MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Applicability of mitochondrial DNA for the identification of Arvicolid species from faecal samples: a case study from the threatened Cabrera's vole

SAMER ALASAAD,*,† RAMÓN C. SORIGUER,* MICHAEL J. JOWERS,* JUAN A. MARCHAL,
† ISMAEL ROMERO† and ANTONIO SÁNCHEZ†
*Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Américo Vespucio S/N 41092 Sevilla, Spain, †Departamento de Biología Experimental, Universidad de Jaén, Campus Las Lagunillas, S/N, E-23071, Jaén, Spain

Abstract

Arvicolid mitochondrial genomes evolve faster than in any other mammalian lineage. The genetic diversity exhibited by these rodents contrasts sharply with their phenotypic homogeneity. Furthermore, faecal droppings from Arvicolid rodents of similar body size are almost undistinguishable on the basis of pellet morphology and content. In this study, we advantaged from their high genetic diversity vs. phenotypic homogeneity to document the applicability of mtDNA extraction from vole droppings for latter identification of such via a rapid and efficient nested PCR-based technique using the threatened *Microtus cabrerae* as a model species. We sequenced the mitochondrial control region from 75 individuals belonging to 11 species of Arvicolinae from Spain, Portugal, Greece and Italy, and an additional 19 sequences from ten *Microtus* species from other countries were downloaded from Genbank. Based on these control region sequences, we successfully designed and applied a nested PCR for *M. cabrerae*-specific and arvicolid-generic mtDNA markers to differentiate Cabrera's vole faecal samples among other species of the Arvicolinae subfamily. Although this study used Cabrera's vole as a model species, similar techniques based on mtDNA sequences may find a broader applicability for noninvasive genetic conservation of vole species and their populations.

Keywords: Arvicolinae, generic primers, *Microtus cabrerae*, Mitochondrial DNA, nested PCR, noninvasive samples, species identification, specific primers, threatened species

Received 17 August 2010; revision received 7 October 2010; accepted 13 October 2010

Recent advances in molecular methods and newly available techniques are rapidly and increasingly changing the way wild populations are studied. Moreover, the use of genetic data from noninvasive samples has become essential in surveys and in conservation management. Mitochondrial DNA is widely genotyped from faecal samples because it tends to be easier to amplify even after very long periods of storage and degradation, mostly because it is usually present in cells in a high number of copies (e.g. Berry & Sarre 2007; O'Reilly *et al.* 2008), while other genetic markers, such as microsatellites (STR) and single nucleotide polymorphisms (SNP), allow individual identification and sex determination. Thus, these nuclear markers are widely used to assess species population structure but are less suitable in species identification (Taberlet *et al.* 1999). Of the ~4500 extant mammalian species, 40% are rodents (Musser &

Carleton 1993). One of the most diverse rodent lineages is the subfamily Arvicolinae (lemmings, muskrats and voles). *Microtus* is one of the most taxonomically diverse rodent genera, including over 60 extant species (Chaline *et al.* 1999). Molecular studies have shown that the Arvicolinae have a fast rate of nucleotide substitution across the entire mtDNA genome as well as at each protein-coding gene implying that their mtDNA genomes evolve more rapidly than any other mammalian lineage (Conroy & Cook 2000; Triant & Dewoody 2006). These findings contrast with the apparent little phenotypic plasticity within the species subfamily. This suggests that genes responsible for morphological variation do not seem to be influenced by either the rapid rate of mtDNA nucleotide substitution or chromosomal rearrangements (Maruyama & Imai 1981; Fink *et al.* 2004; Jaarola & Searle 2004).

The contrasting evidence between the high genetic diversity and the phenotypic homogeneity within this subfamily leads us to prepare a nested PCR-based assessment method to differentiate arvicolid species with

Correspondence: Samer Alasaad, Fax: +34 954621125;
E-mail: samer@ebd.csic.es

a noninvasive method, using the threatened Cabrera's vole (*Microtus cabrerae*) as a model species.

The threatened Iberian vole *Microtus cabrerae* (Thomas 1906) is only found in Portugal and Spain (Blanco & González 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 habitat protection and management if the few 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 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 & Soulé 1986).

Within this context, the use of indirect noninvasive approaches is potentially of great interest, and more specifically, DNA extraction from faeces represents a valuable and powerful tool to use in surveys, in species inventories and in demographic studies (Taberlet & Luikart 1999). Faeces are one of the best noninvasive animal samples available for analyses because they are easy to find in the wild and provide more information (e.g. diet, stress hormone status, parasite infection and animal DNA) than other sample types (Goymann 2005; Luikart *et al.* 2008; Schwartz & Monfort 2008; Pauli *et al.* 2010). Nevertheless, on the basis of just morphological characteristics and content, it is generally difficult to identify the faeces deposited by *M. cabrerae* from those of other sympatric arvicolid species of similar body size (*Arvicola sapidus*, *M. duodecimcostatus*, *M. agrestis* and *M. lusitanicus*).

Fifty-seven *M. cabrerae*, *M. duodecimcostatus* and *Arvicola sapidus* faecal samples were collected from caged animals at Jerez and Granada Zoos and from trapped free-ranging animals from various locations in Andalucia (Spain), between 2009 and 2010. All samples were kept at ambient temperature in the field and were then stored at −20 °C (for more details, see Table 1). Sixty-six tissue samples from six Arvicolinae rodents (*M. cabrerae*, *M. duodecimcostatus*, *M. agrestis*, *M. lusitanicus*, *A. sapidus* and *A. terrestris*) present in the Iberian Peninsula were collected from different locations in Spain and Portugal, between 2005 and 2010. To examine the specificity of the proposed method, and its applicability to other arvicolid species, we included nine specimens from five arvicolid species from Greece (*M. thomasi* and *M. thomasi atticus)*, Italy (*M. savii* and *M. brachycercus)*, and a *M. guentheri* sample from an unknown origin. An additional 16 sequences belonging to ten Arvicolinae vole species from ten different countries worldwide were downloaded from GenBank (for more details, see Table 1), and included in an alignment (Table 2).

For the DNA extraction, three faecal pellets were randomly collected from each animal (Table 1) and their pellet surfaces were washed by incubation for 10 min in a buffer solution (Qiagen). The DNA was extracted from the buffer solution using a blood DNA extraction kit (Qiagen) (Maudet *et al.* 2004; Luikart *et al.* 2008). The DNA was extracted from tissue samples following the standard phenol/chloroform procedures (Sambrook *et al.* 1989). The DNA extractions were carried out in a sterilized laboratory exclusively for low DNA concentration samples. Two blanks (reagents only) were included in each extraction to monitor for contamination (Handt *et al.* 1994).

Control region sequences of all collected arvicolid vole tissue samples were amplified using primers pair Pro+ and Phe+, as described by Haring *et al.* (2000). All the control region sequences together with the others from the GenBank were aligned and used to design a nested PCR with two new primer pairs (arvicolid-generic primers and Cabrera's vole-specific primers). Primers were designed in Primer3 (v. 0.4.0) (Rozen & Skaletsky 2000).

Two consecutive PCRs were performed:

PCR I (a control PCR using arvicolid-generic primers, for the mitochondrial control region fraction amplification of all the studied arvicolid species): the 30-μL PCR mixture contained 2 μL of gDNA (from tissue or faecal samples), 0.25 μM of each primer (Pro+, see Haring *et al.* 2000, and the new reverse MicoMico, 5′-TGGGCGGGTTGCTGGTTTCAC-3′), 0.12 mM of each dNTP, 3 μL of 1× kit-supplied PCR buffer (Bioline), 1.5 mM $MgCl_2$, 0.4% bovine serum albumin (BSA), 1.5 μL DMSO and 0.2 μL (0.2 U/reaction) Taq polymerase (Bioline). Samples were subjected to the following thermal profile for amplification in a 2720 Thermal Cycler PTC-0200 DNA Engine thermal cycler (Bio-Rad): 4 min at 94 °C (initial denaturation), followed by 30 cycles of three steps of 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing) and 50 s at 72 °C (extension), before a final elongation of 5 min at 72 °C. PCR blanks (reagents only) were included with each PCR run.

PCR II (nested PCR using Cabrera's vole-specific primers to amply a fragment of *M. cabrerae's* control region): reagents and concentrations and thermal profile were similar to PCR I, with the exception that 2 μL of PCR I-product was used as template, and sprimers Pro+ and MicoMico were substituted with two new nested primers, MiKa1 (5′-ATTACTCCTTTAAACCATGG-3′) and MiKa2 (5′-CTAATAGACAAAATAGGGATGGG-G-3′) (Table 2). Both sets of primers were tested on all tissue and faecal samples listed in Table 1.

Table 1 Species, countries and localities, number of tissue and faecal samples for each species

Species	Country	Geographical localities	Tissue samples	Feacal samples
Microtus cabrerae	Spain and Portugal	Spain (Jaén, Madrid, Cuenca, Albacete, Cádiz and Granada); Protugal (Bicos)	50	40
M. agrestis	Spain	Navarra	2	0
M. duocecimcostatus	Spain	Granada	4	10
M. lusitanicus	Spain	Burgos	3	0
Arvicola terrestris	Spain	Burgos	3	0
A. sapidus	Spain	Jaén	4	7
M. savil	Italy	Rome	2	0
M. brachycercus	Italy	Isernia	2	0
M. thomasi	Greece	Itea	2	0
M. thomasi atticus	Greece	Kalavryta	2	0
M. guentheri	Unknown	Unknown	1	0

Following the PCRs , 2 μL of each PCR product were cleaned to remove excess primers and dNTPs, using the following enzymatic reaction: 0.2 μL of 10× Antartic phosphatase buffer, 3 units of *E. coli* exonuclease I and 1 unit of Antartic phosphatase in a final volume of 7 μL (New England Biolabs). Samples were subjected to the following thermal profile: 37 °C for 45 min followed by 80 °C for 15 min. Sequencing reactions were obtained, using Pro+ and MicoMico primers, from both directions using the Big Dye® Terminator v1.1 cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems), and labelled fragments were resolved on an automated DNA sequencer (Applied Biosystems 3130xl genetic analyzer). DNA sequences were aligned and edited using the software BioEdit v.7.0.9 (Hall 1999).

The average number of base differences between the studied arvicolid species with the designed primers was 7.5/18 bp for MiKa1 and 8.3/24 bp for MiKa2 (Table 2). The first set of PCR primers (Pro+/MicoMico: generic for the studied rodent species) was used for the first PCR run (PCR test), to evaluate the efficiency of the protocol for faecal DNA extraction and to evaluate the quality of the extracted DNA, because some PCR inhibitors could be presented in the DNA extracted from faeces (Beja-Pereira *et al.* 2009). The amplifications from the generic arvicolid rodent primers were ~300 bp (Fig. 1). The rate of PCR amplification success was 100% for tissue samples ($N = 75$; see Table 1) and 95% for faecal samples ($N = 57$; see Table 1). After the first PCR (test PCR), we ran the nested PCR with the novel *M. cabrerae*-specific primers MiKa1/MiKa2. The resulting amplified fragments were ~140 bp (Fig. 1), and the rate of success of this set of species-specific primers designed to amplify the control region fraction in Cabrera's vole was the same as for the first PCR (PCR test). The failed reactions were likely to be related to the bad conserva-

tion of the faecal samples and/or because of the inefficiency of the used method for DNA extraction from faecal samples. This new nested PCR-based technique was successfully tested in 60 random collected Arvicolid faecal samples form from the National Park of Sierra Segura (Jaén, Spain), from which 23 samples were identified as *M. cabrerae*. Seven haplotypes were identified from the 50 *M. cabrerae* D-loop sequences, and no indels were included in the alignment. Therefore, this marker has a utility to assess intra-population variability in Cabrera's vole.

The success and therefore applicability of our new molecular-based technique as a tool for a simple and efficient identification and differentiation of invasive and noninvasive *M. cabrerae* samples among other arvicolid species lies in (i) the high rate of nucleotide substitution present across the entire mtDNA genome in Arvicolinae, (ii) the high number of copies of the mitochondrial control region and (iii) the use of the nested PCR. The present study shows that this noninvasive nested PCR-based technique allows the direct differentiation and identification of faecal samples of one vole species from other arvicolid species without the need for multiple post-PCR manipulations of samples in sequencing reactions and restriction digests. These manipulations add time and cost to processing of samples and increase the possibility of human error and/or contamination.

In this study, we used Cabrera's vole as a model species, showing that this is a rapid and inexpensive technique likely to have a large applicability to other model vole organisms or even other nongeneric taxa. Arvicolinae species conservation/protection and monitoring programmes may include these noninvasive molecular-based tools with the combination with other molecular markers to provide answers to elucidate new data on long-standing ecological and evolutionary questions.
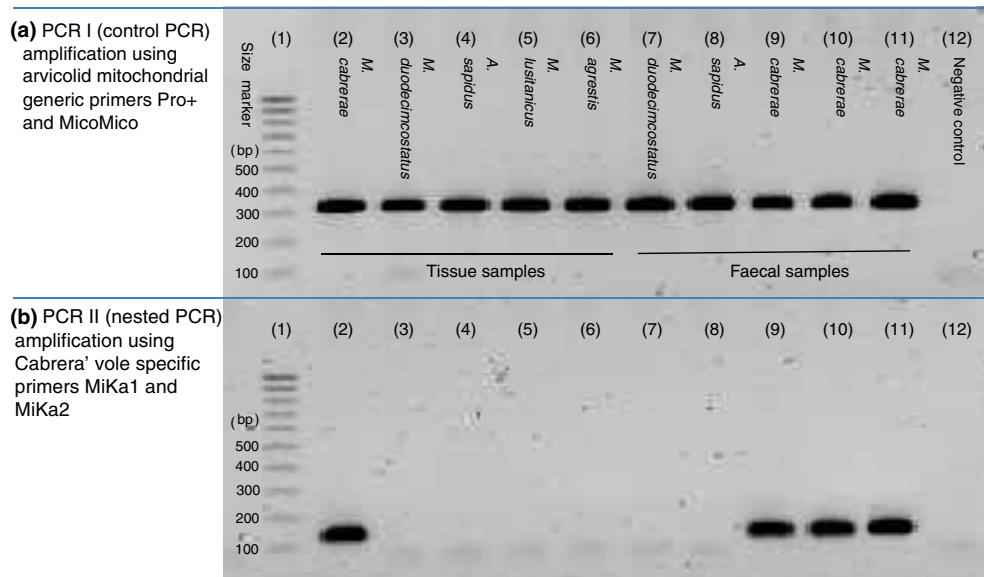
Table 2 Nested PCR primers and their binding sites on the sequence alignment of the mitochondrial control region for the studied specimens. The primer combination Pro+/MicoMico is generic for arvicolid species including *M. cabrerae*. Primer MiKa1/Mika2 are specific for *M. cabrerae*

Primers and binding sites:
- Arvicolid-generic primers
- *M. cabrerae*-specific primers
- Pro+ Forward 5' ACCATCAGCACCCAAAGCTG 3'
- MicoMico Reverse (359-380) 5' GTGAAACCAGCAAACCGCCCCA 3'

Alignment region column headers: MiKa1 Forward (201-222) | MiKa2 Reverse (320-345)

Species	Country	GenBank accession number	References
Microtus cabrerae	Spain and Portugal	FR695397-FR695403	New samples (see Table 1)
Arvicola terrestris	Spain	FR695404	
A. sapidus	Spain	FR695405	
M. duodecimcostatus	Spain	FR695406	
M. savii	Italy	FR695407	
M. guentheri	unknown	FR695408	
M. thomasi	Greece	FR695409	
M. thomasi atticus	Greece	FR695410	
M. brachycercus	Italy	FR695411	
M. lusitanicus	Spain	FR695412	
M. agrestis	Spain	FR695413	
M. subterraneus	Austria	AF267271-AF267273	Haring et al. 2000
M. subterraneus	Austria	AF267274	
M. multiplex	Switzerland	AF267275	
M. bavaricus	Germany	AF267276	
M. liechtensteini	Italy	AF267277	
M. liechtensteini	Greece	AF267278 and AF267279	
M. liechtensteini	Slovenia	AF267280	
M. liechtensteini	Austria	AF267281	
M. nivalis	Slovenia	AF267282	
M. arvalis	Austria	AF267283	
		AF267284	
		AF267285	
M. oeconomus	Hungary	AJ616853	Putnoky et al. Unpublised
M. mexicanus	USA	AF251260	Greenberg et al. Unpublished
M. kikuchii	Taiwan	NC_003041	Lin et al. 2002
M. rossiaemeridionalis	USA	NC_008064	Triant and Dewoody 2006

Dots indicate identical nucleotides to the *M. cabrerae* sequence. Dashes indicate gaps.

(a) PCR I (control PCR) amplification using arvicolid mitochondrial generic primers Pro+ and MicoMico

(b) PCR II (nested PCR) amplification using Cabrera' vole specific primers MiKa1 and MiKa2

Fig. 1 Agarose gel (1%) showing the nested PCR amplification of a partial fragment of the mitochondrial control region from representative species of Arvicolinae. (a) PCR I (control PCR) amplifications using arvicolid mitochondrial generic primers Pro+/MicoMico. (b) PCR II (nested PCR) amplifications using Cabrera' vole-specific primers Mika1/MiKa2 and PCR I-products as templates.

Acknowledgements

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