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Karyological and morphometric variation of the North African green frog *Pelophylax saharicus* (Anura) in north-eastern Africa

Nabil AMOR^{1, 2*}, Sarra FARJALLAH¹, Slim BEN-YACOUB³, Paolo MERELLA⁴,
Khaled SAID¹

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

2 Dipartimento di Biologia Strutturale e Funzionale, Università di Napoli Federico II, Via Cinthia 8, 80126 Naples, Italy

3 Laboratory of Terrestrial and Aquatic Systems Ecology. University BADJI Mokhtar Annaba, Algeria

4 Sezione di Parassitologia e Malattie Parassitarie Dipartimento di Biologia Animale, Università degli Studi di Sassari, Sardegna

Abstract Morphometric variation of *Pelophylax saharicus* was analysed using univariate and multivariate statistics, with both traditional and geometric morphometrics, based on 148 specimens from four different geographical localities in Tunisia and Algeria. The results show the existence of three morphotypes in Tunisia and one in Algeria, and indicate a significant degree of variation in morphometric characters between regions. Specimens from the southernmost region have the smallest body size and the greatest morphometric divergence from other populations. This pattern of morphometric variation probably results from phenotypic plasticity in response to local environmental factors. The results of our chromosomal study (C-, Ag-NOR-, endonuclease digestion, DAPI, CMA₃ and Q-banding) reveal this species to exhibit the plesiomorphic *Pelophylax* karyotype of $2n = 26$ banded chromosomes and NORs on the eleventh pair. Similarities and differences of the North African green frog are discussed in relation to the different forms of data collected (chromosomal, morphometric, ecological) [*Current Zoology* 56 (6): – , 2010].

Key words Green frog, *Pelophylax saharicus*, Karyology, Morphometric variation, Tunisia, Algeria,

The distribution and diversity of the biota of the Mediterranean Basin has been shaped by a complex geological and climatic history, resulting in the formation of several areas of endemism and biodiversity hotspots (De Jong, 1998; Sanmartin, 2003). Among these hotspots, North Africa is of special interest because it harbours a large number of endemic animal species. Previous studies suggest that this region served as glacial refugia, especially for amphibians (Plötner, 1998; García-París et al., 2003; Carranza et al., 2004; Fromhage et al., 2004). However, biogeographic patterns of terrestrial fauna in this region are still poorly known because of a lack of information concerning the distribution and taxonomy of most endemic species. Identifying and defining these taxa may prove pivotal for both discovering and

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* Corresponding author. E-mail: nabil.amor@gmail.com

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interpreting broad-scale biogeographic patterns, and also in setting conservation priorities at the regional level within this biodiversity hotspot (Blackburn and Measey, 2009).

Pelophylax saharicus (Boulenger, 1913) is a largely aquatic species confined to montane and wetland areas, the known altitudinal distribution in Tunisia ranges from sea level up to 1077 m a.s.l. (near Thala, Tell Plateau). Although it is widely distributed in North Africa, this species is characterised by both a disjunct distribution and restricted ecological range (Amor et al., 2009a), which are among the forces that catalyze population variation (Graves, 1985, 1988). Due to the dominance of arid and semi-arid habitats across large parts of North Africa, there are few habitats suitable for long-term breeding populations of amphibians. It is also likely that high temperatures and dry conditions at many sites result in higher adult mortality due to the increased rate of water-loss.

Despite being the most frequently encountered anuran, little is known of intraspecific variation in *P. saharicus*, and comprehensive genetic and morphometric studies across North Africa have been neglected. Prior studies of the distribution of this North African green frog (Boulenger, 1891; Mayet, 1903; Gauthier, 1928; Schneider, 1978; Nourira, 1998, 2001; Romdhane and Missaoui, 2001) were limited in geographical coverage and utilized diagnostic characters based on morphology. Previous studies have not resolved the question of whether one (i.e. *P. saharicus*) or two species (i.e. *P. saharicus* and *P. ridibunda*) exist (Nourira, 1998). In addition, no studies have examined genetic variation in this species, other than for allozyme data (Arano et al., 1998). Subspecies that have been recognized in the past are now considered invalid (Frost et al., 2006).

Analyses of allozymic data revealed East-West differentiation within *P. saharicus*, suggesting the existence of two genetically defined subspecies (*P. saharicus riodeoroi* in Morocco and *P. saharicus saharicus* in Algeria; Buckley et al., 1994, 1996; Arano et al., 1998). Further, Harris et al. (2003) found concordant patterns of genetic variation, consistent with the presence of two subspecies.

Published studies concerning intraspecific variation in *P. saharicus* in Tunisia focus on cytogenetics (Amor et al., 2007) and morphometrics (Amor et al., 2009b). The former described chromosome-banding patterns in Tunisian specimens and the latter supported the existence of three geographically related morphotypes (northern, central and southern morphotypes). No investigations have included population-level data regarding cytogenetic or morphometric variation in populations from adjacent Algeria.

The aims of this study are to: (1) investigate regional variation in external morphology of adults of the North African green frog across Tunisia, especially oasis populations located in the southernmost region of Tunisia, and eastern Algeria (Annaba); (2) determine whether morphological characters differ significantly from one locality to another and, if so, to characterize these differences; and (3) describe and compare the karyological patterns of these populations from Tunisia and Algeria and assess whether these exhibit patterns concordant with patterns of morphometric variation.

1 Materials and Methods

1.1 Sampling

We collected 148 specimens (69 males, 79 females) of *P. saharicus* from 14 populations and four geographical regions during the period 2005–2009 (southern, central and northern Tunisia and north-eastern Algeria; Fig. 1, Table 1). The sampling was performed with the permission of the general direction of forests of the Tunisian ministry of agriculture, hydraulic resources and fisheries. The method used in our work is in conformity with the principles set by

the EC guidelines on the human killing of laboratory animals: European Commission's Publication Euthanasia of Experimental Animals.

1.2 Chromosome analysis

Metaphase spreads were obtained from blood cultures (Nishioka et al., 1993) and a scrapings/air-drying method. For the latter, intestinal epithelium and testicular cells were taken from specimens injected with 1% colchicine at least two hours before anesthetization. The cells were incubated for 30 minutes in a hypotonic solution (0.25 g sodium citrate + 0.28 g KCl in 100 ml H₂O) and fixed in methanol/acetic acid fixative (3:1). In addition to the standard staining method, with 5% Giemsa at pH 7, the following banding techniques were used: Ag-NOR banding (Howell and Black, 1980); chromomycin A₃/methyl green staining (CMA₃) according to Sahar and Latt (1980); Q-banding, as suggested by Schmid (1978a); C-banding, according to Sumner's method (1972); sequential C-banding, CMA₃/DAPI staining (Odierna et al., 1999); and digestion with the restriction enzyme Hae III (Mezzanotte et al., 1983). Standard Giemsa staining was carried out on all specimens investigated, while the other banding treatments were performed on at least four specimens from every population. For karyotyping, four or more Giemsa-stained metaphase plates and two of each of the other treatments were examined.

1.3 Morphometric analysis

Only specimens larger than 36 mm snout-vent length were considered for the morphometric analysis ($n=148$) since smaller individuals are unlikely to be sexually mature (Berger and Rybacki, 1994). Eight measurements were taken using a digital calliper (accuracy: ± 0.01 mm): snout-vent length (SVL); head length (HL), from the posterior margin of the lower jaw to the tip of the snout; tibia length (TL); femur length (FeL); foot length (FoL), from the proximal border of the inner metatarsal tubercle to the tip of the fourth toe; head width (HW), measured at the level of the posterior part of the tympanum; first toe length (FTL); and metatarsal tubercle length (MTL). We used SPSS 16.0 (SPSS Inc., Chicago, USA) for statistical analyses. Data normality was tested using the Shapiro-Wilks' W -test (Shapiro and Wilk, 1965). Data following a non-normal distribution were log-transformed. In addition, frogs were weighed and three descriptive colouration parameters were recorded (dorsal colour, presence/absence of a dorsal stripe and ventral colour). These descriptive parameters were tested for significant differences in the within/between group variation using a one-way ANOVA. The significance of differences among groups for size-corrected values of measurements was tested using one-way ANOVA combined with a Spjotvoll-Stoline *a posteriori* test (Sokal and Rohlf, 1995).

Discriminant analysis was used to describe functions that maximize the probability of correct classification of specimens to their original population. Subsequently, the percentage of proper classifications to geographical regions was computed. This analysis was based on a stepwise discriminant model that involves entering the independent variables into the discriminant function one at a time on the basis of their discriminating power. The selection rule in this procedure maximizes Mahalanobis distance (D^2) between groups (Hair et al., 1998). The quantitative variables involved in the DA test were searched for outliers with the estimation of squared Mahalanobis distances (D^2).

We performed a second discriminant analysis grouping variables by localities and including average annual precipitation as a factor (> 800 mm, 400–800 mm, 200–400 mm, and < 200 mm), to determine the influence of this important environmental factor on morphological variation.

2 Results

2.1 Chromosome analysis

All frogs from Tunisia and Algeria had 26 bichromosomes (Fig. 2). Five chromosome pairs were larger than the eight others, and five chromosome pairs were metacentric (1, 5, 7, 9, 10 and 13), while the others were

submetacentric. Quinacrine stained all chromosomes uniformly (Fig. 2). Ag-NOR and CMA₃ staining revealed interstitial NORs, located on the long arms of the 11th chromosome pair (Fig. 2). C-banding staining, in addition to NOR-associated heterochromatin, also produced tiny centromeric C-bands (Fig. 2). After Hae III/Giemsa treatment, chromosomes were stained uniformly (Fig. 2). These centromeric bands were slightly stained by DAPI, whereas CMA₃ stained only NOR-associated heterochromatin. However, centromeric heterochromatin was not easy to assay and required delicate technical adjustment during barium hydroxide treatment. Suitable results were obtained after exposure at 40°C for 3 min (Fig. 2). No variation in karyotype was observed among individuals.

2.2 Morphometric analysis

The average value and standard error for all variables regarding external morphology are provided in Table 2. Male and female data was pooled because no sexual dimorphism was observed (one-way ANOVA, $F_{1, 146}=0.003$, $P=0.916$). Significant differences in adult body size among the four geographical regions from Tunisia (southern, central, and northern) and Algeria (Annaba) were found (one-way ANOVA, $F_{3,144}=3.8$, $P=0.012$). A clinal variation of SVL in Tunisia (a decrease from north to south) was also observed (Table 2).

Analysis of variance, including geographical location as a factor for all variables revealed that all morphometric variables differed significantly between regions (one-way ANOVA, $F_{3, 144}=5.02$, $P<0.002$). For these variables, an *a posteriori* Spjotvoll-Stoline test demonstrated significant differences between the Algerian and Tunisian samples. The samples from southern Tunisian populations differed from others for all variables examined. However, significant differences in leg measurements were also observed between the northern and central populations (TL, FeL, MTL and FoL; Table 2). A one-way ANOVA for body weight showed significant differences between the four groups ($F_{3,144}=15.08$, $P<0.0001$) and an increase in weight from southern to northern Tunisia. The Algerian samples exhibited the smallest average weight. Descriptive parameters were not significantly different between regions.

The stepwise discriminant analysis developed for the samples correctly classified 85% of specimens to their geographical origin (central Tunisia 96.2%, southern Tunisia 78%, northern Tunisia 71%, and Annaba–Algeria 100%). The plot of the canonical variables CV1 and CV2 is shown in Fig. 3. This indicated a clear separation between the four studied groups of green frog from Tunisia and Algeria (Wilks' lambda = 0.047, $P < 0.0001$).

Correlations between the variables and the canonical variates (CV1 and CV2) showed that all variables were positively correlated with CV1 (85.5% of the total among-groups variance) and that MTM represented the highest correlation score. For CV2 (explained 8.5% of variance), HL was the variable contributing most to overall among-groups differences, but all variables were negatively correlated with this CV (Table 3).

The second discriminant function analysis, developed for localities using average annual precipitation records as a factor, resulted in a lower rate of correct classifications (83.1%, Wilks' lambda=0.087, $P<0.0001$) than the first DA (85%) (Fig. 4). This DA showed a high classification level for specimens from Annaba and Beja (with average annual precipitation > 800 mm).

3 Discussion

This analysis of morphometric variation of the North African green frog *Pelophylax saharicus* using univariate and multivariate statistics reveals significant differences between the surveyed localities in Tunisia and Algeria. Previous studies on poikilothermic animals, especially amphibians, have shown that these variations are caused by a combination of genetic and environmental factors (Berven, 1982; Riha and Berven, 1991; Tryjanowski et al., 2006). In fact, according to the Köppen-Geiger system (Peel et al., 2007), our four localities belong to three climatic regions. First, the transect extending from north-eastern Tunisia to northwestern Algeria, Mediterranean area, Csa (C: warm temperate, s: summer dry, a: hot summer) where annual precipitation ranges between 400–600 mm in the east, and between 800–

1500 mm in the west (Sicilia et al., 2007¹). Second, the central Tunisia steppic plain (Sahel) and plateau (Tell), BSh (B: arid, S: steppe, h: hot arid) where annual precipitation ranges from 200–600 mm (Sicilia et al., 2007). Third, southern Tunisia, the desert zone, BWh (B: arid, W: desert, h: hot arid) characterized by annual precipitation values ranging between 75–200 mm (Sicilia et al. 2007). Differences in precipitation (as revealed by the second discriminant analysis) can explain most of the morphometric variation between samples from Tunisia. However, since samples from Annaba and north Tunisia belong to the same climatic area, precipitation alone cannot completely explain observed morphometric differences. Furthermore, despite sharing the same global conditions, *Pelophylax* populations of the Mediterranean area (Csa) are highly fragmented due to the presence of several mountain formations (Aures and Atlas Mountains) that create microhabitats specific to each locality .

These observations are in agreement with previous findings on morphometric variation in this species from different Tunisian localities (Amor et al., 2009b). These results also agree with those of several studies conducted previously on reptiles and amphibians from North Africa demonstrating insignificant genetic diversity between Algerian and Tunisian forms (Lanza et al., 1986; Mateo et al., 1996).

Our analysis of variation in body length indicates that green frogs from arid and hot oasis habitats in the southernmost region of Tunisia are characterized by larger body sizes than populations from other regions. However, these southernmost samples had the lowest body weight. Ashton (2002) explained the pattern of body size variation in amphibians as being more strongly related to the availability of water (e.g. precipitation and humidity) than to other environmental factors. Ashton (2002) suggested that larger individuals are more commonly found in the driest environments because of their greater tolerance to desiccation. Colder temperatures have also been proposed as a cause for limited activity and short breeding seasons in amphibians, affecting growth and development rates, and resulting in longer larval periods and larger body sizes during and after metamorphosis (Morrison and Hero, 2003). It is likely, therefore, that the ability of the *P. saharicus* to attain a larger body size is due to the southern region experiencing the cold temperatures during metamorphosis. Body weight variation can be explained by differences in stochasticity of environmental conditions, and food availability and quality.

Geographic variation in body shape is due to differences in allometric relationships between body proportion characters and could be explained by the effect of three different processes: natural selection, stochastic evolutionary processes and phenotypic plasticity (Thorpe and Baez, 1987; Lee, 1993; Castellano et al., 1994). Here, observed morphometric variation suggests that climatic and ecological conditions are correlated and may drive differences in overall body shape.

Pelophylax saharicus specimens examined in this study exhibit the same chromosome number ($2n = 26$), chromosome morphology (all biarmed), chromatin characteristics (such as localization of NORs on the 11th pair, and a base pair GC rich NOR-associated heterochromatin) that are commonly observed in all Palaearctic water frogs (Miura, 1995). Furthermore, centromeric heterochromatin was shown to be as scarce and sensitive to barium hydroxide treatment as has been noticed for *P. synklepton esculenta* (Schmid, 1978b). However, in the latter species, centromeric heterochromatin, except that of the 12th chromosome pair that was CMA₃ positive, was indifferently stained both with DAPI and CMA₃ (Gigantino et al., 2002). This was in contrast to *P. saharicus*, where centromeric heterochromatin is

¹ Sicilia A, Marrone F, Sindaco R, Turki S, Arculeo M, 2007. Contribute to the knowledge of Tunisian amphibians: Notes on distribution, habitat features and phenology. Proceedings of the Fifth Symposium for European Freshwater Sciences (SEFS5), Palermo, 8–13 July 2007, p 249.

AT rich, leading to DAPI-positive staining. This data supports the genetic evidence of a close relationship between the green frog populations in Tunisia and Algeria.

Neither the present chromosome analysis nor previous genetic studies (Amor et al., 2007; Martínez-Solano et al., 2007²) revealed patterns of intraspecific variation commensurate to those observed for morphometric differentiation. We posit that phenotypic plasticity is shaping morphometric variation in this species and that variability in the body shape of *P. saharicus* is the plastic response of genotypes to different environments (adaptive phenotypic plasticity).

In conclusion, we have shown the existence of various morphotypes in Tunisia (southern, central and northern) and Algeria (Annaba). Clinal variation in body size and weight as a function of latitude may result from phenotypic plasticity correlated with local environmental factors (e.g. average annual precipitation). However, observed morphometric variation between Tunisian and Algerian populations may be due to more than phenotypic plasticity. Investigation of genetic divergences using more polymorphic markers will likely be necessary to resolve phylogeographic patterns in this species.

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References

- Amor N, Aprea G, Chatti N, Odierna G, Said K, 2007. Karyological analysis of four Tunisian species of Anura (Amphibia). *African Zoology* 42: 268–278.
- Amor N, Kalboussi M, Said K, 2009a. Conservation status of amphibian in Tunisia. In: Heatwole H ed. *Amphibian Biology*. Volume 8C: The Status of Amphibian Decline. Chipping Norton: Surrey Beatty & Sons.
- Amor N, Farjalah S, Said K, 2009b. Morphometric variation in the Tunisian green frog, *Rana saharica* (Anura: Ranidae). *African Zoology* 43 (2): 194–203.
- Arano B, Lorente GA, Montori A, Buckley D, Herrero P, 1998. Diversification in north-west African water frogs: Molecular and morphological evidence. *Herpetological Journal* 8: 57–64.
- Ashton KG, 2002. Patterns of within-species body size variation of birds: Strong evidence for Bergmann's rule. *Global Ecology and Biogeography*, 11: 505–523.
- Berger L, Ryback M, 1994. Growth and maturity of brown frogs *Rana arvalis* and *Rana temporaria* in central Poland. *Alytes* 11: 11–24.
- Berven KA, 1982. The genetic basis of altitudinal variation in the wood frog *Rana sylvatica*. I. An experimental analysis of life history traits. *Evolution* 36: 962–983.
- Blackburn DC, Measey GJ, 2009. Dispersal to or from an African biodiversity hotspot? *Molecular Ecology* 18: 1904–1915.
- Boulenger GA, 1891. A contribution to the knowledge of the races of *Rana esculenta* and their geographical distribution. *Proceedings of the Zoological Society of London* 593–627.
- Buckley D, Arano B, Herrero P, Llorente G, Esteban M, 1994. Moroccan water frogs vs. *R. perezii*: Allozyme studies show their differences. *Zoologica Poloniae* 39(3–4): 377–385.
- Buckley D, Arano B, Herrero P, Llorente G, 1996. Population structure of Moroccan Water Frogs: Genetic cohesion despite a fragmented distribution. *Journal of Zoological Systematics and Evolutionary Research* 34(3): 173–179.
- Carranza S, Arnold EN, Wade E, Fahd S, 2004. Phylogeography of the false smooth snakes, *Macroprotodon* (Serpentes, Colubridae): Mitochondrial DNA sequences show European populations arrived recently from Northwest Africa. *Molecular Phylogenetics and Evolution* 33: 523–532.

² Martínez-Solano I, Buckley D, Velo Anton G, Amor N, Alcobands M et al., 2007. Comparative phylogéographie of *Discoglossus* and *Rana* in North Africa. *Proceedings of the First Mediterranean Herpetological Congress, Marrakech, Morocco, 16–20 April 2007*.

- Castellano S, Malhotra A, Thorpe RS, 1994. Within-island geographic variation of the dangerous Taiwanese snake *Trimeresurus stajnegeri* in relation to ecology. *Biological Journal of the Linnean Society* 32: 365–375.
- De Jong H, 1998. In search of historical biogeographic patterns in the western Mediterranean terrestrial fauna. *Biological Journal of the Linnean Society* 65: 99–164.
- Fromhage L, Vences M, Veith M, 2004. Testing alternative vicariance scenarios in western Mediterranean discoglossid frogs. *Molecular Phylogenetics and Evolution* 31: 308–322.
- Frost DR, Grant T, Faivovich J, Bain RH, Haas A et al., 2006. The amphibians tree of life. *Bulletin of the American Museum of Natural History* 297: 1–370.
- Garcia-Paris M, Buchholz DR, Parra-Olea G, 2003. Phylogenetic relationships of Pelobatoidea re-examined using mtDNA. *Molecular Phylogenetics and Evolution* 28: 12–23.
- Gauthier H, 1928. Recherches sur la faune des eaux continentales de l'Algérie et de la Tunisie. Minerva, Alger Algeria.
- Gigantino R, Apea G, Capriglione T, Guarino FM, Odierna G, 2002. Caratteristiche genomiche dei vertebrati ectotermi del Parco del Matese. I. Risultati dell'analisi cromosomica. In: Odierna G, Guarino FM ed. I vertebrati ectotermi del Parco Regionale del Matese. Naples: Centro stampa dell'Università di Napoli Federico II, 29–55.
- Graves GR, 1985. Elevational correlates of speciation and intraspecific geographical variation in plumage of Andean forest birds. *Auk* 102: 556–579.
- Graves GR, 1988. Linearity of geographic range and possible effect on the population structure of Andean birds. *Auk* 105: 47–52.
- Hair JF, Anderson RE, Tatham RL, Black WC, 1998. *Multivariate Data Analysis*. 5th edn. Upper Saddle River, U.S.A.: Prentice Hall.
- Harris DJ, Batista V, Carretero MA, 2003. Diversity of 12S mitochondrial DNA sequences in Iberian and north-west African water frogs across predicted geographic barriers. *Herpetozoa* 16: 81–83.
- Howell WM, Black DA, 1980. Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: 1-step method. *Experientia* 36: 1014–1015.
- Lanza B, Nascetti G, Capula M, Bullini L, 1986. Les Discoglosses de la région Méditerranéenne occidentale (Amphibia; Anura; Discoglossidae). *Bulletin Société Herpétologique de France*, 40: 16–27.
- Lee JC, 1993. Geographic variation in size and shape of Neotropical frogs: a precipitation gradient analysis. *Occasional Papers of the Museum of Natural History of the University of Kansas*, 163: 1–20.
- Mateo JA., López-Jurado LF, Guillaume CP, 1996. Variabilité électrophoretique et morphologique des lizards ocellés (Lacertidae): un complexe d'espèces de part et d'autre du détroit de Gibraltar. *Comptes Rendus Academie Des Sciences, Paris*, 319: 737–746.
- Mayet V, 1903. *Catalogue raisonné des Reptiles et Batraciens de la Tunisie: Exploration scientifique de la Tunisie*. Paris: Imprimerie Nationale.
- Mezzanotte R, Bianchi U, Vanni R, Ferrucci L, 1983. Chromatin organization and restriction nuclear activity on human metaphase chromosomes. *Cytogenetic and Cell Genetic* 36: 562–566.
- Miura I, 1995. The late replication banding patterns of chromosomes are highly conserved in the genera *Rana*, *Hyla* and *Bufo* (Amphibia: Anura). *Chromosoma* 103: 567–574.
- Morrison C, Hero JM, 2003. Geographic variation in life-history characteristics of amphibians: A review. *Journal of Animal Ecology* 72: 270–279.
- Nishioka M, Miura I, Saitoh K, 1993. Sex chromosomes of *Rana rugosa* with special reference to local differences in sex determining mechanism. *Scientific Report of the Laboratory for Amphibian Biology, Hiroshima University* 12: 55–81.
- Nouira S, 1998. L'Herpétofaune. In: *Etude Nationale de la Diversité Biologique de la Tunisie (Tome 2)*. Tunis: Ministry of Environment and Sustainable Development Press.
- Nouira S, 2001. Conservation des zones humides littorales et des écosystèmes côtiers – Cap Bon. Tunis: Coastal Protection and Planning Agency (internal report), Ministry of Environment and Sustainable Development Press.
- Odierna G, Apea G, Capriglione T, Parisi P, Arribas O et al., 1999. Chromosomal and molecular analysis of some repeated families in *Discoglossus* Otth, 1837 (Anura, Discoglossidae): Taxonomic and phylogenetic implications. *Italian Journal of Zoology* 66: 273–283.
- Peel MC, Finlayson BL, McMahon TA, 2007. Updated world map of the Köppen-Geiger climate classification. *Hydrology and Earth System Sciences* 11: 1633–1644.
- Plötner J, 1998. Genetic diversity in mitochondrial 12S rDNA of western Palearctic water frog (Anura, Ranidae) and implications for their systematics. *Journal of Zoological Systematic and Evolutionary Research* 36: 191–201.
- Riha VF, Berven KA, 1991. An analysis of latitudinal variation in the larval development of the wood frog *Rana sylvatica*. *Copeia* 1991: 209–221.
- Romdhane MS, Missaoui H, 2001. Conservation des Zones Humides Littorales et des Ecosystèmes côtiers du Cap-Bon. Tunis: Coastal Protection and Planning Agency (internal report), Ministry of Environment and Sustainable Development Press.
- Sahar E, Latt SA, 1980. Energy transfer and binding competition between dyes used to enhance staining differentiation in metaphase chromosomes. *Chromosoma* 79: 1–28.

- Sanmartín I, 2003. Dispersal vs. vicariance in the Mediterranean: historical biogeography of the Palearctic Pachydeminae (Coleoptera, Scarabaeoidea). *Journal of Biogeography* 30: 1883–1897.
- Schneider B, 1978. Beitrag zur Herpetofauna Tunesiens 2. *Bufo mauritanicus*. *Salamandra* 14 (1): 33–40.
- Schmid M, 1978a. Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. *Chromosoma* 66: 361–388.
- Schmid M, 1978b. Chromosome banding in Amphibia. II. Constitutive heterochromatin and nucleolar organizer regions in Ranidae, Microhylidae and Rhacophoridae. *Chromosoma* 68: 131–148.
- Shapiro SS, Wilk MB, 1965. An analysis of variance test for normality (complete samples). *Biometrika* 52 (3–4): 591–611.
- Sokal RR, Rohlf FJ, 1995. *Biometry: The Principles and Practice of Statistics in Biological Research*. 3rd edn. New York: W. H. Freeman.
- SPSS Inc, 2007. *Systat for Windows*. Version 16.0. Chicago, Illinois: SPSS Inc..
- Sumner AT, 1972. A simple technique for demonstrating centromeric heterochromatin. *Experimental Cell Research* 75: 304–306.
- Thorpe RS, Baez M, 1987. Geographic variation within an island: univariate and multivariate contouring of scalation, size and shape of the lizard *Gallotia galloti*. *Evolution* 41: 256–268.
- Tryjanowski P, Sparks T, Rybacki M, Berger L, 2006. Is body size of the water frog *Rana esculenta* complex responding to climate change? *Naturwissenschaften* 93: 110–113.

Table 1 Geographic locations and sample sizes (n) for *Pelophylax saharicus* from Tunisia and Algeria

Region	Locality	Geographical coordinates		<i>n</i>
Northern Tunisia	Kelibia	36°50'49.46"N	11° 4'50.09"E	10
	Elmenza	36°52'21.00"N	10°10'54.00"E	12
	Beja	36°50'49.46"N	11° 4'50.09"E	12
	Lebna	36°38'34.22"N	9°12'59.33"E	7
Total			41	
Central Tunisia	Kasserine	35°10'25.93"N	8°49'36.37"E	9
	Kairouan	35°40'0.00"N	10° 5'57.72"E	7
	Sousse	35°45'33.04"N	10°48'49.35"E	10
Total			26	
Southern Tunisia	Douz	33°23'08.87"N	7°55'09.46"E	7
	Nefta	33°52'3.00"N	7°52'46.20"E	9
	Tamerza	34°23'48.87"N	7°56'40.46"E	9
	Chenini	33°52'59.00"N	10° 4'39.68"E	11
	Gafsa	34°25'17.94"N	8°47'11.96"E	10
	Lala	34°23'18.00"N	8°47'50.00"E	11
Total			57	
Northern Algeria	Annaba	36°51'9.48"N	8°28'8.83"E	24

Table 2 Descriptive data for *P. saharicus* from Tunisia and Algeria (body measurements in cm; weight in g)

Region		Mass	SVL	HL	HW	FoL	FeL	TL	MTL	FTL
Northern Tunisia	Mean	33.39	4.68	1.87	1.30	7.90	2.08	2.28	0.90	1.370
	Std. Error of Mean	10.47	1.68	0.72	0.47	2.9	0.76	0.81	0.33	0.78
Central Tunisia	Mean	26.01	3.97	1.33	1.12	6.75	1.90	2.08	0.69	1.22
	Std. Error of Mean	11.89	1.38	0.56	0.35	2.61	0.70	0.72	0.26	0.66
Southern Tunisia	Mean	24.89	5.05	1.78	1.31	8.75	2.41	2.62	0.78	1.65
	Std. Error of Mean	11.70	1.15	0.48	0.30	0.21	0.57	0.63	0.20	0.58
Annaba	Mean	19.54	4.60	1.97	1.66	7.25	1.88	2.21	1.20	1.89
	Std. Error of Mean	4.06	0.44	0.18	0.16	0.82	0.30	0.33	0.21	0.22

Body measurements: snout-vent length (SVL); head length (HL), from the posterior margin of the lower jaw to the tip of the snout; head width (HW), measured at the level of the posterior part of the tympanum; foot length (FoL), from the proximal border of the inner metatarsal tubercle to the tip of the fourth toe; femur length (FeL); tibia length (TL); metatarsal tubercle length (MTL); and first toe length (FTL).

Table 3 Correlations with canonical variates 1–2 (CV1 and CV2) and cumulative proportion of the variance explained for *P. saharicus* from Tunisia and Algeria (geographic location as factor)

Variable	CV1	CV2
MTL	0.231*	-0.181
HL	0.081	-0.398*
SVL	0.013	-0.339*
FoL	0.050	-0.326*
FeL	0.078	-0.279*
TL	0.063	-0.261*
HW	0.166	-0.208*
FTL	0.079	-0.197*
Cumulative %	85.5	94

* Largest absolute correlation between each variable and any discriminant function

Fig. 1 Map of *Pelophylax saharicus* sampling localities from Tunisia and Algeria showing three climatic regions (Csa: warm temperate, summer dry, hot summer; BSh: arid, steppe, hot arid; BWh: arid, desert, hot arid) with isohyets indicating the average annual rainfall measured in millimetres

Fig. 2 Karyotype of *Pelophylax saharicus* from Tunisia and Algeria

a. Giemsa staining, the Ag-NOR and CMA₃ + methyl green-stained 11th chromosome pair is reported above and below. b. C-banding. c. C-banding + DAPI. d. C-banding + CMA₃. e. Q-banding. f. Hae III+DAPI. g. Hae III+CMA₃.

Fig. 3 Ordination on the two canonical variables CV1 and CV2 for Tunisian and Algerian specimens of *Pelophylax saharicus* used in the canonical discriminant function analysis (climatic region as factor)

Fig. 4 Ordination on the two canonical variables CV1 and CV2 for Tunisian and Algerian specimens of *Pelophylax saharicus* used in the canonical discriminant function analysis (average annual precipitation as factor)

Fig.1

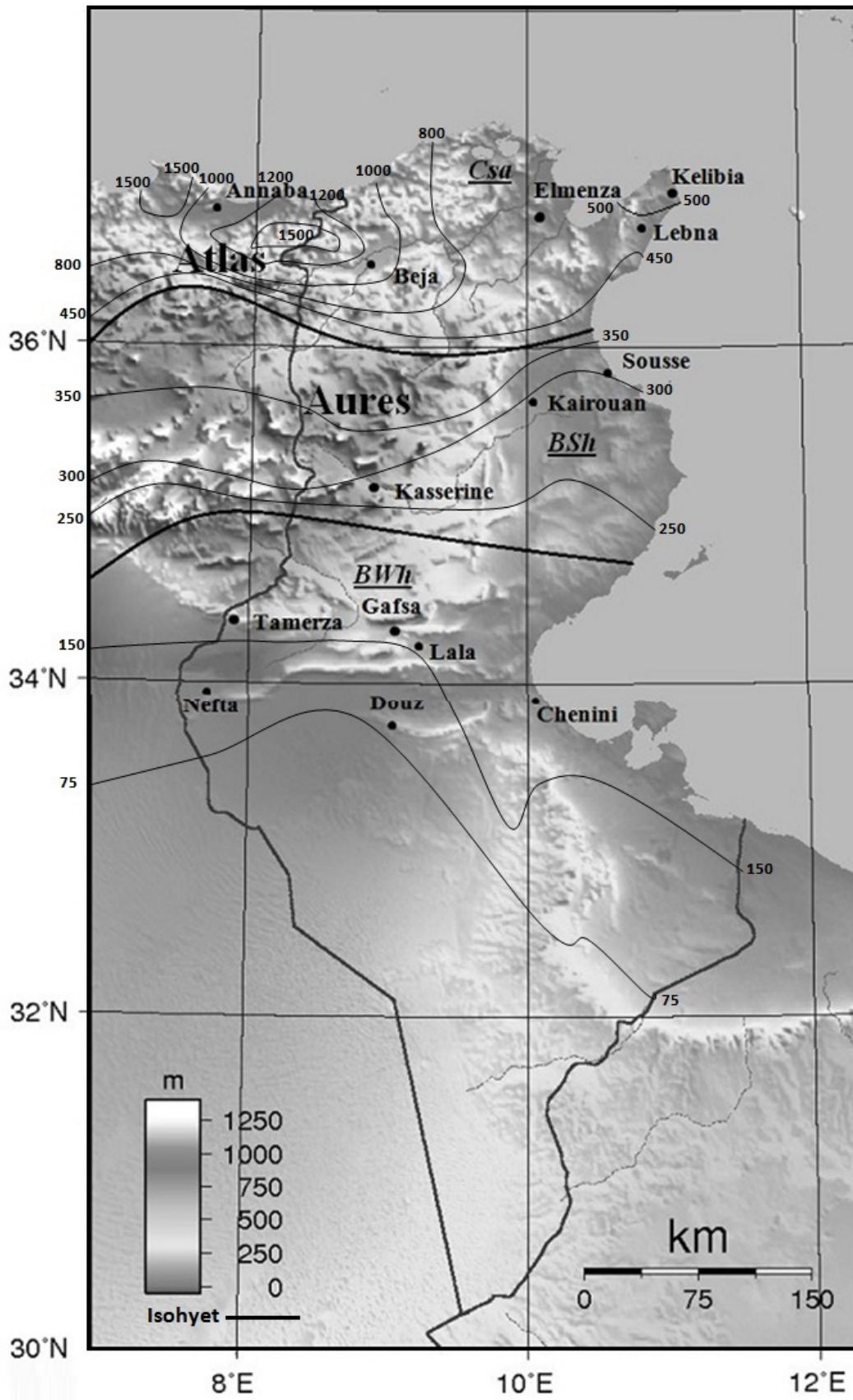


Fig. 2

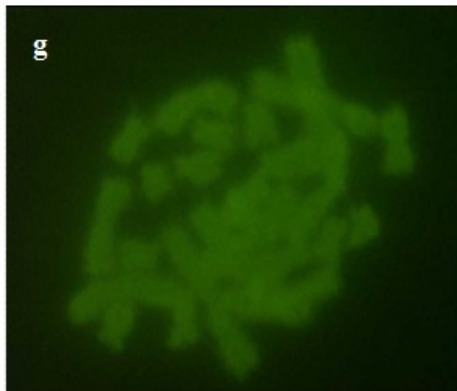
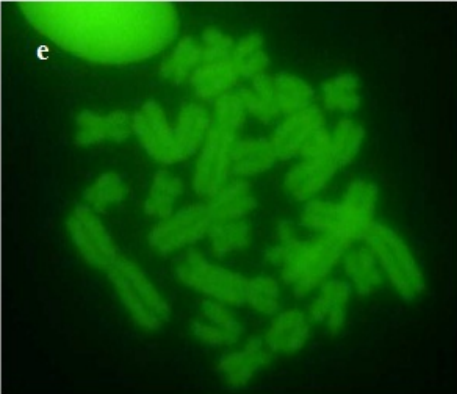
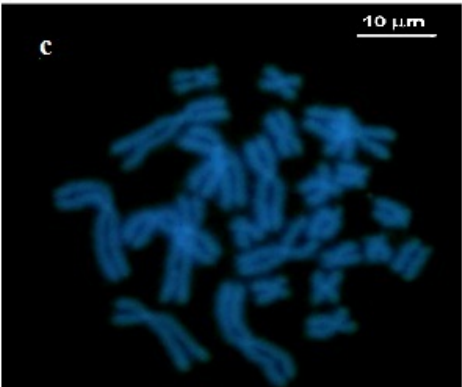
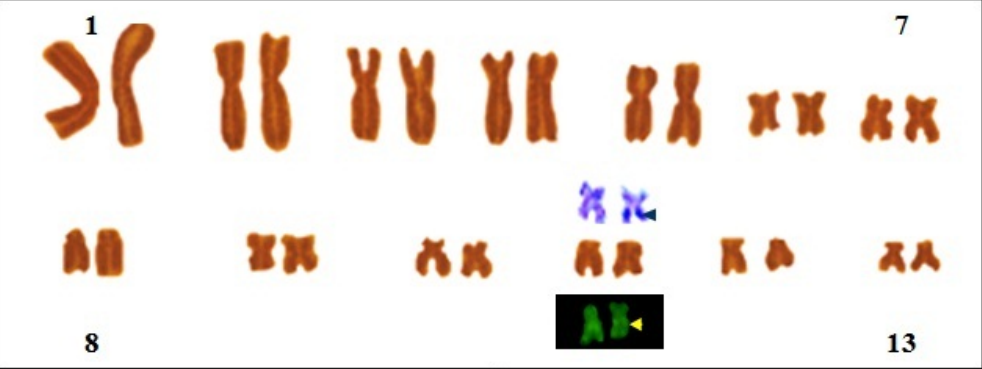


Fig. 3

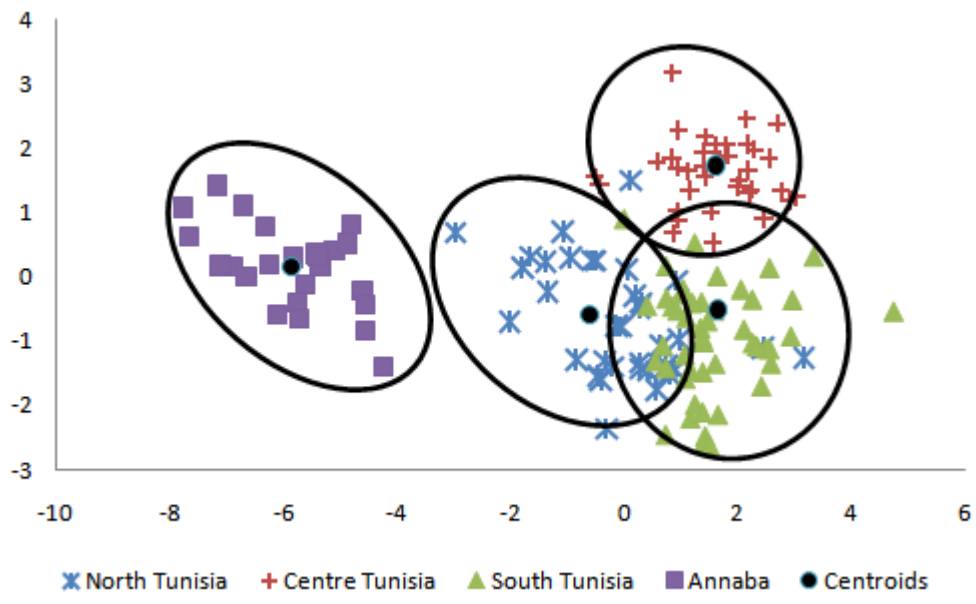


Fig. 4

