

# Red squirrels from south–east Iberia: low genetic diversity at the southernmost species distribution limit

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## Abstract

*Red squirrels from southeast Iberia: low genetic diversity at the southernmost species distribution limit.*— South–east Iberia is the southernmost limit of this species in Europe. Squirrels in the region mainly inhabit coniferous forests of *Pinus*. In this study, we analyzed the pattern of mitochondrial genetic variation of southern Iberian red squirrels. Fragments of two mitochondrial genes, a 350–base pair of the displacement loop (D–loop) and a 359–bp of the cytochrome b (Cytb), were sequenced using samples collected from 88 road–kill squirrels. The genetic variation was low, possibly explained by a recent bottleneck due to historical over–exploitation of forest resources. Habitat loss and fragmentation caused by deforestation and geographic isolation may explain the strong genetic subdivision between the study regions. Six new haplotypes for the D–loop and two new haplotypes for the Cytb fragments are described. A Cytb haplotype of south–east Iberia was found to be present in Albania and Japan, suggesting local extinction of this haplotype in intermediate areas. No significant clustering was found for the south–east of Spain or for the other European populations (except Calabria) in the phylogenetic analysis.

Key words: *Sciurus vulgaris*, Mitochondrial DNA, Genetic diversity, Population bottleneck

## Resumen

*Ardillas rojas del sureste ibérico: baja diversidad genética en el límite austral de la distribución de la especie.*— El sureste ibérico es el límite más austral de la distribución de esta especie en Europa, donde las ardillas habitan principalmente en bosques de *Pinus*. En este estudio, se investigó el patrón de variación genética mitocondrial de las ardillas rojas del sureste ibérico. Se secuenciaron fragmentos de dos genes mitocondriales, 350 pares de bases de la región control (D–loop) y 359 pb del citocromo b (Cytb) utilizando muestras obtenidas a partir de 88 ardillas atropelladas. Se encontró una baja variación genética, lo cual podría explicarse por la existencia de un cuello de botella reciente causado por la sobreexplotación histórica de los recursos madereros de la zona. La pérdida y fragmentación del hábitat debidas a la deforestación y al aislamiento geográfico podrían explicar la fuerte subdivisión genética observada entre las regiones del estudio. Se describen seis nuevos haplotipos para el fragmento D–loop y dos para el Cytb. Un haplotipo encontrado en el sureste ibérico para el Cytb se observó también en Albania y Japón, lo que sugiere una extinción local de este haplotipo en áreas intermedias. En los análisis filogenéticos, no se detectó un agrupamiento significativo de las ardillas del sureste ibérico, ni de ninguna otra población europea (excepto en Calabria).

Palabras clave: *Sciurus vulgaris*, ADN mitocondrial, Diversidad genética, Cuello de botella poblacional

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## Introduction

The red squirrel (*Sciurus vulgaris* Linnaeus, 1758) is widely distributed from Iberia in the west across the Palaearctic to the island of Hokkaido (Japan), and from the UK, Ireland, Scandinavia and Siberia to the Mediterranean (Corbet, 1978; Lee & Fukuda, 1999; Lurz et al., 2005). In the Iberian Peninsula, this native sciurid is continuously distributed from Girona to Galicia and the Northern Iberian Mountain Range, the Northern Plateau and the Central Mountain Range, and southwards to Valencia. It is discontinuously distributed from Cataluña to Andalucía, and widely spread in the Baetic Mountain Ranges, including Murcia, Albacete and Alicante (Valverde, 1967; Purroy, 2014). As the result of recent reintroductions, the species can also be found in central and north Portugal (Mathias & Gurnell, 1998; Ferreira et al., 2001; Ferreira & Guerreiro, 2002) (fig. 1A). Most Iberian squirrels occupy pure pine forest: *Pinus halepensis* in the lower altitudes, *P. pinaster* and *P. nigra* in middle levels, and *P. mugo* in the higher locations (Valverde, 1967). In south-east Iberia, the most common species of pine is *P. halepensis* (Aleppo pine), however, even at relatively medium/high altitudes. This is especially evident in the region of Murcia where red squirrels are found in urban parks and adjacent copses, in small to large villages, and even in cities where Aleppo pine can be found. In these localities, they have even been seen feeding on date palms (pers. obs.).

The species is extremely variable in color. Considerable regional variation is superimposed on a striking polymorphism and equally striking seasonal differences (Corbet, 1978). Many studies of the morphological diversity of Spanish squirrels have been made in the past century, especially in the early nineteenth hundreds (Cabrera, 1905; Miller, 1907, 1909, 1912), which led to an intense taxonomical discussion. More recently, the first researcher to provide new material morphological variation was Valverde (1967). He assigned his samples to four previously described subspecies (*S. v. alpinus* Desmarest, 1822, *S. v. numantius* Miller, 1907, *S. v. infuscatus* Cabrera, 1905 and *S. v. segurae* Miller, 1912) and suggested the existence of a new subspecies, which he named *S. v. hoffmanni* Valverde, 1967 from Sierra Espuña (southeast Spain). However, subsequent authors considered that only two subspecies are present in Iberia: *S. v. fuscoater* Altum, 1876 and *S. v. infuscatus* (Corbet, 1978; Lurz et al., 2005; Sidorowicz, 1971) (fig. 1B).

Valverde (1967) emphasized the importance of the *hoffmanni* subspecies because of its ecological and morphological features. These squirrels represent the southeastern limit of the Iberian distribution of the species in the xerothermic forest-margin of the Iberian Peninsula, where it lives in pure Aleppo pine forest. Moreover, *S. v. hoffmanni* would be the largest of the European red squirrels, with the palest fur. Thus, this form should represent the ecological limit and the most extreme phenotype of Iberian squirrels (Valverde, 1967). According to the author, *S. v. hoffmanni* is restricted to the Regional Park of Sierra Espuña, but currently the *hoffmanni* phenotype can be

easily observed in the Regional Park of Carrascoy–El Valle further south of this region, separated from Espuña by the Guadalentín River. The Regional Park of Sierra Espuña is about 80 km east of the Natural Park of Sierra de Cazorla, Segura y Las Villas and they are connected by a northwestern green corridor (Special Protected Area of Sierra de Burete, Lavia y Cambrón, and the Northwestern Mountains of Murcia). The Natural Park of Sierra de Cazorla, Segura y Las Villas is the largest protected area in Spain, 214,300 ha, and it was designated by UNESCO as a Biosphere Reserve in 1983. There are good-sized red squirrel populations in this area, and they are still under taxonomic discussion (*S. v. baeticus* Cabrera, 1905 = *S. v. segurae* = *S. v. infuscatus*).

Beyond the taxonomical discussion, no recent studies of the ecological characteristics of squirrels from southeast Iberia have been published. Only one study has investigated the genetics of some Iberian populations (Lucas & Galián, 2009), and it found extremely low genetic variation in the population of the Regional Park of Sierra Espuña.

We investigated whether the low genetic diversity found in Sierra Espuña can be considered a pattern in Southeast Iberia or whether it is a peculiarity of this population. In order to study the relationships between the southeastern Iberian squirrels and the other European populations, we compared our results with those in the literature. To achieve these objectives two mitochondrial gene fragments (D-Loop and Cytb) were analyzed using samples from road-kill animals.

## Material and methods

### Sample collection

In southeastern Spain, most natural areas are crossed by roads and frequented by a large number of visitors. As found in other European populations (Shuttleworth, 2010), road-kill squirrels are frequent in the study area both in natural and suburban environments.

The study area was divided into five regions according to geographical and ecological barriers or distance between samples clusters (fig. 2). Samples from CSV and ESP were collected from the reported distribution of the *segurae* and *hoffmanni* subspecies. All of the samples comprised approximately 2 mm<sup>2</sup> of muscle tissue and were preserved in absolute ethanol, then stored at –20°C until DNA purification.

### DNA extraction and sequencing

Total genomic DNA was extracted from tissue samples using a Qiagen DNAeasy Tissue Kit, according to the manufacturer's protocol. A total of 754–bp were amplified from two gene regions of the mitochondrial DNA. A 395–bp fragment of the D-loop was amplified in 12.5–µl reactions, following the protocol described by Hale et al., 2004, using 1 µl of tissue DNA and the red squirrel-specific primers, H16359 (Barratt et al., 1999) and RScnt6 (Hale et al., 2004). A 359–bp region of the Cytb was amplified using the same pro-

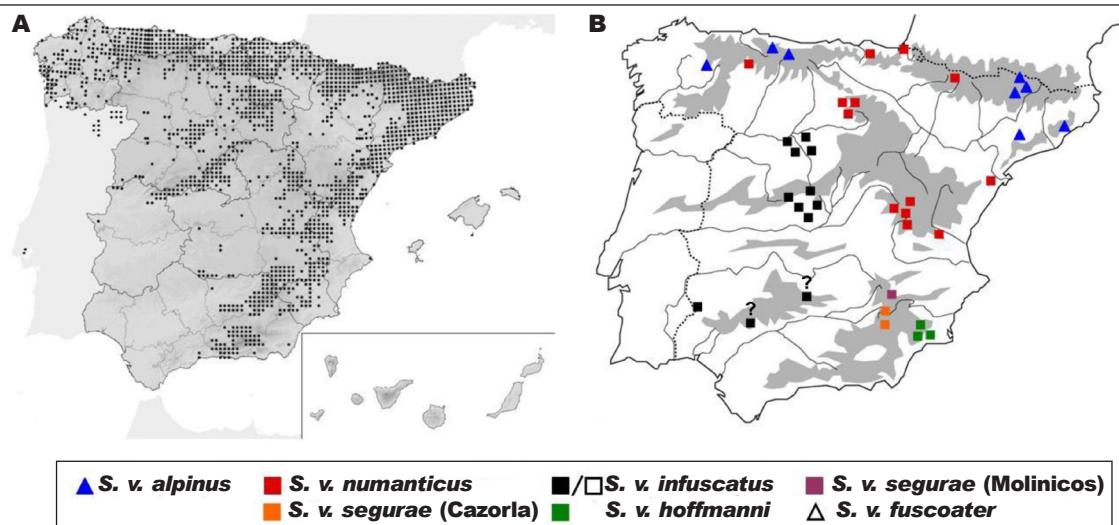


Fig. 1. Map of the species distribution in the Iberian peninsula (A) modified from Palomo & Gisbert (2002). Geographic distribution of red squirrel subspecies (B), obtained from Valverde (1967) and Mathias & Gurnell (1998). Square shape ( $\square$ ) and triangle shape ( $\triangle$ ) refer to the subspecies *infuscatus* and *fuscoater*, respectively, as synonymised in more recent studies (Sidorowicz, 1971; Corbet, 1978; Lurz et al., 2005).

Fig. 1. Mapa de distribución de la especie en la península ibérica (A), modificado de Palomo & Gisbert (2002). Distribución geográfica de las subespecies de ardilla roja (B), a partir de la información de Valverde (1967) y Mathias & Gurnell (1998). Los cuadrados ( $\square$ ) y los triángulos ( $\triangle$ ) hacen referencia a las subespecies *infuscatus* y *fuscoater* respectivamente, sinonimizadas en trabajos más recientes (Sidorowicz, 1971; Corbet, 1978; Lurz et al., 2005).

tol, except that we used the primers SV14226F and SV14647R from Grill et al. (2009). Negative (sterile water) and positive (known squirrel DNA) controls were always used and the products were visualized on 2% agarose gels alongside a 100-bp size standard to determine the success of the amplification. The PCR products were sequenced in both forward and reverse directions for each sample by MacroGen Inc., Korea.

#### Data analysis

Consensus sequences for each individual were obtained by aligning the forward and reverse complementary sequences of each gene (D-loop and Cytb) with Geneious 4.8.3. D-loop sequences were aligned in MUSCLE (Edgar, 2004) and Cytb sequences with ClustalW algorithm (Larkin et al., 2007). The haplotypes were identified with TCS 1.21 (Clement et al., 2000) and compared with those available in the GenBank using BLAST (Altschul et al., 1990). The relative frequencies of the Cytb and D-loop haplotypes were calculated with Arlequin 3.1.2.3 (Excoffier & Lischer, 2010). Haplotype diversity was calculated separately for each gene. Due to the low diversity found in Cytb sequences, both genes were combined to investigate the nucleotide diversity. The molecular diversity indices were determined using DnaSP 5 (Librado & Rozas, 2009).

The pairwise genetic distances between regions, which were measured as  $F_{ST}$ , were calculated from

a distance matrix of D-loop haplotypes based on the Tamura–Nei model (Tamura & Nei, 1993) in Arlequin 3.1.2.3 (Excoffier & Lischer, 2010).

The genealogical relationships between the D-loop haplotypes of southeast Iberia were assessed by constructing a median-joining network in NETWORK 4.6.1 (Bandelt et al., 1999). Haplotype networks including sequences from the GenBank were also calculated for both genes.

Phylogenetic analyses were conducted in MEGA6 (Tamura et al., 2013) using the maximum likelihood (ML) method with the nearest neighbour interchange algorithm. Nucleotide sequences of red squirrels from other European populations (Hale et al., 2004; Grill et al., 2009; Dozières et al., 2012) and of the Japan squirrel *Sciurus lis* (Oshida & Masuda, 2000) were downloaded from GenBank and aligned with our data set. These sequences showed a 100% overlap with the sequences we analysed. The model of nucleotide substitution that best fitted the data set was determined with MEGA6 (Tamura et al., 2013). The stability of the ML tree topologies were tested using 1,000 bootstrap replicates.

#### Results

A total of 88 samples from the five regions were genotyped successfully. Twenty of the samples from ESP were used in previous work (Lucas & Galián,

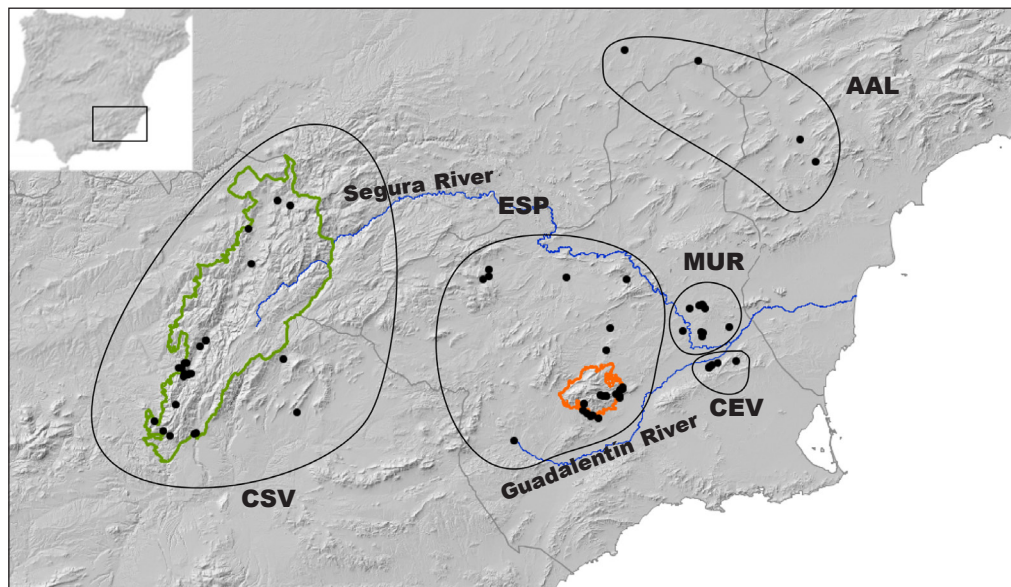


Fig. 2. Map of the study area. The black dots represent *Sciurus vulgaris* specimens. The green line marks the area of the Natural Park of Sierra de Cazorla, Segura y Las Villas, and the orange line delimits the area of the Natural Park of Sierra Espuña. The five regions in the study area are bounded by black lines: CSV. Natural Park of Sierra de Cazorla, Segura y Las Villas and surroundings; ESP. Regional Park of Sierra Espuña and surroundings; MUR. Copses and periurban parks near the city of Murcia; CEV. Regional Park of Carrascoy–El Valle; AAL. Albacete and Alicante.

Fig. 2. Mapa del área de estudio. Los puntos negros representan los individuos de *Sciurus vulgaris*. La línea verde indica el límite del Parque Natural de Sierra de Cazorla, Segura y Las Villas, y la línea naranja delimita el área del Parque Regional de Sierra Espuña. Las líneas negras definen las cinco regiones en las que se divide el área de estudio: CSV. Parque Natural de Sierra de Cazorla, Segura y Las Villas y alrededores; ESP. Parque Regional de Sierra Espuña y alrededores; MUR. Bosquetes y parques periurbanos próximos a la ciudad de Murcia; CEV. Parque Regional de Carrasco y El Valle; AAL. Albacete y Alicante.

2009). Fragments of the D-loop and Cytb (395–bp and 359–bp respectively) were obtained for each sample. As we found that a tRNA was present within the nucleotide spans of the D-loop fragment, they were trimmed to 350–bp to adjust the sequence length to the target gene. Aligned sequence data were submitted to the GenBank database with accession numbers KJ146734–KJ146742. We found a total of six D-loop haplotypes which have never been reported, and a total of three Cytb haplotypes, two of which were also found to be exclusive to the south east of Spain (SvCb2 and SvCb3). SvCb1 was identical to haplotypes previously found in Albania (Grill et al., 2009) and Japan (Oshida et al., 2009).

Three of the six haplotypes identified for the D-loop were found in CSV and two were present throughout the whole study area. One of the three Cytb haplotypes was exclusive to CSV but the others were present in more than one region (table 1).

The concatenated alignment was 709–bp long and contained eight variable positions. These sequences were collapsed into seven haplotypes. The nucleotide ( $\pi$ ) diversity of the concatenated sequence was zero in CEV, low in the ESP region, intermediate in MUR

and AAL, and higher in CSV (table 2). The haplotype diversity ( $H_d$ ) of the two genes varied in the same way when treated separately, although it was lower in the case of the Cytb. Genetic differentiation between regions was high in almost all cases (table 3).

In the haplotype network (fig. 3), three haplotypes were placed as external nodes, two belonging to CSV (one of them unique to this region) and one exclusive to AAL. The two most common haplotypes (SvCR1 and SvCR2) were both placed as internal nodes, as was haplotype SvCR4. This haplotype was exclusive to CSV and located in the center of the network, also being connected to SvCR3 (exclusive to AAL).

Haplotype SvCb1 was placed in the center of the Cytb network (data not shown). The SvCb2 and SvCb3 haplotypes were directly connected to this and both differed in two nucleotide positions. Haplotype networks using sequences from the GenBank (data not shown) did not show any grouping by geographic region. In the Cytb network, the only haplotype that showed a clear differentiation was that found in Calabria by Grill et al. (2009).

A phylogenetic analysis was conducted for the D-loop haplotypes, including haplotypes from Hale

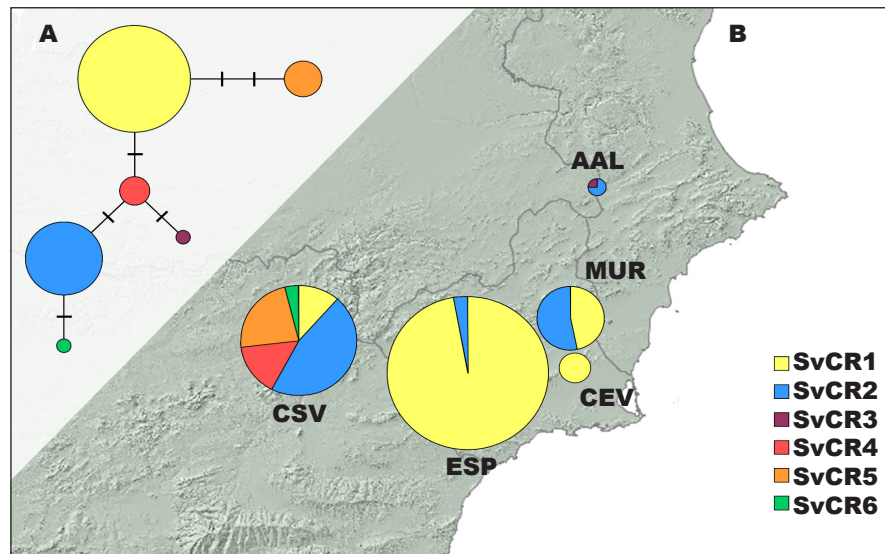


Fig. 3. Median-joining network of the six new D-loop haplotypes (A) and their spatial distribution (B): A. The circles (nodes) in the network represent the haplotypes and the areas of the circles are proportional to the number of samples for each haplotype. The perpendicular short black lines represent mutations; B. Each pie in the distribution map represents the proportion of haplotypes in each region and the size of the pie is proportional to the number of individuals.

Fig. 3. Red haplotípica (basada en el algoritmo de unión de medianas (median-joining) para los seis nuevos haplotipos del fragmento D-loop (A) y distribución espacial de los mismos (B): A. En la red haplotípica, los círculos (nodos) representan los haplotipos y las áreas son proporcionales al número de muestras de cada haplotipo; B. En el mapa de distribución, cada gráfica representa la proporción de haplotipos en cada región y su tamaño es proporcional al número de muestras.

et al. (2004), Grill et al. (2009) and Dozières et al. (2012). A 252-bp alignment was generated. The tree with the highest log likelihood (-762.4389) was obtained in the maximum likelihood analysis of the

D-loop haplotypes (fig. 4A). This phylogenetic tree was conducted under the Hasegawa-Kishino-Yano (HKY85) model (Hasegawa et al., 1985) with rate heterogeneity among sites (gamma distribution shape

Table 1. Haplotype frequencies in the five regions and the overall study area. (For abbreviations see figure 2; SE Spain refers to the overall study samples.)

Tabla 1. Frecuencias haplotípicas en las cinco regiones y en toda el área de estudio. (Para las abreviaturas, véase la figura 2; SE Spain se refiere al total de muestras.)

Haplotype	ESP	MUR	CEV	AAL	CSV	SE Spain
SvCb1	0.972	0.400	1	0.250	0.654	0.761
SvCb2	0.023	0.600	0	0.750	0.192	0.193
SvCb3	0	0	0	0	0.154	0.045
SvCR1	0.972	0.467	1	0	0.115	0.591
SvCR2	0.028	0.533	0	0.750	0.462	0.273
SvCR3	0	0	0	0.250	0	0.011
SvCR4	0	0	0	0	0.154	0.045
SvCR5	0	0	0	0	0.231	0.068
SvCR6	0	0	0	0	0.038	0.011

Table 2. Summary of the diversity indices: N. Number of sequences/individuals;  $\pi$  Nucleotide diversity with standard deviation; h. Number of haplotypes; Hd. Haplotype diversity with standard deviation. (For other abbreviations see figure 2; SE Spain refers to the overall study samples.)

Tabla 2. Resumen de los índices de diversidad: N. Número de secuencias/individuos;  $\pi$ . Diversidad nucleotídica con desviación estándar; h. Número de haplotipos; Hd. Diversidad haplotípica con desviación estándar. (Para las otras abreviaturas, véase la figura 2; SE Spain se refiere al total de muestras.)

	N	$\pi$	$h_{D-loop}$	$h_{Cytb}$	$h_{Combined}$	$Hd_{D-loop}$	$Hd_{Cytb}$	$Hd_{Combined}$
CSV	26	0.00339±0.00030	5	3	6	0.723±0.064	0.532±0.092	0.831±0.032
ESP	36	0.00024±0.00022	2	2	2	0.056±0.052	0.056±0.052	0.056±0.052
MUR	15	0.00226±0.00022	2	2	2	0.533±0.052	0.533±0.052	0.533±0.052
CEV	7	0.00000	1	1	1	0.000	0.000	0.000
AAL	4	0.00212±0.00112	2	2	2	0.500±0.265	0.500±0.265	0.500±0.265
SE Spain	88	0.00222±0.00020	6	3	7	0.576±0.044	0.385±0.055	0.607±0.050

parameter of 0.17). No significant clustering of the haplotypes was found for the southeast of Spain or for the rest of the European populations.

A second analysis was performed for the combined data set, that included nine haplotypes from other European populations (Grill et al., 2009). A 611-bp alignment was generated. The maximum likelihood tree of the combined mtDNA sequences (log likelihood of -1,306.7513) was inferred based on the Tamura 3-parameter model (Tamura, 1992) with invariant sites (fig. 4B). The phylogeny showed a clear differentiation for the Calabrian lineage but not for the rest of the sample. The same result was observed by analyzing the Cytb haplotypes (data not shown). Sequences of *S. lris* were always rooted in the phylogenetic trees.

## Discussion

Capture and manipulation of living red squirrels may imply a high risk for their health, such as heart attack or dorsal spin fracture (Josep Piqué, pers. comm.).

Collecting tissue samples from road-kill squirrels avoids such risk and has been proven a suitable source of quality DNA for molecular studies (Lucas & Galián, 2009; Dozières et al., 2012). However, this kind of sampling does not allow the development of a sampling plan where regions are equally represented. In southeast Spain, this disadvantage can be partially compensated for by the abundance of road-kill animals in rural and suburban areas.

In this study, we found a level of genetic diversity similar to that reported for Spain by Hale et al. (2004) and Grill et al. (2009). However, the extremely low genetic diversity of ESP, described by Lucas & Galián (2009), is the most striking result in this study. This contrasts sharply with the relatively high genetic variation found in CSV, despite its ecological connectivity with ESP.

Anthropogenic effects such as farming or direct human exploitation have decreased the distribution ranges and population sizes of many species in the Iberian peninsula (Gómez & Lunt, 2007). In southeastern Spain, the area occupied by ESP and CSV suffered

Table 3.  $F_{ST}$  values between pairs of regions (below diagonal) and  $P$ -values computed based on 1,000 permutations (upper diagonal): \* $P < 0.05$ , \*\* $P < 0.001$ . (For abbreviations see figure 2.)

Tabla 3. Valores de  $F_{ST}$  entre pares de regiones (diagonal inferior) y valores de  $P$  calculados a partir de 1.000 permutaciones (diagonal superior): \* $P < 0,05$ ; \*\* $P < 0,001$ . (Para las abreviaturas, véase la figura 2.)

	ESP	MUR	CEV	AAL	CSV
ESP	–	–	0.99902 ± 0.0002	–	–
MUR	0.56208**	–	–	0.24805 ± 0.0161	0.19629 ± 0.0111
CEV	-0.07417	0.39655*	–	–	–
AAL	0.89285**	0.13125	0.82554*	–	0.23828 ± 0.0161
CSV	0.45766**	0.02575	0.30762*	0.05825	–

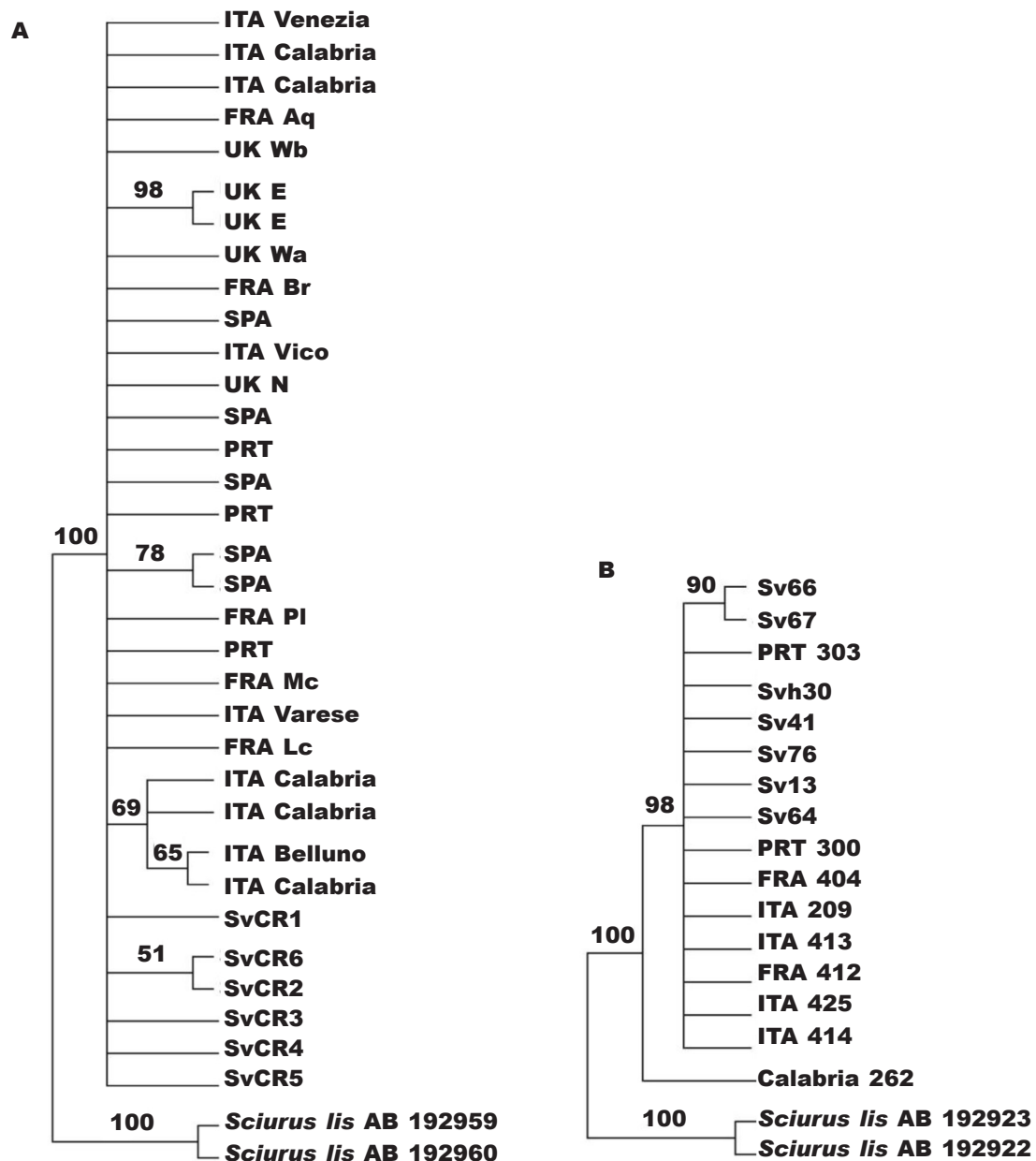


Fig. 4. Condensed maximum-likelihood trees of the D-loop fragment (A) and the combined D-loop and Cyb sequences (B). Branches with less than 50% of bootstrap (1,000 replicates) are collapsed in both trees. The ISO 3166 code is used to designate the country of each sample taken from the literature: A. Taxon labels refer to the D-loop haplotypes from this study (SvCR#) and from other European populations (Hale et al., 2004; Grill et al., 2009); all the French sequences are obtained from Dozières et al. (2012); B. Labels indicate the sample ID of individuals with different combined haplotypes (Sv##, SvH##) and the specimen numbers from Grill et al. (2009). GenBank accession numbers of the outgroups are indicated in the trees.

Fig. 4. Árboles condensados de máxima verosimilitud para el fragmento del D-loop (A) y para las secuencias concatenadas del D-loop y el Cytb (B). Las ramas presentes en menos del 50% de las 1.000 réplicas obtenidas por muestreo con reemplazo (bootstrap) se han condensado en ambos árboles. Se usa el código ISO 3166 para designar el país de procedencia de cada una de las muestras tomadas de la bibliografía: A. Los nombres de los taxones hacen referencia a los haplotipos del D-loop de este estudio (SvCR#) y a aquellos procedentes de otras poblaciones europeas (Hale et al., 2004; Grill et al., 2009); todas las muestras recogidas en Francia se han obtenido de Dozières et al. (2012); B. Los nombres de los taxones indican el código de muestra de individuos con distintos haplotipos de secuencias concatenadas (Sv##, SvH##) y el número del espécimen en Grill et al. (2009). Se indican los números de acceso al GenBank de los grupos externos en ambos árboles.

strong deforestation caused by over-exploitation of forest resources in the 18<sup>th</sup> and 19<sup>th</sup> centuries (Valverde, 1967; Araque, 2013). As of the second half of the 19<sup>th</sup> century, reforestation works have been carried out (Codorniu, 1900; González-Pellejero & Álvarez, 2004), helping to preserve red squirrel populations in this area (Valverde, 1967) to date.

As expected given the previous scenario, the proportion of suitable habitats in the landscape decreased critically, increasing the degree of isolation with increasing habitat fragmentation. This situation may have led to a temporary decline in the local squirrel population, which reduced gene flow (Merriam & Wegner, 1992; Andren & Delin, 1994; Wauters et al., 1994; Amos & Harwood, 1998; Wauters et al., 2010). Therefore, the low genetic variation found in southeast Iberia may be the result of a severe bottleneck, similar to that reported by Trizio et al. (2005) for Alpine squirrels. However, whereas Trizio et al. (2005) found high haplotype diversity but low nucleotide diversity, we found low genetic variation at both levels. This situation contrasts strongly with the high genetic variation found by Gallego & Galián (2008) for the other Pine-specific species *Tomiscus destruens* in the Regional Park of Sierra Espuña.

As in other European populations (Hale et al., 2004; Finnegan et al., 2008; Dozières et al., 2014), we found substantial genetic subdivision between regions (table 3). Habitat loss and fragmentation due to anthropogenic effects and geographical barriers may explain these results. For CEV, where SvCR1 was the only haplotype found, the high haplotype fixation may be explained by the geographical isolation caused by the Guadalentín River or by introduction of animals from other sources such as the Sierra Espuña Regional Park. The strong fixation found in CEV and AAL might also be due to low sample size, which can lead to an overestimation of the  $F_{ST}$  values.

Valverde (1967) emphasized the differentiation of the *hoffmanni* subspecies in Sierra Espuña and its differentiation from the populations of Sierra de Cazorla, Segura y Las Villas (*S. v. segurae*) and the rest of the Iberian Peninsula. This classification was achieved using morphological traits and fur colour. Nevertheless, we found no pattern of genetic variation to support this subspecific classification.

Since the internal nodes of haplotype networks are considered as ancestral and the external nodes as more recent status (Castelloe & Templeton, 1994; Templeton, 1998), and since a reduction in population size results in an accelerated increase in genetic distance in the early generations (Chakraborty & Nei, 1974; Nei, 1976; Takezaki & Nei, 1996), our results may be explained by a scenario where widely distributed ancestral haplotypes became extinct due to a bottleneck events. Thus, haplotype SvCR4 occupying the central node of the D-loop network, but in a very low frequency, is a candidate to be considered an ancestral widely distributed haplotype that became extinct in all areas but CSV, especially in ESP which is the region with the largest sample size.

The finding of a Cytb haplotype (SvCb1) that was previously described in Albania and Japan but not in other Eurasian population suggests an ancestral

wide distribution of this haplotype, followed by local extinction in intermediate areas.

Iberia and Italy have been reported as potential glacial refuges for the red squirrel (Hale et al., 2004; Finnegan et al., 2008; Grill et al., 2009; Dozières et al., 2012) and our results confirm that Iberian samples do not show the expected high levels of genetic diversity (Hewitt, 1996; Taberlet et al., 1998). This finding would be supported by a paper by Dozières et al. (2012) that suggested a postglacial recolonization of Europe from Asia or from the Balkans or, alternatively, a series of recent bottlenecks that reduced the genetic diversity in the Iberian and Italian populations. The finding of haplotype SvCb1 in Iberia, the Balkans and Japan favours the hypothesis of the Iberian Peninsula acting as a glacial refuge. Besides, the low genetic variation found may be explained by the recent bottleneck in these populations.

In contrast with the report by Grill et al. (2009) and Dozières et al. (2012), we found no significant clustering for the squirrels of Calabria in the phylogenetic analysis of the D-loop haplotypes (fig. 4A). However, these individuals were clearly differentiated in the remaining the phylogenetic trees (fig. 4B). Nevertheless, the results of the phylogenetic analysis are largely dependent on the sequence length (number of informative sites) and the number of individuals analysed. Thus, this could be an explanation of the lack of clustering found in this work for the Calabrian squirrels (fig. 4A). None of the squirrels in Spain were separated in these analyses, suggesting that Iberian squirrels have not been isolated from the rest of the European populations, as found by Dozières et al. (2012) for French squirrels. Nonetheless, Grill et al. (2009) emphasised the clear separation of the Iberian squirrels, based on the analysis of eight microsatellite loci. We noticed that the squirrels from ESP did not form a monophyletic clade in the phylogenetic analyses, in contrast with what we found in previous work (Lucas & Galián, 2009). The inclusion of samples from nearby populations (CSV, CEV, ALL and MUR) shows that, in fact, the population of Sierra Espuña is very close to other Iberian populations.

A more extensive study should be carried out to understand the phylogenetic and demographic relationships between the Iberian populations, not only at a mitochondrial level, but also at a nuclear level. The recent development of next-generation sequencing methods offers a wide potential for obtaining complete genomes, allowing more accurate research into the evolutionary relationships at an intraspecific level (McCormack et al., 2013).

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