

# Non-invasive genetic sampling of deer: a method for DNA extraction and genetic analysis from antlers

## Muestreo genético no invasivo en ciervos: Un método para extracción de DNA y análisis genético a partir de las astas

Carlos Venegas<sup>1</sup>, Valeria Varas<sup>1, 2</sup>, Juan Pablo Vásquez<sup>1, 3</sup> & Juan Carlos Marín<sup>1\*</sup>

<sup>1</sup>Laboratorio de Genómica y Biodiversidad, Facultad de Ciencias, Universidad del Bío-Bío, Chillán, Chile.

<sup>2</sup>Instituto de Ciencias Ambientales & Evolutivas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile.

<sup>3</sup>Departamento de Zoología, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile.

\*E-mail: jcmarin@ubiobio.cl

### ABSTRACT

We describe a non-invasive technique to isolate genomic DNA from connective tissue present in the antlers of deer of the genus *Hippocamelus*. This method is simpler and more effective than conventional more-destructive procedures which damage the collection material. This method is applicable to all cervids that annually regenerate their antlers.

**KEYWORDS:** conservation, genetic material, huemul, South America, taruca.

### RESUMEN

Describimos aquí una técnica no-invasiva para aislar ADN genómico desde tejido conectivo presente en astas de ciervos del género *Hippocamelus*. Este método es más efectivo y simple que procedimientos convencionales que dañan el material de colección. Este método es aplicable a todos los cérvidos que renuevan anualmente sus astas.

**PALABRAS CLAVE:** conservación, huemul, material genético, Sudamérica, taruca.

In recent decades biological studies have increasingly relied on the extraction of DNA from wide variety of tissues (Bosch *et al.* 2005). In particular, the genetic evaluation of species that are of conservation or management concern often require a reliable source of biological material for molecular analysis (Wasko *et al.* 2003). As such, population-scale studies are often limited by the difficulty in obtaining samples from sufficient numbers of individuals, especially when they have low population numbers, are difficult to find, and/or difficult to sample. It obtaining an appropriate number of samples, including non-invasive DNA samples, for population studies of endangered species whose value lies precisely in their population decline and high vulnerability.

Genetic analyses through molecular methods are often restricted by DNA availability. The quantity and quality of

the DNA obtained from non-conventional sampling, which can be increased by PCR, is the first obstacle to overcome in population studies. The procedures to obtain DNA include the use of non-invasive sampling, which allows samples to be obtained without the need to anesthetize, modify, or take part from the individual directly (Morin *et al.* 1993; Wasko *et al.* 2003; Bosch *et al.* 2005). These provide a source of DNA sufficient not just for identifying species, but also for differentiating individuals and sexes.

In large mammals, DNA samples can be obtained from fluids, hair, and feces gathered in the field. In museums, the use of skins, bones, horns, and antlers can be a valuable source of non-invasive samples for DNA extraction. However, these types of samples usually provide DNA of low quantity, quality, and integrity, which makes the analysis of some molecular

markers difficult, especially single-copy genes. In deer, different investigations have used the antlers of individuals as a source of DNA for genetic analysis (Wang & Schreiber 2001; Ludt *et al.* 2004; Kuehn *et al.* 2005).

In southern South America, the genus *Hippocamelus* is represented by two extant species: the taruca, *Hippocamelus antisensis*, d'Orbigny, 1834, inhabiting the foothills of southern Peru, western Bolivia, north-western Argentina and northern Chile and the huemul, *Hippocamelus bisulcus*, Molina, 1978, distributed in the sub-Antarctic forest southern Chile.

The huemul, the southern-most Neotropical deer, is the only large herbivore found in the Sub-Antarctic *Nothofagus* forests of Chile and Argentina (Dellafore & Maceira 2001) and the periglacial scrub of the Patagonian steppe (Díaz & Smith-Flueck 2000; CONAF-CODEFF 2001). Currently, there are restricted to only a few isolated populations. The Central Chile population of Nevados de Chillán - Laguna Laja (36° - 37° S) is geographically separated by more than 500 km from the nearest population in Argentina (in Lanin National Park), which is more than 1,200 kms from a second Chile population Southern Patagonia (41° - 54° S) (Aldridge & Montecinos 1998; López *et al.* 1998; Vila *et al.* 2006). As such, the current distribution is substantially lower than in the past by at least 50 % (Perfaur *et al.* 1968; Drouilly 1983; López 2010). The huemul is listed as an endangered species by the book Red of threatened species (IUCN 2007) due to poaching, habitat loss and fragmentation, depredation and disturbance by domestic dogs, overgrazing and transmission of diseases by exotic livestock and isolation of their populations (Povilitis 1998; Smith-Flueck 2000; Serret 2001).

The taruca, similar in appearance, size and body structure, is often called the northern huemul. It is distributed along the Andes from the central-western portion of Peru southwards to the northeast of Argentina and a small population in the northeast Chile (Barrio 2010). Taruca inhabit relatively humid climates of the eastern Andes (Jungius 1974), as well as dry areas in the western Andes (Merkt 1985; Barrio, 1998). Generally, taruca are found above the tree line on mountain slopes characterized by rocky outcrops and cliffs with interspersed grassland (Jungius 1974; Merkt 1985; Barrio 1999), preferably with nearby water sources, small ravines, lagoons or marshes (Barrio 2010).

The taruca is classified as vulnerable in the red book of threatened species (IUCN 2008) due to reduced population size (an estimated 30 % or original size) resulting from poaching and habitat reduction and fragmentation (Barrios 2010). The taruca is a high-profile charismatic species that symbolizes the threatened natural heritage of the Andes Mountains.

Robust and informed conservation efforts are needed to better understand past demographic history of both huemul

and taruca. However, effective long-term data will depend on the development of more efficient tools to assess past population history and to monitor current populations, ideally through non-invasive sampling. Through the extraction of DNA from the horns of huemul and taruca and the use assessment of mitochondrial DNA and nuclear DNA genetic markers, we hope to significantly increase our understanding of past and present *Hippocamelus* species and populations and thus design more-effective conservation strategies and management of the species.

Deer antlers (Fig. 1a), which are made of real bone, are extensions of the skull. They are replaced annually in the males of most *Cervidae* (García *et al.* 1997). Annual renewal of deer antlers is the only case of mammalian appendage regeneration and represents true epimorphic regeneration (Li *et al.* 2009). Each spring, male deer produce a new set of antlers. As the antlers develop, they are covered with a thin sheath of "velvet" which supplies nutrients to the growing bone. During summer, the velvet falls off, leaving mature antlers made of dead bone. After mating season, the antlers fall off.

Antlers are a source of DNA source that, until now, has been rarely used for genetic analysis. The annual renewal of antlers would be a source of non-invasive samples of critical species with conservation issues like the huemul (*Hippocamelus bisulcus*) and the taruca (*Hippocamelus antisensis*).

The area between the antlers and the skull consists of connective tissue that is protected from decomposing. As such it is an excellent source of high molecular weight DNA, even after several years. Many studies have used antlers deposited in museums as a source of DNA. However, in most cases, this involves the partial destruction of the material through the pulverization of fragments of the antler (Taberlet *et al.* 1996; Hofreiter *et al.* 2001; Ludt *et al.* 2004; Bosch *et al.* 2005; Kuehn *et al.* 2005). Here we describe a non-invasive method of obtaining tissues from deer antlers deposited in museum collections or collected in the field that does not damage the antler and that allows the extraction of quality DNA.

Nine antlers of huemul and thirteen antlers of taruca were obtained from field sampling, museum collections, and private collections (Table 1) and were then cleaned and stored in sterile bags. The basal part of the antler was washed with distilled water. After drying, the ring of connective tissue that was adhered to the basal part of the shaft was removed with a sterile scalpel (Fig. 1b). Then, the tissue was collected in sterile eppendorf tubes and before extraction, the pieces were washed by adding 1 mL of 70 % ethanol to the tube. The tube was vortexed for 1 minute with the pieces of connective tissue and ethanol. The alcohol was removed with

a micropipette and the remaining tissue fragments in the tube were dried at 40°C or room temperature before extraction. DNA was extracted from 10 grams of the washed conjunctive tissue. The spraying was done with Pellet pestles. All samples were stored at -70°C in the Laboratorio de Genómica y Biodiversidad, Departamento de Ciencias Básicas, Facultad de Ciencias, Universidad del Bío-Bío, Chillán, Chile. Total genomic DNA was extracted from the tissue using three methods: 1) a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA); 2) digestion with proteinase K, followed by phenol-chloroform extraction and ethanol precipitation (Sambrook *et al.* 1989) and 3) sodium dodecyl sulfate-proteinase K/NaCl extraction and alcohol precipitation (Maniatis *et al.* 1992).

Equal amounts of each sample were extracted using three methods and performed in triplicate. DNA quantity and quality were measured by reading the whole absorption spectrum (220-750 nm) and by electrophoresis 1 % agarose gels. Additionally, each sample was quantified with a Qubit fluorometer. In addition to the precautions detailed in the antler sampling protocol described above, the main protection against contamination depends on the specificity of the primers. Even though the extraction of DNA from connective tissue was a mixture of DNA from a variety of organisms (for example, bacteria and fungi), species-specific primers designed for specific genes were used to amplify only DNA fragments of our species under study.

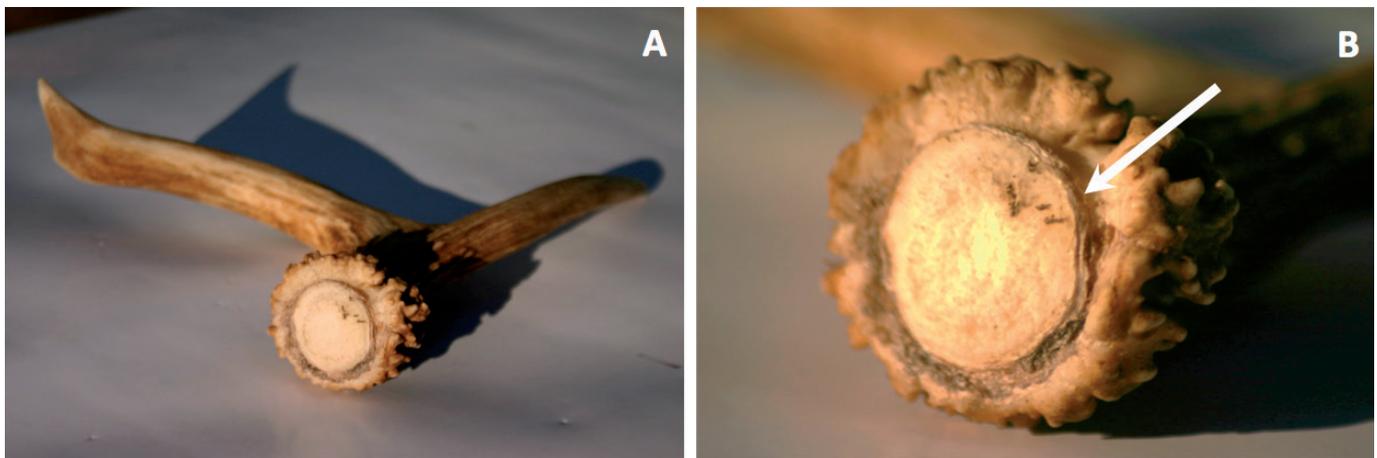


FIGURE 1. Side view of an antler of a male *Hippocamelus bisulcus* from Rio Simpson, Chilean Patagonia (a), view of the base area of the antler showing connective tissue with an arrow (b). / Vista lateral de un asta de *Hippocamelus bisulcus* macho de Río Simpson, Patagonia Chilena (a), vista de la base del asta, indicando con una flecha la presencia de tejido conectivo (b).

TABLE 1. Locality, geographic coordinates, and antler source: field sampling (f), museum collections (m), private source (p) and number of samples *Hippocamelus* samples. / Localidad, coordenadas geográficas y origen de las astas: muestreo de campo (f), colecciones de museos (m), fuentes privadas (p) y número de muestras de *Hippocamelus*.

Localities	Geographic position	Number of samples and collection method
<b><i>Hippocamelus antisencis</i></b>		
Putre, Chile	18°11'28" S, 69°31'30" W	9 from private source
Belén village, Chile	18°29'51" S, 69°31'34" W	4 from field samples
<b><i>Hippocamelus bisulcus</i></b>		
Nevados de Chillán, Chile	36°50'52" S, 71°15'28" W	1 from field samples
Nahuel-Huapi Nacional Parck, Argentina	40°47'58" S, 71°34'35" W	2 from museum
Lago Puelo Nacional Parck, Argentina	42°08'18" S, 71°39'37" W	1 from private source
Mañihuales, Chile	45°13'45" S, 72°12'52" W	1 from field samples
Rio Simpson Nacional Reserve, Chile	45°36'07" S, 72°12'52" W	3 from private source
Los Glaciares Nacional Parck, Argentina	49°37'15" S, 72°55'95" W	1 from private source

To evaluate the success rate and quality of the extracted DNA, six pairs of primers were used to amplify ~ 500 pb from the first third of cytochrome b (Marín *et al.* 2007), and ~ 500 and ~ 600 pb of the left and right domain of the mitochondrial Control Region, using huemul-specific primers (Marín *et al.* 2013). Amplification was performed in 50 µl with ≈ 30 ng genomic DNA, 1 reaction buffer (8 mM Tris-HCl (pH 8.4), 20 mM KCl (Invitrogen Gibco, Life Technologies, Invitrogen Ltd., Paisley, UK), 2 mM MgCl<sub>2</sub>, 25 mM each of deoxyguanosine triphosphate, deoxyadenosine triphosphate, deoxythymidine triphosphate and deoxycytidine triphosphate, 0.5 mM each primer and 0.1 U/µl Taq polymerase (InvitrogenGibco, Life Technologies). Thermocycling conditions were: 95°C for 10 min, followed by 30-35 cycles of 94°C for 45 s, 57-62°C for 45 s, 72°C for 45 s, then 72°C for 5 min. PCR products were purified using the GeneClean Turbo for PCR Kit (Bio101) following the manufacturer's instructions. Products were sequenced in forward and reverse directions using BigDye chemistry on an ABI Prism 377 or 3100 semiautomated DNA analyser. Geneious v.9.1.5 (Biomatters, Auckland, New Zealand) was used to align forward, reverse, and consensus sequences, and the alignments were rechecked by eye. Sequences were confirmed with two independent rounds of amplification and sequencing. GenBank accession codes for the alignments are KY420200-KY420384 and KY420385-KY420569, respectively. From the huemul sequences of the Rio Simpson (RS) and Rio Bravo (RB) populations, genetic and haplotypic diversity were estimated.

Of all the samples (N = 39), DNA was extracted from 9 of the huemul samples and 13 of the taruca samples (Table 1). The quantity of DNA recovered from antlers' connective tissue with the three extraction methods was 0.9-13.4 µg. Overall analysis of variance tests for differences by extraction method were not significant at P<0.005 for all three experimental systems, but were significant at P<0.005 between samples (Fig. 2).

From the antlers' DNA extracted from the huemul it was amplified and sequenced a fragment of ~ 500-450 pb and ~ 600 pb, corresponding to the hypervariable region I and II from the control region (d-loop) of the mitochondrial DNA respectively. The size differences found in the hypervariable region I of huemules and tarucas has to do with the detection of a deletion of 50 pb in taruca, confirmed with sequences made in other tissues. The sampling also amplified the latest one positively. ~ 500 pb from the cytochrome b from both species. A trial made with seven specific markers microsatellites for the huemul (Shafer *et al.* 2012) amplified in the 60 % of the sampling positively.

Among the 22 individuals analysed, we identified 22 variable positions segregated into 13 haplotypes, and total

haplotype (*h*) and nucleotide ( $\pi$ ) diversities of 0.33 and 0.871, respectively. The hypervariable domain II was the most variable region in our data, presenting on average about two-thirds of the polymorphisms. All sequences were deposited in GenBank under the accession numbers JN870923-JN871197. The sequences obtained in the fragment of the gen Cytochrome b were used with sequences obtained from the other tissues in the construction of a phylogenetic tree.

In conservation biology, it is very important to carry out studies of the wild fauna and the environment that surrounds them, that is why in the area of conservation, the collection of samples by means of non-invasive methods of death is the key. These methods allow the extraction of DNA from the samples of species for the recognition of individuals or studies of molecular ecology, phylogenetics or phylogeography (de la Maza & Bonacic 2013) in this way the use of non-invasive samples show a great advantage to obtain samples without causing stress to animals together with minimizes the risk that goes with sampling species with conservation issues, since these methods do not require the animal to be captured, or its environment disturbed. These methods also increase the number of samples per population in species with low numbers of individuals, or ones that are difficult to access because of habitat or behaviour (Taberlet & Luikart 1999; Boch *et al.* 2005). The use of antlers as a source of DNA in population studies of Cervids has been regularly carried out in the past, due to the existence of considerable private and public collections; collections that grow every year thanks to the replacement of antlers that occurs in most cervids. One of the limitations of this type of technique is that these types of samples are not available the whole year in the field, and they restrict the analysis to the male population in the majority of the species, of deer populations, such as huemul and taruca, except for the reindeer, where males and females both show their antlers (Bubenik *et al.* 1997; Lincoln & Tyler 1999; Holand *et al.* 2004). The annual shedding and replacement of the males' antlers provides a method to monitor the same population and individuals.

The use of the bone material from antlers for DNA extraction normally brings with it the loss of a significant portion of the antler; a method that, in most cases, does not produce enough quality DNA. The high amount of deterioration of the DNA extracted through these methods, as well as the presence of inhibitors and contamination with exogenous DNA, can lead to genotyping errors in the subsequent processes of amplification and sequencing (García *et al.* 1997; Bosch *et al.* 2005; Kuehn *et al.* 2005; Price *et al.* 2005) or simply the impossibility of amplifying fragments of medium size (>300 pb). The use of connective tissue, reported by us, although it does not show differences between the

extraction methods, significant differences between samples were evident, presumably due to conservation differences and the antlers' age. Despite these, this method provides an unexplored advantage because it maintains the integrity of the antlers and gives DNA of higher quantity and quality. Since the body of antlers is composed of dead bone, obtaining cells for DNA extraction is more deficient than obtaining DNA from connective tissue. Moreover, the amount of cells per tissue volume is significantly higher in the connective tissue than in the rest of the antler's osseous structures (Colitti *et al.* 2005; Price *et al.* 2005).

The use of antlers has been important in the development of cellular and molecular studies (García *et al.* 1997; Barling & Chong 1999; Colitti *et al.* 2005; Price *et al.* 2005), as well as in evolutionary, conservation, taxonomy, and systemic studies (Wang & Schreiber 2001; Ludt *et al.* 2004). Our results show

that the use of DNA extracted from the base of huemul and taruca antlers by PCR amplification of mitochondrial and nuclear sequences is of good quantity and quality (Table 2, Fig. 2). These fragments can be useful for studies of differentiation, geographic patterns, and historical aspects of both species, and even to formulate and support specific conservation recommendations. In the past, Ludt *et al.* 2004 extracted DNA from the antlers' osseous area and amplified the Cytochrome b gene to study the molecular systematics of the family *Cervidae*. Kuehn *et al.* 2005, using the same tissue and gene, concluded that *Megaloceros giganteus* is more related to its modern regional counterpart, *Cervus elaphus*. These works show, however, low numbers of samples per species and partial destruction of the antlers in order to obtain usable DNA.

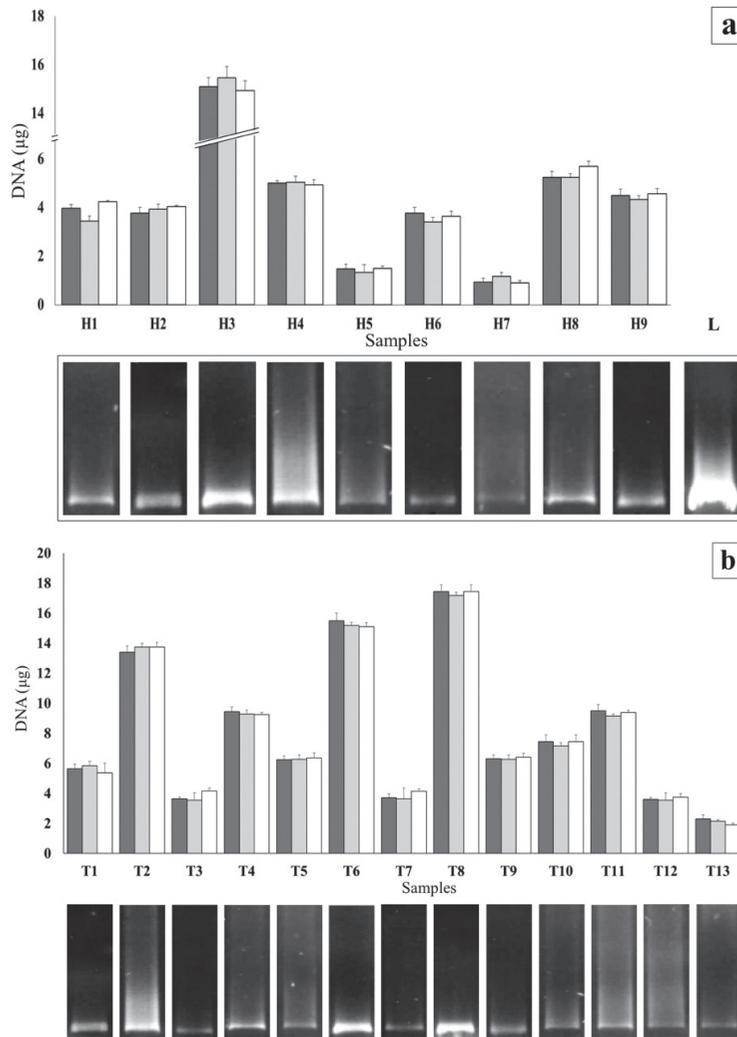


FIGURE 2. Quantity of DNA recovered from antlers' connective tissue in huemul (a) and taruca (b), using three extraction methods. Sample ID is indicated at the bottom. Lane L contains 100 µg of DNA as the reference for normalization. / Cantidad de DNA recuperado del tejido conectivo de astas en huemul (a) y taruca (b), utilizando tres métodos de extracción. La identificación de la muestra se indica en la parte inferior. El carril L contiene 100 µg de DNA como referencia para la normalización.

TABLE 2. Amount of DNA extracted from antlers of huemul and taruca using different extraction methods. / Cantidad de DNA extraído de astas de huemul y taruca utilizando diferentes métodos de extracción.

Huemul				Taruca			
Samples	Methods	Average (ng)	± SD	Samples	Methods	Average (ng)	± SD
H1	A	3.967	0.153	T1	A	5.633	0.322
	B	3.433	0.208		B	5.833	0.289
	C	4.233	0.058		C	5,367	0.635
H2	A	3.767	0.252	T2	A	13.400	0.400
	B	3.933	0.209		B	13,733	0.252
	C	4.033	0.058		C	13.733	0.306
H3	A	15.100	0.361	T3	A	3.633	0.116
	B	15.467	0.451		B	3.533	0.503
	C	14.933	0.404		C	4.167	0.208
H4	A	5.000	0.100	T4	A	9.433	0.306
	B	5.033	0.252		B	9.267	0.252
	C	4.933	0.208		C	9.233	0.153
H5	A	1.467	0.208	T5	A	6.233	0.252
	B	1.333	0.306		B	6.267	0.306
	C	1.500	0.100		C	6.367	0.322
H6	A	3.767	0.252	T6	A	15.500	0.500
	B	3.400	0.200		B	15.167	0.208
	C	3.633	0.208		C	15.100	0.265
H7	A	0.933	0.153	T7	A	3.700	0.265
	B	1.166	0.153		B	3.633	0.710
	C	0.900	0.100		C	4.133	0.153
H8	A	5.233	0.252	T8	A	17.433	0.451
	B	5.233	0.153		B	17.167	0.208
	C	5.700	0.200		C	17.433	0.451
H9	A	4.500	0.265	T9	A	6.300	0.265
	B	4.333	0.153		B	6.267	0.306
	C	4.567	0.208		C	6.400	0.265
				T10	A	7.433	0.451
					B	7.167	0.208
					C	7.433	0.451
				T11	A	9.500	0.400
					B	9.133	0.153
					C	9.367	0.153
				T12	A	3.600	0.100
					B	3.533	0.503
					C	3.733	0.252
				T13	A	2.300	0.265
					B	2.133	0.116
					C	1.900	0.100

The present work demonstrates how DNA extraction from the base of the huemul and taruca antlers constitutes a source of genetic material not previously used and of potentially broad unimaginable applications. However, in some cases we obtained partially deteriorated DNA that could be related to the amount of time that the antlers were susceptible to environmental damage, or to the time and conditions of storage in the collections. We also did not evaluate if there were PCR inhibitory enzymes. However, it is possible that this factor affects all types of samples equally, and the presence of these inhibitors depends more on the origin of the sample than on the type of tissue. In spite of these potential impediments, antlers proved to be a reliable and useful source of DNA that would be a simpler and more effective source of DNA than the conventional procedures used with antlers and which damage the collection material and produce a lower yield.

#### ACKNOWLEDGEMENTS

This research was supported by FONDECYT, Chile Grant 1140785, 11080098, CONICYT PCHA/Magister Nacional/2014-22140806 and DID-UBB (082409\_1/R). We are grateful to SAG and CONAF for collection permits, and Paulo Corti and Rodrigo López. Special thanks are due to Warren Johnson for useful support and English improvement.

#### REFERENCES

- Aldridge, D., Montecinos, L. 1998. Avances en la conservación del Huemul (*Hippocamelus bisulcus*) en Chile. In: Valverde, V. (Eds) La conservación de la fauna nativa de Chile, Logros y perspectivas. Ministerio de Agricultura, CONAF, Chile 178: 133-149 pp.
- Barling, P.M., Chong, K.W. 1999. The involvement of phosphohydrolases in mineralization: studies on enzymatic activities extracted from red deer antler. *Calcified Tissue International* 65: 232-236.
- Barrio, J. 1999. Población y habitat de la taruca en la Zona Reservada Aymara-Lupaca, Perú. In: Fang, T.G., Montenegro O.L., Bodmer, R.E. (Eds) Manejo y Conservación de Fauna Silvestre en América Latina: 453-460. La Paz, Bolivia.
- Barrio, J. 2010. Taruca. *Hippocamelus antisensis* (d'Orbigny 1834). En: Barbanti, J., González, S. (Eds) Neotropical cervidology: biology and medicine of Latin American deer. Jaboticabal, Funep, Brazil. 393 p.
- Bosch, M., Marmi, J., Ferrando, A., López-Giráldez, F., Andrés, O., Gracia-Franquesa, E., Ponsa, M., Kellermann, T., Guallar, B., Bisbal, F., Domingo-Roura, X. 2005. Genotipar sin capturar. *Galemys* 17: 81-102.
- Bubenik, A.G., Schams, D., White, J.R., Rowell, J., Blake, J., Bartos, L. 1997. Seasonal Levels of Reproductive Hormones and Their Relationship to the Antler Cycle of Male and Female Reindeer (*Rangifer tarandus*). *Comparative Biochemistry. Physiology* 2: 269-277.
- CONAF-CODEFF. 2001. Plan para la Conservación del Huemul del Sur (*Hippocamelus bisulcus*) en Chile. CONAF, Departamento Patrimonio Silvestre, Santiago. 40 pp.
- Colitti, M., Allen, S.P., Price, J.S. 2005. Programmed cell death in the regenerating deer antler. *Journal of Anatomy* 207: 339-351.
- De la Maza, M., Bonacic, C. 2013. Manual para el monitoreo de fauna silvestre en Chile. (Eds) Serie Fauna Australis. Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile. 202 pp.
- Díaz, N.I., Smith-Flueck, J.A. 2000. El huemul patagónico. L.O.L.A., Buenos Aires. 156 pp.
- Dellafore, C., Maceira, N. 2001. Los ciervos autóctonos de la Argentina y la acción del hombre. Grupo Abierto de Comunicaciones: 51-56 pp. Buenos Aires.
- Drouilly, P. 1983. Recopilación de antecedentes biológicos y ecológicos del huemul chileno y consideraciones sobre su manejo. Boletín Técnico 5. Corporación Nacional Forestal, Santiago.
- García, R.L., Sagidhi, M., Francis, S.M., Suttie, J.M., Fleming, J.S. 1997. Expression of neurotrophin-3 in the growing velvet antler of the red deer *Cervus elaphus*. *Journal of Molecular Endocrinology* 19: 173-182.
- Hofreiter, M., Jaenicke, V., Serre, D., von Haeseler, A., Pääbo, S. 2001. DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Research* 23: 4793-4799.
- Holand, O., Gjostein, H., Losvar, A., Kumpula, J., Smith, E.M., Roed, H.K., Nieminen, M., Eladji, B. 2004. Social rank in female reindeer (*Rangifer tarandus*): effects of body mass, antlers size and age. *Journal of Zoology* 263(4): 365-372.
- IUCN. 2007. IUCN Red List of Threatened Species. URL: <http://www.iucnredlist.org>.
- IUCN. 2008. *Hippocamelus antisensis*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. URL: <http://www.iucnredlist.org>. Accessed: March 27, 2013.
- Jungius, H. 1974. Beobachtungen am Weisswedelhirsch und an anderen Cerviden in Bolivien. *Zeitschrift Säugetierkunde* 39: 373-383.
- Kuehn, R., Ludt, C., Schroeder, W., Rottmann, O. 2005. Molecular Phylogeny of *Megaloceros gigantus* - the Giant Deer or Just a Giant Red Deer? *Zoological Science* 22: 1031-

- 1044.
- Li, C., Yang, F., Sheppard, A. 2009. Adults Stem Cells and Mammalian Epimorphic Regeneration-Insights from Studying Annual Renewal of Deer Antlers. *Current Stem Cell Research & Therapy* 4: 237-251.
- Lincoln, A.G., Tyler, C.J. 1999. Role of oestradiol in the regulation of the seasonal antler cycle in female reindeer, *Rangifer tarandus*. *Journal of Reproduction and Fertility* 115: 167-174.
- López, R., Serret, A., Faúndez, R., Palé, G. 1998. Estado del conocimiento actual de la distribución del huemul (*Hippocamelus bisulcus*, Cervidae) en Argentina y Chile. Fundación Vida Silvestre Argentina (FVSA), World Wildlife Fund (WWF) y Comité Nacional pro Defensa de la Fauna y Flora (CODEFF), Concepción, Chile. 32 p.
- López, R. 2010. Nevados de Chillán - Laguna de la Laja Wildlife Corridor: Promoting Huemul and Biodiversity Conservation. Survey of Meta-Population of Huemuls (*Hippocamelus bisulcus*) in North Zone Nevados de Chillán Mountains. Comité Nacional Pro Defensa de la Fauna y Flora (CODEFF) and Frankfurt Zoological Society (FZS). Concepción, Chile.
- Ludt, C., Schroeder, W., Rottmann, O., Kuehn, R. 2004. Mitochondrial DNA phylogeography of red deer (*Cervus elaphus*). *Molecular Phylogenetics and Evolution* 31: 1064-1083.
- Maniatis, T., Fritsch, E.F., Sambrook, J. 1992. Molecular cloning. A laboratory manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.
- Marín, J.C., Zapata, B., González, B.A., Bonacic, C., Wheeler, J.C., Casey, C., Bruford, M.W., Palma, R.E., Poulin, E., Allende, M.A., Spotorno, A.E. 2007. Sistemática, taxonomía y domesticación de alpacas y llamas: nueva evidencia cromosómica y molecular. *Revista Chilena de Historia Natural* 80: 121-140.
- Marín, J.C., Varas, V., Vila, A., López, R., Orozco-terWengel, P., Corti, P. 2013. Refugia in Patagonian fjords and the eastern Andes during the Last Glacial Maximum revealed by huemul (*Hippocamelus bisulcus*) phylogeographical patterns and genetic diversity. *Journal of Biogeography* 40: 2285-2298.
- Merk, J. 1985. Social structure of Andean deer (*Hippocamelus antisensis*: Cervidae) in Southern Peru. MSci Thesis. University of British Columbia, Canada. 134 pp.
- Morin, P.A., Wallis, J., Moore, J., Chakrabort, R., Woodruff, D. 1993. Non-invasive sampling and DNA amplification for paternity exclusion, community structure, and phylogeography in wild chimpanzees. *Primates* 34 (3): 347-356.
- Perfaur, J.W., Hermosilla, F., Dicastri, F., González, R.S. 1968. Estudio preliminar de Mamíferos Silvestres Chilenos: Su Distribución, Valor Económico e Importancia Zoonótica. *Revista de la Sociedad de Medicina Veterinaria, Chile* 18 (1-2-3-4): 3-15.
- Povilitis, A. 1998. Characteristics and conservation of a fragmented population of huemul *Hippocamelus bisulcus* in central Chile. *Biological Conservation* 86: 97-104.
- Price, J.S., Allen, S., Fauchaux, C., Althnaian, T., Mount, J.G. 2005. Deer antlers: a zoological curiosity or the key to understanding organ regeneration in mammals? *Journal of Anatomy* 207: 603-618.
- Sambrook, J., Fritsch, E.F., Maniatis, T. (Eds) 1989. Molecular cloning: A laboratory manual. 2<sup>nd</sup> Edition. Cold Spring Harbor Press, Cold Spring Harbor, NY.
- Serret, A. 2001. El huemul: Fantasma de la Patagonia. Zagur y Urrutz Publications, Buenos Aires. 130 pp.
- Shafer, B.A.A., Northrup, M.J., White, S.K., Boyce, S.M., Coté, D.S., Coltman, W.D. 2012. Habitat selection predicts genetic relatedness in an alpine ungulate. *Ecology* 6: 1317- 1329.
- Smith-Flueck, J. 2000. La situación actual del huemul patagónico, pp 67-150. In: Díaz, N.I., Smith-Flueck, J. (Eds) El Huemul Patagónica. Un misterioso cévido al borde de la extinción. Literature of Latin America, Buenos Aires, Argentina. 156 pp.
- Taberlet, P., Griffin, S., Goossens, B., Questiau, S., Manceau, V., Escaravage, N., Waits, L. P., Bouvet, J. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* 26: 3189- 3194.
- Taberlet, P., Luikart, G. 1999. Non-invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society* 68: 41-55.
- Vila, A., López, R., Pastore, H., Faúndez, R., Serret, A. 2006. Current distribution and conservation of the huemul (*Hippocamelus bisulcus*) in Argentina and Chile. *Mastozoología Neotropical* 13: 263-269 pp.
- Schreiber, S., Wang, M. 2001. The impact of habitat fragmentation and social structure on the population genetics of roe deer (*Capreolus capreolus* L.) in Central Europe. *Heredity* 86: 703-715.
- Wasko, A.P., Martins, C., Oliveira, C., Foresti, F. 2003. Non-destructive genetic sampling in fish. An improved method for DNA extraction from fish fins and scales. *Hereditas* 138: 161-165.

Received: 22.12.2017

Accepted: 17.01.2020