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# Phylogeography of *Crocidura suaveolens* (Mammalia: Soricidae) in Iberia has been shaped by competitive exclusion by *C. russula*

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Despite their potential importance, biological processes such as competitive exclusion (CE) have been largely neglected in phylogeographical studies. Here, we analyse the role of glacial events and CE in the evolutionary history of the lesser white-toothed shrew, *Crocidura suaveolens*, in Iberia based on cytochrome *b* sequences. All the Iberian samples grouped together with the rest of western European populations within the previously described clade IV. We identified three distinct evolutionary lineages within this major clade, two of them occurring exclusively in Iberia. Iberian lineage B extends throughout the northwest with a continuous distribution and moderate to high diversity values, whereas Iberian lineage C has a highly patchy distribution and is structured in four sublineages, all having low diversity values. No signs of demographic growth were detected for any of the lineages. The evolutionary history of *C. suaveolens* in Iberia supports the refugia-within-refugia scenario, but ecological studies in areas of sympatry, molecular and fossil datings, and contrasting patterns in the Italian Peninsula suggest that CE exerted by *C. russula* since its arrival in Iberia has been the main factor shaping the distribution, phylogeography and population genetics of lineage C.

ADDITIONAL KEYWORDS: competitive exclusion – *Crocidura suaveolens* – cytochrome *b* – genetic diversity – glacial refugia – phylogeography.

## INTRODUCTION

The geographical distribution and genetic structure and diversity of most European temperate species have been shaped by Quaternary climatic oscillations (Taberlet *et al.*, 1998; Hewitt, 2000, 2004). During glacial periods, species survived in refuge areas located mainly in the peninsulas of Iberia, Italy and the Balkans (Taberlet *et al.*, 1998; Hewitt, 1999). Glacial periods of contraction and isolation were followed by interglacial periods during which recolonization of the continent occurred through northward expansion from the southern refugia (Hewitt, 1999, 2001). Repeated glacial cycles have given rise to divergent phylogeographical clades typically associated with separate glacial refugia (Hewitt, 2004, 2011) and a progressive reduction in diversity with increasing latitude (Hewitt, 1999, 2000).

Recent evidence shows that the southern refugia were a heterogeneous mosaic of suitable habitat isolated by unsuitable habitat that allowed the evolution of separate sublineages within each peninsula (refugia-within-refugia scenario; Gómez & Lunt, 2007;

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Weiss & Ferrand, 2007; Abellán & Svenning, 2014). This resulted not only in high levels of intraspecific diversity, but also of species diversity and endemism when divergence progressed towards speciation, and these are characteristic of the southern refugia (Vega *et al.*, 2010a; Abellán & Svenning, 2014). The description of these intraspecific lineages within European refugia is key for the setting of conservation priorities and for the elaboration of effective management plans.

With the generalized emphasis on the role of Quaternary climatic oscillations, other factors that could have promoted intraspecific divergence have been overlooked. In particular, competitive exclusion (CE) by ecologically similar species, or even conspecific populations, has been largely neglected in phylogeography (but see Waters, 2011; Ranjard *et al.*, 2014). CE is a basic principle of ecological theory, whereby two species cannot coexist in a stable habitat if they compete for the same resources (Gause, 1932; Hardin, 1960; Ayala, 1971). The successful competitor may drive the other towards extinction or promote its evolutionary shift towards a different ecological niche. Recent evidence suggests CE is a widespread phenomenon that can explain the observed phylogenetic overdispersion of mammal communities (Cooper, Rodríguez & Purvis, 2008). Interspecific CE can become an agent of vicariance and cause isolation, eventually resulting in genetic differentiation and lineage divergence (Gutierrez, Boria & Anderson, 2014). Intraspecific CE can contribute to the maintenance of spatially segregated divergent lineages, which explains the genetic homogeneity of recolonized northern European areas (Hewitt, 1996; Ranjard *et al.*, 2014). Furthermore, CE during range expansion can create patterns at neutral loci that mimic those produced by adaptive processes and resemble post-glacial segregation of clades from distinct refugia (Excoffier & Ray, 2008).

The lesser white-toothed shrew, *Crocidura suaveolens* (Pallas, 1811), represents a suitable case study to analyse the relative roles of Quaternary climatic oscillations and CE in the genetic divergence of temperate species in Europe. *Crocidura suaveolens* is widely distributed throughout the Palearctic, extending from the Atlantic coasts of Europe to Siberia (Hutterer, 2005; Palomo *et al.*, 2016). From Central Europe to Asia its distribution is continuous and the species is described as abundant and ubiquitous (Palomo *et al.*, 2016). However, in western Europe, *C. suaveolens* is less common and is absent from large areas, including most of the Iberian Peninsula (Libois, Ramalhinho & Fons, 1999).

Like many other temperate species, *C. suaveolens* retreated to southern Eurasian refugia during Pleistocene glaciations (Taberlet *et al.*, 1998; Hewitt, 1999). Ten phylogeographical clades have been identified across its wide distribution, the Iberian populations grouping in clade IV, which is the most western and basal clade in Europe (Dubey *et al.*, 2006, 2007). However, these previous studies included only two Iberian samples, so any possible internal genetic structure within Iberia might have remained unnoticed. In addition, the most isolated continental populations of European *C. suaveolens* are located in southwestern Iberia, more than 300 km from any other population (Fig. 1) (Román & Ruiz, 2003; Palomo *et al.*, 2016). A plausible hypothesis is that these marginal populations were unintentionally introduced by humans, as demonstrated for some Mediterranean island populations (Dubey *et al.*, 2007); shrews are small, inconspicuous animals and southwestern Iberia has seen huge commercial marine traffic for millennia (Vives, 2015).

An intriguing possibility is that the recent history and current distribution of *C. suaveolens* in western Europe, and especially in Iberia, have been influenced by its congeneric, the greater white-toothed shrew, *C. russula*. *Crocidura russula* reached Iberia from Africa 100 kya (Ruiz-Bustos, 1997; Arsuaga, Baquedano & Pérez-González, 2006; Laplana & Sevilla, 2006; López-García, 2008), and is now a very widespread and common species throughout western Europe (Aulagnier *et al.*, 2016). Several lines of evidence suggest that *C. russula* competitively exclude *C. suaveolens* in areas of sympatry (Niethammer, 1979; Poitevin *et al.*, 1987; Cosson, Pascal & Bioret, 1996; Libois *et al.*, 1999; Kraft, 2000; Román & Ruiz, 2003; Pascal, Lorvelec & Vigne, 2006). CE could thus explain the fragmented distribution of *C. suaveolens* and, if so, it may have also caused genetic isolation leading to intraspecific divergence in western Europe.

Therefore, in the present study we aimed to: (1) infer the evolutionary history of *C. suaveolens* in the Iberian Peninsula by identifying the main mitochondrial evolutionary lineages and estimating divergence times among them; (2) clarify the origin of the southwestern Iberian populations of *C. suaveolens*; and (3) discuss the phylogeographical and demographic processes that have shaped the species genetic variation and its current distribution, given the possible contribution of CE by *C. russula*.

## MATERIALS AND METHODS

### SAMPLE COLLECTION

Samples of 119 specimens of *C. suaveolens* were collected, 55 as bone samples (mandibles or skulls) obtained from owl pellets and 64 as tissue samples from live specimens. Sampling was designed to cover

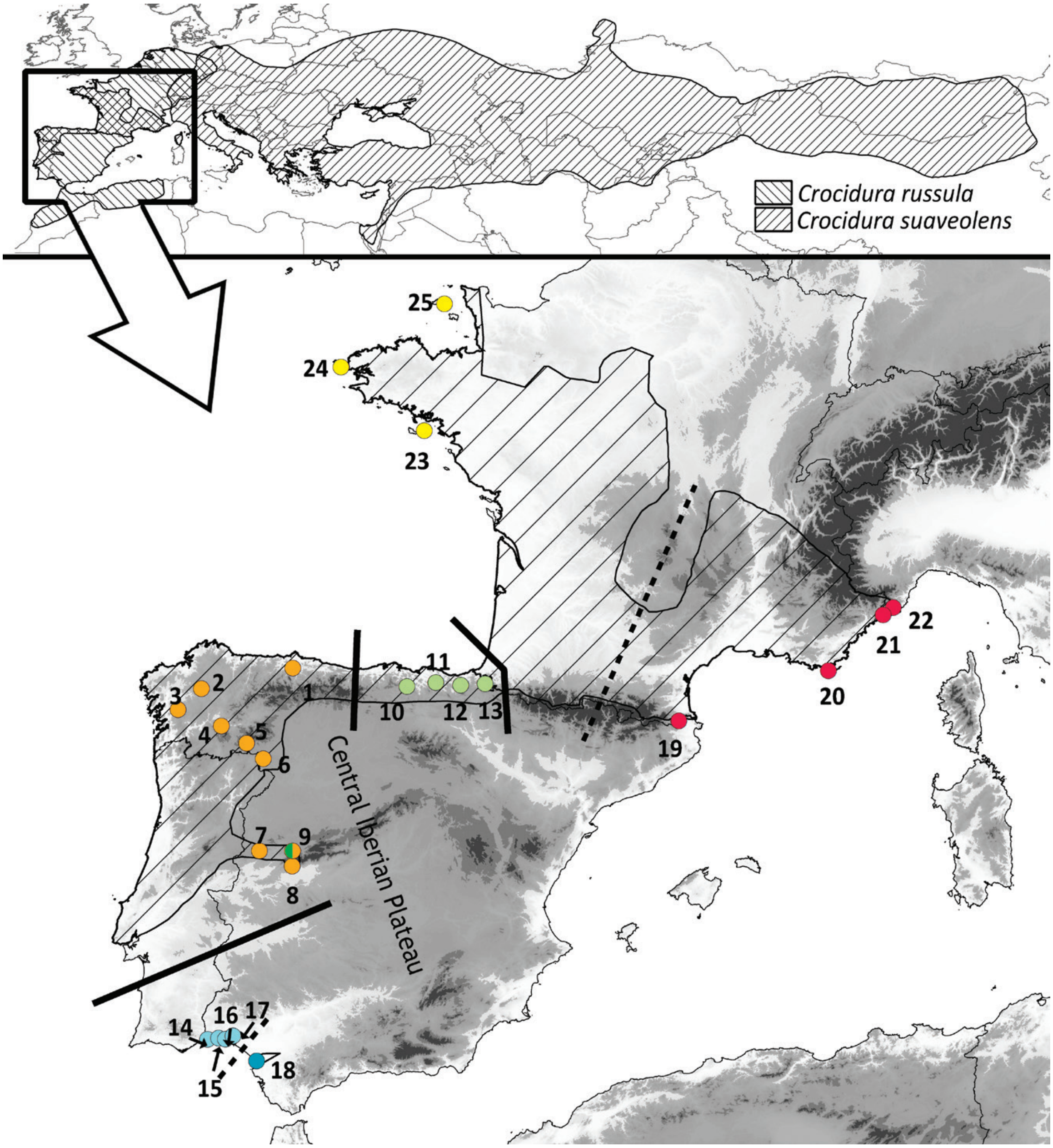

**Figure 1.** Distribution of clade IV of the *Crocidura suaveolens* group in western Europe (striped area), showing collection localities (1–18) and localities of downloaded sequences (19–25) (in colour). Localities in close proximity to each other are represented as a single location; see Supporting Information, Tables S1 and S3 for more details. The colours represent the different lineages/sublineages identified in the phylogenetic analyses, which are also separated by thick black lines/dashed lines on the map. Altitude is shown with a greyscale, lower areas with lighter tones and higher areas with darker tones. The distribution of *C. russula* is also shown with different striped fills. Note that the distribution range of *C. suaveolens* in western Europe is fragmented only where both species are sympatric.

most of the Iberian distribution range of the species, but our attempts to sample the species in Portugal were unsuccessful (Fig. 1; see also Supporting Information, Table S1 for a full description of the collection localities).

### DNA EXTRACTION

DNA was extracted from tissue samples using the NucleoSpin 96 Tissue Kit (Macherey-Nagel & Co. KG) or a 'salting-out' protocol (Müllenbach, Lagoda

& Welter, 1989), as modified by Centeno-Cuadros, Delibes & Godoy (2009). Bone samples were first frozen in liquid nitrogen and homogenized to powder with a ball-mill or with a mortar and pestle (Retsch Mod.MM301). Then, DNA was extracted using a guanidinium silica protocol [method C in Rohland & Hofreiter (2007)]. The final elution volume was 50 µL in all cases. New disposable material was used for extractions from each specimen and the work surface was cleaned with bleach between each sampling to prevent contamination. Extraction blanks were included in every round of extraction and bone samples were extracted in a laboratory used exclusively for low quality DNA sources.

### PCR amplification and sequencing

A fragment of 1251 bp including the mitochondrial cytochrome *b* gene (*Cytb*) was PCR amplified and sequenced. For tissue samples, primers L14734/H15985 (Ohdachi *et al.*, 2001) were used; however, for degraded bone extracts, we designed five pairs of internal partially overlapping primers (Supporting Information, Table S2). Sequences were trimmed, edited and assembled using SEQUENCHER 4.9 (Gene Codes Corporation), and aligned with the Geneious alignment in GENEIOUS version 8.1.5 (http://www.geneious.com; Kearse *et al.*, 2012). All novel sequences were deposited in GenBank (accession numbers: MF987937–MF988055).

### Phylogenetic and network analyses

We reconstructed a Bayesian phylogenetic tree in BEAST 2.4.1 (Bouckaert *et al.*, 2014) using 119 sequences obtained in this study (Supporting Information, Table S1), together with 122 additional sequences downloaded from GenBank (Supporting Information, Table S3) (Dubey *et al.*, 2006, 2007). Sequences of *C. nigripes* and *C. brunnea* were used as outgroups. We also obtained a maximum likelihood (ML) tree for this same alignment and assessed support with 100 non-parametric bootstrap replicates in MEGA 7 (Kumar, Stecher & Tamura, 2016). To further explore the internal structure of clade IV, a phylogenetic network was constructed in NETWORK 4.6, using a median-joining (MJ) algorithm (Bandelt, Forster & Röhl, 1999) and the haplotypes identified by the program DNASP 5.10 (Librado & Rozas, 2009) from all sequences grouping within clade IV.

To estimate the arrival date of *C. russula* in Iberia and to evaluate the possible influence of CE on the evolutionary history of *C. suaveolens* in this area, we constructed a Bayesian phylogenetic tree in BEAST 2.4.1 (Bouckaert *et al.*, 2014) with a set of 81 *Cytb* published sequences of *C. russula* (Brändli *et al.*, 2005; Cosson *et al.*, 2005; Gargan *et al.*, 2016).

### Divergence time estimates: StarBEAST2

We dated the times of divergence between lineages within clade IV in StarBEAST2, using the same sequences, partitions and substitution models previously employed in the phylogenetic tree reconstruction, setting clades as independent populations. The mean clock rate used was the *Cytb* substitution rate that we estimated for the *C. suaveolens* branch with a Bayesian relaxed clock analysis in BEAST2 (Supporting Information, Fig. S1), as described by Igea *et al.* (2015) with some minor modifications, including additional calibrations (Supporting Information, Table S4). Similarly, we estimated the time of the split between Marrocan and European populations of *C. russula* from the phylogenetic tree of *C. russula*, setting the European and Moroccan samples of *C. russula* as predefined populations and the substitution rate estimated for the *C. russula* branch as the mean clock rate (Supporting Information, Fig. S1), which happened to be the same as that of *C. suaveolens*.

### Genetic structure and diversity

We used spatial analysis of molecular variance (SAMOVA 2.0; Dupanloup, Schneider & Excoffier, 2002) to infer groups of populations. Samples were grouped *a priori* in 12 geographical populations generally corresponding to separate sampling localities (Fig. 1; Supporting Information, Tables S1, S3). SAMOVA was run for 10 000 iterations from each of 100 random initial conditions, and we tested a predefined number of groups ($K$) ranging from two to 12. The inferred hierarchical structure was then used for the analysis of molecular variance (AMOVA), genetic diversity estimates and pairwise comparisons based on $F_{ST}$ (using only haplotype frequencies) or $\Phi_{ST}$ (using also nucleotide distances among haplotypes) in ARLEQUIN v3.11 (Excoffier, Laval & Schneider, 2005). The numbers of sequences, polymorphic sites, haplotypes and average number of nucleotide differences, as well as haplotype and nucleotide diversities, were estimated for each population, for population groups suggested by SAMOVA, and for lineages of clade IV using ARLEQUIN v3.11 (Excoffier *et al.*, 2005).

### Demographic history

We investigated the demographic history of clade IV lineages with the mismatch distribution of pairwise nucleotide differences, Harpending's raggedness index (Rogers & Harpending, 1992), Tajima's $D$ (Tajima,

1989) and Fu's $F_S$ (Fu, 1997) neutrality statistics using ARLEQUIN v3.11 (Excoffier *et al.*, 2005). In addition, Fu and Li's $F^*$ and $D^*$ (Fu & Li, 1993) and $R^2$ (Ramos-Onsins & Rozas, 2002) statistics were estimated and tests were performed using DNASP 5.10 (Librado & Rozas, 2009). We also generated Bayesian skyline plots (BSPs; Drummond *et al.*, 2005) with BEAST2.

Further details of the methods can be found in the Supplementary Information.

## RESULTS

All 119 specimens sequenced in this study grouped within clade IV of the *C. suaveolens* group (Dubey *et al.*, 2006, 2007), including those sampled in the southern populations. The novel sequences formed a highly supported clade together with the other 12 pre-existing sequences of this clade [posterior probability (PP) = 1.00 and 100% bootstrap; Supporting Information, Figs S2, S3]. The 131 *Cytb* sequences in clade IV included 95 polymorphic sites, 74 of which were phylogenetically informative, and defined 45 haplotypes (Supporting Information, Table S5).

Both phylogenetic analyses (Bayesian and ML) revealed three main phylogenetic lineages within clade IV occurring in different areas of the species range (see Supporting Information, Figs S2, S3 for Bayesian and ML phylogenetic trees; see Fig. 2 for a dated phylogeny of clade IV and Fig. 1 for the geographical distribution of each lineage). One lineage was located mainly in France (lineage A), within which two sublineages were differentiated. Sublineage A1 was found on the French Mediterranean coast, including samples ranging from the province of Gerona, in Spain, to the northwest of Italy (sublineage A1; PP = 0.90/66% bootstrap), and sublineage A2 grouped shrews from the different islands of the northwestern coast of France and the Channel Islands (sublineage A2; PP = 1.00/70 % bootstrap). The other two main lineages within clade IV (lineages B and C) were strictly Iberian.

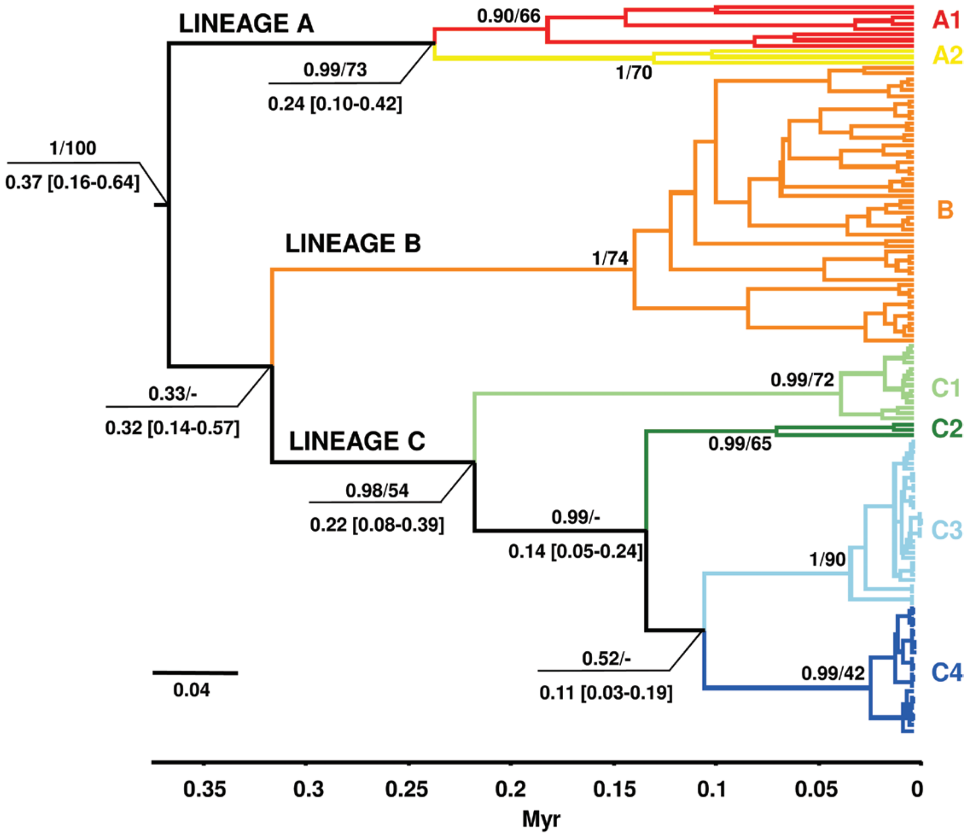

**Figure 2.** Dated tree of clade IV of the *Crocidura suaveolens* group with the main lineages (A, B and C) and sublineages (A1 and A2, C1–C4) identified herein. Bayesian posterior probabilities/bootstrap support values (above branch) were obtained for the Bayesian and maximum likelihood (ML) trees, respectively (Supporting Information, Figs S2, S3). Bootstrap support values of the nodes that were not present in the ML tree are represented by a dash (-). Times of divergence and credibility intervals (in square brackets) estimated in *BEAST are also indicated for each node.

Lineage B was widely distributed and located in the northwest of the Iberian Peninsula [from the province of Oviedo, in the north of Spain, to the mountain ranges of the Iberian Central System (lineage B; PP = 1.00/74% bootstrap]. The other Iberian lineage, lineage C, was more structured, with four differentiated sublineages. One of these sublineages was located in the north-central area of Iberia (sublineage C1; PP = 0.99/72% bootstrap). Interestingly, a second sublineage was found in Candelario (locality 9 in Fig. 1 and Supporting Information, Tables S1, S3), at the eastern edge of the Iberian Central System (sublineage C2; PP = 0.99/65% bootstrap); the co-occurrence of haplotypes of sublineage C2 and lineage B identifies this locality as a secondary contact zone. The other two remaining sublineages were located in southwestern Iberia. One grouped individuals sampled in several river mouths in the province of Huelva (sublineage C3; PP = 1.00/90% bootstrap), whereas the other included individuals sampled exclusively at the Guadalquivir River mouth (sublineage C4; PP = 0.99/42% bootstrap).

The three lineages and their respective sublineages were also clearly distinguished in an MJ network (Fig. 3). Sublineages A1 and A2 are clearly separated, and the rather large haplotype divergence within sublineages could indicate further subdivision in France, although the sparse sampling there impedes any strong conclusion on this issue. Lineage B included a large number of haplotypes (*h* = 22) with similar frequencies, whereas lineage C altogether presented fewer haplotypes (*h* = 13) with more heterogeneous frequencies (Table 1; Supporting Information, Table S5). Indeed, haplotypes H15, H31 and H3 of sublineages C3, C4 and C1 were clearly predominant within clade IV, with 25, 15 and 11 occurrences, respectively.

### DIVERGENCE TIMES

The substitution rate for the *Cytb* gene in *C. suaveolens* was estimated at 0.0377 substitutions/site/Myr (95% confidence interval: 0.0220–0.0577; Supporting Information, Fig. S1). Using this rate, the splits that separate the French lineage A and Iberian lineages

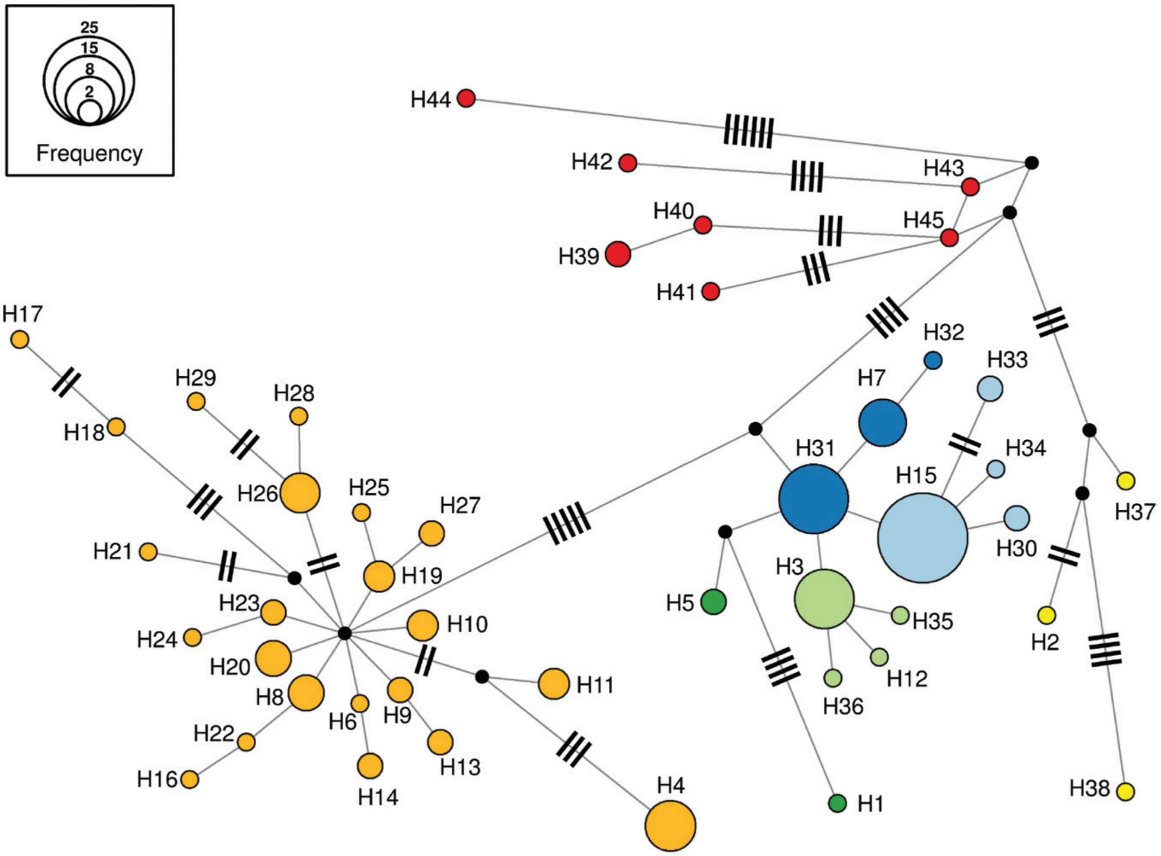

**Figure 3.** Median-joining network of cytochrome *b* clade IV haplotypes of *Crocidura suaveolens*, coloured according to lineage (A1, red; A2, yellow; B, orange; C1, light green; C2, dark green; C3, light blue; C4, dark blue). The diameter of the circles represents the number of sampled individuals with that haplotype. Black dots indicate unsampled intermediary haplotypes.

**Table 1.** Genetic diversity and demographic statistics for lineages of clade IV of the *Crocidura suaveolens* group

| | | Genetic diversity | | | | | Mismatch distributional analysis | | | Neutrality test | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lineage | $N$ | $h$ | $S$ | $H$ | Pi | $K$ | SSD | HRI | Tau | Tajima $D$ | Fu's $F_S$ | Fu and Li's $D$ | Fu and Li's $F$ | $R_2$ |
| A1* | 8 | 7 | 26 | 0.964 | 1.026 | 9.75 | 0.034<br>($P$ = 0.465) | 0.070<br>($P$ = 0.588) | 7.607 | −0.146<br>($P$ = 0.448) | −0.505<br>($P$ = 0.304) | −0.420<br>($P$ > 0.10) | −0.413<br>($P$ > 0.10) | 0.1428<br>($P$ = 0.211) |
| A2* | 3 | 3 | 15 | 1.000 | 1.026 | 10 | 0.000 | 0.000 | 0 | 0.000<br>($P$ = 0.689) | 1.139<br>($P$ = 0.478) | n.a. | n.a. | n.a. |
| B | 50 | 22 | 41 | 0.948 | 0.693 | 5.55 | 0.010<br>($P$ = 0.211) | 0.026<br>($P$ = 0.227) | 6.515 | −1.342<br>($P$ = 0.06) | −6.046<br>($P$ = 0.034) | −0.075<br>($P$ > 0.10) | −0.592<br>($P$ > 0.10) | 0.0672<br>($P$ = 0.084) |
| C1 | 14 | 4 | 3 | 0.396 | 0.063 | 0.429 | 0.005<br>($P$ = 0.684) | 0.168<br>($P$ = 0.63) | 0.505 | −1.671<br>**($P$ = 0.026)** | −2.288<br>**($P$ = 0.005)** | −2.091<br>(0.10 > $P$ > 0.05) | −2.255<br>(0.10 > $P$ > 0.05) | 0.1368<br>($P$ = 0.138) |
| C2* | 3 | 2 | 5 | 0.667 | 0.402 | 3.333 | 0.392<br>($P$ = 0.068) | 1.000<br>($P$ = 0.545) | 5.881 | 0.000<br>($P$ = 0.782) | 2.357<br>($P$ = 0.824) | n.a. | n.a. | n.a. |
| C3 | 30 | 4 | 4 | 0.306 | 0.049 | 0.453 | 0.005<br>($P$ = 0.494) | 0.286<br>($P$ = 0.566) | 3 | −1.416<br>($P$ = 0.07) | −1.436<br>($P$ = 0.087) | −0.608<br>($P$ > 0.10) | −1.050<br>($P$ > 0.10) | 0.0631<br>**($P$ = 0.000)** |
| C4 | 23 | 3 | 2 | 0.502 | 0.071 | 0.561 | 0.013<br>($P$ = 0.272) | 0.154<br>($P$ = 0.313) | 0.697 | 0.080<br>($P$ = 0.622) | 0.109<br>($P$ = 0.452) | −0.646<br>($P$ > 0.10) | −0.514<br>($P$ > 0.10) | 0.1564<br>($P$ = 0.563) |
| Total | 131 | 45 | 95 | 0.936 | 1.214 | 9.589 | 0.008<br>($P$ = 0.547) | 0.011<br>($P$ = 0.497) | 13.617 | −1.441<br>($P$ = 0.054) | −11.101<br>($P$ = 0.028) | −1.889<br>($P$ > 0.10) | −2.123 (0.10 > $P$ > 0.05) | 0.0447<br>**($P$ = 0.037)** |

*N*, sample size; h, number of haplotypes; S, number of polymorphic sites; H, haplotype diversity; Pi, nucleotide diversity (expressed as percentages, i.e. 0.001 = 0.1%); K, average number of pairwise nucleotide differences. HRI, Harpending's raggedness index; n.a., not available (for lineages with a small number of samples); SSD, sum of square deviation. Tau is the mode of the curve of the mismatch distribution when a signal of demographic expansion is detected and it is proportional to the time since expansion. Values in bold show significant results for SSD, HRI, Tajima's *D*, Fu and Li's tests and $R_2$ statistics ($P < 0.05$), and for Fu's $F_S$ test ($P < 0.02$). *Lineages including haplotypes downloaded from GenBank obtained from an unknown number of samples (assumed here to be one).

B and C were dated to *c*. 0.37 Ma (95% confidence interval: 0.16–0.64 Ma) and 0.32 Ma (95% confidence interval: 0.14–0.57 Ma), respectively, although the low support for this latter node suggests a simultaneous division for the three lineages in clade IV (Fig. 2). The split between sublineages A1 and A2 occurred around 0.24 Ma (95% confidence interval: 0.10–0.42 Ma), and subsequent splits giving rise to sublineages C1, C2, C3 and C4 were dated to *c*. 0.22 Ma (95% confidence interval: 0.08–0.39 Ma; PP = 0.96), 0.14 Ma (95% confidence interval: 0.05–0.24 Ma; PP = 0.99) and 0.11 Ma (95% confidence interval: 0.03–0.19 Ma; PP = 0.59), respectively.

The *Cytb* substitution rate estimated for the *C. russula* branch was identical to that for *C. suaveolens* (Supporting Information, Fig. S1). Application of this rate yielded an estimate for the split of European and African populations of *C. russula* of 126 ka (41–200 ka; Supporting Information, Fig. S4).

### GENETIC STRUCTURE AND DIVERSITY

SAMOVA analyses suggested a spatial subdivision of the Iberian distribution area into eight population groups (Table 2; see also Supporting Information, Table S1). Only two groups were formed by more than one population: the north-central Iberian Peninsula (NC-IP), formed by the populations of Burgos and the combined population of País Vasco and Navarra, and the southwestern Huelva (SW-Huelva), grouping all populations of southwestern Iberia, except Guadalquivir. Groups suggested by SAMOVA generally corresponded to lineages and sublineages, with two exceptions: (1) the Eastern Iberian Central System (Eastern-CS) included samples of lineages B and C2 and (2) lineage B was subdivided into four genetic groups [Oviedo, Galicia, Zamora and Western Central System (Western-CS)]. The first represents the only occurrence of secondary contact and the second indicates some hierarchical genetic structure within lineage B, which could be due both to historical isolation and to current restrictions to gene flow.

The AMOVA with this hierarchical structure suggested by SAMOVA showed that the majority of the total mitochondrial DNA variation was attributed to differences among groups (77.45%), whereas a very low and negative percentage of variation was due to differences among populations within groups (−1.96%). Slightly negative variance can result from random variance or from genes from different populations being more closely related to each other than genes from the same population, as occurs in Eastern-CS, where a secondary contact area was detected (locality 9 in Fig. 1 and Supporting Information, Table S1). Pairwise comparisons based both on differences between sequences ($\Phi_{ST}$) and on haplotype frequencies only ($F_{ST}$) were consistent in showing that neighbouring populations or populations grouped within the same phylogenetic lineage displayed lower differentiation between them (Supporting Information, Table S6; see also Supporting Information, Table S1 and Fig. 1). However, differentiation between populations of the Iberian Central System (Eastern-CS and Western-CS, populations 3 and 4 in Supporting Information, Table S6, respectively) was somewhat larger than expected given their proximity ($\Phi_{ST}$ = 0.307; $F_{ST}$ = 0.476), which

**Table 2.** Genetic diversity indices in Iberian populations of *Crocidura suaveolens*

| Population group ID | Population | Localities | *N* | *h* | *S* | *H* | Pi | *K* |
|---|---|---|---|---|---|---|---|---|
| 1 | **Oviedo** | 1 | 13 | 6 | 15 | 0.821 | 0.483 | 5.077 |
| 2 | **Galicia** | 2, 3, 4 | 13 | 9 | 17 | 0.910 | 0.531 | 4.410 |
| 3 | **Zamora** | 5, 6 | 10 | 4 | 7 | 0.800 | 0.399 | 3.022 |
| 4 | **Western-CS** | 7 | 6 | 2 | 6 | 0.600 | 0.434 | 3.600 |
| 5 | **Eastern-CS** | 8, 9 | 11 | 3 | 21 | 0.473 | 0.968 | 8.036 |
| 6 | **NC-IP** | 10, 11, 12, 13 | 14 | 4 | 3 | 0.396 | 0.063 | 0.429 |
|  | Burgos | 10 | 5 | 3 | 2 | 0.700 | 0.117 | 0.800 |
|  | País Vasco and Navarra | 11, 12, 13 | 9 | 2 | 1 | 0.222 | 0.029 | 0.222 |
| 7 | **SW-Huelva** | 14, 15, 16, 17 | 30 | 4 | 4 | 0.306 | 0.049 | 0.453 |
|  | Guadiana | 14 | 6 | 2 | 2 | 0.533 | 0.115 | 1.067 |
|  | Piedras | 15 | 8 | 1 | 0 | 0.000 | 0.000 | 0.000 |
|  | Odiel | 16 | 7 | 3 | 2 | 0.667 | 0.071 | 0.762 |
|  | Tinto | 17 | 9 | 1 | 0 | 0.000 | 0.000 | 0.000 |
| 8 | **Guadalquivir** | 18 | 23 | 3 | 2 | 0.502 | 0.071 | 0.561 |
|  | Total |  | 120 | 35 | 63 | 0.923 | 1.031 | 8.146 |

*N*, sample size; *h*, number of haplotypes; *S*, number of polymorphic sites; *H*, haplotype diversity; Pi, nucleotide diversity (expressed as percentages, i.e. 0.001 = 0.1%); *K*, average number of pairwise nucleotide differences. Eastern-CS, Eastern Central System; NC-IP, North-Central Iberian Peninsula; SW-Huelva, Southwestern Huelva; Western-CS, Western Central System. Groups of populations suggested by SAMOVA are indicated in bold.

may again be due to the co-occurrence of haplotypes of different lineages in Eastern-CS. Guadalquivir also showed wide differentiation from the other populations in SW-Huelva (populations 8 and 9–12 in Supporting Information, Table S6, respectively), despite their relative proximity, which is consistent with long-term isolation resulting in divergent sublineages (C3 and C4; Figs 1, 2).

Lineage B of northwestern Iberia showed the highest haplotype and nucleotide diversity among Iberian lineages ($H$ = 0.948 and Pi = 0.693), whereas all C sublineages showed low diversity (sublineage C2 of Candelario was too poorly sampled to draw any conclusion) (Table 1). The lowest values were obtained for sublineage C3 of SW-Huelva ($H$ = 0.306 and Pi = 0.049), followed closely by sublineage C1 of north-central Iberia ($H$ = 0.396 and Pi = 0.063) and sublineage C4 of Guadalquivir River ($H$ = 0.502 and Pi = 0.071).

Very similar patterns were obtained for population groups suggested by SAMOVA, with those of the northwest and centre of the Iberian distribution range of *C. suaveolens* showing moderate or high diversity values (population groups 1–5 in Table 2 and localities 1–9 in Fig. 1), whereas the rest of the Iberian population groups presented low diversity values. Galicia showed the highest values of haplotype diversity ($H$ = 0.910) and Eastern Central System the highest values of nucleotide diversity (Pi = 0.968), the latter reflecting the co-occurrence of highly divergent haplotypes in Candelario (locality 9 in Fig. 1 and Supporting Information, Tables S1, S3). By contrast, *C. suaveolens* populations of the Piedras and Tinto rivers, belonging to the SW-Huelva group, were fixed for a single haplotype each. SW-Huelva was the population group with the lowest values of diversity ($H$ = 0.306 and Pi = 0.049), followed closely by the NC-IP group, with low values for the combined population of País Vasco and Navarra ($H$ = 0.222 and Pi = 0.029).

### HISTORICAL DEMOGRAPHY

No sign of demographic expansion was detected for clade IV or any of the sublineages (Table 1), with the only exception being sublineage C1 in north-central Iberia, which showed significant negative values of Tajima's $D$ and Fu's $F_S$ test, as well as not significant values for both, Fu and Li's test ($F^*$ and $D^*$). Nevertheless, the $R^2$ statistic, one of the most reliable statistical measures for small populations (Ramos-Onsins & Rozas, 2002), was not significant and the BSP did not reveal a clear sign of demographic growth for this sublineage (Supporting Information, Fig. S5). On the other hand, sublineage C3 of SW-Huelva showed a significant $R^2$ value; however, all other calculated statistics were not significant and, as for sublineage C1, the BSP did not show clear evidence of population expansion (Supporting Information, Fig. S5).

## DISCUSSION

### EVOLUTIONARY HISTORY OF *C. SUAVEOLENS* IN IBERIA

Our phylogenetic results revealed that all *C. suaveolens* populations in Iberia belong to clade IV of western Europe (Figs. 2; Supporting Information, Figs S2, S3), refuting an introduction by humans in this area as the origin of southern populations, and revealing a sharp internal phylogeographic substructure.

According to our analysis, the split that separated clade IV of the nucleus comprising clades V–X took place 1.41 Ma (95% confidence interval: 0.71–2.45 Ma; Supporting Information, Fig. S2a), a date similar to that provided by Dubey *et al.* (2006) [1.72 Ma (95% confidence interval: 1.40–2.23 Ma)], and overlapping the Donau glaciation (1.4–1.8 Ma) (Penck & Brückner, 1909). In agreement with this, palaeontological studies estimate the arrival of *C. suaveolens* in Iberia at least 1–1.3 Ma, in the Lower/Middle Pleistocene (Montoya *et al.*, 2001; Rofes & Cuenca-Bescós, 2011). Once established in Iberia, the combination of subsequent glacial cycles, separate refugia and CE by *C. russula* has probably been responsible for further intraclade divergence, as discussed below.

We show here a well-defined internal structure within clade IV of western Europe with three main lineages, two of them occurring exclusively in Iberia (lineages B and C; Figs 1, 2). Lack of a more intensive sampling and the uncertain distribution of the species in France hampered the assessment of the internal structure of lineage A. However, the two Iberian lineages are clearly distributed in different parts of the distribution range and show contrasting internal patterns. Lineage B in the northwest forms a single widespread monophyletic group, concordant with its more continuous distribution in this area, whereas lineage C is divided into several sublineages, each corresponding to separate distribution patches within Iberia. Interestingly, sublineage C2 coexists with lineage B in a single locality (Candelario, Salamanca province; locality 9 in Fig. 1), providing the only instance of secondary contact between divergent lineages within Iberia. Therefore, the phylogeographical discontinuities within Iberia provide support for the 'refugia within refugia' hypothesis (Gómez & Lunt, 2007; Weiss & Ferrand, 2007; Abellán & Svenning, 2014).

Given the similarity of their dating, it is likely that a single event produced the two oldest splits within clade IV (Fig. 2), the split that gave rise to the French lineage A, estimated at 370 ka (95% confidence interval: 160–640 ka), and the split that separated the Iberian lineages B and C, dated at 320 ka (95% confidence interval: 140–570 ka), probably the Mindel glacial period (390–580 ka). During glacial maxima, habitats favourable for temperate species such as *C. suaveolens* would be at lower altitude separated by inhospitable habitats at high altitude zones (Gómez & Lunt, 2007). Therefore, lineage A sheltered in the south of France, a known refuge area described for other temperate species of small mammals (Yannic, Basset & Hausser, 2008; Vega *et al.*, 2010b; Feuda *et al.*, 2015), whereas lineages B and C might have diverged within Iberia in subrefugia separated by the Central Iberian Plateau. This elevated and large plateau could have acted as a barrier for this temperate small mammal because it was dominated by cold and arid steppe landscapes during the Pleistocene glaciations (González-Sampériz *et al.*, 2010). In fact, cold and dry climate limits the current distribution of the species in northern Eurasia (Palomo *et al.*, 2016). A similar phylogeographical divide separate lineages of another Iberian small mammal (*Microtus cabrerae*; Barbosa *et al.*, 2017), and two closely related species, *Microtus lusitanicus* and *Microtus duodecimcostatus* (Jaarola *et al.*, 2004).

The exact location of Iberian subrefugia cannot be directly inferred from our data, but the geographical ranges of these lineages coincide with areas of refuge proposed for other species in Iberia. On the one hand, the distribution range of lineage B coincides with a well-known area of speciation in Iberia, where numerous species of endemic vertebrates occur, including the small mammals *Sorex granarius* and *M. lusitanicus*. Furthermore, northwestern Iberia has been previously proposed as subrefugium for small mammals, such as *Arvicola sapidus* and *Galemys pyrenaicus* (Centeno-Cuadros *et al.*, 2009; Igea *et al.*, 2013), whereas a region in central Portugal has been proposed as one of the main subrefugia for *Microtus agrestis* (Jaarola & Searle, 2004). It is therefore highly likely that these areas had adequate habitats for *C. suaveolens* during Pleistocene glaciations. Unfortunately, our lack of sampling from a potential subrefugium in Portugal may have led us to miss the origin of B clade, as suggested by the absence of a basal central haplotype for the star-like lineage B (Fig. 3). On the other hand, the fragmented range of lineage C suggests either several separate subrefugia in the eastern half of Iberia, as suggested for other small mammals (Jaarola & Searle, 2004; Centeno-Cuadros *et al.*, 2009; Igea *et al.*, 2013), or a single refugium that expanded and was subsequently isolated into at least three separate patches. In this latter scenario, the basal position of haplotype H31 sampled in southwestern Iberia (Fig. 3) suggests the possibility that this region – an important glacial refuge area described for other European mammals (Randi, 2007) – acted as the main eastern refugium for lineage C. In the former scenario, a subsequent climatic event, probably the Riss glacial period (140–200 ka), may have caused a new isolation event resulting in the formation of Iberian sublineages C1, C2, C3 and C4, as well as French sublineages A1 and A2 (Fig. 2). However, fragmentation of these sublineages may have had a non-climatic cause. Thus, according to our own dating (Supporting Information, Fig. S4), approximately at the end of the Riss glacial and beginning of the Riss–Wurm interglacial (80–140 ka), a new and a highly competitive species of *Crocidura*, *C. russula*, colonized Iberia from Africa. Previous studies (Brändli *et al.*, 2005; Cosson *et al.*, 2005) estimated somewhat more recent dates for the arrival of *C. russula* during the Wurm glacial (11–80 ka). Nevertheless, we estimated the arrival date for *C. russula* at 126 ka (41–200 ka) (Supporting Information, Fig. S4), a confidence interval with substantial overlap with the internal diversifications of lineages A and C. Furthermore, *C. russula* seems to be present in the Iberian fossil record since at least 100 ka (Ruiz-Bustos, 1997; Arsuaga *et al.*, 2006; Laplana & Sevilla, 2006; López-García, 2008). Therefore, an intriguing possibility is that the isolation driving the divergence of sublineages A and C was caused by the CE exerted by *C. russula* since its arrival. *Crocidura russula* would have outcompeted *C. suaveolens* in the hotter and drier habitats of Iberia, more similar to habitats in North Africa where *C. russula* evolved (Poitevin *et al.*, 1986; González & Román, 1988). The same seems to be occurring in some areas of sympatry (Niethammer, 1979; Poitevin *et al.*, 1987; Cosson *et al.*, 1996; Libois *et al.*, 1999; Kraft, 2000; Román & Ruiz, 2003; Pascal *et al.*, 2006). In the Mediterranean climate region of Iberia (Supporting Information, Fig. S6), *C. suaveolens* has been able to withstand the pressure of *C. russula* only in relatively wetter habitats (Rey, 2007), such as the marshy areas in the southwest (sublineages C3 and C4; Fig. 1) (Román & Ruiz, 2003) and high-altitude wooded areas in the mountain ranges of the Iberian Central System (sublineage C2 and localities 7, 8 and 9 of lineage B; Fig. 1). On the other hand, the more humid and cold climate in the Atlantic region of Iberia (Supporting Information, Fig. S6) might have favoured *C. suaveolens* in its competition with *C. russula*, allowing the persistence of sublineage C1 in the north-central area as well as a more extensive and continuous distribution of lineage B in the northwest (Fig. 1).

The recent contraction of the Iberian range of *C. suaveolens* is supported by numerous citations of this shrew in Pleistocene fossil deposits located in areas where the species is currently absent (Ruiz-Bustos *et al.*, 1984;

Arribas, 1994; Guillem-Calatayud, 1995a, b, 2000, 2001; Montoya *et al.*, 2001; Barroso Ruiz & Desclaux, 2006; López-García, 2008). Moreover, in other European areas of Mediterranean climate where *C. russula* is absent, *C. suaveolens* appears to have a more continuous distribution and to occupy a wider range of habitats (Palomo *et al.*, 2016). Particularly relevant is the contrasting case of the Italian Peninsula, given its climate similarity with Iberia and its role as providing glacial refugia. Here *C. suaveolens* is distributed continuously throughout the peninsula, including the most Mediterranean and dry habitats (Mortelliti & Boitani, 2009). Furthermore, no phylogeographical substructure was found in a recent study of *Crocidura suaveolens* (Castiglia *et al.*, 2017), with all Italian populations belonging to a single evolutionary lineage of Italo-Balkan origin (clade VII of Dubey *et al.*, 2006, 2007), which expanded around 60–149 kya, coinciding with the arrival of *C. russula* in Iberia. Therefore, in Iberia a similar population expansion from subrefugia may have been impeded, or the resultant range may have subsequently fragmented into isolated patches by CE with *C. russula*, specifically in areas of Mediterranean climate (Supporting Information, Fig. S6), giving rise to the divergence of sublineages within lineages A and C (Fig. 2). Furthermore, a rather long history of small population size and isolation caused by CE can explain the low diversity and high differentiation observed for isolated populations of lineage C when compared to populations of the widespread and more continuous lineage B (Tables 1, 2).

### TAXONOMIC AND CONSERVATION IMPLICATIONS

Despite its wide divergence with respect to other clades of the *C. suaveolens* group, clade IV was classified conservatively as subspecies *C. suaveolens iculisma*, mainly because the degree of reproductive isolation from its neighbouring clade, clade VII of the south and centre of Europe, is unknown (Dubey *et al.*, 2006). Our results reveal several lineages and sublineages within clade IV of *C. suaveolens*, but further analysis is need to show if there is reproductive isolation between them that justifies any taxonomic split within this clade. Analysis in secondary contact areas, such as in Candelario between lineage B and sublineage C2, as well as the use of nuclear markers, could help to clarify the taxonomy in this region. Irrespective of the degree of reproductive isolation, lineages and sublineages of clade IV identified here should be conservatively considered as separate Evolutionary Significant Units for conservation purposes, because of the clade's independent evolutionary history and the potential for local adaptation.

According to the IUCN Red List of Threatened Species, *C. suaveolens* is a species classified as Least Concern (LC) (Palomo *et al.*, 2016), but the species is classified as Data Deficient (DD) in the Spanish Red List (Palomo, Gisbert & Blanco, 2007), and it is Not Evaluated (NE) in Portugal (Cabral *et al.*, 2005). This study thus provides a first approximation to the genetic status of the species in Iberia and encourages further studies of the species in western Europe.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Methods

**Table S1.** *Crocidura suaveolens* samples collected for this study.

**Table S2.** Primers used for amplification of the cytochrome *b* gene.

**Table S3.** *Crocidura suaveolens* cytochrome *b* sequences downloaded from GenBank.

**Table S4.** Calibration constraints, in Myr, used as priors in the BEAST2 analysis of cytochrome *b* of soricids.

**Table S5.** Populations of occurrence and frequencies of haplotypes found within clade IV of the *Crocidura suaveolens* group.

**Table S6.** Matrix of population pairwise comparisons for Iberian populations of *Crocidura suaveolens.*

**Figure S1.** Bayesian relaxed clock tree reconstructed with cytochrome *b* sequences of soricids.

**Figure S2.** (a) Bayesian phylogenetic tree of the *Crocidura suaveolens* group. (b) Amplified view of clade IV subtree.

**Figure S3.** (a) Maximum likelihood phylogenetic tree of the *Crocidura suaveolens* group. (b) Amplified view of clade IV subtree.
**Figure S4.** Dated tree of *Crocidura russula.*
**Figure S5.** Bayesian skyline plots showing demographic histories of the different lineages/sublineages identified within clade IV.
**Figure S6.** Distributions of *Crocidura suaveolens* and *Crocidura russula* in different European biogeographical regions in western Europe.