



# Lessons from a complex biogeographical scenario: morphological characters match mitochondrial lineages within Iberian *Coronella austriaca* (Reptilia: Colubridae)

GUSTAVO A. LLORENTE<sup>1</sup>, MARTA VIDAL-GARCÍA<sup>1</sup>, NÚRIA GARRIGA<sup>1</sup>, SALVADOR CARRANZA<sup>2</sup>, JUAN M. PLEGUEZUELOS<sup>3</sup> and XAVIER SANTOS<sup>1,4\*</sup>

<sup>1</sup>*Departament de Biologia Animal, Universitat de Barcelona, Avinguda Diagonal 643, E-08028 Barcelona, Spain*

<sup>2</sup>*Instituto de Biología Evolutiva (CSIC-UPF), Paseo Marítimo de la Barceloneta 37-39, E-08003 Barcelona, Spain*

<sup>3</sup>*Departamento de Zoología, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain*

<sup>4</sup>*CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal*

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Molecular evidence suggests that climatic fluctuations in the peninsulas of southern European during the Miocene and Pleistocene resulted in considerable genetic differentiation of organisms, probably following a ‘refugia within refugia’ model. We examined morphometric and meristic characters in museum specimens from the three Iberian clades of the smooth snake *Coronella austriaca*, previously described by molecular markers. We found sexual and interclade differences in scale counts and head shape. Sexes were dimorphic in the number of ventral and subcaudal scales, although sexual disparities in scale counts differed among clades. Moreover, discriminant and canonical analyses showed higher interclade differences in males than in females. The results obtained in the present study match those from molecular markers and confirm the population structure identified within Iberian *C. austriaca*. The observed sex discrepancy in this pattern suggests that males and females are subjected to different selective pressures along their Iberian distribution. In light of the above, *C. austriaca* may offer a useful model system in which to explore phylogeographical patterns in southern Europe, as well as the conflicts between processes driving morphological sexual divergence. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **106**, 210–223.

**ADDITIONAL KEYWORDS:** fecundity selection – interclade variation – morphometric – phylogeography – sexual dimorphism.

## INTRODUCTION

A significant aspect of phylogeographic research in Europe is the role of the southern peninsulas – in other words, Iberia, Italy, and the Balkans (together with the Caucasus region). During the glacial periods these localities acted as refugia for a range of organisms prior to their recolonisation of northern

latitudes as the ice retreated (Taberlet *et al.*, 1998; Hewitt, 2004; Ursenbacher *et al.*, 2005; Horn *et al.*, 2006). Arribas & Carranza (2004) have suggested that, at least in some reptilian species, speciation processes occurred during the Messinian Salinity Crisis (5.3–5.9 Mya BP), a short but crucial period, when the Mediterranean Sea desiccated almost completely, thereby producing a wide-ranging and drastic increase in aridity around the Mediterranean Basin (Hsü *et al.*, 1977; Krijgsman *et al.*, 1999;

\*Corresponding author. E-mail: xsantossantiro@gmail.com

Duggen *et al.*, 2003). This increased aridity forced mesic species to retreat within moister Atlantic-influenced areas and mountainous regions (García-Antón *et al.*, 2002). Later, during the Pleistocene, populations differentiated as a result of fragmentation during the glacial cycles of the Quaternary (Haffer, 1969). This fluctuating scenario has produced a complex biogeographical model that has been referred to as a 'refugia within refugia' model (Gómez & Lunt, 2007).

The phylogeographical processes that occurred during these periods in Europe have been examined for a number of organisms (Taberlet *et al.*, 1998; Hewitt, 2003, 2004), especially amphibians and reptiles, which have been used as model groups to illustrate these evolutionary processes in southern European peninsulas (Paulo *et al.*, 2001; Veith, Kosuch & Vences, 2003; Arribas & Carranza, 2004; Martínez-Solano *et al.*, 2006; Pinho, Ferrand & Harris, 2006; Arnold, Arribas & Carranza, 2007). The particular natural-history traits (i.e. limited dispersion capacity), environmental dependence (e.g. to temperature), and wide distribution ranges of these species, have all contributed to the high levels of diversification observed throughout regions of high climatic variability, such as the Mediterranean Basin (Veith *et al.*, 2003; Arnold *et al.*, 2007; Santos *et al.*, 2009).

The European smooth snake *Coronella austriaca* Laurenti, 1768 is a small ovoviviparous colubrid that primarily feeds on lizards and small mammals, uses ambush and active foraging tactics (Spellerberg & Phelps, 1977; Galán, 1998), exhibits a delayed sexual maturity (at the fourth year; Reading, 2004), and has low vagility, with higher dispersal rates in males than in females (Pernetta *et al.*, 2011). It shows sexual dimorphism because females have longer snout-vent lengths (SVL) and shorter tails than males (Goddard, 1984). The species has an impressive distribution across the Palearctic (Strijbosch, 1997). Towards the south, it reaches the Balkan, Italian, and Iberian peninsulas, where it shows a rather more fragmented distribution than the homogeneous distribution found in central and northern Europe (Strijbosch, 1997). Despite its large distribution range, only one subspecies, namely *Coronella austriaca acutirostris* Malkmus, 1995, from the north-western Iberian Peninsula (type locality: Serra da Estrela, north Portugal), has been described to date. The nominal subspecies *Coronella austriaca austriaca* (type locality: Vienna, Austria) occupies the rest of the range of this species. *Coronella a. acutirostris* differs from the nominal subspecies in terms of its shorter total length, narrow head with pointed snout, and a larger number of scales in the head and neck regions. A third subspecies, namely *Coronella austriaca fitz-*

*ingeri* (Bonaparte, 1840), restricted to southern Italy and Sicily (type locality: Sicily), and characterized by its small body size and less contrasted dorsal pattern, is not currently recognized (Sindaco *et al.*, 2006).

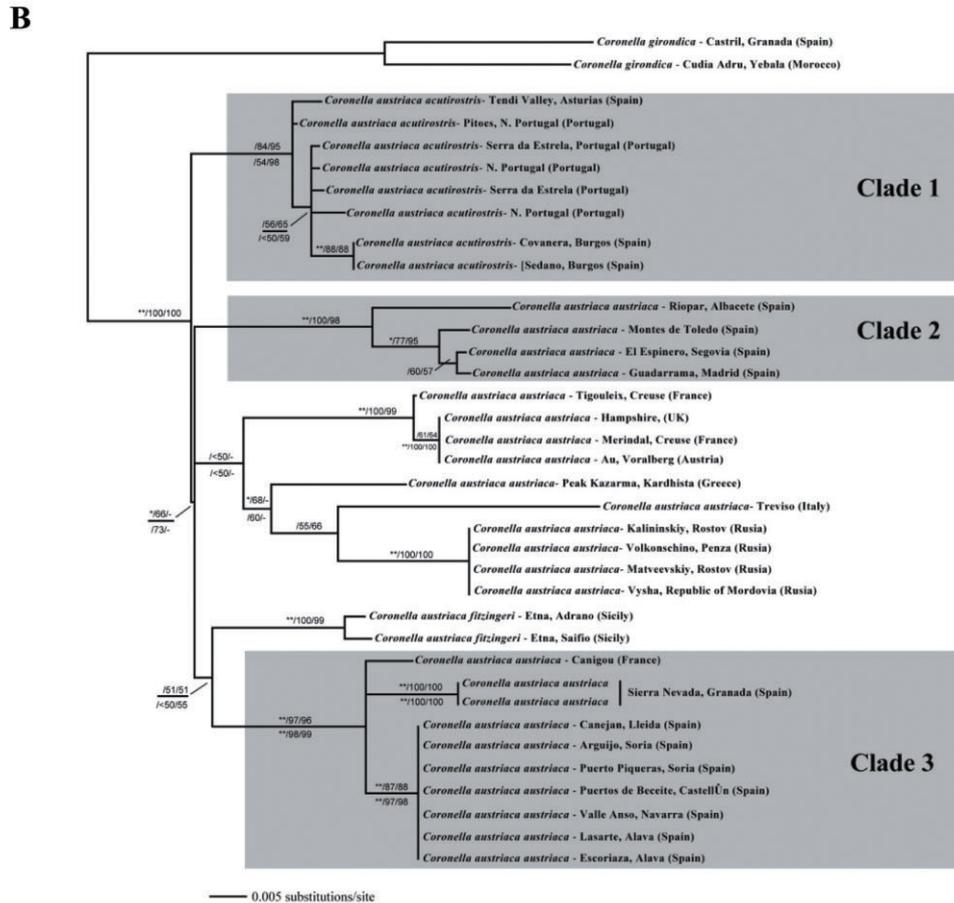
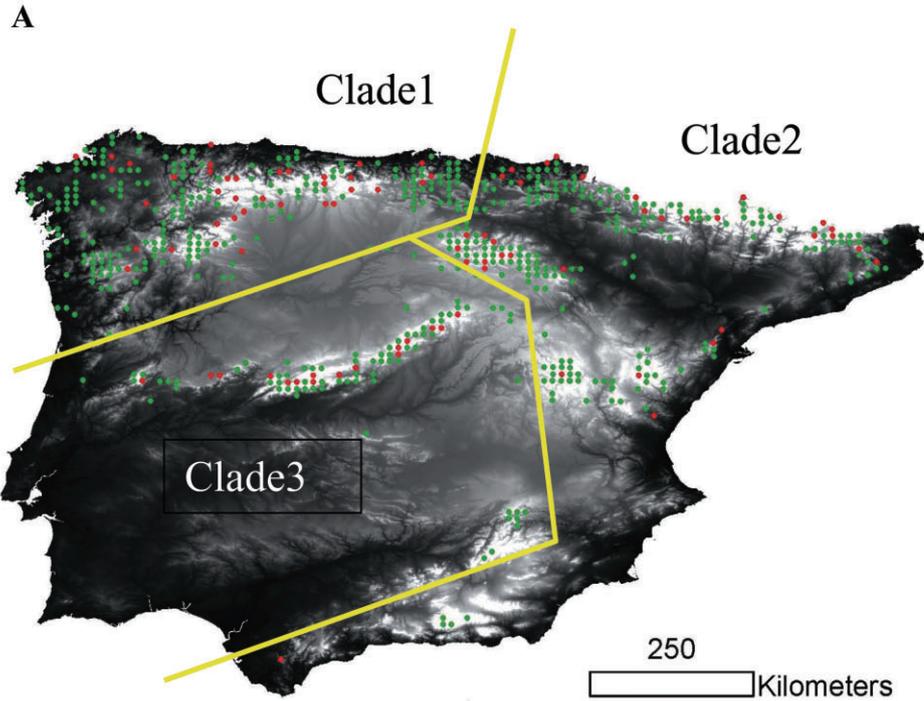
Recent molecular studies demonstrated that, at least in Iberia, *C. austriaca* has a complex phylogeographical pattern, with three mitochondrial (mt)DNA lineages that may have originated during the Messinian Salinity Crisis (Santos *et al.*, 2008). It was also reported that subsequent range shifts within each clade, facilitated by Pleistocene climate fluctuations, created a complex scenario of 'refugia within refugia'. To date, specific references to examples of 'refugia within refugia' for reptiles have only been supported by molecular data (Paulo *et al.*, 2001; Carranza *et al.*, 2004; Pinho *et al.*, 2006). However, it is widely accepted that combining morphology and molecular data in phylogenetics has an important role in modern systematics (Giribet *et al.*, 2008). Indeed, evolution can often be better understood by evaluating the dispersal and speciation processes, and a combination of molecular and morphological approaches can lead to the discovery of cryptic speciation patterns (Guil & Giribet, 2009). Interestingly, *C. a. acutirostris*, which was first described on the basis of its morphology, was recently confirmed by mtDNA analyses (Santos *et al.*, 2008).

The present study aimed to determine whether the complex genetic structure observed in Iberia (three main clades; Santos *et al.*, 2008) is confirmed by morphology using meristic and morphometric characters, and also to assess the effect of environmental variables as potential factors of geographical differentiation. Isolated Iberian populations of *C. austriaca* are restricted to marginal habitats (mostly to high mountains), thus making these populations vulnerable to extinction by several threats, including climate change, anthropogenic impact, and stochastic events (Santos *et al.*, 2009). The detection of coherent molecular and morphological units within *C. austriaca* will facilitate the revision of its systematics and the subsequent design of conservation strategies in those areas where populations are isolated and threatened.

## MATERIAL AND METHODS

### STUDY AREA AND SAMPLES

The present study was based on specimens from several herpetological collection (see Acknowledgements), originally collected from most of its distribution range in the Iberian Peninsula (Fig. 1A). According to the biogeography and evolution of *C. austriaca* in the Iberian Peninsula (Santos *et al.*, 2008) (Fig. 1B), specimens were classified into three



**Figure 1.** A, Distribution of *Coronella austriaca* in Universal Transverse Mercator 10 × 10 km squares across the Iberian Peninsula and range of the three Iberian clades, *sensu* Santos *et al.* (2008). Red points indicate specimens used for morphological analyses within each clade. B, maximum-likelihood (ML) tree adapted from Santos *et al.* (2008). The tree was inferred with the mitochondrial cytochrome *b* gene. Posterior probability (*pp*) values for the Bayesian analyses and bootstrap support values for the ML and maximum parsimony (MP) analyses are shown above the branches in this order from left to right. An asterisk indicates that the Bayesian *pp* > 0.95. If no asterisk is shown it means that the *pp* ≤ 0.94 or lower. Numbers and asterisks below the branches are in the same order than above the branches (Bayesian/ML/MP) but are the result of the combined analysis of *cytb* and 16S rRNA mitochondrial genes. The symbol ‘–’ indicates that this particular method did not support the topology presented in the figure. The three Iberian clades are highlighted. Further details on the phylogenetic analyses performed and the geographical precedence of the samples please are provided in Santos *et al.* (2008).

clades with precise range limits: clade 1 ranges through the North-western corner of Iberia (Galicia, Cantabria, Northern Portugal), and corresponds to *C. a. acutirostris* (Malkmus, 1995); clade 2 includes individuals from the Sistema Central chain, Montes de Toledo and Alcaraz; and clade 3 includes populations from the mountain chains surrounding the Ebro Valley (Pyrenees, Sistema Ibérico) and isolated populations from Sierra Nevada (Santos *et al.*, 2008).

A former molecular study (Santos *et al.*, 2008) and the present morphological one, have been conducted with different samples. Most samples for molecular studies were collected from road kills, which renders them unusable for morphological analyses. On the other hand, most vouchers analyzed for morphology were previously fixed in formalin, which precludes molecular studies. After removing unsexed specimens and poorly preserved vouchers, we included 174 snakes: 87 specimens (49 males, 38 females) from clade 1, 37 specimens (18 males, 19 females) from clade 2, and 50 specimens (26 males, 24 females) from clade 3.

#### CHARACTERS

For each specimen, we measured meristic and morphometric characters, and several head-shape variables based on geometric morphometric procedures.

##### *Meristic characters*

The twenty-one meristic characters counted included the number of ventral scales (V), subcaudal scales (SBC), midbody dorsal rows (Dor), right and left supralabial scales (SupraLabR, SupraLabL), infralabial scales (InfralabR, InfralabL), nasal scales (NasR, NasL), loreal scales (LorR, LorL), preocular scales (PreocR, PreocL), supraocular scales (SupraocR, SupraocL), postocular scales (PosOcR, PosOcL), anterior temporal scales (TemAntR, TemAntL), and posterior temporal scales (TemPosR, TemPosL).

##### *Morphometric measurements and indices from the head*

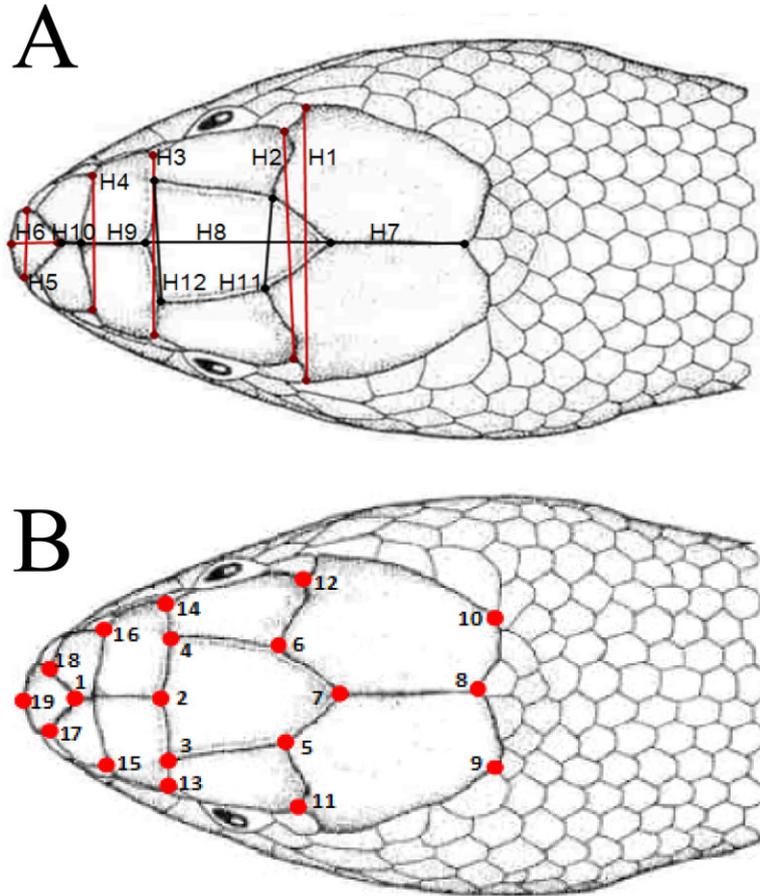
The 19 linear morphometric measurements were recorded with a string and a digital calliper: snout–

vent length (SVL), tail length (TL), head length (distance from tip of the snout to posterior end of mandible) (LHead), maximum head width (WHead), maximum head height (HHead), external width of the pileus (Head1), internal width of the pileus (between supraocular scales) (Head2), pileus width (between prefrontal scales) (Head3), internasal scales width (Head4), rostral scale width (Head5), rostral length (Head6), parietal scales internal length (Head7), frontal scale length (Head8), prefrontal scales length (Head9), internasal scales internal length (Head10), frontal scales posterior width (Head11), frontal scales anterior width (Head12), and pileus length (PL, distance from the tip of the snout to the dorsal edge between the parietal scales) (Fig. 2A). Body measurements were obtained to the closest mm, and head measurements to the closest 0.1 mm. Some head characters (Head7 to Head12) were also measured from photographs of the upper side of the head. Both manual and digital measurements were compared to assess any possible error capturing different measurements with the calliper, and no differences were found (*t*-test; *P* > 0.05).

We also calculated several indices that estimated the shape of some head plaques: rostral scale shape (RSS, length divided by maximum width of the rostral scale), frontal scale shape (FSS, frontal scale length divided by frontal scale anterior width), pileus shape (PS, pileus length divided by pileus internal width), snout shape (SS, measured as Head3/(Head6 + Head9 + Head10), rostral–prefrontal relative distance (RPD, length of the internasal scales divided by the pileus length), and pileus relative length (PRL, pileus length divided by SVL).

##### *Geometric morphometric procedures*

We captured 19 landmarks from digital photographs of the head (Fig. 2B) using TPSDIG, version 2.11 (Rohlf, 2006). Configurations of landmarks were translated, scaled, and rotated to the centroid size unit, bringing all landmark configurations into the same coordinated system, aiming to ensure that differences among groups were only related to dif-



**Figure 2.** Morphometric head measurements (A) and landmarks (B) of Iberian *Coronella austriaca* captured by means of digital photographs in the morphometric analyses of head shape.

ferences in shape, then removing other unrelated information such as specimen's size, position, and orientation. This configuration was carried out using a generalized least-square Procrustes superimposition procedure (Rohlf & Slice, 1990) performed with TPSRELW, version 1.45 (Rohlf, 2007). Resulting from the Procrustes procedure, all the data inherent in all the landmarks were transformed into components of a weight matrix (shape variables of specimens) and a relative warps matrix (equivalent to a principal components analysis of the weight matrix). The weight matrix was used to evaluate whether clades showed general differences in shape, performing a discriminant analysis (DA). As we used both sides of the head for our analysis, we checked fluctuant asymmetry in the snake head using MORPHOJ (Klingenberg, 2008). We performed a multivariate regression of the full set of shape variables on log SVL (TPSREGR, version 1.31; Rohlf, 2005) to study the relationship between head shape and body size (Klingenberg, 1996). Morphological shape differences among the consensus form of the three clades were dis-

played by wireframe comparisons using MORPHOJ (Klingenberg, 2008).

#### STATISTICAL ANALYSIS

To account for allometry, biometric variables (log transformed) were regressed against log SVL and the standardized residuals were used for further multivariate analyses. In a first step, we tested whether the biometric characters differed between molecular clades and sexes by general linear models (GLM). Although ventral and subcaudal scale counts are meristic characters, we analyzed them by GLM because the high dispersion of scores in these variables. Bonferroni correction to multiple tests was applied. In a second step, all characters that significantly differed among clades were used in discriminant and canonical analyses. Because male and female *C. austriaca* are dimorphic (Engelmann, 1993), discriminant and canonical analyses were performed for both sexes separately.

We explored for differences in two environmental variables (annual rainfall and summer temperature) among the ranges of the three Iberian clades. These variables are relevant to the distribution of other viviparous snakes, also ranging in mountains, from the Western Mediterranean (Santos *et al.*, 2006; Brito *et al.*, 2008; Martínez-Freiría *et al.*, 2008). Values for these variables were acquired from the European Environment Agency (period 1986–1996; available at: <http://www.eea.europa.eu>), using a geographical information system (SAGA; Conrad, 2005). The resolution of the data was standardized to a grid cell size of  $10 \times 10$  km for matching the resolution of Iberian observations available in atlases from Portugal and Spain (Galán, 2002; Malkmus, 2008).

## RESULTS

### DIFFERENCES IN BIOMETRIC CHARACTERS AMONG CLADES AND SEXES

The pholidotic characters of head and dorsal scale counts showed a very low interclade and sexual variation. Almost all these characters had modal scores accounted by more than 75% of the specimens examined, except the number of posterior temporal scales. In this case, the percent of smooth snakes with two and three temporal posterior scales was similar, though without sexual or clade bias (see Supporting information, Table S1).

GLM analyses detected several characters for which we found interclade and sexual differences (Table 1; see also Supporting information, Table S2). After Bonferroni correction, head length (LHead) and five measures of the frontal part of the head, showed significant interclade differences. For shape indices of the head, the rostral–prefrontal relative distance (RPD), the rostral shape (RS), and the relative pileous length (RPL) differed between clades. These differences indicate that specimens from clade 2 have a longer head, especially in the frontal part (snout), than specimens from clade 3. The members of clade 1 present intermediate scores (Table 1). The rostral–prefrontal relative distance measures how the rostral scale reaches deep between the internasalia, a character easily observed with the naked eye in specimens. The results indicate that the rostral scale penetrates between the internasalia more in clade 2 than clade 1, and more in the latter clade than in clade 3. We also found sexual differences in seven head measurements (Head1, Head2, Head3, Head7, Head8, Head11, and Head12), all of them located in the centre of the head (Fig. 2A). All the post-hoc comparisons showed lower scores in females, suggesting that males have a more robust head.

Two meristic traits, the number of ventral and subcaudal scales, differed between clades in *C. aus-*

*triac* (Table 1). Clade 2 showed higher scale counts than clade 1, which, in turn, had higher scale counts than clade 3 for both characters. These characters also showed sexual dimorphism, with males having more subcaudals and females more ventral scales. The total number of scales (V + SBC) also showed interclade and sexual differences (Fig. 3A, Table 1). Interestingly, the intersex disparity in terms of V + SBC differed between clades, with clade 3 showing the highest disparity, followed by clade 1, with clade 2 showing the lowest intersex differences (Fig. 3A). The coefficient of variation for V + SBC scale counts was higher in males than in females (4.76 versus 3.10), thus indicating marked variability in this character in males.

The geographical areas with specimens belonging to the three Iberian clades were found to differ in their annual rainfall ( $F_{2,448} = 82.62$ ,  $P < 0.00001$ ; Fig. 3B) and summer temperature ( $F_{2,448} = 26.20$ ,  $P < 0.00001$ ; Fig. 3C). Comparisons between all possible pairs of clades for the climatic variables were significant (Sheffé post-hoc test,  $P < 0.05$ ).

### GEOMETRIC MORPHOMETRIC RESULTS

The geometric morphometric analysis generated 16 non-uniform partial warps and one uniform component of the shape represented in the weight matrix, whereas relative warp analysis of the weight matrix produced 34 relative warps. The first ten relative warps showed more than 75% of the shape variation. The multivariate regression of the W matrix against log SVL was not significant (Wilks' lambda = 0.773,  $F_{34,118} = 1.018$ ,  $P = 0.453$ ) because there were no shape changes related to body size. We also performed a DA with the weight matrix. The first function of the DA for males showed 62.1% of the variability, indicating significant differences between the three clades (Wilks' lambda = 0.156,  $F_{68,98} = 2.211$ ,  $P < 0.001$ ), and there was correct classification of 85.9% of the specimens. On the other hand, the first function of the DA for females was 55.2%, showing a lower variability than in males. The DA showed marginal significant differences between the three clades (Wilks' lambda = 0.218,  $F_{68,82} = 1.377$ ,  $P = 0.08$ ), although with correct classification of 85.7% of the individuals.

The wireframe modifications showed interclade head shape differences for both sexes that mainly involved the anterior part of the head (Fig. 4). Clade 1 presents the flattest snout shape, whereas snout shape is wider in clade 3 than in clades 1 and 2. In clade 2, the rostral scale penetrates between the internasals deeper than in the other two clades, and the frontal scale widens on the posterior part, acquiring a pentagonal shape. Our results agree with Malkmus (1995), who described *C. a. acutirostris*

**Table 1.** Results of the general linear model (GLM) analyses on the morphological characters of the three Iberian genetic clades of *Coronella austriaca*, according to sex

	GLM results ( <i>F</i> scores and <i>P</i> )			Post-hoc comparisons	
	Clade	Sex	Interaction	Clades	Sex
SVL	6.126 <sup>a</sup>	3.436	0.262		
LHead	14.945*	4.114 <sup>a</sup>	0.284	C12 > C11 > C13	
WHead	3.649 <sup>a</sup>	2.728	0.072		
HHead	1.490	4.843 <sup>a</sup>	0.298		
V	19.615*	166.279*	2.755	C12 > C11 > C13	F > M
SBC	26.860*	66.322*	4.857 <sup>a</sup>	C12 > C11 > C13	M > F
V + SBC	33.812*	19.702*	5.888 <sup>a</sup>	C12 > C11 > C13	F > M
Head1	1.369	18.877*	1.769		M > F
Head2	0.202	20.431*	0.534		M > F
Head3	3.095 <sup>a</sup>	22.634*	1.461		M > F
Head4	8.901*	10.734 <sup>a</sup>	0.474	C13 > C11 and C12	
Head5	12.313*	4.501*	0.468	C13 > C11 and C12	
Head6	14.947*	2.091	1.986	C12 > C11 and C13	
Head7	4.195 <sup>a</sup>	27.563*	0.024		M > F
Head8	4.647 <sup>a</sup>	13.425*	2.410		M > F
Head9	2.435 <sup>a</sup>	6.984 <sup>a</sup>	0.025		
Head10	9.658*	0.064	0.793	C11 and C13 > C12	
Head11	21.199*	16.922*	2.237	C12 > C11 > C13	M > F
Head12	2.703	26.220*	0.153		M > F
RSS	14.367*	0.459	0.980	C11 and C12 > C13	
FSS	0.088	1.514	0.665		
PS	5.411 <sup>a</sup>	0.179	2.277		
SS	3.879 <sup>a</sup>	3.598	2.341		
RPD	17.153 <sup>a</sup>	3.861	0.092	C13 > C11 > C12	
PRL	7.980*	9.094 <sup>a</sup>	0.182	C12 > C11 and C13	

For each factor and the interaction, we included *F*-values. <sup>a</sup>Characters with no significant differences after Bonferroni correction ( $0.05 > P > 0.0001$ ). \* $P < 0.0001$ . Scheffé post-hoc pairwise tests were included for characters with significant differences after that correction. SVL, snout-vent length; LHead, head length; WHead, maximum head width; HHead, maximum head height; V, ventral scales; SBC, subcaudal scales; Head1, external width of the pileus; Head2, internal width of the pileus (between supraocular scales); Head3, pileus width (between prefrontal scales); Head4, internasal scales width; Head5, rostral scale width; Head6, rostral length; Head7, parietal scales internal length; Head8, frontal scale length; Head9, prefrontal scales length; Head10, internasal scales internal length; Head11, frontal scales posterior width; Head12, frontal scales anterior width; RSS, rostral scale shape; FSS, frontal scale shape; PS, pileus shape; SS, snout shape; RPD, rostral-prefrontal relative distance; PRL, pileus relative length.

(clade 1 in our study), although our results also extend these characters to clade 2.

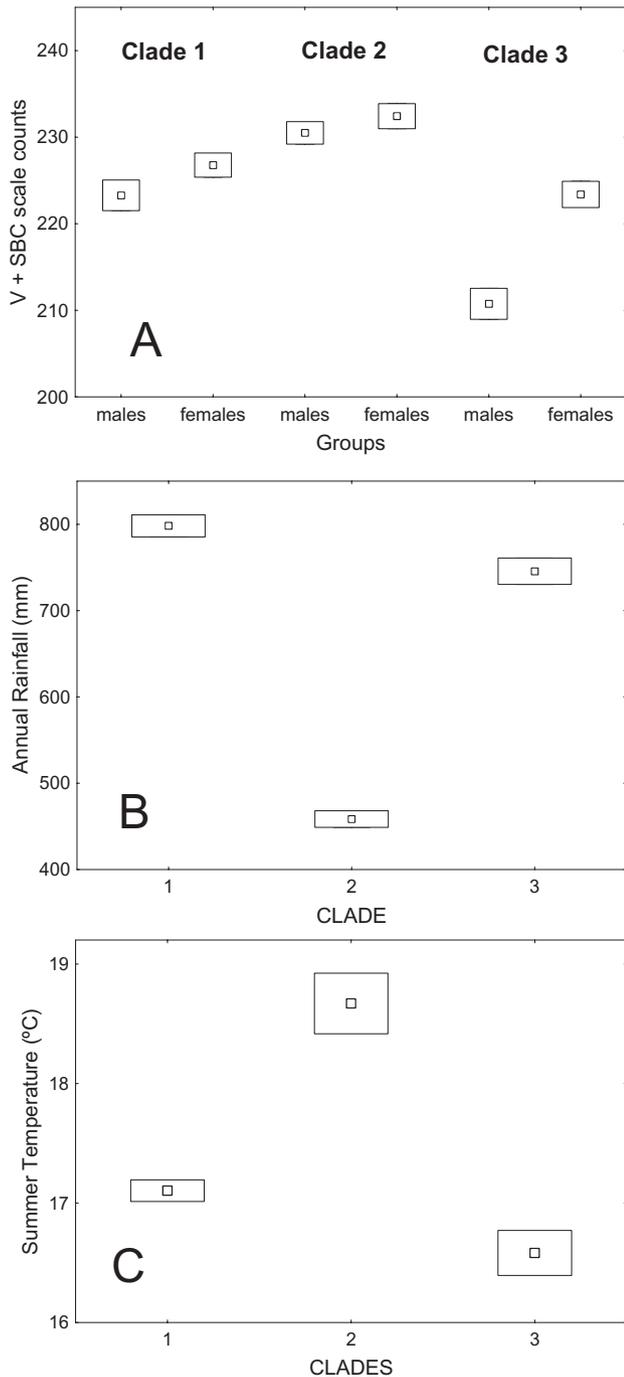
An asymmetry analysis based on head morphometrics showed nonsignificant differences ( $F = 0.55$ ,  $P = 0.98$ ), thus indicating that specimens did not present fluctuating asymmetry.

#### DISCRIMINANT AND CANONICAL CORRESPONDENCE ANALYSES

The DA showed that the overall morphological discrimination between clades was significant both in males (Wilks' lambda = 0.31,  $F_{16,166} = 8.37$ ,  $P < 0.00001$ ) and females (Wilks' lambda = 0.35,

$F_{20,138} = 4.76$ ,  $P < 0.00001$ ). According to the stepwise discriminant procedure, in males, the significant partial Wilks' lambda scores ( $P < 0.05$ ) included in the final model were ventral and subcaudal scales, and residuals of LHead, Head4, Head6, Head10, and Head11, whereas only residuals of Head5 and Head11 were included in females, indicating smaller inter-clade discriminant power of morphological characters for this sex.

The DA derived from the canonical axes reclassifies male and female specimens with similar rates. However, the percentage of correct classifications was higher in males than females, with overall rates of 84% and 75%, respectively (Table 2). Clade 1 achieved



**Figure 3.** Sexual disparities for the three Iberian clades of *Coronella austriaca* related to: (A) ventral plus subcaudal scale counts; (B) annual rainfall; (C) summer temperature. Symbols and boxes indicate the mean  $\pm$  SE for each group. V, ventral scales; SBC, subcaudal scales.

the highest correct-classification rates for both males and females, whereas clade 2 had the lowest rates. Post-hoc comparison in the GLM analyses showed that clade 1 often had the intermediate scores for

**Table 2.** Percentage of correct classification and number of individuals classified in each group after discriminant analysis for the three Iberian *Coronella austriaca* clades

	Percent correctly classified	Clade 1	Clade 2	Clade 3
<b>♂♂</b>				
Clade 1	95.9	47	1	1
Clade 2	61.1	7	11	0
Clade 3	76.9	5	1	20
Total	83.9	59	13	21
<b>♀♀</b>				
Clade 1	84.2	32	3	3
Clade 2	57.9	8	11	0
Clade 3	75.0	6	0	18
Total	75.3	46	14	21

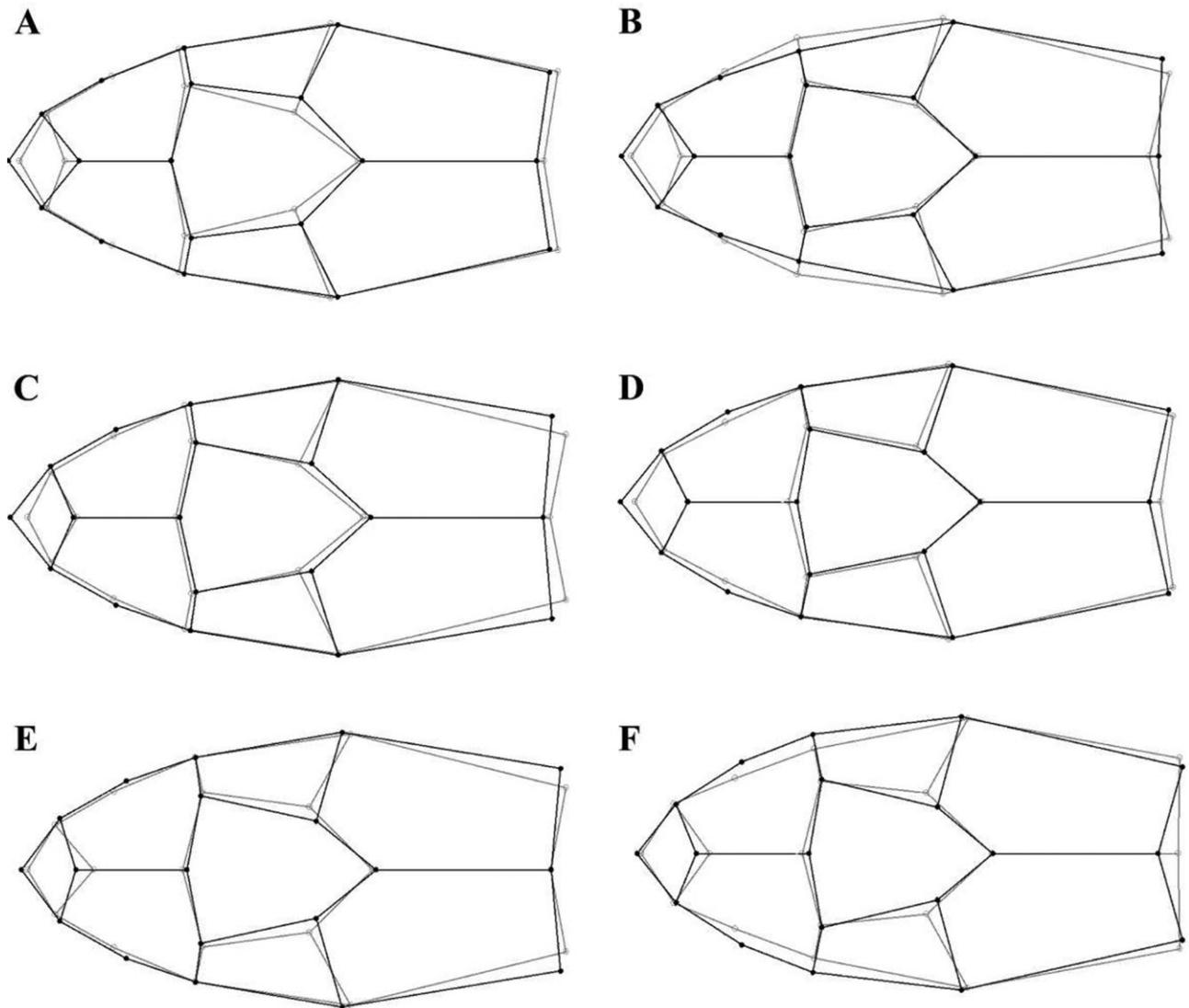
Males and females were analyzed separately as a result of sexual differences in morphology.

most significant characters. As a result, few or no specimens were incorrectly classified between clades 2 and 3. Consequently, we conclude that these two clades show the more dissimilar morphologies.

In the canonical analysis, a visual inspection of the two scatter plots shows a better discrimination in males than in females (Fig. 5). In males, the three clades are well distributed in the graphical representation, especially along the first axis (Fig. 5A). The first canonical discriminant function axis (eigenvalue of 1.57 and accounts for 85.5% of the intersample variability) separates the three clades in both its negative and positive parts. Morphological variables with the highest weight over the first canonical axis were number of ventral scales and Head11 in the negative values (Table 3). In females, the first canonical axis (eigenvalues of 1.19% and 79.4% of explained interclade variability) barely discriminates between clades 1 and 2 (Fig. 5B). According to the standardized canonical scores, the rostral–prefrontal relative distance in negative scores and LHead and Head11 in positive scores are the characters that better explain the interclade differences in the first canonical axis (Table 3).

## DISCUSSION

In agreement with the molecular results (Santos *et al.*, 2008), we found that the three Iberian clades of the European smooth snake *C. austriaca* also show morphological differences, namely several characters of the head and the body. This is one of the first evolutionary studies to report the coincidence between molecular and morphological results among

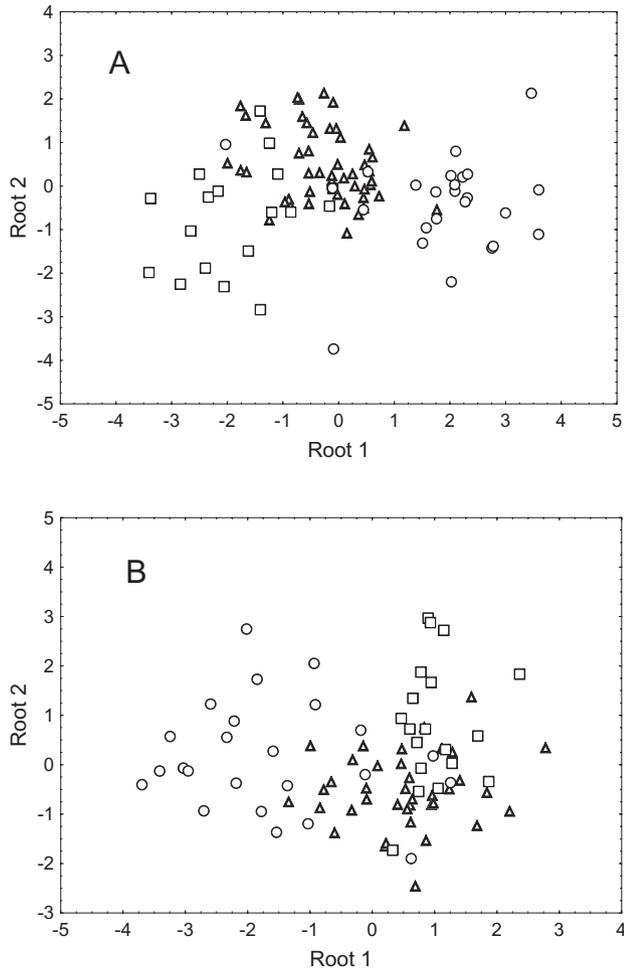


**Figure 4.** Head shape differences between the three Iberian clades of *Coronella austriaca* represented by wireframes for both sexes. Clade 1 as reference and 2 as target for males (A) and females (B); clade 1 as reference and 3 as target for males (C) and females (D); clade 2 as reference and 3 as target for males (E) and females (F). Differences are exaggerated three times for a better visualization. Colours: grey (reference), black (target).

Iberian reptile species. The morphological characters that show interclade differences may be used as diagnose characters to recognize specimens, delimitate distribution limits or contact areas, and therefore to define operational taxonomic units (OTUs) for management and conservation purposes. Moreover, this approach may track characters across phylogenies or phylogeographical groups previously defined by molecular markers (Kaliontzopoulou *et al.*, 2011), and allow an independent definition of OTUs, a weakness of many morphometric studies (Carretero, 2008).

Our findings in *C. austriaca* support the importance of the Iberian Peninsula as a source of evolutionary differentiation processes as a result of its

geographical position in the Mediterranean Basin and diverse climate and topography (Paulo *et al.*, 2001; Carranza & Arnold, 2004; Carranza *et al.*, 2004; Martínez-Solano *et al.*, 2006; Pinho *et al.*, 2006). According to the concept of refugia within refugia model (Gómez & Lunt, 2007), southern European peninsulas divided species ranges into a patchwork of suitable areas within a more limited space for organisms to survive during critical periods (Nieto Feliner, 2011). This process was driven by the varied topography, which favoured altitudinal displacements instead of large latitudinal shifts (Hewitt, 2001). In accordance with this general model, both genetic



**Figure 5.** Relative position of individual male (A) and female (B) *Coronella austriaca* specimens of the three Iberian genetic clades in the projection of the two canonical variates. Triangles, clade 1; squares, clade 2; circles, clade 3.

and morphology differences observed among Iberian *C. austriaca* clades are the result of population isolation as a result of fragmentation, which occurred during the Messinian Salinity Crisis. This extremely arid period forced mesic species to retreat to moister Atlantic-influenced areas and mountainous regions (García-Antón *et al.*, 2002). After isolation, local adaptation to different environmental conditions can be the ecological underlying mechanisms that have produced morphological variation among Iberian *C. austriaca* clades.

The present study demonstrated morphological differences not only among the clades described by molecular data (Santos *et al.*, 2008), but also between sexes. Unexpectedly, some head characters that varied among clades were located in the snout, whereas sexually dimorphic characters were located

**Table 3.** Standardized coefficients for two canonical variates from the discriminant analysis of the morphological characters of the three Iberian *Coronella austriaca* clades

	Males		Females	
	CV1	CV2	CV1	CV2
V	-0.576	0.200	0.275	0.011
SBC	-0.426	0.212	0.224	0.514
LHead	-0.449	0.154	0.528	0.012
Head4	0.396	-0.707	-0.431	0.078
Head5			-0.396	0.564
Head6	0.102	-0.772	-0.336	0.567
Head10	0.317	0.478	0.491	-1.205
Head11	-0.535	0.036	0.568	-0.039
PRL	0.0906	-0.503	-0.146	0.565
RPD			-0.680	0.944
Eigenvalues	1.576	0.267	1.186	0.307
Variance	85%	15%	79%	21%

Only characters with significant interclade differences in the general linear models analyses were included. The discriminant analysis was performed for males and females separately. CV, coefficient of variation; V, ventral scales; SBC, subcaudal scales; LHead, head length (distance from tip of the snout to posterior end of mandible); Head4, internasal scales width; Head5, rostral scale width; Head6, rostral length; Head10, internasal scales internal length; Head11, frontal scales posterior width; PRL, pileus relative length; RPD, rostral–prefrontal relative distance.

in the central and posterior part of the pileus (Fig. 2). The possible adaptive value for larger and narrower snouts, as presented in snakes from clades 1 and 2, is unknown. On the other hand, sexual dimorphism in relative head size and shape has been primarily related to sex differences in diet (Shine, 1993). This is especially conspicuous in feeding structures for those species where males and females have evolved to ingest different sizes of prey (Camilleri & Shine, 1990). According to Galán (1998), males and females of Iberian *C. austriaca* consume the same size prey, although Luiselli, Capula & Shine (1996), in a study of *C. austriaca* from Italy, reported that females consume larger prey. Consequently, the proximate mechanism responsible for sexual differences in the relative measurements of the central and posterior part of the pileus appears to be unrelated to feeding habits. It is important to note that, during mating, the male bites on the female's neck (Streck, 1965). This behaviour may be why males evolved more robust structures than females in the central part of the head, as reported for males of many lizard species that use their jaws to hold females before and during copulation (Anderson, McBrayer & Herrel, 2008).

Interestingly, two characters that were found to best discriminate among the three clades, the ventral and subcaudal scale count, showed sexual dimorphism (higher V in females and higher SBC in males). Ventral and subcaudal scales match the number of vertebrae within snakes (Saint-Girons, 1978), correlate positively to body size (Lindell, Forsman & Merilä, 1993; Zuffi & Bonnet, 1999), and the former is a character subjected to selection in snakes (Shine, 2000). Interclade differences in ventral and subcaudal scale counts in *C. austriaca* suggest that isolated populations in geographical refugia during the Messinian Salinity Crisis might have evolved adaptations to specific environmental conditions. In the present study, we have found that the highest average number of scale counts corresponds to the clade found in the area with the highest summer temperatures (Fig. 3). These results agree with Fox's (1948) experiments in which *Thamnophis elegans* females exposed to warm temperatures produced broods with a higher number of scale counts in the head and trunk than those exposed to cooler temperatures. However, Fox (1948) reviewed geographical differences from several snake species in the USA and observed that some species followed the reverse of the pattern found experimentally. Indeed, the relationship between body scale counts and environmental factors, such as temperature, is a complex issue that has been related to Bergmann's rule (1847), and is also reported in ectothermic species (Atkinson, 1994; Van Voorhies, 1996), with some reptile groups following the Bergmann's rule or the opposite (Ashton & Feldman, 2003). Ashton (2001) has proposed climatic seasonality and predation pressure as processes that can drive the formation of size-related geographical clines in crotaline snakes. Shine *et al.* (1997) reported that the incubation temperature in *Liasis fuscus* eggs is responsible for the size of newborn individuals. These examples, although based on species phylogenetically and geographically distant to *C. austriaca*, suggest that temperature can cause differences in scale count and body size in snakes. Fox (1948) discussed whether geographical variation in the scale characters of reptiles might be a result of temperature rather than genetic differences. Based on the results obtained for *C. austriaca* in the Iberian Peninsula, we consider that the three Iberian clades originated from isolated refugia (Santos *et al.*, 2008) and were probably subjected to different environmental conditions that produced morphological differences in scale counts. Because climatic differences persist currently, the three genetic clades maintain morphological differences. More experimental assays should be required to disentangle the roles of environmental and genetic factors in snake scale counts.

In brief, we found both sexual dimorphism in some characters such as ventral and subcaudal scale counts, and geographical differences in the sex disparity for the same characters (Fig. 3A). Indeed, males varied more in body scale counts among clades than females (see a similar pattern for body size in *Vipera berus*; Forsman, 1991). These results suggest that males and females were subjected to different evolutionary forces in the same geographical scenario, a conclusion also reached by Benton (1980) for the snake *Thamnophis sirtalis*. Ventrals and SBC are highly correlated with trunk and tail length in snakes (Shine, 2000), and these characteristics are subjected to strong selection pressure (Shine, 1993): males tend to be larger than females in species with male–male combat during the mating periods (i.e. sexual selection), whereas females tend to be larger than males to increase clutch size (i.e. fecundity selection). In agreement with the fecundity selection hypothesis, female *C. austriaca* present more ventral scales, and thus a relatively larger trunk than males to increase fecundity. This constraint probably reduces intraclade female variability, which is maintained in males. An alternative hypothesis suggests that sexual size dimorphism may prevent feeding competition between sexes (Shetty & Shine, 2002), although sexual dietary differences have not been documented in Iberian *C. austriaca* (Galán, 1998).

Our analyses partially support the arguments used by Malkmus (1995) to describe *C. a. acutirostris* from northwest Iberia (i.e. narrow head and the rostral scale penetrating deep between the internasalia, characteristics of clade 1). However, the characters that define clade 1 are also observed in clade 2, with even higher scores. After the former genetic study (Santos *et al.*, 2008) and the current morphological one, we conclude that *C. austriaca* has a fragmented distribution in the Iberian Peninsula, with three clades that have rather well defined limits (Galán, 2002; Malkmus, 2008; Santos *et al.*, 2008). Only the limit between clades 1 and 3, in the eastern part of the Cantabrian Mountains, is not clear. However, based on mtDNA (Santos *et al.*, 2008), and taking into account the distribution of other species in this region, we suggest that this limit lies on a north–south axis (10–20 km wide), from Bilbao to Miranda de Ebro, along the valleys of the Nervión (Atlantic watershed) and Bayas (Mediterranean watershed) rivers. The Nervión valley has been reported to be a corridor for Mediterranean plant species approaching the Cantabrian seashore (Aseginolaza *et al.*, 1989). We can thus speculate that it may also act as an environmental barrier for Eurosiberian animals such as *C. austriaca*, which avoid Mediterranean conditions. Further molecular analysis in this region should confirm the accuracy of this hypothesis.

The present study supports the validity of the subspecies described by Malkmus (1995). Based on the phylogeographical and morphological results, two new taxa should be described for the Iberian Peninsula, aiming to resolve the taxonomy of *C. austriaca* (Santos *et al.*, 2008). The preliminary molecular results of Santos *et al.* (2008) also suggested a complex scenario outside the Iberian Peninsula; therefore, new combined molecular and morphological studies that include the entire distribution range of the species are needed to elucidate the intraspecific variability of *C. austriaca*.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Modal values and percentage of individuals for the pholidotic characters of the head.

**Table S2.** Mean and standard error values in each clade and sex for biometric characters of the head, and ventral (V) and subcaudal (SBC) scale counts.

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