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CASE REPORT

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Application of DNA Forensic Techniques for Identifying Poached Guanacos (*Lama guanicoe*) in Chilean Patagonia*

ABSTRACT: Guanaco (*Lama guanicoe*) is a protected and widely distributed ungulate in South America. A poacher, after killing guanacos in Valle Chacabuco, Chilean Patagonia, transported and stored the meat. Samples were retrieved by local police but the suspect argued that the meat was from a horse. Mitochondrial cytochrome b gene (774 pb), 15 loci microsatellites, and SRY gene were used to identify the species, number of animals and their population origin, and the sex of the animals, respectively. Analysis revealed that the samples came from a female (absence of SRY gene) Patagonian guanaco (assignment probability between 0.0075 and 0.0282), and clearly distinguishing it from sympatric ungulates (*E*-value = 0). Based on the evidence obtained in the field in addition to forensic data, the suspect was convicted of poaching and illegally carrying fire arms. This is the first report of molecular tools being used in forensic investigations of Chilean wildlife indicating its promising future application in guanaco management and conservation.

KEYWORDS: forensic science, DNA analysis, species identification, sex identification, South American camelids, poaching

The guanaco (*Lama guanicoe*) is the most widely distributed ungulate in South America. It is found from the arid desert regions along the Pacific Ocean, in El Chaco, through the Andes mountain range extending east to the Patagonian and Fueguinean steppes and reaching as far as the Atlantic coast (1–4). Across its range, population status varies widely (5). While some populations have recovered in number in some protected areas, other guanaco populations are critically endangered and close to extinction (4,5). Intensification of farming, livestock herding, habitat degradation, and hunting pressure are the main threats, and currently less than 3% of the original total population survives (reviewed in [4]). This critical situation led to the protection of guanacos at both national and international levels, and the species is listed in Convention of International Trade of Endangered Species II (6). Peru, Bolivia, Argentina, and Chile have given the species full legal protection against poaching, while sustainable use of wild herds is promoted in Chile and Argentina (6–8).

Molecular markers have recently been applied to South American camelids. DNA studies have been used to answer biological questions about taxonomic relationships among and within camel species (9–13) and for detecting variation among populations (14–17). Despite their potential for high resolution and accuracy, genetic analyses have not been applied in forensic issues in South American camelids contrasting with other countries where molecular tools have played a crucial role in solving cases of poaching and to support local wildlife law application (18–23). In this work, we report the first contribution of molecular techniques in the resolution of a judicial case involving the poaching of guanacos in Chilean Patagonia.

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Materials and Methods

Case Report

Two poachers driving through a private protected area for wildlife located in the Valle Chacabuco Patagonia (47° 36′ Lat S and 72° 27′ Long W), illegally hunted a total of five guanacos using an unlicensed firearm. On 30 November 2006, one of the poachers shot two guanacos which they placed in their truck before fleeing from the area. This incident was observed by volunteers that were camping nearby, who informed the administration of the protected area. Local police were notified and located the poachers at their home, where fresh meat and a rifle were confiscated. During the trial, the poacher argued that the confiscated meat samples were from a domestic horse (*Equus caballus*). Between 19 and 24 January of 2007, three more guanacos were found dead in the protected area. A 9 mm bullet was found inside the carcass of one of these animals. The bullet matched one of the poacher's firearms confiscated at his home in the nearest village; therefore, the poacher was rapidly identified and brought to trial. The local tribunal found him guilty of all charges and he was sentenced to pay a relatively low fine of $353,562 Chilean pesos (*c*. US$ 760), considering the crimes of illegal bearing of a firearm, invasion of private property, and the illegal killing of protected wildlife.

Specimen Collection and Species Identification

Material suitable for DNA analysis was collected from the confiscated meat found in the poacher's home, which had been taken to the police station near Cochrane, Chilean Patagonia. The samples were sent to the Laboratorio de Genómica y Biodiversidad, Departamento de Ciencias Básicas, Universidad del Bío-Bío. Genomic DNA was extracted from three muscle samples using standard proteinase K digestion, phenol/chloroform extraction procedures (24).

To identify the species from the samples, polymerase chain reaction (PCR) amplification was performed using a partial sequence of cytochrome b gene and the primer: LGlu ARTIO: 5′-TCTAACCACGACTAATGACATG-3′ and HThr ARTIO: 5′-TCCTTTTTCGGCTTACAAGACC-3′ (12). These primers were designed by aligning consensus sequences obtained from domestic ungulate taxa available in GeneBank (25) and located in the tRNA for glutamine and the tRNA for tryptophan, respectively.

PCR containing 10 ng of DNA were amplified in a total volume of 50 μL, using a 5 min denaturing step at 95°C, followed by 30 cycles of 95°C for 45 sec, 58°C for 30 sec, 72°C for 45 sec, and a final extension at 72°C for 5 min. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN®, Crawley, UK). A total of 1140 bp of cytochrome b was sequenced using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned using the programs Clustal X (26), and by sight. Aligned sequences were translated using the software MEGA (27) for the genetic code of the mammal mitochondrial DNA.

Statistical confidence of sequence similarity within and among species was further assessed using a Basic Local Alignment Search Tool (BLAST) (28) searching within the evidence sequence. Specifically, we used the Nucleotide BLAST program option to find regions of local similarity between sequences using the nucleotide collection (nr/nt) and highly similar sequences (megablast). To assess whether a given sequence alignment constituted evidence for homology, we used the max indent and expectation values (*E*-value) to evaluate how strong an alignment can be expected from chance alone. The *E*-value gives an indication of the statistical significance of a given pairwise alignment and reflects the size of the database and the scoring system used. The lower the *E*-value, the more significant was the hit.

Sex Determination

Sex-specific amplification of SRY gene was performed to identify the sex of the confiscated samples. Currently, the most common tests to sex mammals is by amplification of Y-specific gene such as SRY (21,29). The SRY system, however, produces only male-specific amplifications. The SRY gene (≈175 bp) was amplified using the camel's specific primers SryB5 (5′-TGAATGCTTTCATTGTATGGGC-3′) and SryB3 (5′-GCCAGTAGTCTCTGTG CCTCCT-3′) (modified after 30). The amplification was performed in 50 μL with *c*. 20 ng genomic DNA, 1x reaction buffer (8 mM Tris–HCl, pH 8.4, 20 mM KCl [InvitrogenGibco, Life Technologies™, Paisley, UK], 2 mM $MgCl_2$, 25 μM each of dGTP, dATP, dTTP, and dCTP, 0.5 μM each primer, and 1 U Taq polymerase [InvitrogenGibco, Life Technologies™]). Thermocycling conditions were: 95°C for 10 min, followed by 30–35 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec, and then 72°C for 5 min. The PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide (0.5 mg/mL), and observed under a UV transilluminator.

Determination of Individuals

A panel of 15 highly polymorphic microsatellites from South American camelids was used to amplify the sample DNA, to establish whether the respective DNA profiles matched. The primer pairs used were: YWLL08, YWLL29, YWLL36, YWLL38, YWLL43, YWLL44, YWLL46, LCA5, LCA19, LCA22, LCA23, LCA65, LCA82, LGU49, and LGU68 (14,31,32). PCR reactions contained 10 ng of total DNA, 2.5 μL of 10x buffer Gold, 200 mM of each dNTP, 10 pm of each primer (with the forward primer fluorescently labeled), 2 mM $MgCl_2$, and 1.5 U of AmpliTaq Gold polymerase (Applied Biosystems) in 25 mL total volume. Loci YWLL36, LCA22, LCA23, LCA65, LGU49, LGU68, as well as loci YWLL08, YWLL43, YWLL44, LCA5, LCA82, as well as loci YWLL29, YWLL38, YWLL46, and LCA19, were coamplified in two single reactions, respectively. The remaining loci were individually amplified. Samples were heated to 95°C for 15 min, followed by 35 cycles at 94°C for 30 sec, an annealing temperature of 60°C for 1 min 30 sec, and 72°C for 1 min. The final synthesis step was extended to 30 min at 60°C. PCR products were multiplex pooled in individual tubes into groups of four to six loci depending on the expected product size and fluorescent dye labeled. Pools were heated at 95°C for 2 min, placed on ice, and loaded onto an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems). Fragment sizing was performed using Genescan Software Version 3.1.4 (Applied Biosystems).

The statistical certainty of assignment or exclusion for individuals in their reference populations was evaluated by their multilocus genotype using a likelihood-based Bayesian technique implemented in GENECLASS version 2 (33). We performed an assignment test with a maximum likelihood approach to reassign the evidence to a guanaco's population based on allelic composition of the originally defined populations (34).

Results and Discussion

We obtained the partial sequence (774 pb) of cytochrome b in the mitochondrial gene from the three confiscated meat samples. In addition, we obtained the same sequence of the mitochondrial cytochrome b from samples from the two species of wild ungulates inhabiting the Chacabuco Valley (47° 36′ S y 72° 27′ W), guanaco and huemul deer (*Hippocamelus bisulcus*). Once the aligned sequences were totally translated, using the genetic code of the mammal mitochondrial DNA (27), they neither showed terminal codons nor changes in the frame reading of the sequences. Sequences for horse, cow, and pig were obtained from the GeneBank (their respective access numbers were NC001640, DQ124418, and EF545593) and added to analyses. The sequences obtained from the confiscated samples presented total similarities, favoring the probability of belonging to the same individual.

Pairwise differences and alignment analysis of cytochrome b indicated strongly that the source of confiscated material was from *Lama guanicoe*. Considering all the taxa, 495 characters (63.95%) were constants and 279 sites (36.05%) were variables. Among the variables sites, 161 (20.8%) were phylogenetically informative. The comparison of these sequences showed between 6 and 176 different nucleotides among pairwise species; six corresponded to differences in the sequence between the evidence and *Lama guanicoe* (Table 1). The alignment analysis through Megablast revealed high identity levels (98% and 97%) between the sequences of cytochrome b of *Lama guanicoe* available and the sequence provided by the evidences; all with *E*-value = 0, indicating high significance in the results corroborating the identity of the samples obtained from the

TABLE 1—*Number of variable sites (above diagonal) and percent similarity (below diagonal) amongst cytochrome b sequences generated by primers LGlu ARTIO and HThr ARTIO from the evidences no. 1, 2, and 3 with sequences of reference animals.*

	Evidences/reference animals	1	2	3	4	5	6	7	8
1	Evidence 1	–	0	0	6	153	156	161	176
2	Evidence 2	100.00	–	0	6	153	156	161	176
3	Evidence 3	100.00	100.00	–	6	153	156	161	176
4	Guanaco (*Lama guanicoe*)*	99.20	99.20	99.20	–	150	150	157	171
5	Horse (*Equus caballus*)†	80.23	80.23	80.23	80.62	–	138	144	165
6	Pig (*Sus scrofa*)†	79.84	79.84	79.84	80.62	82.17	–	141	148
7	Cow (*Bos taurus*)†	79.20	79.20	79.20	79.72	81.39	81.78	–	129
8	Huemul (*Hippocamelus bisulcus*)*	77.26	77.26	77.26	77.91	78.68	80.87	83.33	–

*Sequences obtain in the laboratory.
†Sequences obtained from NCBI GeneBank (25).

evidence that belonged to guanaco. The cytochrome b sequence obtained from evidences numbers one, two, and three is 99.2% (six variations) similar to *Lama guanicoe* cytochrome b sequence, indicating that the source of material is *Lama guanicoe*.

Analysis of the sex marker showed no amplification of SRY gene in any of the three confiscated samples, indicating that the samples came from female guanacos. Together with the confiscated samples, we amplified a sex-known male guanaco gene to show the efficacy of the marker and PCR. The same results were obtained with PCR at alignment temperatures. This marker has shown consistency in the determination of sex in more than 200 guanacos of known sex (Marín, unpublished data). However, conducting another test with a complementary sex marker such as AMELX-AMELY or ZFX-ZFY would allow the discounting of the absence of gene amplification due to mutations in the primer link site.

Finally, analysis of the microsatellite set markers amplified for each sample extracted from the evidence, showed the presence of the same alleles for each locus. This is strong evidence that the samples taken at the police station corresponded to the same individual, even though the poachers had been observed killing five guanacos. This result is completely consistent with the mitochondrial (cytochrome b) and sex (SRY) marker sequences that showed a similar pattern of no variation. In addition, from the loci microsatellite analyzed and based on the Bayesian method, the evidence was correctly assigned to its population of origin by using a direct approach, whereas the accuracy of assignment probability of individuals from three closer Patagonian localities Bosques Petrificados, Pali-Ayke, and Monte Leon were 0.0282, 0.0239, and 0.0075, respectively.

To our knowledge, this is the first report where molecular tools were applied in forensic investigation in solving a wildlife poaching case in Chile and applied to a wild South American camelid. DNA evidence was used to identify species, number of individuals, possible origin population, and the sex of the illegally hunted animals. Nevertheless, more accuracy is needed to determine gender of guanacos due to the fact that the SRY gene had been shown to give false negatives.

We recommend that DNA forensics protocols be linked with management and conservation plans in guanacos. Recently, one guanaco population in Chile was legally incorporated into a culling program in Tierra del Fuego, and the meat was exported to Europe or sold in local markets. DNA-based molecular methods would have been valuable to differentiate meat coming from legal and illegal harvests. Moreover, genotype identification may be a tool to identify guanaco meat produced in farms or ranches in Argentina and Chile as a new alternative for animal sanitary control (6,7). These techniques can be applied at intermediary markets and a local level to distinguish between meat harvested legally from ranches from meat harvested illegally from other localities.

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