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Discordant Patterns of Nuclear and Mitochondrial Introgression in Iberian Populations of the European Common Frog (*Rana temporaria*)

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Abstract

Amphibians often show complex histories of intraspecific and interspecific genetic introgression, which might differ in mitochondrial and nuclear genes. In our study of the genetic differentiation of the European common frog, *Rana temporaria* (159 specimens from 23 populations were analyzed for 24 presumptive allozyme loci; 82 specimens were sequenced for a 540-bp fragment of the mitochondrial 16S rRNA gene), multilocus correspondence analysis (CA) and Bayesian assignment tests of the nuclear data were concordant in identifying 2 population groups corresponding to 1) the Pyrenees in the east and 2) the Galicia and Asturias regions in the west, the latter corresponding to the subspecies *R. temporaria parvipalmata*. Geographically intermediate populations were genetically intermediate in the allozyme CA and, less clearly in the Bayesian assignment, with mitochondrial haplotypes exclusively belonging to the *parvipalmata* group. This indicates different degrees of introgression in the mitochondrial and nuclear genomes. Although Pyrenean high-altitude populations are morphologically distinct from low-altitude populations, these 2 groups were not separate clusters in any analysis. This suggests that the morphological differences may be due to fast adaptation to elevational gradients, likely under maintenance of gene flow, and that the underlying genetic changes are not detectable by the analyzed markers. We argue that a parsimonious explanation for the observed pattern along the east–west axis in northern Spain may be competition between invading and resident populations, with no need to invoke selection. However, in order to conclusively rule out selective processes, additional and finer scale data are required to test for asymmetric mating preference/behaviour, sex-biased gene flow, or sex-biased survival of potential hybrids.

Key words: *amphibia, Iberian Peninsula, introgression, phylogeography,* Rana temporaria

In the northern hemisphere, the distribution, genetic structure, and subdivision of populations have been fundamentally shaped by past climatic changes that occurred during the Pliocene and Pleistocene (e.g., Taberlet et al. 1998; Hewitt 2004; Waltari et al. 2007; Weiss and Ferrand 2007). Early hypotheses assumed that extant species largely formed during the Pleistocene, and these speciation processes were driven by glacial cycles. However, molecular clock analyses have provided evidence that the origin of most northern hemisphere species predates the Pleistocene (e.g., Klicka and Zink 1997; Avise et al. 1998). However, the subdivision of species into major phylogeographic units has in many cases been influenced by the isolation of species in glacial refugia, with or without subsequent range expansions (Taberlet et al. 1998; Hewitt 2004).

Several amphibians and reptiles have ranges that include a narrow northern stretch of the Iberian Peninsula and much of central and western Europe. Besides the common frog, *Rana temporaria*, these include the palmate newt, *Lissotriton helveticus*, and the lizards, *Podarcis muralis*, *Lacerta viridis*, and *Zootoca vivipara*. The fire salamander (*Salamandra salamandra*) is more widespread in Iberia, but different genetic lineages usually named as subspecies are found in different regions; the Pyrenees, Cantabria, Asturias, and much of northern Galicia are populated by several striped phenotype populations (subspecies *fastuosa* and *bernardezi*) of complex molecular relationships (García-París et al. 2003).

Recent phylogeographic studies revealed concordant patterns of genetic differentiation in species that occur along a north–south axis in western Spain and Portugal, such as the salamander *Chioglossa lusitanica* (Sequeira et al. 2005), the newt *Lissotriton boscai* (Martínez-Solano et al. 2006), and the lizard *Lacerta schreiberi* (Godinho et al. 2006). In these species, populations of highest genetic diversity occur in the southern part of their ranges, suggesting that their northern populations originated by range expansions from southern refugia in climatically favorable periods. Other species clearly have their highest genetic diversity in Iberia and have expanded from this refuge northeastward, as is the case in the midwife toad, *Alytes obstetricans* (Martínez-Solano et al. 2004), and in the natterjack, *Bufo calamita* (Rowe et al. 2006).

In contrast, studies of species occurring across northern Iberia usually have revealed a more complex pattern (Guillaume et al. 2000). In fact, the northern regions of the Iberian Peninsula have been heavily affected by climatic changes in the Pleistocene (Crowley and North 1991). The central part of the Cantabrian mountain range remained glaciated above 750 m above sea level, leading to isolation of numerous animal and plant populations which survived in coastal refuges or in deep valleys, where more temperate climatic conditions persisted (Uzquiano 1995; García-París et al. 2003). The distribution and genetic structure of many species currently populating this area has been shaped by patterns of colonization from these diverse refugia (e.g., Gómez and Lunt 2007).

The European common frog, *R. temporaria*, is one of the most widespread European amphibians, occurring from western Spain to northern Scandinavia in the West and to western Siberia in the East (Grossenbacher 1997). Despite this large distribution area, there are only few strongly differentiated regional sublineages, although on the other hand, there is also a distinct genetic subdivision among local populations (e.g., Reh and Seitz 1990; Hitchings and Beebee 1997; Palo, Schmeller, et al. 2004; Schmeller and Merilä 2007). Besides a genetically divergent group of populations in northwestern Iberia, often considered as subspecies, *R. temporaria parvipalmata* (Veith et al. 2002 and references therein), the species can be divided chiefly into a western and an eastern genealogical lineage (Palo, Schmeller, et al. 2004).

In Iberia, *R. temporaria* occurs along the Pyrenean and Cantabrian mountain ranges into the westernmost region of Galicia, from where the subspecies *R. temporaria parvipalmata* has been described, originally on the basis of morphological features, such as reduced webbing and small body size (see Galán Regalado and Fernández Arias 1993; Esteban and García-Paris 2002). In general, the morphological variability of *R. temporaria* in the Pyrenees (viz. the Basque country and Navarra) and adjacent regions is surprisingly high and has led to the hypothesis of possible cryptic species inhabiting these areas (e.g., Dubois 1982). For example, besides the morphologically deviant *R. temporaria parvipalmata* populations from Galicia, in many high elevation areas of the Pyrenees, these frogs are relatively small, with short hindlimbs and often a coloration with multiple black spots. Such specimens in the past led to descriptions of new taxa, which currently are considered as synonyms or subspecies, such as *R. temporaria canigonensis* from Mont Canigou in the eastern French Pyrenees or *R. (temporaria) aragonensis* from Respomuso in the Spanish Pyrenees (Dubois 1982, 1983; Veith et al. 2002). In contrast, in French lowlands adjacent to the Pyrenees, specimens are more slender and often larger, with extremely long hindlimbs, the tibiotarsal articulation surpassing the tip of the snout as typically only in the related *R. dalmatina*, and with larger relative tympanum sizes. These specimens have been referred to as "Gasser's frog" (Dubois 1983) and erroneously reported as "*R. temporaria gasseri*" by Veith et al. (2003), this constituting a nomen nudum because a description of such a taxon has never been published.

Preliminary allozyme and mitochondrial DNA (mtDNA) sequence analyses (Arano et al. 1993; Veith et al. 2002, 2003) found no genetic differentiation among these morphologically diverse Pyrenean populations. Rather the evidence pointed to the existence of an eastern (Pyrenean) cluster of populations and a western (Galician–Asturian) cluster of populations, with geographically intermediate populations from the Basque country being also genetically intermediate and providing a first hint at introgression between the 2 lineages.

Here, we complement previous allozyme data (Veith et al. 2002) with a data set of mitochondrial sequences and include further crucial populations. Our goal was to combine different population genetic and phylogeographic approaches to test 1) whether morphologically divergent populations from the French lowlands adjacent to the Pyrenees may be genetically distinct and 2) whether a geographically more complete sampling along the Cantabrian ridge and inclusion of an additional mitochondrial marker support the existence of an intermediate area of introgression.

Materials and Methods

Sample Sites

Specimens were collected from 23 populations from west to east Iberian Peninsula (Spain) and in Germany (Table 1). Samples from 9 populations were added to Veith et al. (2002).

Four population groups were a priori defined on the basis of allozyme data published by Veith et al. (2002) and

Table 1 Sampling localities and numbers of specimens of populations studied

Country and region	No.	Locality	Group	Coordinates	*N* Individuals	
					Allozymes	DNA
Spain						
Galicia	1	Serra da Capelada	1	43°44′N/07°56′W	5*	3
	2	Serra dos Ancares	1	42°50′N/07°00′W	4*	5
Asturias	3	Puerto de Somiedo	1	43°11′N/06°17′W	14*	5
	4	Espina near Salas, Los Porcinos	1	43°24′N/06°19′W	5*	5
	5	Near Picos de Europa	2	43°17′N/04°56′W	6*	5
	6	Rio Sella	2	43°07′N/05°01′W	10	8
	7	Rio Saja	2	43°05′N/04°15′W	3	2
Euskadi	8	Puerto de Altube	2	43°19′N/02°52′W	5*	4
Navarra	9	Zugarramurdi	4	43°16′N/01°32′W	10	5
Aragon	14	Between Oza and Aguas Tuertas	3	42°51′N/00°40′W	11*	4
	15	Aguas Tuertas	3	42°49′N/00°35′W	8*	3
	16	Upper valley Canal Roya	3	42°47′N/00°30′W	8*	4
	17	Pico de Anayet	3	42°46′N/00°26′W	3*	2
	18	Between Formigal and Portalet	3	42°47′N/00°24′E	2*	2
	19	Respomuso, Circo de Piedrafita	3	42°49′N/00°17′W	9*	4
	20	Ibones de la Facha	3	42°48′N/00°15′W	15*	4
	21	Barranco Ordiso, Bujaruelo	3	42°43′N/00°9′W	6*	4
France						
Pyrénées-Atlantiques	10	Lapitzurri (Ainhoa)	4	43°10′N/01°30′W	10	2
	11	Espelette	4	43°20′N/01°27′W	1	1
	12	Iraty	4	43°02′N/01°05′W	10	4
Hautes-Pyrénées	13	Near Gerde	4	43°03′N/00°10′E	0	1
Pyrénées-Orientales	22	Mont Canigou	3	42°29′N/02°27′E	10	4
Germany						
Nordrhein-Westfalen	23	Wahner Heide near Bonn		50°53′N/07°09′E	4*	1
Σ					159	82

Samples and markers studied already by Veith et al. (2002) are marked by asterisks. The column group gives a priori grouping of populations: 1 = *Rana temporaria parvipalmata*, 2 = geographically intermediate, 3 = high-altitude Pyrenean, and 4 = low-altitude Pyrenean population groups. Abb. = abbreviation of locality names as used in the text and in the figures.

on the basis of morphological data (Vences M, unpublished data). In the following, populations will in the text be referred to with a combined acronym including their numbers as in the distribution maps and Structure analysis and an abbreviation of the locality name as in the multivariate plot. 1) *Rana temporaria parvipalmata*, defined as populations occurring in Galicia and the eastern part of Asturias (populations 1–4); 2) geographically intermediate populations (5–8); 3) low-altitude Pyrenean populations that include Gasser's frog and other long-legged populations (9–13); and 4) high-altitude Pyrenean frogs including "aragonensis" and "canigonensis" (14–22). For convenience, these groups will in the following be named the *parvipalmata* group, intermediate group, low-altitude Pyrenean group, and high-altitude Pyrenean group, respectively.

DNA Sequencing and Sequence Alignment

We extracted DNA from frozen samples of muscle using the QIAmp tissue extraction kit (Qiagen). An approximately 600-bp section of the mitochondrial 16S rRNA gene was amplified using primers16Sar-L (light chain; 5′-CGC CTG TTTATC AAAAAC AT-3′) and 16Sbr-H (heavy chain; 5′-CCG GTC TGAACT CAG ATC ACG T-3′) of Palumbi et al. (1991). PCR cycling procedure was as follows: initial denaturation step: 90 s at 94 °C; 33 cycles: denaturation 45 s at 94 °C, primer annealing for 45 s at 55 °C; extension for 90 s at 72 °C. PCR products were purified using Qiaquick purification kits (Qiagen). We sequenced single-stranded fragments using an automatic sequencer (ABI 377). We consistently obtained approximately 540 bp for 82 specimens. Sequences were aligned automatically using the "Clustal" option of the sequence navigator (Applied Biosystems) and subsequently adjusted by eye.

Allozyme Electrophoresis and Population Statistics

Tissue sampling and allozyme processing were according to Veith et al. (2002). Stained allozyme systems coded for a total of 24 presumptive loci in the 159 specimens analyzed. Allele frequencies and population genetic variability estimates (mean heterozygosity, average number of polymorphic loci, and average number of alleles) were calculated for all samples using G-Stat (Sigismund 1997).

Identification of Introgressed Individuals and Hybrid Populations

To test for panmixia, we calculated deviations of population-specific observed genotype frequencies from respective ideal Hardy–Weinberg (HW) proportions (χ^2 test; rare alleles were pooled to avoid expected genotype counts below

1; G-Stat of Sigismund 1997). Such deviations from panmixia would be expected when reproductively isolated lineages coexist within populations (e.g., morphotypes that indicate different species). We corrected for multiple tests within populations and across polymorphic loci via sequential Bonferroni correction as outlined by Rice (1989).

Previous analyses (Veith et al. 2002) suggested a main subdivision of populations into a western (*R. temporaria parvipalmata*) and eastern (Pyrenean) group of populations, with some geographically intermediate populations showing introgression. To identify individuals potentially intermediate between these lineages, we submitted individual multilocus allozyme genotypes to a correspondence analysis (CA) using reciprocal averaging (Jongman et al. 1995), with alleles coded as present (1) or absent (0) (Community Analysis Package; Henderson and Seaby 2002). Because rare alleles may have a disproportionately large effect on the result of a CA ordination, their abundance, once lower than the abundance of the commonest allele divided by 5, was downweighted in proportion to their frequencies. Ordination was started with a principle component analysis, and CA was subsequently optimized via varimax rotation; by default 4 axes were extracted. We expected specimens from intermediate populations to lie between the western and the eastern clusters in multidimensional allele space.

In the case of intergradation, populations from the geographically intermediate area should show signs of intergradation, with individual multilocus genotypes assigning members of theses populations to all potential source groups. We therefore tested for the existence of 4 distinct population groups by applying the likelihood ratio test of Paetkau et al. (1995) for assignment of individual multilocus genotypes to our 4 predefined groups (*parvipalmata* group, intermediate group, low-altitude Pyrenean group, and high-altitude Pyrenean group) using G-Stat (Sigismund 1997). This procedure assumes HW proportions. Besides assignment of individuals to all groups of populations, it also tests if a given assignment can significantly be rejected.

To support results gained from the likelihood ratio test of Paetkau et al. (1995), we applied Bayesian assignment using Structure (version 2.3.3; Pritchard et al. 2000). This allows identification of genetically homogeneous populations or groups of populations with or without predefinition and to judge assignment of specimens to such groups on the basis of multilocus genotypes. To detect the most likely number of K clusters of individuals, we calculated the log likelihoods for $1 \leq K \leq 22$ clusters (number of populations). Because the estimated log probabilities do not provide a correct estimation of K, we calculated ΔK as recommended by Evanno et al. (2005). For each K, we ran 10 replications with 10 iterations each (1 000 000 generations, with the burn-in set to 100 000). We applied the admixture ancestry model and the noncorrelated allele frequency model. Additional Structure runs with populations 1–8 and populations 10–22, respectively, were analyzed in order to be sure that the differentiation of the 2 subspecies does not superimpose a pattern that prevents detection of fine scale genetic structure within the 2 subgroups. If the a priori population groups were valid, we would expect $K = 2$ with specimens from populations 1–4 and 5–8 forming genetically homogeneous clusters for the first additional run. In the same way, $K = 2$ was our expectation for the second additional run, with specimens from low-altitude (9–13) and high-altitude (14–22) populations forming the 2 respective clusters.

Haplotype Network

We constructed a haplotype network on the basis of statistical parsimony using TCS (version 1.21; Clement et al. 2000), which we converted into a nested clade design (Templeton et al. 1987). We used the nested clade design as an additional tool to further specify patterns that had emerged from our population genetic analyses and to deduce the root of the network. Because no a priori root was available, we additionally determined it via midpoint rooting.

Results

Allozyme Variability

In 24 studied loci, we identified 48 different alleles among our 23 population samples (see Supplementary Material). Observed and expected heterozygosities ranged between 0.042 and 0.153 and between 0.056 and 0.147, respectively, with no apparent geographical pattern. Our test for HW equilibrium per locus and population identified only 9 of 153 cases of polymorphic loci (=6%) being not in HW equilibrium. Again, no conspicuous geographic pattern emerges. *Rana temporaria parvipalmata* and the Pyrenean populations share allozyme alleles at all loci, and all those loci that dominate the Pyrenean populations are also present in the *R. temporaria parvipalmata* populations, although sometimes at a distinctly lower frequency (e.g., idh1-a, ldh1-d pepD2-c, pgm-a).

Individual Assignments Based on Allozymes

Only individuals of the geographically intermediate population group were assigned to all other groups (the likelihood ratio test of Paetkau et al. (1995); Table 2 and Supplementary Material). For 10 of 24 specimens, assignment to the wrong group could not be rejected. Single specimens from all other groups were assigned to the intermediate group, again indicating the intermediate genetic composition of their allozyme multilocus genotypes. *Rana temporaria parvipalmata* specimens were assigned with a high probability (96%) to the correct group, and none of them was assigned to any of the Pyrenean population groups. Although also low- and high-altitude Pyrenean frogs could be assigned to a high proportion to the correct population group, assignment to the respective other group could not be rejected in numerous specimens. However, no specimen of the Pyrenean groups could be assigned to the *parvipalmata* group.

Table 2 Individual assignment of common frogs to population groups based on allozyme multilocus genotypes using the likelihood ratio test of Paetkau et al. (1995)

	(1)	(2)	(3)	(4)
N individuals	28	24	62	41
Target group				
(1) *Rana temporaria parvipalmata* (%)	**96**	13	0	0
(2) Geographically intermediate (%)	4	**79**	3	0
(3) Pyrenees low altitude (%)	0	4	**82**	22
(4) Pyrenees high altitude (%)	0	4	15	**78**

Values indicate the percentage of individuals from each population (source group) to all populations (target groups); assignments to the source groups are printed in bold, whereas other values indicate the number of specimens whose assignment to a given population cannot be rejected (the likelihood ratio test).

ΔK unambiguously identified $K = 2$ as the most probable level of population structure in the Bayesian assignment of individuals based on allozyme multilocus genotypes (see Supplementary Material). Specimens of populations 1–7 were assigned to one (*parvipalmata*) group, and those of populations 9–22 to the second (Pyrenean) group (Figure 1). Only population 8 contains specimens assigned to either group in more or less equal amounts but with a higher assignment probability to the Pyrenean group in all but one individual. When further exploring the Structure results, we found $K = 5$ to be the next probable number of clusters. Two additional runs performed with either only populations 1–8 or populations 9–22 resulted in $K = 2$ and $K = 4$, respectively, as the most likely number of groups. In the *parvipalmata* group, no geographic structure of the obtained subgroups was apparent, with only 3 individuals from populations 5 and 7 forming a group of their own. In the Pyrenees, the westernmost lowland populations 9 and 10 formed a somewhat homogeneous and separated subgroup, and the same was true for the easternmost population 22 from Mont Canigou (Supplementary Material).

The first 4 CA axes of the ordination analysis explain 13.3%, 7.0%, 6.8%, and 5.3% of the total variance, respectively. In the 2D CA plot of axes 1 and 2 *R. temporaria*, individuals from the high- and low-altitude Pyrenean population groups are clearly separated from those of the *parvipalmata* group (Figure 2). Axis 1 clearly separates specimens from the 2 Pyrenean groups from those of the *parvipalmata* and intermediate groups, whereas axis 2 separates the *parvipalmata* from the intermediate group. As expected under an introgression scenario, the specimens from the geographically intermediate population group bridge the gap between more western and more eastern populations, albeit with no overlap. Populations 4 and 5 clearly emerge as allozymatically intermediate, together with populations 6 and 7. Population 8 seems to be more similar to the 2 Pyrenean groups. Populations from low- and high-altitude Pyrenean groups totally overlap in the CA plot. Specimens from single high-altitude populations are more separated from each other in the CA plot despite their partial geographic proximity. Two-dimensional CA plots of further combinations of the first 4 axes (not shown) show no clear pattern.

mtDNA Variability

We found 13 16S rRNA haplotypes (H1–H13; GenBank accession numbers JF299194–JF299206), one of which (H8) only occurred in the German population. In contrast to allozymes, the *parvipalmata* group does not share haplotypes with the 2 Pyrenean population groups. The westernmost population from the Serra da Capelada is characterized by 2 private haplotypes (H12 and H13) (Supplementary Material). The geographically intermediate populations share the derived haplotypes H6 and H9 with the *parvipalmata* populations. Within the Pyrenees, a pattern of few widely distributed and abundant haplotypes (H1 and H2) and some rare and locally restricted haplotypes is much more obvious than in the *parvipalmata* group.

Haplotype Network

In the initial minimum spanning network (data not shown), a second equally parsimonious pathway connected H11 and H2 via an undetected haplotype. Because coalescence theory predicts that rare haplotypes are more likely connected to frequent haplotypes than to singletons (frequency criterion of Pfenninger and Posada 2002), we kept the connection of

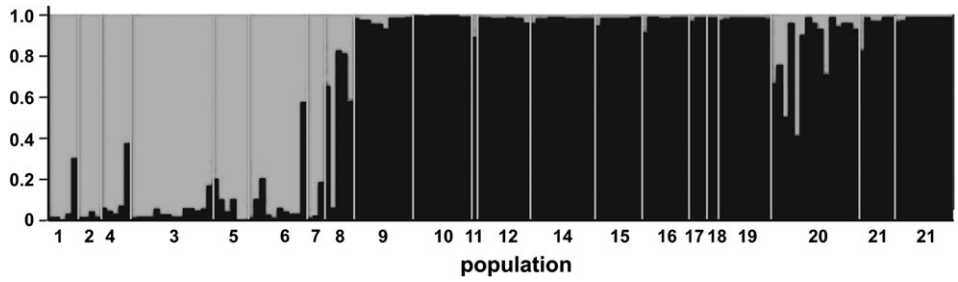

Figure 1. The Bayesian assignment of specimens to $K = 2$ population groups based on allozyme multilocus genotypes. Populations are ordered from left to right according to increasing distance from population 1.

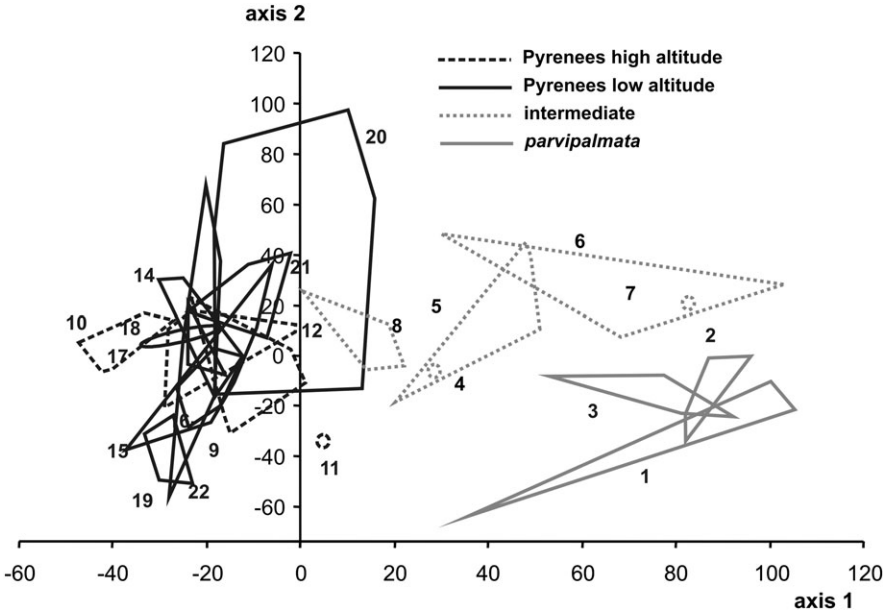

Figure 2. Two-dimensional representation of multilocus genotypes of Iberian *Rana temporaria* specimens along the first 2 axes of a CA on presence/absence of allozyme alleles; minimum convex polygons mark population borders. Specimens are separated into the 4 a priori groups: *R. t. parvipalmata* population group (grey, solid line), geographically intermediate group (grey, dotted line), low altitude (black, solid line) and high altitude (black, dotted line) Pyrenean populations of *R. t. temporaria*.

H11 to H2 via H1. This resulted in a fully resolved network (Figure 3).

The subnetworks of the *parvipalmata* and the Pyrenean population groups are separated by 2 substitutions only. The nested clade design and midpoint rooting identified the connecting missing haplotype as the root of the entire network. The major difference between these 2 subnetworks is that in the Pyrenees 2 central haplotypes are common and widespread, whereas all others are rare and locally restricted, a pattern typical for a recent range expansion with subsequent local differentiation. In the *parvipalmata* subnetwork, haplotypes are more evenly abundant and locally restricted, with the most abundant H9 being a terminal one.

Discussion

Pyrenean Low- and High-Altitude Morphs Do Not Constitute Different Evolutionary Lineages

Lowland Pyrenean common frogs have repeatedly been described as morphologically divergent from other Pyrenean populations. Some of them have been named even as distinct taxa. However, our mitochondrial and nuclear genetic data consistently show that long-legged low-altitude and short-legged high-altitude populations share the same gene pool. Populations with long-legged frogs are, for instance, populations 11, 13, and, to a lower degree, 9 and 10. In most of the other high-altitude populations, frogs have distinctly shorter legs. Nevertheless, there is no differentiation among these populations in the CA analysis of allozyme data (Figure 2), and they share most of their mitochondrial haplotypes (Figure 3). This agrees with the hypothesis of fast and recurrent adaptation to elevational gradients in the common frog on the basis of only a limited number of candidate loci (Bonin et al. 2006), which might account for a high degree of local adaptation in larval life history (Brand and Grossenbacher 1979; Richter-Boix et al. 2010) and breeding period (Phillimore et al. 2010). It is remarkable that within the high- and low-elevation Pyrenean groups, it is the population from the highest altitude (population 20) that has the highest allelic diversity (Figure 2 and Supplementary Material).

From a taxonomic perspective, the data presented here include populations from respective type localities and thus provide a solid basis to affirm that they are not differentiated at the subspecies or species level from other Pyrenean populations (for details, see Supplemental Material).

Discordant Patterns of Introgression

Allozymes and mtDNA consistently distinguish populations from the regions of Galicia and Asturias (our *parvipalmata* group) from Pyrenean populations (Figures 1–3). They are discordant in that mtDNA clearly assigns the geographically "intermediate" populations to the *R. temporaria parvipalmata* group (black color in the haplotype network, Figure 3), whereas allozymes characterize them as intermediate between the *R. temporaria parvipalmata* and the Pyrenean populations, albeit to different degrees depending on the analytical approach (black-colored polygons in Figure 2,

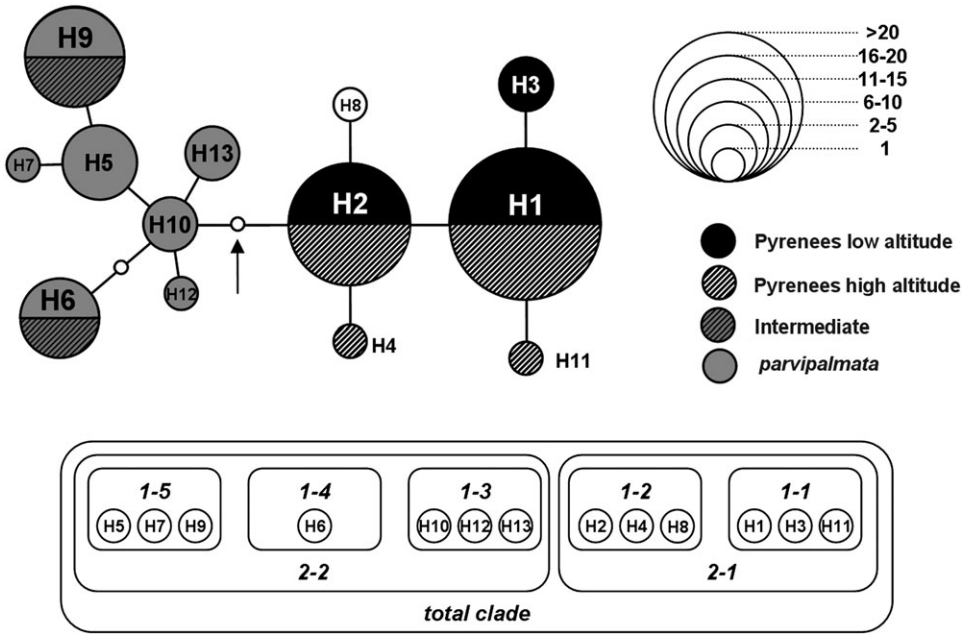

Figure 3. Minimum spanning haplotype network (upper left), haplotype abundance (upper right), and nested clade design (lower) of 12 Iberian *Rana temporaria* 16S mtDNA haplotypes (H1–H7 and H9–H13; H8 was only found in the German outgroup population); midpoint rooting and the nested clade design consistently identify the unobserved haplotype between H2 and H10 as the root. For occurrence of haplotypes in single populations, see Supplementary Material.

especially populations 4, 5, and 8. Although in the CA, all geographically intermediate populations bridge the gap between the *R. temporaria parvipalmata* and the Pyrenean populations in the 2D parameter space of axes 1 and 2 (Figure 2), the Bayesian approach with 2 clusters of individuals (Figure 1) assigns specimens of this area to *R. temporaria parvipalmata*, except most from population 8 (see also Veith et al. 2002).

Rana temporaria temporaria and *R. temporaria parvipalmata* are supposed to have diverged during the Pleistocene approximately 1.1 Ma (Veith et al. 2003). The *R. temporaria temporaria* lineage later on diverged into 2 sublineages, of which the western one is restricted to the Pyrenees and adjacent regions of western and central Europe (Palo, Schmeller, et al. 2004). It is sound to assume that during the last glacial maximum *R. temporaria parvipalmata* was restricted to a western refugium in Galicia (Galán et al. 2009), whereas the western *R. temporaria temporaria* lineage supposedly survived in an area close to the Pyrenees from where it postglacially expanded northward into central Europe.

In Iberia, these postglacial range expansions must have brought both lineages into secondary contact somewhere between the eastern Cantabria and the Basque country, with a differential pattern of introgression for the 2 marker systems studied herein (Figure 4). *Rana temporaria parvipalmata* haplotypes and allozyme alleles occur eastward until the Basque Country (population 8). The geographical distribution of the derived haplotypes H6 and H9 in relation to the more ancestral *R. temporaria parvipalmata* haplotypes meets the expectation of a range expansion from the Galicia region to the East. The 2 sets of markers show, however, differences in their distribution from a Pyrenean perspective: after the initial differentiation in refuges and subsequent range expansion with secondary contact of *parvipalmata* and Pyrenean populations, nuclear alleles of the Pyrenean populations penetrated far westward into the area occupied by *parvipalmata* populations, whereas Pyrenean mitochondrial markers remained stuck inside the Pyrenees. Such an exclusive spread of nuclear alleles as found in this study primarily hints at male-biased dispersal although so far the available evidence points to female-based dispersal in *R. temporaria* (Palo, Lesbarrres, et al. 2004).

The pattern encountered in this study resembles that described for a contact zone of fire salamander lineages in the same region, albeit geographically in the opposite direction (García-París et al. 2003). In the salamander example, *Salamandra salamandra terrestris* had expanded from the Pyrenees deep into the Cantabrian Mountains. After forming a zone of secondary contact with the Cantabrian *S. salamandra bernardezi*, nuclear alleles of *bernardezi* spread via eastward range expansion of the western lineage. Local selection was invoked to explain the fast success of nuclear genes of the western lineage over those from the eastern lineage (García-París et al. 2003). While in the European common frog, allozyme alleles of both lineages co-occur across a large geographic range and in fire salamanders, one nuclear genome outcompeted the other due to its presumed selective advantage.

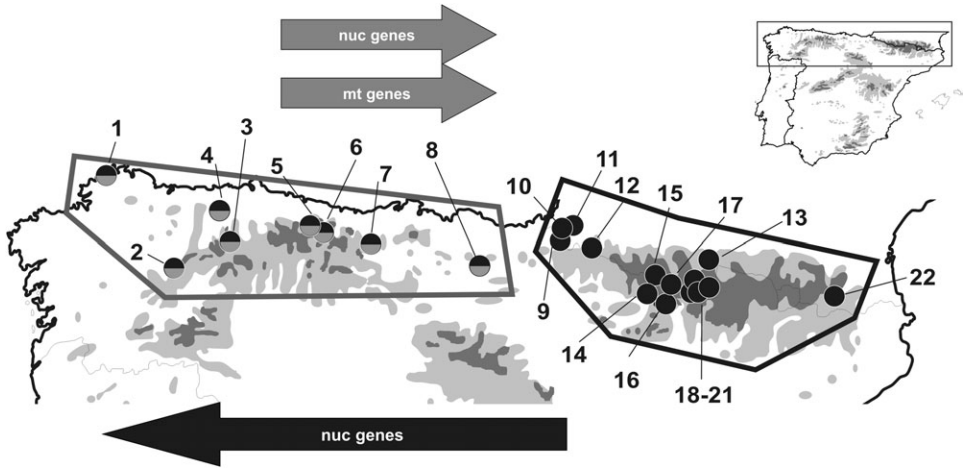

Figure 4. Assignment of populations to *Rana temporaria parvipalmata* (grey) and *R. temporaria temporaria* (black; comprising the 2 Pyrenean population groups). Polygons encircle populations characterized by the mitochondrial haplotypes of the *parvipalmata* group or of the 2 Pyrenean groups (no haplotype sharing observed among populations of grey vs. black polygons. Circles schematically indicate the presence of nuclear alleles typical for either the *parvipalmata* group (grey) or the 2 Pyrenean groups (black); the predominant alleles of the Pyrenees are also found in all or most *parvipalmata* populations, which in addition have alleles that are completely or largely missing in the Pyrenean groups. Arrows indicate presumptive postglacial range expansions of nuclear and mitochondrial alleles during or after the secondary contact and admixture of frogs that initially diverged in a Pyrenean and a Galician refugium. The assumed eastward expansion of mitochondrial and nuclear alleles is based on the exclusive occurrence of mitochondrial haplotypes of the *parvipalmata* group in all geographically intermediate populations and the intermediate placement of these populations along first axis of the CA analysis of allozyme allele occurrence (Figure 2). The assumed westward expansion of nuclear alleles of the Pyrenean group of population is based on the fact that none of the alleles that dominate the Pyrenean populations is absent from the *parvipalmata* populations. Bicolored circles represent populations with *parvipalmata* and *temporaria* alleles at allozyme loci.

Recent simulation studies have shown that under resource competition between residents and invaders, range expansion of an invader into the area of a local population may result in a replacement of the invaders genome by the local genome (Currat et al. 2008). This pattern does not need to involve selection and is strongest in markers experiencing reduced gene flow and enhanced effects of genetic drift, due to a reduced effective population size, as is the case with mtDNA (see reviews by Birky 2001; Ballard and Whitlock 2004; White et al. 2008). A mitochondrial genetic bottleneck may even accelerate intraindividual segregation processes (e.g., Cree et al. 2008), eventually leading to a very fast fixation of haplotypes in populations, Consequently, neither the assumption of a selective advantage of one mitochondrial haplolineage over the other nor sex-biased dispersal have to be invoked to explain discordance of nuclear and mitochondrial introgression patterns of *R. temporaria temporaria* nuclear alleles and mitochondrial haplotypes throughout the Cantabrian Mountains (Figure 4). However, additional evidence from other marker systems, such as microsatellites, is needed before other explanations such as selective processes (Ballard and Whitlock 2004; Whitney et al. 2006), asymmetric mating preference or behavior (Patton and Smith 1993; Roca et al. 2005), sex-biased gene flow (Petit et al. 2004), or sex-biased survival of hybrids (Haldane's rule) can be ruled out as being responsible for the observed pattern.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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