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Phylogeographical and speciation patterns in subterranean worm lizards of the genus *Blanus* (Amphisbaenia: Blanidae)

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Abstract

The peculiar lifestyle of subterranean reptiles must determine their modes of speciation and diversification. To further understand the evolutionary biology of subterranean reptiles, we studied the phylogeny of worm lizards of the genus *Blanus* and the phylogeography of its Iberian representatives. We used mitochondrial (ND4 and 16S rRNA) and nuclear (anonymous) partial gene sequences to resolve phylogenetic relationships within *Blanus*. The Eastern Mediterranean *Blanus strauchi* was recovered as sister group of Western Mediterranean species. Iberian and North African *Blanus* were recovered as reciprocally monophyletic groups. The same genes were used to determine phylogeography of 47 populations of *Blanus cinereus*. Mitochondrial and nuclear sequence data recovered two highly supported Iberian clades. Parapatry and high sequence divergences between them suggest that these clades may represent independent taxonomic units. A molecular clock was calibrated considering that the split between Iberian and North African *Blanus* was due to the re-opening of the Betic Strait in the Upper Tortonian (8–9 million years ago). Differentiation between the two Iberian clades was estimated to date back to 5.2 million years ago. The Central Iberian clade included five mitochondrial haplotype lineages (A–E). Geographical ranges of two of them broadly overlap in the central Iberian plateau. After testing alternative hypotheses, the most likely explanation for this striking phylogeographical pattern involves recent dispersal of one of the lineages (C) over the geographical range of the other (B). The inferred recent dispersal of this fossorial reptile is explained in terms of demographic advantages associated to underground lifestyle.

Keywords: Amphisbaenia, *Blanus*, Iberian Peninsula, molecular clock, mtDNA, speciation

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Introduction

Vertebrates with a subterranean lifestyle are rather secretive, and many aspects of their evolutionary biology including prevalent modes of speciation, patterns of diversification, and geographical structuring of population genetic diversity, are still poorly understood. Although fossorial lineages are found in amphibians, reptiles, and mammals, thus far the majority of the evolutionary studies have centred in the latter group (Nevo 1979; Nevo *et al*. 1982; Patton & Sherwood 1983; Reig *et al*. 1990; Lacey *et al*. 2000; Cutrera *et al*. 2005). Studies focused on fossorial rodents (e.g. *Thomomys*, *Spalax*, *Ctenomys*) have shown that speciation modes are highly diversified in subterranean taxa, ranging from rapid, and almost sympatric, chromosomic speciation (e.g. *Ctenomys*, Reig *et al*. 1990; Massarini *et al*. 2002) to classical allopatric speciation following geographical isolation (e.g. *Thomomys*, Daly & Patton 1990; Steinberg & Patton 2000). At the population level, genetic diversification and corresponding geographical structuring of temperate subterranean rodents (Daly & Patton 1990; Cutrera *et al*. 2005) seem to conform generally to the phylogeographical patterns shown by surface-dwelling temperate small mammals with limited dispersal ability such as, for example hedgehogs and shrews (Taberlet *et al*. 1998; Hewitt 2000). Outside mammals, no information on modes of speciation and phylogeographical patterns of amphibian fossorial lineages (i.e. caecilians) is available, and it is very limited for subterranean reptiles (Bezy *et al*.

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1977; Pearse & Pogson 2000; Macey *et al*. 2004; Mulvaney *et al*. 2005). The large proportion of studies focused on mammals clearly biases our current knowledge on the prevalent mode of speciation, patterns of diversification, and population genetic structure in subterranean vertebrates, and data on nonmammalian taxa is particularly needed.

Studies on burrowing, semisubterranean amphibians (e.g. *Ambystoma*, Routman 1993; Spear *et al*. 2005) and reptiles (e.g. *Anniella*, Bezy *et al*. 1977; Pearse & Pogson 2000) have shown that phylogeographical signal is generally strong in these groups. However, the generalization of these findings to truly subterranean taxa is not straightforward, and the role of, for example chemical communication (López & Martín 2001; Zenuto *et al*. 2004) together with a suite of adaptations to underground life might impose restrictions to both patterns of colonization and processes of population isolation and differentiation. In this regard, a recent study on the Florida worm lizard, *Rhineura floridana* (Baird, 1858) (Rhineuridae: Amphisbaenia), which is an obligated burrower, showed evidence of strong genetic structure (Mulvaney *et al*. 2005).

Worm lizards of the genus *Blanus* (Blanidae: Amphisbaenia) are a natural group of strict subterranean limbless and blind reptiles with a circum-Mediterranean distribution (Kearney & Stuart 2004). The taxonomic history of the genus has been troublesome since morphological differentiation is limited compared with genetic divergences. Hence, early morphological studies (Vandelli 1797; Bedriaga 1884) recognized two species corresponding to Eastern and Western Mediterranean, respectively. Further morphological studies subdivided these two taxa into several subspecies (Bons 1963; Alexandre 1966) whereas allozyme analyses found large genetic distances among western subspecies, which could support their species status (Busack 1988). As a result, *Blanus* is currently composed of four recognized species: *B. cinereus* (Vandelli 1797) inhabiting the Iberian Peninsula, *B. tingitanus* Busack (1988), and *B. mettetali* Bons (1963) distributed over Northern and Western Morocco, respectively, and *B. strauchi* (Bedriaga 1884) with three subspecies distributed in Turkey, Kos, Rhodos and Cyprus islands, and the Middle East: Lebanon, Palestine, Eastern Iraq (Alexandre 1966). The Iberian worm lizard, *B. cinereus* may be a particularly suitable model system to study patterns of diversification, and population genetic structure in subterranean reptiles. This species inhabits along most of the Iberian Peninsula (Martín *et al*. 1991; Barbadillo *et al*. 1999), a rocky ancient region, deeply modified by palaeogeological activity, where barriers to dispersal such as deep canyons, rivers, and high mountain chains, may have played an important role shaping the phylogeography of these subterranean reptiles.

The main aim of this study was to determine phyletic diversification and phylogeographical structure in *Blanus*, a natural group of truly subterranean reptiles, and to identify the genetic consequences of fossoriality in this group. In particular, two competing hypotheses can be tested. The reduction in gene flow due to limited dispersal capacity that is implicit in fossoriality would lead to strong interpopulation genetic structure (Poulson & White 1969). Alternatively, the stability of subterranean environments combined with lower predation risk may favour the maintenance of large population sizes (Buhay & Crandall 2005), which might counteract the effects of limited gene flow. The particular objectives are (i) to propose a robust phylogenetic hypothesis for the relationships among the species of *Blanus* based on mtDNA and nuclear sequence data, (ii) to date major cladogenetic events in the evolutionary history of *Blanus*, and (iii) to identify levels of genetic diversification and phlylogeographical patterns in Iberian *Blanus*.

Materials and methods

Sampling design

We collected individuals from populations representing most of the distribution of *Blanus cinereus* (Fig. 1). Between one and four individuals per population were captured, adding up to a total of 72 individuals from 47 populations (Table 1). Fieldwork was conducted mainly between March and June 2001–2003, but some individuals were captured during autumn in warmer areas. The animals were found under stones in sandy soils, mainly in open Holm oak (*Quercus ilex*) forests. Localities of origin of all samples and the resulting haplotypes per locality are listed in Table 1. In addition to the *B. cinereus* samples, we used two individuals of *Blanus tingitanus* from Ceuta (Northern Africa), one individual of *Blanus mettetali* from Central Morocco, and one specimen of *Blanus strauchi* from Turkey.

DNA isolation, amplification and sequencing

Total genomic DNA was purified from fresh or recently preserved (ethanol 80–96%) small amounts of tissue (muscle or liver) using standard proteinase K/SDS digestion, phenol–chloroform extraction, and ethanol precipitation (Kocher *et al*. 1989). Standard polymerase chain reactions (PCR) containing 67 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 2.5 μM of each primer, template DNA (10–100 ng), and *Taq* DNA polymerase (1 U, BioTools) in a final volume of 25 μL were subjected to 35 cycles of denaturing at 94 °C for 1 min, annealing at 50–58 °C for 1 min and extending at 72 °C for 7 min. A fragment of the mitochondrial 16S rRNA gene was amplified by PCR using the '16Sar' and '16Sbr' primers (Palumbi 1996). In addition, a portion of the mitochondrial ND4 and HIS-tRNA genes was amplified with primers ND4 and LEU (Arévalo *et al*. 1994). An anonymous nuclear locus was also amplified using primers Bla F (5′-TATCAAGTAATTCCACATTCT-3′)

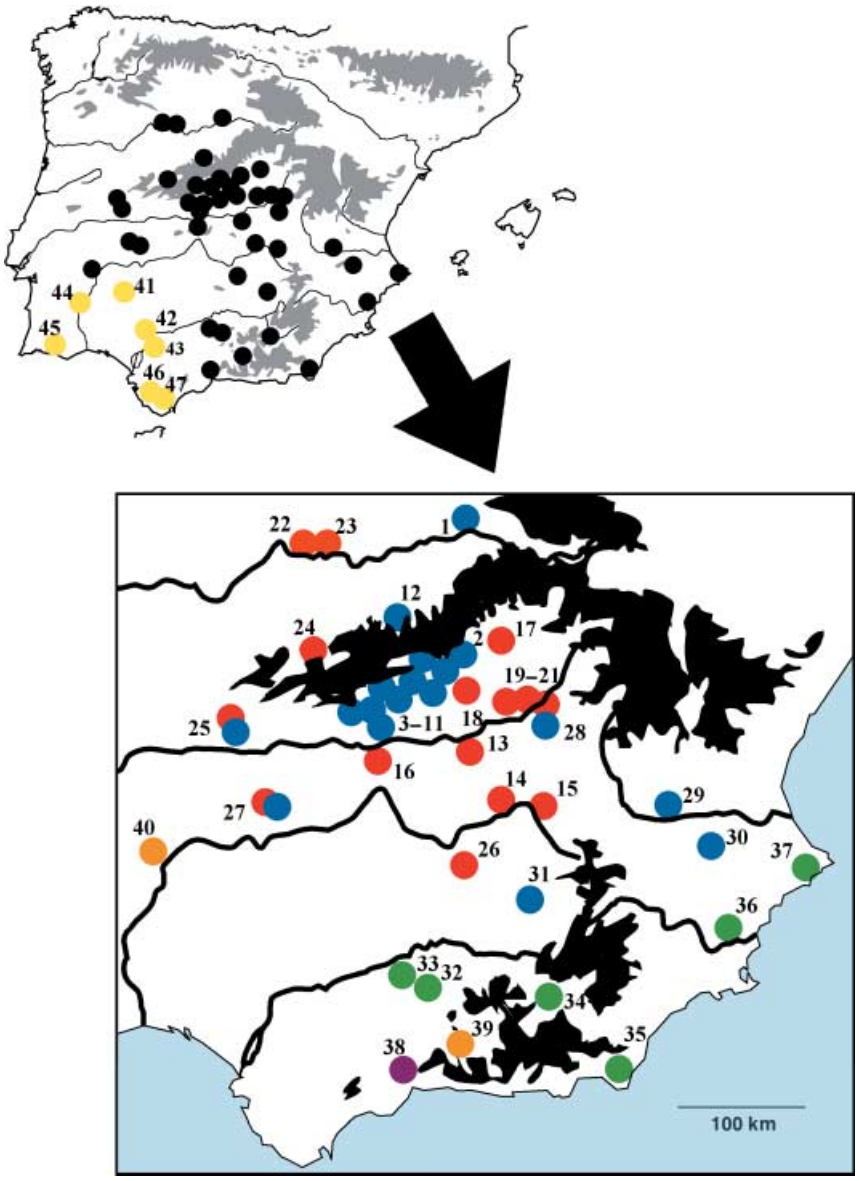

Fig. 1 Map of the Iberian Peninsula showing sampling localities of *Blanus cinereus*. Grey shades correspond to mountain ranges. Colours in the map indicate Iberian main clades (southwestern, yellow; central, black). The enlarged portion of the map includes all populations sampled from the central Iberian clade; colours represent main lineages (A, orange; B, blue; C, red; D, green; E, purple; see Fig. 2). Population numbers correspond to those on Table 1.

and Bla R (5′-GAGCGCTCCCTGTTTATTTGG-3′). PCR products were ethanol precipitated, and directly sequenced using the corresponding PCR primers. The amplified PCR products were cycle-sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (version 3.0) in an automated Applied Biosystems PRISM 3700 DNA sequencer following manufacturer's protocols. For each fragment, sequences from both strands were obtained. Nucleotide sequences here reported were deposited in GenBank under the accession nos EF036315–EF036466 and EI011512–EI011566.

Phylogenetic analyses

Sequences were read and aligned to each other by eye with SEQUENCE NAVIGATOR (Applied Biosystems). No ambiguous alignments were found within the ND4 gene and the nuclear anonymous locus, and 20 gapped positions in the 16S rRNA gene were excluded from further analyses. Phylogenetic analyses were performed on a mitochondrial data set that included ND4 and 16S rRNA sequences of all studied *Blanus* specimens, and on a nuclear data set that included sequences of 55 selected specimens of *B. cinereus*, one of *B. mettetali*, and two of *B. tingitanus* (the designed primers failed to amplify the anonymous locus in *B. strauchi*). Haplotypes (Table 1) were extracted from the two data sets with COLLAPSE version 1.1, available from http://darwin.uvigo.es/software/collapse.html. A total of 25 mitochondrial and four nuclear haplotypes were analysed.

The amphisbaenian mitochondrial sequences available in GenBank correspond to species only distantly related to

Table 1 Samples used in this study. Specimen code identifies each individual sequenced. All specimens are deposited in the Museo Nacional de Ciencias Naturales, Madrid

Population (Fig. 1)	Specimen code	Mitochondrial haplotype	Locality
Blanus strauchi	Bs		Turkey
Blanus mettetali	Bm		Morocco: Rabat
Blanus tingitanus	BT68		Spain: Ceuta (NW Africa)
Blanus tingitanus	BT69		Spain: Ceuta (NW Africa)
1	BC70	XIX	Spain: Burgos: Valdecondes
2	BC43	I	Spain: Madrid: Colmenar Viejo
2	BC44	I	Spain: Madrid: Colmenar Viejo
3	BC45	I	Spain: Madrid: Villalba
4	BC1	I	Spain: Madrid: Colmenarejo
4	BC54	I	Spain: Madrid: Villanueva de la Cañada
5	BC14	I	Spain: Madrid: Santa María de la Alameda
5	BC15	I	Spain: Madrid: Santa María de la Alameda
6	BC53	I	Spain: Madrid: Pelayos de la Presa
7	BC51	I	Spain: Madrid: Arroyomolinos
7	BC52	I	Spain: Madrid: Arroyomolinos
8	BC10	I	Spain: Avila: Casavieja
9	BC19	I	Spain: Toledo: Navalcán
9	BC22	I	Spain: Toledo: Parrillas
10	BC11	I	Spain: Toledo: Pelahustán
10	BC55	I	Spain: Toledo: El Real de San Vicente
10	BC56	I	Spain: Toledo: El Real de San Vicente
11	BC2	I	Spain: Madrid: Fresnedillas
11	BC3	I	Spain: Madrid: Fresnedillas
12	BC87	I	Spain: Avila: El Barraco
13	BC32	III	Spain: Toledo: Mazarambroz
13	BC33	III	Spain: Toledo: Mazarambroz
14	BC37	X	Spain: Toledo: Cortijos de Abajo
15	BC39	III	Spain: Toledo: Urda
16	BC21	III	Spain: Toledo: La Estrella
17	BC50	III	Spain: Madrid: Torremocha de Jarama
18	BC9	III	Spain: Madrid: El Pardo
19	BC28	III	Spain: Madrid: Arganda
20	BC7	III	Spain: Guadalajara: Illana
20	BC8	IV	Spain: Guadalajara: Illana
21	BC83	III	Spain: Guadalajara: Almoguera
21	BC85	III	Spain: Guadalajara: Almoguera
22	BC72	III	Spain: Zamora: Toro
23	BC71	III	Spain: Zamora: Venialbo
24	BC29	III	Spain: Salamanca: El Tejado de Béjar
24	BC30	III	Spain: Salamanca: El Tejado de Béjar
25	BC59	III	Spain: Cáceres: Garciaz
25	BC60	V	Spain: Cáceres: Garciaz
26	BC77	XX	Spain: Ciudad Real: Fuencaliente
27	BC88	III	Spain: Ciudad Real: Puebla de Don Rodrigo
27	BC89	III	Spain: Ciudad Real: Puebla de Don Rodrigo
27	BC90	XXII	Spain: Ciudad Real: Puebla de Don Rodrigo
27	BC86	I	Spain: Ciudad Real: Puebla de Don Rodrigo
28	BC13	V	Spain: Cuenca: Almendros
29	BC31	V	Spain: Albacete: Río Júcar
30	BC61	XVI	Spain: Valencia: Ayora
30	BC62	XVI	Spain: Valencia: Ayora
31	BC16	V	Spain: Jaén: Aldeaquemada
32	BC75	VI	Spain: Córdoba: Baena
32	BC76	VI	Spain: Córdoba: Baena
33	BC78	XXI	Spain: Córdoba: Doña Mencia
33	BC79	XXI	Spain: Córdoba: Doña Mencia

Table 1 *Continued*

Population (Fig. 1)	Specimen code	Mitochondrial haplotype	Locality
34	BC17	VI	Spain: Granada: Guadahortuna
34	BC18	VI	Spain: Granada: Guadahortuna
35	BC63	XVII	Spain: Almería: San José del Valle
36	BC57	VI	Spain: Alicante: Rojas
37	BC35	IX	Spain: Alicante: Sella
37	BC36	IX	Spain: Alicante: Sella
38	BC48	XIV	Spain: Málaga: Teba
39	BC4	II	Spain: Granada: Puerto de Zafarraya
39	BC5	II	Spain: Granada: Puerto de Zafarraya
39	BC6	II	Spain: Granada: Puerto de Zafarraya
40	BC40	XI	Portugal: Campo Maior
40	BC41	XII	Portugal: Campo Maior
41	BC25	VIII	Spain: Badajoz: Oliva de la Frontera
42	BC93	XVIII	Spain: Badajoz: Pallares
43	BC58	XV	Spain: Sevilla: Alanis
44	BC74	XIII	Portugal: Evora
45	BC42	XIII	Portugal: Loulé
46	BC64	VII	Spain: Cádiz: Alcalá de los Gazules
47	BC23	VII	Spain: Cádiz: San José del Valle
47	BC24	VII	Spain: Cádiz: San José del Valle

Blanus (Kearney 2003; Kearney & Stuart 2004). Therefore, in a preliminary phylogenetic analysis under maximum parsimony (MP), *Rhineura floridana* and *Diplometopon zarudnyi* (Trogonophidae: Amphisbaenia) were used as outgroup to determine which is the most basal lineage of *Blanus*. With a 100% bootstrap support, *B. strauchi* was recovered as sister group to the remaining *Blanus* species, and was used as outgroup in further analyses.

The mitochondrial haplotype data sets were subjected to four commonly used methods of phylogenetic inference: MP, minimum evolution (ME), maximum likelihood (ML) and Bayesian inference (BI). Unweighted MP analyses were performed with PAUP* version 4.0b10 (Swofford 2002) using the heuristic search algorithm with 100 random stepwise additions of taxa, and tree-bisection–reconnection (TBR) branch swapping. MODELTEST version 3.6 (Posada & Crandall 1998) was used to find the model of evolution that best fit our data for ME, ML, and BI. ME was performed using PAUP* and ML using PHYML version 2.4.3 (Guindon & Gascuel 2003). Non-parametric bootstrapping with 1000 (MP and ME) and 500 (ML) pseudoreplicates was used to assess the robustness of internal branches.

BI was performed using MRBAYES version 3.0b4 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) with four simultaneous chains, each of 2×10^6 generations, sampled every 100 generations. Trees sampled before the cold chain reached stationarity (as judged by plots of ML scores) were discarded as 'burn-in'. Runs were repeated twice. Support of the recovered BI trees was evaluated with Bayesian posterior probabilities (BPP).

A haplotype network was reconstructed using the parsimony criterion with TCS version 1.13 (Clement *et al*. 2000). The minimum number of steps necessary to link lineages was set to 30 in order to recover a single network.

Molecular-clock calibration

A likelihood-ratio test (LRT) was performed to assess whether the molecular-clock hypothesis could be rejected. The LRT showed that the molecular-clock-constrained tree based on mitochondrial haplotype sequence data was not significantly worse than the corresponding ML tree (unconstrained tree, –log likelihood = 3502.7139; clock-enforced tree, –log likelihood = 3515.5775; $P > 0.05$), and hence, the ML tree was directly transformed into a linearized tree.

Dating (mean and confidence intervals) of the main cladogenetic events within *Blanus* was performed using a Bayesian divergence dating analysis (Kishino *et al*. 2001; Thorne & Kishino 2002). We used the ML topology that was inferred based on the mitochondrial haplotype data set as the starting phylogeny. Following Thorne & Kishino (2002), PAML version 3.14 (Yang 1997) was employed to estimate ML parameters using a discrete gamma distribution with five rate categories, and the F84 model of nucleotide substitution. Branch lengths of the inferred topology were estimated using the ESTBRANCHES program (Kishino *et al*. 2001; Thorne & Kishino 2002). Subsequently, the MULTIDIVTIME program was used to estimate divergence times. Bayesian method requires the specification of prior distributions for parameters. The prior assumption for the

Table 2 Uncorrected *p* distances among ND4 haplotype lineages

	Blanus strauchi	*Blanus tingitanus*	*Blanus mettetali*	*Blanus cinereus* Southwestern	*B. cinereus* Central
B. strauchi	—				
B. tingitanus	0.2018	—			
B. mettetali	0.1942	0.1229	—		
B. cinereus					
Southwestern	0.2018–0.2049	0.1563–0.1624	0.1290–0.1335	0.0015–0.0091	
Central	0.1866–0.2018	0.1381–0.1502	0.1275–0.1320	0.1047–0.1244	0.0015–0.0410
	Central				
Central	A	B	C	D	E
A	0.0121				
B	0.0242–0.0349	0.0015–0.0182			
C	0.0273–0.0319	0.0319–0.0410	0.0015–0.0030		
D	0.0228–0.0319	0.0319–0.0379	0.0349–0.0379	0.0015–0.0045	
E	0.0167–0.0197	0.0228–0.0303	0.0303–0.0319	0.0273–0.0288	—

mean and standard deviation of the time of the ingroup root node (rttm) was set to 6.05 time units, where 1 time unit in this analysis represents 10 million years. This value was obtained based on the age of the rhineurid fossil *Plesiorhineura* (Sullivan 1985), which dates back to the Palaeocene [Torrejonian; minimum age of 60.5 million years ago (Ma)]. The standard deviation of the prior distribution was set to its maximum value (equal to the mean) to avoid violation of the definition of a prior. Because the data follow a molecular clock, the brownmean was set to zero. The Markov chain Monte Carlo (MCMC) method was employed to approximate both prior and posterior distributions (Kishino *et al*. 2001). The Markov chain was sampled every 100 cycles until a total of 2×10^6 generations (the burn-in was of 200 000 cycles).

In order to calibrate the molecular clock we used the split between the African and European taxa, and two independent time estimates associated to palaeographic events: (i) the re-opening of the Betic corridor after the marine transgression of the Upper Tortonian around 8–9 Ma (Weijermars 1991; Meulenkamp & Sissingh 2003) and (ii) the opening of the Strait of Gibraltar after the Messinian Salinity Crisis between 5.3 Ma and 5.6 Ma (Hsü *et al*. 1977; Krijgsman *et al*. 2000; Duggen *et al*. 2003). In order to discern which of the two competing palaeogeographical scenarios provides a more realistic calibration, we compared ND4 divergence rates obtained for each scenario to previously published data (Zamudio & Greene 1997; Malone *et al*. 2000; Pang *et al*. 2003).

Results

Sequence data

The mitochondrial haplotype data set without gaps produced an alignment of 1094 positions (817 sites were invariant and 164 positions were parsimony informative). The nuclear data set produced an alignment of 173 positions with 169 invariant sites and only one parsimony-informative position. The model of evolution that best fit the combined mitochondrial data set according to the Akaike information criterion was the TIM+G (α: 0.16). ND4 haplotype sequence divergences among lineages are summarized in Table 2. Uncorrected *p* distances for ND4 between southwestern and central populations of *Blanus cinereus* (10.5–12.4%) are of the same magnitude as those found between *Blanus mettetali* and *Blanus tingitanus* (12.3%). The nuclear sequences showed little differentiation among taxa (max. 2.3% divergence) compared to mitochondrial data (Table 2), likely as a consequence of its slower evolutionary rate.

Phylogenetic relationships within Blanus

The ML tree based on mitochondrial haplotypes (–log likelihood = 3502.7139) is shown in Fig. 2. MP (one tree, L = 420 steps, CI = 0.78, RI = 0.86), ME (two trees, score = 0.74), and BI (–log likelihood = 3518.5460) phylogenetic analyses arrived at the same majority-rule consensus topology, with high statistical support in most relevant nodes (Fig. 2). In all phylogenetic analyses, northwestern African *Blanus* (*B. tingitanus* and *B. mettetali*) form a monophyletic group, which is the sister group of another clade including all Iberian *B. cinereus* haplotypes (Fig. 2). Within the Iberian clade, two distinct monophyletic groups were recovered with high statistical support (Fig. 2). One, the 'southwestern' clade, included the populations of Cádiz, Sevilla, Badajoz, Huelva and Algarve, whereas the other, the 'central' clade, included the remaining populations (Figs 1 and 2). The central clade could be further divided into five lineages (A–E) supported by high bootstrap and posterior probability values (except lineage A) (Fig. 2).

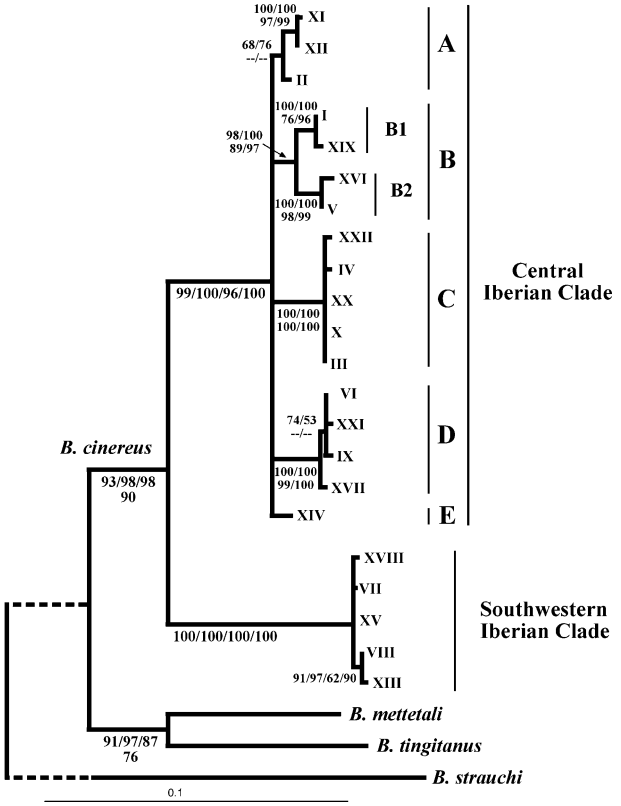

Fig. 2 Phylogeny of *Blanus*. The ML phylogram inferred based on ND4 + 16S haplotypes is shown. Numbers at each node represent from top to bottom, ML bootstrap values, BI posterior probabilities, MP and ME bootstrap values. Nodes with either ML or MP bootstrap values above 70% are shown, otherwise are collapsed.

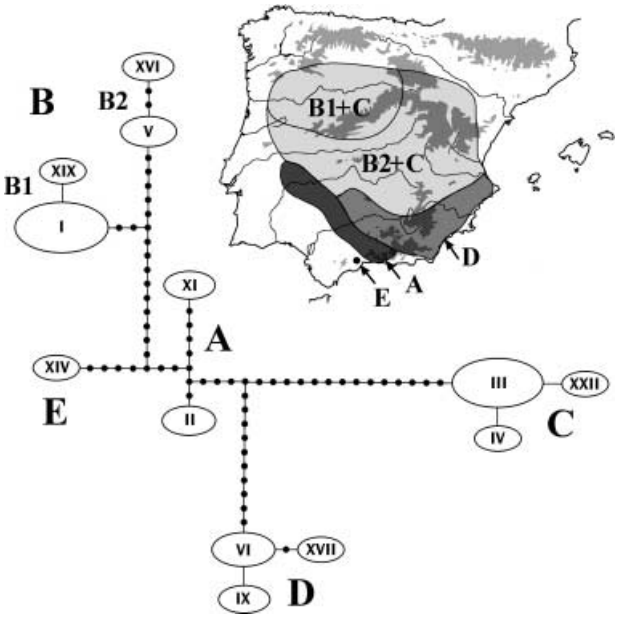

Fig. 3 Mitochondrial ND4 haplotype network of the main lineages in the central Iberian clade. The size of the ellipsoids (haplotypes) is proportional to the number of individuals. The approximate geographical distribution of each main lineage in the Iberian Peninsula is shown.

Moreover, lineage B could be further divided into two distinct highly supported sublineages, B1 and B2.

Variability of the nuclear anonymous locus among Iberian specimens was limited to two haplotypes, which are geographically constrained to the southwestern and central regions, respectively. Each of the African species had its own haplotype. The analysed nuclear fragment has a fixed difference (four variable sites, one parsimoniously informative site) that acts as a single-nucleotide polymorphism (SNP) separating the two African from the two Iberian haplotypes, and thus, supporting the finding based on mitochondrial data.

To further understand evolutionary patterns within the central clade, a network among haplotypes was reconstructed using statistical parsimony (Fig. 3). Three lineages (B, C and D) were outside the confidence limits of parsimony. Lineage A is poorly defined in the network in agreement with its lower statistical support in the phylogenetic tree. The sublineages B1 and B2 are also recovered as distinct groupings in the network. Geographical distribution of lineages A, B1, B2, D, and E is mostly parapatric (Fig. 3). Lineage C overlaps over most of its geographical range with B1 and B2, and in some populations the overlap results in strict syntopy (some individuals were found under the same stone) (Fig. 3). Despite the extensive geographical overlap of lineages B and C, none of the two lineages could be depicted as directly related to the other (because A, D or E are connected in between).

Molecular clock and divergence estimates

The evolution of the mitochondrial haplotype data set conforms to a molecular clock according to the LRT. The molecular-clock-constrained ML tree is shown in Fig. 4. In order to calibrate this linearized tree, two independent time estimates associated to palaeographic events could be used as a time constraint for the split between the African and European taxa: (i) the re-opening of the Betic Strait around 8–9 Ma, and (ii) the opening of the Strait of Gibraltar around 5.3 Ma. The two datings applied to Iberian/northwestern African *Blanus* ND4 gene pairwise sequence divergences (Table 2) imply substitutuion rates of 1.63% and 2.62% per million years, respectively. Previous calibrations of the ND4 molecular clock in reptiles provided estimations including 0.47–1.32% per million years for Viperidae (Zamudio & Greene 1997), 0.77% for Iguanidae (Malone *et al*. 2000), 1.13–2.04% for Agamidae (Pang *et al*. 2003). Hence, we consider that using the re-opening of the Betic Strait as calibration point is more realistic since the estimated divergence rate is included

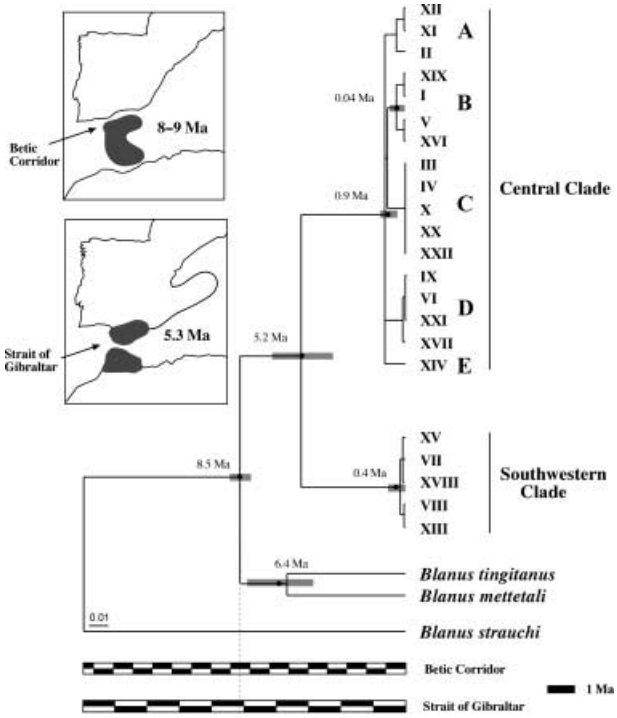

Fig. 4 Molecular-clock-enforced ML tree. Timescales represent two independent calibrations based on the opening of the Gibraltar Strait at 5.3 Ma and the re-opening of the Betic Corridor between 8 Ma and 9 Ma (simplified scenarios depicted in upper left corner), respectively. Calibration point is indicated by a dashed line. Divergence dates (mean and 95% confidence intervals) based on the Bayesian divergence dating analysis obtained with MULTIDIVTIME, and the re-opening of the Betic Corridor at 8–9 Ma as calibration point are depicted onto the relevant nodes of the linearized tree.

well within previously published estimates. Divergence dates (mean and confidence intervals) were estimated using a Bayesian divergence dating analysis based on the ML topology and the re-opening of the Betic Strait at 8–9 Ma as calibration point. According to these datings, separation of *B. mettetali* and *B. tingitanus* may have occurred around 6.4 Ma and the splitting between the two main Iberian clades, central vs. southwestern, dates back to 5.2 Ma. The radiation of the central clade in the Iberian Peninsula took place around 0.9 Ma.

Discussion

Phylogeny and Biogeography of Blanus

Recent phylogenetic analyses based on morphological characters concluded that *Blanus* is distantly related to other genera of Amphisbaenia (Kearney 2003). The genus was placed in a separate family, Blanidae, and phylogenetic relationships within *Blanus* awaited further investigation. The fully resolved molecular phylogeny for the genus *Blanus* recovered in the present study places eastern Mediterranean *Blanus strauchi* as sister group of Iberian + northwestern African taxa. The splitting between eastern and western Mediterranean *Blanus* may be the consequence of the extinction of populations located in the geographically intermediate regions (Alexandre 1966). This hypothesis is further supported by a widespread distribution of Miocene fossil remains of *Blanus* along the Mediterranean shores (Delfino 1997, 2003). The oldest fossil record of *Blanus* correponds to the Eocene (55 Ma) of England (Milner *et al*. 1982). Fossil remains are also known from the mid-Miocene of Germany (*Blanus antiquus* Schleich 1985) and France (*Blanus* sp., Augé & Rage 2000). The extinction event separating eastern and western Mediterranean clades within *Blanus* cannot be thus considered older than the mid-Miocene, the dating of the most recent fossil record known from Central Europe, which is not morphologically ascribed to either *Blanus cinereus* or *B. strauchi*.

Within the Western Mediterranean clade, Iberian and Northwestern African *Blanus* are recovered as reciprocally monophyletic groups (Fig. 2). Diversification of each clade into two main lineages is ancient (Table 2), and almost contemporary (6.4–5.2 Ma) (Fig. 4). While our large sampling of Iberian *Blanus* provides a strong support for their monophyly, the definitive recognition of the monophyly of the Northwestern African clade awaits an extended sampling in Morocco that ensures that no ancient paraphyletic lineage has been overlooked. In addition, a larger sampling could unravel any putative recent (natural or anthropogenic) colonization of Northwestern Africa from the Iberian Peninsula, as is the case of the salamander *Pleurodeles waltl* (Carranza & Arnold 2004; Veith *et al*. 2004).

Three independent lines of evidence support that the most recent vicariant event that could be responsible for the main splitting within the Western Mediterranean clade was the re-opening of the Betic Strait in the Upper Tortonian (8–9 Ma): (i) the estimated ND4 sequence divergence rate obtained using the Upper Tortonian calibration point is consistent with previously published estimated ND4 divergence rates, whereas the calibration based on the opening of the Gibraltar strait would imply an accelerated substitution rate of the gene in *Blanus*; (ii) fossil remains from the Medas Islands (eastern Spain) ascribed to *B. cinereus* (Bailón 1991) are dated back to the Upper Pliocene supporting that the splitting of Iberian and northwestern African *Blanus* predated the Messinian salinity crisis; and (iii) the cladogenetic pattern shown by *Blanus* is not congruent with diversification patterns found in lineages that split upon the opening of the Strait of Gibraltar such as, for example *Alytes* (*Baleaphryne*) (Martínez-Solano *et al*. 2004), *Discoglossus* (Fromhage *et al*. 2004; Martínez-Solano 2004; but see Zangari *et al*. 2006 for an allozyme perspective), *Rana* (*Pelophylax*) (Beerli *et al*. 1996), and the Betic lineage of *Podarcis hispanica* (Pinho *et al*. 2006).

Large-scale genetic differentiation among Iberian populations

Central and southwestern individuals of *Blanus* clustered into two highly divergent clades based both on mitochondrial and nuclear evidence (Figs 1 and 2). No single event of introgression was detected among the 52 studied specimens. The geographically concordant mitochondrial and nuclear patterns support that each clade could represent an independent taxon. Morphological and ecological studies will be necessary in order to confirm whether these two clades represent independent species. Further studies of the contact zone between the two clades using rapidly evolving nuclear markers are needed to determine whether there is current gene flow between them.

No contemporaneous geographical barrier account for the large genetic divergence observed between southwestern and central clades. Hence, assuming vicariant differentiation of the two clades, an old geographical barrier no longer at place needs to be invoked. In fact, other vertebrate Iberian taxa show deep genetic differentiation at the southwestern region, including endemics (e.g. Arntzen & García-París 1995; Mesquita *et al*. 2005; Martínez-Solano *et al*. 2006). However, datings for those taxa are generally older than our estimate of 5.2 Ma for the split between the southwestern and central clades. The complex geological history of the Algarve region is poorly documented (Mesquita *et al*. 2005), and precludes further understanding of the biogeographical events that triggered cladogenetic events in the region. Nevertheless, it is important to note that at 5.3 Ma, the Mediterranean region underwent the Messinian Salinity Crisis (Hsü *et al*. 1977; Krijgsman *et al*. 2000; Duggen *et al*. 2003).

After this manuscript was submitted, Vasconcelos *et al*. (2006) reported a study related to ours. In their work, partial mitochondrial ND4 gene sequences from 16 *B. cinereus* individuals of 13 populations were analysed. Their results are congruent with ours in grouping Iberian samples into two different clades. However, the limits of the geographical distribution of the two clades proposed by Vasconcelos *et al*. (2006) differ from ours due to the assignation of one sample from Cádiz (B17) to the Central clade. Our data contradict this result since we were unable to tind any individual assignable to the Central clade in either Cádiz or western Sevilla areas despite our sampling in the region (populations 43, 46 and 47, Fig. 1).

Patterns of genetic diversification in the Iberian central clade

Haplotype diversity within the central Iberian clade is high, and at least three lineages (B, C and D) felt outside the confidence limits of statistical parsimony in the haplotype network (Fig. 3). The lineages are well differentiated (Table 2), and according to our time estimate, the split among them dates back to the Pleistocene (0.9 Ma), possibly as a consequence of the extreme climatic fluctuations. Diversification within the central Iberian clade was likely a consequence of bottlenecked populations surviving in isolation within Iberian refugia. The internal structuring of the Iberian clade is in agreement with the hypothesis of 'refugia within refugia' during the Pleistocene glaciations (Gómez & Lunt 2006), which is shown by many temperate amphibians and reptiles. Those taxa exhibit a pattern of mtDNA diversification in which clades are strongly structured on a geographical basis (Busack 1986; García-París *et al*. 1998; Paulo *et al*. 2001; Crochet *et al*. 2004).

However, the hypothesis of 'refugia within refugia' cannot explain the extensive geographical overlap of central Iberian lineage C with sublineages B1 and B2, and other evolutionary processes need to be invoked to account for this complex geographical pattern not shown by the other lineages (Figs 1 and 3). Sublineage B1 is mostly distributed in the siliceous northwestern range whereas sublineage B2 mostly occurs in the calcareous southeastern range (Fig. 3). Lineage C shows little differentiation across its entire geographical distribution, which includes both siliceous and calcareous substrates. For instance, specimens found at the opposite edges of lineage C range share the same ND4 haplotype (locality 22 in Zamora separated by 415 km across main mountain system and rivers, from locality 26 in Ciudad Real).

Retention of ancestral polymorhism is generally invoked to explain extensive geographical overlap of highly divergent lineages (Neigel & Avise 1986; Moran & Kornfield 1993; Morando *et al*. 2004). This evolutionary process would imply simultaneous migration of lineage C with sublineages B1 and B2 from a common refuge into an extended single geographical unit. Instead, the observed outcome is the separation of B1 and B2 sublineages into two disjunct geographical units (Fig. 3), clearly rejecting the abovementioned hypothesis.

Two alternative hypotheses may explain the geographical overlap between the divergent B and C haplotype lineages.

1 Lineage C could represent a nuclear copy of the ND4 lineage B, originated prior to the diversification of lineage B (Fig. 5a). Therefore, the observed pattern would result from random alternative amplification of the nuclear or the mitochondrial copy. Although C sequences showed clear mitochondrial characteristics, we only definitively rejected this hypothesis after designing specific primers for each potential copy (differing in two divergent positions, one of them in the 3' end), and testing them under stringent conditions. No C copy was amplified from B individuals and vice versa (not shown).

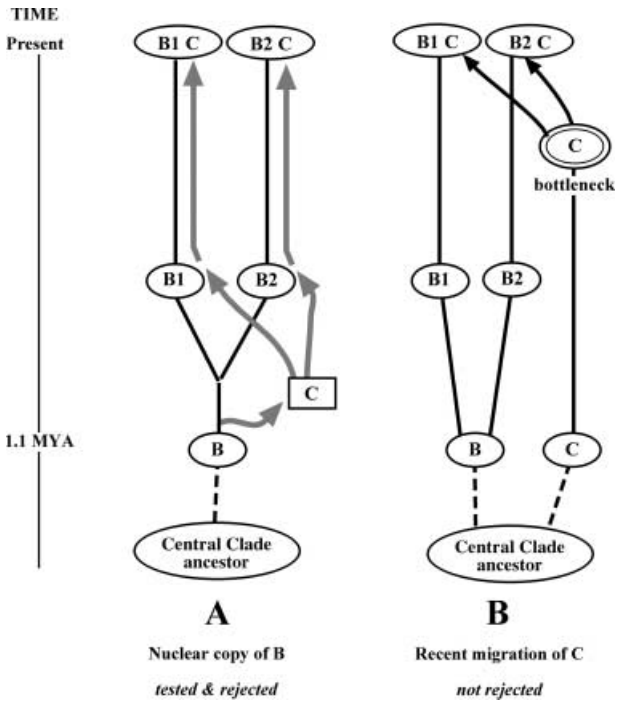

Fig. 5 Evolutionary scenarios that could account for the syntopic presence of divergent ND4 haplotype lineages B1, B2, and C over the Iberian Plateau.

2 Independent recent colonization by lineage C over the geographical ranges of B1 and B2 (Fig. 5b) may best explain the observed pattern. This hypothesis requires that lineage C suffered at least a recent bottleneck followed by an extensive geographical dispersal.

Speciation and phylogeographical patterns in fossorial taxa

The biogeographical structuring shown by *Blanus* at deeper phylogenetic levels, with highly divergent taxa showing almost nonoverlaping distributions, conforms to a classic model of allopatric speciation after geographical isolation. This is also the common case of subterranean mammals that do not follow speciation by chromosomal rearrangements (Daly & Patton 1990; Steinberg & Patton 2000). In this respect, the subterranean environment apparently does not impose any particular restrictive condition, and speciation seems to follow the same prevalent model than in nonsubterranean species.

At the population level, Iberian *Blanus* populations show overall high levels of genetic diversification with strong geographical structuring. Limited fecundity (females of *B. cinereus* normally lay a single egg per year; Díaz-Paniagua *et al*. 1995; Salvador 1998), locomotion adapted to a subterranean lifestyle, together with blindness, suggest that movements of Iberian *Blanus* are likely restricted within areas with a particularly suitable substrate. This is the same phylogeographical pattern exhibited by the Florida worm lizard, *Rhineura floridana* (Mulvaney *et al*. 2005) and temperate subterranean rodents (*Thomomys*, Steinberg & Patton 2000), and matches the patterns shown by small surface dwelling vertebrates with limited dispersal ability (Jockusch & Wake 2002; Parra-Olea 2002). Hence, we can conclude that the underground lifestyle allows the long-term accumulation of genetic diversity in fossorial taxa in a similar manner that surface barriers do in taxa with low dispersal (Buhay & Crandall 2005).

A striking peculiarity of the phylogeographical pattern of Iberian *Blanus* is the broad large-scale geographical overlap found between two divergent lineages (B and C) in Central Spain, which mimics absence of any geographical structure (Fig. 1). Limited geographical structuring, resulting from large overlap among lineages due to secondary contact, is a common pattern shown by homeothermic flying species such as bats or birds (Ditchfield & Burns 1998; Ditchfield 2000) that can cover long distances in a relatively short period of time, but it is virtually absent in surface dwelling ectothermic reptiles or amphibians. Among alternative hypothesis that could explain the geographical overlap of central Iberian lineages of *Blanus*, the most favoured involves a bottleneck in lineage C (inferred from its reduced intralineage diversification) and its relatively rapid dispersal over the geographical areas occupied by sublineages B1 and B2. The existence of bottlenecks in subterranean species is widely documented for many taxa (Poulson & White 1969), but extensive geographical dispersal is not so easily incontestable unless the demographic advantages associated to underground lifestyle are considered. Living underground benefits from avoiding surface predators (mostly birds in the case of *Blanus*), which associated with the environmental stability of the subterranean habitat, allows for the steady increasing and maintenance of large population sizes, which in turn may result in long-term contiguous range expansions (Buhay & Crandall 2005). Under these circumstances, specimens of lineage C would have achieved their relatively large current distribution in around 390 000 years (the time since the split between B1 and B2).

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