Pakistan J. Zool., vol. 44 (4), pp. 901-907, 2012.

Pattern of Genetic Diversity of North African Green Frog *Pelophylax saharicus* (Amphibia) in Tunisia

Sarra Farjallah, [1*] Nabil Amor,[1] Paolo Merella,[2] and Khaled Said[1]

[1]*Unité de Recherche, Génétique, Biodiversité et Valorisation des Bioressources UR/09-30, Institut Supérieur de Biotechnologie de Monastir, Tunisia*

[2] *Sezione di Parassitologia e Malattie Parassitarie, Dipartimento di Biologia Animale, Università degli Studi di Sassari, Sardaigne, Italy.*

Abstract.- Genetic variation among 39 samples from 16 populations of the North African green frog *Pelophylax saharicus* from Tunisia was investigated, using 513 bp partial sequences of the mitochondrial gene cytochrome b. *P. saharicus* exhibits little genetic differentiation, with overall nucleotide diversity Pi = 0.002 and haplotype diversity Hd = 0.7. The intraspecific pairwise divergence was low (mean 0.2 %). The results from AMOVA analyses indicate that there is no evidence of genetic structuring related to the three studied groups (north, center, and south Tunisia). As shown by the mismatch distributions, significantly negative values of Fu's Fs and Tajima's D statistics, this reduced genetic variability may be due to a recent population expansion from a smaller founder population.

Key words: *Pelophylax saharicus*, Tunisia, cytb, genetic variation.

INTRODUCTION

Tunisia lies in North Africa, between the Mediterranean Sea and the Sahara Desert. While some patches of forest occur in the centre of the country (Tell or Tunisian Dorsal), broadly-forested areas are limited to the north. Almost one-third of Tunisia's surface area is covered by the Sahara desert (Grand Erg Oriental), so major portion of the country is dominated by arid climate characterized by annual and seasonal variation in rainfall (Sicilia *et al*., 2007; Amor *et al*., 2009a).

The North African green frog *Pelophylax saharicus* (Boulenger 1913), is the most abundant amphibian species in Tunisia (Amor *et al*., 2007, 2009a). The systematic status of this species has been confused in the past (Steinwarz and Schneider, 1991; Nouira, 2001) and its Tunisian populations have been considered to be composed of two species *P. perezi* (Seoane, 1885) or *P. ridibundus* **(**Pallas, 1771).

Although it is widely distributed in North Africa, this species is characterized by its disjunct distribution and restricted ecological breadth (Amor *et al*., 2009a), which are among the forces that catalyze population variation (Graves, 1985, 1988). Therefore, significant population genetic structure is expected, especially over spatial environmental heterogeneity (Mayr, 1963; Smith and Green, 2005). In fact, recent phylogenetic study, based on mitochondrial DNA, supported this expectation and found significant differentiation among Tunisian populations of *P. saharicus* (Lymberakis *et al*., 2007). However, using a different cytochrome b fragment we observed low genetic variation and the absence of structure within *P. saharicus* in Tunisia (Amor *et al*., 2010b).

In the present study, we carried out a comparison of the sequence of a mitochondrial gene, cytochrome b, in *Pelophylax saharicus* specimens collected from several Tunisian localities to characterize their genetic variation and to identify geographical patterns in their molecular variation. The sequences for this gene are combined with previously published sequences (Lymberakis *et al*., 2007) in order to examine the validity of the pattern of genetic variability described previously, and evaluate alternative models of the biogeographic history of *Pelophylax* in this area.

MATERIALS AND METHODS

Thirty-nine samples of *P. saharicus* were

* Corresponding author: sarra_farj@yahoo.fr

0030-9923/2012/0004-0901 $ 8.00/0

collected from 16 localities across Tunisia (Fig. 1, Table I). Total genomic DNA was extracted from ethanol-preserved tissues using a proteinase K and phenol-chloroform procedure (Sambrook *et al*., 1989). The quantity and quality of the extracted DNA were estimated on 1% agarose gels stained with ethidium bromide (EB). The polymerase chain reaction (PCR) was used to amplify the mitochondrial part *cytb* fragment (513 bp). PCR amplifications were conducted in 25 µl reactions containing, containing $MgCl_2$ (2.5 mM), buffer reaction (1x, Biotools Edmont, Alberta, Canada), dNTPs (0.2 mM), forward L14850 and reverse H15410 primers (0.2 mM each) (Tanaka *et al*., 1994), Taq polymerase (Biotools, 5 U/ml) and DNA template (10–100ng). Amplification was carried out using the following conditions: initial denaturation for 5 min. at 94°C, followed by 35 cycles (denaturation for 30 s at 94°C, annealing for 47 s at 47°C and extension for 60 s at 72°C) and a final extension for 10 min. at 72°C.

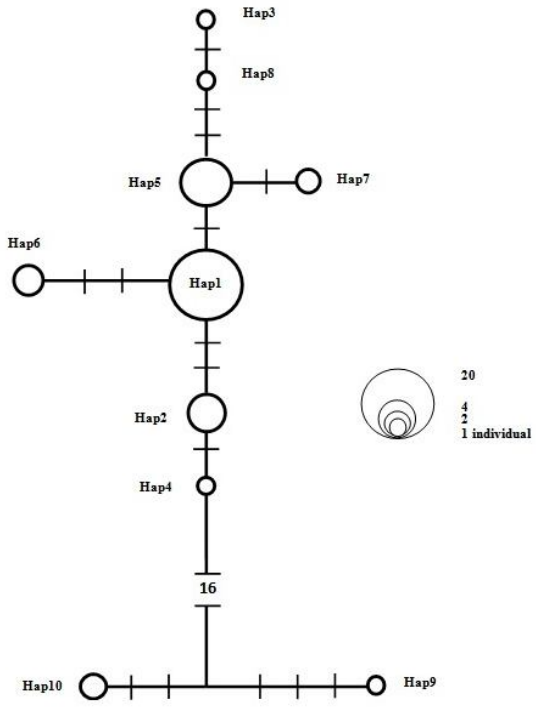

Fig. 1. Maps showing sampling localities for *Pelophylax saharicus*. Shading indicates elevation (see legend).

PCR products were purified using the *Jena Biosciences* purification kit, and sequenced in an automated DNA sequencer (Abi Prism 3700) using the BigDye Deoxy Terminator cycle-sequencing kit (Applied Biosystems).

The DNA sequences were aligned by ClustalX (Thompson *et al*., 1997), and checked via ocular inspection. The full alignment comprised 513 bp. *Pelophylax saharicus* sequences, recovered from GenBank (DQ474177, DQ474179, DQ474179; Lymberakis *et al*., 2007), were added to our dataset. MEGA version 3.1 (Kumar *et al*., 2004) was used to estimate genetic distances and calculate sequence statistics. Haplotype (Hd) and nucleotide (Pi) diversity (Nei, 1978) were estimated for each species using the software DnaSP ver. 4.1 (Rozas *et al*., 2003).

Intraspecific relationships among the mtDNA haplotypes were inferred using statistical parsimony with the software TCS ver. 1.21 (Clement *et al*., 2000). Analyses of molecular variance (AMOVA) were performed in Arlequin ver. 3.1 (Excoffier *et al*., 2005) to test the geographic divisions among populations. We grouped sampling localities from Tunisia in three distinct groups (Grp_1: north, Grp_2: center and Grp_3: south) (Fig. 1, Table I) based on topography of the studied area and morphotypes described previously in *P. saharicus* to test the correspondence of these morphotypes with genetic data (Amor *et al*., 2009b, 2010a). Levels of significance of statistics characterizing variation at different hierarchical levels were assessed through 10,000 permutations.

We computed pairwise nucleotide mismatch distributions among haplotypes and tested the data against the population sudden-expansion model (Rogers and Harpending, 1992) using the programs Arlequin ver. 3.1 (Excoffier *et al*., 2005) and DnaSP ver. 4.1 (Rozas *et al*., 2003). A scenario of a recent demographic expansion or bottleneck is expected to generate a unimodal distribution of pairwise nucleotide differences between haplotypes, whereas in populations at demographic equilibrium, the pairwise mismatch distribution curve is expected to be multimodal (Rogers and Harpending, 1992; Harpending *et al*., 1998).

We validated the conformity of our data to the chosen population model using Tajima's D (Tajima, 1989) and Fu's FS (Fu, 1997) statistics.

Table I.- ***Pelophylax saharicus*** **sample information, including haplotypes observed (Fig. 1), voucher numbers, groups, localities and latitude/longitude coordinates. NA, Nabil Amor personal tissue collection.**

ID	Group	Voucher	Locality	Haplotype	Latitude	Longitude
1	Group 1 (Grp_1)	NA311	Ichkeul	Hap1	37°07'33.47"N	9° 40'20.65"E
2		NA312	Ichkeul	Hap1	37°07'33.47"N	9° 40'20.65"E
3		NA313	Ichkeul	Hap1	37°07'33.47"N	9° 40'20.65"E
4		NA319	Tabarka	Hap1	36°57'20.20"N	8° 43'51.88"E
5		NA320	Tabarka	Hap3	36°57'20.20"N	8° 43'51.88"E
6		NA335	Medjerda	Hap5	36°29’03.22”N	8°35’04.42” E
7		NA336	Medjerda	Hap8	36°29’03.22”N	8°35’04.42” E
8		NA337	Kelibia	Hap1	36°50’58.75”N	11°6’49.49” E
9		NA338	Kelibia	Hap2	36°50’58.75”N	11°6’49.49” E
10		NA339	Tunis	Hap1	36°52'21.00"N	10°10'54.00"E
11		NA340	Tunis	Hap1	36°52'21.00"N	10°10'54.00"E
12		NA344	Beja	Hap1	36°43'06.93"N	09° 13'16.76"E
13		NA345	Beja	Hap5	36°43'06.93"N	09° 13'16.76"E
14		NA346	Beja	Hap1	36°43'06.93"N	09° 13'16.76"E
15	Group 1 (Grp_2)	NA327	Kasserine	Hap7	35°10'25.93"N	08°49'36.37"E
16		NA328	Kasserine	Hap1	35°10'25.93"N	08°49'36.37"E
17		NA333	Monastir	Hap2	35°45'33.04"N	10°48'49.35"E
18		NA334	Monastir	Hap1	35°45'33.04"N	10°48'49.35"E
19		NA341	Kairouan	Hap1	35°40'0.00"N	10° 5'57.72"E
20		NA342	Kairouan	Hap7	35°40'0.00"N	10° 5'57.72"E
21		NA343	Kairouan	Hap5	35°40'0.00"N	10° 5'57.72"E
22		NA347	Sidibouzid	Hap5	35°02'48.94"N	09° 33'12.34"E
23		NA348	Sidibouzid	Hap5	35°02'48.94"N	09° 33'12.34"E
24		NA349	Sidibouzid	Hap5	35°02'48.94"N	09° 33'12.34"E
25	Group 1 (Grp_3)	NA314	Tozeur	Hap2	33°51'15.34"N	8° 7'57.72"E
26		NA315	Tozeur	Hap1	33°51'15.34"N	8° 7'57.72"E
27		NA316	Nefta	Hap1	33°52'24.36"N	7° 52'51.32"E
28		NA317	Nefta	Hap1	33°52'24.36"N	7° 52'51.32"E
29		NA318	Nefta	Hap1	33°52'24.36"N	7° 52'51.32"E
30		NA321	Tamerza	Hap4	34°23'58.80"N	07°59'20.78"E
31		NA322	Tamerza	Hap1	34°23'58.80"N	07°59'20.78"E
32		NA323	Tamerza	Hap1	34°23'58.80"N	07°59'20.78"E
33		NA324	Tamerza	Hap5	34°23'58.80"N	07°59'20.78"E
34		NA325	kebili	Hap6	33°45'25.93"N	08°49'36.37"E
35		NA326	kebili	Hap6	33°45'25.93"N	08°49'36.37"E
36		NA329	Gabes	Hap6	33°54'00.73"N	10°05'19.18"E
37		NA330	Gabes	Hap6	33°54'00.73"N	10°05'19.18"E
38		NA331	Gafsa	Hap1	34°25'17.94"N	08°47'11.96"E
39		NA332	Gafsa	Hap1	34°25'17.94"N	08°47'11.96"E

RESULTS AND DISCUSSION

No insertions, deletions, or stop codons were observed in the final alignment. Haplotypes sequences have been deposited in GenBank (accession nos. HM470196-HM470203).

Despite our increased sample sizes and geographic coverage compared to previous studies, including fragmented habitats, we observed extremely low genetic variation (0.2%) with overall nucleotide diversity, Pi = 0.002 and haplotype diversity, Hd = 0.7. These observations are in accordance with the low values of genetic diversity previously reported within *P. saharicus* by Amor *et*

al. (2010b). Also, previous studies focused on the North African green frog from the westernmost region of Africa (Buckley *et al*., 1994, 1996; Arano *et al*., 1998; Harris *et al*., 2003). Using different molecular markers, they observed the same pattern of genetic variation in *P. saharicus.*

Our data show that all *P. saharicus* samples are from a single haplotype group (8 haplotypes) (Fig. 2), which is widespread across Tunisia, and presents low value of intraspecific sequence divergence (0.2%). This uniform genetic diversity results in low genetic differentiation among populations. Few population-specific haplotypes exist because of exchanges of genetic information. The observed low variability might reflect adaptation of this green frog to a wide variety of biotopes across Tunisia, from mountainous forests in the northern and central area to oases in the southernmost localities (Amor *et al*., 2009a, 2010b).

Contrary to what would be expected, haplotypes Hap1–Hap8 were very different from those described previously by Lymberakis *et al*. (2007) (Hap9 and Hap10). Even though, we used the same cytb fragment as in this study combined with a dense sampling covering all Tunisia, especially from the two populations of Tamerza and Ichkeul, not one of these haplotypes or even a similar one was found and four amino acids differ between these two datasets.

Despite these observations, *P. saharicus* may face more changes in populations' structures and thus severe restrictions to gene flow, as a result of the increase threats due to natural and human causes (Amor *et al*., 2009a, 2010b).

Considering the large geographical distance, three large mountain chains (Atlas, Aures and Orbata) and also Chott el Jerid (5360 km^2, the largest depression in Tunisia separating the southernmost area from the other regions (Gueddari *et al*., 1983; Millington *et al*., 1989) within the studied region, it is somewhat surprising that we found geographically unstructured patterns of genetic variation in *P. saharicus*. The hierarchical AMOVA indicated that there is no evidence of genetic structuring related to these three groups (northern, central and southern Tunisia) (Φ_{CT} = 0.06, P > 0.01, Table II). Relatively low levels of mtDNA variation can result from range expansions, where

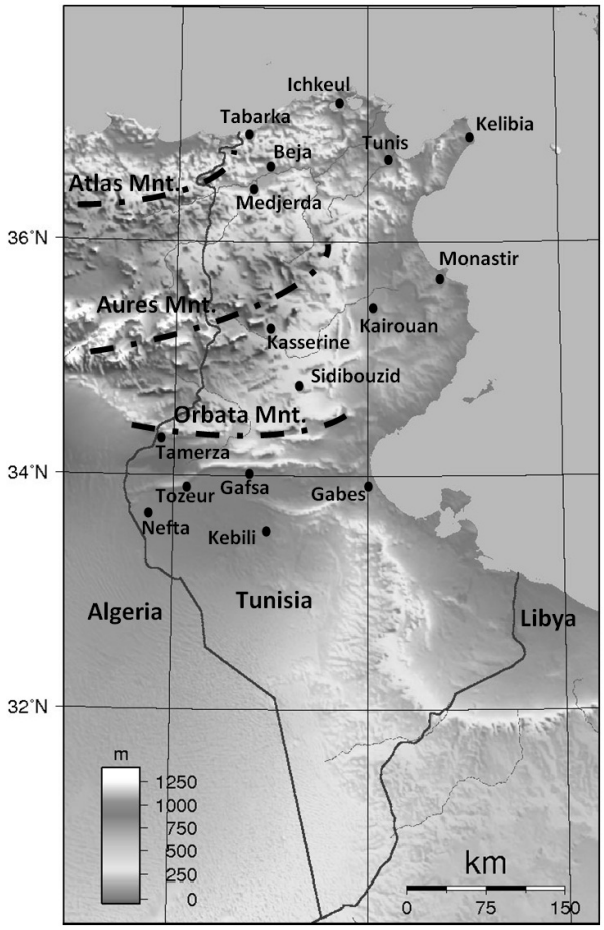

Fig. 2. Network presentation (TCS) for *Pelophylax saharicus* mtDNA haplotypes. Symbol size corresponds to approximate frequency of identified haplotypes (Table II). Circles indicate Tunisian haplotypes. Dashes between symbols correspond to one mutation step. 16 is the number of changes between our new sequences and Lymberakis *et al.* (2007) ones.

the smaller effective population size of mtDNA genes can enhances founder effects. The low nucleotide diversity, unimodal mismatch distributions (Fig. 3), and significantly negative values of Fu's Fs and Tajima's D statistics (Fs = -1.9, P<0.01, D= - 0.63, P<0.01)all support a recent population expansion from a smaller founder population as the most plausible explanation for the observed significant deviations from neutrality in the North-East African green frog populations.

Table II.- AMOVA results for *Pelophylax saharicus* populations in Tunisia. We assessed hierarchical patterns of genetic structure at three levels (among groups, among populations within groups and among individuals within populations) in both species. d.f. = degrees of freedom; SS = sum of square deviations; *P* = *p*-value (99%).

Variation source	d.f.	SS	AMOVA Variance	TOTAL Variation (%)	*P*
Among groups	2	3.439	0.046	6.09	*0.114*
Among populations within groups	13	14.01	0.258	33.95	*0.015*
Within populations	23	10.50	0.456	59.96	*0.003*
Total	38	27.949	0.761		

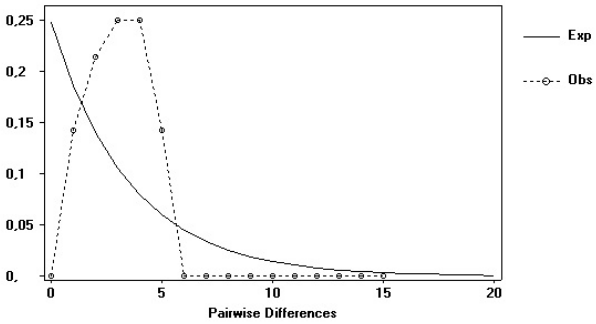

Fig. 3. Observed (• • • • •) and expected (——) pair-wise mismatch distributions in the studied populations of Pelophylax saharicus under the sudden population expansion model. The number of pair-wise nucleotide differences between haplotypes is represented on the abscissa whereas their frequencies are represented on the ordinate axis.

It is well known that environmental changes in Northern Africa, since the last glacial period, have played a major role in determining species' distributions and their modern genetic diversity (De Jong, 1998; Sanmartin, 2003). Also, Algerian-Tunisian Tell area was described as one of the potential North African glacial refuges (Guiller and Madec, 2010). Thus, Tunisian *P. saharicus* are probably originated from the same source population which, following environmental amelioration, have underwent a range expansion leading to the low local-scale genetic structure. Also, this may account for the East-West differentiation in North Africa, reported previously (Buckley *et al*., 1994, 1996; Arano *et al*., 1998; Harris *et al*., 2003). In fact, a second lineage of green frog localized in the western refugium (Morocco) is probably the source of the actual Moroccan and west Algerian *Pelophylax*.

The lack of genetic structure found here contradicts with high morphological differentiation described previously (Amor *et al*., 2009b). This suggests that the pattern of morphometric variation observed results from phenotypic plasticity correlated with local environmental factors, rather than from different evolutionary histories (Walther *et al*., 2002; Tryjanowski *et al*., 2003).

In conclusion, Tunisian *P. saharicus* surveyed here showed low variation in mitochondrial coding gene sequence and herewith this study gives new evidence geographic unstructured genetic patterns, despite its fragmented habitat. Combined with previous studies using other types of genetic data (16S, allozymes), these results are consistent with patterns of population expansion of the North African green frog. As an extension of this study, an extensive sampling especially across Algeria might be useful in distinguishing factors which influence genetic variation patterns, both on a macrogeographic scale as well as microgeographic scale among habitat types.

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(Received 22 February 2011, revised 19 January 2012)