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# Cross-amplification of nonspecific microsatellites markers: a useful tool to study endangered/ vulnerable species of southern Andes deer

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**ABSTRACT.** Thirty-nine microsatellite loci that are highly conserved in red deer, sika deer, reindeer, Soay sheep, and other artiodactyls were tested in two vulnerable and endangered Neotropical deer (pudu: *Pudu puda* and huemul: *Hippocamelus bisulcus*) with the aim of producing a standardized set of markers that can be used successfully in noninvasive samples from these species. We also compared these nonspecific loci against eight polymorphic loci that were recently developed for huemul to determine whether the nonspecific markers could reflect the huemul's genetic variation that was observed with the specific loci. We identified 10 suitable loci, six of which constitute a standardized set for the two species and can be used to identify them in the absence of phenotypic data. The expected heterozygosity per locus for the panel of

 

six loci ranged from 0.461 to 0.889 (average 0.665), and the maximum probability of identity value was 6.9 x $10^{-6}$ and 3.2 x $10^{-4}$ in pudu and huemul, respectively. This set of loci has potential applications in evolutionary, ecological, forensic, and conservation studies in pudu and huemul.

**Key words:** Cervidae; Huemul; Pudu; Short tandem repeat; Nonspecific microsatellites; Species genetic determination

## INTRODUCTION

Microsatellite markers are highly polymorphic nuclear DNA loci, which carry valuable information about species' evolutionary history. Although these markers have been isolated for multiple species, it is typical to isolate new markers for genetically undescribed taxa. This is a costly and time-consuming process. The cross-amplification of nonspecific markers (i.e., markers designed for a different species but can be amplified in a taxon of interest) may reduce genotyping costs, allowing the development of population genetic studies in otherwise uncharacterized taxa. A high rate of cross-amplification has already been reported for many animal species, such as tortoises (Orozco-terWengel et al., 2013), apes (Deka et al., 1994), canids (Fontoura-Rodrigues et al., 2008), and ungulates (Leite et al., 2007; Shafer et al., 2012). The rate of cross-amplification in Artiodactyla is surprisingly high, even between different families (e.g., Cervidae and Bovidae), indicating high genome similarity (Leite et al., 2007).

For conservation biology, genetic data can be a useful tool to determine a specie's threat status. For this purpose, the successful amplification of microsatellite markers from noninvasive samples (e.g., faeces) is of utmost importance for species genetic characterization when the taxa are elusive, rare, or cannot be approached for invasive sample collection (e.g., blood). The cross-amplification of microsatellites has been shown using canid fecal samples (Fontoura-Rodrigues et al., 2008) and invasive samples in deer (Cosse et al., 2007; Leite et al., 2007). In the case of pudu and huemul, there is no information about the cross-amplification of polymorphic loci. Only eight microsatellites have been reported in huemul (Shafer et al., 2012), and none have been reported for pudu.

*Hippocamelus bisulcus* (huemul) is the world's southernmost large deer species, and it inhabits southern Chile and Argentina's Andean forest and Patagonian fjords. Huemul is an endangered species (IUCN, 2012) mostly due to habitat loss and population fragmentation. This is exacerbated by the consequent population isolation, poaching, and disturbance and predation by domestic dogs. This condition has led to a contraction of its historic distribution (34 to 54°S latitude) over the last 200 years. Currently, a population of 1000-2000 extant huemul inhabits the far south of Chile and Argentina (Serret, 1992) with the exception of a single isolated population that occurs between 36 and 37°S (central Chile) at Nevados de Chillán (Povilitis, 1998). Nevados de Chillán is likely a refugial population whose genetic richness is yet unknown.

*Pudu puda* (pudu) is one of the smallest deer in the world, with adults reaching just 40 cm in height and weighing less than 10 kg (Silva-Rodríguez et al., 2010). Pudus are solitary, humid forest dwellers that are endemic to the Andean lowlands (Wemmer, 1998). Their geographical distribution ranges from 36 to 49°S in Chile and from 39 to 43°S in Argentina

 

(Meier and Merino, 2007), where approximately 10,000 animals live (Wemmer, 1998). Alterations in the habitat (the replacement of native trees and shrubs by plantations of introduced trees), death from feral dogs, frequent poaching, and introduction of non-native deer species (such as red deer, fallow deer, and roe deer) are the main causes of the steady decline of pudu's wild populations (Hershkovitz, 1982). The pudu is categorized as vulnerable on the IUCN Red List (IUCN, 2012), and it is included in Appendix I of the Convention on International Trade of Endangered Species of Wild Fauna and Flora.

This study aimed to test a panel of microsatellites that were isolated in deer, sika deer, reindeer, Soay sheep, and other artiodactyls (Slate et al., 1998; Wilson and Strobeck, 1999; Cronin et al., 2006) in pudu and huemul and to compare nonspecific microsatellite loci against huemul-specific loci. This was performed in order to identify loci that i) successfully amplify, ii) are polymorphic in both species, and iii) generate short amplification products (<350 bp), facilitating their use in DNA that is obtained from feces and hairs. We evaluated 39 microsatellites with forward primers containing a 5′-M13 tail (Boutin-Ganache et al., 2001). Both species are partially co-distributed in the temperate forests in Chile and Argentina and are lesser-known South American deer. Therefore, it is important to produce a standardized set of markers that can be successfully used in noninvasive samples from these species.

## MATERIAL AND METHODS

Huemul and pudu samples were collected throughout their current distribution range (Table 1) following guidelines of the American Society of Mammalogists (Sikes et al., 2011). DNA samples were obtained using three methods: 1) skin samples from adults obtained with biopsy darts (Dan-Inject Biopsy Needle, Børkop, Denmark), 2) muscle or skin tissue from dead animals found during field work, and 3) blood samples following chemical immobilization from wild-caught freeranging individuals that were sent to wildlife rehabilitation centers.

**Table 1.** Summary of the samples used in the analyses.

| Location, Country | Geographic position | Samples |
|---|---|---|
| *Pudu puda* | | N = 44 |
| Hualqui, Región del Bío-Bío, Chile | 36°57'10"S, 72°55'20"W | 1 |
| Coihueco, Región del Bío-Bío, Chile | 36°38'00"S, 71°45'50"W | 2 |
| Pucón, Villarrica National Park, Región de la Araucanía, Chile | 39°15'50"S, 71°54'00"W | 6 |
| Loncoche, Región de la Araucanía, Chile | 39°22'00"S, 72°40'60"W | 2 |
| Valdiva, Región de los Ríos, Chile | 39°49'10"S, 73°11'50"W | 23 |
| Puyehue, Puyehue National Park, Región de los Ríos, Chile | 40°43'20"S, 72°19'40"W | 4 |
| Chiloé, Chiloé National Park, Región de los Lagos, Chile | 42°37'30"S, 74°06'00"W | 6 |
| *Hippocamelus bisulcus* | | N = 55 |
| Río Simpson National Reserve, Región de Aysen, Chile | 45°36'07"S, 72°12'52"W | 5 |
| Cerro Castillo, National Reserve, Región de Aysen, Chile | 45°58'55"S, 71°55'43"W | 7 |
| Lago Cochrane National Reserve, Región de Aysen, Chile | 47°13'06"S, 72°29'46"W | 12 |
| Tortel cove, Región de Aysen, Chile | 47°49'51"S, 73°18'23"W | 5 |
| Bravo river, Región de Aysen, Chile | 48°02'37"S, 73°01'04"W | 5 |
| Bernado lagoon, B. O'Higgins National Park, Región de Aysen, Chile | 48°35'33"S, 73°54'28"W | 13 |
| Torres del Paine National Park, Región de Magallanes, Chile | 51°07'43"S, 73°07'07"W | 8 |

Genomic DNA was extracted from blood or tissue samples with a phenol/chloroform protocol and from fresh faeces using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia,

CA, USA). Thirty-nine autosomal dinucleotide microsatellite loci were tested in both species (Table 2). After establishing the polymerase chain reaction (PCR) product size, the primers where labelled with FAM, NED, or HEX fluorescent dyes for multiplexing. PCRs were performed in 10 µL containing 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.2 µM reverse primer and fluorescent M13 primer, 0.013 µM forward primer, 0.25 U *Taq* polymerase (Invitrogen), and 10-50 ng DNA. The conditions were the same for all loci to maximize standardization. PCR profiles included initial denaturing at 94°C for 3 min; 10 touchdown cycles of 94°C for 45 s, annealing at 60°-50°C (-1°C/cycle) for 45 s, and 72°C for 1 min 30 s; 30 additional cycles with constant annealing temperature at 50°C; and final extension at 72°C for 30 min. PCR products were genotyped using an ABI Prism 377 or 3100 semi-automated DNA analyzer.

**Table 2.** Cross-amplification and characterization of 39 dinucleotide microsatellites in pudu and huemul.

| Locus | Reference | *Pudu puda* | | *Hippocamelus bisulcus* | |
|---|---|---|---|---|---|
| | | No. of alleles | Observed size (bp) | No. of alleles | Observed size (bp) |
| BBJ2 | Wilson and Strobeck (1999) | 6 | 185-195 | 4 | 187-195 |
| BBJ11 | Wilson and Strobeck (1999) | 5 | 144-152 | 2 | 195-201 |
| BBJ24 | Wilson and Strobeck (1999) | 2 | 276-278 | * | - |
| RT7 | Wilson et al. (1997) | 2 | 153-155 | 2 | 223-225 |
| RT5 | Wilson et al. (1997) | * | - | 6 | 184-212 |
| RT10 | Wilson et al. (1997) | * | - | * | - |
| RT13 | Wilson et al. (1997) | * | - | 2 | 300-302 |
| RT27 | Wilson et al. (1997) | 5 | 172-182 | 6 | 194-204 |
| RT30 | Wilson et al. (1997) | 16 | 187-223 | 2 | 212-214 |
| BM121 | Bishop et al. (1994) | * | - | 1 | 152 |
| BM848 | Bishop et al. (1994) | * | - | 1 | 227 |
| BM6506 | Bishop et al. (1994) | 11 | 205-229 | 3 | 205-213 |
| BM6438 | Bishop et al. (1994) | 1 | 270 | 2 | 271-273 |
| BM1225 | Bishop et al. (1994) | 1 | 264 | 2 | 231-235 |
| BM203 | Bishop et al. (1994) | 10 | 244-264 | 4 | 234-240 |
| BM4025 | Bishop et al. (1994) | * | - | 1 | 146 |
| BM4107 | Bishop et al. (1994) | * | - | 1 | 185 |
| ABS012 | Bishop et al. (1994) | 1 | 150 | * | - |
| BL25 | Bishop et al. (1994) | 1 | 196 | 5 | 194-202 |
| ABS012 | Bishop et al. (1994) | 1 | 150 | * | - |
| BL6 | Grosz et al. (1997) | * | - | 2 | 198 |
| Cervid 1 | DeWoody et al. (1995) | * | - | * | - |
| Cervid 2 | DeWoody et al. (1995) | * | - | * | - |
| Cervid 3 | DeWoody et al. (1995) | 2 | 330-332 | * | - |
| Cervid 14 | DeWoody et al. (1995) | 8 | 226-262 | 7 | 202-240 |
| INRA011 | Vaiman et al. (1992) | * | - | 2 | 203-205 |
| INRA 107 | Vaiman et al. (1992) | * | - | * | - |
| INRA 121 | Vaiman et al. (1992) | * | - | * | - |
| NVRT 16 | Røed and Midthjell (1998) | 1 | 220 | * | - |
| NVRT 22 | Røed and Midthjell (1998) | 2 | 166-168 | * | - |
| NVHRT 30 | Røed and Midthjell (1998) | * | - | * | - |
| N | Jones et al. (2000) | 9 | 337-383 | 7 | 299-345 |
| Q | Jones et al. (2000) | * | - | 2 | 271-275 |
| BDC01 | Oliveira et al. (2008) | * | - | * | - |
| BDC04 | Oliveira et al. (2008) | * | - | * | - |
| BDC15 | Oliveira et al. (2008) | 2 | 150-152 | * | - |
| BDC28 | Oliveira et al. (2008) | * | - | * | - |
| BDC65 | Oliveira et al. (2008) | 1 | 148 | 1 | 147 |
| CSS043 | Moore et al. (1994) | * | - | * | - |

*No amplification.

For each sampled population of the two focal species, we tested the Hardy-Weinberg (HW) equilibrium, linkage disequilibrium (LD), and probability of identity (PI) using Ge-

 

nAlEx 6.4.1 (Peakall and Smouse, 2006). Finaly, in huemul, we also compared the amplification results of seven nonspecific microsatellite loci against eight polymorphic loci that were recently developed for the species (Shafer et al., 2012).

## RESULTS AND DISCUSSION

We initially tested 10 individuals of each species from different localities, and markers with adequate PCR product sizes were screened in 44 pudu samples and 55 huemul samples (Table 1). This screening process yielded six loci that showed all of the desired characteristics (Table 3). Four other microsatellites (two for each species) were found to be potentially useful for population studies despite not meeting all the target criteria for all species. Loci RT30 and BBJ11 presented the highest levels of allelic richness in pudu (A = 16, $H_E$ = 0.904, and $H_O$ = 0.487 and A = 5, $H_E$ = 0.692, and $H_O$ = 0.571, respectively) but low polymorphism in huemul. The RT5 locus presented moderate levels of allelic richness in huemul (A = 6; $H_E$ = 0.736; $H_O$ = 0.181), but it was not amplified in pudu. Finally, locus BL25 was monomorphic in pudu and moderately polymorphic in huemul (Table 2). Consequently, we suggest that although these loci may not be ideal for studies where non-invasive samples from both species are analyzed simultaneously, they should be considered for species-specific analyses (Table 2).

**Table 3.** Genetic diversity measures of the common set of six microsatellites in pudu and huemul.

| Locus | *Pudu puda* | | | | | *Hippocamelus bisulcus* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | A | Size (bp) | $H_E$ | $H_O$ | N | A | Size (bp) | $H_E$ | $H_O$ |
| BBJ2 | 44 | 6 | 185-195 | 0.684** | 0.651 | 52 | 4 | 187-195 | 0.505* | 0.615 |
| RT27 | 14 | 5 | 172-182 | $0.889^{ns}$ | 0.400 | 54 | 6 | 194-204 | 0.552** | 0.333 |
| BM6506 | 42 | 11 | 205-229 | $0.812^{ns}$ | 0.785 | 26 | 3 | 205-213 | 0.645** | 0.080 |
| BM203 | 30 | 10 | 244-264 | 0.876** | 0.620 | 55 | 4 | 234-240 | $0.522^{ns}$ | 0.436 |
| Cervid 14 | 24 | 8 | 226-262 | 0.546* | 0.500 | 44 | 7 | 202-240 | 0.688** | 0.250 |
| N | 39 | 9 | 337-383 | 0.739* | 0.717 | 49 | 7 | 299-345 | 0.461** | 0.142 |

N = sample size; A = number of observed alleles; size range of the PCR products (Size), $H_E$ = expected heterozygosity; $H_O$ = observed heterozygosity, for microsatellite loci in two southern Andes deer. Asterisks in the $H_E$ column indicate loci that exhibited departure from Hardy-Weinberg equilibrium ($P < 0.05$) in at least one population of the indicated species (ns = not significant; *$P < 0.05$; **$P < 0.001$).

No significant LD was detected among any of the pairwise comparisons of loci in either of the two species. This supports the hypothesis that these loci segregate independently in each species' genome. Three loci presented a significant deviation from HW equilibrium after Bonferroni's correction (pudu: RT27 and BM6506; huemul: BM203), but it is likely that this observation reflects an underlying pattern of population structure that was not yet assessed in these samples. The estimated PI values in the analysis of the six loci simultaneously were $6.9 \times 10^{-6}$ in pudu and $3.2 \times 10^{-4}$ in huemul (Figure 1), indicating a high probability of successful individual identification using these markers. The mean number of alelles per locus was 8.87 ± 1.67 and 5.25 ± 1.64, and the mean expected heterozygosity was 0.759 and 0.570 in pudu and huemul, respectively. The average expected heterozygosity for all six loci was 0.665, and the mean polymorphism information content value was 0.605. No significant differences in expected heterozygosity were found between the two species using the six loci (Fisher's exact test P = 1).

 

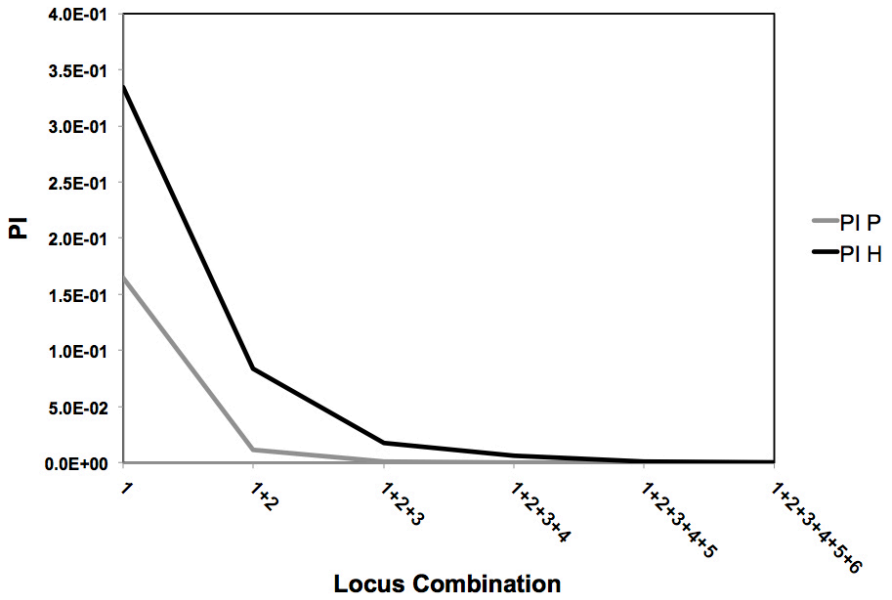


**Figure 1.** Probability of identity (PI) in *Pudu puda* (PI P) and *Hippocamelus bisulcus* (PI H) using the selected panel of six microsatellite loci.

Additionally, we compared eight huemul-specific microsatellites against seven loci that were developed in other ungulates. The goal of this comparison was to determine whether the markers that were developed in other ungulates presented similar levels of polymorsphism as the huemul-specific markers. Interestingly, these two sets of loci did not differ statistically in terms of expected heterozygosity (Welch *t*-test P = 0.9). Consequently, we suggest that these seven markers can be used to complement the eight huemul-specific loci to increase the marker set that is available for this species.

The markers that were identified here show great potential for population and evolutionary studies of Neotropical deer, including the use of noninvasive sampling and comparative analyses across species. This set of primers proved to have sufficient levels of polymorphism and heterozygosity to differentiate between pudu and huemul on the basis of the alleles that were found in each species. Furthermore, the availability of a common set of polymorphic markers in these species allows testing of the population genetic parameters that are required to establish the conservation status of natural populations without needing to develop species-specific markers. We also showed that the differences in allele frequency distributions between these markers have value for forensic cases when species determination is necessary in the absence of phenotypic data (e.g., Marín et al., 2009). These microsatellite markers will be useful for resolving conservation problems related to management programs for these southern Andes deer.

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