Genetic uniformity of *Rana pyrenaica* Serra-Cobo, 1993 across its distribution range: a preliminary study with mtDNA sequences

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Abstract. The genetic variability of the Pyrenean frog *Rana pyrenaica* was assessed using DNA sequences of the cytochrome *b*, COI and 12S rRNA mitochondrial genes. The results show that, despite inhabiting an area of mountainous terrain with high mountain peaks and deep isolated valleys, *R. pyrenaica* is genetically very homogeneous. The extremely low level of genetic variability observed, even in the fast evolving cytochrome *b* and COI genes, suggests that *R. pyrenaica* may have colonized most of its present range very rapidly after the last Würm glaciation from a refuge area in the Prepyrenees. This is the first attempt to establish the level of genetic variability of this endangered Pyrenean endemism and, as a result, it has major implications for its conservation.

Keywords: biogeography, conservation, dispersal, mitochondrial DNA, postglacial colonization.

The Pyrenean frog *Rana pyrenaica* Serra-Cobo, 1993, is the smallest European member of the so called "brown frogs" (Genus *Rana*, subgenus *Rana*, constituting the *Rana temporaria* group of this speciose genus; Frost et al., 2006), with adults reaching a maximum size of 5.5 cm. *Rana pyrenaica* is endemic to the Pyrenean mountain range, where it is usually found in cool, well-oxygenated, mountain streams of fast-running water. It lives from 780 m to 2050 m, being more abundant between 1200 and 1800 m (Serra-Cobo, 1993, 1997; Llamas, Martinez-Gil and Arribas, 1995; Vieites and Vences, 2003; García-París, 2004; Serra-Cobo et al., 2006). Its distribution is restricted to the central and western parts of the southern slopes of the Pyrenees, from the Ordesa and Monte Perdido National Park in the East, to the Irati area in the West, where it enters the French territory (fig. 1) (Serra-Cobo, 1993; Llamas, Martinez-Gil and Arribas, 1995, 1998; Llamas and Martínez-Gil, 2005). It is more abundant in the Central Pyrenees than in the extreme Western Pyrenees, where some populations have been found as low as 780 m, in the Irati forest area (Llamas and Martínez-Gil, 2005).

Phylogenetic studies using allozymes (Veith et al., 2002) and DNA sequences (Veith, Kosuch and Vences, 2003) suggest that *R. pyrenaica* is a very deep lineage, probably sister to *R. temporaria*. This genetic differentiation is also supported by its unique karyotype, which exhibits $2n = 26$ chromosomes, like other european "brown frogs" but distributed in five large pairs (with minute pericentromeric bands, distinctive to this species) and 8 relatively smaller pairs, with NORs in the short arm of the 6th chromosome. By contrast, in other European "brown frogs", heterochromatin appears in all or nearly all of the chromosomes as heavy centromeric or pericentromeric bands, and NORs in 3rd or 10th chromosome pairs (Odierna et al., 2001).

Despite appearing as endangered in the 2007 IUCN Red List of Threatened Species (Bosch et al., 2006) and the existence of several conservation programs intended to halt the gradual decline of *R. pyrenaica* in its area of occupancy, there is still no data regarding the degree of genetic variability of this species across its distribution range. Its presence in areas of the Pyrenees that were heavily glaciated during the last Wurm glacial maximum (see Arribas, 2004 for a general account on the Pyrenean glaciations but also Pallas et al., 2006) suggests that it

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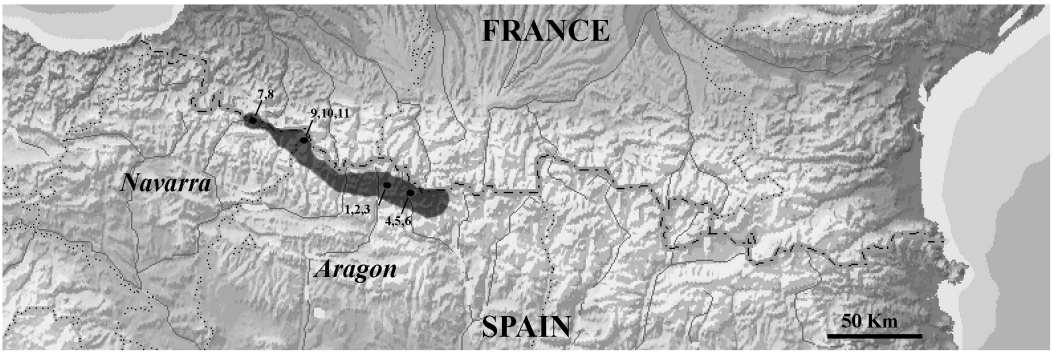

Figure 1. Map showing the distribution range of *Rana pyrenaica* (dark area) and the localities of the 11 samples included in the mtDNA study.

Table 1. Details of material used in the present study. Numbers after species names identify the samples shown in fig. 1.

Taxa	Locality	Codes
R. pyrenaica-1	Yésero-Puerto de Cotefablo (Spain) N 42.61694 / W 0.20972	E19067.5
R. pyrenaica-2	Yésero-Puerto de Cotefablo (Spain) N 42.61694 / W 0.20972	E31006.2
R. pyrenaica-3	Yésero-Puerto de Cotefablo (Spain) N 42.61694 / W 0.20972	E1967.4
R. pyrenaica-4	Bujaruelo Valley, Huesca (Spain) N 42.66333 / W 0.10889	E19067.2
R. pyrenaica-5	Bujaruelo Valley, Huesca (Spain) N 42.66333 / W 0.10889	E19067.1
R. pyrenaica-6	Bujaruelo Valley, Huesca (Spain) N 42.66333 / W 0.10889	E14067.1
R. pyrenaica-7	Irati, Navarra (Spain) N 42.99833 / W 1.10083	E14067.2
R. pyrenaica-8	Irati, Navarra (Spain) N 42.99833 / W 1.10083	E19067.3
R. pyrenaica-9	Between Zuriza and Isaba, Navarra (Spain) N 42.87696 / W 0.82250	E31006.10
R. pyrenaica-10	Between Zuriza and Isaba, Navarra (Spain) N 42.87696 / W 0.82250	E31006.11
R. pyrenaica-11	Between Zuriza and Isaba, Navarra (Spain) N 42.87696 / W 0.82250	E31006.12

probably dispersed into the core of this mountain range very recently from refuge areas at lower latitude. However, data from other cold-blooded Pyrenean endemic vertebrates like, for instance, the lacertid lizards of the genus *Iberolacerta*, suggest that the pattern might be different. Molecular phylogenies suggest that these lizards survived up to the glacial cycles of the Pleistocene in or near to the main mountain axis of the Pyrenees (Mayer and Arribas, 2003; Carranza, Arnold and Amat, 2004; Arribas, Carranza and Odierna, 2006). These different hypotheses would produce different patterns of genetic variability, which can be tested by using molecular studies. From a conservation point of view, the analysis of the genetic variability of *R. pyrenaica* must be taken into account when deciding future strategies for preserving the declining populations of this interesting Pyrenean endemism.

In order to assess the level of genetic variability of *R. pyrenaica*, mitochondrial DNA sequences of the fast-evolving mitochondrial coding genes cytochrome *b* and cytochrome oxidase I and the mitochondrial ribosomal 12S rRNA were analyzed for 11 individuals covering most of its distribution range (see fig. 1 and table 1). DNA extractions and PCR amplifications of the mitochondrial fragment were carried out according to methods described elsewhere (Carranza et al., 1999, 2000). The primers used for both amplification and sequencing were S1F 5′-TTC AAC TAC AAA AAC CTA ATG AAC C-3′ and cytochrome *b*2 (Kocher et al., 1989) for the cytochrome *b*; LCOI and HCOI (Folmer et al., 1994) for the cytochrome oxidase I and 12Sa and 12Sb (Kocher et al., 1989) for the 12S rRNA.

In total, 1423 base pairs (bp) of mitochondrial DNA (398 bp of the cytochrome *b*, 658 bp of the cytochrome oxidase I and 377 of the 12S rRNA) were sequenced for this study. Of these, only position number 3 of the cytochrome *b* alignment was variable. It was a cytosine in two specimens from Yésero-Puerto Cotefablo (specimens 2 and 3 from table 1) and one specimen from the Bujaruelo Valley (specimen 4) and an

adenine in all the remaining samples, including two specimens from the Bujaruelo Valley and one from Yesero-Cotefablo. The Genbank accession numbers for the two cytochrome *b* haplotypes were EU746404 and EU746403, respectively. The accession numbers for the single 12S rRNA haplotype found in the present study is EU746401 and for the single cytochrome oxidase I haplotype is EU746402. These analyses indicate that, despite being an old lineage (see Veith et al., 2002, 2003) distributed across an area of mountainous terrain with high mountain peaks and deep, isolated valleys, *R. pyrenaica* is genetically a very homogeneous species at the mitochondrial DNA level. These results are concordant with the low level of genetic variability shown by another highly aquatic amphibian from the same area, the urodele *Calotriton asper* (Carranza and Amat, 2005), and contrast with the relatively high level of genetic variability shown by the lacertid lizards of the genus *Iberolacerta* (Mayer and Arribas, 2003; Carranza, Arnold and Amat, 2004; Arribas, Carranza and Odierna, 2006).

Most of the current distribution range of *R. pyrenaica* was completely covered by ice during the coldest glacial phases of the Pleistocene, especially during the last glacial maximum. During the Würm, ravines and streams at the sides of the main river valleys presently inhabited by Pyrenean frogs, were continuously under the level of the ice tongues and only became free of glaciers during the last glacial retreat, occurred 16 000-10 000 years ago (Bordonau, 1992; Pallàs et al., 2006). As suggested by Veith et al. (2002, 2003), *R. pyrenaica* has a long independent history from all other "brown frogs" and, like *C. asper*, it presents many adaptations for life in mountain streams (see introduction), which would indicate that it has been probably around its present distribution area at least during part of the Neogene and especially during the Pleistocene. The extremely low level of genetic variability even in the fast evolving cytochrome *b* and COI gene fragments analyzed, suggests that *R. pyrenaica* might have recolonized the Pyrenees very rapidly after the last Würm glaciation from a refuge further south. In fact, *R. pyrenaica* may have been able to survive the glaciations by seeking refuge in the lower and westernmost areas of its current distribution range (Irati and areas nearby belonging to the Ebro watershed), as glaciated areas in the Pyrenees were absent west of the Roncal Valley (Arribas, 2004). After the Würm glaciation, the species probably colonized the Western and Central Pyrenees very quickly from its refuge areas, arriving at the heart of the mountain range through the formerly glaciated valleys of Roncal, Ansó, Hecho, Aragón, Gállego and Broto-Bujaruelo among others. This can be deduced from the distribution pattern of *R. pyrenaica*, which suggests that this species mainly follows watercourses and does not cross high mountain crests, such as, for instance, the mountains that separate the southern and northern slopes of the Pyrenees.

Although further analyses including more samples and maybe nuclear molecular markers like, for instance, microsatellites or nuclear introns (Sequeira et al., 2006) may give us a more detailed picture of the genetic variability of *R. pyrenaica*, the analysis of 1423 bp of mitochondrial DNA indicates that variability within this species is very low. These results, together with future studies on highly variable nuclear markers, might also be useful for ongoing and future conservation programs in *R. pyrenaica*, such as, for instance, captive breeding programs, reintroductions and translocations of populations, for which prior knowledge would be necessary regarding the genetic status of the different populations across the species' distribution range.

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