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Morphological Variation of the African Green Toad, *Bufo boulengeri* (Amphibia: Anura) in Tunisia

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Abstract.- This study describes patterns of geographic variation in morphometric characters of the African green toad *Bufo boulengeri*, from Tunisia (11 populations). Body size differences of 89 specimens were analysed with univariate statistics, and body proportions with multivariate methods. Among-groups differences in morphometric characters included both size and shape. Southern populations were found to have larger body size than northern ones, and there was an association between climatic factors and morphological characters. The observed clinal variation in both body size and weight may result from phenotypic plasticity correlated with local environmental factors.

Key words: *Bufo boulengeri*, African green toad, morphometric variation, Tunisia.

INTRODUCTION

The African green toad *Bufo boulengeri* Lataste, 1879 is distributed in North Africa, from western Morocco to eastern Egypt (Stöck *et al.*, 2006, 2008). Previously, it was considered a member of the Palearctic green toad (*Bufo viridis* complex). The different degrees of genetic divergence found within *Bufo viridis* complex and the occurrence of three bisexually reproducing ploidy levels (2n=22, 2n=33 and 2n=44) makes these green toad a uniquely interesting vertebrate group and led various authors to propose new taxonomic arrangement (Odierna *et al.*, 2004; Stöck *et al.*, 2006). Consequently, Stöck *et al.* (2006, 2008), using morphological and molecular approaches, reassigned the African green toad to the status of independent specie.

Bufo boulengeri is widely distributed throughout Tunisia, and inhabits forests, meadows, and steppes. It is resistant to both drought and saline conditions, it prefers open land near watercourses, but in arid areas it lives close to irrigation ditches, springs, oases, and other moist areas (Sicilia *et al.*, 2007; Amor *et al.*, 2009a). The altitudinal distribution of the species in Tunisia ranges from sea level up to 1077 m a.s.l. (near Thala, Tell plateau) (Sicilia *et al.*, 2007). According to Salvador (1996), in Tunisia the African green toad usually breeds from February to May, though another breeding period in August and September is also recorded.

Data on the African green toad variation in Tunisia have been mainly restricted to faunistics (Salvador, 1996; Nouira, 2001) or analyses of few samples (Stöck *et al.*, 2006, 2008). Recent chromosomes study (Amor *et al.*, 2007) revealed that the Tunisian populations of the African green toad share chromosome number and morphology, and chromatin characters (NOR phenotype, localization and composition of heterochromatin) with other North African (Morocco and Egypt) populations. In particular, the presence of a paracentromeric, Quinacrine-positive, heterochromatic band on the short arms of the smallest six chromosome pairs groups together North African populations of green toads and indicates that they derived from the same stock (Stöck *et al.*, 2005, 2006; Odierna *et al.*, 2004).

Morphological differentiation of amphibian taxa at subspecific and even specific rank is often very small and involves mainly differences in body proportions (Babik and Rafinski, 2000). However, in the case of green toads, Stöck *et al.* (2006, 2008)

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demonstrated the power of this tool to distinguish individuals belonging to different geographic groups (Sicily, North Africa, Corsica and Sardinia, and the Apennine Peninsula). Moreover, Castellano and Giacoma (1998) described geographic variation in morphometric characters of the green toad in the western part of its distribution (Italian Peninsula and the islands of Sardinia and Corsica). These authors found that among-population differences in morphometric characters include both size and shape, and that were due to both genetics and the environment.

In this paper we describe the geographic pattern of morphometric variation of *B. boulengeri* in Tunisia to determine whether or not morphological characters differ significantly from one locality to another, and to characterize these differences. The study area was divided into three climatic regions according to the Köppen-Geiger system: the northern (Mediterranean area of the country), the central (steppic plain, Sahel, and plateau, Tell) and the southern (the desert zone) (Sicilia *et al*., 2007). Body size differences were analysed with univariate statistics, and body proportions with multivariate methods such as principal component analysis (PCA); canonical variate analysis (CVA) and discriminant analysis (DA). Average annual precipitation records were considered as an environmental variable, since this variable seems potentially effective for selecting the size and shape of green toads.

MATERIALS AND METHODS

Only adult male toads were included in the analysis (Castellano and Giacoma, 1998). A total of 89 specimens of *B. boulengeri* were collected and analysed from 11 populations from three geographical regions: southern (n=33) where annual precipitation is inferior to 100 mm in the southernmost localities (Kebili, Tozeur and Tamerza) and ranges between 100 and 200 mm in Gafsa and Gabes, central (n=29) where the annual precipitation ranges from 200 to about 400 mm (Monastir, Kairouan and Sidi Bouzid) and northern (n=27) which is characterized by annual precipitation higer than 400 mm (Nabeul, Beja and Hammamet) (Fig. 1).

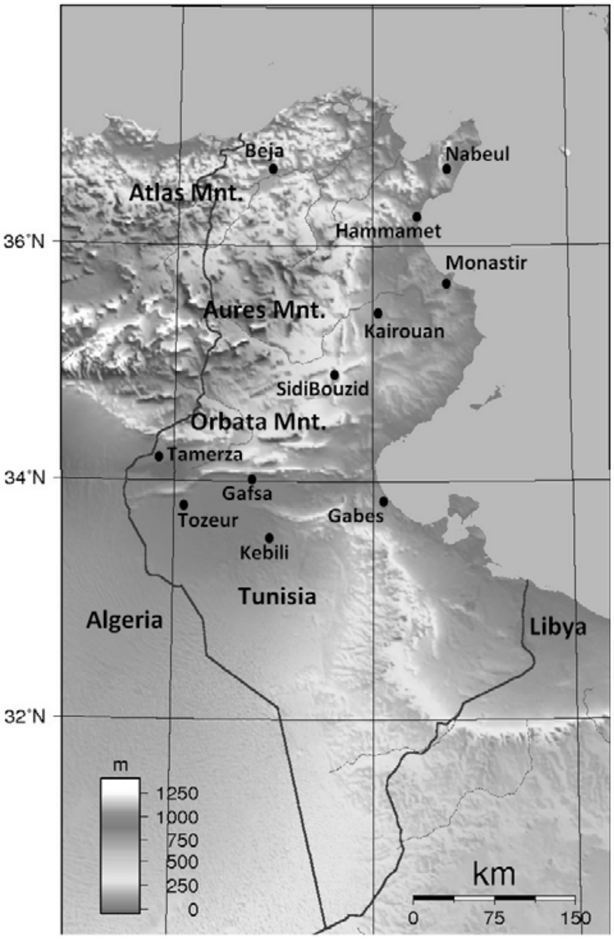

Fig. 1. Map of Tunisia showing sampling localities of *B. boulengeri*.

Ten measurements were taken using a digital calliper (accuracy 0.01 mm): snout-urostyle 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; distance between nostril and anterior corner of eye (NED); distance between eyes (DE); 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; first toe length (FTL); and metatarsal tubercle length (MTL). Weight was measured with an accuracy of 0.5 g using a spiral dynamometer.

SPSS 16.0 (2007) was used for the statistical analysis. Normality of the data was tested using the Shapiro-Wilks' *W*-test (Shapiro and Wilk, 1965). Data not normally distributed were log-transformed in order to meet the assumption of normality. The significance of differences among groups for size-corrected values of measurements was tested by means of one-way ANOVAs complemented with a Spjotvoll-Stoline *a posteriori* test (Sokal and Rohlf, 1995). Size measurements were also subject to PCA, with subsequent ANOVA on the PC 1 and PC 2 scores.

DA was used to describe the 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).

A second discriminant analysis was performed grouping variables by localities and using average annual precipitation records as a factor (>400 mm, 200–400 mm, 100–200 mm and <100 mm), to determine the influence of a possible important environmental factor on morphological variation.

RESULTS

The means and relative standard deviations of the morphometric characters of the populations of African green toads examined are shown in Table I. Significant differences in the size of body were found between the three Tunisian geographical regions (southern, centre and northern) (one-way ANOVA, d.f=2, $P<0.001$). The mean body sizes of the three regions suggest a trend of increase from north to south.

Analysis of variance, including geographic location as a factor for all the analysed variables, showed that all the morphometric variables differed significantly between the regions (Table II). For all these variables an *a posteriori* test of Spjotvoll-Stoline showed a significant difference between the three geographical regions.

Table I.- Descriptive statistics for *B. boulengeri* from Tunisia (body measurements in cm; weight in g).

Variable*	North Tunisia	Centre Tunisia	South Tunisia
Mass	29.9±3.42	32.2±3.5	35.73±5.43
SVL	5.77±1.99	6.6±0.2	6.74±1.1
HL	2.3±0.79	2.59±0.27	2.74±0.63
HW	2.48±0.33	2.06±0.08	2.06±0.39
DNE	0.37±0.05	0.35±0.01	0.4±0.03
DE	0.47±0.04	0.52±0.03	0.6±0.01
FoL	6.85±1.7	7.57±0.4	8.25±0.21
FeL	2.04±0.39	2.1±0.15	2.61±0.37
TL	2.06±0.71	2.32±0.12	2.69±0.3
MTL	0.98±0.74	1.18±0.08	1.27±0.28
FTL	1.76±0.55	1.82±0.06	2.05±0.48

*Abbreviations: DE, distance between eyes; FeL, femur length; FoL, foot length; FTL, first toe length; HL, head length; HW, head width; MTL, metatarsal tubercle length; NED, distance between nostril and anterior corner of eye; SVL, snout-urostyle length; TL, tibia length.

Table II.- Results of one-way ANOVAs for *B. boulengeri* from Tunisia showing differences in means of size-adjusted variables among regions.

Variable*	$F_{2.71}$	P
SVL	5.66	0.000
HL	4.8	0.001
HW	4.48	0.001
NED	11.08	0.000
DE	9.44	0.000
FoL	9.43	0.000
FeL	10.41	0.000
TL	9.01	0.000
MTL	6.02	0.000
FTL	5.37	0.000

[1] For abbreviations seen Table I.

One-way ANOVA of weight showed significant differences between the three groups ($F = 4.66$; $P < 0.01$) and also an increase of this measure from north to south.

Shape differences among groups were analysed by PCA. The first principal component (PC1) explained 74.3% of the total variance; 98.0 % of the total variance was explained by as many as five principal components. For PC1, NED and DE showed the lowest positive loadings. The second principal component (PC2) differentiated most efficiently frogs from the three geographic groups.

This is shown by the highly significant result of ANOVA on PC2 scores (PC2: F=106.9, p=<0.0001; PC1: F = 6.9, p = 0.002). The distance between eyes (DE) showed high positive loadings for PC2.

The stepwise discriminant analysis correctly classified 94% of specimens to geographical regions. The plot of the canonical variables CV1 and CV2 is shown in Figure 2. This showed a clear separation between the three studied groups of green toad from Tunisia (Wilks' lambda = 0.05, $P < 0.0001$).

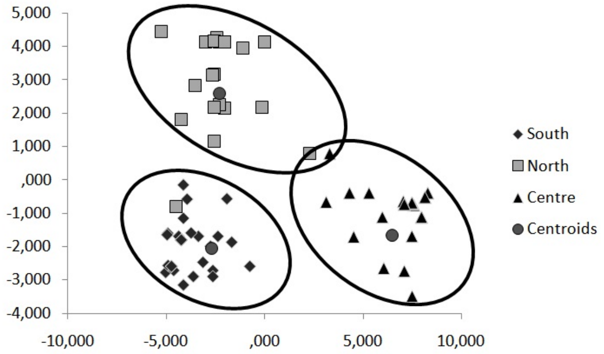

Fig. 2. Ordination on the two canonical variables CV1 and CV2 for Tunisian of *B. boulengeri* used in the canonical discriminant function analysis (climatic region as factor).

The correlations between the variables and the canonical variates (Table III) showed which variables contributed most to the overall among-groups differences. The first canonical axis (CV1) accounted for 82.1% of the total among-groups variance. All measurements were positively correlated with the CV2 (which explained 17.9% of variance) except HW. DE showed the highest positive loading for CV2.

The second discriminant function analysis (DA) for localities, using the average annual precipitation records as a factor, resulted in a lower rate of correct classifications (80.9%) than the first stepwise discriminant analysis. However, reducing annual precipitation records to only three classes (> 400 mm, 200–400 mm and < 200 mm) resulted in a higher rate of correct classifications (96.6%). Correlations between the variables and the canonical variates showed that CV1 accounted for 81.2% of the total among-groups variance.

Table III.- Correlations of variables with canonical variates 1–2 and cumulative proportion of the variance explained for *B. boulengeri* from Tunisia (geographical location as a factor).

Variable[1]	CV1	CV2
SVL	0.38	0.11*
HL	0.023	0.10*
HW	-0.08	-0.19*
DNE	-0.06	0.16*
DE	0.038	0.93*
FoL	0.021	0.18*
FeL	-0.10	0.33*
TL	0.009	0,17*
MTL	0.021	0.09*
FTL	0.09	0.112*
Cumulative %	82.1	100

[1] For abbreviations seen Table I.

*. Largest absolute correlation between each variable and any discriminant function.

DISCUSSION

The analysis of morphometric variation in *B. boulengeri* using univariate and multivariate statistics revealed significant differences between the Tunisian regions and confirm earlier studies, which demonstrate the power of this tool to distinguish between individuals of green toads belonging to different geographic groups (Castellano and Giacoma, 1998; Stöck *et al.*, 2008). Differences in precipitation between regions can also explain most of the morphometric variation between Tunisian samples, as revealed by the second DA. Previous studies on Tunisian anurans (*Discoglossus pictus* and *Pelophylax saharicus*) have shown identical patterns of morphological variations (Amor *et al.*, 2009b; Amor *et al.*, 2010a,b). However, in the case of *B. boulengeri* this variation seems to be more noticeable.

Several studies on poikilothermic animals, especially green toads, have shown that morphometric variations are caused by a combination of genetic and environmental factors (Castellano and Giacoma, 1998; Tryjanowski *et al.*, 2006; Stöck *et al.*, 2008).

The present data on body weight variation showed significant differences between the three groups, with an increase of weight from north to south. This observation can be explained by differences in stochasticity of environmental conditions, and food availability and quality.

Principal component analysis revealed that the differences in body shape among the three groups of populations resulted from differences in both head and leg size.

Analysis of green toads' body length showed significant variation between regions with an increase from northern to southern localities. In fact, the investifated localities belong to three climatic regions: northern Tunisia, Mediterranean area (warm temperate, summer dry, hot summer); central Tunisia, steppic plain (Sahel) and plateau (Tell, arid steppe), and southern Tunisia, the desert zone (arid desert).

Furthermore, Tunisian green toad populations are highly fragmented due to the presence of several mountain formations (Aures, Atlas and Orbata) that create specific microhabitats and morphometric variations. In fact, 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. Consequently, larger individuals are favoured in the driest environment because of a greater tolerance to desiccation (Ashton, 2002).

According to Castellano and Giacoma (1998) the correlation between body proportion characters and climatic conditions is related to the association between larger sized animals and warmer and dryer climates. Larger specimens have a smaller surface-to-volume ratio than smaller specimens (Castellano and Giacoma, 1998). Nevo (1973) suggested that a positive correlation between large body size and a hot and dry climate can result from selection against desiccation. However, a reduction of the surface to volume ratio can be also achieved by proportionally reducing limb length (Nevo, 1973).

Our analysis showed that frogs from southern populations had on average relatively longer legs than individuals from the northern and central regions. Many adaptive explanations for leg size variation have been proposed. Tejedo *et al.* (2000) explained this variation by differences in larval density in ponds. He observed that anurans raised at low density (in our case southern localities) were characterised by larger legs than those raised at high densities (northern and central localities).

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

Furthermore, comparing islands and mainland populations, Castellano and Giacoma (1998) found that green toads from both southernmost populations tend to be larger than those from the northern ones. They suggested that this parallelism supports the hypothesis of an ecological cause for this pattern. Thus, body size differences among populations are the result of differences in growth rate.

During several trips, between 2004 and 2009, to more than thirty localities including different aquatic and terrestrial habitats, we observed that a large numbers of amphibians, mainly toads (*B. boulengeri* and *B. mauritanicus*), are killed on roads during the breeding season and after rainy events. Amphibians are constrained to move within their home ranges and must often cross roads, exposing them to vehicular traffic. There is no system of tunnels allowing safe movement from one place to another along the national network of routes and highways (Amor *et al.*, 2009a). Consequently, the negative impact of road mortality has to be considered as a major factor disturbing local population structure and even decline.

In conclusion, the present study showed the existence of three morphotypes of African green toads in Tunisia (southern, central and northern). As expected under the adaptive hypothesis, larger specimens are associated with dryer habitat conditions. The observed morphometric variation suggests that climatic and ecological conditions are correlated with each other and thus may drive differences in overall body shape. Clinal variation in both body size and weight may result from phenotypic plasticity correlated with local environmental factors (*e.g.*, average annual precipitation). In terms of future research, our results indicate a need for basic and applied studies to lay a foundation for standardized and informative amphibian road mortality surveys. Investigation of genetic divergences using unlinked molecular markers (both mtDNA and nucDNA) will likely be necessary to resolve phylogeographic patterns in this species.

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