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

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**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, $CMA_3$ and Q-banding) reveal this species to exhibit the plesiomorphic *Pelophylax* karyotype of 2n = 26 biarmed 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): 678–686, 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 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

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studies of the distribution of this North African green frog (Boulenger, 1891; Mayet, 1903; Gauthier, 1928; Schneider, 1978; Nouira, 1998, 2001[1]; Romdhane and Missaoui, 2001[2]) 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 (Nouira, 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 $H_2O$) 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 $A_3$/methyl green staining ($CMA_3$) according to Sahar and Latt (1980); Q-banding, as suggested by Schmid (1978a); C-banding, according to Sumner's method (1972); sequential C-banding, $CMA_3$/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

[1] 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.

[2] 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.

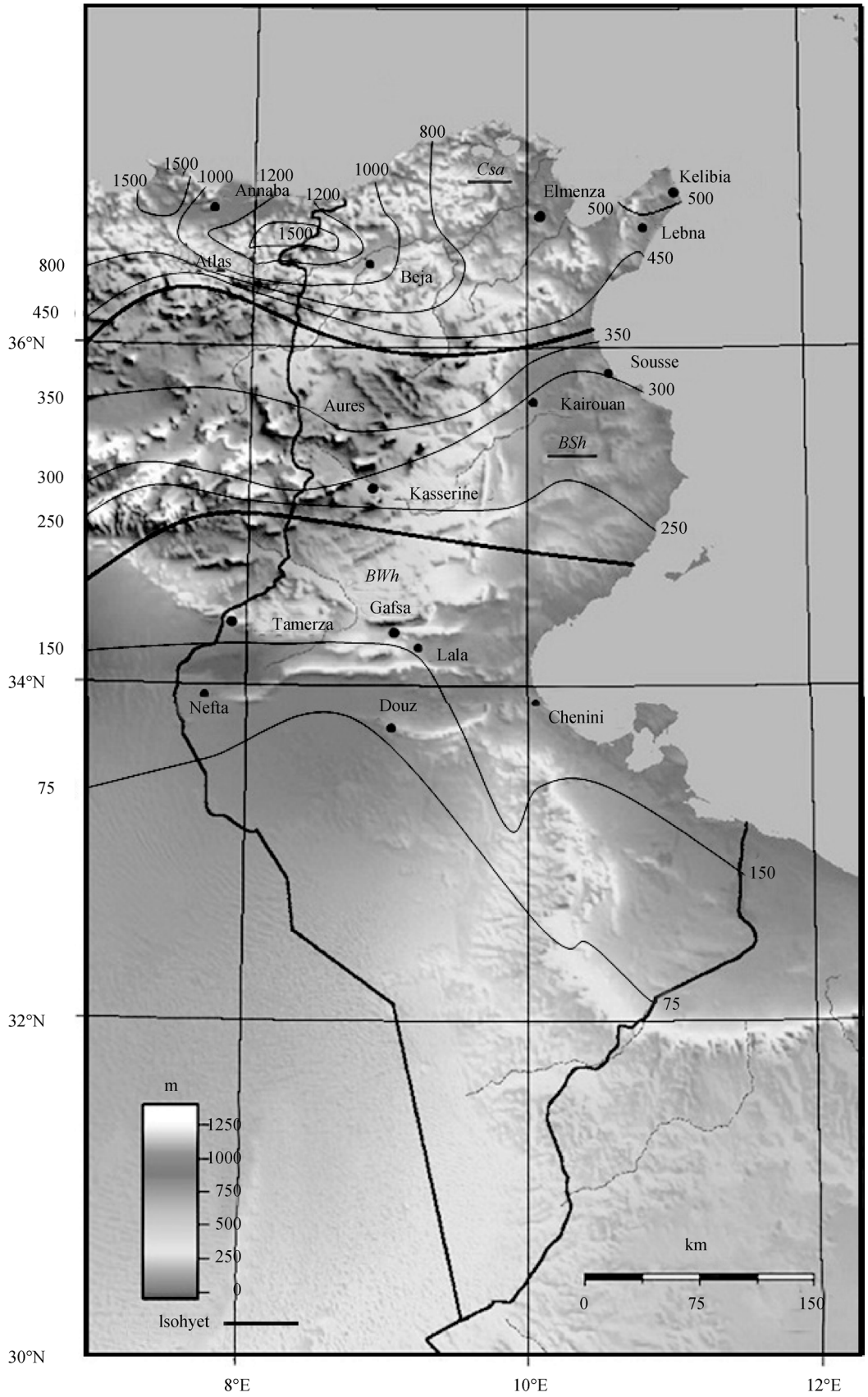


**Fig. 1 Map of *Pelophylax saharicus* sampling localities from Tunisia and Algeria showing three climatic regions with isohyets indicating the average annual rainfall measured in millimetres**

(Csa: warm temperate, summer dry, hot summer; BSh: arid, steppe, hot arid; BWh: arid, desert, hot arid).

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

| Region | Locality | Geographical coordinates | | *n* |
|---|---|---|---|---|
| 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 |

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 athis important environmental factor on morphological variation.

## 2 Results

### 2.1 Chromosome analysis

All frogs from Tunisia and Algeria had 26 biarmed chromosomes (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

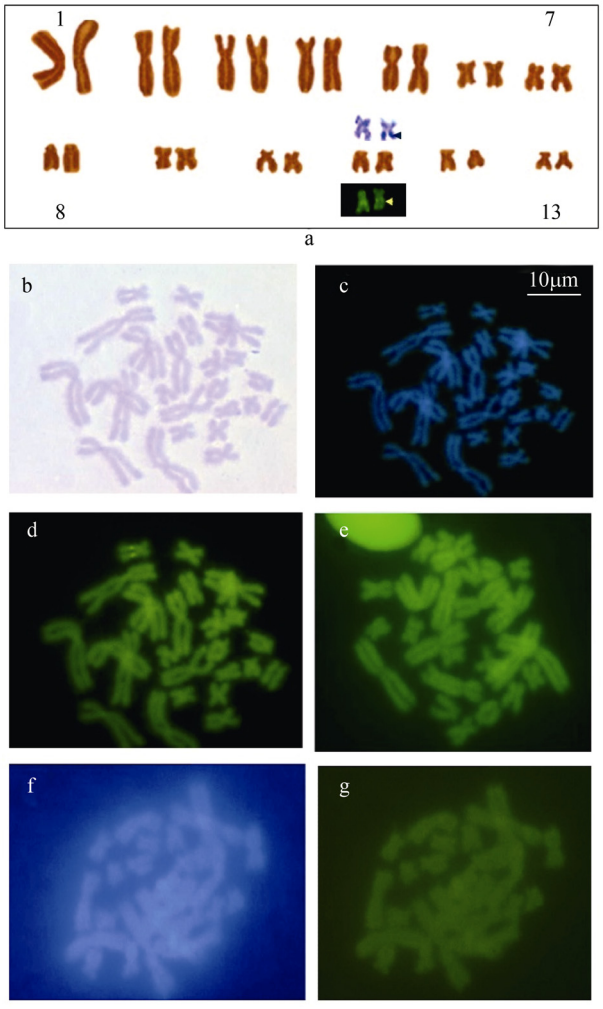


**Fig. 2 Karyotype of *Pelophylax saharicus* from Tunisia and Algeria**

a. Giemsa staining, the Ag-NOR and $CMA_3$ + methyl green-stained 11$^{th}$ chromosome pair is reported above and below. b. C-banding. c. C-banding + DAPI. d. C-banding + $CMA_3$. e. Q-banding. f. Hae III+DAPI. g. Hae III+$CMA_3$.

were submetacentric. Quinacrine stained all chromosomes uniformly (Fig. 2). Ag-NOR and $CMA_3$ 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 $CMA_3$ 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 40ºC 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).

**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).

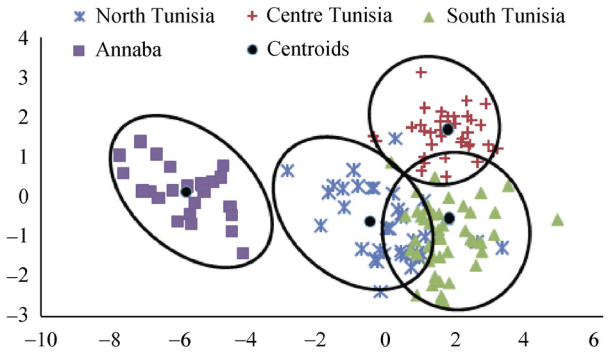


**Fig. 3 Ordination on the two canonical variables CV1 and CV2 for Tunisian and Algerian specimens of *Pelophylax saharicus* used in the canonical discriminant function analysis (climatic region as factor)**

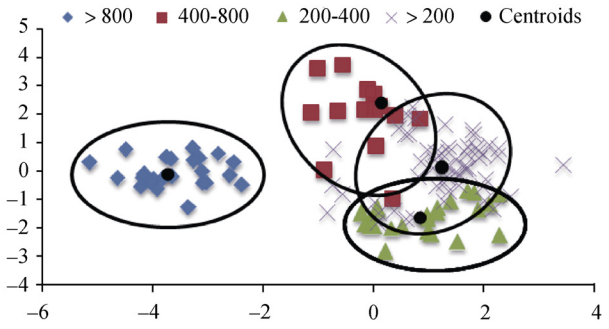


**Fig. 4 Ordination on the two canonical variables CV1 and CV2 for Tunisian and Algerian specimens of *Pelophylax saharicus* used in the canonical discriminant function analysis (average annual precipitation as factor)**

**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

## 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., 2007[3]). 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

[3] 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.

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 11$^{th}$ 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 12$^{th}$ chromosome pair that was $CMA_3$ positive, was indifferently stained both with DAPI and $CMA_3$ (Gigantino et al., 2002). This was in contrast to *P. saharicus*, where centromeric heterochromatin is 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., 2007[4]) 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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