HERPETOLOGICAL JOURNAL, Vol., pp. 57-64 (1998)

DIVERSIFICATION IN NORTH-WEST AFRICAN WATER FROGS: MOLECULAR AND MORPHOLOGICAL EVIDENCE

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We have assessed the consistency of allozyme and morphometric data sets in discriminating water frogs at inter- and intraspecific level. Twenty allozyme loci and 14 morphometric characters were used in a study on Iberian and North African water frogs. The results from the morphometric analysis, using PCA, confirmed the interspecific differences between *Rana perezi* from the Iberian Peninsula and *Rana saharica* from North-west Africa previously detected by allozyme analysis. Allozyme and morphometric data were also consistent in discerning between Algerian and Moroccan populations of *R. saharica*, pointing to the presence of at least two subspecies in the Maghreb: *R. saharica saharica* from Algeria and *R. saharica riodeoroi* from Morocco. A possible paleobiogeographical scenario of the divergence between the two groups is discussed.

INTRODUCTION

The taxonomy of Palearctic water frogs has been disputed in recent years following the work by Berger (1968,1973,1977). This has yielded a new view of the classification of the group (Dubois & Ohler, 1994*a*) and in some cases created a debate over nomenclature (Dubois, 1991; Hotz, Uzzell, Beerli & Guex, 1996). The main reason for this complexity stems from the capacity of several species to hybridize. The reproductive mechanism used by some of these hybridizing species has largely added to the confusion. This mechanism, known as hybridogenesis, yields fertile hybrid progeny which are capable of hemiclonal reproduction after excluding one of the parental genomes (Schultz, 1969; Graf & Polls-Pellaz, 1989). However, it seems evident that these hybridogenetic hybrids only occur in certain areas of the water frog distribution, and they all carry the genome of *R. ridibunda* (Hotz, Mancino, Bucci-Innocenti, Ragghianti, Berger, & Uzzell, 1985), capable of inducing exclusion. The most widespread hybrid is *R. esculenta*, which arises from hybridization between the Mendelian species *R. lessonae* and *R. ridibunda*. The distributions of the other hybrids such as *R. grafi* or *R. hispanica* are constrained to smaller areas (Graf &Polls-Pellaz, 1989; Crochet, Dubois, Ohler & Tunner, 1995).

It is only recently that the taxonomic status of the remaining Mendelian water frog species is beginning to be understood, especially in the case of the Aegean (Beerli, Hotz, Tunner, Heppich & Uzzell, 1994) and Middle East water frogs (Schneider, Sinsch & Nevo, 1992). The Maghrebian region has posed problems after Hemmer, Konrad & Bachman (1980) indicated the presence of a hybrid complex in North Africa and Steinwarz & Schneider (1991) extended the range of *R. perezi* beyond the Iberian Peninsula into Morocco, Algeria and Tunisia. Both propositions seem unlikely in the light of the latest findings based on allozyme differentiation (Arano, Llorente, Herrero & Sanchíz, 1994; Beerli,1994; Buckley, Arano, Herrero, Llorente & Esteban, 1994), which confirm that hybridogenetic populations are not found beyond the north-east of the Iberian Peninsula and that the range of *R. perezi* does not extend to the north of Africa. On the contrary, the species present in the north of Africa would be *Rana saharica*. The differences between *Rana perezi* and *Rana saharica* are further supported by data on larval morphology (Llorente, Arano, Carretero, García-Paris, Herrero & Esteban, 1996).

However, *Rana saharica* is more diversified than originally thought. This is what the allozyme studies by Buckley *et al.* (1994) disclosed in a preliminary survey comparing Moroccan and Algerian water frog populations, suggesting that a different subspecies should be attributed to each country. Nevertheless, differentiation at a molecular level did not seem complemented by clearly discriminating morphological characters. Hence, the purpose of this paper is to make a comparative study at molecular and morphological levels, trying to assess the consistency of both approaches in discriminating at species (*R. perezi* and *R. saharica*) and subspecies level (Moroccan and Algerian *R. saharica*). A parallel aim of the study is to find consistent morphological characters which can be used to differentiate these taxa in the field.

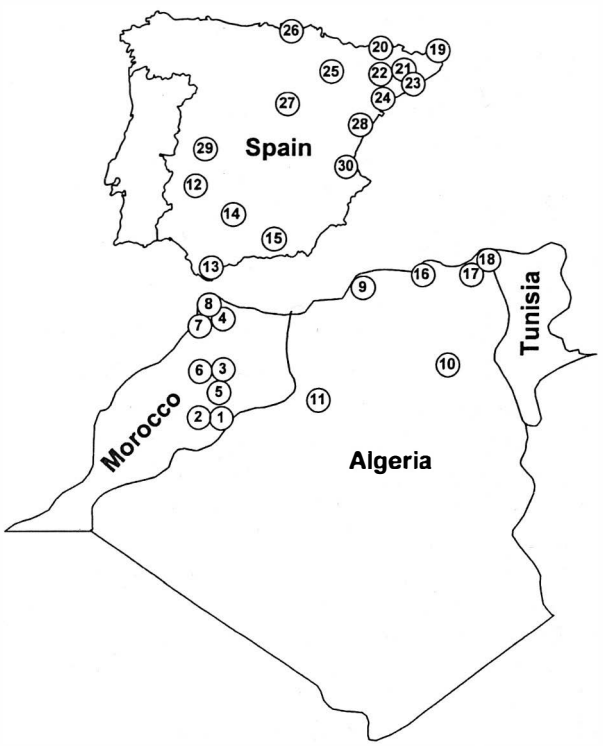

FIG. 1 Localities used in the allozyme and/or morphometric analyses. Allozyme and morphometric analysis: 1 Maadid, 2 Krair, 3 Annoceur, 4 Souk el Arba des beni Hassan, 5:N'Zala, 6 Dayed Aaoua, 7 Larache, 8 Zinat, 9 Chiffa River, 10 Tegernina, 11 Namous, 12 Mérida, 13 Tarifa, 14 Córdoba, 15 Nerja. Morphometric analysis only (sample size in brackets): 16 Djurjura (4), 17 El Tarf (4), 18 Tabarka (3), 19 Port Bou (5), 20 Pont de Suert (2), 21 Moianés (3), 22 Lleida (3), 23 Barcelona (6), 24 Tarragona (6), 25 Huesca (2), 26 Euskadi (2), 27 Soria (2), 28 Castellón (12), 29 Cáceres (6), 30 Valencia (6).(Although Tabarka lies in Tunisia, on the limit with the Algerian border, we refer to it as "Algerian" in the text to avoid semantic confusion.)

MATERIALS AND METHODS

Fig. 1 shows the localities used in both the electrophoretic and morphometric studies. In the case of the electrophoretical survey, samples of heart, liver, muscle and stomach were removed in the field, after animals were anaesthetised with MS222 (Sandoz), frozen and stored at -70°C. Tissues were later homogenized, and centrifuged, and the supernatant was used on standard horizontal starch gel electrophoresis (see Buckley *et al.*, 1994, for details on electrophoretic conditions). A total of 20 presumptive loci were examined: aspartate aminotransferase (AAT, EC 2.6.1.1.), alcohol-deshydrogenase (ADH, EC 1.1.1.1), adenylate-kinase (AK, EC 2.7.4.3), esterases (EST, EC 3.1.1.-.), glucosephosphate-isomerase (GPI, EC 5.3.1.9), glucose-6-phosphate-dehydrogenase (G6PD, 1.1.1.49), alpha-glycerophosphate-deshydrogenase (G3PDH, EC 1.1.1.8), isocitrate-deshydrogenase (IDH, EC 1.1.1.42), lactate-deshydrogenase (LDH, EC1.1.1.27), malate-deshydrogenase (MDH, EC 1.1.1.37), mannosephosphate-isomerase (MPI, EC 5.3.1.8), Peptidase-C (with leucine-alanine as substrate, PEP-C, EC 3.4.13.*), peptidase-D (with phenyl-proline as substrate, PEP-D, EC 3.4.13.9), phosphogluconate deshydrogenase (6PGDH, EC 1.1.1.44), phosphoglucomutase (PGM, EC 2.7.5.1) and superoxyde-dismutase (SOD, EC 1.15.1.1.). Allele frequencies were used in the computation of Nei's (1978) and Rogers' (1972) genetic distances by means of the BIOSYS-1 (Swofford & Selander, 1981) program. UPGMA and distance Wagner dendrograms were constructed using the distance coefficients.

Only adults were used in the morphological analysis. The measurements used were: LCC: body length (head-urostyle); MA: forelimb length; MP: hindlimb length; F: femur length; TM: length of metatarsal tuberculum; T: tibial-fibular length; P: foot length; ASE: head width; IO: interocular distance; DN: distance between nasal apertures; NO: eye-nasal aperture distance; O: eye diameter; OT: eye-tympanum distance; T: tympanum diameter.

A univariate analysis was carried out on the distribution of each variable. The presence of intersexual or interspecific differences was contrasted by means of a two-way ANCOVA (main factors: species and sex), using LCC as covariate. Logarithmic transformed data were used in the analyses. A canonical Principal Components Analysis (PCA) was used to study the variation and divergence within the morphometric characters. The variables used for this analysis were obtained from a previous factorial PCA. Using more representative variables, a discriminant function was obtained that was capable of differentiating between *R. perezi* and *R. saharica* and between the two *R. saharica* types. These functions were estimated using raw values in order to facilitate their use in the field. Morphometric relationships were corroborated by means of Mahalanobis' distances calculated between each group centroids.

RESULTS

The results of the allozyme analysis are shown in Table 1, where the allele frequencies for all the populations examined are given. Diagnostic loci for *R. perezi* and *R. saharica* were pointed out in Buckley *et al.* (1994). In the case of the north African species, Sod is a diagnostic locus for the Moroccan populations while Pep-D is diagnostic for those from Algeria (Table 1). These latter populations are also characterized by several exclusive alleles such as Est-1 a, G3pdh d, Idh-1 a, Ldh-A c, Ldh-B g and Mdh d and e. These differences explain the genetic distances found between Moroccan and Algerian populations (Buckley *et al.*, 1994 and Table 2). The Distance Wagner tree obtained using Rogers' genetic distances and *R. ridibunda* from Greece as an outgroup (Fig. 2a), shows two clearly distinct groups corresponding to *Rana perezi* and *Rana saharica*. However, within the latter grouping there are two distinct clusters corresponding to the Moroccan and Algerian populations. All distance/clustering method combinations yielded equal results, with only slight differences in the resolution of the internal branches of the Moroccan group.

TABLE 1. Allele frequencies across populations. Locality numbers correspond to those in Fig. 1. (N)= number of individuals

	POPULATIONS														
	Morocco									Algeria		Spain			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
LOCI															
AAT															
(N)	19	45	6	10	17	8	29	9	2	4	6	5	5	9	3
A	-	-	-	-	.029	-	-	-	-	-	-	-	-	-	-
B	1.000	1.000	1.000	1.000	.971	1.000	1.000	1.000	1.000	1.000	1.000	.700	.600	.667	.667
C	-	-	-	-	-	-	-	-	-	-	-	.300	.400	.333	.333
ADH-1															
(N)	13	40	10	7	13	8	9	9	2	4	6	5	6	8	5
A	.038	.038	-	.286	-	.062	-	-	-	-	-	.200	-	-	-
B	.962	.962	1.000	.714	1.000	.938	1.000	1.000	1.000	1.000	1.000	.800	1.000	1.000	1.000
ADH-2															
(N)	10	38	10	2	10	8	9	7	2	1	1	4	6	7	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	-	.132	-	-	.200	-	-	.143	-	-	-	1.000	1.000	1.000	1.000
C	1.000	.868	1.000	1.000	.800	1.000	1.000	.857	1.000	1.000	1.000	-	-	-	-
AK															
(N)	29	5	5	9	7	3	16	3	1	4	5	3	5	7	1
A	.018	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	.948	1.000	1.000	1.000	1.000	1.000	.906	1.000	1.000	1.000	.900	1.000	.800	1.000	1.000
C	-	-	-	-	-	-	.063	-	-	-	.100	-	.200	-	-
D	.034	-	-	-	-	-	.031	-	-	-	-	-	-	-	-
EST-1															
(N)	16	17	3	8	11	1	3	2	2	4	6	4	3	4	3
A	-	-	-	-	-	-	-	-	-	.500	-	-	-	-	-
B	.781	.882	.833	.250	.591	-	.667	1.000	.250	.500	.250	-	-	-	-
C	-	-	-	-	.045	-	-	-	.500	-	-	.750	1.000	.750	.667
D	.219	.118	.167	.750	.364	1.000	.333	-	.250	-	.750	.250	-	.250	.333
EST-2															
(N)	17	13	15	10	4	7	22	8	2	4	5	5	6	6	5
B	.853	.346	.200	.450	.375	.357	.909	1.000	-	.500	-	.200	.750	.417	.800
C	.147	.615	.767	.550	.625	.643	.091	-	1.000	.500	1.000	.500	.250	.333	.200
D	-	.038	.033	-	-	-	-	-	-	-	-	.300	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-	-	.250	-
G6PD															
(N)	19	5	5	7	9	7	4	3	2	1	1	5	6	5	1
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
G3PDH															
(N)	22	45	11	9	17	8	25	9	2	4	6	5	6	9	5
B	-	-	.273	-	-	-	.020	-	-	-	-	-	-	-	-
C	1.000	1.000	.727	1.000	1.000	1.000	.980	1.000	1.000	.875	1.000	1.000	1.000	1.000	1.000
D	-	-	-	-	-	-	-	-	-	.125	-	-	-	-	-
GPI															
(N)	23	37	18	10	17	7	18	9	2	3	5	5	6	9	5
A	-	-	-	-	-	-	.028	-	-	-	-	-	-	-	-
B	.804	.986	1.000	1.000	1.000	1.000	.972	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	.196	.014	-	-	-	-	-	-	-	-	-	-	-	-	-
IDH-1															
(N)	22	36	17	10	17	8	27	9	2	4	6	5	6	9	5
A	-	-	-	-	-	-	-	-	.500	-	.583	-	-	-	-
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.500	1.000	.417	1.000	1.000	1.000	1.000
IDH-2															
(N)	22	41	16	10	17	8	29	9	2	4	5	5	5	9	5
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.900	.900	1.000	.700
B	-	-	-	-	-	-	-	-	-	-	-	.100	.100	-	.300
LDH-A															
(N)	21	29	13	7	6	8	24	6	2	4	6	5	5	8	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	.119	.155	-	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000
C	-	-	-	-	-	-	-	-	.500	-	-	-	-	-	-
D	.881	.845	1.000	1.000	1.000	1.000	1.000	1.000	.500	1.000	1.000	-	-	-	-
LDH-B															
(N)	37	45	18	10	19	8	29	8	2	4	6	5	6	9	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E	.081	.200	-	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000
G	-	-	-	-	-	-	-	-	.500	-	-	-	-	-	-
H	.919	.800	1.000	1.000	1.000	1.000	1.000	1.000	.500	1.000	1.000	-	-	-	-
MDH															
(N)	27	44	18	10	19	8	29	9	2	4	6	5	6	9	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.100
B	.056	.023	-	-	.105	-	-	-	.500	-	.167	-	-	-	-
C	.944	.977	1.000	1.000	.895	1.000	1.000	1.000	.500	.625	.583	1.000	1.000	1.000	.900
D	-	-	-	-	-	-	-	-	-	.375	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	.250	-	-	-	-
MPI															
(N	21	44	15	9	17	7	29	9	2	4	6	5	5	9	5
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	1.000	.417	.900	.800	.611	1.000
C	-	.011	-	-	-	-	-	-	-	-	-	.100	.200	.389	-
D	.048	.045	.067	-	.118	-	.086	-	.750	-	.500	-	-	-	-
E	.952	.943	.933	1.000	.882	1.000	.914	1.000	.250	-	.083	-	-	-	-
PEP-C															
(N)	14	27	5	8	8	4	17	3	1	3	2	2	5	7	5
A	.786	.833	1.000	1.000	.562	1.000	.941	1.000	1.000	-	.250	.250	.800	.429	.800
B	-	.130	-	-	.063	-	.059	-	-	1.000	.750	-	.200	.571	.200
C	.214	.037	-	-	.375	-	-	-	-	-	-	.750	-	-	-
PEP-D															
(N)	13	22	11	4	9	2	8	9	2	4	3	1	6	2	3
A	-	-	-	-	-	-	-	-	1.000	1.000	1.000	-	-	-	-
C	1.000	1.000	1.000	1.000	.944	1.000	1.000	1.000	-	-	-	1.000	1.000	1.000	1.000
D	-	-	-	-	.056	-	-	-	-	-	-	-	-	-	-
6PGDH															
(N)	17	38	15	10	19	8	23	9	2	4	5	3	5	6	4
A	-	-	-	-	-	-	-	-	-	-	-	.500	.500	.500	.500
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	.500	.500	.500	.500
PGM															
(N)	26	41	18	10	17	8	27	5	2	4	6	5	6	9	5
A	-	.146	.167	-	.147	-	.019	.100	-	-	.083	-	-	-	-
B	1.000	.854	.833	1.000	.853	1.000	.981	.900	1.000	1.000	.917	1.000	1.000	1.000	1.000
SOD-1															
(N)	20	46	13	10	17	6	12	4	2	4	6	5	6	9	5
A	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-	-	-	-

TABLE 2. Mean genetic distances between groups of populations: Nei: Nei's (1972) genetic distance; Nei*: Nei's (1978) unbiased genetic distance; Rogers: Rogers' modified genetic distance; Cavalli: Cavalli-Sforza & Edwards arc distance; Cavalli*: Cavalli-Sforza & Edwards chord distance.

	NEI	NEI*	ROGERS	CAVALLI	CAVALLI*
Morocco	0.03	0.026	0.161	0.177	0.173
Algeria	0.131	0.100	0.190	0.352	0.335
Spain	0.034	0.015	0.167	0.183	0.183
Morocco-Algeria	0.231	0.224	0.425	0.432	0.403
Morocco-Spain	0.453	0.441	0.570	0.592	0.541
Algeria-Spain	0.453	0.437	0.553	0.586	0.539

TABLE 3. Results of factorial Principal Components Analysis.

	Factor 1	Factor 2	Factor 3
LOGLCC	0.96653	-0.172026	-0.011013
LOGMA	0.94673	-0.154758	-0.129836
LOGMP	0.95507	-0.208568	-0.122346
LOGF	0.97469	-0.041224	-0.054902
LOGT	0.95505	-0.219451	-0.083720
LOGP	0.94251	-0.177952	-0.142242
LOGASE	0.96283	-0.052926	-0.017892
LOGIO	0.86543	0.405423	0.064863
LOGDN	0.82380	0.289684	0.071100
LOGNO	0.86353	0.239011	0.246404
LOGO	0.84143	0.360570	-0.219881
LOGOT	0.79587	-0.236919	0.506620
LOGTI	0.91366	0.076275	-0.011488
Expl. vara.	10.76965	0.678739	0.437644
Prp. total	0.82843	0.052211	0.033665

TABLE 4. Standardized coefficients for canonical variables.

Variable	Root 1	Root 2
Factor 1	0.133	0.238
Factor 2	1.105	0.022
Factor 3	0.317	-0.122
Factor 4	0.261	0.410
Factor 5	-0.007	-0.301
Factor 6	0.240	-0.150
Factor 7	0.056	0.329
Factor 8	0.138	-0.584
Factor 9	-0.062	0.421
Factor 10	0.113	-0.369
Factor 11	-0.053	-0.462
Factor 12	0.327	-0.129
Factor 13	0.059	-0.124
Eigenvalue	4.128	0.430
Cum. prop.	0.906	1.000

TABLE 5. Discriminant prediction at interspecific level. %: Percentage of correctly classified individuals.

Species	%	*R. perezi*	*R. saharica*
Rana perezi	100.0	58	0
Rana saharica	94.7	2	36
Total	97.92	60	36

TABLE 6. Discriminant prediction at intraspecific level. %: Percentage of correctly classified individuals. *Rs*M: *Rana saharica* from Morocco; *Rs*A: *Rana saharica* from Algeria.

Species	%	*Rs*M	*Rs*A
*Rs*M	92.6	25	2
*Rs*A	81.8	2	9
Total	89.47	27	11

Due to the low number of individuals per locality, especially in the case of the Algerian sample, populations were pooled into three groups, Iberian Peninsula, Morocco and Algeria, for the morphometric study. This pooling was also supported by the results of the allozyme analysis. Previous to the populational morphometric analysis, an ANOVA showed no differences in the LCC between sexes of each species. Likewise, a two-way MANCOVA (sex-species), carried out for *Rana perezi* and *R. saharica*, did not show significant differences between sexes (Wilks λ =0.863, P=0.255) nor for the sex-species interaction (Wilks λ = 0.873, P=0.317), although it did show significant differences between species (Wilks λ =0.265, P<0.001). These differences were significant at P<0.05 for variables F, ASE, DN, NO, O and TI. The same type of analysis for *R. perezi* and the two *R. saharica* varieties showed similar results (Wilks λ =0.895, P=0.515; Wilks λ =0.772, P=0.364; Wilks λ =0.218 P<0.001), differences being significant at P<0.05 for variables MP, F, T, P, ASE, IO, DN, NO, O and TI. Since intersexual differences were not significant, the sexes were pooled for the remaining analyses.

Using the first three factors, factorial PCA explains 91.43% of the variance (Table 3). The factors extracted from this analysis have been used in a canonical PCA on the 13 morphological variables considered. The first factor was not eliminated, since the ANOVA using LCC of the three OTUs did not show significant differences ($F_{2,90}$=2.096, P=0.129). Results show that each group (*R. perezi* -*Rp*-, Moroccan *R.saharica* -*Rs*M- and Algerian *R.saharica* -*Rs*A-) is significantly different from the others, as indicated by the F statistics associated with Mahalanobis' distances (*Rp*-*Rs*M 21.04, P<0.0001; *Rp*-*Rs*A 9.91, P<0,0001; *Rs*M-*Rs*A, 2.57 P=0.005). Subsequent classifications using this same

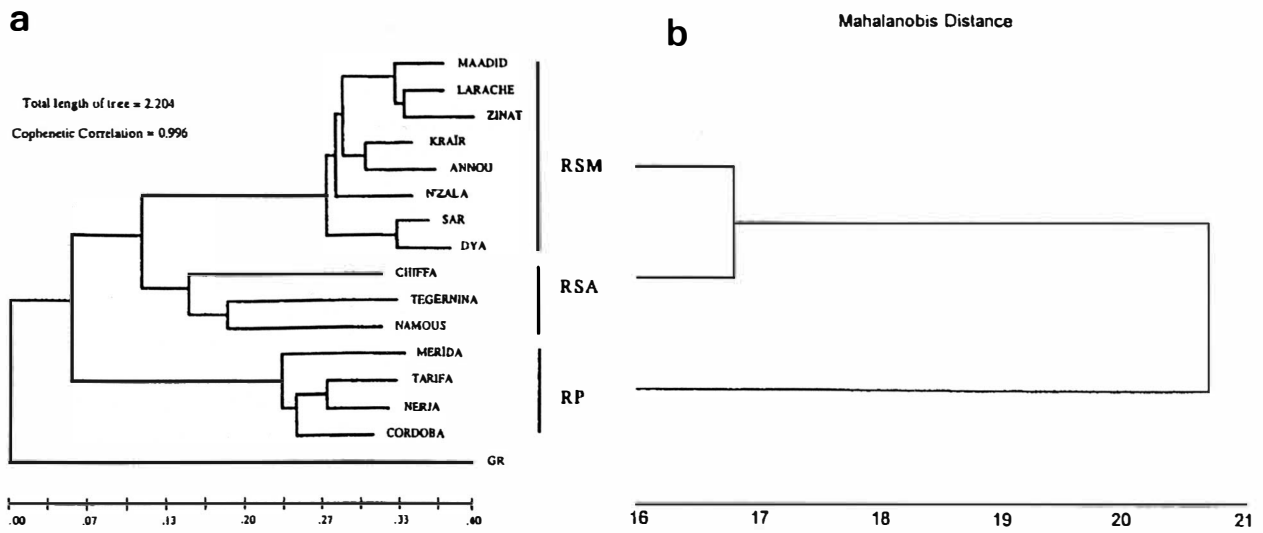

FIG. 2. (a) Wagner Distance tree built using modified Rogers' genetic distances. Outgroup corresponds to Greece (GR); (b) UPGMA cluster using Mahalanobis' distance.

TABLE 7. Discriminant function coefficients for the inter- and intraspecific analyses. ASE, head width; IO, interocular distance; LCC, body length (head-urostyle); O, eye diameter; P, foot length.

Variables	*R. perezi*	*R. saharica*	*Rs*M	*Rs*A
ASE	-	-	-1.205	-2.272
IO	2.662	7.661	-	-
LCC	-0.045	-0.942	1.055	0.307
O	3.436	7.050	-	-
P	-	-	0.783	2.144
Coefficient	-19.012	-31.375	-34.833	-32.125

distance were correct in 91.39% of the cases (79.17-98.28%). Errors were mainly at the intraspecific level (*Rs*M vs. *Rs*A). The two canonical axes explain a variance of 90.56% and of 9.44% respectively with values of 4.13 and 0.43 (Table 4). Fig. 3 shows the graphic representation of the canonical analysis with a 95% confidence interval. Standardized coefficients for both canonical variables are shown in Table 4. Root 1 of the canonical analysis clearly separates the two species *R. perezi* and *R. saharica*, while root 2 separates the Moroccan and Algerian populations. The characters involved in root 1 are components for shape of the head. Hence, both *R. perezi* and *R. saharica*, would differ in IO, O and DN, larger in *R. saharica* than in *R. perezi*. The axis (root 2) separates the two groups of *R. saharica* with less resolution. The characters involved, DN, MA, ASE, T and P, are larger in Morocco than in the Algerian group of *R. saharica*. The UPGMA cluster for the three groups RP, *Rs*M and *Rs*A using Mahalanobis' distances (Fig. 2b) is consistent with that obtained using Rogers' genetic distances (Fig. 2a).

A discriminant analysis among species using non-transformed variables yields a function with variables

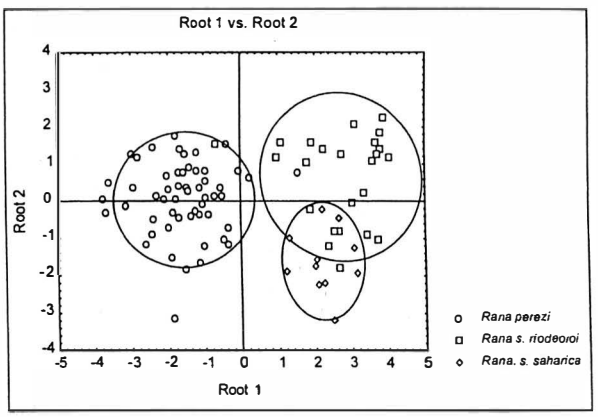

FIG. 3. Graphic representation of the canonical PCA for the three groups considering the two roots. Circles indicate the confidence intervals at 95% from each group centroid.

LCC, IO and O, which classifies 97.92% of the cases correctly (Table 5). A second function was obtained for the North-west African populations using variables LCC, P and ASE with 89.47% correct classifications (Table 6). Coefficients for both discriminant functions are shown in Table 7.

The length of the metatarsal tuberculum (TM) has been used traditionally as a discriminant character for Central European water frog species. However, in our study this character shared neither inter- nor intraspecific differences (ANCOVAinter: F=1.688, P=0.195; ANCOVAintra: F=0.7171, P=0.400). For this reason, it was excluded from subsequent analyses.

DISCUSSION

Buckley *et al.* (1994) proposed that new analyses were necessary to identify all the different forms of north African water frogs. As we previously pointed out, recent studies in the Middle East (Schneider *et al.*,

1992) and the Aegean islands (Beerli *et al.*, 1994) have revealed new species where only *R. ridibunda* was thought to be present. These findings were based on non-morphological characters. A combination of several character sets is needed to detect hidden differentiation in this group, since morphology alone is not conclusive enough to distinguish among different water frog taxa.

Despite claims that molecular and morphological data can be in conflict, many systematists are currently understanding the value of multidisciplinary studies (Larson, 1989). Some data sets are useful to unravel the phylogenetic relationships among closely related species, whereas others are more suitable when dealing with species in a larger temporal scale.

Our results offer two kinds of data which are not in conflict since clear genetic differentiation is congruent with morphological differences. Allozyme data suggest the taxonomic separation of Algerian and Moroccan water frogs at subspecific level. This view is supported by clear differences in loci Sod and Pep-D, as well as by the genetic distances obtained (DN*=0.224). According to Avise & Aquadro (1982), distance values of 0.2-0.5 can be found at both intra- and interspecific level.

When multivariate techniques such as PCA are used with size and shape components, morphological data confirm the taxonomic differentiation between *R. perezi* and *R. saharica*. Likewise, these analyses clearly discriminate between the Algerian and Moroccan water frogs. Based on both data sets, we propose a subspecific status for each *R. saharica* group.

We searched into the nomenclature history of North African water frogs in order to avoid further confusion when assigning names to the new subspecies. In 1913 Boulenger (Hartert, 1913) described *R. esculenta* var. *saharica* from the Saharan oases in the South of Algeria. More recently, Salvador & Peris (1975) described *R. ridibunda riodeoroi*, its type locality being Rio de Oro (western Sahara). In the latest revisions of the group, Dubois & Ohler (1994*a*; 1994*b*) and Salvador (1996) did not consider *R. ridibunda riodeoroi* a valid subspecies but as a synonym of *R. saharica*. This implies that *R. saharica* is considered by these authors as the only valid name for the North African water frogs. Despite this, the differences found between Algerian and Moroccan populations indicate that they should be considered distinct subspecies and be named as such. Hence, their formal nomenclatural denomination should be *Rana saharica saharica* for the Algerian populations and *Rana saharica riodeoroi* for the Moroccan ones. However, these denominations should be used cautiously until similar studies are carried out on the type localities. According to Bons & Geniez (1996), southern Moroccan populations would be different from Northern ones, and possibly more similar to the Algerian *Rana*. It is therefore possible that more differentiation is still to be detected within *R. saharica*.

A widely accepted hypothesis is that the separation between *R. perezi* and *R. saharica* can be related to the separation of the Iberian Peninsula from the north of Africa due to the Strait of Gibraltar opening (Busack, 1986). The south of the Iberian Peninsula and the north of Africa were part of the Betic-Riffian plate, which was separated from both continents. During the Messinian several factors led to shifting of the three plates (African, Iberian and Betic-Riffian) together, the sea connections became interrupted and the subsequent salinity crisis caused the Mediterranean desiccation (Hsü *et al.*, 1977). The collapse of the Betic-Riffian arch connected both continents, and allowed the contact between their respective faunas. The later and final opening of the Gibraltar Strait during the Early Pliocene contributed to interrupted gene flow between the two water frog groups leading to a speciation process, where the ancestral groups for *R. perezi* and *R. saharica* would have inhabited the Iberian Peninsula and the north of Africa respectively.

Presumably, the two taxa presently found in Morocco and Algeria would have evolved from the *R. saharica* ancestral pool. According to our data (Buckley, Arano, Herrero & Llorente, 1996) this would have taken place approximately 2 my ago in the period between the upper Pliocene and the Pleistocene. Again the separation can be related to palaeogeographical events, although the case is not as strong as with the Gibraltar Strait opening. According to Weijermars (1988), the coast lines of both the Iberian Peninsula and the North of Africa did not acquire their present configuration until the beginning of the Pleistocene (2 my). Until then, the sea continued to flood land masses which had emerged during the collision of the three plates (Fig. 4). The former Betic and Riffian channels, that had connected the Atlantic and the Mediterranean before the collapse of the Gibraltar arch, were partially flooded, contributing to the isolation of the Betic and Riffian blocks from both continents. In the case of the Riffian block, the connection between the Atlantic and the Mediterranean was never re-established.

The fact that the Riffian block was isolated to a certain extent from the African continent through the

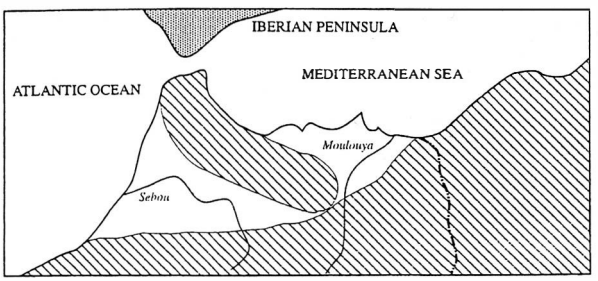

FIG. 4. Map of the approximate coastline of the northwestern corner of Africa during the Plio-Pleistocene boundary (approx. 2 my ago) (following Weijermars, 1988; Benson *et al.*, 1992). Diagonal shading represents emerged land. The discontinuous line represents the current Algerian-Moroccan border.

marine transgression could be related to the isolation of both taxa. This transgression would have lasted long enough to contribute to the differentiation of the Riffian population into *R. saharica riodeoroi* and the continental one into *R. saharica saharica*. Once the continents acquired their present topography during the Pleistocene, each taxon would have dispersed towards the south, reaching their present distribution. Studies on population structure of *R. saharica riodeoroi* (Buckley *et al.*, 1996) suggest that the expansions could have followed a pattern of extinction and recolonization cycles, linked to climatic conditions.

Although the distribution limits of both taxa are still to be established, we are inclined to consider the River Moulouya basin as a cause of discontinuity between them. This river appears to be a natural barrier preventing gene flow among many other species of amphibians and reptiles from North Africa (Lanza, Nascetti, Capula & Bullini, 1986; Mateo, 1990). More recently, Steinfarz, Joger & Barrio (in prep.) have found further evidence of gene flow interruption in two groups of urodeles: *Pleurodeles waltl* (Morocco)/ *P. poireti* (Algeria/Tunisia) and between the western and eastern subspecies of *Salamandra algira*. Although it seems unlikely that a river could act as a barrier for amphibians, it is necessary to bear in mind that the courses of North African rivers would have become established after the Plio-Pleistocene, following previous sea introgressions (Doadrio, 1994). In the case of the Moulouya river its course corresponds to the Betic-Riffian channel and the Pliocene marine transgression zones. Interestingly, the area between the Moulouya valley and the Algerian border, represents the most arid coastal strip in Mediterranean Northwest Africa, and has been named the "Moulouya steppe" by Bons (1960). Hence, the present river can be considered as the reflection of a previous palaeogeographical barrier that is actively interrupting gene flow between amphibian populations and contributing to the morphological and genetic differentiation processes observed.

ACKNOWLEDGEMENTS

The authors are indebted to M. A. Carretero, Marisa Esteban, M. García Paris, M. Herrero, B. Kelly and C. Martín Albadalejo for field assistance in Morocco. I. Doadrio, A. I. Perdices and J. A. Mateo provided the frog samples from Algeria. Suggestions made by U. Joger, J. Castanet and M. Girondot, greatly improved the final version. We are particularly grateful to T. R. Halliday for his corrections and critical comments on an earlier draft of the manuscript. Financial support was provided by projects DGICYT PB91-01150-CO2 and DGICYT PB92-0091. DB was supported by a studentship (FP93) from the Spanish Ministry of Education and BA by a "Marie Curie" Fellowship from the European Union (Training and Mobility of Researchers)

REFERENCES

Arano, B., Llorente, G. A., Herrero, P., & Sanchíz, B. (1994). Current studies on Iberian water frogs. *Zoologica Poloniae* **39**, 365-375.

Avise, J. C. & Aquadro, C. F. (1982). A comparative summary of genetic distances in the Vertebrates. *Evol. Biol.* **15**, 151-185

Beerli, P. (1994). Genetic isolation and calibration of an average molecular clock in western Palaearctic water frogs of the Aegean region. PhD Dissertation, Universität Zürich.

Beerli, P., Hotz, H., Tunner, S., Heppich, S. & Uzzell, T. (1994). Two new water frog species from the Aegean islands Crete and Karpathos (Amphibia, Salientia, Ranidae). *Notulae Naturae* **470**, 1-9.

Benson, R. H., Rakic-El Beid, K., Bonaduce, G. (1992). An important current reversal (influx) in the Riffian Corridor (Morocco) at the Tortonian-Messinian boundary: the end of the Tethys Ocean. *Notes et M. Sev. géol. Maroc, Rabat* **336**, 115-146.

Berger, L. (1968). Morphology of the F_1 generation of various crosses within *Rana esculenta* complex. *Acta Zool. Cracov.* **13**, 301-324.

Berger, L. (1973). Systematics and Hybridization in European green frogs. *J. Herpet.* **7**, 1-10.

Berger, L. (1977). Systematics and Hybridization in the *Rana esculenta* Complex. In: *The Reproductive Biology of Amphibians*, 367-388. Taylor D.H. (Ed.). New York: Plenum Press.

Bons, J. (1960). Aperçu sur le peuplement herpétologique du Maroc oriental. *Bull. Soc. Sci. Nat. Phys. Maroc* **40**, 53-75

Bons, J. & Gèniez, P. (1996). Amphibiens et Reptiles du Maroc. Asociación Herpetológica Española, 320pp

Buckley, D., Arano, B., Herrero, P. & Llorente, G. A. (1996). Population structure of Moroccan water frogs: genetic cohesion despite a fragmented distribution. *J. Zoo. Syst. Evol. Research* **34**, 173-179

Buckley, D., Arano, B., Herrero, P., Llorente, G. A. & Esteban, M. (1994). Moroccan water frogs vs. *R. perezi*: Allozyme studies show up their differences. *Zool. Poloniae* **39**, 377-386.

Busack, S. D. (1986). Biogeographic analysis of the herpetofauna separated by the formation of the strait of Gibraltar. *National Geographic Research*, **2** (1), 17-36

Crochet, P. A., Dubois, A., Ohler A. & Tunner, H. (1995). *Rana (Pelophylax) ridibunda* Pallas 1771, *Rana (Pelophylax) perezi* Seoane 1885 and their associated klepton (Amphibia:Anura): morphological diagnoses and description of a new taxon. *Bull. Mus. Natl. Hist. Nat. 4e sér.* **17**, 11-30.

Doadrio, I. (1994). Freshwater fish fauna of North Africa and its biogeography. *Ann. Mus. Afr. Centr. Zool.* **275**, 21-34

Dubois, A. (1991). Nomenclature of parthenogenetic, gynogenetic and "hybridogenetic" vertebrate taxons: new proposals. *Alytes* **8**, 61-64.

Dubois, A. & Ohler A. (1994*a*). Frogs of the subgenus *Pelophylax* (Amphibia, Anura, genus *Rana*): A catalogue of available and valid scientific names, with comments on name-bearing types, complete synonymies, proposed common names, and maps showing all type localities. *Zool. Poloniae* **39,**139-204.

Dubois, A. & Ohler A. (1994*b*). Catalogue of names of frogs of the subgenus Pelophylax: a few additions and corrections. *Zool. Poloniae* **39,** 205-208.

Graf, J. D. & Polls-Pellaz, M. (1989). Evolutionary genetics of the *Rana esculenta* complex. In: *Evolution and Ecology of Unisexual Vertebrates* Bull. 466, 289-301. Dawley, R.M. and Bogart, J.P. (Eds). Albany: N.York State Museum.

Hartert, E. (1913). Reptiles and Batrachians. In: *Expedition to the central western Sahara.* Hartert E. (Ed). *Novitates zoologicae* **20,** 76-84

Hemmer, H., Konrad, A. & Bachman, K. (1980). Hybridization between the *Rana esculenta* complex of North Africa. *Amphibia-Reptilia* **1**,41-48

Hotz, H., Mancino, G., Bucci-Innocenti, S., Ragghianti, M., Berger, L. & Uzzell, T. (1985). *Rana ridibunda* varies geographically in inducing clonal gametogenesis in interspecific hybrids. *J. Exp. Zool.* **236,**199-210.

Hotz, H., Uzzell, T., Beerli, P. & Guex, G-D. (1996). Are hybrid clonals species? A case for enlightened anarchy. *Amphibia-Reptilia*, **4**, 315-320.

Hsü, K. J., Montadert, L., Bernoulli, D., Cita, M. B., Erickson, A., Garrison, R.E., Kidd, R.B., Mélierés, F., Müller, C. & Wright, R. (1977). History of the Mediterranean salinity crisis. *Nature* **267**, 399-403

Lanza, B., Nascetti, G., Capula, M. & Bullini, L. (1986). Les Discoglosses de la région méditerranéenne occidentale (Amphibia: Anura: Discoglossidae*) Bull. Soc. Herp. Fr.* **40,**16-27

Larson, A. (1989). The relationship between speciation and morphological evolution. In: *Speciation and its consequences* , 579-598. Otte, D., and Endler, J.A. (Eds.). Sinauer Asooc. Sunderland, Mass.

Llorente, G. A., Arano, B., Carretero, M. A., García-Paris, M., Herrero, P., & Esteban, M. (1996). Descripción de la larva de *Rana saharica* Boulenger, 1913. *Bol. Asoc. Herpetol. Esp.* **7,**19-23.

Mateo, J. A. (1990). Taxonomy and evolution of the North African ocellated lizard, *Lacer pater* (Lataste, 1880) (Sauria:Lacertidae). *Bonn. zool. Beitr.* **41,** 203-212.

Nei, M. (1972). Genetic distance between populations. *Am. Nat.* **106**, 283-292.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89,**583-590

Rogers, J. S. (1972). Measures of genetic similarity and genetic distance. *Stud. Genet. VII. Univ. Texas Publ.* **7213**, 145-153

Salvador, A. (1996). Amphibians of Northwest Africa. *Smithsonian Herpetological Information Service,* **109**, 43 pp

Salvador, A. & Peris, S. (1975). Contribución al estudio de la fauna herpetológica de Rio de Oro. *Bol. Estación Central de Ecología* **4,**49-60.

Schneider, H., Sinsch, U. & Nevo, E. (1992). The lake frog in Israel represents a new species. *Zool. Anz.* **228,** 97-106

Schultz, R. J. (1969). Hybridization, unisexuality and poliploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. *Amer. Natur.* **103,** 605-619

Steinwarz, D. & Schneider H. (1991). Distribution and bioacustic of *R. perezi* Seoane 1885 (Amphibia: Anura: Ranidae) in Tunisia. *Bonn. Zool. Beitr.* **42,**283-297

Swofford, D. L. & Selander, R. B. (1981). Biosys-1: a Fortran program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J. Hered.* **72,** 281-283.

Weijermars, R. (1988). Neogene tectonics in the Wetern Mediterranean may have caused the Messinian Salinity Crisis and associated glacial events. *Tectonophysiscs* **148,** 211-219

Accepted: 24.10.97