

Full Length Research Paper

## RAPD-PCR molecular analysis of the threatened Cabrera's vole populations in the Iberian Peninsula

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**Optimal management and conservation programs of the threatened Cabrera's vole require investigating potential molecular genetic markers in the genomic background, if the few remaining fragile populations are to be conserved. A collection of 30 Cabrera's vole representing four populations in Spain and Portugal was characterized by 134 RAPD-PCR markers. Molecular analyses did not detect low level of the genetic diversity or population bottlenecks in all studied populations, in discordance with the expectation of low level of viability of the Cabrera's vole. The results described Cabrera's vole populations as a single genetic unit with slightly restricted gene flow. Phylogenetic reconstruction suggested genetic differentiation between northern and southern Cabrera's vole populations, with the basal branches formed by the southern populations, which may be an evidence of the southern origin of Iberian vole ancestral population. To our knowledge, this is the first study on the genetic diversity of *Microtus cabreræ*, which may have further application for the conservation programs of this threatened species of Iberian vole.**

**Key words:** *Microtus cabreræ*, RAPD-PCR, Spain, Portugal, gene flow, genetic diversity, bottleneck, conservation.

### INTRODUCTION

Development of an adequate strategy for conservation of rare and endangered species is based on knowledge of their current genetic and ecological state. This information is insufficient for many animal species included in the Red List (<http://www.iucnredlist.org/details/13418/0>). The Iberian vole *Microtus cabreræ* Thomas 1906 is among such species (Soriguer and Amat, 1988; Feliu et al., 1991). The near threatened Iberian vole is only found in the

Iberian Peninsula (Spain and Portugal) (Blanco and Gonzalez, 1992; Cabral et al., 2005) and is currently listed under the European Community Habitats Directive (92/43/EEC) and the Berne Convention (82/72/CEE). This species' habitat requirement is very specific and it is always found in small populations in habitat patches that require protection and management if the remaining fragile populations are to be conserved (Primack, 1993). *M. cabreræ* is very difficult to monitor in the wild and

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**Table 1.** Species, countries, localities, and number of animals of the studied Cabrera's vole and out group samples

Species	Country	Locality	Number of animal
<i>M. cabrerae</i>	Spain	Valdemorillo de la Sierra (Cuenca)	4
		Paterna de Madera (Albacete)	18
		Siles (Jaen)	4
<i>M. agrestis</i>	Portugal	Bicos (Odemira)	4
	Spain	Navarra	1
<i>M. duodecimcostatus</i>	Spain	Granada	1
<i>Chionomys nivalis</i>	Spain	Sierra Nevada	1

hence conventional approaches such as trapping or photography are mostly inefficient. Studies of this threatened species are still needed if effective conservation efforts are to be implemented to identify the key factors that are currently subjecting populations at risk (Gilpen and Soulé, 1986). Alassad and his colleagues conducted molecular investigation on *M. cabrerae*, however according to our knowledge no molecular study was reported on the genetic diversity of this elusive species (Alasaad et al., 2010, 2011b, 2012). Due to its reduced population size and its fragmented habitat in the Iberian Peninsula, together with the phylopatric behavior of this vole species, it was expected that the persisting populations have high genetic differentiation and absence of gene flow (Blanco and Gonzalez, 1992; Cabral et al., 2005).

RAPD-PCR technique has found a wide range of application in many biological areas because of the simplicity and low cost (Hadrys et al., 1992). This technique relies on neutral polymorphic markers throughout the genome, which can be revealed easily with agarose/acrylamide gel, without the need of the time-consuming and labor-intensive nucleotide sequencing (Welsh and McClelland, 1990; Grosberg, 1996; Curtsinger et al., 1998). This technique has been proven useful to study the genetic diversity of many rodent species (Spiridonova et al., 2004, 2005; Atopkin et al., 2007).

The aim of the present study was to apply RAPD-PCR markers: (i) to explore, for the first time, the genetic diversity of the threatened Cabrera's vole throughout its distribution in the Iberian Peninsula, (ii) to test for the extent of genetic separation and absence of gene flow between its populations, and (iii) to reveal the possible geographical origin of the current populations.

## MATERIALS AND METHODS

### Sample collection and DNA extraction

Thirty (30) tissue samples of *M. cabrerae* were randomly collected from different locations in Spain (18 from Albacete, 4 from Cuenca and 4 from Jaén) and Portugal (4 from Bicos), between 2007 and 2009 (Table 1 and Figure 1), and 3 isolated DNA samples of the snow vole (*Chionomys nivalis*), the field vole (*Microtus agrestis*) and

the Mediterranean pine vole (*M. duodecimcostatus*) from Spain were used as out-groups. The DNA was extracted following the standard phenol/chloroform procedures (Sambrook et al., 1989). Two blanks (reagents only) were included in each extraction to monitor for contamination. Extract concentrations were measured with Nanodrop® (ND-1000 Spectrophotometer).

### RAPD-PCR amplification

The 20 µl PCR reaction mixture contained 10 ng of genomic DNA, 0.5 µM RAPD-primer (for primer names and sequences (Table 2), 0.2 mM of each dNTP, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1.5 mM MgCl<sub>2</sub> and 1 U/reaction Taq polymerase (Sibenzyme). Samples were subjected to the following thermal profile for amplification in a UNOII Thermocycler (Germany): 2 min at 94°C (initial denaturation), followed by 40 cycles of four steps of 45 s at 92°C (denaturation), 30 s at 37°C (first annealing), 15 s at 45°C (second annealing) and 2 min at 72°C (extension), before a final elongation of 10 min at 72°C. PCR blanks (reagents only) were included with each PCR run. RAPD-PCR products were subjected to electrophoresis in 3% agarose gels stained with ethidium bromide (5 mg/ml).

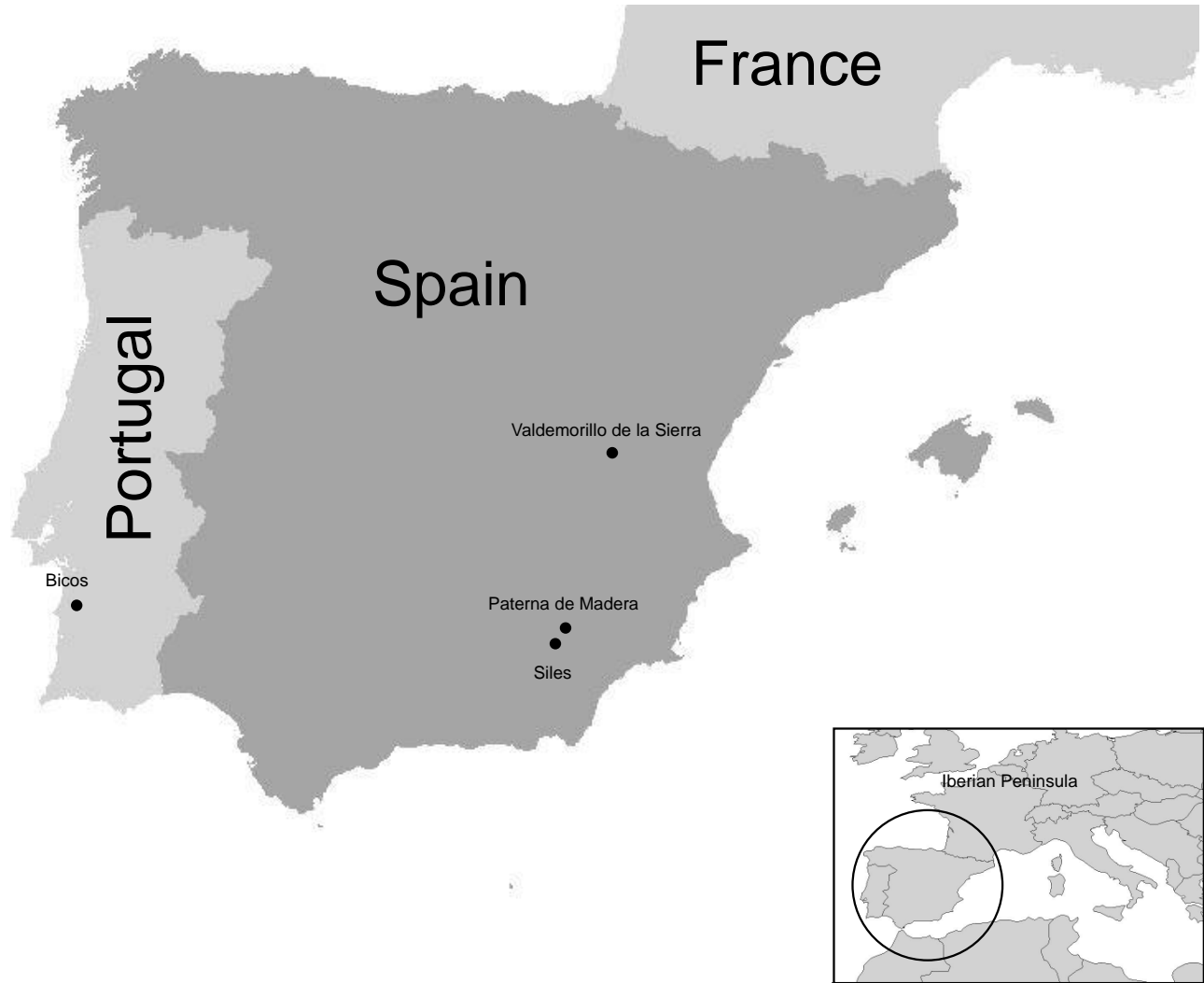
### Molecular analysis

POPGENE 1.31 (Yeh et al., 1999) and TFGPA 1.3 (Miller, 1997) software packages were used to estimate the number of loci ( $N$ ), the number of polymorphic loci ( $N_p$ ), the average number of observed ( $na$ ) and effective ( $ne$ ) alleles per locus (Hartl and Clark, 1989), the percentage of polymorphic loci ( $P$ ) within each population, the unbiased expected heterozygosity ( $H_{un}$ ) (Nei, 1978) and the Shannon-information index of phenotypic diversity ( $I$ ) (Shannon and Weaver, 1949). Nei (1987) gene diversity statistics and Nei (1978) unbiased population genetic distances were used to estimate the distribution of genetic variation among *M. cabrerae* populations. Monte-Carlo approximation of exact test (Raymond and Rousset, 1995) was also performed to evaluate interpopulation differentiation. Global test over loci to determine the overall significance was implemented using Fisher's combined probability test (Sokal and Rohlf, 1995).

Un-weighted pair-group method with arithmetic mean (UPGMA) and phylogenetic neighbour-joining (NJ) dendrograms were constructed using genetic distance values for all pair Cabrera's vole specimens, as implemented by TREECON with one million replicated (Van de Peer and De Wachter, 1994).

## RESULTS

In this study, RAPD technique was used to characterize



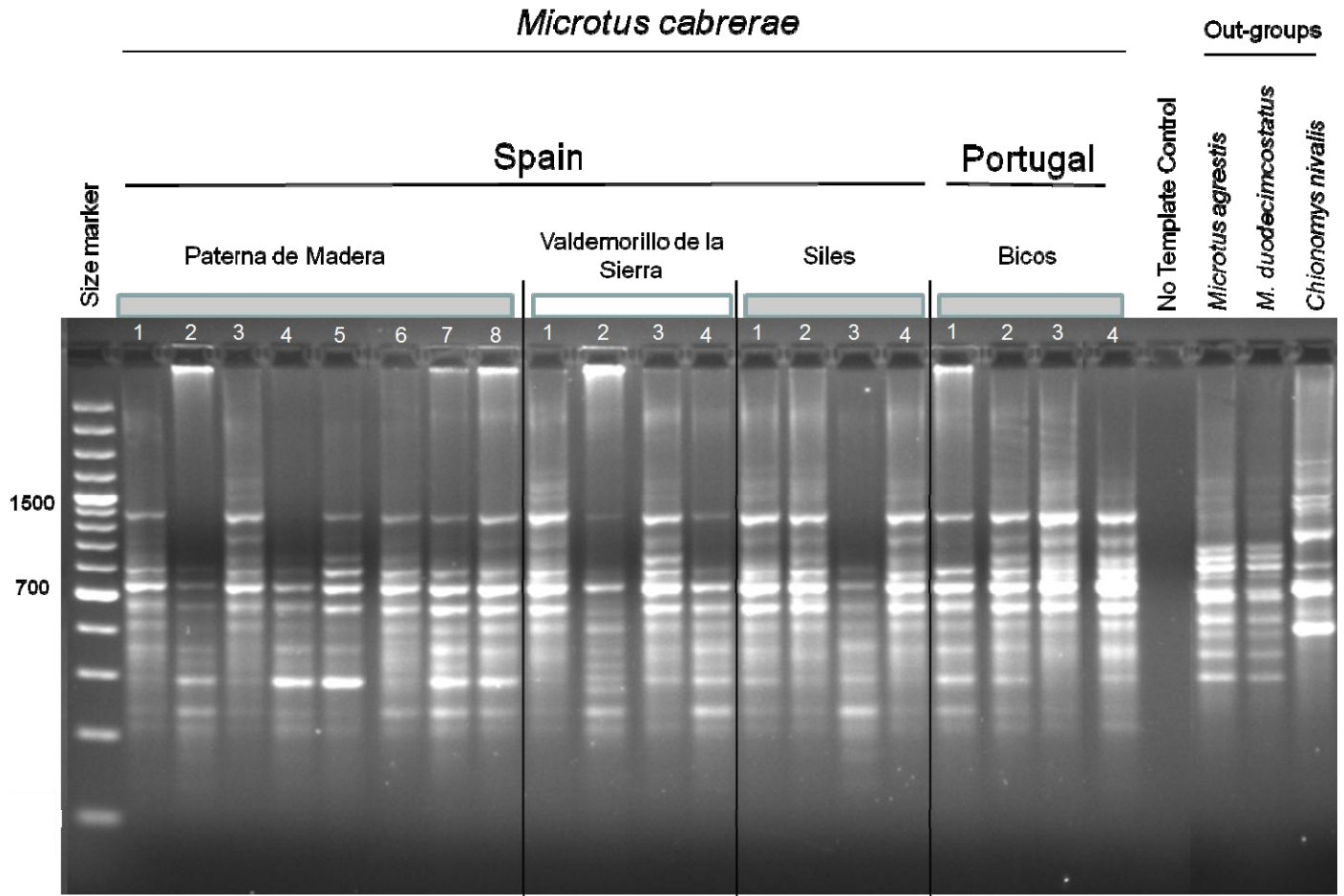
**Figure 1.** Iberian Peninsula map showing approximate sites for *M. cabreræ* samples collection (Table 1).

**Table 2.** Sequence information of the eight primers used for RAPD-PCR analysis.

<b>RAPD Primer</b>	<b>Sequence (5'-3')</b>
OPA-07	GAAACGGGTG
OPA-10	GTGATCGCAG
OPA-20	GTTGCGATCC
OPC-02	GTGAGGCGTC
OPF-07	CCGATATCCC
OPF-08	GGGATATCGG
OPF-12	ACGGTACCAG
OPF-15	CCAGTACTCC

*M. cabreræ* samples randomly collected from four populations in Spain and Portugal. For this purpose, 8 arbitrary 10-mer primers, which produced consistently reproducible patterns among samples and repeated PCR

runs were used (Table 2). A total of 134 different DNA fragments (considered as alleles located at 134 loci) were revealed by means of the selected primers. Allelic size ranged between 200 and 1500 bp (Figure 2). The number



**Figure 2.** Representative SRAP profiles produced for 20 representative Cabrera's vole samples using primer OPC-10 (Table 1 and Figure 1).

of alleles per primer varied in narrow range, between 13 (F-08 and F-12) and 17 (F-15). Of the total primers used, only C-10 generated monomorphic bands across all the samples. The most variable profiles of DNA fragments were generated with A-20 and F-07. Other primers revealed single differences between specimens under comparison, including both presence and absence of the majority of amplified DNA bands, but no locus was a molecular marker for a single population. From the 134 loci examined, 87 (65%) were polymorphic across all the studied samples, reflecting the usefulness of the selected primers in the molecular studies of this vole species.

According to Ewens-Watson test (Manly, 1985), all the RAPD loci were under neutral selection. The total number of observed ( $n_a$ ) and effective ( $n_e$ ) alleles per locus was 1.65 and 1.41, respectively. The population unbiased heterozygosity  $H_{un}$  was 0.25. The value of Shannon information index ( $I$ ) was 0.35. The level of RAPD variability in the three Cabrera's vole populations with equal number of specimens (Valdemorillo de la Sierra, Siles and Bicos) was very similar. Increasing the sample size from 4 to 18 (in the case of Paterna de

Madera population) did not lead to significant increase in the values of the genetic parameters. Similarly, differences between the values of genetic parameters between Paterna de Madera population (the population with the largest sample size) and the pooled samples from all populations were not significantly different (Table 3). The results of the hierarchical partitioning of the RAPD diversity are presented in Table 4. The value of total RAPD diversity ( $H_t = 0.23$ ) was close to that of within population diversity ( $H_s = 0.18$ ). Therefore, the mean RAPD diversity between the different populations ( $D_{st}$ ) was not high (that is, 0.05) which accounts approximately to 21% of the total RAPD diversity, while almost 80% was due to within-population component diversity. The proportion of genetic variation ( $G_{st}$ ) based on the RAPD data was 0.22, which correspond to slightly restricted gene flow ( $Nm$ ), that is, 1.7 migrants per generation. Therefore, the exact test over all loci identified Cabrera's vole populations as a single genetic unit ( $\chi^2 = 207$ ,  $df = 268$  and  $p = 0.998$ ).

The mean genetic distances between the four studied *M. cabreræ* populations was 0.063, ranging from 0.048

**Table 3.** Genetic variability of *M. cabreræ* populations from Spain and Portugal based on RAPD data.

Population	N	na	ne	I	Hun	P (%)	P <sub>95</sub> (%)
Paterna de Madera	18	1.61	1.42	0.35	0.24	61.2	58.9
Valdemorillo de la Sierra	4	1.43	1.28	0.24	0.19	43.3	-
Siles	4	1.37	1.27	0.22	0.17	37.3	-
Bicos	4	1.35	1.28	0.22	0.17	35.1	-
Total	30	1.65	1.41	0.35	0.25	64.9	63.4

N, sample number; na, observed number of alleles; ne, effective number of alleles; I, Shannon's Information index; Hun, unbiased heterozygosity; P, polymorphism (without criterion); P<sub>95</sub>, polymorphism with 95% criterion.

**Table 4.** Genetic differentiation of *M. cabreræ* populations based on RAPD data.

Cabrera's vole population	N	HT	HS	Dst	Gst	Nm	Exact test ( $\chi^2/df/p$ )
Paterna de Madera/ Valdemorillo de la Sierra	22	0.23	0.2	0.03	0.13	3.5	140/268/1.0
Paterna de Madera/Siles	22	0.22	0.19	0.03	0.12	3.8	125/268/1.0
Paterna de Madera/Bicos	22	0.22	0.19	0.03	0.12	3.8	113/268/1.0
Valdemorillo de la Sierra/Siles	8	0.2	0.16	0.04	0.21	1.9	53/268/1.0
Valdemorillo de la Sierra/Bicos	8	0.2	0.16	0.04	0.22	1.9	56/268/1.0
Siles/Bicos	8	0.19	0.15	0.04	0.19	2.2	41/268/1.0
Total	30	0.23	0.18	0.05	0.22	1.7	207/268/0.998

N, sample size; Ht, total genetic diversity; Hs, genetic diversity within populations; Dst, allelic diversity among populations; Gst, gene fixation coefficient; Nm, number of migrants per generation and Exact test, including  $\chi^2$  (chi-square), df (degrees of freedom) and p (probability).

(between Paterna de Madera and both Siles and Bicos) to 0.084 (between Valdemorillo de la Sierra and Bicos), (Table 5). When *M. cabreræ* populations were compared with the other out-group vole species, genetic distances were strongly increased ( $D = 0.287$  to  $0.772$ ) in accordance with their phylogenetic relationships (Jaarola et al., 2004).

Both UPGMA (data not shown) and NJ reconstructions (Figure 3), generated using genetic distances between pairs of *M. cabreræ* specimens, did not reveal any genetic-geographical clustering. However, the basal branches in NJ tree were dominated by specimens from southern Spain and Portugal. The UPMGA dendrogram, constructed based on the genetic distances between population pairs (Figure 4) showed clear genetic separation between *M. cabreræ* populations; *M. cabreræ* populations from the south of the Iberian Peninsula (Paterna de Madera, Siles and Bicos) were closer to each other ( $D = 0.054$ ), with higher differentiation between them and the northern population of Valdemorillo de la Sierra ( $D = 0.073$ ).

## DISCUSSION

Many rare and endangered species have passed through population bottleneck, and this event determines the maximum loss in their genetic variation (Luikart et al., 1998; Alasaad et al., 2011a). A considerable demographical decrease in the population size also took

place with *M. cabreræ* (Blanco and Gonzalez, 1992; Cabral et al., 2005). Cabrera's vole lives near the superficial water tables, which emerge to the ground. At these places, local plant communities with rushes sedges and perennial grass supply green permanent food to the Cabrera's voles (Soriguer and Amat, 1988). One of the main factors affecting the *M. cabreræ* population connectivity has been the reduction of the preference habitat patches during the last decades (Soriguer and Amat, 1988).

The geographical isolation of the persisting Cabrera's vole populations in the Iberian Peninsula was suspected to have caused a restricted intra-species gene flow (Blanco and Gonzalez, 1992; Cabral et al., 2005). Nonetheless, no molecular study has been carried out to test the extent of this hypothesis.

The heterozygosity is one of the main genetic characteristics of animal populations, and an increase of heterozygosity improves the chances of population survival (O'Brien, 1994; Reed et al., 2003). It is difficult to precisely determine a value of this parameter according to RAPD markers because of the dominant character of the RAPD loci which does not allow distinguishing between dominant homozygotes and heterozygotes. The approximate estimations of the expected heterozygosity in our study did not indicate a considerable deficiency in heterozygosity of the Iberian vole, which suggest a high level of viability. The reason behind this high genetic diversity in the studied populations could be attributed to the underestimation of the real population size and to the



**Table 5.** Nei's unbiased measures of genetic distances (Nei, 1978) between *Microtus cabreræ* populations.

Parameter	Population	Spain			Portugal	Out-group		
		Paterna de Madera	Valdemorilo de la Sierra	Siles	Bicos	<i>M. agrestis</i>	<i>M. duodecimcostatus</i>	<i>Chinomys nivalis</i>
Spain	Paterna de Madera	-						
	Valdemorilo de la Sierra	0.056	-					
	Siles	0.048	0.080	-				
Portugal	Bicos	0.048	0.084	0.065	-			
Out-group	<i>M. agrestis</i>	0.303	0.364	0.287	0.337			
	<i>M. duodecimcostatus</i>	0.511	0.601	0.519	0.542	0.406	-	
	<i>Chinomys nivalis</i>	0.689	0.772	0.728	0.764	0.755	0.570	-

presence of adequate ecological corridors between these populations. There are different approaches to detect an evidence of recent population bottleneck; among them allelic richness which was proven to be more sensitive to short and severe bottleneck than heterozygosity. In comparison with heterozygosity, allelic richness may be more successful in reflection long-term population evolution (Leberg, 2002).

Our data did not detect limited allelic diversity, which means that *M. cabreræ* did not suffer from potential decreased evolutionary pattern or strong bottleneck events. This is in concordance with our previous conclusion about high level of heterozygosity and viability of *M. cabreræ* in the Iberian Peninsula. An important characteristic of natural populations is the level of its genetic isolation. Isolated populations usually show low level of genetic variation and restricted gene flow (Wroblewska et al., 2003). Since RAPD analysis is an indirect method for gene flow estimation, the interpretation of the results is based on three categories: (i) when  $Nm < 1$ , the gene flow is too small to prevent genetic differentiation because of genetic drift; (ii) if  $Nm > 5$ , then the gene flow will

prevent genetic differentiation (the effect of gene flow will be stronger than the effect of genetic drift); and (iii) when  $1 < Nm < 5$ , the gene flow may be enough or not to prevent drift effect depending on different reasons (Gurdebeke et al., 2003). A population is genetically isolated; if during one generation, less than one effective migrant is able to give offspring (Lande and Berrouklaf, 1989). Thus, the studied populations of *M. cabreræ* are not differentiated enough.

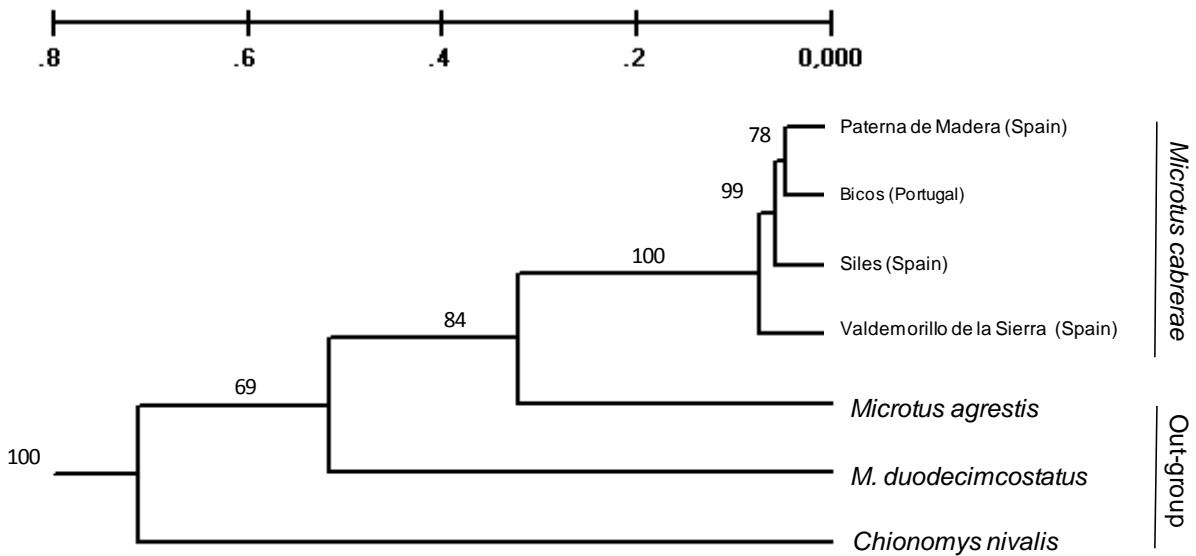
The values of the main genetic variation parameters for the studied Iberian vole populations are comparable to those obtained by the same method for other rodent species: for example, the house mouse (Spiridonova et al., 2004), ground squirrels (Spiridonova et al., 2005) and field mouse (Atopkin et al., 2007). The genetic diversity is equally distributed among the studied populations. Phylogenetic reconstructions suggest genetic differentiation between northern and southern *M. cabreræ* populations. The basal branches in the NJ reconstruction were formed by Cabrera's vole specimens from the southern Iberian Peninsula, which may be an evidence of the southern origin of Iberian vole ancestral

population, with south-north migration route. Such biogeographical division in the Iberian Peninsula has already been described for many animal species, as large *Psammmodromus*, *Psammmodromus algirus* (Busack et al., 2006, Carranza et al., 2006), European Rabbit, *Oryctolagus cuniculus* (Branco et al., 2000) and different species of the genus *Discoglossus* (García-París and Jockusch, 1999).

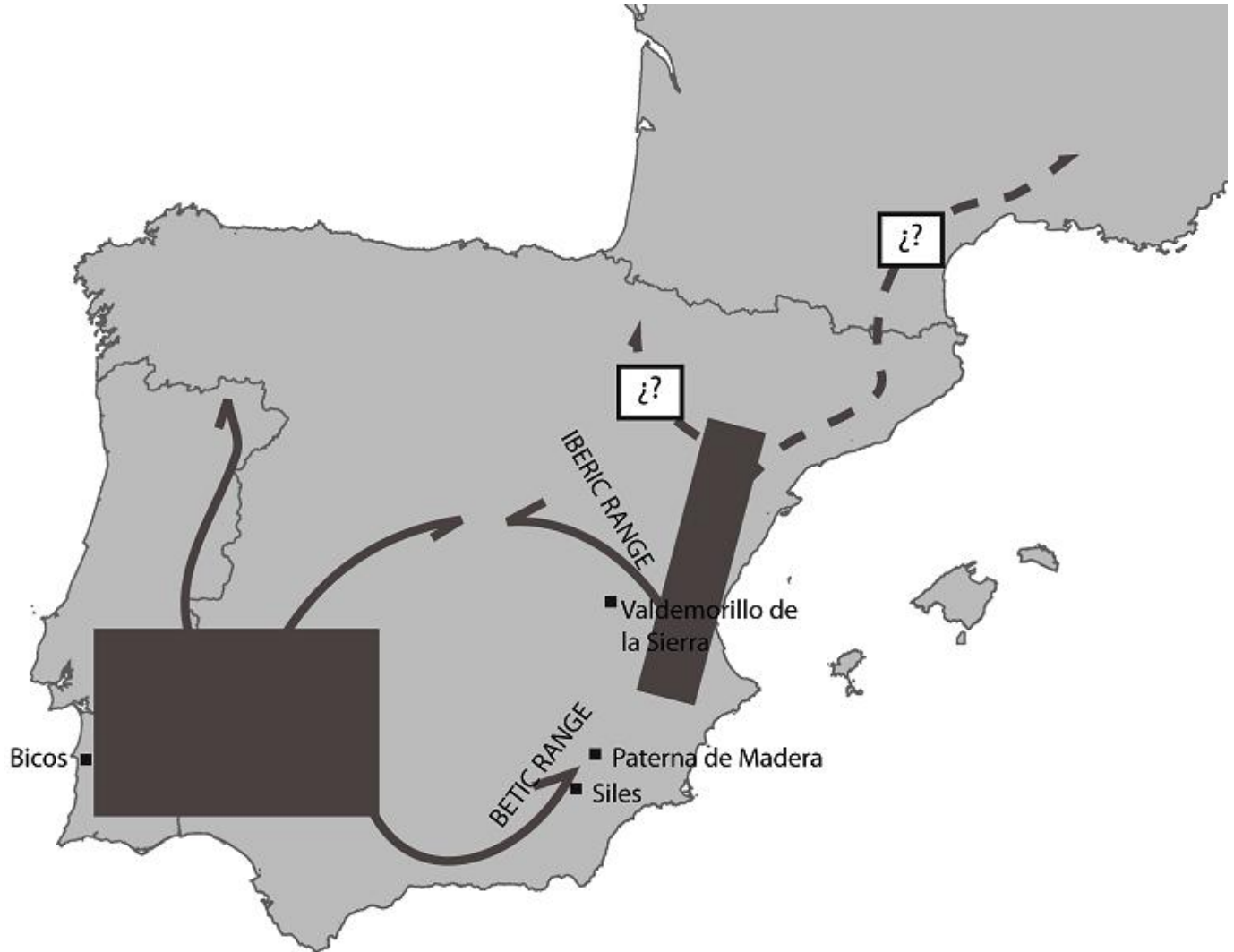
*M. cabreræ* habitat is formed by perennial grass, supplying green permanent food, which need mean annual temperature higher than 8°C (Lorite et al., 2003; Fernández-Salvador, 2007). In the last glacial period, the mean annual temperature in the Iberian Peninsula was between 9 and 11°C, which is lower than the actual mean annual temperature (Peyron et al., 1998). Hence, the distribution area of Cabrera's vole had been reduced and fragmented during the last glacial period, and consequently there were two main refuges for *M. cabreræ* in the south and north of the Iberian Peninsula, namely the Iberic and the Betic Ranges. The Mediterranean costs were divided in southwest and east parts, while the land between two Ranges were occupied by big



**Figure 3.** Phylogenetic neighbour-joining (NJ) tree reconstructed based on Nei's unbiased genetic distance (Nei, 1987) for Cabrera's vole individuals from Spain and Portugal. Numbers before nodes represent the proportion of similar replicates out of 1000000 permutations conducted.



**Figure 4.** Distance tree (UPGMA) based on Nei's unbiased genetic distance (Nei, 1987) for Cabrera's vole populations from Spain and Portugal. Numbers before nodes represent the proportion of similar replicates out of 1000000 permutations conducted.



**Figure 5.** Hypothetical scenario of *M. cabreræ* migration route to explain the phylogeographic structuring found in our study. Squares indicate refuge areas during the last glacial period. Arrows indicate expansion routes during the Holocene. Discontinuous lines with question marks indicate putative migration routes of the extinct populations of Cabrera's vole.

extensions of high land (including Sierra Nevada mountain, 3484 m) under continental climate conditions (Peinado and Rivas-Martínez, 1987; Costa et al., 1998). During the Holocene period, *M. cabreræ* from the southwest refuge of the Iberian Peninsula expanded to occupy most of Portugal, and central and southern Spain, including Jaén and Albacete, while the refuge of the east Mediterranean coast expanded to the Iberic system (Cuenca), and possible to the Pyrenees and southeastern France, where Cabrera's vole survived until the Romanian period (Pascal et al., 2006) (Figure 5).

New molecular markers with higher evolutionary rates, and additional samples of the Iberian vole over all distribution area should be used in further studies. However, the results presented in our study could be of vital interest for the appropriate Cabrera's vole management and conservation plans.

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