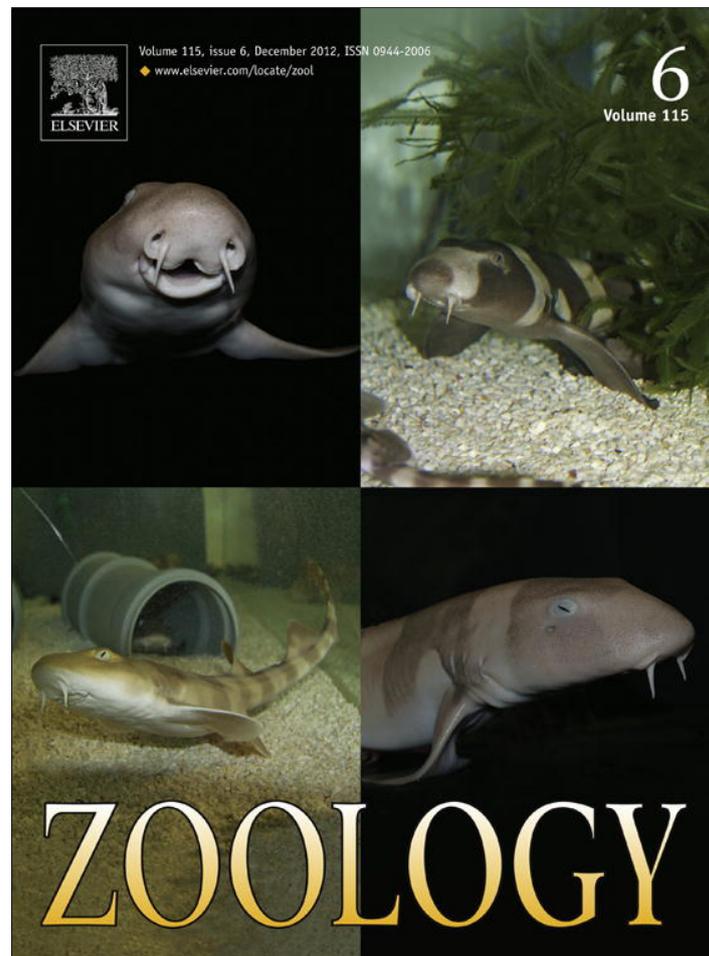


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ZOOLOGY

Morphology matches mtDNA lineages in the southern smooth snake (*Coronella girondica*) from Iberia

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ABSTRACT

The rise of molecular techniques in the study of evolutionary histories has resulted in a gradual abandonment of morphological characters as the only sources of phylogenetic inference. However, morphological characters may be valuable for phylogenetic reconstruction, especially for tracking adaptive changes across phylogeographic groups defined by genetic markers. We examined the discriminative power of morphological characters between four mitochondrial clades covering almost the entire distribution area of the smooth snake *Coronella girondica* in the Western Mediterranean. We detected three characters showing sexual dimorphism (relative tail length, number of ventral and of subcaudal scale counts) and, more interestingly, two characters (number of subcaudal and of dorsal rows) displaying interclade differences. Almost all *C. girondica* examined had 21 dorsal rows except those from a narrow coastal belt in the south-eastern Iberian Peninsula, which had 19 dorsal rows. The distribution of these specimens matches a mitochondrial clade that originated approximately 1.4–2.0 million years ago. Both of these morphological characters support a Betic lineage with a rather well-defined contact zone with the other Iberian lineage, which has been maintained even without the existence of current geographic barriers. The long-term survival of the Betic lineage throughout the Pleistocene climatic oscillations suggests a systematic revision within *C. girondica*.

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1. Introduction

The Western Mediterranean is known to be a region that has undergone complex geographic shifts (Benson et al., 1991; De Jong, 1998; Gómez et al., 2000; Blondel et al., 2010) and severe climatic fluctuations such as the Messinian salinity crisis and the Pleistocene glaciations (Krijgsman et al., 1999; Duggen et al., 2003). The co-occurrence of these processes strongly shaped the distribution of boreal organisms, highlighting the Western Mediterranean as a place for the origin of several endemisms and speciation by vicariant events (Hewitt, 2000; Sanmartín, 2003). Reptiles, as ectothermic organisms, are sensitive to temperature oscillations (Pough, 1980), and hence are prone to change their distribution

range, to be confined to glacial refugia, and to display genetic isolation patterns due to geographic barriers (Lynch, 1989). In recent decades, the development of molecular techniques has increased the number of phylogeographic studies focusing on Western Mediterranean reptile species (e.g., Carranza et al., 2004, 2006; Guicking et al., 2008; Miraldo et al., 2011). These studies have shown intricate phylogeographic structures in agreement with the palaeogeographic and palaeoclimatic complexity of the Strait of Gibraltar and the Iberian Peninsula (Pleguezuelos et al., 2008). The Iberian Peninsula, in particular, did not act as a single and homogeneous glacial refugium, but consisted of multiple ones organised in a “refugia within refugia” system (Gómez and Lunt, 2007).

The rise of molecular techniques in the study of evolutionary history has resulted in a major change in our understanding of the patterns of evolution (Avise, 1994), resulting in a gradual abandonment of morphological characters as unique sources of phylogenetic inference in favour of evidence from genetic markers (Scotland et al., 2003). Certainly, morphology provides a much lower number of unambiguous characters and it is not free of

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homoplasy (Scotland et al., 2003). Recent works debate whether morphological characters are still valuable for phylogenetic reconstruction, and if so, to what extent their quality varies among traits and taxa (de Queiroz and Good, 1997; Wiens, 2001, 2004). While methods combining molecular and morphological characters have been proposed (Giribet et al., 2008), a simple but promising approach is to track characters across phylogenies or across phylogeographic groups defined by genetic markers (Kaliontzopoulou et al., 2012), which will allow an independent definition of operational taxonomic units (OTUs), a weakness of many morphometric studies (Carretero, 2008). This strategy may be useful not only for taxonomical purposes (Padial et al., 2010), but also in applied terms since once phylogenetic groups are defined through molecular markers, there may be a chance to recognise them morphologically, saving time and effort when other studies (e.g., biogeographic or conservational) are conducted. Nevertheless, thorough investigations on the correspondence between genotypes and morphotypes are required prior to drawing such conclusions widely. Regarding European reptiles, only a few morphological studies have demonstrated an agreement between morphology and phylogenetic clades (Sá-Sousa et al., 2002; Brito et al., 2006, 2008; Llorente et al., 2012; Kaliontzopoulou et al., 2012).

Coronella girondica (Daudin, 1803) is a small oviparous colubrid, secretive and shy, which feeds mainly on lizards and more rarely on other snakes and small rodents and inhabits rocky, shrubby ecotone areas where it finds prey and shelter. It occurs all across the Western Mediterranean, in south-western Europe (northern and central Italy, southern France and almost the entire Iberian Peninsula; Dusej, 1997) as well as in western North Africa (Morocco, Algeria and Tunisia; Schleich et al., 1996). In Europe, its distribution is almost continuous; it is absent only from the highest, coolest areas of high mountains (Dusej, 1997). In North Africa, the picture is partially the reverse. In Morocco, it has a discontinuous distribution, being restricted to the mountainous Riff, and the Middle and High Atlas, and absent from the low and coastal areas (Bons and Geniez, 1996), whereas its detailed distribution in Tunisia and Algeria is unknown (Schleich et al., 1996).

Despite this wide distribution, no subspecies are currently recognised. While Boettger (1881) described *C. g. amaliae* from Morocco based on its snout shape, further studies did not support this differentiation (Domergue, 1962). In a preliminary analysis of the morphological variation of this species, Santos and Pleguezuelos (2003) grouped specimens into OTUs according to geographic units, since no previous phylogenetic analysis was available. However, the results did not support population differentiation, neither for the African populations (*C. g. amaliae*) nor for other areas, suggesting monotypy of the species, although a clinal variation in some morphological characters, such as the number of ventral scales, was found (Santos and Pleguezuelos, 2003). More recently, an analysis using mitochondrial genes has revealed a complex phylogeographic structure within this species (Santos et al., 2012); the genealogy shows a deep polytomy during the Plio-Pleistocene transition (1.4–2.0 million years ago), with three main clades, namely in the Betic range, in the rest of south-western Europe (the Iberian Peninsula, southern France, and Central and Northern Italy) and in north-western Africa, and a substructure within these clades associated with more recent (Pleistocene) climate oscillations. The lineage detected in the Betic range in fact occurs only in a narrow coastal belt; this region is part of the Betic-Riffian Massif, which is known as the place of origin of endemisms within multiple taxa (Oosterbroek and Arntzen, 1992).

Given the complex and ancient phylogeography of *C. girondica*, the objective of this study is to re-analyse its morphological variability across the whole distribution range, with special emphasis on differences found between lineages defined by mtDNA markers,

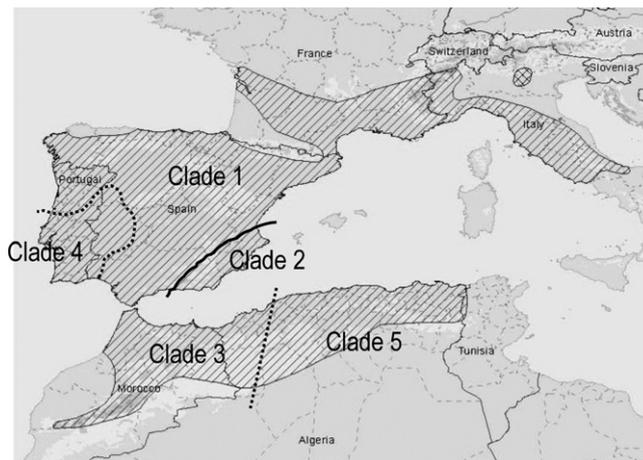


Fig. 1. Distribution of *Coronella girondica* and possible boundaries of the five mitochondrial clades (Santos et al., 2012). Dashed lines represent unknown limits of contact zones.

Map modified from Sá-Sousa et al. (2009).

i.e., to assess whether morphology matches the mtDNA lineages found for this snake.

2. Materials and methods

This study was conducted using 282 preserved specimens from several university and museum collections (see “Acknowledgements” section for a complete list), and we also used morphological data from Liguria (Italy, $n=31$) reported by Capocaccia (1959). Specimens were georeferenced at 10 km \times 10 km UTM grid cells, according to the information provided by museum labels.

Each specimen was sexed following Feriche et al. (1993) or by means of anatomical examination of gonads when possible. For each specimen, we measured snout–vent length (SVL) and tail length (TL) with a measuring tape to the nearest 1 mm, and recorded nine meristic characters that, when bilateral, were considered on the left (L) and the right (R) sides. The meristic characters were: number of ventral scales (V) following Saint-Girons (1978); subcaudal scale pairs (SBC); scale rows at mid-body (D); preocular scales (PreOc); postocular scales (PostOc); anterior temporal scales (TemAnt); posterior temporal scales (TemPost); supralabial scales (SupraLab); and infralabial scales (InfraLab).

According to mitochondrial phylogeography, *C. girondica* presents three divergent and well-supported lineages (Santos et al., 2012): clade 1 includes samples from the coastal belt south of the Sierra Nevada mountains (Granada province), ranging from Murcia to Málaga provinces in the south-eastern Iberian Peninsula; clades 2 and 3 include specimens from eastern Algeria and Tunisia (clade 2) and Morocco (clade 3); clades 4 and 5 correspond to all the samples from the south-western Iberian Peninsula, roughly including central and southern Portugal, and the closest regions in Spain (clade 4), as well as the geographically most widespread group, i.e., the remaining part of the Iberian Peninsula, southern France, and north-western Italy (clade 5) (see Fig. 1 for the clades’ distribution ranges). As the boundaries between clades 4 and 5 are poorly defined, snakes from potential contact zones were excluded from the analyses. Unfortunately, no specimens were available from clade 2 (Algeria and Tunisia) to be examined for this morphological study. The boundaries and contact zones between clades 1 and 5 were tentative, based on the exact location of specimens examined for the mtDNA analysis and according to the relief of the External Betic Chain. Thus, the animals found on the southern slope of this

Table 1
Frequency of head scalation characters (in %) and dorsal number of scale rows in samples of *Coronella girondica* classified according to four genetic clades throughout its area of distribution.

	Males (n = 114)	Females (n = 87)	Clade 1 (n = 27)	Clade 3 (n = 35)	Clade 4 (n = 15)	Clade 5 (n = 203)
D						
19	19.3	10.5	100	0	0	6.0
20	2.63	4.7	0	0	0	2.5
21	76.3	83.7	0	100	93.3	88.1
22	1.8	1.2	0	0	6.7	3.5
PreOcl						
1	99.1	100	100	100	100	99.5
2	0.9	0	0	0	0	0.5
PreOclR						
1	99.1	100	100	100	100	99.5
2	0.9	0	0	0	0	0.5
PostOcl						
1	0	2.4	0	0	0	2.5
2	99.1	97.7	100	100	100	97.0
3	0.9	0	0	0	0	0.5
PostOclR						
1	0	1.2	0	0	0	1.5
2	100	98.9	100	100	100	98.5
TemAntL						
1	2.7	2.4	3.7	0	0	4.0
2	96.4	95.2	92.6	96.8	100	94.5
3	0.9	2.4	3.7	3.2	0	1.5
TemAntR						
1	5.5	0	3.7	0	0	3.5
2	92.7	97.7	88.9	97.0	100	94.4
3	1.8	2.3	7.4	3.0	0	2.0
TemPostL						
1	0	1.2	0	0	0	0.5
2	11.9	15.7	11.5	3.2	40	14.1
3	85.3	78.3	80.8	90.3	60	80.8
4	2.8	4.8	7.7	6.5	0	4.6
TemPostR						
1	0.9	0	0	0	0	0.5
2	12.8	23.3	7.4	9.1	42.9	18.2
3	79.8	72.1	81.5	90.9	57.1	75.3
4	6.4	4.7	11.1	0	0	6.1
SupraLabL						
7	0.9	0	3.7	0	0	2.5
8	99.1	97.6	96.3	100	100	95.9
9	0	2.4	0	0	0	1.5
SupraLabR						
7	0.9	0	3.7	0	6.7	2.1
8	98.1	97.6	96.3	100	93.3	96.4
9	0.9	2.4	0	0	0	1.6
InfraLabL						
7	4.9	0	13.0	0	0	2.7
8	3.7	10	17.4	3.5	6.7	6.1
9	90.2	82.9	69.6	89.7	86.7	88.5
10	1.2	7.1	0	6.9	6.7	2.7
InfraLabR						
7	5.9	0	12.0	0	0	2.6
8	2.4	5.6	20.0	3.2	0	5.3
9	91.8	85.9	68.0	93.6	86.7	86.8
10	0	7.0	0	3.2	6.7	5.3
11	0	1.4	0	0	6.7	0

D, dorsal scale rows at mid-body; InfraLab, infralabial scales; PostOc, postocular scales; PreOc, preocular scales; SupraLab, supralabial scales; TemAnt, anterior temporal scales; TemPost, posterior temporal scales; L, left side; R, right side.

chain and in the Gérgal Corridor between the Sierra Nevada and the Sierra de Baza were classified as clade 1 and the rest as clade 5.

Given the phylogeography of the species (Santos et al., 2012) and the existence of sexual dimorphism in several characters (Santos and Pleguezuelos, 2003), analyses were conducted taking into account the factors clade and sex in order to check for geographic and sexual differences simultaneously. The unbalanced sample sizes for the four clades (clade 1, $n = 27$; clade 3, $n = 35$; clade 4, $n = 15$; clade 5, $n = 205$) are related to the extension of each clade (see Fig. 1), but also to the logistic constraints for sampling in some areas such as western North Africa. Therefore, clade 5 was randomly

resampled to 30 specimens to balance the four clades in statistical analyses. Sample size varied between analyses since some animals were partially damaged (e.g., those coming from road kills) and not all variables could always be recorded.

Given the discrete distribution of data for the pholidotic characters of the head, we checked for sexual and interclade differences by log-linear analysis. This statistical procedure tests for association between several categorical variables in a multidimensional contingency table. In the model to be tested, we included only the interactions of the head character with sex and mitochondrial clade. Characters which did not show sexual and interclade

variation (more than 90% of individuals had the same phenotype) were excluded from the analyses, due to the high number of cells with zero value.

Using general linear models (GLMs) we tested whether the biometric (SVL and TL) and three meristic (D, V and SBC) characters differed between molecular clades and sexes. We used SVL as the covariate to prevent allometric biases related to body size.

3. Results

Most pholidotic characters of the head showed no sexual or interclade differences, as 90% or more of the individuals had the same phenotype (Table 1). Only the numbers of temporal posterior (TempPost) and infralabial (InfraLab) scales were included in the log-linear analyses. The partial and marginal association tests between sex and head character, and between clade and head character, were not significant ($P > 0.05$). Thus, we found no sexual or interclade differences in the pholidotic characters of the head within *C. girondica*.

GLM analyses showed that TL, V, and SBC varied according to sex, whereas SBC and D showed interclade differences (Tables 2 and 3). Post hoc Scheffé tests ($P < 0.05$) indicated that males had longer tails, more SBC and fewer V. Furthermore, pairwise post hoc comparisons between clades indicated that clades 1 and 3 had more SBC than clade 5, and clade 1 had fewer D than did the other three clades. No interactions between sex and clade factors were detected ($P > 0.05$).

The number of scale rows at mid-body (D) was the only character that discriminated the Betic clade (clade 1) from snakes occurring in the surrounding areas hypothetically belonging to clade 5. The distribution map of specimens with 19 and 21 dorsal rows in Iberia showed a consistent geographic separation between the two phenotypes (Fig. 2A and B). Both maps clearly show a coastal belt from Alicante to Málaga, corresponding to the southern slopes of the Internal Betic Chain, where almost all the animals had 19 dorsal rows. Far from the coast, in a natural corridor between the mountain ranges of the Sierra Nevada and the Sierra de Baza (Gérgal

Corridor; Fig. 2B), most snakes also had 19 dorsal rows. Individuals with 19 and 21 dorsal rows were found in sympatry in particular 10 km × 10 km UTM squares within this region (Fig. 2B).

4. Discussion

Despite the reduced number of morphological characters available for the analysis, some discriminated correctly between sexes and between clades. The latter fact contrasts with the lack of differentiation found when specimens were grouped according to geographic units (Santos and Pleguezuelos, 2003). As expected, tail length (TL) and number of subcaudal scales (SBC) were larger in males, because of the existence of hemipenises in the base of the tail (Shine, 1993; Feriche et al., 1993), whereas the number of ventral scales (V) was higher in females, in agreement with the fecundity selection theory, which predicts that female snakes with larger abdominal volume will be selected for due to the advantages of increased clutch or litter size (Shine, 1993).

We found interclade differences in SBC, but we failed to find differences in V. Body-scale counts, i.e., V and SBC, have been reported as useful characters to discriminate among phylogenetic groups in European snakes (e.g., *Vipera aspis*, Zuffi and Bonnet, 1999; *Coronella austriaca*, Llorente et al., 2012). Indeed, these characters are correlated with the number of vertebrae and body size (Voris, 1975; Saint-Girons, 1978; Lindell et al., 1993; Shine, 2000) and are likely to be under ongoing selection. In a previous study regarding the geographic variability of *C. girondica* across a latitudinal gradient, Santos and Pleguezuelos (2003) found a marked decline in the number of ventral scales from the south (lowest scores in the Middle and High Atlas, Morocco) to the north (highest scores in southern France and Italy). The results of the present study clearly differ since specimens were grouped according to the mtDNA lineages found by Santos et al. (2012) instead of making an approximation to a priori geographic groupings of snakes, as performed by Santos and Pleguezuelos (2003). This suggests that variation in ventral scale count is determined primarily by environmental conditions (see also Martínez-Freiría et al., 2009; Llorente et al., 2012),

Table 2

Mean, standard error and sample size (in brackets) of biometric and pholidotic variables of *Coronella girondica* throughout its area of distribution. Groups were formed according to sex and genetic clades.

		Clade 1	Clade 3	Clade 4	Clade 5
Males	SVL	417.7 ± 16.9 (12)	426.5 ± 24.7 (15)	418.0 ± 0 (1)	406.8 ± 11.0 (73)
	TL	112.1 ± 4.0 (12)	92.0 ± 11.0 (2)	107.0 ± 0 (1)	103.2 ± 3.0 (69)
	V	179.3 ± 1.5 (12)	179.9 ± 1.2 (15)	180.0 ± 0 (1)	183.6 ± 0.8 (72)
	SBC	65.6 ± 0.9 (12)	65.8 ± 0.7 (15)	67.0 ± 0 (1)	63.42 ± 0.4 (67)
	D	19.0 ± 0 (12)	21.0 ± 0 (15)	21.0 ± 0 (1)	20.8 ± 0.1 (75)
Females	SVL	383.7 ± 26.0 (7)	379.3 ± 17.9 (15)	382.4 ± 30.3 (7)	384.2 ± 12.3 (52)
	TL	84.4 ± 6.7 (7)	74.8 ± 2.5 (4)	79.5 ± 8.5 (6)	82.9 ± 2.6 (51)
	V	182.0 ± 2.1 (6)	184.0 ± 1.7 (14)	185.3 ± 2.2 (7)	185.5 ± 0.7 (52)
	SBC	58.6 ± 1.3 (7)	57.6 ± 0.8 (14)	59.0 ± 0.9 (6)	56.5 ± 0.6 (49)
	D	19.0 ± 0 (7)	21.0 ± 0 (15)	21.0 ± 0 (7)	20.9 ± 0.1 (51)

D, dorsal scale rows at mid-body; SBC, subcaudal scale pairs; SVL, snout–vent length; TL, tail length; V, ventral scales.

Table 3

Generalised linear modelling (GLM) results of morphological characters recorded in *Coronella girondica* using clade (C1, C3, C4 and C5) and sex (males, M and females, F) as factors. Significant results were checked for pairwise differences by post hoc Scheffé tests.

	Clade			Sex			Clade × sex	
	F	P	Post hoc	F	P	Post hoc	F	P
SVL	0.14	n.s.		1.39	n.s.		0.22	n.s.
TL	0.58	n.s.		25.51	<0.0001	M > F	0.58	n.s.
D	296.20	<0.0001	C1 < C3, C4, C5	0.01	n.s.		0.14	n.s.
V	0.89	n.s.		5.73	0.02	F > M	0.08	n.s.
SBC	4.86	0.004	C1 and C3 > C5	53.07	<0.0001	M > F	0.30	n.s.

D, dorsal scale rows at mid-body; SBC, subcaudal scale pairs; SVL, snout–vent length; TL, tail length; V, ventral scales.

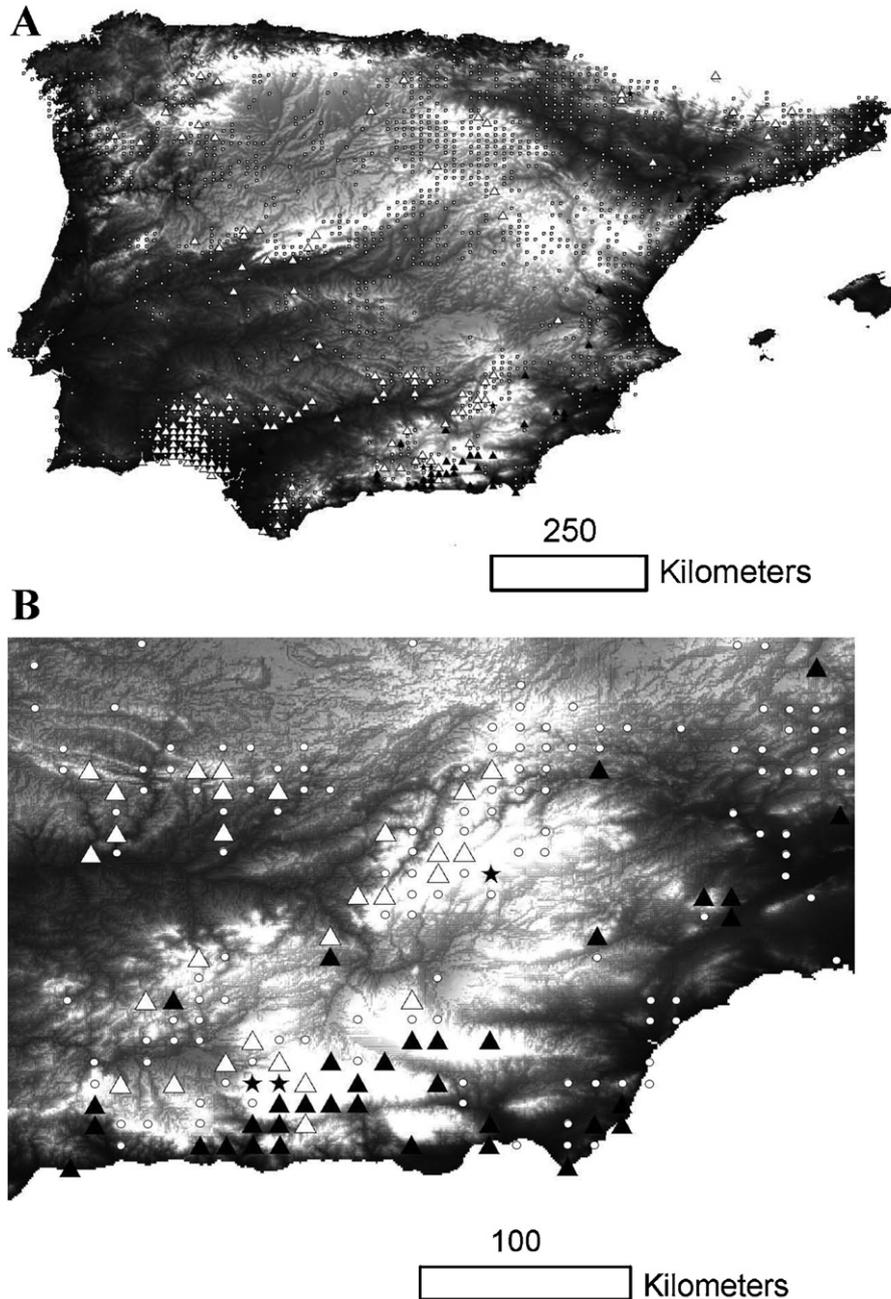


Fig. 2. (A) Distribution of *Coronella girondica* in the Iberian Peninsula (dots) in 10 km × 10 km UTM squares, including specimens with 19 (black triangles) and 20, 21 and 22 (white triangles) dorsal scale rows. Stars indicate squares with at least one specimen each with 19 and with 21 dorsal rows. (B) Detailed distribution of *Coronella girondica* in the Betic range (south-eastern Iberian Peninsula) in 10 km × 10 km UTM squares. White dots represent squares with citations but without information on the number of dorsal scale rows, black triangles represent recordings of specimens with 19 dorsal scale rows, white triangles those with 20–22 dorsal scale rows, and stars indicate squares with sympatric snakes with 19 and 21 dorsal rows.

although allopatric processes may also be involved where isolated populations are under contrasting environmental conditions.

Dorsal scale rows clearly differed between clade 1 and the rest of the clades. All specimens within this clade had 19 dorsal rows, and only a few *C. girondica* other than those from clade 1 had the same number of dorsal rows. According to the *C. girondica* phylogeography, clade 1 is an ancient lineage that originated 1.4–2.0 million years ago (Santos et al., 2012), which coincides roughly with the Plio–Pleistocene transition, a period characterised by a sudden increase in climate variability, inducing an intensification and southward expansion of glaciation in Europe (Lisiecki and

Raymo, 2007). This scenario is consistent with previous phylogeographic studies as this area has been reported to have high rates of endemism (Rivas-Martínez et al., 1997; Fonseca et al., 2009; Kaliontzopoulou et al., 2011; Miraldo et al., 2011). According to Fox's (1948) experiments, the number of dorsal rows is a character modulated by environmental conditions (i.e., temperature), since this author experimentally demonstrated that offspring born from gravid female *Thamnophis sirtalis* exposed to warm temperatures had more scale rows than those exposed to cool temperatures. However, our results do not match Fox's (1948) experiments since clade 1 is located in the warmest European region, and *C. girondica*

from this area had the lowest number of scale rows. These results highlight the intriguing interaction between genes and environment in evolutionary processes.

The boundary of specimens with 19 scale rows corresponds only to the southern slopes of the Internal Betic Range as well as to some valleys representing natural lowland corridors across it. However, the existence of some 10 km × 10 km UTM squares with 19- and 21-scale-row specimens suggests that individuals of both clades are currently sympatric. According to the concept of the refugia-within-refugia model (Gómez and Lunt, 2007), Southern European peninsulas produced subdivisions of species within a patchwork of genomes as these regions offered a great number of suitable areas for organisms to survive during critical periods (Nieto Feliner, 2011). This scenario is relevant not only insofar as it explains recent vicariant events, but also as a determining factor of range shifts for clades that originated prior to the glaciations, namely during the late Miocene or the Pliocene. This process was favoured by the varied topography, which allowed altitudinal displacements instead of large latitudinal shifts (Hewitt, 2001). According to Hewitt (1993, 1996), when climatic cycles forced migration and contact between genomes previously differentiated in allopatry, hybrid zones protected those genomes from merging and only allowed permeability of some genes and traits. This model could be applied to *C. girondica* in the Betic Mountains, as clade 1 has been preserved in a small area despite being an old lineage (Santos et al., 2012) which presumably has been forced to patterns of range expansion/contraction during the Pleistocene. Further studies using nuclear DNA markers would be needed to corroborate this hypothesis.

Long-term survival of clade 1 throughout Pleistocene climatic oscillations suggests the need for a systematic revision within *C. girondica*. Furthermore, we consider the designation of a protected area within the distribution of clade 1 of high interest for the conservation of this genetic and morphologic evolutionary unit, as well as another previously described endemism (e.g., Joger and Steinfartz, 1994; Miraldo et al., 2011).

Hybrid zones have been considered to be natural laboratories to study evolutionary processes (Harrison, 1990; Barton and Hewitt, 1985). Unfortunately, the impossibility of extracting DNA from museum vouchers in the present study prevented further insights into the evolutionary processes that occurred in the Betic contact zone and highlights the need of further sampling and new molecular analyses with multiple nuclear markers. In snakes, molecular markers may reveal that subspecies determined by colour patterns and scale counts do not always represent distinct evolutionary lineages (e.g., *Elaphe obsoleta* – Burbrink et al., 2000; *Hypsiglena torquata* – Mulcahy, 2008; *Lampropeltis getula* – Pyron and Burbrink, 2009). This discordance between groups based on colour or morphology and evolutionary lineages also suggests that convergence in external characters could mask phylogenetic diversification. Therefore, the combined use of molecular markers and morphological characters is an excellent approach to identify evolutionary lineages; it saves time and effort in defining their ranges and helps to discern the adaptive processes driving morphological variation. This combination has recently been used in the Iberian populations of the congeneric *C. austriaca* populations (Llorente et al., 2012) and also seems useful in *C. girondica*. The description of ancient and well-supported lineages, be they designated as a subspecies or not, illustrates putative incipient species and provides important taxonomic resolution for environmental managers and policy makers (Mulcahy, 2008).

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