Biogeography and evolution of the Smooth snake *Coronella austriaca* (Serpentes: Colubridae) in the Iberian Peninsula: evidence for Messinian refuges and Pleistocenic range expansions

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Abstract. The European Smooth snake, *Coronella austriaca* Laurenti, 1768, presents a continuous distribution range across central and northern Europe, with isolated fragmented populations in the southern European peninsulas. Up to 814 bp of mitochondrial DNA from 34 specimens were used to determine the phylogeny and biogeography of this species within the Iberian Peninsula. The results indicate that *Coronella austriaca* presents at least three mtDNA lineages in Iberia that may have originated during the Messinian Salinity Crisis, 5.3-5.9 Ma. Posterior range shifts within the independent clades were facilitated by climatic fluctuations that occurred during the Pleistocene Ice Ages and, in the Iberian Peninsula, created a complex scenario of refugia-within-refugia. The formation of old allopatric lineages during the Messinian followed by shifts in the distribution range during the Pleistocene coincide with what has been reported for other Iberian reptiles and amphibians and highlights the importance of the Southern European Peninsulas as "old" hotspots of biodiversity. Our results also support the more recent view that the accentuated environmental instability occurred during the Pleistocene did not lead to speciation in many animal groups. The subspecific status of *C. a. acutirostris* is confirmed by the mtDNA analyses.

Keywords: *Coronella austriaca*, Iberian Peninsula, Messinian refugia, mitochondrial DNA, phylogeography, Pleistocene distribution shifts, snakes.

Introduction

The genetic consequences of the glacial and interglacial cycles in Europe have been studied for many organisms (Taberlet et al., 1998; Hewitt, 2003, 2004). One of the most interesting points of research is the role of the southern European Peninsulas, i.e. Iberian, Italian and Balkan, and the Caucasus region as glacial refugia (Hewitt, 2004; Ursenbacher et al., 2005; Horn et al., 2006). Although the Pleistocene glacial refugium hypothesis proposed that populations differentiated as a result of fragmentation during the glacial cycles of the Quaternary (Haffer, 1969), a more recent view suggests that the accentuated environmental instability did not lead

to increased speciation rates, with many species and populations predating the Pleistocene and proceeding through the Quaternary (Paulo et al., 2001; Tzedakis et al., 2002). Despite increasing evidence pointing towards Pre-Pleistocene lineages originating in the Late Miocene–Early Pliocene epoch (Paulo et al., 2001; Arnold et al., 2007; Martínez-Solano et al., 2006; Pinho et al., 2006), the possible causes of their origin and maintenance have not been elucidated yet. One possibility suggested by Arribas and Carranza (2004) is that population fragmentation in some lizards from the Iberian Peninsula was produced by the onset of the Messinian Salinity Crisis, a short (600 000 years) but crucial period that occurred between 5.9 and 5.3 Ma during which the Mediterranean Sea desiccated almost completely, producing a general and drastic increase in aridity around the Mediterranean Basin (Hsü et al., 1977; Blondel and Aronson, 1999; Krijgsman et al., 1999; Duggen et al., 2003). This increased aridity produced the desiccation of the internal lakes present in many European depressions, forcing the mesic species to retreat to the moister Atlantic-influenced areas and to

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the mountainous regions (García-Antón et al., 2002).

As a result of their particular physiological characteristics (they are ectothermic animals) relatively high number of species and wide distribution patterns, reptiles and amphibians are excellent model organisms to assess the relative role that the Pre-Quaternary and Quaternary major climatic events have played in the origin, evolution and distribution of species (Paulo et al., 2001; Veith et al., 2003; Arribas and Carranza, 2004; Escoriza et al., 2006; Martínez-Solano et al., 2006; Pinho et al., 2006). Among the reptiles, the European Smooth snake *Coronella austriaca* Laurenti, 1768 is an outstanding model. It is a small colubrid with an almost homogeneous distribution in central and north Europe although fragmented towards the south (fig. 1). Its geographic range extends from the Ural Mountains to Portugal, and northwards to parallel 64°. Towards the south it reaches the Italian, Balkan, and Iberian Peninsulas, and also some Mediterranean islands such as Sicily (Engelmann, 1993; Strijbosch, 1997).

In spite of the large distribution range of the European Smooth Snake, only two subspecies are recognized to date: *C. a. acutirostris* Malkmus, 1995, from the northwest quadrant of the Iberian Peninsula (type locality Serra da Estrela) and differentiated from the nominal subspecies by its lower total length, narrow head with pointed and projected snout and an increasing number of scales in the head and neck region; and *C. a. fitzingeri* (Bonaparte, 1840), restricted to southern Italy and Sicily (type locality Sicily), characterized by its small body size and the less contrasted dorsal pattern. The nominal subspecies, *C. a. austriaca* (type locality Vienna, Austia) occupies all the rest of the large distribution range of the species (see fig. 1). According to Tortonese and Lanza (1968), the subspecies *C. a. fitzingeri* is of doubtful validity.

In Iberia, *Coronella austriaca* lives preferably in the Euro-Siberian region, although it is also present in the Mediterranean climatic region, where it is often restricted to the highest parts of several mountain chains (Galán, 2002) (fig. 1b). In southern Iberia, this snake becomes extremely rare in the light of the very low number of records: a single record from the Montes de Toledo (Donaire et al., 2001) and from the Sierra de Cazorla (Rubio and Vigal, 1988), six records from the Sierra de Alcaraz (Rubio et al., 2000), thirteen records from Sierra Nevada (Meijide, 1987; unpublished data from the authors), and one record from the Sierra del Aljibe (Donaire et al., 2001). In these mountains, *C. austriaca* has been found from 1000 up to 2700 m. a.s.l., usually in cold and wet habitats.

The main goal of our study is to infer the phylogenetic relationships of Iberian *Coronella austriaca* using mitochondrial DNA sequences in order to reconstruct its biogeography and to assess the role that the major Pre-Quaternary and Quaternary climatic events have played in the distribution of the Iberian clades.

Material and methods

Study species and sampling

Following the main goal of this study, we sequenced a total of 22 individuals of *Coronella austriaca* covering most of its distribution range in Iberia (see fig. 1). Of these, eight specimens belonged presumably to the subspecies *C. a. acutirostris* and 14 to the nominal subspecies *C. a. austriaca*. Furthermore, 12 specimens from the rest of its distribution range (two *C. a. fitzingeri* and 10 *C. a. austriaca*) were included in our analyses, although the lack of samples from some European areas precluded a complete phylogenetic overview of the species. Two individuals of *Coronella girondica* (Daudin, 1803) were used to root the tree. Specimen data are given in table 1.

Extraction of DNA and PCR amplification

Some samples were obtained from tail tips of living specimens that were later released back in the field. As a result of the secretive habits of the Smooth snake in many parts of its distribution range, many of the samples were obtained from road-kills. Moreover, in most of these cases the snakes had been on the road for days or weeks. As a result of that, the DNA easy extraction kit (Quiagen), which has proven very efficient to extract DNA from dried specimens of *Chalcides* from Egypt (S. Carranza, pers. obs.), was used to extract genomic DNA from all the samples following the manufacturers' instructions. Primers used in both amplification and sequencing were CBV14846F1 (Lenk et al., 1999) and cytochrome b2 (Kocher et al., 1989) for the cytochrome b
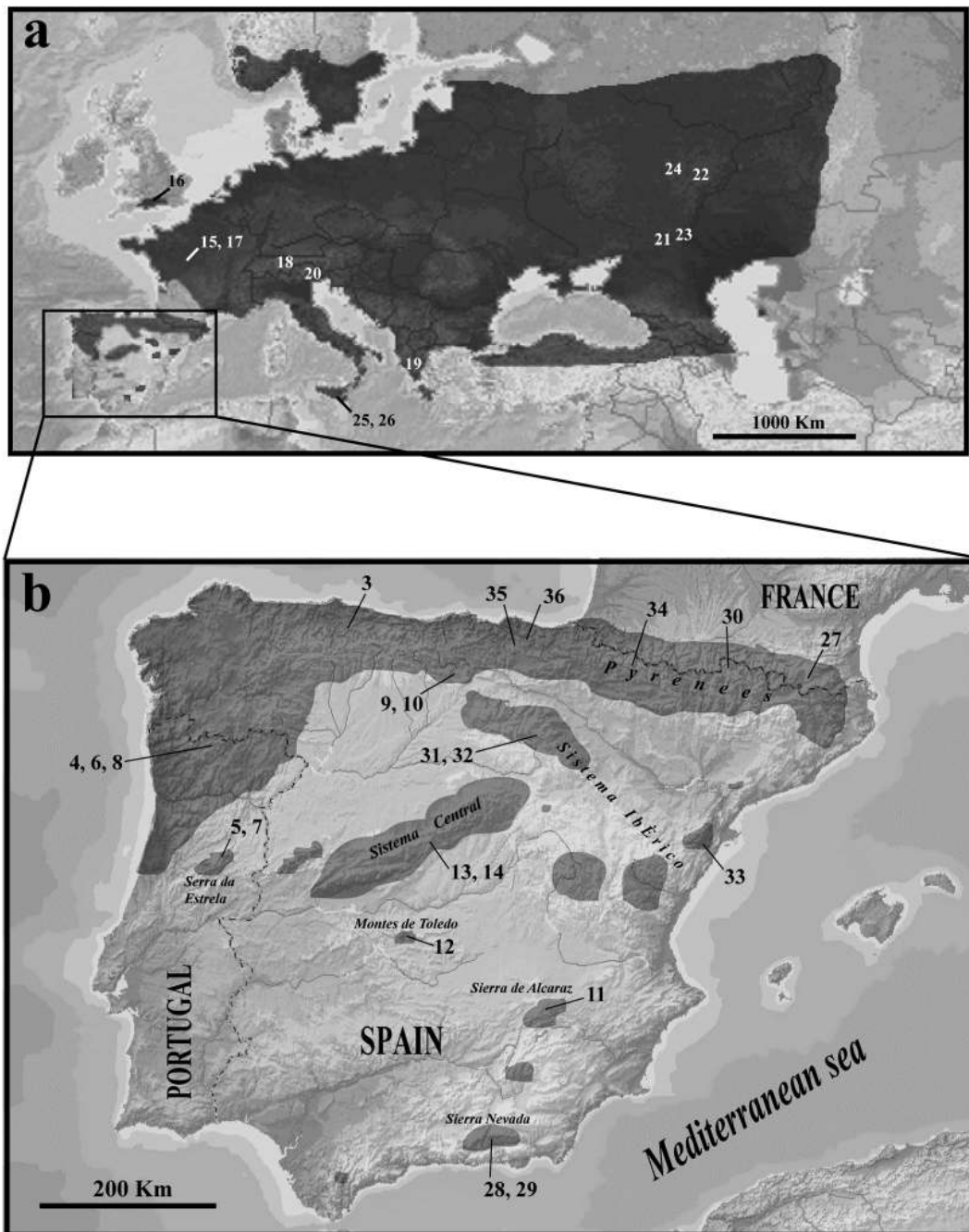
Figure 1. Distribution map of the European Smooth Snake *Coronella austriaca* (shadowed area) in Europe (a) and the Iberian Peninsula (b). Numbers refer to specimen localities sampled for this study (see table 1).

(cytb) gene and L2510 and H3062 for the 16S rRNA gene (Lenk et al., 1999). The two gene fragments were amplified using PCR procedures described by Carranza et al. (1999, 2000) and processed with an ABI 377 automated sequencer following the manufacturer's protocols. Despite the use of different PCR conditions and more specific PCR primers (data not shown), the 16S rRNA could only be amplified in 16 out of 34 specimens (see table 1). This might be explained as a result of the poor DNA recovery in some of the road-kill samples. However, the two mitochondrial gene

Table 1. Details of material and new sequences used in the present study. Number after species name refers to locality shown in fig. 1.

Taxa	Locality	Accession Numbers Cyt b/16SrRNA	Codes
Coronella girondica – 1	Castril, Granada (Spain)	EU022676	E27104.7
Coronella girondica – 2	Cudia Adru, Yebala (Morocco)	EU022677/EU022641	E5121.20
Coronella austriaca acutirostris – 3	Tendi Valley, Asturias (Spain)	EU022665/	E3010.3
Coronella austriaca acutirostris – 4	Pitoes (Portugal)	EU022669/EU022638	E27104.1
Coronella austriaca acutirostris – 5	Serra da Estrela (Portugal)	EU022662/	E2026.1
Coronella austriaca acutirostris – 6	N. Portugal (Portugal)	EU022663/	E2026.7
Coronella austriaca acutirostris – 7	Serra da Estrela (Portugal)	EU022664/	E2026.2
Coronella austriaca acutirostris – 8	N. Portugal (Portugal)	EU022668/EU022637	E2026.8
Coronella austriaca acutirostris – 9	Covanera, Burgos (Spain)	EU022666/	E2026.3
Coronella austriaca acutirostris – 10	Sedano, Burgos (Spain)	EU022667/	E2026.11
Coronella austriaca austriaca – 11	Riopar, Albacete (Spain)	EU022675/EU022640	E2405.2
Coronella austriaca austriaca – 12	Montes de Toledo, Toledo (Spain)	EU022674/	E2026.6
Coronella austriaca austriaca – 13	El Espinero, Segovia (Spain)	EU022672/	E1406.30
Coronella austriaca austriaca – 14	Sierra de Guadarrama, Madrid (Spain)	EU022673/	E2026.5
Coronella austriaca austriaca – 15	Tigouleix, Creuse (France)	EU022649/	E1511.9
Coronella austriaca austriaca – 16	Hampshire (UK)	EU022648/	E27104.2
Coronella austriaca austriaca – 17	Merindal, Creuse (France)	EU022650/EU022628	E1110.10
Coronella austriaca austriaca – 18	Au, Vorarlberg (Austria)	EU022651/EU022629	E27104.0
Coronella austriaca austriaca – 19	Peak Kazarma, Kardhista (Greece)	EU022647/EU022627	E27104.3
Coronella austriaca austriaca – 20	Treviso (Italy)	EU022646/	E3010.1
Coronella austriaca austriaca – 21	Kalininskiy, Rostov (Rusia)	EU022642/	E17116.3
Coronella austriaca austriaca – 22	Volkonschino, Penza (Rusia)	EU022643/	E17116.5
Coronella austriaca austriaca – 23	Matveevskiy, Rostov (Rusia)	EU022644/	E17116.6
Coronella austriaca austriaca – 24	Vysha, Republic of Mordovia (Rusia)	EU022645/EU022626	E17116.2
Coronella austriaca fitzingeri – 25	Etna, Adrano, Sicily (Italy)	EU022670/EU022639	E3026.16
Coronella austriaca fitzingeri – 26	Etna, Saifio, Sicily (Italy)	EU022671/	E3026.17
Coronella austriaca austriaca – 27	Canigou (France)	EU022654/EU022632	E3010.4
Coronella austriaca austriaca – 28	Sierra Nevada, Granada (Spain)	EU022652/EU022630	E27104.4
Coronella austriaca austriaca – 29	Sierra Nevada, Granada (Spain)	EU022653/EU022631	E2007.1
Coronella austriaca austriaca – 30	Canejan, Lleida (Spain)	EU022655/	E18124.1
Coronella austriaca austriaca – 31	Arguijo, Soria (Spain)	EU022656/	E2606.1
Coronella austriaca austriaca – 32	Puerto Piqueras, Soria (Spain)	EU022657/	E2606.3
Coronella austriaca austriaca – 33	Puertos de Beceite, Castellón (Spain)	EU022658/EU022633	E3010.5
Coronella austriaca austriaca – 34	Valle de Anso, Navarra (Spain)	EU022659/EU022634	E27014.5
Coronella austriaca austriaca – 35	Lasarte, Alava (Spain)	EU022660/EU022635	E2505.2
Coronella austriaca austriaca – 36	Escoriaza, Alava (Spain)	EU022661/EU9022636	E2026.4

fragments were sequenced for at least one representative of each one of the main groups recovered in the phylogenetic analyses (see results).

Phylogenetic analyses

DNA sequences were aligned using ClustalX (Thompson et al., 1997) with default parameters (gap opening = 10; gap extension = 0.2). The cytb alignment included no gaps and no stop codons were observed when the sequences were translated into amino acids using the vertebrate mitochondrial code, suggesting that all the cytb sequences analyzed were functional. Only four gaps had to be postulated to unambiguously align all the 16S rRNA sequences and therefore all the positions were included in the phylogenetic analyses. The incongruence length difference (ILD) test (Mickevich and Farris, 1981; Farris et al., 1994) was used to check for incongruence between the two gene fragments. In this test, 10 000 heuristic searches were made and invariable characters were removed beforehand (Cunningham, 1997).

Three methods of phylogenetic analysis were employed for all the analyses of the two separate fragments and for the analyses in which they were combined. These were: maximum-likelihood (ML), maximum-parsimony (MP) and Bayesian analysis. Modeltest (Posada and Crandall, 1998) was used to select the most appropriate model of sequence evolution for the ML and Bayesian analyses under the Akaike Information Criterion. This was the TrN model taking into account the shape of the gamma distribution (G) for the cytb dataset; the Kishino-Hasegawa model taking into account the number of invariable sites (I) for the 16S rRNA dataset; and the Kishino-Hasegawa model taking into account both the shape of the gamma distribution and

the number of invariable sites for the two gene fragments together (cytb + 16S rRNA).

Both ML and MP analyses were performed in PAUP* 4.0b10 (Swofford, 1998) and included searches involving tree bisection and reconnection (TBR) branch swapping with 10 and 100 step-wise additions of taxa, respectively. In the MP analyses gaps were treated as a fifth state. In the MP analyses transitions and transversions were given the same weight. Bayesian analyses were performed on MRBAYES v. 3.1.2 (Huelsenbeck and Ronquist, 2001). For the cytb + 16S rRNA combined analysis each partition had its own model of sequence evolution and model parameters (see above). Four incrementally heated Markov chains with the default heating values were used. All analyses started with randomly generated trees and ran for 2×10^6 generations in two independent runs with samplings at intervals of 100 generations that produced 20 000 trees. After verifying that stationarity had been reached, both in terms of likelihood scores and parameter estimation, the first 5000 trees were discarded in both independent runs of the cytb and the combined analyses and a majority rule consensus tree was generated from the remaining 15 000 post-burnin trees. The frequency of any particular clade among the individual trees contributing to the consensus tree represents the posterior probability of that clade (Huelsenbeck and Ronquist, 2001); only values equal or above 95% were considered to indicate sufficient support (Wilcox et al., 2002).

Estimation of divergence times

In order to assess the age of speciation events, molecular clock assumptions were incorporated. To do so we first checked if the branch lengths of our ML phylogenetic tree were evolving clock-like using the likelihood ratio test (Huelsenbeck and Crandall, 1997). A recent study by Guicking et al. (2006) calibrated the evolutionary rate of three snake species of the genus *Natrix* (*N. maura* (Linnaeus, 1758), *N. natrix* (Linnaeus, 1758) and *N. tessellata* (Laurenti, 1768)) for a combination of four mitochondrial coding genes (cytb, ND1, ND2 and ND4) and suggested that the molecular clock rate ranged between 1 and 1.35% per million years. This calibration was based on the assumption that the European and African populations of *N. maura* split 5.3 Ma, as a result of the opening of the Strait of Gibraltar at the end of the Messinian Salinity Crisis (Krijgsman et al., 1999; Duggen et al., 2003). Although it has been shown that the opening of the Strait of Gibraltar was a vicariant event for some groups, especially amphibians, recent studies on reptiles suggests that several taxa dispersed across the Strait of Gibraltar after its formation (see Carranza et al., 2006a). If this was the case in *Natrix maura*, it would suggest that the evolutionary rate might have been underestimated and therefore we would expect higher evolutionary rates for the cytb. However, according to Guicking et al. (2006), divergence time estimates using the Strait of Gibraltar were congruent with time estimates obtained using a different method based on amino acid distances and several calibration points. As a result of that, and the lack of other available calibrations in snakes for the cytb region used in our analyses, we have used Guicking et al. (2006) rate to

infer the dates of the main cladogenetic events in our phylogeny.

Basic sequence statistics and genetic distances were calculated with the program MEGA v. 2.1 (Kumar et al., 2001).

Results

In order to infer the phylogenetic relationships within *Coronella austriaca*, two independent data sets were created. Data set 1 contained cytb sequences of 36 specimens (34 ingroup sequences and two outgroup sequences). Of the 302 bp analyzed, 81 positions were variable and 59 parsimony-informative. The results of the ML, MP and Bayesian analyses of data set 1 are summarized in fig. 2. Data set 2 included all the specimens for which the two mitochondrial genes (cytb + 16S rRNA) were sequenced (16 *C. austriaca* and 1 *C. girondica*; see table 1). The results of the ILD test showed that the two gene partitions were congruent ($P = 0.35$) and therefore a combined phylogenetic analysis was carried out. Data set 2 contained 814 bp (302 bp of cytb and 512 bp of 16S rRNA) of which 105 were variable (66 of cytb and 39 of 16S rRNA) and 51 parsimony-informative (34 of cytb and 17 of 16S rRNA). The results of the ML, MP and Bayesian analyses were all very similar and largely supported the topology obtained with cytb only (see fig. 1). In view of that, and to simplify the phylogenetic conclusions, we decided to incorporate the bootstrap support and posterior probability values of the combined analyses directly into fig. 2.

The topology of the phylogenetic tree shown in fig. 2 indicates that *C. austriaca* is genetically quite variable. However, the relationships at the base of the tree are uncertain (see fig. 2) and could not be resolved with the present data set. This might be as a result of the low number of informative positions in our study or because the clades split within a very short period of time. The samples from Iberia are grouped into three well-supported clades (Clades I-III), which show a relatively high level of genetic differentiation (table 2). Especially if one takes
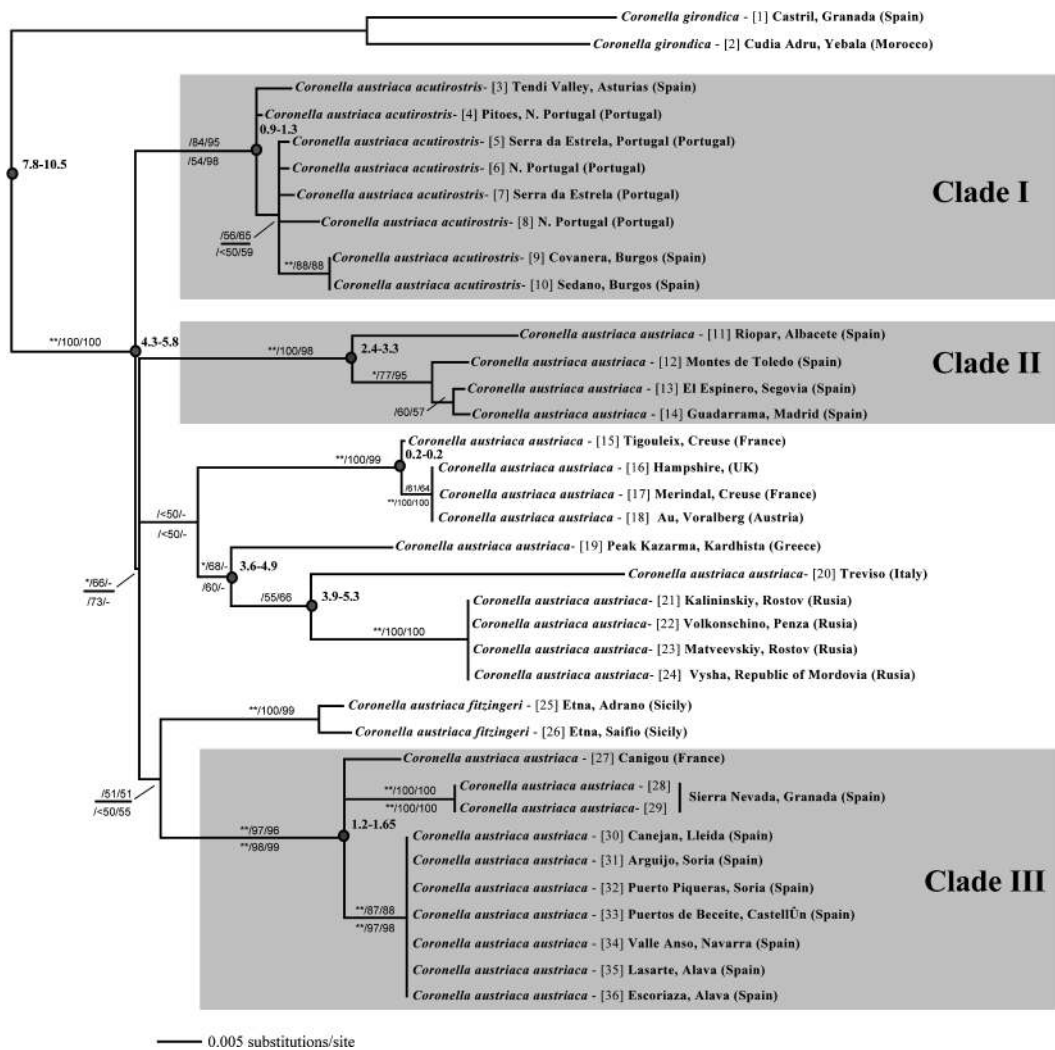
Figure 2. Maximum-likelihood tree (TrN + G; − log likelihood = 1059.6421) inferred with the mitochondrial cytb gene. Numbers in square brackets after species names are the locality codes as represented in fig. 1. Posterior probability (pp) values for the Bayesian analyses and bootstrap support values for the ML and MP analyses of the cytb gene are shown above the branches. An asterisk indicates that the Bayesian pp value is above 0.95. If no asterisk is shown it means that the pp value is 0.94 or lower. The number in the middle indicates the bootstrap support for the cytb analysis with ML (TrN + G) and the number to the right indicates the bootstrap support for the MP (ts = tv) analysis. 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 genes (814 bp). Numbers situated above the dots present in some nodes are the estimated range of the ages (in millions of years) of the speciation events concerned. The symbol "−" indicates that this particular method did not support the topology presented in fig. 2. The three Iberian clades are highlighted.

Table 2. Uncorrected genetic distances for the cytochrome b gene among all three Iberian clades of *Coronella austriaca* (see fig. 1). All values are given in %. Numbers below diagonal indicate genetic distances, and numbers above diagonal indicate standard error.

	Clade I	Clade II	Clade III	Intraspecific variability
Clade I (*C. a. acutirostris*)	–	±1.2	±1.1	0.7 ± 0.3
Clade II (*C. a. austriaca*)	5.9	–	±1.3	0.2 ± 0.2
Clade III (*C. a. austriaca*)	4.8	6.8	–	0.9 ± 0.3

into account that two of these clades (Clades II and III) include specimens from the same subspecies (see fig. 2). The low level of genetic variability within Clade I (0.7% ± 0.3), which includes specimens assigned to the subspecies *C. a. acutirostris*, suggests snakes from this clade are genetically very homogeneous despite their wide geographical range that extends from Serra da Estrela (Portugal) (type locality of *C. a. acutirostris*) to Burgos (Spain) (see fig. 1). The monophyly of Clade II is very well supported and consists of specimens from three mountain chains: Sistema Central, Montes de Toledo and the Sierra de Alcaraz (fig. 1). Genetic divergence between the sample from Montes de Toledo and the two samples from Sistema Central is rather low (1% uncorrected genetic distance for the cytb) but the genetic distance between these three individuals and the sample from Sierra de Alcaraz is much higher (3.3%). Clade III is also very well supported in all the analyses. It is the Iberian clade with the largest geographic range, extending from Sierra Nevada in SE Spain through Beceite and Soria (the northern and southern extremes of the Sistema Ibérico) up to the Pyrenees (see fig. 1 and table 1). Despite the large geographical area occupied by Clade III, the different samples included are genetically very homogeneous, with samples from localities around the Ebro Valley being identical (see figs 1 and 2). The only exceptions are: 1. – the two specimens from Sierra Nevada, in the extreme SE of the distribution range of *C. austriaca* in Iberia, which present uncorrected cytb genetic distances of 2% from all the remaining individuals of Clade III; 2. – the sample from Canigou (France) in the northern side of the Pyrenees which differs by 1.3% in the cytb from the other Pyrenean samples.

Despite the relatively large geographical distance between the samples from Austria, central France and the UK (fig. 1), they have identical cytb sequences (fig. 2). The few samples from Greece, NE Italy and Rusia form a clade, although the support in both cytb and combined analyses is very low (see fig. 2).

When the log likelihood value of the ML tree from fig. 2 (cytb tree; −1059.64) was compared with the log likelihood of the same tree constructed under molecular clock assumptions (−1072.63), there was no significant difference between the two (likelihood ratio test statistic $-2 \log \Delta = 25.97$, which approximates to a X^2_{35} distribution under the null hypothesis; $P < 0.05$). The topology of the ML tree shown in fig. 2 was therefore used to estimate approximate dates based on Guicking et al. (2006) calibration of the cytb gene of *Natrix* (see material and methods).

Discussion

Biogeography and evolution of C. austriaca

The phylogenetic study of *Coronella austriaca* within the Iberian Peninsula evidenced the existence of a complex scenario of multiple refugia within this area. According to our calibrations (see above) the split between the main clades might have occurred approximately at the end of the Miocene beginning of the Pliocene, some 4.3-5.8 Ma. These dates coincide roughly with the Messinian Salinity Crisis (MSC), a major geophysical event that produced the almost complete desiccation of the Mediterranean Sea for a period of 600 000 years (Hsü et al., 1973, 1977; Blondel and Aronson, 1999; Krijgsman et al., 2000; Duggen et al., 2003). One of the principal climatic consequences of the desiccation was the increase in the aridity of the whole Mediterranean basin. For instance, it has been shown that the Vienna woods were replaced by steppes and that palm trees grew in Switzerland (see Blondel and Aronson, 1999). This increased aridity might have also affected animals that were adapted to cold and moist habitats like *C. austriaca*. This is specially so for those populations in the Iberian Peninsula, southern Italy, Sicily and Greece, where climatic conditions were probably more extreme.

Fragmentation within the Iberian Peninsula was especially important with three highly divergent clades originating roughly at this time. Using the same markers, the high level of genetic variability found in *C. austriaca* contrasts with the low level of genetic variability found in other Iberian snake groups studied to date (Carranza et al., 2004, 2006a) and suggests that *C. austriaca* has been in the Iberian Peninsula at least since the Upper Miocene (see fig. 2). The oldest fossils from Iberia that could be unambiguously identified as *C. austriaca* date back to the Lower Pleistocene of Atapuerca 0.8-1.0 Ma (Blain, 2005), but there are fossils of *Coronella* sp. from the Upper Pliocene of the Illes Medes, NE Spain (Bailón, 1991).

The isolated population of *C. austriaca* from Sierra Nevada belongs to Clade III, a monophyletic group that also includes populations from both extremes of the Sistema Ibérico, as well as both extremes of the Pyrenees (fig. 1). According to the molecular clock, the population from Sierra Nevada diverged from all the remaining members of Clade III approximately 1.2-1.65 million years ago. This might have been as a result of fragmentation of its distribution range during the Pleistocene. Fragmentations of the distribution ranges of species during Pleistocenic ice cycles have been widely reported for many species (Zagwijn, 1992; Coope, 1994; Williams et al., 1998; Hewitt, 2003; Finlayson and Carrión, 2007) and this might also be the case for *C. austriaca*. During glaciations, Iberian mountain tops were covered by ice making them unavailable for reptiles, which were mainly confined to coastal regions and valleys (Williams et al., 1998; Arribas, 2004; Huntley and Allen, 2004). This situation probably facilitated the colonization of the southernmost parts of Iberia by Eurosiberian species like *C. austriaca*. On the contrary, during the interglacial warm periods the Eurosiberian species could not survive in the valleys and Mediterranean coastal habitats and had to retreat to high altitude areas of the surrounding mountain chains, with the consequent fragmentation

in their distribution range. Barroso and Bailón (2003) found Pleistocenic fossil records in Zafarraya, 50 km to the south of Sierra Nevada, in an area where *C. austriaca* is not actually present. This finding supports the idea that this species extended their range to lower altitudes during cold periods. More support for this biogeographical scenario comes also from the comparison of individuals located around the Ebro River valley, which are genetically identical although *C. austriaca* is not present now in the valley (probably a recent Holocenic extinction).

Coronella austriaca might have used the eastern Mediterranean coastal regions and more interior valleys for dispersal and to establish contact between the different populations of Clade III. The eastern Mediterranean corridor has probably been very favorable for faunal dispersal, as several other species of reptiles and amphibians like for instance the lacertid lizards *Psammodromus algirus* (Linnaeus, 1758) (Carranza et al., 2006b) and *Acanthodactylus erythrurus* (Harris et al., 2004), the newt *Pleurodeles waltl* Michaelles, 1830 (Carranza and Arnold, 2004; Carranza and Wade, 2004), and the Whip snake *Hemorrhois hippocrepis* (Linnaeus, 1758) (Carranza et al., 2006a) all seem to have used this corridor at different times to move from north to south or vice-versa.

The phylogeny from fig. 2 shows that the population from Sierra de Alcaraz is more closely related to populations from the Montes de Toledo and Sistema Central than to the population from Sierra Nevada. Therefore, these two areas that are part of the same mountain system (Sistema Bético) and are approximately 200 km apart have been colonized from two different stocks, following different routes and at different times. A similar situation is only found in *Salamandra salamandra* (Linnaeus, 1758), with the subspecies *S. s. longirostris* Joger and Steinfartz, 1994 inhabiting the SW of the Sistema Bético and the subspecies *S. s. morenica* Joger and Steinfartz, 1994 the NE (Sierra de Alcaraz and Sierra de Cazorla) (Garcia-París et al., 2004; Escoriza et al., 2006).

The genetic uniformity of the specimens from France, Austria and the UK and the topology from fig. 2 indicate that the present populations from England have colonized this area very recently. This data is in agreement with Strijbosch (1997), who suggests that the actual populations of *C. austriaca* from southern England and Scandinavia arrived there by 8000-7500 B.C. However, the fossil remains of *C. austriaca* from the Middle Pleistocene of Westbury-Sub-Mendip cave in Somerset, England (Holman, 1998) found together with fossils of extralimital continental forms like *Emys orbicularis* (Linnaeus, 1758), indicates that milder climates than the present one during some periods of the Pleistocene of Britain might have propitiated the expansion of *C. austriaca* and other species into Central and North Europe prior to the last Würm glaciation (Stuart, 1979, 1982). These findings suggest that shifts in species distribution ranges during the Pleistocene might have been very dynamic, with multiple expansions and retreats (Coope, 1994; Hewitt, 2000; Finlayson and Carrion, 2007).

In summary, our study indicates that genetic variability of Iberian *C. austriaca* is relatively high compared to other snake taxa and might have been facilitated by the existence of complex mountainous refugial areas in Iberia. According to the age of the first historical splits, we hypothesize that deep cladogenesis in *C. austriaca* was triggered by the onset of the Messinian Salinity Crisis, which fragmented the distribution ranges of many species inhabiting the Iberian Peninsula such as the lacertids of the genus *Iberolacerta* (Arribas and Carranza, 2004; Arribas et al., 2006), *Podarcis* (Harris et al., 2002; Pinho et al., 2006), *Lacerta* (Paulo et al., 2001), the Amphisbaenian *Blanus cinereus* (Vasconcelos et al., 2006), and the newt *Lissotriton boscai* (Lataste, 1879) (Martínez-Solano et al., 2006) among others, highlighting the importance of the Iberian Peninsula as an "old" hotspot of biodiversity.

Conservation remarks

Most of the Iberian populations that have been included in this study are severely constrained to small areas on mountain tops. The low number of records (see introduction) suggests that *C. austriaca* has very low population densities in such areas (Kuning, 1998). These very reduced and isolated populations are very exposed to stochastic factors that could difficult their future viability. Another important factor that might jeopardize the future of the southernmost Iberian populations of *C. austriaca* is the effect that the global warming of the planet is having on European reptiles and amphibians (IPPC, 1994; Jones et al., 2001; Araujo et al., 2006). One direct effect of global warming is the progressive aridification of the habitats where *C. austriaca* lives, and their transformation into unsuitable places for this Eurosiberian species. An indirect effect of global warming on the long-term survival of *C. austriaca*, is that the progressive mediterraneization of the Southern Peninsulas is producing the expansion of xeric adapted snake species like *Coronella girondica*, which are moving into the already reduced and fragmented habitats of *C. austriaca* and competing with it for similar resources. In fact, the occurrence of *C. girondica* in localities with historical records of *C. austriaca* in Sierra de Cazorla and Sierra del Aljibe in Cadiz (personal observations) could be a signal of extinction of the latter species. In Sierra Nevada (see table 1 and fig. 1), the Mediterranean snake *Malpolon monspessulanus* (Hermann, 1804) is expanding altitudinally, and has recently reached some of the mountain localities of *Coronella austriaca* (personal observation). *Malpolon monspessulanus* is an effective predator, rather ophidiophagous, which would contribute to the rapid decline of the European Smooth snake. The extremely low sightings of *C. austriaca* during recent field sampling in Sierra Nevada in comparison to several sightings of *C. girondica*, point towards the same conclusions. The two congeneric species have different reproductive modes (oviparity in *C. girondica* versus vivipar-

ity in *C. austriaca*) and they are not sympatric in central Italy (Capula et al., 1995) or southern Iberia (Galán, 2002). When they are sympatric, like for instance in rainy areas of the NW Iberian Peninsula (Soares et al., 2005), *C. austriaca* selects more forested, humid and colder habitats, therefore reducing interespecific competition between these two similar lizard predators (Galán, 2002). Some particular life-history traits make snakes more vulnerable to local extinction (Santos et al., 2007). For example, viviparity and biennal or trienal frequency of reproduction (Andrén and Nilson, 1976; Spellerberg and Phelps, 1977; Luiselli et al., 1996; Reading, 2004) might explain why *C. austriaca* seems to be outcompeted by *C. girondica*.

The genetic singularity of the southernmost Iberian populations of *C. austriaca* from Sierra Nevada and Sierra de Alcaraz increases their interest (see fig. 2). Regarding its range, *C. austriaca* is well adapted to cool and humid habitats (Engelmann, 1993; Galán, 1998), hence suggesting that these southern populations are clearly under suboptimal climatic conditions. As these populations are scarce, fragmented, vulnerable in terms of intrinsic factors sensu IUCN (several life-history traits) and possible competition with *C. girondica* (see above), we predict their future extinction. Extinction of these Iberian populations could reduce intraspecific genetic variability, hence affecting evolutionary processes that originate biodiversity.

Taxonomic implications

Our data supports the validity of the Iberian subspecies *C. austriaca acutirostris* Malkmus, 1995; a subspecies that had been previously described on morphological grounds. According to our results, *C. austriaca acutirostris* has a larger distribution range than previously suggested by Malkmus, 1995, including populations from its type locality in Serra da Estrela (Portugal) to Burgos in N. Spain (see fig. 1 and table 1). Future studies combining morphological and molecular markers (both mitochondrial and nuclear) might prove useful to examine

the boundaries between the different clades of *C. austriaca* in Iberia and to assess their taxonomic status.

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