Folia Zool. – 51(4): 307–318 (2002)

Genetic differentiation and population structure within Spanish common frogs (*Rana temporaria* complex; Ranidae, Amphibia)

Michael VEITH[1]*, Miguel VENCES[2], David R. VIEITES[3], Sandra NIETO-ROMAN[3] and Antonio PALANCA[3]

[1] *Zoologisches Institut der Universität Mainz, Saarstraße 21, D-55099 Mainz, Germany; e-mail: michael@oekologie.biologie.uni-mainz.de*

[2] *Zoologisches Forschungsinstitut und Museum Alexander Koenig, Adenauerallee 160, 53113 Bonn, Germany; e-mail: m.vences@t-online.de*

[3] *Laboratorio de Anatomía Animal, Facultade de Ciencias Biolóxicas, Universidade de Vigo, Apdo. 874, 36200 Vigo (PO), Spain; e-mail: apalanca@uvigo.es*

Received 2 February 2001; Accepted 23 April 2002

A b s t r a c t . Genetic differentiation of *Rana temporaria* from the Pyrenean and Cantabrian mountains in Spain was studied by means of allozyme electrophoresis. 24 loci were analysed in 104 specimens from 15 populations: nine populations from the Pyrenean massif, five populations from the area of the Cantabrian mountain chain (regions of Galicia, Asturias, and Basque Country), and one population from Germany. Three distinct clusters were distinguished by phenetic analysis: (a) the Pyrenean samples and the single population from the Basque Country, (b) the populations from Galicia and Asturias) and (c) the German population. Ordination (PCA) resulted in one principle component (PC1) that separated Cantabrian from Pyrenean populations, and in a second one (PC2) that separated the single German population from the Iberian ones. PC1 indicated introgression that was corroborated by west-east clines in several alleles along the Cantabrian chain. The rather clear separation of the Cantabrian and Pyrenean clusters (mean genetic distance 0.121) suggests that two genetically different subspecies of *R. temporaria* may be distinguished in Spain. The absence of fixed allelic differences between populations refutes recent hypotheses of the existence of syntopic sibling species within *R. temporaria* in Spain. Biogeographically, the Pyrenean and Cantabrian populations possibly originated in two separate colonisation events starting from different glacial refuges. The strong morphological differentiation of Pyrenean *R. temporaria* populations is not paralleled by genetic divergence, and may better be explained by ecological factors such as climate, altitude and vegetation.

Key words: population genetics, taxonomy, allozymes, Spain, *Rana temporaria*

Introduction

The systematics of the Iberian brown frogs, subgenus *Rana (Rana)* according to D u b o i s (1992), has long been discussed. Especially the species affiliation of populations from the Pyrenean mountain range has not been satisfactorily studied in the past as shown by the recent discovery and description of a new species, *Rana pyrenaica* Serra-Cobo, 1993, which is well differentiated by adult and larval morphology, and ecology (S e r r a - C o b o 1993, S t r i j b o s c h 1996, V e n c e s et al. 1998b).

Currently, four brown frog species are recognised from the Iberian Peninsula. (1) *Rana pyrenaica* is, as far as is known, restricted to the Pyrenean mountain range (S e r r a - C o b o 1997). (2) *Rana iberica* Boulenger, 1879, an endemic, brook-dwelling species occurs mainly in north-western Spain and northern Portugal (E s t e b a n 1997a). (3) *Rana dalmatina* Bonaparte, 1840, a mainly Central European species, occurs in a restricted area in

* Corresponding author

the Basque Country and Navarra (G o s á 1997); records from Catalonia and possibly from the French Pyrenees are due to misidentification (see L l o r e n t e et al. 1995, D u b o i s 1998). (4) *Rana temporaria* Linnaeus, 1758, is a species with a vast distribution area including almost all European countries (G r o s s e n b a c h e r 1997); in Spain it is restricted to a northern stretch largely corresponding to the Pyrenean and Cantabrian mountain ranges (E s t e b a n 1997b).

Geographic variation of *Rana temporaria* on the Iberian Peninsula and adjacent regions has so far not been sufficiently studied. However, present taxonomy indicates a remarkable differentiation. Populations from the Basses Alpes in France (referable to the taxon *R. t. honnorati*) differ from German populations in tadpole morphology and mean nuclear DNA content (S p e r l i n g et al. 1996). In north-western Spain, populations attributed to the subspecies *R. t. parvipalmata* (Seoanne, 1885) have a slightly different advertisement call and a reduced foot webbing (G a l á n 1989, V e n c e s 1992) as compared to German populations. They are allozymatically well differentiated from other Spanish and from Central European populations (A r a n o et al. 1993). The status of the taxon *R. t. canigonensis* Boubée, 1833, from Mont Canigou in the French Pyrenees, is unsolved (see D u b o i s 1983). Recently, P a l a n c a et al. (1995) defined morphotypes of brown frogs from the Spanish Pyrenees of Aragon; one of these morphotypes was named *Rana aragonensis* Palanca Soler, Rodriguez Vieites et Suárez Martínez, 1995, unintentionally constituting a valid taxon description due to the lack of explicit statement that the name just referred to a morphotype. A lectotype of *R. aragonensis* was later designated (V e n c e s et al. 1998a), but the status of the taxon remains uncertain.

The by now single study on allozyme variation in Iberian brown frogs (A r a n o et al. 1993) did not comprise Pyrenean *R. temporaria* populations. These authors were thus unable to clarify the status of the populations inhabiting this massif as well as their relation to *R. t. parvipalmata* and *R. t. temporaria*.

The aim of the present study is to test whether there exists more than one *R. temporaria*-like species in the western Pyrenees. This was (i) assumed as one possible explanation of the observed morphological divergence among Pyrenean populations (V e n c e s et al. 1998b), and (ii) deduced from the co-existence of two seemingly separated taxa at localities in the Aragonese Pyrenees (P a l a n c a et al. 1995). To test this hypothesis we analysed the genetic differentiation of the Pyrenean *R. temporaria* populations by means of allozyme studies.

Material and Methods

Specimens were collected by opportunistic day and night searching. They were sacrificed using chlorobutanol. Femur muscle tissue and liver was removed from freshly dead specimens and frozen at -80°C for electrophoresis. Specimens were fixed in 5% formaldehyde or 95% ethanol, and stored in 70% ethanol. Vouchers were deposited in the collections of the Zoologisches Forschungsinstitut und Museum Alexander Koenig (ZFMK), Bonn, and the Muséum National d'Histoire Naturelle (MNHN), Paris.

Specimens of *Rana temporaria* were collected at the following localities (Fig. 1) from West to East Iberian Peninsula (Spain), and in Germany. *Galicia*: (1) Serra da Capelada, province of La Coruña (CAP; 43°44'N/7°56'W; 5 specimens; no vouchers preserved); (2) Serra dos Ancares, province of Lugo, (ANC; 42°50'N/7°00'W; 4 specimens; MNHN 1998.136-138, ZFMK 68854); *Asturias:* (3) Puerto de Somiedo, province of Oviedo (PSO;

43°11'N/6°17'W; 14 specimens; ZFMK 68361-68374); (4) Espina near Salas, Los Porcinos (ESP; 42°24'N/6°19'W; 5 specimens; ZFMK 68402-68405); (5) near Picos de Europa (PIC; 43°17'N/4°56'W; 6 specimens; ZFMK 68379-68384); *Basque Country:* (6) Puerto de Altube, province of Álava (PAL; 42°19'N/2°52'W; 5 specimens; ZFMK 68393-68397); *Aragón (Huesca province)*: (7) between Oza and Aguas Tuertas, (OAT; 42°51'N/0°40'W; 11 specimens; ZFMK 65399-65409); (8) Aguas Tuertas, (AGU; 42°49'N/0°35'W; 8 specimens; ZFMK 65410-65416, 65437); (9) upper Canal Roya valley, (CRO; 42°47'N/0°30'W; 8 specimens; ZFMK 65419-65426); (10) Pico de Anayet, (ANA; 42°46'N/0°26'W; 3 specimens; ZFMK 65427-65429); (11) between Formigal and Portalet, (FOR; 42°47'N/0°24'E; 2 specimens; ZFMK 65417-65418); (12) Respomuso, Circo de Piedrafita (RES; 42°49' N/0°17'W; 8 specimens; ZFMK 65430-65436); (13) Ibones de la Facha (FAC; 42°48'N/0°15'W; 15 specimens; ZFMK 68347-68360); (14) Barranco Ordiso, Bujaruelo (BUJ; 42°43'N/0°9'W; 6 specimens; ZFMK 65439-65444); *Germany:* (15) environments of Bonn, (BON; 50°53'N/7°9'E; 4 specimens; MNHN 1998.135).

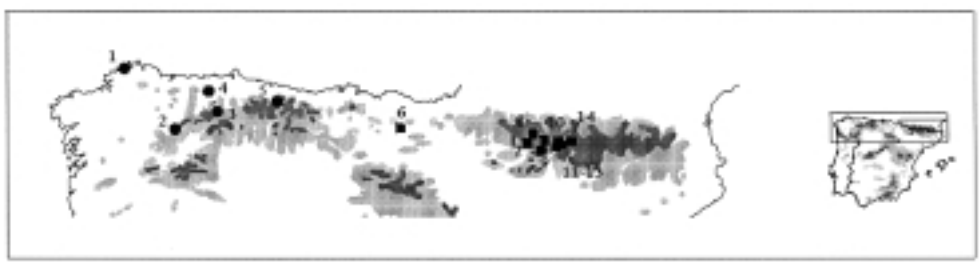

Fig. 1. Map showing sample locations. Circles, populations of the Cantabrian cluster; squares, populations of the Pyrenean cluster.

Samples of *Rana iberica* (RIBE; Salas, Asturias, Spain; 42°25'N/6°16'W; 6 specimens; ZFMK 68875-78), *Rana pyrenaica* (RPYR; Zuriza, Aragón, Spain; 42°54'N/0°48'W; 2 specimens; ZFMK 65447-65449) and *Rana macrocnemis* (RMAC; Tavas, Turkey; 37°42'N/29°03'E; 4 specimens; vouchers are preserved in the Musée National d'Histoire Naturelle, Paris, MNHN 2000.660-2000.663) were used for hierarchical outgroup rooting.

Pieces of muscle and liver were homogenised in Pgm buffer (Hebert & Beaton 1993). Electrophoresis was run on cellulose acetate (CA) plates from Helena Diagnostics, Texas. We used four different buffer systems for separation of allozymes (Table 1): Phosphate buffer, pH 7.2 (PP 7.2); tris-maleic buffer, pH 7.0 (TM 7.0); tris-citric buffer, pH 7.2 (TC 7.2); tris-glycine buffer, pH 8.5 (TG 8.5). Twenty enzyme systems provided data on 24 presumptive gene loci (Table 1). Allozyme loci and alleles were numbered according to their electrophoretic mobility, either anodal or cathodal, with the fastest being 1 or a, respectively.

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). We tested for syntopic occurrence of different taxa by calculating deviations of observed genotype frequencies from ideal Hardy-Weinberg proportions χ^2-Test; rare alleles were pooled to avoid expected genotype frequencies below 1.0; G-STAT). Subsequently, we corrected within populations for multiple tests across polymorphic loci (sequential Bonferroni correction as outlined by Rice 1989). Inbreeding parameters according to Wright's (1951) *F*-statistics were calculated with G-STAT for ingroup samples with $n \geq 4$ by the procedure described in

Table 1. Enzyme systems, enzyme commission (E.C.) number, buffer systems and tissues used in electrophoresis.

enzyme system	loci	E.C. number	buffer system	tissue
aspartate aminotransferase	*aat1, aat2*	2.6.1.1	PP 7.2	muscle
adenosyl-homocysteine hydrolase	*ahh*	3.3.1.1	PP 7.2	liver
arginine phosphokinase	*apk*	2.7.3.3	PP 7.2	liver
creatine kinase	*ck*	2.7.3.2	TC 8.2	liver
fumarate hydratase	*fum*	4.2.1.2	TC 8.2	muscle
glucose dehydrogenase	*gldh*	1.1.1.47	TM 7.0	liver
glyceraldehyde-3-phosphate dehydrogenase	*gapd*	1.2.1.12	TM 7.0	muscle
glucose-phosphate isomerase	*gpi*	5.3.1.9	TG 8.5	muscle
isocitrate dehydrogenase	*idh1, idh2*	1.1.1.42	TM 7.0	liver
lactate dehydrogenase	*ldh1, ldh2*	1.1.1.27	PP 7.2	muscle
NAD-dependent malate dehydrogenase	*mdh*	1.1.1.37	TM 7.0	liver
NADP-dependent malate dehydrogenase (malic enzyme)	*me*	1.1.1.40	TM 7.0	liver
mannose-phosphate isomerase	*mpi*	5.3.1.8	TM 7.0	muscle
dipeptidase with alanine-leucine as substrate	*pepA*	3.4.11/13	TC 8.2	liver
tripeptidase with glycine-leucine-leucine as substrate	*pepB*	3.4.11/13	PP 7.2	liver
dipeptidase with phenylalanine-proline as substrate	*pepD1, pepD2*	3.4.11/13	TC 8.2	liver
6-phosphogluconate dehydrogenase	*6pgd*	1.1.1.44	TM 7.0	liver
phosphoglucomutase	*pgm*	5.4.2.2	TC 8.2	muscle
pyruvate kinase	*pk*	2.7.1.40	TM 7.0	liver
threhalase	*tre*	3.2.1.28	PP 7.2	liver

Weir & Cockerham (1984). Variances of these estimators were obtained by jackknifing populations. Inbreeding estimates deviating ±1.96 standard deviation (SD) from zero were regarded as significant.

We used Nei's (1972) standard genetic distance to build an UPGMA tree. Both calculations were performed with NTSYS (Rohlf 1990). 1000 bootstrap replicates (Felsenstein 1985) were run using the subroutine SEQBOOT as implemented in PHYLIP 3.5c (Felsenstein 1993). Since UPGMA cluster analysis hardly allows for the detection of intergraded populations (it forces all populations into a dichotomic branching pattern) and *a priori* information on intergradation was not available we used a principle component analysis (PCA) using the alleles as characters and their frequencies as states in order to detect potentially intergraded populations relative to pure populations of the detected lineages.

Results

In 24 studied loci, we identified 77 different alleles among the samples (Table 2). In the UPGMA phenogram (Fig. 2) based on Nei's (1972) standard genetic distances as shown in Table 3, the Spanish populations were clearly separated into two geographic clusters. One included the Galician and Asturian samples (referred to subsequently as the Cantabrian cluster), and a second cluster composed of all Pyrenean samples and the single population from the Basque Country (Pyrenean cluster). The German sample was basal to the Iberian samples.

The PCA detected only two principle components (Eigenvalue > 1) that accounted for ca. 40% of the total variance. PC1 explained 23.6 % of the total variance and discriminated among the Spanish samples. The position of the Basque sample was intermediate between the Galicia/Asturias and the Pyrenean samples in a two-dimensional plot of PC1 and PC2 (Fig. 3). PC2 explained 16.8 % of the total variance, and clearly separated the German sample from the Spanish samples (Fig. 3).

Table 2. Allele frequencies, observed (H_o) and expected (H_e) heterozygosity, average number of polymorphic loci (P_{95}; 95% criterion) and average number of alleles (A) per locus for 15 *R. temporaria* and three outgroup samples (*RIBE*, *R. iberica*; *RPYR*, *R. pyrenaica*; *RMAC*, *R. macrocnemis*).

locus ↓	CAP 5	ANC 4	PSO 14	ESP 5	PIC 6	PAL 5	OAT 11	AGU 8	CRO 8	ANA 3	FOR 2	RES 8	FAC 15	BUJ 6	BON 4	RIBE 6	RPYR 2	RMAC 4
aat1	d(1.000)	d(1.000)	d(1.000)	b(0.100) d(0.900)	d(1.000)	a(0.100) d(0.900)	d(1.000)	d(1.000)	d(1.000)	d(1.000)	d(1.000)	d(1.000)	d(1.000)	d(1.000)	d(1.000)	c(1.000)	d(1.000)	e(1.000)
aat2	c(1.000)	c(1.000)	a(0.107) c(0.893)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	d(1.000)
ahh	b(0.500) c(0.500)	b(0.250) c(0.750)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	a(0.038) b(0.962)	b(1.000)	b(1.000)	d(1.000)	b(1.000)	a(1.000)
fum	b(1.000)	b(1.000)	b(0.929) c(0.071)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	b(0.375) c(0.625)	b(1.000)	b(1.000)	b(1.000)
gpi	b(1.000)	b(1.000)	b(0.893) c(0.107)	b(1.000)	a(0.250) b(0.750)	b(1.000)	b(0.864) c(0.136)	b(0.938) c(0.063)	b(0.857) c(0.143)	b(1.000)	b(1.000)	b(0.938) c(0.063)	b(1.000)	b(0.917) c(0.083)	b(1.000)	b(1.000)	b(1.000)	b(1.000)
idh1	a(0.200) b(0.800)	a(0.625) b(0.375)	a(0.214) b(0.786)	a(0.200) b(0.800)	a(0.500) b(0.500)	a(0.600) b(0.400)	a(0.682) b(0.318)	a(0.429) b(0.571)	a(0.813) b(0.188)	a(0.667) b(0.333)	a(1.000)	a(0.750) b(0.250)	a(0.700) b(0.300)	a(1.000)	b(1.000)	a(1.000)	a(1.000)	a(1.000)
idh2	c(1.000)	b(0.125) c(0.875)	c(1.000)	b(0.100) c(0.900)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	b(0.083) c(0.917)	c(1.000)	a(1.000)	c(1.000)	c(1.000)
ldh1	c(0.800) d(0.200)	c(0.625) d(0.375)	c(0.143) d(0.857)	c(0.700) d(0.300)	c(0.083) d(0.917)	d(1.000)	a(0.136) d(0.864)	d(1.000)	d(1.000)	d(1.000)	d(1.000)	d(1.000)	a(0.200) d(0.800)	d(1.000)	d(1.000)	d(1.000)	b(1.000)	d(1.000)
mdh	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	c(1.000)	a(1.000)	b(1.000)
me	a(1.000)	a(1.000)	a(1.000) d(0.036)	a(0.900) b(0.100)	a(1.000)	a(0.700) b(0.300)	a(0.864) b(0.136)	a(0.875) b(0.125)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(0.933) b(0.067)	a(0.750) b(0.250)	a(1.000)	a(1.000) 0	a(1.000) 0	c(1.000) 0
mpi	d(0.700) e(0.200) g(0.100)	d(0.875) e(0.125)	d(0.571) e(0.357(g(0.036)	b(0.300) d(0.700)	d(0.750) e(0.167) g(0.083)	d(0.800) e(0.100) g(0.100)	d(0.409) e(0.318) g(0.273)	c(0.250) d(0.375) e(0.188) g(0.188)	d(0.438) e(0.500) g(0.063)	c(0.667) d(0.333)	c(0.500) d(0.250) g(0.250)	d(0.625) e(0.313) g(0.063)	c(0.133) d(0.567) e(0.233) g(0.067)	d(0.917) e(0.083)	b(0.167) d(0.333) e(0.500)	f(1.000)	d(0.250) e(0.750)	h(1.000)
pepA	b(0.700) d(0.300)	b(0.625) d(0.375)	b(0.700) c(0.200) d(0.100)	b(0.900) c(0.100)	b(1.000)	b(0.375) c(0.250) d(0.375)	b(0.350) d(0.650)	b(0.286) d(0.714)	b(0.214) d(0.786)	d(1.000)	d(1.000)	d(1.000)	b(0.071) d(0.929)	b(0.500) d(0.500)	c(1.000)	b(1.000)	a(1.000)	c(1.000)
pepB	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	b(0.063) c(0.938)	c(1.000)	c(1.000)	c(1.000)	b(0.188) c(0.813)	b(0.300) c(0.700)	c(1.000)	c(1.000)	c(1.000)	a(1.000)	d(1.000)
pepD1	b(1.000)	b(1.000)	b(0.917) c(0.083)	b(1.000)	a(0.250) b(0.583) c(0.167)	b(0.900) c(0.100)	a(0.500) b(0.500)	b(1.000)	b(1.000)	b(1.000)	b(1.000)	a(0.125) b(0.875)	a(0.100) b(0.900)	a(0.167) b(0.833)	b(1.000)	b(1.000)	b(1.000)	b(1.000)
pepD2	a(0.300) b(0.400) c(0.300)	b(1.000)	a(0.077) b(0.769) c(0.154)	b(0.100) c(0.900)	b(0.600) c(0.400)	a(0.400) c(0.600)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	b(0.533) c(0.467)	c(1.000)	a(0.833) b(0.167)	d(1.000)	c(1.000)	c(1.000)

Table 2. Continued.

locus ↓	CAP 5	ANC 4	PSO 14	ESP 5	PIC 6	PAL 5	OAT 11	AGU 8	CRO 8	ANA 3	FOR 2	RES 8	FAC 15	BUJ 6	BON 4	RIBE 6	RPYR 2	RMAC 4
6pgd	c(1.000)	c(1.000)	c(1.000)	c(1.000)	a(0.333) c(0.667)	a(0.100) c(0.900)	a(0.091) c(0.864) d(0.045)	a(0.286) c(0.643) d(0.071)	a(0.143) c(0.786) d(0.071)	a(0.333) c(0.500) d(0.167)	a(0.250) c(0.750)	a(0.313) c(0.625) d(0.063)	a(0.400) c(0.500) d(0.100)	a(0.167) c(0.750) d(0.083)	a(1.000)	b(1.000)	c(1.000)	c(1.000)
pgm	a(0.100) b(0.900)	a(0.250) b(0.750)	a(0.250) b(0.750)	a(0.600) b(0.400)	a(0.417) b(0.583)	a(0.900) b(0.100)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)	a(1.000)
pk	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	a(0.063) c(0.938)	a(0.167) c(0.833)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	c(1.000)	b(1.000)	b(1.000)	b(1.000)
H_e	0.135	0.123	0.131	0.115	0.144	0.147	0.132	0.115	0.086	0.091	0.056	0.092	0.145	0.092	0.064	0.000	0.019	0.000
H_o	0.100	0.115	0.134	0.125	0.104	0.113	0.113	0.112	0.072	0.069	0.042	0.068	0.129	0.087	0.066	0.000	0.021	0.000
P_{95}	0.292	0.292	0.417	0.375	0.292	0.375	0.333	0.292	0.250	0.167	0.083	0.250	0.375	0.292	0.125	0.000	0.042	0.000
A	1.375	1.292	1.583	1.375	1.375	1.458	1.417	1.417	1.333	1.208	1.125	1.333	1.542	1.333	1.167	1.000	1.042	1.000

Table 3. Pairwise genetic distances (N e i 1972) between *R. temporaria* populations and outgroup samples.

	CAP	ANC	PSO	ESP	PIC	PAL	OAT	AGU	CRO	ANA	FOR	RES	FAC	BUJ	BON	RIBE	RPYR
ANC	0.029																
PSO	0.044	0.058															
ESP	0.046	0.092	0.055														
PIC	0.071	0.076	0.057	0.057													
PAL	0.102	0.117	0.066	0.060	0.056												
OAT	0.137	0.153	0.100	0.081	0.071	0.031											
AGU	0.126	0.150	0.189	0.071	0.076	0.025	0.020										
CRO	0.140	0.146	0.100	0.090	0.084	0.030	0.017	0.013									
ANA	0.166	0.176	0.133	0.115	0.114	0.051	0.041	0.015	0.024								
FOR	0.173	0.170	0.140	0.124	0.117	0.048	0.033	0.022	0.018	0.010							
RES	0.152	0.158	0.118	0.106	0.096	0.039	0.021	0.014	0.008	0.019	0.017						
FAC	0.133	0.117	0.092	0.110	0.079	0.042	0.039	0.027	0.029	0.031	0.033	0.018					
BUJ	0.146	0.139	0.109	0.081	0.068	0.021	0.022	0.030	0.020	0.048	0.033	0.023	0.042				
BON	0.216	0.216	0.193	0.193	0.165	0.126	0.182	0.137	0.168	0.167	0.199	0.165	0.142	0.198			
RIBE	0.534	0.534	0.490	0.490	0.451	0.437	0.464	0.445	0.436	0.454	0.449	0.468	0.456	0.426	0.556		
RPYR	0.308	0.308	0.248	0.248	0.282	0.223	0.207	0.216	0.187	0.228	0.214	0.204	0.217	0.211	0.379	0.531	
RMAC	0.604	0.664	0.573	0.573	0.639	0.503	0.539	0.513	0.523	0.537	0.536	0.536	0.561	0.538	0.633	0.693	0.531

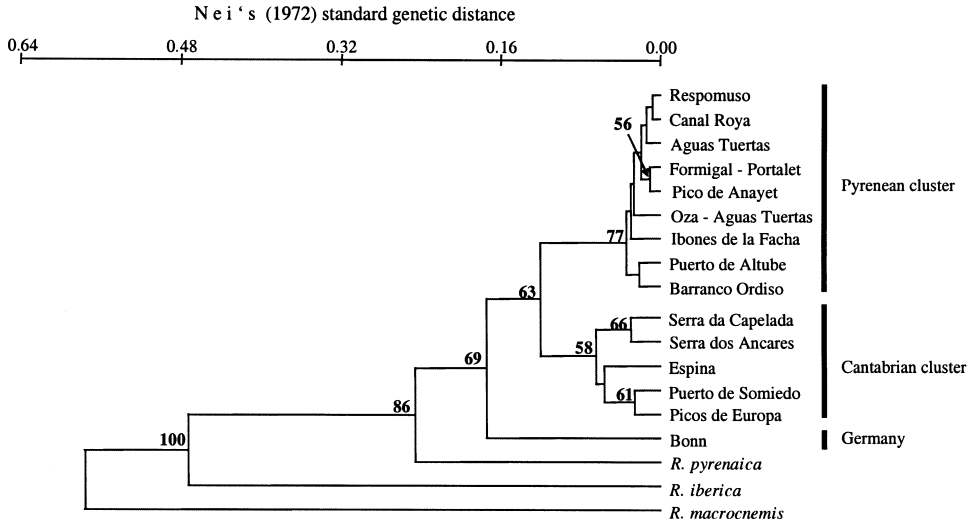

Fig. 2. UPGMA phenogram of all samples based on N e i ' s (1972) standard genetic distances; bootstrap p-values >50% for 1000 replicates are shown.

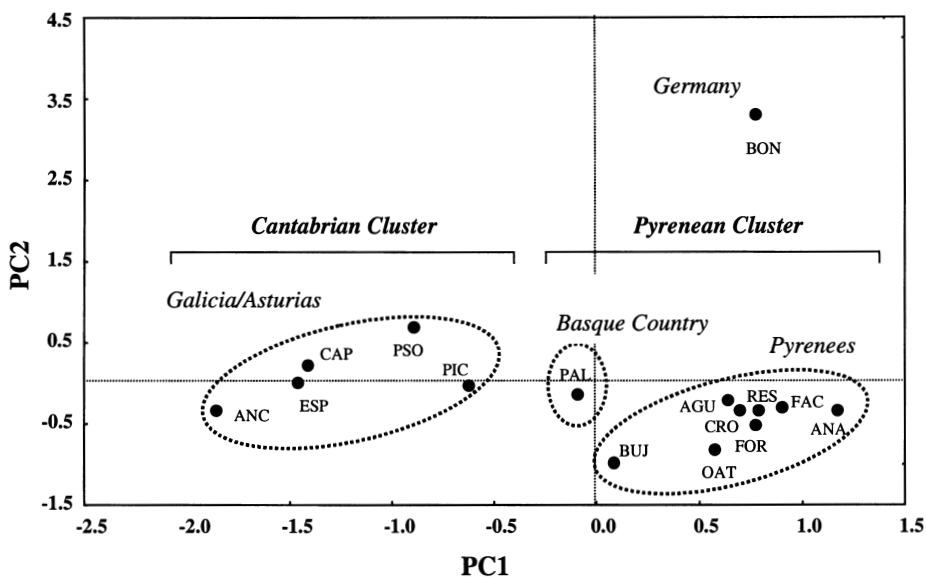

Fig. 3. Scatterplot of the first two principal components of genetic variance.

The mean genetic distance of $D = 0.121$ between the Pyrenean and the Cantabrian cluster (Tables 3 and 4) was not due to fixed allelic differences. The genetic distances to the German sample were considerably higher ($D = 0.161$ and 0.197, respectively). Of the three outgroup species, *R. pyrenaica* showed the closest affinities to *R. temporaria* (*D* ranged from 0.212–0.379).

In only five populations one out of 6–11 polymorphic loci deviated from Hardy-Weinberg proportions at the 5% level (*gpi* in OAT, *idh1* in PSO, *pepB* in FAC, *pepD1* in RES, and *pepD2* in PIC). However, after Bonferroni correction none deviated significantly.

Table 4. Mean ± SD of N e i ' s (1972) genetic distances between clusters and taxa (minimum and maximum distances are given in parentheses). The Pyrenean cluster of *R. temporaria* includes the populations from Euskadi and Aragon; the Cantabrian cluster includes those from Galicia and Asturias (see Fig. 2).

Taxon	*R.temporaria* (Pyrenean cluster)	*R.temporaria* (Cantabrian cluster)	*R.temporaria* (Germany)	*R.pyrenaica*	*R.iberica*
R. temporaria (Pyrenean cluster)	**0.027** ± 0.011 (0.008-0.051)				
R. temporaria (Cantabrian cluster)	**0.121** ± 0.032 (0.056-0.189)	**0.059** ± 0.018 (0.029-0.092)			
R. temporaria (Germany)	**0.161** ± 0.024 (0.126-0.199)	**0.197** ± 0.021 (0.165-0.216)	-----		
R. pyrenaica	**0.212** ± 0.013 (0.187-0.228)	**0.279** ± 0.030 (0.248-0.308)	**0.379**	-----	
R. iberica	**0.451** ± 0.012 (0.436-0.468)	**0.500** ± 0.035 (0.451-0.534)	**0.556**	**0.531**	-----
R. macrocnemis	**0.531** ± 0.018 (0.503-0.561)	**0.623** ± 0.046 (0.573-0.664)	**0.633**	**0.531**	**0.693**

Thirty percent of the total genetic variance of all samples was due to within population variation (F_{IS} = 0.122). Seventy percent (F_{ST} = 0.284) was distributed among populations (Table 5). In the Cantabrian cluster the situation was similar, whereas samples from the Pyrenean cluster were much more homogeneous (only 44% of genetic variance distributed among populations). The degree of population subdivision was also less pronounced (F_{ST} = 0.109) in the Pyrenean cluster than in the Cantabrian cluster (F_{ST} = 0.195).

Six loci (*apk, ck, gldh, gapd, ldh2, tre*) were monomorphic among all studied brown frog species (Table 2). Of the remaining loci only *mdh* was monomorphic among *R. temporaria* samples. None of the 17 loci polymorphic within *R. temporaria* was diagnostic for either population or geographic cluster. Several alleles that were present in more than one population of one cluster were absent or almost completely absent in the other: (a) allele a of *6pgd,* allele d of *6pgd*, allele c of *gpi*, allele b of *me2* and allele c of *mpi* were characteristic for the Pyrenean cluster; (b) allele c of *ldh1*, allele b of *pepD2* and allele b of *pgm2* were present in all or almost all populations of the Cantabrian cluster but almost entirely absent in all populations of the Pyrenean cluster. The mean frequency of the latter three alleles decreased from West to East along the Cantabrian chain (Fig. 4). In the Galician and Asturian populations, their combined average frequency ranged from 0.55 to 0.4, followed by a steep decrease between the easternmost Asturias population (PIC) and the single population from the Basque country (PAL). The latter was clearly grouped within the Pyrenean cluster (mean genetic distance to other populations of this cluster: 0.036). However, PAL also showed a low genetic distances to PIC which is the geographically nearest population of the Cantabrian cluster (D = 0.056).

Table 5. W e i r & C o c k e r h a m ' s (1984) *F*-statistics averaged over 17 polymorphic loci of all *R. temporaria* populations with n ≥ 4, and of subsamples from the Cantabrian and Pyrenean clusters. Standard deviations (SD) were obtained by jackknifing samples. Populations FOR and ANA were excluded from the analysis due to their low sample size.

Samples	*n*	F_{IT}	SD(F_{IT})	F_{IS}	SD(F_{IS})	F_{ST}	SD(F_{ST})
Cantabrian cluster	6	0.277	0.056	0.102	0.070	0.195	0.044
Pyrenean cluster	7	0.233	0.024	0.139	0.023	0.109	0.028
all samples	12	0.371	0.033	0.122	0.028	0.284	0.039

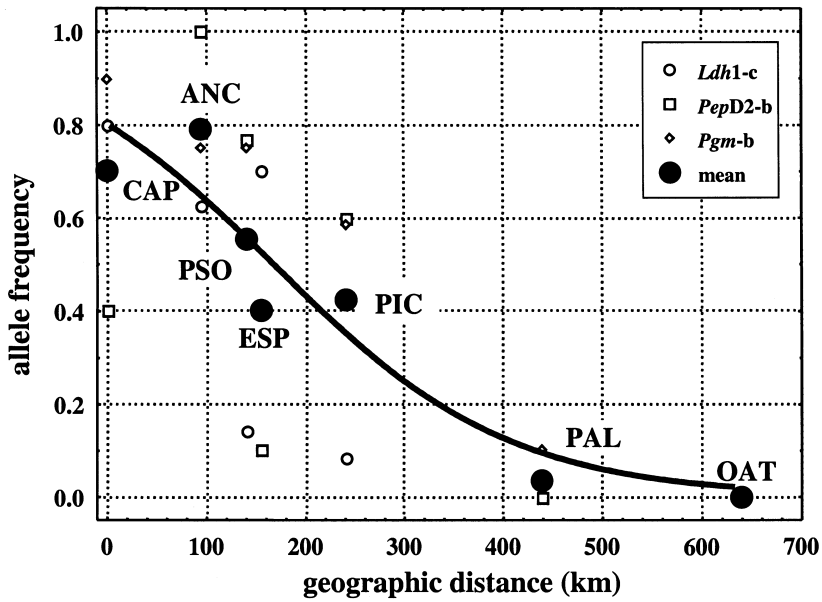

Fig. 4. Changes in frequencies of "typical" *R. t. parvipalmata* alleles (*ldh1*-c, *pepD*2-b and *pgm*-b) from the easternmost population (CAP) to population OAT in the West; we fitted a logistic regression model ($p < 0.05$) to describe the decrease of average allele frequencies from East to West.

Discussion

Our data demonstrate the existence of two geographically separated genetic lineages of *R. temporaria* in Iberia. The lack of fixed allelic differences between these clusters contradicts the results of A r a n o et al. (1993). They found fixed allelic differences between a *R. t. parvipalmata* and an Alava-Barcelona-Germany cluster at two loci, *icd-2* and *lcd-2*, which should be homologous to our loci *idh1* and *ldh1*. In our study, both loci show frequency differences between the Cantabrian and the Pyrenean clusters, but no fixed alternative alleles. This may at least partially be explained by the small size of some samples of A r a n o et al. (1993; only two specimens in the geographically intermediate Asturias and Alava samples) with a low chance to detect comparatively rare alleles.

C l i n a l v a r i a t i o n i n a l l e l e f r e q u e n c i e s

The lack of fixed allelic differences between the two clusters may account for either a short separation time or for gene flow following secondary contact. Indication for the latter may be the decrease of mean frequency of *R. t. parvipalmata* alleles from West to East which resembles a clinal pattern (discordant among alleles, but evident when averaging allele frequencies over loci). No such cline exists for typical Pyrenean alleles.

In principle, several scenarios may account for this pattern: (1) an initial polymorphic but homogeneous population broke up into local populations; (2) an initial isolation-by-distance structure produced frequency clines at single loci; afterwards it broke up into local populations; (3) two populations evolved divergently in isolation and formed a cline after secondary contact; gene flow among local populations subsequently broke up again.

After the break up of a homogeneous polymorphic population into isolated subpopulations (scenario 1) drift would produce a geographically irregular pattern of allele frequencies, being clinal neither at single loci nor on average. An initial isolation-by-distance pattern with subsequent isolation of local populations (scenario 2) would well explain the irregular frequency pattern of single alleles. Again there is no rationale to assume that on average a cline would result since isolation-by-distance would produce non-parallel clines at different loci.

We therefore prefer scenario 3. It well explains (i) clinal variation when averaging loci, since two populations that differentially evolved in isolation would always produce a cline when hybridising after secondary contact, and (ii) the irregular frequencies pattern at single loci due to genetic drift after subsequent isolation. The high inbreeding coefficients (F_{ST}) indicate that the degree of isolation among populations is still high, even within geographical clusters. Whether there exists a transition zone between the two clusters with ongoing gene flow remains open. If gene flow still does occur between clusters, it is likely to take place somewhere between PIC and PAL.

Large parts of the montane areas of the Pyrenees and of the Cantabrian mountain chain which are currently densely populated by *R. temporaria* did not constitute suitable amphibian habitats during glaciation periods. The genetic differentiation of Spanish *R. temporaria* populations as found in the present study could be explained by a scenario in which two separated groups of populations, one in Galicia/Northern Portugal (where refuges of deciduous forests existed during glaciation maxima; see Barbadillo et al. 1997), and one more to the east, remained in isolation during considerable time. At the end of the glaciations, the two populations, meanwhile genetically differentiated, came into secondary contact. It may well be that since the Pleistocene such a process of range expansion and retreat may have occurred repeatedly, resulting in a geographical mosaic of allele frequencies as is discussed for Spanish *Salamandra salamandra* (Alcobendas et al. 1994, 1996) and for many organisms in general (e.g., Taberlet et al. 1998). However, the observed pattern in Spanish *R. temporaria* would not be in conflict with the much simpler scenario of gene flow through a single secondary contact and genetic drift subsequently altering allele frequencies at random in isolation.

Taxonomic conclusions

Rana pyrenaica, which is morphologically and ecologically rather similar to the (as far as known allopatrically distributed) *R. iberica*, showed a much closer genetic affinity to *R. temporaria* (Table 4). Its specific differentiation is for the first time corroborated on genetic grounds, however, a detailed discussion of its relationships to other western palearctic brown frogs will be given elsewhere.

The total absence of fixed alleles characterising any Spanish population or cluster together with the low genetic distance between the Cantabrian and the Pyrenean cluster (see Veith 1996 for a review of species-specific genetic distances among European amphibians) support Arano's et al. (1993) conclusion that the Cantabrian and the Pyrenean *R. temporaria* populations are differentiated at the subspecies level without any further taxonomically relevant substructure. Therefore, the hypotheses of Vences et al. (1998a, b) that more than one species of the *Rana temporaria* complex may occur in the Pyrenees can be rejected. We also reject the theory of Palanca et al. (1995) who assumed co-existence of two separated taxa at localities in the Aragonese Pyrenees (Circo de Piedrafita), which corresponds to our locality RES. This and all other populations are in

Hardy-Weinberg equilibrium, which is not expected when different taxa are pooled into one sample. In addition, ongoing morphological studies of the Circo de Piedrafita population, including more than 1000 specimens, failed to discriminate two separate morphs (M. V e n c e s , pers. obs. 1998 and 1999). A specific status of the taxon *R. aragonensis* from the Circo de Piedrafita can thus be excluded.

On the other hand, it remains true that the different Pyrenean populations of *Rana temporaria* included in this study are markedly heterogeneous in their external morphology. For example, the population from Valle de Bujaruelo (BUJ) is composed of very large specimens (males 78–86 mm, females 77–100 mm SVL; N=2/3), whereas only a minor proportion of specimens of other populations reach a similar size (only 12 out of 828 males from Respomuso (RES) reached a SVL >78 mm, and none exceeded 80 mm; V e n c e s et al. 1999). Many specimens from the high-altitude populations (e.g. RES) had a large number of black dorsal markings (R i o b ó et al. 2000) which were absent in the lower-altitude population PAL. Relative hindlimb length was also variable among populations (M. V e n c e s pers. obs.), and within the Cantabrian cluster important differences are found between the small, long-legged and poorly webbed CAP specimens and the larger PIC specimens, several of which have relatively short legs and more extensive webbing. This variation may better be explained by ecological parameters.

The CAP population lives close to the type locality of *R. temporaria parvipalmata* (Seoane, 1885). The Cantabrian cluster therefore corresponds to this taxon. In contrast, the Pyrenean cluster may correspond to *R. t. canigonensis* Boubee, 1833. The fact that in the study of A r a n o et al. (1993) the easternmost included *R. temporaria* population from the Montseny massif in Catalonia clustered close to a population from Basque Country makes it likely that the whole Pyrenees between Catalonia and Basque Country are inhabited by a genetically homogeneous group of populations. Consequently, the taxon *aragonensis* is to be seen as junior synonym of *R. t. canigonensis*.

A c k n o w l e d g e m e n t s

Thanks are due to Pedro Galan R e g a l a d o (La Coruña) for his help in the field, and Alain Dubois (Paris) for important advice. Nicolas P a l a n c a (Vigo) helped during collection of specimens in Spanish high-altitude habitats. Thanks to Dagmar K l e b s c h for laboratory assistance. We are indebted to the regional authorities of Galicia, Asturias, Euskadi, and Aragon for the permit of collection of specimens.

L I T E R A T U R E

ALCOBENDAS, M., DOPAZO, H. & ALBERCH, P., 1994: Genetic structure and differentiation in *Salamandra salamandra* populations from the Northern Iberian Peninsula. In: Greven, H. & Thiesmeier, B. (eds), Biology of *Salamandra* and *Mertensiella. Deutsche Gesellschaft für Herpetologie und Terrarienkunde, Bonn: 7–23.*

ALCOBENDAS, M., DOPAZO, H. & ALBERCH, P., 1996: Geographic variation in allozymes of populations of *Salamandra salamandra* (Amphibia: Urodela) exhibiting distinct reproductive modes. *J. Evol. Biol., 9: 83–102*.

ARANO, B., ESTEBAN, M. & HERRERO, P., 1993: Evolutionary divergence of the Iberian brown frogs. *Ann. Sci. Nat., Zool., 14: 49–57.*

BARBADILLO, L. J., GARCÍA-PARÍS, M. & SANCHIZ, B., 1997: Orígenes y relaciones evolutivas de la herpetofauna ibérica. In: Pleguezuelos, J. M. (ed.), Distribución y biogeografía de los anfibios y reptiles en España y Portugal. *Granada, Universidad de Granada and AHE: 47–100*.

DUBOIS, A., 1983: Notes sur les grenouilles brunes (Groupe de *Rana temporaria* Linné, 1758). II. Les Grenouilles du Mont Canigou (Pyrenees Orientales). *Alytes, 2: 19–26*.

DUBOIS, A., 1992: Notes sur la classification des Ranidae (Amphibiens Anoures). *Bull. mens. Soc. linn. Lyon*, *61: 305–352*.

DUBOIS, A., 1998: Mapping European amphibians and reptiles: collective inquiry and scientific methodology. *Alytes, 15: 176–204*.

ESTEBAN, R., 1997a: *Rana iberica.* In: Pleguezuelos, J.M. (ed.), Distribución y biogeografía de los anfibios y reptiles en España y Portugal. *Granada, Universidad de Granada and AHE: 161–163.*

ESTEBAN, R. 1997b: *Rana temporaria.* In: Pleguezuelos, J.M. (ed.), Distribución y biogeografía de los anfibios y reptiles en España y Portugal. *Granada, Universidad de Granada and AHE: 169–171.*

FELSENSTEIN, J., 1985: Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39: 783–791*.

FELSENSTEIN, J., 1993: PHYLIP, version 3.5. *Washington*.

GALÁN, P., 1985: Morfología y fenología del tritón palmeado, *Triturus helveticus* (Razoumowsky, 1789) en el noroeste de la Península Ibérica. *Alytes (Spain), 3: 31–50*.

GOSÁ, A., 1997: *Rana dalmatina.* In: Pleguezuelos, J.M. (ed.), Distribución y biogeografía de los anfibios y reptiles en España y Portugal. *Granada, Universidad de Granada and AHE: 158–160.*

GROSSENBACHER, K., 1997: *Rana temporaria* Linnaeus, 1758. In: Gasc, J. P., Cabela, A., Crnobrnja-Isailovic, J., Dolmen, D., Grossenbacher, K., Haffner, P., Lescure, J., Martens, H., Martínez-Rica, J. P., Maurin, H., Oliveira, M. E., Sofianidou, T. S., Veith, M. & Zuiderwijk, A. (eds), Atlas of Amphibians and Reptiles in Europe. *Societas Europaea Herpetologica and Muséum National d'Histoire Naturelle (IEGB/SPN), Paris: 158–159*.

HEBERT, P. D. N. & BEATON, M. J., 1993: Methodologies for allozyme analysis using cellulose acetate electrophoresis. *Helena Laboratories, Beaumont*.

LLORENTE, G., MONTORI, A., SANTOS, X. & CARRETERO, M. A., 1995: Atlas dels Amfibis i Rèptils de Catalunya i Andorra. *Edicions El Brau, Barcelona.*

NEI, M., 1972: Genetic distance between populations. *Amer. Nat*., *106: 283–292*.

PALANCA SOLER, A., RODRIGUEZ VIEITES, D. & SUÁREZ MARTÍNEZ, M., 1995: Contribución al estudio anatómico del géneo *Rana* L., 1758 en el alto Aragón. *Lucas Mallada (Huesca), 7: 227–247*.

RICE, W. R., 1989: Analyzing tables of statistical tests. *Evolution, 43: 223–225*.

RIOBÓ, A., REY, J., PUENTE, M., MIRAMONTES, C. & VENCES, M., 2000: Ontogenetic increase of black dorsal pattern in *Rana temporaria*. *Brit. Herpetol. Soc*. *Bull., 70*: *1–6*.

ROHLF, F J., 1990: NTSYS: Numerical taxonomy and multivariate analysis system, version 1.60. *Setauket, NY*.

SERRA-COBO, J., 1993: Descripción de una nueva especie europea de rana parda (Amphibia, Anura, Ranidae). *Alytes, 11: 1–15.*

SERRA-COBO, J., 1997: *Rana pyrenaica.* In: Pleguezuelos, J.M. (ed.), Distribución y biogeografía de los anfibios y reptiles en España y Portugal. *Granada, Universidad de Granada and AHE: 167–168.*

SIGISMUND, H. R., 1997: GSTAT, version 3. *München*.

SPERLING, P., VENCES, M. & BÖHME, W., 1996: Vorläufige Bemerkungen zum taxonomischen Status von *Rana temporaria honnorati* Heron-Royer, 1881. *Salamandra, 32*: *99–112*.

STRIJBOSCH, H., 1996: Pas ontdekt: *Rana pyrenaica,* de Pyreneeën-kikker. *Lacerta, 54: 53–55*.

TABERLET, P., FUMAGALLI, L., WUST-SAUCY, A.-G. & COSSON, J.-F., 1998: Comparative phylogeography and postglacial colonization routes in Europe. *Mol. Ecol.*, *7: 453–464.*

VEITH, M., 1996: Molecular markers and species delimitation: examples from the European batrachofauna. *Amph.-Rept., 17: 303–314*.

VENCES, M., 1992: Zur Biologie der nordwestspanischen Braunfrösche *Rana iberica* Boulenger, 1879 und *Rana temporaria parvipalmata* Seoane, 1885. *Salamandra, 28: 61–71*.

VENCES, M., PALANCA SOLER, A., RODRIGUEZ VIEITES, D. & NIETO ROMAN, S., 1998a: Designation and description of a lectotype of *Rana aragonensis* Palanca Soler et al., 1995 (Anura: Ranidae). *Herpetozoa, 10: 129–134*.

VENCES, M., KUPFER, A., LLORENTE, G.A., MONTORI, A. & CARRETERO, M.A., 1998b: Description of the larval stages of the Pyrenean frog, *Rana pyrenaica* Serra-Cobo, 1993 (Amphibia: Ranidae). *Boll. Mus. reg. Sci. nat. Torino*, *15: 1–23*.

VENCES, M., PIQUÉ, N., LOPEZ, A., PUENTE, M., MIRAMONTES, C. & RODRIGUEZ, D., 1999: Summer habitat population estimate and body size variation in a high altitude population of *Rana temporaria. Amph.-Rept., 20: 440–443*.

WEIR, B. S. & COCKERHAM, C. C., 1984: Estimating *F*-statistics for the analysis of population structure. *Evolution*, *38: 1358–1370*.

WRIGHT, S., 1951: The genetical structure of populations. *Ann. Eugenics*, *1: 323–354*.