academicJournals

Vol. 12(26), pp. 4065-4073, 26 June, 2013
DOI: 10.5897/AJB12.739
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

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

Samer Alasaad[1,2,3]*, Konstantin V. Rozhkovan[1], Dmitry M. Atopkin[1], Antonio Sánchez[2], José Garrido-García[3], Ismael Romero[2], Ramón C. Soriguer[3] and Galina N. Chelomina[1]

[1]Institute of Biology and Soil Science, Far Eastern Branch of the Russian, Academy of Sciences, 690022 Vladivostok, Russia.
[2]Departamento de Biología Experimental, Universidad de Jaén, Campus Las Lagunillas, s/n, E-23071, Jaén, Spain.
[3]Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Américo Vespucio s/n 41092 Sevilla, Spain.

Accepted 28 June, 2013

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 cabrerae*, which may have further application for the conservation programs of this threatened species of Iberian vole.

Key words: *Microtus cabrerae*, 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 cabrerae* 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. cabrerae* is very difficult to monitor in the wild and hence conventional approaches such as trapping or photography are mostly inefficient. Studies of this

*Corresponding author. samer@ebd.csic.es; chelomina@ibss.dvo.ru.

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

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

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 $MgCl^2$ 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 electrophorese in 3% agarose gels stained with ethidium bromide (5 mg/ml).

Molecular analysis

POPGENE 1.31 (Yeh et al., 1999) and TFPGA 1.3 (Miller, 1997) software packages were used to estimate the number of loci (*N*), the number of polymorphic loci (*Np*), 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 (*Hun*) (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 neigbour-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

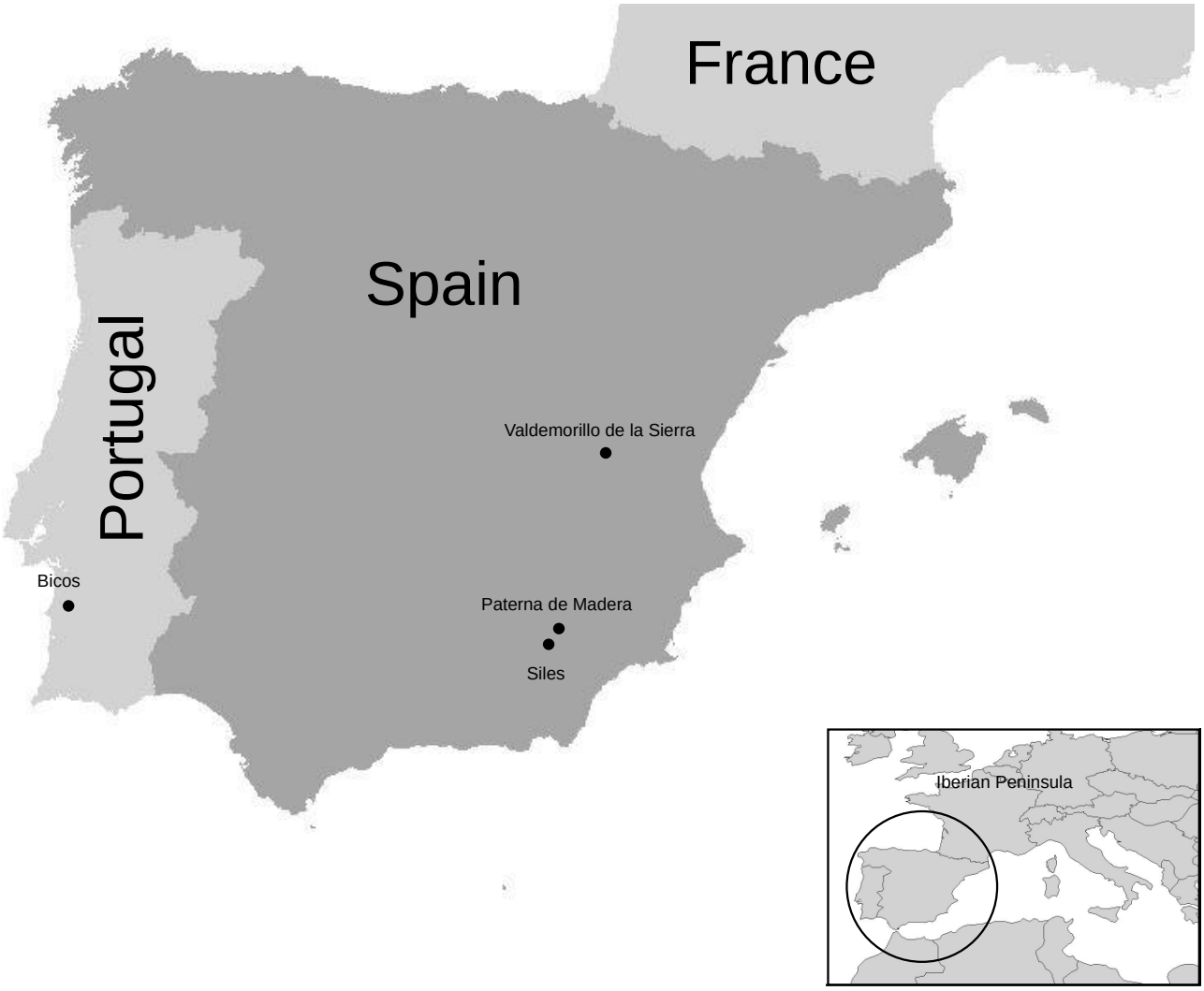

Figure 1. Iberian Peninsula map showing approximate sites for *M. cabrerae* samples collection (Table 1).

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

RAPD primer	Sequence (5'-3')
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. cabrerae samples randomly collected from four populations in Spain and Portugal. For this purpose, eight 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

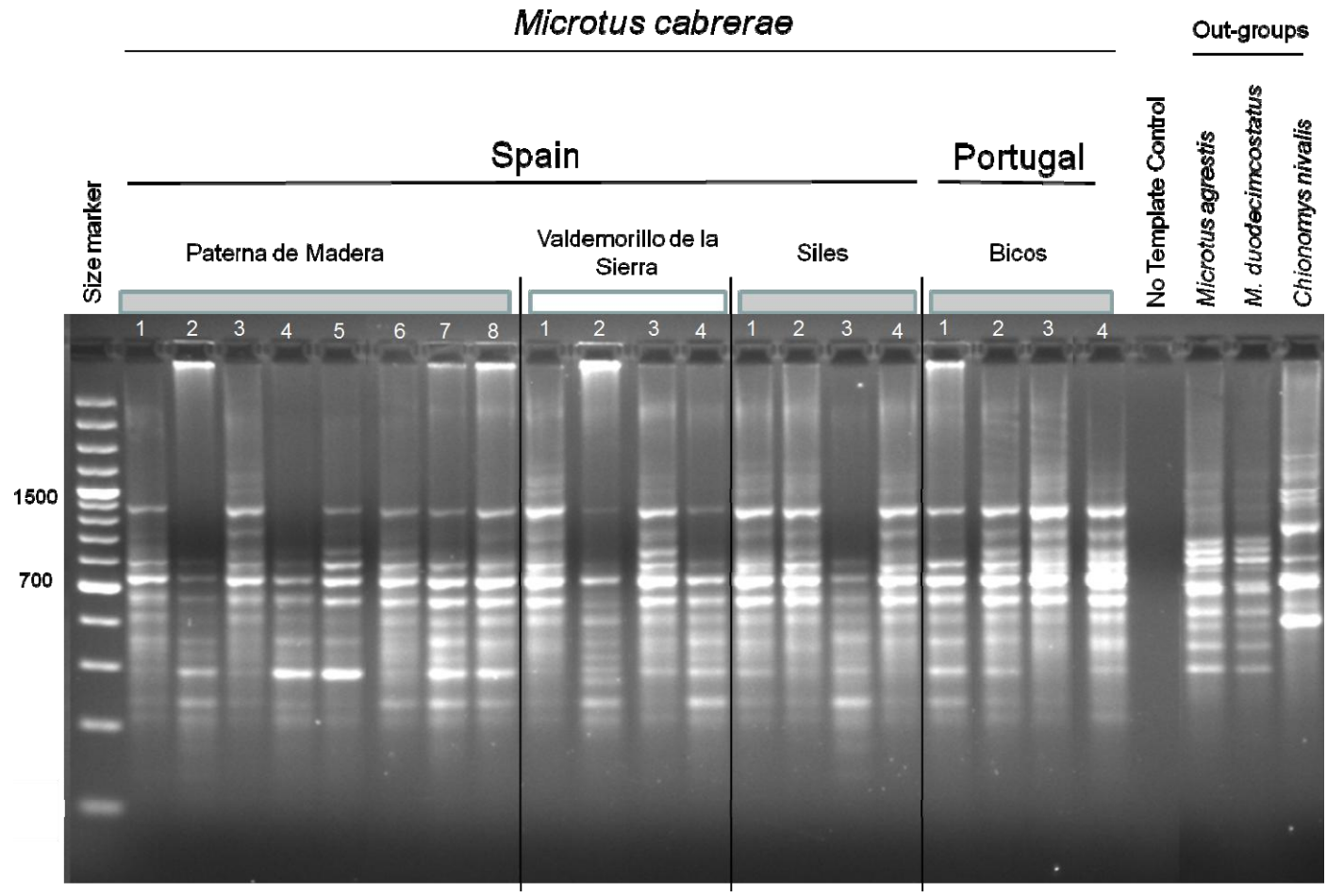

Figure 2. Representative SRAP profiles produced for 20 representative Cabrera's vole samples using primer OPC-10 (Table 1 and Figure 1).

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-Waterson test (Manly, 1985), all the RAPD loci were under neutral selection. The total number of observed (*na*) and effective (*ne*) alleles per locus was 1.65 and 1.41, respectively. The population unbiased heterozygosity *Hun* 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 (*Ht* = 0.23) was close to that of within population diversity (*Hs* = 0.18). Therefore, the mean RAPD diversity between the different populations (*Dst*) 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 (*Gst*) 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 (χ^2 = 207, *df* = 268 and *p* = 0.998).

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

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

Population	N	*na*	*ne*	*I*	*Hun*	*P* (%)	P_{95} (%)
Paterna de Madera	18	1.61	1.42	0.35	0.24	61.2	58.9
Valdemorilo 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_{95}, polymorphism with 95% criterion.

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

Cabrera's vole population	*N*	*HT*	*HS*	*Dst*	*Gst*	*Nm*	Exact test (χ^2/df/p)
Paterna de Madera/ Valdemorilo 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
Valdemorilo de la Sierra/Siles	8	0.2	0.16	0.04	0.21	1.9	53/268/1.0
Valdemorilo 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 χ^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. cabrerae* 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. cabrerae* 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. cabrerae* populations; *M. cabrerae* 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. cabrerae* (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. cabrerae* 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 cabrerae* populations.

Parameter		Spain			Portugal	Out-group		
	Population	Paterna de Madera	Valdemorilo de la Sierra	Siles	Bicos	*M. agrestis*	*M. duodecimcostatus*	*Chinomys nivalis*
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	*M. agrestis*	0.303	0.364	0.287	0.337			
	M. duodecimcostatus	0.511	0.601	0.519	0.542	0.406	-	
	Chinomys nivalis	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. cabrerae* 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. cabrerae* 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 (ii) 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. cabrerae* 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. cabrerae* 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 *Psammodromus*, *Psammodromus 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. cabrerae 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. cabrerae* 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

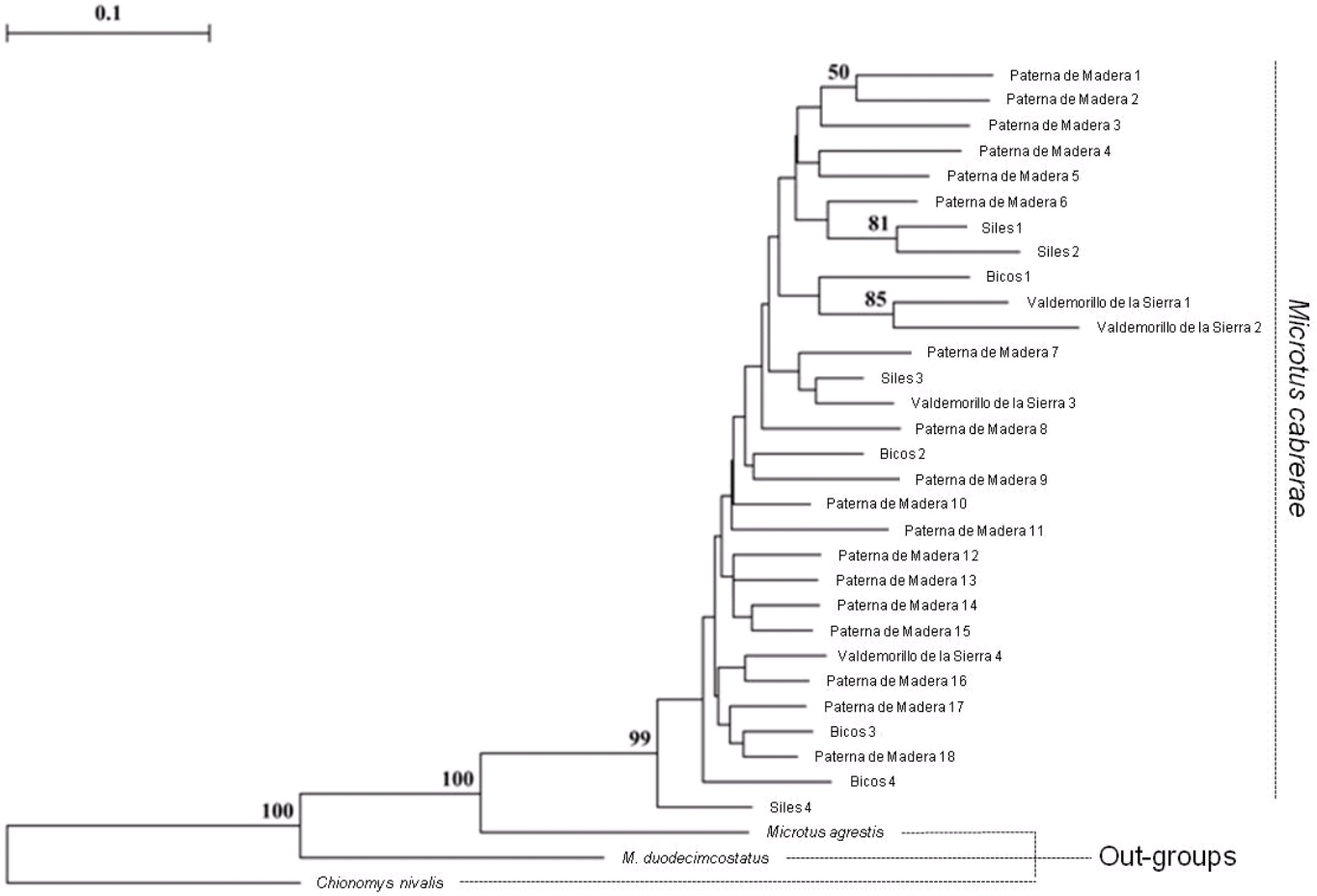

Figure 3. Phylogenetic neigbour-joining (NJ) tree reconstructed based on Nei's unbiased genetic distance (Nei, 1987) for Cabrera's vole individuals from Spain and Portugal. Numbers before nodes represent the proportion of similar replicates out of 1000000 permutations conducted.

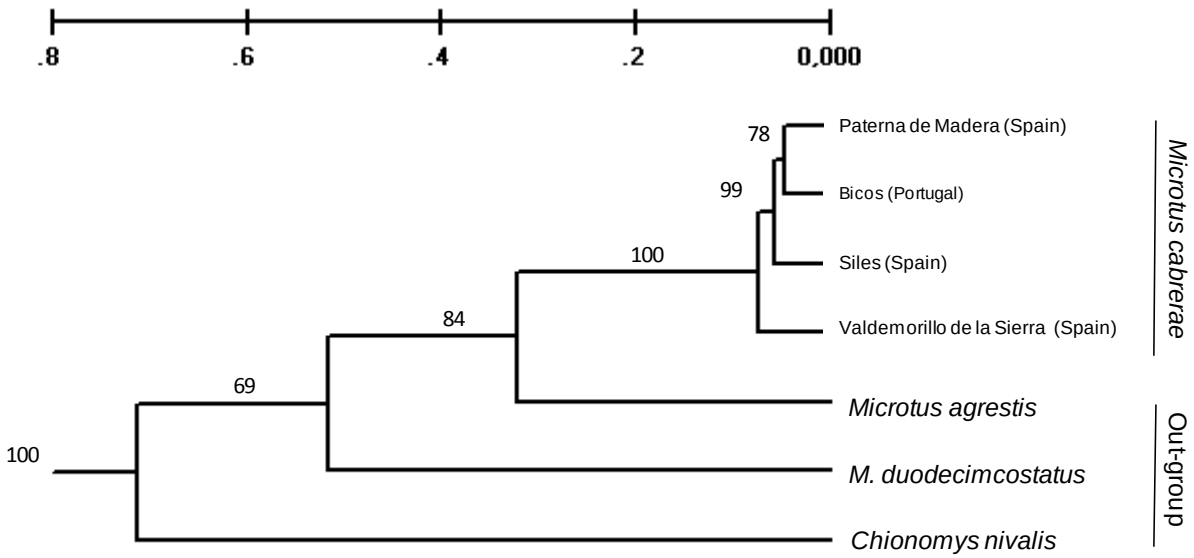

Figure 4. Distance tree (UPGMA) based on Nei's unbiased genetic distance (Nei, 1987) for Cabrera's vole populations from Spain and Portugal. Numbers before nodes represent the proportion of similar replicates out of 1000000 permutations conducted.

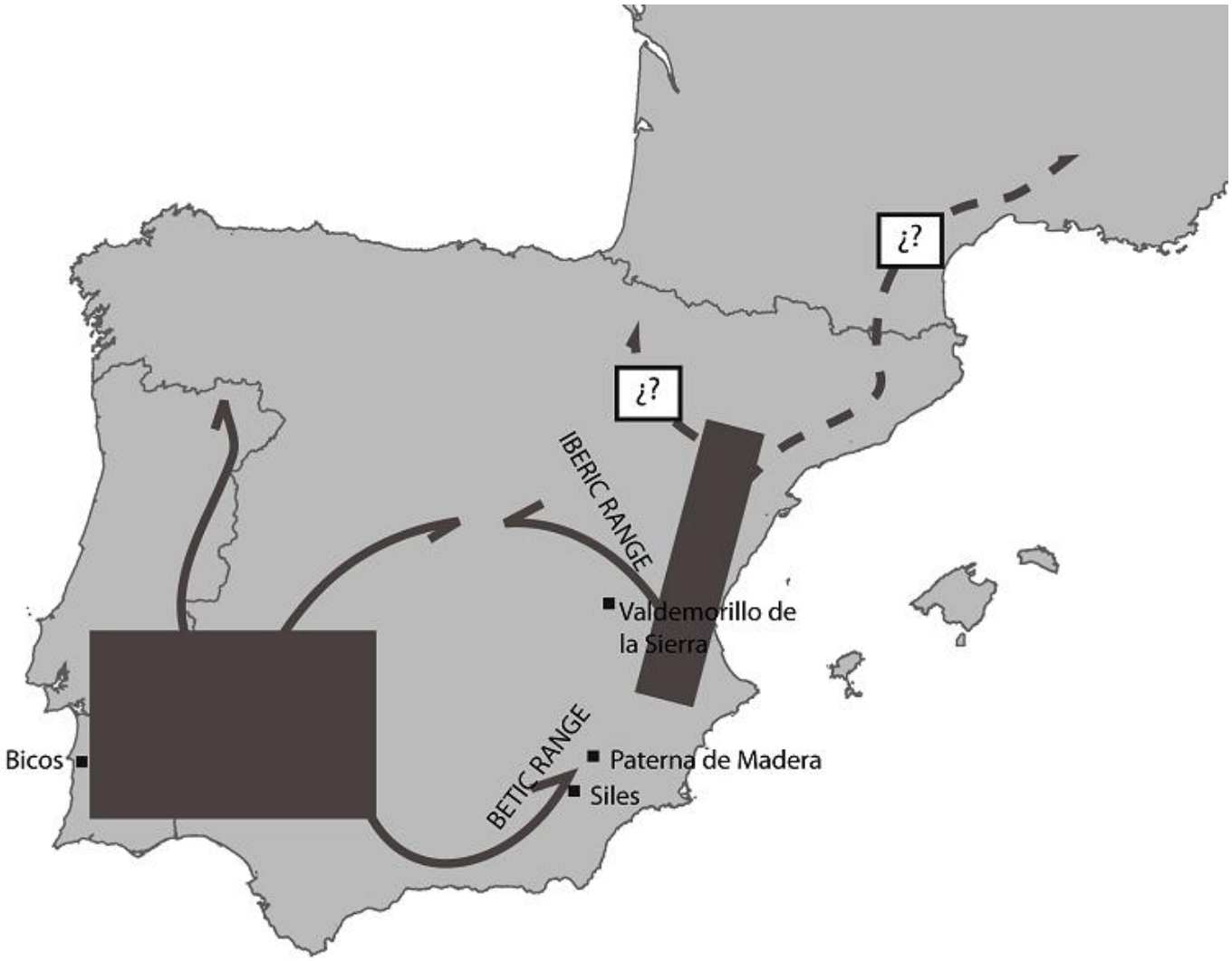

Figure 5. Hypothetical scenario of *M. cabrerae* migration route to explain the phylogeographic structuring found in our study. Squares indicate refuge areas during the last glacial period. Arrows indicate expansion routes during the Holocene. Discontinuous lines with question marks indicate putative 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. cabrerae* 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 cost expanded to the Iberic system (Cuenca), and possible to the Pyrenees and south-eastern 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.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Junta de Andalucia, Junta de Castilla-La Mancha and Instituto da Conservação da Natureza (Portugal) for capture permits of *M. cabrerae*. This work was supported by the Spanish Ministerio de Ciencia y Tecnología through project number: CGL2009-07754 (cofunded by the European Regional Development Fund) and by the Junta de Andalucía throughout the programme "Ayudas a grupos de investigación", groups numbers: RNM118, CVI 220 and NET829160 (Egmasa).

REFERENCES

Alasaad S, Sánchez A, Marchal JA, Píriz A, Garrido-García JA, Carro F, Romero I, Soriguer RC (2010). Efficient identification of *Microtus*

cabrerae excrements using noninvasive molecular analysis. Conserv.Genet. Resour. 3:127-129.
Alasaad S, Sánchez A, García-Mudarra JL, Pérez JM, Marchal JA, Romero I, Garrido-García JA, Soriguer RC (2012). Single-tube HotSHOT technique for the collection, preservation and PCR-ready DNA preparation of faecal samples: the threatened Cabrera's vole as a model. Eur. J. Wildl. Res. 58:345-350.
Alasaad S, Soriguer RC, Chelomina G, Sushitsky YP, Fickel J (2011a). Siberian tiger's recent population bottleneck in the Russian Far East revealed by microsatellite markers. Mammal. Biol. 76:722-726.
Alasaad S, Soriguer RC, Jowers MJ, Marchal JA, Romero I, Sánchez A (2011b). Applicability of mitochondrial DNA for the identification of Arvicolid species from faecal samples: a case study from the threatened Cabrera's vole. Mol. Ecol. Resour. 11:409-414.
Atopkin DM, Bogdanov AS, Chelomina GN (2007). Genetic variation and differentiation in striped field mouse *Apodemus agrarius* inferred from RAPD-PCR Analysis. Genetika 43:804-817.
Blanco JC, González JL (1992). V. Fichas descriptivas de ls especies y subespecies amenazadas: Mamiferos: 515-681. In: Blanco JC, González JL (Eds.), Libro Rojo de los Vertebrados de España. Instituto para la Conservación de la Naturaleza, Madrid, pp.714.
Branco M, Ferrand N, Monnerot M (2000). Phylogeography of the European rabbit (*Oryctolagus cuniculus*) in the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene. Heredity 85:307-317.
Busack SD, Salvador A, Lawson R (2006). Two news species in the genus *Psammodromus* (Reptilia: Lacertidae) from the Iberian peninsula. Ann. Carnegie. Mus. 75:1-10.
Cabral MJ (coord.). Almeida J, Almeida PR, Dellinger T, Ferrand de Almeida N, Oliveira ME, Palmeirim JM, Queiroz AI, Rogado L, Santos-Reis M (2005). Livro Vermelho dos Vertebrados de Portugal. Lisboa, Instituto de Conservação da Natureza.
Carranza S, Harris DJ, Arnold EN, Batista V, Gonzalez de la Vega JP (2006). Phylogeography of the lacertid lizard, Psammodromus algirus, in Iberia and across the Strait of Gibraltar. J. Biogeogr. 33:1279-1288.
Costa M, Morla C, Sáinz H (Eds.) (1998). Los bosques ibéricos. Una interpretación biogeográfica. Planeta, Barcelona p.597.
Curtsinger JW, Fukui HH, Resler AS, Kelly K, Khazaeli AA (1998). Genetic analysis of extended life span in *Drosophila melanogaster*. I. RAPD screen for genetic divergence between selected and control lines. Genetica 104:21-32.
Feliu C, Torres J, Miquel J, Gisbert J, Garcia-Perea R (1991) Helminthfauna of *Microtus* (*Microtus*) *cabrerae* (Thomas, 1906) (Rodentia: Arvicolidae) in the Iberian peninsula: faunistic and ecological considerations. Ann. Parasitol. Hum. Comp. 66:121-125.
Fernández-Salvador R (2007). *Microtus cabrerae* Thomas, 1906. Topillo de Cabrera. Pp. 429-433 in Palomo JL, Gisbert J, Blanco JC (Eds.): Atlas y Libro Rojo de los mamíferos terrestres de España. DGCNA - TRAGSA - SECEM - SECEMU. Madrid.
García-París M, Jockusch EL (1999). A mitochondrial DNA perspective on the evolution of Iberian Discoglossus (Amphibia: Anura). J. Zool. 248:209-218.
Gilpen ME, Soulé ME (1986). Minimum viable populations: processes of species extinction. In: Soulé ME (Ed.), Conservation Biology: the Science of Scarcity and Diversity. Sinauer Associates, Sunderland, Massachusetts pp. 19-34.
Grosberg RK (1996). Characterization of genetic structure and genealogies using RAPD-PCR markers: a random primer for the novice and nervous. In (Ferraris JD and Palumbi SR eds.) A John Willey and Sons, Inc, Publication, New York, Chichester, Brisbane, Toronto, Singapore. Mol. Zool. pp. 67-100.
Gurdebeke S, Maelfait JP, Backeljau T (2003). Constructing allozyme and RAPD variation in Spider populations from patchy forest habitats. Genet. 119:27-34.
Hadrys H, Balick M, Schierwater B (1992). Application of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. 1:55-63.
Hartl DL, Clark AG (1989). Principles of population genetics. Sinauer Associates, Sunderland, MA.
Jaarola M, Martínková N, Gündüz I, Brunhoff C, Zima J, Nadachowski A, Amori G, Bulatova NS, Chondropoulos B, Fraguedakis-Tsolis S, González-Esteban J, José López-Fuster M, Kandaurov AS, Kefelioğlu H, da Luz Mathias M, Villate I, Searle JB (2004). Molecular phylogeny of the species vole genus *Microtus* (Arvicolinae, Rodentia) inferred from mitochondrial DNA sequences. Mol. Phylogenet. Evol. 33:647-663.
Lande R, Berrouklaf JF (1989). Genetic variation and their use in population management. In: Viability of populations (Suley M. Ed.), Moskow: Mir.
Leberg PL (2002). Estimation allelic richness: effects of sample size and bottlenecks. Mol. Ecol. 11:2445-2449.
Lorite J, Valle F, Salazar C (2003). Síntesis de la vegetación edafohigrófila del Parque Natural y Parque Nacional de Sierra Nevada. Monogr. Fl. Veg. Béticas, 13:47-110.
Luikart G, Sherwin WB, Stelle BM, Allendorf FW (1998). Usefulness of molecular markers for detecting population bottlenecks via monitoring genetic change. Mol. Ecol. 7:963-974.
Manly BFJ (1985). The statistics of neutral selection on animal populations. Chapman and Hall (London, New York). pp.272-282.
Miller MP (1997). Tools for Population Genetics Analyses (TFPGA). 1.3. Department of Biological Sciences, Northern Arizona University, Flagstaff.
Nei M (1972). Genetic distance between populations. Am. Nat. 106:283-292.
Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genet. 76:379-390.
O'Brien SJ (1994). A role for molecular genetics in biological conservation. Proc. Natl. Acad. Sci. USA. 91:5748-5755.
Pascal M, Lorvelec O, Vigne JD (2006). Invasions biologiques et extinctions. 11000 ans d'histoire des vertébrés en France. Belin, Paris. p. 352.
Peinado M, Rivas-Martínez S (Eds.) (1987). La vegetación de España. Universidad de Alcalá de Henares, Madrid. p. 544.
Peyron O, Guiot J, Cheddadi R, Tarasov P, Reille M, Bauleu JL, Bottema S, Andrieu V (1998). Climatic reconstruction in Europe for 18000 yrBP from pollen data. Quaternary. Res. 49:183-196.
Primack RB (1993). Essentials of conservation biology. Sinauer Associates, Sunderland, Massachusetts.
Raymond ML, Rousset F (1995). An exact test for population differentiation. Evol. 49:1280-1283.
Reed D, Lowe EH, Briscoe DA, Frankham R (2003). Inbreeding and extinction: effects of rate of inbreeding. Conserv. Genet. 4:405-410.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
Shannon CE, Weaver W (1949). The Mathematical Theory of Communication. University of Illinois Press, Urbana.
Sokal R, Rolf FJ (1995). Biometry. 3rd edition. Freeman WH, New York.
Soriguer RC, Amat JA (1988). Feeding of Cabrera vole in west-central Spain. Acta. Theriol. 33:589-593.
Spiridonova LN, Chelomina GN, Moriwaki K, Yonegawa H, Bogdanov AS (2004). Genetic and taxonomic diversity of the house mouse *Mus musculus* from the asian part of the former Soviet Union. Genetika 40:1378-1388.
Spiridonova LN, Chelomina GN, Starikov VP, Korablev VP, Tsvirka MV, Liapunova EA (2005). RAPD-PCR analysis of ground squirrels from the Tobol-Ishim interfluve: evidence for interspecific hybridization between ground squirrel species *Spermophilus major* and *S. erythrogenys*. Genetika 41:1210-1221.
Van de Peer Y, De Wachter R (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Applic. Biosci. 10:569-570.
Welsh J, McClelland M (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic. Acids. Res. 18:7213-7218.
Wroblewska A, Brzosko E, Czrnecka B, Nowosielski J (2003). High levels of genetic diversity in populations of Iris aphylla L. (Iridaceae), an endangered species in Poland. Botanical. J. Linn. Soc. 142:65-72.
Yeh FC, Yang RC, Boyle TBJ, Ye ZH, Mao JX (1999). POPGENE 3.2. The user-friendly shareware for population genetics analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton.