Efficient identification of Microtus cabrerae excrements using noninvasive molecular analysis

Samer Alasaad · Antonio Sánchez · Juan Alberto Marchal · Ana Píriz · José A. Garrido-García · Francisco Carro · Ismael Romero · Ramón C. Soriguer

Abstract Noninvasive sampling is a potentially cost-effective and efficient means of monitoring wild animals that precludes the need for captures and avoids undue disturbance. Nevertheless, it is generally difficult to separate faeces deposited by Microtus cabrerae from that of other sympatric rodents of similar body size on the basis of just morphological features and content. Species identification methods in many noninvasive studies involve mtDNA analysis. Here we report the first use of species-specific primers targeting the SRY gene in a noninvasive molecular identification of Cabrera's vole. This noninvasive molecular-based technique may thus provide us with a potential tool for further genetic and ecological study of this threatened species of Iberian vole.

Keywords Microtus cabrerae Species identification Noninvasive samples SRY gene Repetitive DNA Semi-nested PCR Threatened species

Cabrera's vole (Microtus cabrerae, Thomas 1906) is a threatened rodent with a world distribution limited to Portugal and Spain (Blanco and González 1992; Cabral et al. 2005) that is listed in the European Community Habitats Directive (92/43/EEC) and the Berne Convention (82/72/CEE). It is always found in small populations in habitat patches that require protection and management, if the few remaining fragile populations of this vole are to be conserved (Primack 1993). Due to a number of environmental and logistical factors, this species is very difficult to approach in the wild and hence conventional approaches such as trapping or photography are not efficient. Studies of this threatened species are still needed if effective conservation efforts are to be implemented and in order to identify the factors that are currently putting its populations at risk (Gilpen and Soulé 1986). Within this context, the use of indirect noninvasive approaches is potentially of great interest and, specifically, the extraction of DNA from faeces represents an extremely valuable and powerful tool for the study of this species (Taberlet and Luikart 1999). Faeces analysis is one of the most commonly used noninvasive techniques in animal sampling given that excrements are easy to find in the wild and provide more information than other sample types (Kohn and Wayne 1997; Schwartz and Monfort 2008; Beja-Pereira et al. 2009).

In our previous studies we reported the peculiarity of the SRY gene in this vole, which has multiple SRY copies in both males and females instead of a single copy gene located on the Y chromosomes as is the case in most mammals (Fernández et al. 2002; Marchal et al. 2004, 2008). In terms of the number of copies, the SRY gene in M. cabrerae is half-way between mitochondrial DNA (mtDNA) and single-copy nuclear DNA (scnDNA). The standard first step for species identification methods in many noninvasive studies often involves mtDNA analysis (e.g. Berry and Sarre 2007; O'Reilly et al. 2008) and here, based on the peculiarity of this vole's SRY gene, we use species-specific primers for the noninvasive molecular identification of Cabrera's vole.

S. Alasaad (&) A. Píriz J. A. Garrido-García F. Carro R. C. Soriguer
Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC),
Avda. Américo Vespucio s/n, 41092 Sevilla, Spain
e-mail: samer@ebd.csic.es

S. Alasaad A. Sánchez J. A. Marchal I. Romero
Departamento de Biología Experimental, Universidad de Jaén,
Campus Las Lagunillas S/N, 23071 Jaén, Spain

M. cabrerae and M. duodecimcostatus faecal samples were collected from sex-known caged animals in Granada and Jerez Zoos (30 M. cabrerae) and from trapped free-ranging animals (20 M. cabrerae and 4 M. duodecimcostatus) from various locations in Andalusia (Southern Spain). Faecal samples were collected 1–2 days after defecation and remained at field temperature for 2–3 days before submitting to our laboratories. Tissue samples from male and female voles of five species (M. cabrerae, M. duodecimcostatus, M. agrestis, Chionomys nivalis and Arvicola sapidus) were collected from different geographical locations in Spain. These vole species are sympatric to Cabrera's vole and it is difficult to separate faeces deposited by M. cabrerae from those of other sympatric rodents using only morphological features and/or content. Then, the DNA was extracted from faecal (Maudet et al. 2004; Luikart et al. 2008) and tissue samples (Sambrook et al. 1989). Extractions were carried out in a separate laboratory exclusively dedicated to low copy number DNA samples. Two blanks (reagents only) were included in each extraction to monitor for contamination (Handt et al. 1994).

To overcome problems associated with low-quantity DNA we applied a semi-nested PCR. The semi-nested PCR increases genotyping success and specificity by amplifying only the target locus given that the internal primers (as well as the initial external primers) are locus specific. Likewise, the semi-nested PCR increases the amount of low copy number template because the products of the first amplification are used as templates for the subsequent PCR (Lau et al. 2003; Bellemain and Taberlet 2004).

PCR I (pre-amplification): each 30 µl PCR reaction mixture consisted of 2 µl of gDNA (from tissue or faecal samples), together with the PCR mixture containing primer pairs SRY-X1 (5′-TCCCCATACTGGAGTGTGTTAC-3′) and SRY-F (5′-GTAAAACGACGGCCAGT-3′), 10 pmol of each primer (Marchal et al. 2008), 0.12 mM of each dNTP, 3 µl of 10× kit-supplied PCR buffer, 1.5 mM $MgCl_2$, 0.4% 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 denaturing), followed by 40 cycles of three steps of 1 min at 94°C (denaturation), 1 min at 57°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 experiment.

PCR II (semi-nested): we used 2 µl of the PCR-product of PCR I as a template for PCR II, using primers SRY-X1 and SRY-inv3 (5′-CTGAGGCTGTAATCATGGACTG-3′), with the PCR mixture content and the thermal profile as in PCR I. The annealing temperature was modified to 57°C. PCR blanks (reagents only) were included with each PCR experiment.

PCR I (~500 bp amplicons) apparently only worked for DNA extracted from the tissue samples of M. cabrerae, as there were failed reactions for the remaining samples

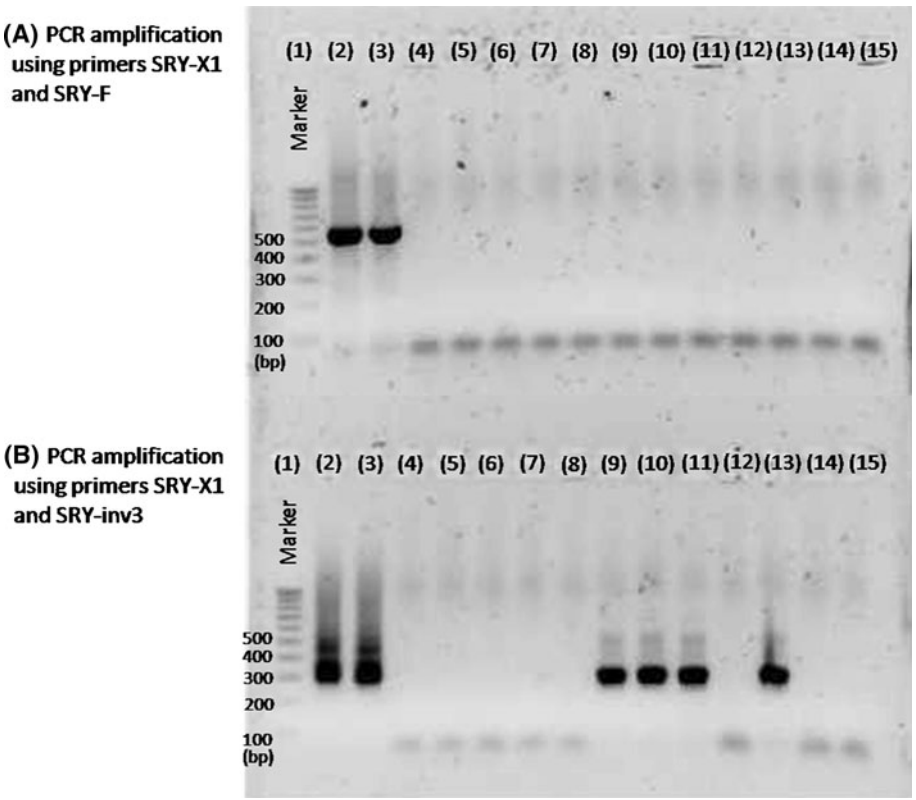

Fig. 1 PCR amplification of a partial fragment of the SRY gene in 1% agarose gel from representative rodent tissue and faecal samples: two positive control DNA from Microtus cabrerae male and female (DNA from tissue samples, lanes 2 and 3, respectively); M. duodecimcostatus (DNA from tissue sample, lane 4); Arvicola sapidus (DNA from tissue sample, lane 5); Chionomys nivalis (DNA from tissue sample, lane 6); M. agrestis (DNA from tissue sample, lane 7); M. duodecimoscostatus (DNA from faecal sample, lane 8); M. cabrerae (DNA from faecal samples, lanes 9–13); negative controls (lanes 14 and 15). Lane 1 represents a DNA size marker (ordinate values in bp). a PCR I amplification using primers SRY-X1 and SRY-F. b PCR II amplification using primers SRY-X1 and SRY-inv3

(using DNA extracted from tissue and faecal samples from other vole species and from faecal samples of M. cabrerae). PCR II (semi-nested PCR) gave positive amplifications (*300 bp) for the DNA extracted from tissue and faecal samples of M. cabrerae (with a success ratio of 100% for tissue samples and 85% for faecal samples), while for the DNA extracted from tissue and faecal samples of other rodent species no amplifications were detected (Fig. 1).

Sequencing analysis demonstrated that the SRY sequences amplified in our study were identical to those reported byby Marchal et al. (2008) (GenBank accession numbers EF601002, EF601010-EF601012, EF601020, EF601028-EF601030, EF601032, EF601022 and EF601018).

The strength of our new molecular-based noninvasive technique for the specific identification of M. cabrerae lies in the high copy number of the repetitive SRY gene in both males and females of this vole and in the appropriate use of the semi-nested PCR. This noninvasive technique permits the direct identification of faecal samples of Cabrera's vole without the need for multiple post-PCR manipulations of samples in sequencing reactions and restriction digests. These manipulations add time and cost to sample processing and increase the possibility of human error and/or contamination. This noninvasive molecular-based technique may thus provide investigators with new affordable approaches that can be included in M. cabrerae protection programmes and provide answers to long-standing ecological and evolutionary questions regarding this rare and elusive species of vole.

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