, and that proposed by Anderson and Thompson (2002), implemented in the program NEWHYBRID 1.1b3.

The method of Pritchard *et al*. (2000) was used to infer the most likely number of populations (K) in our dataset. The model uses unlinked multilocus genotypes to define clusters of individuals in which Hardy-Weinberg and linkage disequilibria are minimized. We used the admixture model, which assumes gene flow between populations and assigns a proportion of each individual's genome (q_i) to each inferred population, thus providing estimates of individuals' admixture proportions. Five independent runs were carried out for each value of K (K from 1 to 16) to check for convergence. For each run, 300 000 iterations were carried out with a burn-in length of 30 000 iterations. The most likely value of K was determined using the absolute values of the second order rate of change of the likelihood distribution divided by the SD of the likelihoods (ΔK), following Evanno *et al*. (2005).

The clustering method of Anderson and Thompson (2002) was used to assign individuals to 'pure' parental populations and to check for the presence of different classes of hybrids (F_1, F_2 or backcrosses). Using Markov's Chain Monte Carlo procedure, the method estimates the Bayesian posterior probability that an individual in a sample belongs to each of the different hybrid classes, while simultaneously estimating allelic frequencies for parental species. The data set was analysed several times, with various overdispersed starting values, lengths of burn-in period, and numbers of sweeps, as recommended by the authors. The F_1, F_2 and backcross classes were specified. The different simulations were highly congruent for the estimation of posterior probabilities. Thus, results of a final simulation with 500 000 sweeps are shown.

Finally, to obtain an estimate of the time of divergence between the two species based on the protein clock calibrated by Beerli *et al*. (1996) for European water frogs, we re-analysed the data of Nascetti *et al*. (1995) concerning allopatric populations of *H. intermedia* and *H. arborea* to calculate Nei's (1978) genetic distance as modified by Hillis (1984) (D_{Nei}), using the software POPDIST 1.1.1 (Guldbrandtsen *et al*. 2000). According to the calibration of Beerli *et al*. (1996), the pace of allozymic divergence is $0.10 D_{Nei}$/Myr.

mtDNA

Sequences were aligned using CLUSTALX (1.81) (Thompson *et al*. 1997). Sequence polymorphism analysis and pairwise sequence divergence (uncorrected *p*-distance) were computed using the program MEGA 2.1 (Kumar *et al*. 2001). The same program was also used to test the null hypothesis of constant substitution rate, by means of Tajima's relative rate tests (1993). A rough estimate of the time of divergence between the two species was obtained using a cytochrome-*b*-specific substitution rate of 3.6% of sequence divergence per million years, as proposed by Babik *et al*. (2004) based on previous works of Veith *et al*. (2003) and Beerli *et al*. (1996).

Results

Allozymes

Neither significant deviations from the expected Hardy-Weinberg equilibrium nor linkage disequilibria were observed (all $p > 0.05$). Seven out of the nine studied loci showed bi-allelic (*Icdh-1*, *Xdh*, *Sod-1*, *Pep-1*, *Pep-4*) or mostly bi-allelic variation (*Est-2*, *Ada*), whereas three and four alleles were observed at loci *Pep-2* and *Aat-2*, respectively (table of allele frequencies available upon request). Estimates of population genetic variation based on the nine analysed loci are presented in Table 1. Mean allelic richness varied between 1.0 observed at Tarvisio and Veglia to 1.7 observed at Isonzo and Preval, while average gene diversity ranged from 0.00 to 0.22 at

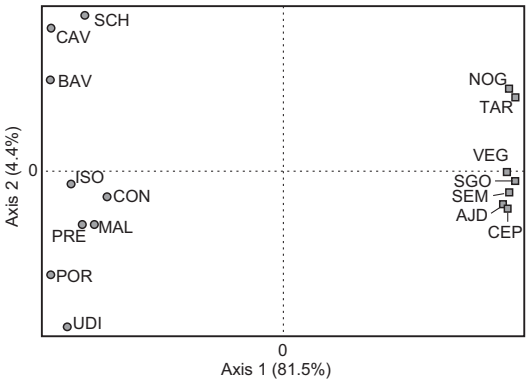

Fig. 2. Two-dimensional plot derived from a factorial correspondence analysis of the 16 sampled populations of the European treefrogs *Hyla arborea* (squares) and *H. intermedia* (circles), based on allozyme data. Samples are encoded as in Table 1.

Veglia and Schiavetti respectively. Particularly high estimates of population genetic variability were observed for a group of five samples of *H. intermedia* located at the easternmost portion of the species range (Malina, Isonzo, Preval, Cona and Schiavetti).

The first two factors obtained by the FCA explained 81% and 4% of the overall variation respectively. When these two factors were plotted against each other (Fig. 2), the populations clearly separated into the two species, with no overlap. The degree of scattering appeared wider among *H. intermedia* than among *H. arborea* populations, indicating the former as a more heterogeneous group of samples.

The geographic distribution of pooled frequencies of *H. arborea* and *H intermedia* alleles at the nine discriminant loci is illustrated in Fig. 3. Among the *H. intermedia* samples, alleles typical of *H. arborea* were observed at all loci, although with frequencies varying across samples and loci. By contrast, among the *H. arborea* samples, alleles typical of *H. intermedia* were observed at only five of the nine discriminant loci analysed (*Icdh-1*, *Xdh*, *Aat-2*, *Est-2*, *Pep-2*). Within *H. intermedia* the most introgressed locus was *Sod-1*, whose levels of introgression varied greatly among samples, ranging from 46% observed at Cona to 0% observed at Cavarzere, Bavaria and Cordenons. Similarly, frequencies of the *arborea* allele $Est\text{-}2^{105}$ in *H. intermedia* samples ranged from 22% at Cona

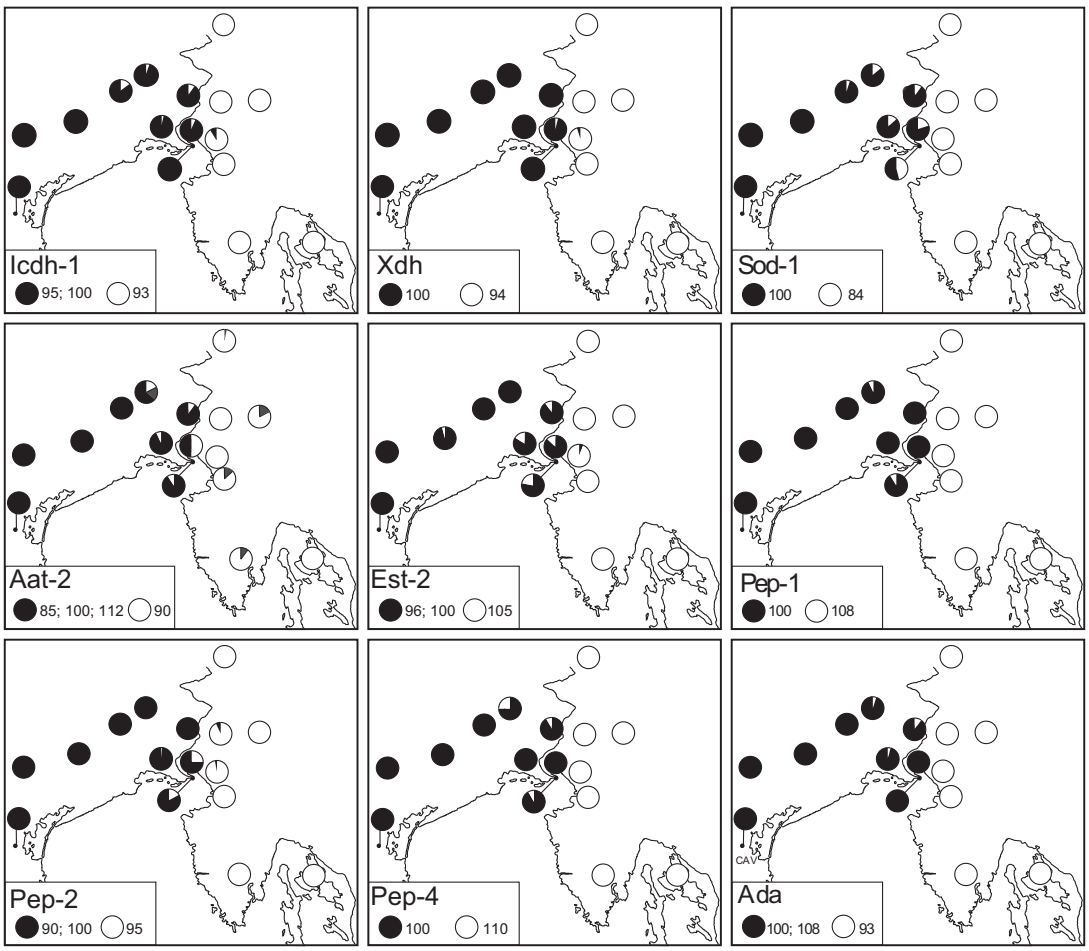

Fig. 3. Pie diagrams showing the geographic variation of allele frequencies at the nine diagnostic loci between *Hyla arborea* and *H. intermedia*. *Hyla arborea* alleles are in white, whereas *H. intermedia* alleles are in black. Samples are encoded as in Table 1.

to 0% at Cavarzere, Bavaria, Brazzacco and Malina. The loci *Xdh* and *Pep-1* were the least introgressed loci within the Italian taxon, *H. arborea* alleles having been observed in only one (Schiavetti) and two samples (Malina, Cona) respectively, with frequencies never above 9%. A wide variation in levels of introgression was also observed among loci within the same locality. For example the sample from Cona showed a 46% frequency of the *H. arborea* allele $Sod\text{-}1^{84}$ while showing no signs of introgression at loci *Icdh-1*, *Xdh* and *Ada*.

Levels of introgression of *H. intermedia* alleles into populations of *H. arborea* were notably lower. Indeed, at three out of the five loci showing traces of introgression (*Icdh-1*, *Xdh*, *Est-2*), *H. intermedia* alleles were observed only in the single sample of Sgonico, with a frequency not exceeding 9%, whereas at locus *Pep-2* the *H. intermedia* allele $Pep\text{-}2^{100}$ was also observed at Sempas with a frequency of 7%. Among the *H. arborea* samples, the only locus showing levels of introgression comparable to that observed among *H. intermedia* samples was *Aat-2*, where the foreign allele $Aat\text{-}2^{85}$ was observed in four *H. arborea* samples, with a frequency reaching 17% at Ajdovscina.

Cluster analysis carried out using the procedure implemented by the program STRUCTURE indicated that the genetic structure maximizing ΔK, and thus the most likely for the data, consisted of two clusters ($K = 2$; *see* Fig. 4), with

individuals of each species assigned to one cluster or the other (Fig. 5). Within *H. intermedia* the average score was 0.99 ± 0.02, with a minimum value of q = 0.87 assigned to one individual from the population of Preval and to one from Malina. Within *H. arborea* the mean value for q was 0.99 ± 0.00, the lowest score being q = 0.96 assigned to three individuals from the population of Sgonico.

When individuals' genotypic data were analysed with NEWHYBRID, the program failed to identify with confidence individuals belonging to F_1, F_2 or the backcrosses of various generations. Indeed, one individual from Malina and one from Preval (the ones that received q = 0.87 by STRUCTURE analysis) were assigned a probability of being third (or later) generation backcrosses of 2% and 5% respectively, whereas all other individuals were assigned their pure parental species with a probability above 99%.

Nei's mean genetic distance between allopatric samples of the two treefrog species was D_{Nei} = 0.55. Based on the molecular clock calibrated by Beerli *et al*. (1996), this value leads to an estimated time of divergence between the two species of 5.5 Myr.

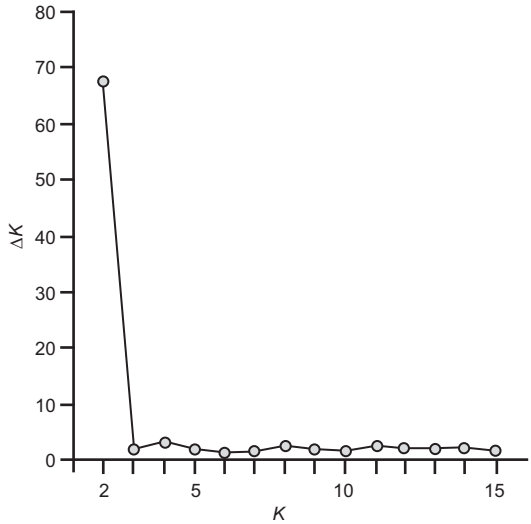

Fig. 4. Magnitude of Δ*K* (Evanno *et al.* 2005) as a function of *K*, estimated based on five replicates for each value of *K*.

mtDNA

Of the four endonucleases used, *Rsa*I produced three restriction profiles, while *Alu*I, *Hae*III and *Taq*I produced two each. Three different composite RFLP-haplotypes were found overall. Within *H. intermedia* two haplotypes were found: the most common was n1 observed at high frequencies in all sampled populations of this species, whereas haplotype n2 was observed only at Bavaria, Cordenons and Isonzo, at frequencies of 6%, 13% and 2%, respectively. The haplotype a1 was the only one observed in samples of *H. arborea*, besides having been found in three individuals of *H. intermedia*, two at Malina and one at Isonzo. The allozyme genotypes of these three individuals were assigned a probability of $q > 0.95$ of belonging to their group by the programme STRUCTURE.

Two samples for each restriction haplotype were sequenced (Genebank Accession Numbers:

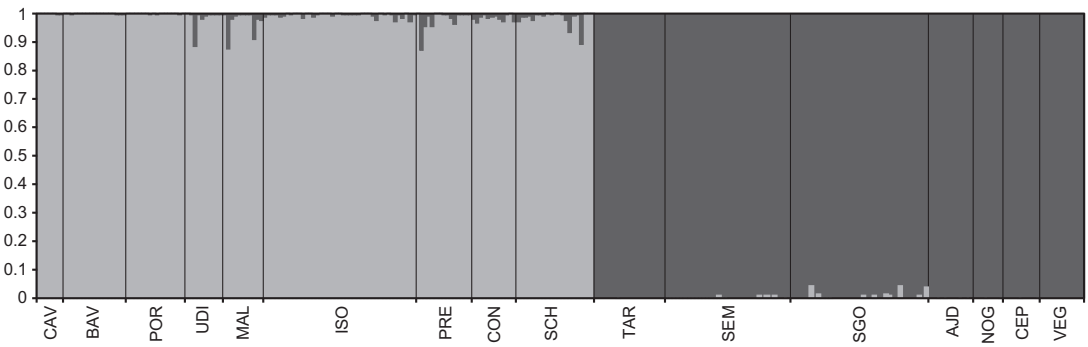

Fig. 5. Best clustering option (K = 2) for the 282 individual treefrogs sampled, according to the Structure procedure. Each individual is represented by a single vertical line, whose colour is partitioned according to the proportion of its membership coefficient for the K cluster. Samples are encoded as in Table 1.

AY093690, AY093693, AY093694). Balkanic and Italian haplotypes showed an average of 14% of sequence divergence. On the other hand, within *H. intermedia*, n1 and n2 are differentiated by just one substitution.

The null hypothesis of constant substitution rate cannot be rejected according to Tajima's (1993) relative rate tests, as all these were not significant. Based on a substitution rate of 3.6% of sequence divergence per Myr (Babik *et al.* 2004), the divergence time between mitochondrial haplotypes of the two species was estimated at 3.9 Myr.

Discussion

The two treefrog species *Hyla arborea* and *H. intermedia* were never found in either syntopy or sympatry. In fact, they are distributed along a zone of parapatry east and west of the Isonzo river, respectively, with the exception of Schiavetti, a population of *H. intermedia* found to the east of the Isonzo. This zone of parapatry falls within one of the proposed European "suture zones" (Taberlet *et al.* 1998, Hewitt 1999) where it is supposed that, during interglacials, multiple taxa expanding from their southern glacial refugia met, forming zones of secondary contact (Schmitt & Seitz 2001 and references therein). Our data fit comfortably within this scenario.

The two kinds of markers analysed give similar estimates of the time of divergence between the two species, the overall range being 5.5–3.9 Myr. Even taking account of the vagaries of the molecular clocks (Ayala 1997, 1999, Gibbons 1998, Welch & Bromham 2005, Ho & Larson 2006), these time estimates strongly suggest that the evolutionary split of the two lineages predates the Quaternary climatic oscillations. The study of the intraspecific genetic variation among populations of the Italian treefrog (Canestrelli *et al.* 2007a, 2007b) suggested that it survived the Pleistocene climatic oscillations in three major distinct refugia, two located in peninsular Italy south of the northern Apennines, the other spanning the entire Padanovenezian plain. Following the glacio-eustatic sea level oscillations, the extension of this latter geographic area varied considerably. At the last pleniglacial, the northern Adriatic sea coastline was located several hundreds kilometres southeast of its present location (Corregiari *et al.* 1996, Amorosi *et al.* 1999), and it has been suggested that during this period *H. intermedia* populations were widely distributed in the area (Canestrelli *et al.* 2007a, 2007b). The northern Adriatic sea coastline reached its present location, following the interglacial sea level rising, approximately 5000 years BP. (Tortora *et al.* 2001). These events led to the formation of the present corridor between the Italian peninsula and the Balkans (i.e. the land strip located between the northern mountainous and the southern marine regions) upon which the zone of parapatry between the two treefrog species is located. When also considering the proposed pleniglacial distribution of the Italian treefrog, if the secondary contact between the two species had pre-dated the formation of such corridor, one would expect the putative contact zone to be further east of its present location (but *see* e.g. Dasmahapatra *et al.* 2002 and references therein). We thus hypothesize that the secondary contact between the two species — and thus the evolutionary processes that it primed (*see* below) — have occurred within the last few thousand years.The fact that no F_1 or F_2 hybrids or backcrosses were found in the study area, suggests that there is no ongoing gene-flow between the two species. The presence of exogenous alleles at varying frequencies at discriminant loci, in parapatric populations of both species, appears therefore attributable to past events of introgressive hybridisation. A possible alternative explanation for the presence of alleles, common in one taxon, at low frequencies in a neighbouring sister species, is that they are isoelectrophoretic alleles, genetically distinct but electrophoretically indistinguishable. However, given that isoalleles are by definition experimental artefacts, their distribution within the species range is expected to be random, whereas in our study they were found only in proximity to the contact zone in both species. Moreover, the fact that they were observed at all diagnostic loci and, as in the case of *Icdh1*, *Xdh*, *Est2* and *Pep2*, in both species reciprocally, makes this hypothesis dismissible. Furthermore, the same arguments also allow us to reject the existence of shared ancestral polymorphisms as an alternative

explanation to introgression (*see* also Urbanelli & Porretta 2008).

Levels of introgression vary among loci (*see* Fig. 3). This phenomenon has also been observed in other organisms (e.g. Rieseberg *et al*. 1999, Martinsen *et al*. 2001, Cianchi *et al*. 2003). It has mostly been interpreted as a consequence of differential selective filtering acting on different loci, because of structural or functional properties of the enzymes (e.g. Eanes 1999), or because of alleles having different selective values (or being tightly linked to alleles with such characteristics), as determined by the internal genetic environment (e.g. Hunt & Selander 1973, Avise 1994). The apparent asymmetry observed with mitochondrial markers may be the consequence of non random interspecific mating patterns and/or hybrid progeny differential viability or sterility. Unfortunately, the overall observed introgression at mtDNA is too low to test for species asymmetric introgression and/or differences between markers. Given that mtDNA effective population size is 1/4 of that of nuclear genes (Birky *et al*. 1989), and mtDNA introgression being so low, introgressed haplotypes in *H. arborea* may easily have been lost as a consequence of genetic drift.

The analysis carried out with the program STRUCTURE shows that no individuals received a *q* value smaller than 0.87, while all other analyses indicated the full bimodality of the genotypes in the dataset. These results further support the inference of lack of ongoing gene flow, and suggest that introgression has had little effect on the gene pools of both species, which therefore appear as hardly permeable systems of co-adapted gene complexes. The absence of ongoing gene flow, coupled with evidence of past hybridization and introgression, has at least two non-mutually exclusive explanations. First, the central portion of the former contact zone (i.e. the 'sympatric area') could have recently disappeared, due to intervening unsuitable environmental conditions. A particularly intensive sampling effort was made in search of the 'sympatric area'. Nevertheless, we failed to find admixed populations even among those most closely located to the area of parapatry (no calling treefrogs during the breeding season, no responses to playbacks, no tadpoles). Since the past existence of such an area is a necessary premise for hybridization and introgression to have occurred, and given that we also inspected all known potential breeding sites in the area (each more than once), it can be reliably argued that this area has recently disappeared. Obviously, the ultimate causes of such an event cannot be indicated with confidence, and further analyses is being carried out with this aim. But in light of the physiographic features of the underlying geographic area and the harshness of recent anthropogenic alterations to the habitat, we hypothesize that several causes concurred to bring about such an event. The area is indeed surrounded by a karsic region to the north and the Adriatic sea to the south, and its core has been heavily human-impacted during the last few decades, mainly through industrialization and intensive agriculture. Its present arrangement could therefore have led to the disappearance of the 'sympatric area'.

A second possible explanation for the lack of ongoing gene-flow coupled with evidences for past events of introgressive hybridization, could be that mechanisms of reproductive isolation between the two species are more effective at present than when they came into contact, which would be indirect evidence of reinforcement. One of the most serious theoretical challenges to the idea of reinforcement is the possibility that the necessary association between loci controlling sex choice and hybrid fitness is broken up by recombination (Butlin 1989). However, in bimodal hybrid zones, as the zone under study probably was, this problem is overcome because, with the two genomes hardly mixing at all, there is almost complete linkage between conspecifc traits (Liou & Price 1994, Jiggins & Mallet 2000). Analysis of the advertisment call of the two species (Castellano *et al*. 2002) has shown significant differences in both static and dynamic properties of the mating call between samples collected in allopatric areas. This could suggest that some assortative mating could have been already active when they first came into secondary contact, an occurrence which would make more probable the hypothesis of reinforcement (Liou & Price 1994). Nevertheless, to strengthen this hypothesis it remains to be clarified whether there are any differences at the intraspecific level

between mating calls of allopatric and parapatric populations of each species. Preliminary data obtained by our research group suggest that mating calls are particularly divergent between the two species in the area of parapatry (G. Nascetti unpubl. data).

Acknowledgements

We are grateful to Claudio Bagnoli, Paola Bellini and Luca Lapini for kind help during field sampling and manuscript preparation, and to Mark Eltenton who reviewed the English. This work was funded by MIUR (Italian Ministry of University and of Scientific and Technological Research).

References

Amorosi, A., Cotalongo, M. L. & Fusco, F. 1999: Glacio-eustatic control of continental-shallow marine cyclicity from late Quaternary deposits of the southeastern Po plain, northern Italy. — *Quaternary Research* 52: 1–13.

Anderson, E. C. & Thompson, E. A. 2002: A model-based method for identifying species hybrids using multilocus genetic data. — *Genetics* 160: 1217–1229.

Arnold, M. L. 1992: Natural hybridization as an evolutionary process. — *Annual Review of Ecology Systematics* 23: 237–261.

Avise, J. C. 1994: *Molecular markers, natural history and evolution*. — Chapman & Hall, New York.

Ayala, F. J. 1997: Vagaries of the molecular clock. — *Proceedings of the National Academy of Science USA* 94: 7776–7783.

Ayala, F. J. 1999: Molecular clock mirages. — *Bioassays* 21: 71–75.

Babik, W., Branicki, W., Sandera, M., Litvinchuk, S., Borkin, L. J., Irwin, T. & Rafinski, J. 2004: Mitochondrial phylogeography of the moor frog, *Rana arvalis*. — *Molecular Ecology* 13: 1469–1480.

Barton, N. H. 1979: Gene flow past a cline. — *Heredity* 43: 333–339.

Barton, N. H. 2001: The role of hybridization in evolution. — *Molecular Ecology* 10: 551–568.

Barton, N. H. & Hewitt, G. M. 1981: Hybrid zones and speciation. — In: Atchley, W. R. & Woodruff, D. S. (eds.), *Evolution and speciation: essays in honor of M. J. D. White*: 109–145. Cambridge University Press, Cambridge.

Barton, N. H. & Hewitt, G. M. 1985: Analysis of hybrid zones. — *Annual Review of Ecology and Systematics* 16: 113–148.

Beerli, P., Hotz, H. & Uzzell, T. 1996: Geologically dated sea barriers calibrate a protein clock for Aegean water frogs. — *Evolution* 50: 1676–1687.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. & Bohomme, F. 1996–2004: *Genetix 4.05, logiciel sous Windows™ pour la génétique des populations*. — Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France).

Bigelow, R. S. 1965: Hybrid zones and reproductive isolation. — *Evolution* 19: 449–458.

Birky, C. W. J., Fuerst, P. & Maruyama, T. 1989: Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. — *Genetics* 121: 613–627.

Butlin, R. K. 1989: Reinforcement of premating isolation. — In: Otte, D. & Endler, J. (eds.), *Speciation and its consequences*: 158–179. Sinauer Associates, Sunderland, Massachusetts.

Canestrelli, D., Verardi, A. & Nascetti, G. 2007a: Genetic differentiation and history of populations of the Italian treefrog *Hyla intermedia*: lack of concordance between mitochondrial and nuclear markers. — *Genetica* 130: 241–255.

Canestrelli, D., Cimmaruta, R. & Nascetti, G. 2007b: Phylogeography and historical demography of the Italian treefrog *Hyla intermedia* reveals multiple refugia, range expansions and secondary contacts within the Italian peninsula. — *Molecular Ecology* 16: 4808–4821.

Castellano, S., Cuatto, B., Rinella, R., Rosso, A. & Giacoma, C. 2002: The advertisement call of the European treefrogs (*Hyla arborea*): a multilevel study of variation. — *Ethology* 108: 75–89.

Cianchi, R., Ungaro, A., Marini, M. & Bullini, L. 2003: Differential patterns of hybridization and introgression between the swallowtails *Papilio machaon* and *P. hospiton* from Sardinia and Corsica islands (Lepidoptera, Papilionidae). — *Molecular Ecology* 12: 1461–1471.

Corregiari, A., Roveri, M. & Trincardi, F. 1996: Late Pleistocene and Holocene evolution of the North Adriatic Sea. — *Il Quaternario* 9: 697–704.

Dasmahapatra, K. K., Blum, M. J., Aiello, A., Hackwell, S., Davies, N., Bermingham, E. P. & Mallett, T. 2002: Inferences from a rapidly moving hybrid zone. — *Evolution* 56: 741–753.

Dobzhansky, T. 1940: Speciation as a stage in evolutionary divergence. — *American Naturalist* 74: 312–321.

Eanes, W. F. 1999: Analysis of selection on enzyme polymorphisms. — *Annual Review of Ecology and Systematics* 30: 301–326.

El Mousadik, A. & Petit, R. J. 1996: High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. — *Theoretical and Applied Genetics* 92: 832–839.

Evanno, G., Regnaut, S. & Goudet, J. 2005: Detecting the number of clusters of individuals using the software structure: a simulation study. — *Molecular Ecology* 14: 2611–2620.

Faivovich, J., Haddad, C. F. B., Garcia, P. C. A., Frost, D. R., Campbell, J. A. & Wheeler, W. C. 2005: Systematic review of the frog family Hylidae, with special reference to Hylinae: phylogenetic analysis and taxonomic revision. — *Bulletin of the American Museum of Natural History* 294: 1–240.

Gibbons, A. 1998: Calibrating the mitochondrial DNA clock. — *Science* 279: 38–39.

Goudet, J. 2001. *FSTAT, a program to estimate and test gene diversities and fixation indices (ver. 293)*. — Available at http://www.unil.ch/izea/softwares/fstat.html.

Grant, V. 1963: *The origin of adaptations*. — Columbia University Press, New York.

Guldbrandtsen, B., Tomiuk, J. & Loeschcke, V. 2000: POPDIST, ver. 1.1.1: a program to calculate population genetic distance and identity measures. — *Journal of Heredity* 91: 178–179.

Harrison, R. G. 1993: *Hybrid zones and the evolutionary process*. — Oxford University Press, Oxford.

Hedges, S. B. 1986: An electrophoretic analysis of Holarctic hylid frog evolution. — *Systematic Zoology* 35: 1–21.

Hewitt, G. M. 1996: Some genetic consequences of ice ages, and their role in divergence and speciation. — *Biological Journal of the Linnean Society* 58: 247–276.

Hewitt, G. M. 1999: Postglacial re-colonization of European biota. — *Biological Journal of the Linnean Society* 68: 87–112.

Hewitt, G. M. 2000: The genetic legacy of the Quaternary ice ages. — *Nature* 405: 907–913.

Hillis, D. M. 1984: Misuse and modification of Nei's genetic distance. — *Systematic Zoology* 33: 238–240.

Ho, S. Y. & Larson, G. 2006: Molecular clocks: when times are a-changin'. — *Trends in Genetics* 22: 79–83.

Howard, D. J. 1993: Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. — In: Harrison, R. G. (ed.), *Hybrid zones and the evolutionary process*: 46–69. Oxford University Press, New York.

Hunt, W. G. & Selander, R. K. 1973: Biochemical genetics of hybridisation in European house mice. — *Heredity* 31: 11–33.

Jiggins, C. D., King, P., McMillan, W. O. & Mallet, J. 1997: The maintenance of species differences across a *Heliconius* hybrid zone. — *Heredity* 79: 495–505.

Jiggins, C. D. & Mallet, J. 2000: Bimodal hybrid zones and speciation. — *Trends in Ecology and Evolution* 15: 250–255.

Kawamura, T. & Nishioka, M. 1977: Aspects of reproductive biology of Japanese anurans. — In: Tylor, D. H. & Guttman, S. I. (eds.), *Reproductive biology of Amphibians*: 103–139. Plenum Press, New York.

Kocher, T. D., Thomas, W. K., Meyer, A., Pääbo, S., Villablanca, F. X. & Wilson, A. C. 1989: Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. — *Proceedings of the National Academy of Science USA* 86: 6196–6200.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. 2001: MEGA2: Molecular Evolutionary Genetics Analysis software. — *Bioinformatics* 17: 1244–1245.

Liou, L. W. & Price, T. D. 1994: Speciation by reinforcement of premating isolation. — *Evolution* 48: 1451–1459.

Littlejohn, M. J. 1981: Reproductive isolation: a critical review. — In: Atchley, W. R. & Woodruff, D. S. (eds.), *Evolution and speciation: essays in honor of M. J. D. White*: 298–334. Cambridge University Press, Cambridge.

Mallet, J. 2005: Hybridization as an invasion of the genome. — *Trends in Ecology and Evolution* 20: 229–237.

Martinsen, G. D., Whitham, T. G., Turek, R. J. & Keim, P. 2001: Hybrid populations selectively filter gene introgression between species. — *Evolution* 55: 1325–1335.

Moore, J. A. 1957: An embryologist's view of the species concept. — In: Mayr, E. (ed.), *The species problem*: 325–338. American Association for the Advancement of Science, Washington DC.

Nascetti, G., Capula, M., Lanza, B. & Bullini, L. 1985: Ricerche elettroforetiche su anfibi della regione mediterranea: aspetti tassonomici ed evolutivi. — In: *Riassunti del IV congresso associazione Ghigi*: 45. Associazione A. Ghigi per la Biologia dei Vertebrati, Grottammare.

Nascetti, G., Lanza, B. & Bullini, L. 1995: Genetic data for the specific status of the Italian treefrog (Amphibia: Anura: Hylidae). — *Amphibia-Reptilia* 16: 215–227.

Nei, M. 1978: Estimation of average heterozygosity and genetic distance from a small number of individuals. — *Genetics* 83: 583–589.

Nei, M. 1987: *Molecular evolutionary genetics*. — Columbia University Press, New York.

Paillette, M. 1967: Valeur taxonomique des émissions sonores chez les *Hyla* (Amphibiens, Anoures) de la faune française. — *Comptes Rendus de l'Academie des Sciences Paris, séries D* 264: 1626–1628.

Paterson, H. E. H. 1978: More evidence against speciation by reinforcement. — *South African Journal of Science* 74: 369–371.

Phelan, P. L. & Baker, T. C. 1987: Evolution of male pheromones in moths: reproductive isolation through sexual isolation? — *Science* 235: 205–207.

Pritchard, J. K., Stephens, M. & Donnelly, P. J. 2000: Inference of population structure using multilocus genotype data. — *Genetics* 155: 945–959.

Raymond, M. & Rousset, F. 1995: GENEPOP (ver. 1.2): population genetics software for exact tests and ecumenicism. — *Journal of Heredity* 86: 248–249.

Remington, C. 1968: Suture-zones of hybrid interaction between recently joined biotas. — *Evolutionary Biology* 2: 321–428.

Riehl, C. J., Lell, J. T. & Maxson, L. R. 1995: Relationships among Palearctic *Hyla*: Insights from Immunology. — *Biochemical Systematics and Ecology* 23: 245–249.

Rieseberg, L. H., Whitton, J. & Gardner, K. 1999: Hybrid zones and the genetic architecture of a barrier to gene flow between two wild sunflower species. — *Genetics* 152: 713–727.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989: *Molecular cloning: a laboratory manual, 2nd ed*. — Cold Spring Harbor Laboratory Press.

Schmitt, T. & Seitz, A. 2001: Allozyme variation in *Polyommatus coridon* (Lepidoptera: Lycaenidae): identification of ice-age refugia and reconstruction of post-glacial expansion. — *Journal of Biogegraphy* 28: 1129–1136.

Schneider, H. 1974: Structure of mating calls and relationship of the European tree frogs (Hylidae, Anura). — *Oecologia* 14: 99–110.

Seehausen, O. 2004: Hybridization and adaptive radiation. — *Trends in Ecology and Evolution* 19: 198–207.

Servedio, M. R. 2004: The what and why of research of reinforcement. — *PLoS Biology* 2(e420): 2032–2035.

Taberlet, P., Fumagalli, L., Wust-Saucy, A. & Cosson, J. 1998: Comparative phylogeography and postglacial colonization routes in Europe. — *Molecular Ecology* 8: 1923–1934.

Tajima, F. 1993: Simple methods for testing the molecular evolutionary clock hypothesis. — *Genetics* l35: 599–607.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997: The ClustalX Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. — *Nucleic Acid Research* 25: 4876–82.

Tortora, P., Bellotti, P. & Valeri, P. 2001: Late-Pleistocene and Holocene deposition along the coasts and continental shelves. — In: Martini, P. & Vai, G. B. (eds.), *Anatomy of an orogen: the Apennines and adjacent Mediterranean basin*: 455–478. Kluwer Academic Publisher, Dordrecht-Boston-London.

Urbanelli, S. & Porretta, D. 2008: Evidence of reinforcement of premating isolation between two species of the genus *Ochthebius* (Coleoptera: Hydraenidae). — *Evolution* 62: 1520–1527

Veith, M., Kosuch, J. & Vences, M. 2003: Climatic oscillations triggered post-Messinian speciation of Western Palearctic brown frogs (Amphibia, Ranidae). — *Molecular Phylogenetics and Evolution* 26: 310–327.

Welch, J. J. & Bromham, L. 2005: Molecular dating when rates vary. — *Trends in Ecology and Evolution* 20: 320–327.