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## Complex phylogeography in the Southern Smooth Snake (*Coronella girondica*) supported by mtDNA sequences

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### Abstract

Palaearctic reptiles with wide distribution through the Western Mediterranean are expected to display genetic substructuring because of the combining effects of current or past geographic barriers and climate fluctuations. We have examined this issue by sequencing cytochrome *b* and 16S rRNA mitochondrial fragments of 80 individuals of the snake *Coronella girondica* from 71 localities, covering the range of the species across Tunisia, Algeria, Morocco, Spain, Portugal, southern France and north-western Italy. According to the obtained genealogy, *C. girondica* is structured into three divergent and well-supported clades (north-western Africa, Betic range and Iberia–France–Italy), which greatly match other phylogeographies already published for this region. Our estimations suggest that the divergence among the three clades took place approximately 1.4–2.0 Ma, which roughly coincides with the Plio-Pleistocene transition, characterized by an increase in climate variability. The existence of a clade in a narrow belt of south-eastern Iberia represents another example of the high endemism rate of the region, with a key geographical situation and an important role in vicariant processes. Since the split among the three major lineages would be take place after the opening of the Strait of Gibraltar, overwater dispersal is here suggested. The subsequent genetic substructuring of these clades during the Pleistocene fits within the refugia-within-refugia model, highlighting the importance of the region as a scenario for multiple vicariant events.

**Key words:** phylogeography – mitochondrial DNA – snake – Betic range – Western Mediterranean.

### Introduction

The Western Mediterranean is one of the 25 hotspots of biodiversity in the world (Myers et al. 2000). Several geographical, historical and environmental factors have turned this temperate region into one of the most interesting scenarios to study phylogeographic processes (De Jong 1998). The region comprises the contact zone between two continents, across the Strait of Gibraltar, that has been affected during the last 15 million years by complex interactions between geological plates (Benson et al. 1991; Gómez et al. 2000; Blondel et al. 2010), and severe climatic fluctuations as the Messinian Salinity Crisis, the Pliocenic aridification and the Pleistocenic Glaciations (Krijgsman et al. 1999; Duggen et al. 2003; Jiménez-Moreno et al. 2010). By promoting multiple range shifts and vicariance processes of Palaearctic organisms, these geological and climatic events are certainly responsible for the high geographical substructuring, for the frequent speciation processes and, ultimately, for the high biodiversity and strong levels of endemism in this region (Ronquist 1997; Hewitt 2000; Sanmartín 2003).

Reptiles, as ectothermic organisms, are sensitive to temperature oscillations (Pough 1980) and, hence, prone to modify their distribution ranges and to be confined to thermal suitable refugia; namely, during the Pleistocenic glacial periods. Less frequently mentioned but equally important, many reptiles depend on a certain degree of humidity, either because of their physiology (Lillywhite 2006) or indirectly through the micro-

habitat selected, hence, responding to changes in precipitation regimes. Likewise, reptiles are prone to genetic isolation by vicariance, because of the appearance of geographic barriers with unsuitable environmental characteristics for them (Lynch 1989). Perhaps because of these reasons, reptiles have been used frequently as model organisms to study the biogeography of the Western Mediterranean (Paulo 2001; Guicking et al. 2006). The first phylogeographic study analysing the genetic differences between reptiles separated by the Strait of Gibraltar, assumed that this gap was an important geographic barrier for these organisms (Busack 1986). However, recent studies suggest that the Strait of Gibraltar acted as a dispersive channel, rather than an impermeable barrier for many reptiles (Carranza et al. 2004, 2006a, 2008; Pinho et al. 2006), and even for some organisms much less capable of oversea dispersion like amphibians (Carranza and Arnold 2004; Recuero et al. 2007; García-Porta et al. 2012). As a result, the Iberian Peninsula is the area with the highest percentage of African reptiles in Europe, whereas north-western Morocco harbours the highest percentage of European reptiles in Africa (Pleguezuelos et al. 2010).

Snakes constitute an adequate group to examine the biogeography of the Western Mediterranean, because a number of species inhabiting the region have wide ranges, covering north-western Africa and the Iberian Peninsula (Schleich et al. 1996; Gasc et al. 1997). Phylogeographic studies investigating the snake dispersal across the Strait of Gibraltar indicate a diversity of patterns, with old, recent and null dispersal, depending on the species (Mateo et al. 2003; Carranza et al. 2004, 2006a; Guicking et al. 2008). In addition to these intercontinental relations, intraspecific variability is also found among populations within the Iberian Peninsula (e.g. *Coronella austriaca* [Santos et al. 2008]; *Natrix maura*

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[Guicking et al. 2008]) and north-western Africa (e.g. *Malp-olon monspessulanus* [Geniez et al. 2006; Carranza et al. 2006a]; *Psammophis shokari* [Rato et al. 2007]). This diversity of patterns remarks the importance of Western Mediterranean for snake biogeography and encourages for further studies on other species in the region. Therefore, the present study focussed on *Coronella girondica* (Daudin 1803) is intended to provide another piece of evidence to understand the main processes that have shaped the biodiversity of this area. *Coronella girondica* is a small oviparous colubrid snake distributed across south-western Europe (almost all of the Iberian Peninsula, the southern half of France, and the northern half of Italy) and north-western Africa (north-central Morocco, northern Algeria and north-western Tunisia). In Europe, it presents a continuous distribution, with the only exception of the highest mountain ranges (Dusej 1997). In contrast, in north-western Africa its distribution is not well known in Algeria and Tunisia (Schleich et al. 1996), and in Morocco, it shows a patchy distribution, associated with the mountain chains of the Rif and the Middle and High Atlas Mountains, being absent from the lowland and coastal regions (Bons and Geniez 1996). Preliminary morphological studies failed to detect any geographically related pattern, stating the monotypy of the species (Santos and Pleguezuelos 2003). Much earlier, Boettger (1881) described *Coronella amaliae* from Morocco, although the hemipenis and other morphological characters did not support this differentiation (Domergue 1962; Santos and Pleguezuelos 2003). The goal of this study was to assess the phylogeographic pattern of *C. girondica* using mtDNA markers across the range of the species, inferring the events of vicariance and dispersal through this biogeographically complex region.

## Materials and Methods

### Sampling, PCR amplification and sequencing

Eighty individuals of *C. girondica* from 71 localities covering a significant part of the species' distribution range were included in

the present study with the exception of western France, as well as central and eastern Italy (Fig. 1). Four samples of *Coronella austriaca* from four different clades (Santos et al. 2008) were used as outgroups. Precise information on the geographic locality, specimen codes and GenBank accession numbers of all the samples is given in Table 1.

Two mitochondrial fragments were included in the present study, 302 bp of the cytochrome *b* (*cytb*) protein coding gene and 514 bp of the ribosomal 16S rRNA (16S) gene (primers size excluded). The *cytb* fragment was amplified using primers *cytb1* (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') and *cytb2* (5'-CCC TCA GAA TGA TAT TTG TCC TCA-3') modified primers from Kocher et al. (1989) and the 16S using primers 16Sa (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16Sb (5'-CCG GTC TGA ACT CAG ATC ACG T-3') modified primers from Palumbi (1996). The PCR cycle programme consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30" at 94°C, 45" min at 45°C and 1 min at 72°C. The cycling was ended with 10 min sequence extension at 72°C. All specimens were sequenced directly from the PCR products using the primers mentioned earlier on an ABI 377 automated sequencer.

### Phylogenetic analyses and divergence dating

The obtained sequences (302 bp of *cytb* and 514 bp of 16S) were aligned using MAFFT v.5.861 (Katoh 2008) with default parameters (gap opening penalty = 1.53; gap extension penalty = 0.123; progressive method = FFT-NS-2). All the *cytb* sequences had the same length, and only a few gaps (2–3, all of them unambiguous) had to be postulated to align the 16S sequences. As a result of that, all the positions were included in the phylogenetic analyses. The software JMODELTEST v. 0.1.1 (Posada 2008) was used to infer the best model of sequence evolution for each one of the two mitochondrial partitions. The aligned DNA data set was analysed using both maximum-likelihood (ML) and Bayesian inference (BI) methods. The ML analysis was carried out using the computer program RAxML (Stamatakis 2006) with the two gene partitions defined and with 100 independent tree searches. Reliability of the ML trees was assessed by bootstrap analysis (Felsenstein 1985), involving 1000 replications. The BI analysis was conducted using MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001). Both runs began with a random starting tree and ran for 2 x 10<sup>6</sup> generations, saving one tree each 100 generations. Convergence and appropriate sampling were confirmed examining the standard deviation of the split frequencies between the two

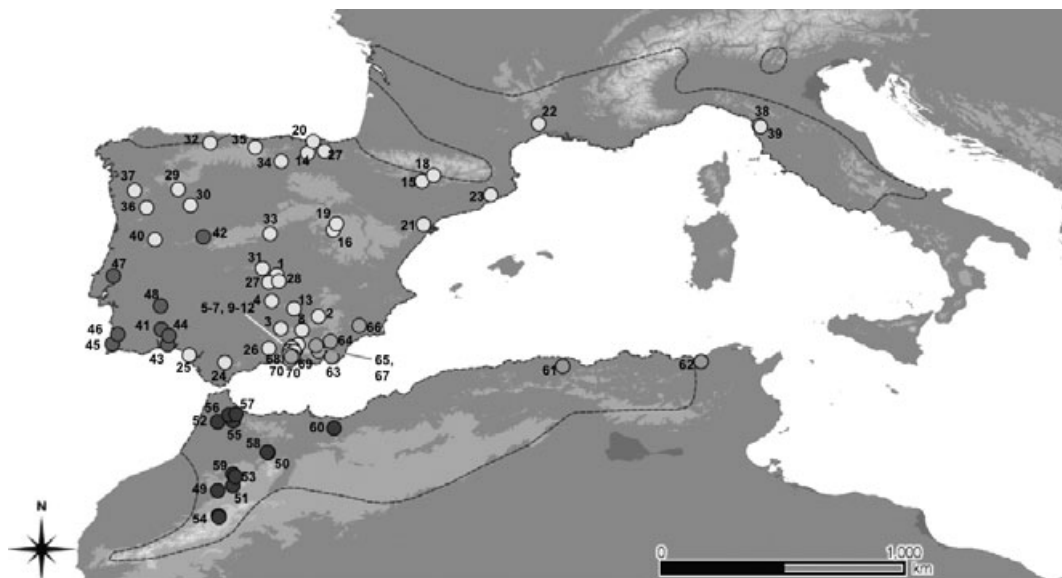


Fig. 1. Distribution map of the smooth snake *Coronella girondica* and localities with samples used for this study (see details in Table 1). The dashed red line indicates limits of the distribution range of the species

Table 1. Samples and sequences of *Coronella girondica* used in the present study. Numbers besides locality names (column 'Map') refer to localities shown in Fig. 1. Codes are individual codes used also in the trees (Figs. 1 and S1)

Taxa	Locality	Map	Accession Numbers Cyt b/16SrRNA	Codes
<i>Outgroups</i>				
<i>C. a. austriaca</i>	Merindal, Creuse, France		EU022650/EU022628	E1110.10
<i>C. a. austriaca</i>	El Espinar, Segovia, Spain		EU022672/	E1406.30
<i>C. a. acutirostris</i>	Ponte, ASE, Serra da Estrela, Portugal		JQ904296/JQ904298	DB2710
<i>C. a. austriaca</i>	Miraflores de la Sierra, Madrid, Spain		JQ904297/JQ904299	DB2682
<i>Ingroup</i>				
<i>C. girondica</i>	Sierra Luenga, Ciudad Real, Spain	1	JQ837590/JQ837513	DB2680
<i>C. girondica</i>	Pontones, Jaén, Spain	2	JQ837632/JQ837553	SPM001974
<i>C. girondica</i>	Cuevas Paria, Jaén, Spain	3	JQ837633/JQ837554	SPM001975
<i>C. girondica</i>	Mestanza, Ciudad Real, Spain	4	JQ837629/JQ837550	JP3
<i>C. girondica</i>	El Chorro-Vadillo, N. S. Nevada, Granada, Spain	5	JQ837617/JQ837540	DB3252
<i>C. girondica</i>	El Chorro-Vadillo, N. S. Nevada, Granada, Spain	5	JQ837619/JQ837542	DB3254
<i>C. girondica</i>	Monachil, N. of Sierra Nevada, Granada, Spain	6	JQ837624/JQ837546	DB3259
<i>C. girondica</i>	La Peza, N. of Sierra Nevada, Granada, Spain	7	JQ837615/JQ837538	DB3250
<i>C. girondica</i>	Sierra de Larva, Jaén, Spain	8	JQ837585/JQ837508	DB2668
<i>C. girondica</i>	Güéjar Sierra, N. Sierra Nevada, Granada, Spain	9	JQ837618/JQ837541	DB3253
<i>C. girondica</i>	Quéntar, N. of Sierra Nevada, Granada, Spain	10	JQ837623/JQ837545	DB3258
<i>C. girondica</i>	Quéntar, N. of Sierra Nevada, Granada, Spain	10	JQ837601/JQ837524	DB2693
<i>C. girondica</i>	Fuente del Hervidero, La Zubia, Granada, Spain	11	JQ837621/JQ837544	DB3256
<i>C. girondica</i>	Fuente del Mirlo, N. S. Nevada, Granada, Spain	12	JQ837620/JQ837543	DB3255
<i>C. girondica</i>	La Zimbarra, Jaén, Spain	13	JQ837586/JQ837509	DB2669
<i>C. girondica</i>	Orduña, Vizaya, Spain	14	JQ837612/JQ837535	DB2720
<i>C. girondica</i>	Puerto de Monrepós, Huesca, Spain	15	JQ837606/JQ837529	DB2705
<i>C. girondica</i>	Villanueva de Alcorcón, Guadalajara, Spain	16	JQ837573/JQ837499	DB2645
<i>C. girondica</i>	Villanueva de Alcorcón, Guadalajara, Spain	16	JQ837575/JQ837501	DB2647
<i>C. girondica</i>	Aretxabaleta, Guipúzcoa, Spain	17	JQ837583/JQ837507	DB2665
<i>C. girondica</i>	Sopeira, Huesca, Spain	18	JQ837597/JQ837520	DB2689
<i>C. girondica</i>	Cobeta, Guadalajara, Spain	19	JQ837572/JQ837498	DB2644
<i>C. girondica</i>	Presa de Gamboa, Vitoria, Spain	20	JQ837611/JQ837534	DB2715
<i>C. girondica</i>	Els Ports de Beseit, Tarragona, Spain	21	JQ837608/JQ837531	DB2711
<i>C. girondica</i>	Claret, France	22	JQ837614/JQ837537	DB2752
<i>C. girondica</i>	Santa Fe del Montseny, Barcelona, Spain	23	JQ837584/	DB2666
<i>C. girondica</i>	Benamahona, Sierra del Pinar, Cádiz, Spain	24	JQ837613/JQ837536	DB2722
<i>C. girondica</i>	Doñana, Huelva, Spain	25	JQ837567/JQ837493	DB11
<i>C. girondica</i>	Loja, Granada, Spain	26	JQ837627/JQ837548	JP1
<i>C. girondica</i>	Porzuna, Ciudad Real, Spain	27	JQ837591/JQ837514	DB2681
<i>C. girondica</i>	Malagón, Ciudad Real, Spain	28	JQ837588/JQ837511	DB2678
<i>C. girondica</i>	Malagón, Ciudad Real, Spain	28	JQ837587/JQ837510	DB2677
<i>C. girondica</i>	Parque Natural do Montesinho, Portugal	29	JQ837634/JQ837555	SPM001976
<i>C. girondica</i>	Atenor, Miranda do Douro, Portugal	30	JQ837569/JQ837495	DB1392
<i>C. girondica</i>	Montes de Toledo, Toledo, Spain	31	JQ837635/JQ837556	SPM001978
<i>C. girondica</i>	San Esteban, Asturias, Spain	32	JQ837574/JQ837500	DB2646
<i>C. girondica</i>	El Escorial, Madrid, Spain	33	JQ837589/JQ837512	DB2679
<i>C. girondica</i>	Covana, Burgos, Spain	34	JQ837610/JQ837533	DB2714
<i>C. girondica</i>	Sierra de la Liébana, Cantabria, Spain	35	JQ837607/JQ837530	DB2707
<i>C. girondica</i>	Alvão, Portugal	36	JQ837595/JQ837518	DB2686
<i>C. girondica</i>	Geres, Portugal	37	JQ837582/JQ837506	DB2664
<i>C. girondica</i>	Molina di Quosa, S. Giuliano Terme, Pisa, Italy	38	JQ837571/JQ837497	DB1913
<i>C. girondica</i>	Asciano, S. Giuliano Terme, Pisa, Italy	39	JQ837570/JQ837496	DB1788
<i>C. girondica</i>	Manteigas, Portugal	40	JQ837594/JQ837517	DB2685
<i>C. girondica</i>	Paymogo, Huelva, Spain	41	JQ837637/JQ837557	SPM1983
<i>C. girondica</i>	La Alberca, Salamanca, Spain	42	JQ837596/JQ837519	DB2687
<i>C. girondica</i>	Cartaya, Huelva, Spain	43	JQ837631/JQ837552	SPM001973
<i>C. girondica</i>	Alosno, Huelva, Spain	44	JQ837628/JQ837549	JP14
<i>C. girondica</i>	Aljezur, Alentejo, Portugal	45	JQ837580/JQ837504	DB2662
<i>C. girondica</i>	Odemira, Alentejo, Portugal	46	JQ837581/JQ837505	DB2663
<i>C. girondica</i>	Lamarosa, Ribatejo, Portugal	47	JQ837566/JQ837492	CGI4
<i>C. girondica</i>	Monsaraz, Portugal	48	JQ837599/JQ837522	DB2691
<i>C. girondica</i>	Azizga, Khenifra, Morocco	49	JQ837640/JQ837560	SPM004525
<i>C. girondica</i>	5 Km E. of Bakrit, Middle Atlas, Morocco	50	JQ837604/JQ837527	DB2698
<i>C. girondica</i>	Close to Bakrit, Middle Atlas, Morocco	51	JQ837638/JQ837558	SPM004523
<i>C. girondica</i>	15 Km S. of Ain Lahcen, Tanger, Morocco	52	JQ837642/JQ837562	SPM004527
<i>C. girondica</i>	2 Km from Ouina, Azrou, Morocco	53	JQ837644/JQ837564	SPM004529
<i>C. girondica</i>	Lake Tislit, Imilchil, High Atlas, Morocco	54	JQ837626/JQ837547	DB98
<i>C. girondica</i>	Lake Tislit, Imilchil, High Atlas, Morocco	54	JQ837568/JQ837494	DB131
<i>C. girondica</i>	Lake Tislit, Imilchil, High Atlas, Morocco	54	JQ837603/JQ837526	DB2697
<i>C. girondica</i>	Lake Tislit, Imilchil, High Atlas, Morocco	54	JQ837643/JQ837563	SPM004528

Table 1. (Continued)

Taxa	Locality	Map	Accession Numbers Cyt b/16SrRNA	Codes
<i>C. girondica</i>	5 Km S. of Bab Taza, Morocco	55	JQ837593/JQ837516	DB2684
<i>C. girondica</i>	5 Km S. of Bab Taza, Morocco	55	JQ837600/JQ837523	DB2692
<i>C. girondica</i>	5 Km S. of Bab Taza, Morocco	55	JQ837592/JQ837515	DB2683
<i>C. girondica</i>	Cudia Adru, Yebala, Morocco	56	JQ837630/JQ837551	MC2Cg1
<i>C. girondica</i>	Talassemtane, Rif, Morocco	57	JQ837609/JQ837532	DB2713
<i>C. girondica</i>	Jebel Tazekka, Taza, Morocco	58	JQ837641/JQ837561	SPM004526
<i>C. girondica</i>	Jebel Hebri, Ifrane, Morocco	59	JQ837639/JQ837559	SPM004524
<i>C. girondica</i>	Ain Almou, Beni Sanassen, Morocco	60	JQ837605/JQ837528	DB2703
<i>C. girondica</i>	Akfadou, El-Kseur, Kabylia, Algeria	61	JQ837602/JQ837525	DB2695
<i>C. girondica</i>	Hotel les Chenes, Ain Draham, Tunisia	62	JQ837565/	SPM000756
<i>C. girondica</i>	Cortijada Murcias de Arriba, Nijar, Almería, SP.	63	JQ837579/JQ837503	DB2656
<i>C. girondica</i>	Agua Amarga, Almería, Spain	64	JQ837578/JQ837502	DB2654
<i>C. girondica</i>	Montenegro, S. Sierra Nevada, Granada, Spain	65	JQ837616/JQ837539	DB3251
<i>C. girondica</i>	Sierra Espuña, Murcia, Spain	66	JQ837598/JQ837521	DB2690
<i>C. girondica</i>	Sierra de Baza, Granada-Almería, Spain	67	JQ837576/	DB2649
<i>C. girondica</i>	Señora del Cañar, S. S. Nevada, Granada, Spain	68	JQ837636/	SPM001980
<i>C. girondica</i>	Puente Palo, S. of Sierra Nevada, Granada, Spain	69	JQ837577/	DB2651
<i>C. girondica</i>	Vélez de Benaudalla, S. S. Nevada, Granada, Spain	70	JQ837625/	DB3261
<i>C. girondica</i>	Lanjarón, S. of Sierra Nevada, Granada, Spain	71	JQ837622/	DB3257

simultaneous runs and the Potential Scale Reduction Factor diagnostic. Burn-in was performed discarding the first 5000 trees of each run, and a majority-rule consensus tree was generated from the remaining trees.

Topological constraints to test alternative topologies were compared with optimal topologies using the Approximately-Unbiased (AU) (Shimodaira 2002) and Shimodaira-Hasegawa tests implemented in CONSEL (Shimodaira and Hasegawa 2001). Genetic distances were calculated with MEGA4 (Tamura et al. 2007).

The lack of internal calibration points in *Coronella* precluded the direct estimation of the time of the cladogenetic events in our phylogeny. Alternatively, the substitution rate of the same cytb mitochondrial region calculated for other squamate groups could be used for this purpose. Mean substitution rates and their standard errors for the cytb used in the present study were extracted from Carranza and Arnold (in press) and were based in fully calibrated phylogenies of various squamate groups from the Canary islands: *Tarentola* sp. (Gekkonidae) (Carranza et al. 2000, 2002), *Gallotia* sp. (Lacertidae) (Cox et al. 2010) and *Chalcides* sp. (Scincidae) (Brown and Pestano 1998; Carranza et al. 2008; Brown and Yang 2010). These values were used as informative priors in all divergence dating analyses. Specifically, we set a normal distribution prior for the ucl.d.mean parameter of the cytb partition based on the combined meanRate posteriors (mean and standard error) of all three independent calibration analyses of *Tarentola*, *Gallotia* and *Chalcides* ( $0.0228 \pm 0.00806$ ; Carranza and Arnold, in press).

We used BEAST v.1.6.1 (Drummond and Rambaut 2007) to estimate dates of the cladogenetic events from the concatenated data set including only the ingroup sequences (*C. girondica*). Analyses were run twice for  $5 \times 10^7$  generations with a sampling frequency of 10000. Models and prior specifications applied were as follows (otherwise by default): GTR+I+G (cytb); HKY+G (16S); Relaxed Uncorrelated Lognormal Clock (estimate) for both cytb and 16S; coalescent constant size process of speciation; random starting tree; alpha Uniform (0, 10); ucl.d.mean of cytb Normal (initial value: 0.0228, mean: 0.0228, Stdev: 0.00806).

## Results

The concatenated data set included 816 bp, of which 80 were variable (58 of the cytb and 22 of the 16S) and 58 parsimony-informative (40 of the cytb and 18 of the 16S) when only *C. girondica* specimens were compared. Uncorrected (*p*) genetic distances of the cytb and 16S mtDNA genes among and within the five Clades recognized in the present study (see Fig. 2) are shown in Table 2. The results from jModeltest showed that the most appropriate model of sequence evolution

for the cytb fragment was the HKY (Hasegawa et al. 1985) + I (invariant sites) + G (gamma distribution) and the HKY+G for the 16S.

Both ML and BI analyses produced almost identical tree topologies and consequently, only the ML tree is shown with the corresponding bootstrap values and Bayesian posterior probability values on the nodes (Fig. 2). According to the obtained genealogy, *C. girondica* presents three divergent and well-supported lineages: Clade 1, includes all the samples from the coastal belt south of the Sierra Nevada mountain, ranging from Murcia to Málaga provinces in south-eastern Iberia; Clades 2+3, includes specimens from eastern Algeria and Tunisia (Clade 2) and Morocco (Clade 3); Clades 4+5 includes all the samples from south-western Iberia (Clade 4) and the geographically most widespread group, that is, the remaining of the Iberian Peninsula, southern France and north-western Italy (Clade 5). To further confirm the parphyly of the Iberian samples of *C. girondica*, a topological test was carried out in which Clades 1, 4 and 5 were forced to be monophyletic, and the resulting constrained topologies were compared with our obtained topology from Fig. 2, using the SH and AU tests (see Materials and Methods section). The results indicate that our data set rejects the alternative hypothesis of monophyly of the European *C. girondica* (AU:  $p < 0.001$ , SH:  $p < 0.014$ ).

The results of the node estimation analysis using BEAST are shown in Supplementary Materials Fig. S1 and indicate that divergence between the main clades occurred between 1.4 and 2.0 Ma [0.3 and 3.8 if one considers the 95% highest posterior density (HPD) intervals]. All the nodes of the tree have high posterior probability values with the only exception of the node that separates Clade 1 from the monophyletic group formed by Clades 2+3.

## Discussion

The results of the phylogenetic analyses shown in Fig. 2 and Supplementary Materials Fig. S1 suggest that the relationships among the three major groups, namely the Betic range (Clade 1), Africa (Clades 2 and 3) and remaining Europe (Clades 4 and 5), are not well supported and may be the result of a rapid

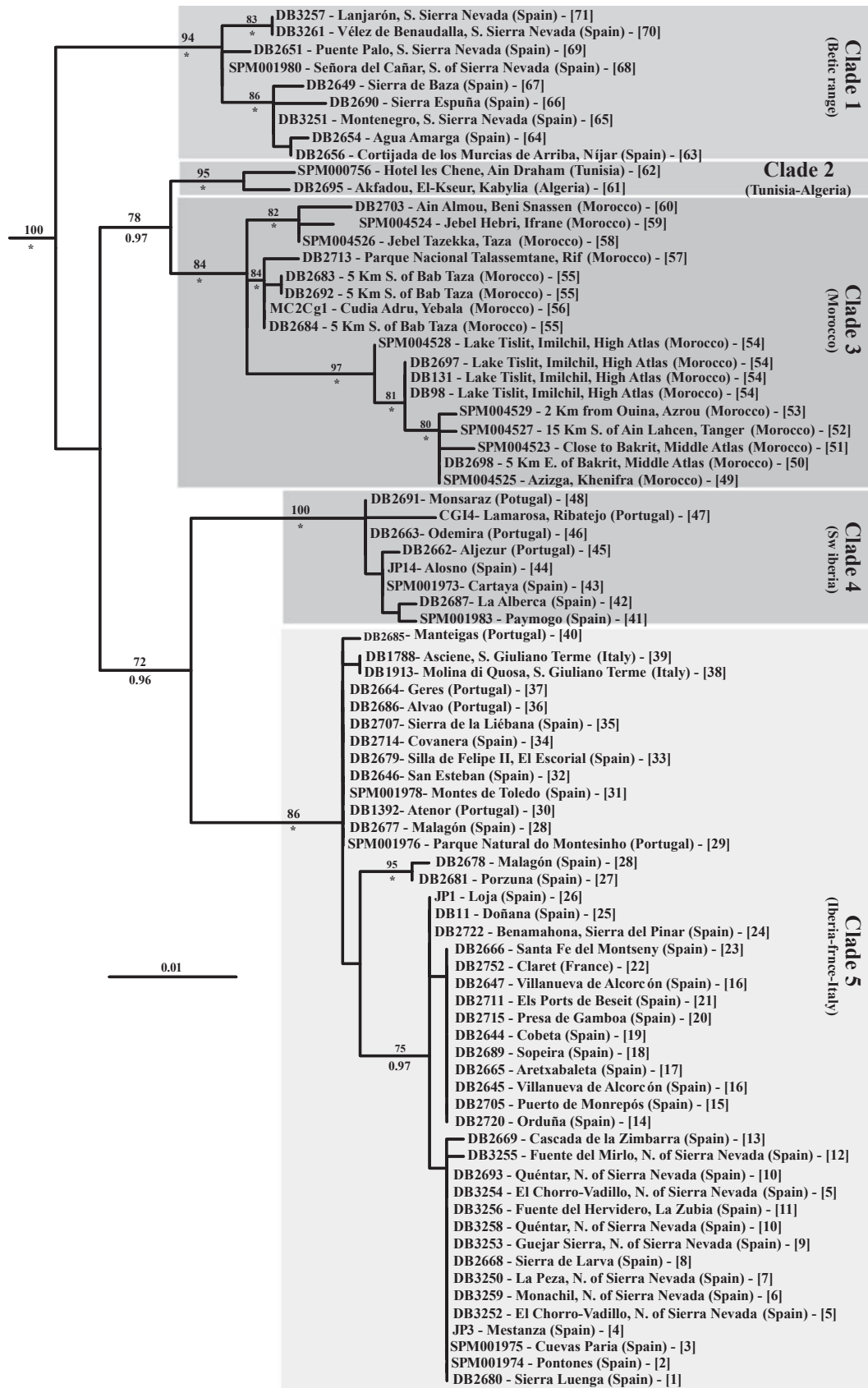


Fig. 2. Maximum-likelihood (ML) tree inferred from the mitochondrial cytb and 16S genes. Numbers in square brackets after the locality names are the locality codes represented in Fig. 1. Bootstrap support values for the ML analysis are shown above the branches and posterior probability (pp) values for the Bayesian analysis are shown below the branches. An asterisk indicates that the Bayesian pp value is equal to 1.0. Posterior probability values below 0.96 are not shown. The five main clades recovered in the phylogenetic analyses have been highlighted using the same colour codes as in Fig. 1

Table 2. Uncorrected  $p$ -distances between the different clades and within each clade for the cytb (A) and 16S (B) mitochondrial fragments analysed in the present study. Lower-left genetic distances; upper-right standard errors. Distances within clades are given in the last column

	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Within
<b>(A)</b>						
Clade 1	–	1.20	1.15	1.43	1.29	1.14
Clade 2	5.55	–	0.92	1.39	1.29	1.14
Clade 3	5.22	3.64	–	1.26	1.27	1.43
Clade 4	6.82	6.41	5.78	–	1.1	0.68
Clade 5	5.99	5.81	6.06	3.71	–	0.93
<b>(B)</b>						
Clade 1	–	0.51	0.52	0.57	0.58	0.10
Clade 2	1.33	–	0.32	0.49	0.61	0.00
Clade 3	1.56	0.70	–	0.43	0.55	0.34
Clade 4	1.82	1.33	1.16	–	0.54	0.09
Clade 5	1.84	2.00	1.67	1.65	–	0.26

split during the Plio-Pleistocene transition, a period characterized by a sudden increase in climate variability, inducing an intensification and southwards expansion of glaciation in Europe (Lisiecki and Raymo 2007). According to the distribution range of *C. girondica*, the split among the three major lineages occurred when the Strait of Gibraltar was already open (opening of the Strait of Gibraltar in its current location occurred 5.33 My ago), suggesting overseas dispersal. This was favoured by a decrease in sea level (130 m) during some glacial periods, which reduced the distance between both sides of the Strait (from the current 14 km to 4–5 km; Brandt et al. 1996; Zazo 1999) facilitating the dispersal across the emerged stepping-stones (Carranza et al. 2006a).

The distribution of the two species of the genus *Coronella* in Europe and Asia suggests that the direction of this dispersion across the Strait of Gibraltar was from North to South. The presence of currently isolated populations of *C. austriaca* in southern Spain suggests that this species was probably more widely distributed in this area during the colder phases of the Pleistocene (Santos et al. 2008). Given that *C. austriaca* is a species with quite similar habitat and dietary requirements than *C. girondica* but inhabiting colder environments (Santos et al. 2009), it might have displaced *C. girondica* to warmer regions in the South during cold periods, hence promoting both its isolation in Southern Iberia and the dispersal across the Strait of Gibraltar.

Several phylogeographic studies on Western Mediterranean snakes have demonstrated that dispersal across the Strait of Gibraltar is a common process in this group of squamates (Carranza et al. 2004, 2006a; Guicking et al. 2008). Earlier on, during the Messinian Salinity Crisis, some species of snakes crossed the Strait from North Africa to Europe (e. g. *Natrix maura*, Guicking et al. 2008; *Malpolon* sp., Carranza et al. 2006a; and members of genus *Eryx* and *Naja*, Szyndlar and Schleich 1994; Szyndlar and Rage 1990). The same pattern has been observed for other reptiles, such as the lizard *Acanthodactylus erythrurus* (Harris et al. 2004) and the skink *Chalcides bedriagae* (Carranza et al. 2008). However, up to now, and still with no data available for *Vipera latastei*, *Natrix natrix* and *Coronella girondica*, there is not a single well-documented example of any snake that crossed the Strait of Gibraltar in a North-South direction during the Messinian. This dispersal direction has only been documented for the ocellated lizards of the genus *Timon* (Paulo et al. 2008), and for some amphibians, such as *Discoglossus* (Fromhage et al. 2004; Zangari et al. 2006), *Salamandra* (Escoriza et al. 2006; Beukema et al. 2010),

*Alytes* (Martínez-Solano et al. 2004); *Pelobates* (García-París et al. 2003), *Pelophylax* (Beerli et al. 1996) and *Pleurodeles* (Carranza and Arnold 2004). Moreover, three snakes of African origin crossed the Strait of Gibraltar much more recently, probably during the upper Pleistocene (*Malpolon monspessulanus*, *Hemorrhhois hippocreppis* and *Macroprotodon brevis*; Carranza et al. 2004, 2006a). Likewise, recent phylogeographic studies carried out in the Iberian Peninsula and north Africa have indicated that several species such as *Podarcis* lizards clearly crossed the Strait of Gibraltar twice, at least once after the late Miocene led to the refilling of the Mediterranean (Kaliontzopoulou et al. 2011), and *Psammotromus algirus* also crossed after the reopening of the Strait of Gibraltar (Carranza et al. 2006b).

Interestingly, one of the three clades found in *C. girondica* exclusively occurs in the Betic range. Recent morphological analyses also support the differentiation of this clade, with specimens from this area having 19 dorsal scale rows at mid-body, in contrast to specimens from the remaining clades that have 21 scale rows (Santos et al. in press). Although no other Iberian snake shows consistent independent lineages in the Betic region, several reptile species have well-supported lineages in this region, namely the terrapins *Mauremys leprosa* (Fritz et al. 2006) and *Emys orbicularis* (Velo-Antón et al. 2008), and the lizards *Acanthodactylus erythrurus* (Fonseca et al. 2009), *Podarcis* sp. (Kaliontzopoulou et al. 2011) and *Timon lepidus* (Miraldo et al. 2011). However, at least in the case of *T. lepidus*, the Betic lineage was older and initiated by overseas dispersal between what was then the Iberian mainland and the Betic Massif that existed as an archipelago between Iberia and north Africa, approximately 9.4 Ma, with subsequent contact with other Iberian populations because of the closing of the north Betic marine transgression (Paulo et al. 2008; Miraldo et al. 2011). A similar distribution pattern to that of Clade 1 has been also reported for two amphibians, *Salamandra salamandra longirostris* (García-París et al. 1998) and *Alytes dickhilleni* (Arntzen and García-París 1995). These patterns observed in a number of amphibian and reptile species confirm that the Betic Mountain range acted as a motor of vicariant differentiation during several periods (since the Messinian to the Pleistocene), supporting its role as one of the ten Mediterranean hotspots of biodiversity, with a high level of endemism (Médail and Quézel 1997).

Within the North African range of the species, we find two clades, a Western one, corresponding to the populations in Morocco and an Eastern one, corresponding to the populations from eastern Algeria and north-western Tunisia. According to the calibration, the cladogenesis of these two lineages occurred approximately 1 Ma ago (0.3–1.9 95% HPD), in the middle Pleistocene. During this period, the effects of the Ice Ages on the species' ranges varied according to latitude and topography, with extensive extinction and recolonization at higher latitudes, and altitudinal shifts and complex refugia closer to the tropics (reviewed by Hewitt 2004). There was also an increase in aridity, reducing considerably the tropical forests and deserts such as the Sahara in North Africa expanded. All these climatic and habitat changes throughout the Pleistocene have left a present day genetic signature on the populations, as the patterns recovered for *C. girondica* from North Africa. The genetic structuring between western and eastern North Africa in *C. girondica* matches those found in other Maghrebian species studied to date, both among amphibians (e.g. *Pleurodeles* sp., Carranza and Arnold 2004; *Discoglossus* sp. Fromhage

et al. 2004) and reptiles (e.g. *Mauremys leprosa*, Fritz et al. 2006; *Acanthodactylus erythrurus*, Fonseca et al. 2009; *Podarcis vaucheri*, Lima et al. 2009; *Chalcides* sp. Carranza et al. 2008; *Mesalina guttulata*, Kapli et al. 2008; *Psammophis schokari*, Rato et al. 2007; *Macroprotodon* sp., Carranza et al. 2004; *Malpolon* sp., Carranza et al. 2006a; *Natrix maura*, Guicking et al. 2008; *N. natrix*, S. Carranza, unpublished results). The exact geographic border between both North African *C. girondica* clades is yet unknown, because of the sociopolitical situation of Algeria that has precluded field sampling for the last two decades. However, at least two herptile species, *Hyla meridionalis* (Recuero et al. 2007) and *Testudo graeca* (Fritz et al. 2009) show much more complicated genetic patterns in this region, suggesting that the east-west differentiation reported for many species could be simply due to a sampling bias caused by missing samples from Algeria.

In Morocco, although the species is currently distributed in isolated populations along the main mountain ranges such as the Rif, Middle and High Atlas (Bons and Geniez 1996), we failed to observe a genetic substructuring corresponding to these isolated areas, suggesting that this distribution pattern, currently restricted to mountains, could be relatively recent and fragmented by extinction in dry and hot lowland areas as observed for another Maghrebian species of European origin, such as *Emys orbicularis* (Fritz et al. 2007) and *Vipera latastei* (Brito et al. 2011). The lack of genetic substructuring within Morocco, together with the morphological studies by Saint Girons (1956) and Domergue (1962), do not support the existence of the taxon *C. amaliae*, described by Boettger (1881) based on a single morphological character of the rostral part of the head with specimens collected in the Tingitane Peninsula (north Morocco).

In Iberia, we found two more clades, which also diverged during the early Pleistocene [1.4 Ma (0.4–2.7 95% HPD)]; one lineage occurs in the south-western part of the Iberian Peninsula (Clade 4), and the other one is present across Spain, northern Portugal, southern France and north-western Italy (Clade 5). Several vertebrate taxa show genetic differentiation in south-western Iberia (Arntzen and Garcia-Paris 1995; Mesquita et al. 2005; Martínez-Solano et al. 2006; Albert et al. 2007), although these lineages are apparently older than our time estimates for those of *C. girondica*. The Iberian diversification in *C. girondica*, however, predates the Pleistocene glaciations, which separated the populations within multiple Iberian refugia (Gómez and Lunt 2007). Clade 5 seems to have expanded North reaching southern France and north-western Italy. The low genetic diversity and extensive geographic range of this clade suggests a bottleneck effect preceded by a recent expansion from South to North because of climate warming creating environments more suitable for the species (see a similar pattern in French *Natrix maura* populations, Guicking et al. 2002). Further sampling is needed to establish accurately the large contact zone between the south-western and central Iberian clades.

The genetic variation of Iberian *C. girondica* suggests that populations of this species were confined to refugia during glaciations, allowing them to evolve in the absence (or low levels) of gene flow, creating a substructured phylogeographic pattern. The special topography of the Iberian Peninsula offered a great number of suitable areas for organisms to survive during critical periods (Nieto Feliner 2011). The process was favoured by the varied geomorphology, allowing altitudinal displacements instead of large latitudinal shifts (Hewitt 2001), and hence, the survival of old Iberian lineages

in species such as *C. girondica*. This *refugia-within-refugia* concept was primarily described for the Iberian Peninsula and is supported by several phylogeographic patterns observed in many organisms ranging from vertebrates and invertebrates to plants (reviewed in Gómez and Lunt 2007).

This study is a new contribution to the understanding of the phylogeographic patterns of organisms in the Western Mediterranean and highlights the importance of the region as scenario for multiple vicariant events from Miocene to Pleistocene. The investigation of these phylogeographic patterns coupled with node age estimation, allows the placement of the cladogenesis events in time, and a better understanding of the evolutionary history of the species and of the region. The assessment of each population's genetic diversity would be of great interest for determining evolutionary significant units, designing protected areas and implementing conservation measures that take into account the genetic dimension of biodiversity.

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### Resumen

*Compleja filogeografía en la culebra lisa meridional (Coronella girondica) confirmada con secuencias de ADN mitocondrial*

Para los reptiles paleárticos con amplia distribución a lo largo del Mediterráneo Occidental, cabe esperar una estructura genética de acuerdo con las barreras geográficas actuales o pasadas y con las fluctuaciones climáticas. Se han estudiado fragmentos de ARN mitocondrial de los genes citocromo b y 16S de 80 individuos de *Coronella girondica* de 71 localidades, que abarcan la distribución de la especie en Túnez, Argelia, Marruecos, España, Portugal, sur de Francia y noroeste de Italia. De acuerdo con la genealogía obtenida, *C. girondica* se estructura en tres clados divergentes (Noroeste de África, Cordillera Bética e Iberia-Francia-Italia) que en gran medida coinciden con otras filogeografías ya publicadas para esta región. Nuestros cálculos sugieren que la divergencia entre los tres clados tuvo lugar aproximadamente hace 1.4-2.0 Ma, durante la transición Pliopleistocénica, coincidiendo con un incremento de la variabilidad climática. La existencia de un clado restringido a una estrecha franja costera del sureste de Iberia (clado Bético) representa un nuevo ejemplo del elevado grado de endemismo propio de la región, confirmando su papel clave en procesos vicariantes debido a su situación geográfica. La separación entre los tres clados ocurrió tras la apertura del Estrecho de Gibraltar, lo cual sugiere que la expansión de la especie se produjo por colonización a través del mar. La subestructura genética de los tres clados durante el Pleistoceno se ajusta al modelo de 'refugio dentro de refugio', lo cual corrobora la importancia de la región como escenario de múltiples eventos vicariantes.

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## Supporting Information

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

**Figure S1.** Time-calibrated tree inferred with BEAST (see materials and methods for explanation on the calibrations and other parameters used in the analysis). Blue bars correspond to the 95% highest posterior density (HPD) intervals. Values above some of the selected nodes refer to the calibration and the 95% HPD. Posterior probability (pp) values for the BEAST analysis are shown below the branches. An asterisk indicates that the pp value is equal to 1.0. Posterior probability values below 0.96 are not shown.

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