Molecular Ecology Resources (2009) 9, 754–758 doi: 10.1111/j.1755-0998.2008.02390.x

MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Identification of the endangered small red brocket deer (*Mazama bororo*) using noninvasive genetic techniques (Mammalia; Cervidae)

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Abstract

The small red brocket deer *Mazama bororo* is one of the most endangered deer in the Neotropics. The great morphological similarities with three other sympatric brocket deer species, coupled with the fact that they inhabit densely forested habitats complicate detection and prevent the use of traditional methodologies for accurate identification of species. The ability to determine the presence of this endangered species in an area is crucial for estimating its distribution range, and is critical for establishing conservation management strategies. Here we describe a fast and reliable noninvasive genetic method for species identification of *Mazama* species from faeces. We designed a primer set that amplifies a short 224-bp fragment of the cytochrome *b* and demonstrate its effectiveness in successful amplification of DNA isolated from both tissue and faecal samples. This fragment contains a *BSTNI/ECORII* digestion site that is unique to the endangered *M. bororo*. The digested polymerase chain reaction products yielded a 160-bp fragment that is clearly visible in a 2% agarose gel. Two other diagnostic sites were identified to differentiate the other three sympatric species, *Ssp*I *(M. gouazoubira)* and *Afl*III (*M. americana*, and *M. nana*).

Keywords: cryptic species, *Mazama*, noninvasive sampling, small red brocket deer, species identification

Received 3 April 2008; revision accepted 9 June 2008

Brocket deer (Genus *Mazama*) are widely distributed in the Neotropics from southern Mexico to Argentina (Weber & González 2003). The taxonomy, distribution, ecology, and status of every species in the genus *Mazama* remain unclear (Wemmer 1998). Brocket deer occupy a broad range of forest ecosystems but in general are found in closed environments such as forests and thickets, only occasionally venturing into open areas. They are very secretive and difficult to detect and observe, especially in their densely vegetated habitats (Vogliotti & Duarte in press).

The different species vary in size from small to medium but they share morphological characters in common, such as the presence of spiked antlers in males, and the overall skull morphology. However, pelage colouration varies greatly from light brown, reddish brown to grey, and the body size ranges from less than 10 to 40 kg (Duarte & Jorge 1996; Duarte & Merino 1997).

The genus *Mazama* is highly diverse and is composed of 10 species with a wide geographical range from southern Mexico to Argentina (Weber & González 2003; Wilson & Reeder 2005). The recent discovery (or rediscovery) of

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three new species has raised considerable interest in the taxonomic status and phylogenetic relationships of members of the genus *Mazama* (Duarte & Merino 1997; Medellín *et al.* 1998; Duarte & Jorge 2003; Duarte *el al.* 2008). There are four species of brocket deer known to occur in sympatry in the Atlantic rainforest: the red brocket deer *M. americana*, the brown brocket deer *M. gouazoubira*, the Brazilian dwarf brocket deer *M. nana*, and the recently described small red brocket deer *M. bororo* (Duarte & Jorge 2003). This species is one of the most endangered Neotropical deer inhabiting small and highly isolated fragments of the remaining Brazilian Atlantic forest (Duarte & Jorge 2003; Weber & González 2003). This area is one of the most important biodiversity hotspots (Myers *et al.* 2000) but it is quickly disappearing due to intensive habitat fragmentation by human activity. Because of the very close morphological similarity between the endangered small red brocket deer *M. bororo* and the more common red brocket deer *M. americana*, information on their distribution and demography was until recently inaccurate and very sparse.

The different species of brocket deer are morphologically nearly indistinguishable but they have striking karyotypic differences (Duarte & Jorge 1996, 2003). It was only through the karyotypical characterization that the small red brocket deer *M. bororo* was discriminated and described as a distinct species from the red brocket deer *M. americana* (Duarte & Jorge 2003). Since then, karyotyping has been proposed as one of the most reliable tools for species identification and distinguishing *M. bororo* from other sympatric species of brocket deer (Duarte & Jorge 1996, 2003; Duarte & Merino 1997; Vogliotti & Duarte in press). However, this method is impractical, since it requires the capture of individuals to collect fresh and adequate amounts of blood samples to conduct the cytogenetic technical procedures. This secretive species is extremely difficult to capture and sampling procedures can be highly stressful to the animals. Efforts to locate and capture animals can also be expensive and time-consuming. For instance, it took 2 years of intensive fieldwork to locate and capture the first wild *M. bororo* (Vogliotti & Duarte in press). This first record prompted the urgent need to explore alternative methods such as noninvasive sampling in combination with molecular genetic methods that would allow faster and more efficient strategies for detection and determination of the geographical range of this very rare and elusive species.

Molecular markers provide powerful tools with many potential applications for conservation biology and wildlife management (Kohn *et al.* 1999). For example, noninvasive faecal DNA sampling has been extremely useful in other numerous studies for identifying species and individuals in an area, evaluating distribution, sex ratio and estimating population size, without having to capture, handle or observe individuals (Paxinos *et al.* 1997; Taberlet *et al.* 1997; Kohn *et al.* 1999; Eggert *et al.* 2003; Bellemain *et al.* 2005; Smith *et al.* 2006). Another application involves the development of fast and reliable methods to identify species from shed integument and faeces (Kohn *et al.* 1999). Developing a better method to distinguish brocket deer species will enhance the conservation management strategies that target this threatened species. Genetic methods can be used to identify species more accurately when more conventional, visual techniques of species identification prove inconclusive (Smith *et al.* 2006; Rudnick *et al.* 2007; van Vliet *et al.* 2007). We used the mitochondrial cytochrome *b* gene (*cyt b*) to develop a quick, inexpensive and robust assay for discriminating among the three brocket deer species that can potentially occur in the same areas with *M. bororo* (Duarte & Jorge 2003).

We first used reference samples of known species origin to characterize the patterns of variation in the mitochondrial DNA (mtDNA) *cyt b* region, both within and among these brocket deer species. We then developed a new set of primers specifically designed from several species of *Mazama* and a system of restriction enzyme digestion of polymerase chain reaction (PCR)-amplified mtDNA that can be easily and reliably applied to distinguish the four species that can potentially occur in the same geographical range of *M. bororo*. The restriction fragment length polymorphism (RFLP) assay was then tested on mtDNA extracted from deer faeces that were collected in the potential distribution range of this species.

Reference sample

Blood samples used for marker development and for screening were collected over a 14-year period (1990–2004) and were stored at the cell bank of the Deer Research and Conservation Center at the Universidade Estadual Paulista. Sample information regarding morphology, cytogenetics, biochemical genetics and geographical location was taken into account in the selection process. We attempted to maximize the use of samples that could only potentially occur in the same areas with *M. bororo*. A sample of 49 animals with known species designations that inhabit the potential areas of sympatry was selected. The sample consisted of seven *M. americana*, 27 *M. gouazoubira*, eight *M. nana* and seven *M. bororo*.

Extraction, amplification and mtDNA sequencing

DNA extraction from tissue samples followed procedures modified from Medrano *et al.* (1990). PCR amplifications of a 480-bp fragment of the 3′ end of the *cyt b* gene were performed with primers L14724 and H15149 following conditions in Maldonado *et al.* (1995). In addition, we amplified a 660-bp fragment of the 5′ end using primers Far H and Far L and conditions previously described in Duarte *et al.* (2008). Purified PCR products were sequenced using

the ABI BigDye ready reaction kit and ran on an ABI 377 and 3100 automated sequencers (Applied Biosystems). The sequences were obtained by concatenating data from the two fragments using the internal primers L14724 and H15149 for the 3′ end of the *cyt b* and Far H and Far L for the 5′ end of the *cyt b* and totalled 934 bp in length. Sequences were analysed using Sequencher 4.1 (Gene Codes Corp.) and aligned by eye.

Molecular species identification

A total of 49 individuals from the four *Mazama* species were sequenced for both *cyt b* gene regions, yielding a 934-bp fragment. We identified five haplotypes in *M. americana* (Accession nos DQ789201, DQ789209, DQ789212, DQ789215, DQ789230), 12 in *M. gouazoubira* (Accession nos DQ789179, DQ789181, DQ789183, DQ789184, DQ789185, DQ789186, DQ789188, DQ789189, DQ789200, DQ789202, DQ789203, DQ789229), three in *M. nana* (Accession nos DQ789210, DQ789214, DQ789227) and three in *M. bororo* (Accession nos DQ789187, DQ789228, DQ789231). However, because the fragments amplified with the previously published *cyt b* primers are large, they are unsuitable for screening samples with degraded DNA from faeces. Therefore, we analysed a 934 bp fragment of the *cyt b* gene region and designed a set of internal primers that amplified a shorter fragment size of 224 bp but that included recognizable and diagnostic restriction sites that allowed us to discriminate the small red deer among the three sympatric species. The two internal primers (IDMAZ224 L 5′-CATCCGACACAATAACAGCA-3′ and IDMAZ H 5′-TCCTACGAATGCTGTGGCTA-3′) were designed using the program Primer 3 (Rozen & Skaletsky 1996). PCR amplifications were performed in a final volume of 25 μL and contained 1× Invitrogen buffer, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 1 pM/μL of each primer, 1 U *Taq* DNA Polymerase (Invitrogen), and approximately 50–100 ng of DNA. The thermal profile included an initial denaturation step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 58 °C for 30 s, and 72 °C for 50 s. A final extension step of 72 °C for 7 min concluded the profile.

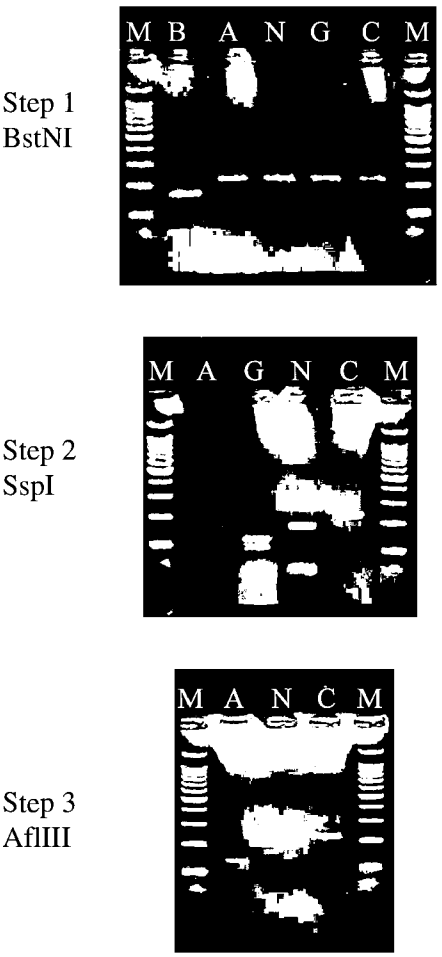

Fig. 1 A gel image of *cyt b* PCR-RFLP profiles for the four brocket deer species under consideration. In step 1, the *cyt b* fragment is digested with *BSTNI* in the small red brocket deer sample in lane B. Uncut PCR products are indicated by A, the red brocket deer; G, brown brocket deer; N, Brazilian dwarf brocket deer; and C, positive PCR control (224 bp). The ladder is indicated by M. In step 2, the *cyt b* fragment is digested with *Ssp*I and the brown brocket deer sample is assigned in lane G. In step 3, the *cyt b* fragment is digested with *Afl*III and the red brocket deer is discriminated in lane A from the Brazilian dwarf brocket deer sample in lane N.

Developing a method for species identification

The 224-bp fragment amplified using primers IDMAZ224 L and IDMAZ H contains an *ECORII* or *BSTNI* digestion site in position 160 that is unique to the endangered *M. bororo*. As expected, samples from *M. bororo* yielded a clear band of 160 bp and one faint band of 64 bp in a 2% agarose gel (Fig. 1, step 1). We also identified two other diagnostic sites using *Ssp*I enzyme. One digestion site was located in position 100 only in *M. gouazoubira* (expected band sizes = 100/124), and another restriction site for this same enzyme was located in position 176 in the red brocket *M. americana* and the Brazilian dwarf brocket *M. nana* (expected band sizes = 176/48) (Fig. 1, step 2). Finally, in order to distinguish from red and Brazilian dwarf brockets, an *Afl*III digestion site was identified in position 122 only in the red brocket (expected band sizes = 122/102) and is absent in the dwarf brocket (Fig. 1, step 3).

In order to test the reliability of the newly designed primers and the RFLP reactions, we tested the DNA of the entire reference sample (49 individuals representing four species), and we had a 100% PCR amplification rate and accuracy rate of species determination. We then conducted field surveys in order to collect deer faecal samples in the

Sample		*N*	PCR
Percentage of total sample amplified		228/238 (96%)	224 bp
Genotyped species	*M. bororo*	108/110 (98%)	160–64 bp *BSTNI/ECORII*
	M. gouazoubira	60/62 (97%)	100–124 bp *Ssp*I
	M. americana	26/29 (89%)	176–48 bp *Ssp*I
	M. nana	19/19 (100%)	122–102 bp *Afl*III

Table 1 The detailed number of individuals of each *Mazama* species amplified including the percentage successfully genotyped for all of the samples and for each species. The expected sizes of the digested *cyt b* fragments in the four species are given in number of base pairs (bp) followed by the restriction enzyme that produced the fragments

protected areas from the south and southeast regions in Sao Paulo and Paraná States of Brazil. We collected 238 faecal samples with the aid of a dog specifically trained to find deer scat. Faecal DNA extractions were performed using a DNeasy kit (QIAGEN). Extractions were carried out in a separate pre-PCR room to prevent contamination.

We then tested the reliability and effectiveness of this new protocol on all deer scat samples. In all reactions, we included an extraction negative control to detect any possible contamination during the DNA extraction and a control without DNA to detect contamination during the PCR stage. Of the 238 samples collected, 228 (96%) were successfully amplified (Table 1). We detected no evidence of contamination during the extraction and the PCR stages.

The PCR-RFLP reaction was successfully used to genotype 210 samples. From these, 108 samples were identified as small red brocket deer *M. bororo*, 60 samples were identified as grey brocket deer, 26 as red brocket deer, and 19 as the Brazilian dwarf brocket deer. Eighteen samples resulted in very weak PCR amplifications due to the poor quality of the isolated faecal DNA and the products were not strong enough to visualize using the RFLP reactions. We therefore performed cycle sequencing reactions on these and 10 of them resulted in good sequences. Analysis of the sequence data for the 224-bp fragment of these 10 samples resulted in two *M. bororo*, two *M. gouazoubira*, and six *M. americana*. Furthermore, in one of the two sequences that were determined to be *M. bororo*, we detected a transition mutation (A/G) in the recognition site for the enzyme explaining the RFLP failure. This mutation, however, was only present in 0.9% of the sample and suggests that when using this protocol, a small number of samples that do not digest for any of the three restriction enzymes may need to be sequenced for confirmation and validation.

In addition to the four species of brocket deer, we used these new primers to test PCR amplifications and sequences from faecal samples of other endangered Neotropical deer species such as the pampas deer (*Ozotoceros bezoarticus*: *DQ789190, DQ789191, DQ789192, DQ789193, DQ789194, DQ789195, DQ789196, DQ789197, DQ789198, DQ789199*), the huemul (*Hippocamelus bisulcus*: *DQ789177, DQ789178*), the marsh deer (*Blastoceros dichotomus*: *DQ789173, DQ789174, DQ789175, DQ789176*) as well as for the introduced Asiatic axis deer (*Cervus axis*: *AY456910.1*) and the domestic sheep (*Ovis aries*: *DQ903227.1*). Sequence analysis of this *cyt b* fragment demonstrated enough informative variation to discriminate among these nine species tested using a simple BLAST (Altschul *et al.* 1990)-based comparison.

The combination of noninvasive field sampling methodologies with quick, reliable and economic molecular tools such as the one we have developed here will help provide necessary information for management and conservation strategies for this endangered species. Since *Mazama* is one of the most poorly known Neotropical deer genera with the highest species diversity in the region, this methodological approach can be potentially useful to discriminate among species in other geographical areas throughout their distribution range. Further research is needed to measure the prospective of this genetic approach to explore the scantily known Neotropical deer diversity and to help determine and map the distribution of rare and elusive brocket deer species.

Acknowledgements

Our study was funded by FAPESP, CNPq, PROBIO/MMA, from Brazil, CSIC-UdelaR; PEDECIBA, from Uruguay, and the Smithsonian Center for Conservation and Evolutionary Genetics, Friends of the National Zoo and Wildlife Trust Alliance. We thank the 'Instituto Brazileiro do Meio Ambiente e dos Recursos Naturais Renováveis' and Secretaria do Meio Ambiente do Parana for granting permits for sample collections. M. Cosse assisted in laboratory techniques and K. Maldonado provided comments that greatly enhanced the quality of this manuscript.

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