Transferability of microsatellite loci from exotic Cervidae to Brazilian brocket deer (*Mazama* spp, Mammalia: Cervidae)

A.M.B. Mantellatto, E.A.G. Carnelossi and J.M.B. Duarte

Departamento de Zootecnia, Núcleo de Pesquisa e Conservação de Cervídeos,
Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal,
Universidade Estadual Paulista, Jaboticabal, SP, Brasil

Corresponding author: A.M.B. Mantellatto
E-mail: alinemeira22@hotmail.com

ABSTRACT. Transferability of microsatellite loci between closely related species has been reported in several species. This helps reduce costs involved with the development of primers for newly investigated species. Fifteen microsatellite primers developed for *Rangifer tarandus*, *Cervus elaphus*, *C. axis*, and *Moschus berezovskii* were tested on five species of Brazilian brocket deer of the genus *Mazama* (*M. americana*, *M. bororo*, *M. gouazoubira*, *M. nana, and M. nemorivaga*). These primers were tested with DNA extracted from blood samples of two individuals of each species obtained from the Núcleo de Pesquisa e Conservação de Cervídeos (NUPECCE) of Universidade Estadual Paulista (UNESP). Fourteen of the 15 primers tested amplified microsatellite regions of all five species of *Mazama*, confirmed by sequencing of the amplified fragments. We conclude that these primers could be used for population studies of brocket deer.

Key words: Microsatellite; Heterologous primers; Brocket deer; *Mazama*

INTRODUCTION

Mazama is the second most numerous genus within the deer of the world (Duarte and Merino, 1997). From cytogenetic (Duarte and Merino, 1997) and morphological (Rossi, 2000) evidence, five species are recognized in Brazil: *M. americana*, *M. bororo*, *M. gouazoubira*, *M. nana*, and *M. nemorivaga*. The distribution of the genus extends from southern Mexico to central Argentina, where they occupy various types of habitat such as mountain forests, lowland rain forests and savanna. *Mazama* occur in almost the entire national territory of Brazil (Eisenberg and Redford, 1999). Illegal hunting and increased destruction of habitat threatens the survival of significant populations of species such as *M. nana* and *M. bororo* (IBAMA, 2003; Abril and Duarte, 2008; Duarte, 2008). These conditions usually lead to a decrease in genetic variability due to the absence of gene flow between different populations and due to the effects of genetic drift, which can dramatically reduce the genetic diversity of species (Chesser, 1983), making them more susceptible to environmental changes. Microsatellite loci have been primarily used for the detection of fluctuations in genetic variability. Transferability of microsatellite loci between related species is possible due to the conservation of flanking regions of simple repetitive sequences (Schlötterer and Pemberton, 1994; Rafalski et al., 1996; Ferreira and Grattapaglia, 1996; Zane et al., 2002). This feature allows reduction in labor and costs in the development of primers for each species of interest (Ferreira and Grattapaglia, 1996). Conservation of microsatellite loci between closely related species has been demonstrated in cows, sheep (Moore et al., 1991), primates (Morin and Woodruff, 1992), dogs (Roy et al., 1994), cetaceans (Schlötterer et al., 1991), and turtles (FitzSimmons et al., 1995). The family Cervidae is one of the most studied, by means of microsatellite markers, in the order Artiodactyla. The main factor for this large volume of studies is the extensive number of primers described.

A large number of studies have used primers of mainly bovine origin (*Bos* sp) (Oliveira, 2005), indicating the conservation of both the flanking regions of the primer*s* and the microsatellite region. Within this context, this study aimed to select heterologous primers for the five Brazilian brocket deer species. We selected 15 microsatellite loci described in the literature for the species *Rangifer tarandus* (8), *Moschus berezovskii* (5), *Cervus axis* (1), and *C. elaphus canadensis* (1). We obtained a set of microsatellite loci with the potential application for characterization of genetic variability and for population studies in Brazilian species of *Mazama*.

MATERIAL AND METHODS

Animals

Pairs of each species, *M. americana*, *M. bororo*, *M. gouazoubira*, *M. nana*, and *M. nemorivaga*, housed at the Núcleo de Pesquisa e Conservação de Cervídeos (NUPECCE) of São Paulo State University (UNESP), were used for this experiment.

DNA extraction and purification

DNA was extracted from blood cells by digestion with proteinase K, purification with phenol-chloroform and precipitation with absolute ethanol (Sambrook et al., 1989).

Markers tested

Fifteen primers were tested (Table 1): 8 developed for *R. tarandus*, 5 for *M. berezovskii*, 1 for *C. axis*, and 1 for *C. elaphus canadensis*, selected from the literature with the criteria of close phylogenetic linkage, structure of microsatellite and transferability to other deer species.

Table 1. Characteristics of the 15 primers and their respective microsatellite loci, tested in five Brazilian species of the genus *Mazama*.

Primer	Concentration	Repeat motif	Species	Temperature (°C)	Observed size (bp)	Expected size (bp)
CA71[1]	0.1 µM	$(CT)_{12}$	*Cervus axis*	58	300-310	308-318
Mber25F[2]	1.0 µM	$(AC)_{15}$	*Moschus berezovskii*	-	-	166-186
Mber39B[2]	1.0 µM	$(AC)_{16}$	*Moschus berezovskii*	49	180-210	199-235
Mber43B[2]	1.0 µM	$(AC)_{20}$	*Moschus berezovskii*	53	200-210	204-238
Mber710B[3]	15.0 µM	$(AC)_{24}$	*Moschus berezovskii*	55.5	180-200	175-194
Mber99B[3]	15.0 µM	$(CA)_{27}$	*Moschus berezovskii*	58	270-280	285-311
NVHRT16[4]	0.02 µM	$(CA)_5TA(CA)_5$ $(TG)_2CG(CA)_{19}$	*Rangifer tarandus*	57	180-200	152-192
RT01[5]	0.16 µM	$(GT)_{22}$	*Rangifer tarandus*	55	220-230	222-240
RT05[5]	0.16 µM	$(GT)_{22}$	*Rangifer tarandus*	55.5	150-160	143-171
RT06[5]	0.16 µM	$(GT)_{23}$	*Rangifer tarandus*	55	120-130	105-117
RT07[5]	0.16 µM	$(GT)_{18}$	*Rangifer tarandus*	55	190-200	216-234
RT09[5]	0.16 µM	$(GT)_{21}$	*Rangifer tarandus*	55	120-130	116-128
RT13[5]	0.16 µM	$(GT)_{13}$	*Rangifer tarandus*	55	290-295	293-314
RT30[5]	0.16 µM	$(GT)_{21}$	*Rangifer tarandus*	55.5	200-220	190-220
T156[6]	0.3 µM	TAGA	*Cervus elaphus canadensis*	58	110-120	134-234

[1]Gaur et al. (2003), [2]Xia et al. (2006), [3]Zou et al. (2005), [4]Roed and Midthjell (1998), [5]Wilson et al. (1997), [6]Jones, et al. (2002). Temperature (°C), annealing temperature standardized for *M. americana, M. gouazoubira, M. bororo, M. nana*, and *M. nemorivaga*.

The polymerase chain reaction (PCR) conditions were standardized to a final volume of 15 µL containing 10 mM *Taq* buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM MgCl$_2$), 120 µM dNTP, 0.3 U *Taq* polymerase, 45 ng genomic DNA and 0.08 µM of each primer. The concentration of each primer is described in Table 1. The amplifications for 13 primers (RT01, RT05, RT06, RT07, RT09, RT13, RT30, Mber99B, Mber43B, Mber39B, T156, NVHRT16, CA71) were performed in a T1 Thermocycler under the following conditions: 94°C for 5 min, 94°C for 1 min, 49º to 58°C for 1 min (according to each primer), 72°C for 1 min and 72°C for 10 min. The amplification for primer Mber710B was carried out in a gradient thermocycler. Despite the success obtained for the 14 loci, the standardization of PCR conditions for the primer Mber25F was not achieved due to the occurrence of many nonspecific fragments, and therefore, this primer was discarded. The locus Mber39B showed the amplification of a few nonspecific regions, and therefore, excision of the fragment of interest was performed, in order to obtain only the region that was the specific size of the microsatellite described for this primer. To achieve this, the elution and reamplification of the fragment were performed from the electrophoresis of the initial PCR product, on a 2% low-melting gel. The band of interest was excised, and placed in a tube with 300 µL water. This tube was then placed in a water bath at 73°C for 20 min. Finally, a new PCR was carried out, using 3 µL of the eluted product, obtaining only the fragment of interest, which was confirmed by visualization of this product on a 2% agarose gel.

The amplified products of the 14 primers were separated by electrophoresis on a 2% agarose gel. Next, the PCR products were applied to a 10% denaturing polyacrylamide gel, stained with ethidium bromide and analyzed. From the visualization of the amplified fragments on agarose and polyacrylamide gels, it was possible to determine whether the size of the amplified product coincided with the size of the microsatellite locus described in the literature for the primer concerned.

The products were purified by the removal of nucleotides with exonucleases. For this, 0.5 μL *Exo*I, 0.5 μL shrimp alkaline phosphatase (SAP), 1.0 μL SAP buffer, 1.0 μL water, and 7 μL PCR product were placed in each tube. The solutions were placed in the thermocycler: 37°C for 1 h, 80°C for 10 min and finally 4°C for an undetermined time. Next, the samples were sequenced in the ABI PRISM® 3100 Applied Biosystems automatic sequencer. The sequencing reaction was performed with the BI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit. The sequences obtained were analyzed using the Sequence Scanner V1.0 program, to compare the amplified regions with those deposited in GenBank for the species of origin. This made it possible to confirm that the primers amplified the microsatellite locus of the original species and to see the levels of homology both in the microsatellite region and the flanking regions.

RESULTS

From the sequencing of a sample of the amplification product of each of the 14 primers, the amplification of the same microsatellite region of the original species for which the primer was developed in the five Brazilian species of the genus *Mazama* was confirmed.

DISCUSSION

There have been many studies carried out on the transferability of heterologous primers in various species, including Artiodactyla (Engel et al., 1996), Canidae (Gotelli et al., 1994), Mustelidae (O'Connell et al., 1996), Cetacea (Valsecchi and Amos, 1996), Pinnipedia (Coltman et al., 1996), and Primates (Kayser et al., 1996). According to a study by Barbará et al. (2007), the highest percentage of amplification of polymorphic heterologous markers is observed between species of the same genus, in reptiles, birds, mammals, and invertebrates. Transferability between species of the same family shows less significant results for invertebrates and birds, when compared to mammals and reptiles (over 40% of successful transfer in mammals, over 25% in fish, and over 10% in birds).

Of the 15 primers selected for this study, 14 amplified the region of interest in the five Brazilian species of the genus *Mazama*. Although the primers selected were not developed for *Mazama*, but for other species of the same family (Cervidae), transferability was high, much higher than the minimum expected of 40% (Barbará et al., 2007).

Transferability obtained for 93.3% of animals is a result that can be considered significant. We attributed this success to the criteria used for selection of markers, which provided us with primers with the potential for use in the study species.

The molecular analysis results indicate that *Mazama* is a polyphyletic genus and, therefore, not a valid genus (Pitra et al., 2004; Gilbert et al., 2006; Duarte et al., 2008). The red brockets differ from grey brockets by at least 5 million years, which suggests an important molecular difference (Duarte et al., 2008). Even so, the primers showed homology in different species of *Mazama*, which suggests the conservation of these regions analyzed, making them

potentially important in studies with other South American deer genera that are closely related to the grey brockets (*M. gouazoubira* and *M. nemorivaga*), such as *Blastocerus*, *Ozotoceros* and *Hippocamelus*, or close to the red brockets (*M. americana*, *M. bororo*, *M. nana*), such as *Odocoileus* (Duarte et al., 2008).

The sequencing of the amplified regions settles any doubt about their constitution and their similarity with the regions described in the original species, guaranteeing that these regions were truly conserved during the cervid evolutionary process. This is the case in both the region of the primer and the microsatellite region itself.

The reason the locus Mber25F did not produce positive results is possibly due to the occurrence of a mutation in the binding site of the primer in the common ancestor of the Brazilian species, since this locus was developed for *Moschus berezovskii*, a species that displays 13.5 million years of evolutionary divergence with the genus *Mazama* (Gilbert et al., 2006). However, other primers developed for *M. berezovskii* amplified the microsatellites of *Mazama*, which demonstrates a high level of conservation of the flanking regions, where the primer sequences are situated.

The description of this panel of primers for studies in Brazilian deer opens an immense field for population studies in these species, some threatened with extinction (IBAMA, 2003). This has the potential to help these endangered populations in both the diagnosis of problems, as well as in making recommendations regarding management for their maintenance.

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