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Case Report

South American camelid illegal traffic detection by means of molecular markers

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ABSTRACT

South American camelids comprise the wild species guanaco and vicuña and their respective domestic relatives llama and alpaca. The aim of the present study was to determine by DNA analysis to which of these species belong a herd of camelids confiscated from a llama breeder but alleged to be alpacas by the prosecution, and to evaluate the usefulness of mitochondrial and autosomal DNA markers to solve judicial cases involving camelid taxa. Cytochrome b and cytochrome oxidase I mitochondrial genes and 7 STR were analyzed in 25 confiscated samples. Mitochondrial results were inconclusive because 18 of the sequestered samples presented haplotypes that corresponded to the guanaco haplogroup and the remaining seven belonged to a vicuña linage. Microsatellite data of casework samples and llama reference samples revealed different genetic profiles by the presence of private alleles at two microsatellites suggesting that the confiscated animals could be alpaca, or at least alpaca hybrids instead of pure llama.

1. Introduction

In the last two decades, genetics tools have been developed to examine wildlife DNA and to protect plants and animals natural populations. Most countries have laws that regulate the protection of endangered species, but usually it is difficult to obtain evidence to incriminate persons suspected of illegal appropriation or trafficking of these species [1–3]. In this context, molecular genetic methods have an increasing role in identifying the origin of biological materials such as meat, hairs, ivory, bones, teeth, leather, or eggs. DNA-based species identification techniques have been successfully used to solve animal traffic caseworks [4–6].

Camelids are classified into the Artiodactyla order, within the Camelidae family. This group was first originated in the North American continent around late Eocene. About three million years ago, camelid ancestors migrated to Eurasia and South America, where they evolved into the Old World and New World camelids, respectively. Today, South American camelids comprise the wild species guanaco (*Lama guanicoe*) and vicuña (*Vicugna vicugna*), and domesticated relatives llama (*Lama glama*) and alpaca (*Lama pacos*) [7]. The origin of the two latter by domestication of the guanaco and vicuña and the genetic relationships among the wild and domesticated forms has been investigated by archaeozoological [8] and molecular studies [9–11]. Currently the most accepted hypothesis is that llama was originated from guanaco domestication while alpaca is the domestic derivative of the vicuña. Molecular genetics studies have confirmed the occurrence of extensive hybridization between llamas and alpacas, probably beginning at the time of the European colonization and continuing today. The first generations of crossbred animals are easy to recognize by their phenotypic characteristics, but later they are impossible to distinguish as hybridization has been an ongoing process.

Illegal trafficking of domestic animals, including South American camelids, is common in the borderlands of South America countries due to their expanses and isolation. These actions carry serious risk for human and animal health, and bear negative consequences for camelid species conservation.

The aim of the present study was to determine by DNA-based methods the species origin of a herd of camelids confiscated by the Federal Court of the Jujuy Province from a llama flock under the accusation of illegal traffic from Bolivia to Argentina. The usefulness of mitochondrial and autosomal DNA markers to solve judicial cases involving camelids of South America was evaluated.

2. Materials and methods

2.1. Studied casework samples

Twenty-five camelids purported to be alpacas were sequestered from the "Puesto Grande" farm located in Yoscava, Santa Catalina

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Department, Jujuy Province at Northwest Argentina. Blood samples from these animals were submitted to the IGEVET laboratory by the Federal Court of Jujuy Province.

2.2. Genotyping of genetic markers

Total genomic DNA was isolated from blood with the DNAzol® kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Casework samples were genotyped for two mitochondrial gene fragments and seven autosomal microsatellites. Universal oligonucleotide primers L14816 5′-CCATCCAACATCTCAGCATGATGAAA-3′ and H15173 5′-CCCCTCAGAATGATATTTGTCCTCA-3′ were used to PCR amplify a 358 bp mitochondrial fragment of the cytochrome b (cyt b) and primers COX1 Fw 5′-gaacttgcaattcaatgtgt-3′ and COXI Rv 5′-GTGGGAGATTATTCCAAAGC-3′ were used to amplify a 872 bp of cytochrome oxidase I (COXI) gene [12–15]. PCR products were purified with QIAquick columns (QIAGEN, Hilden, Germany) or with polyethylene glycol 8000. All purified DNA samples were sequenced for both DNA strands using the DYEnamic ET Dye Terminator Kit (GE Healthcare, USA) in 10 μl volume containing approximately 40 ng of DNA and 5 pmol of primer, using a MegaBACE 1000 automated sequencer (GE Healthcare).

Casework samples and 22 wild vicuña reference samples (from Jujuy Province, captured for seasonal shearing and then released, were genotyped by PCR analysis of seven microsatellites LAB7, LAB13, LAB15, LAB17, GLM4, GLM5, and GLM6, previously characterized by our group in llama and guanaco populations. PCR reactions were carried out using the primers and conditions reported by Bustamante et al. and Maté et al. [16–18]. Amplicons were run in 6% (19:1) 1× TBE denaturing polyacrylamide gels and allele bands were detected by silver nitrate staining. In each run, DNA with known genotype was used to standardize the molecular size of alleles.

2.3. Sequences analysis

Raw mitochondrial sequences were edited by using MegaBACE Sequence Analyzer (GE Healthcare). The resulting sequences were aligned with 11 COXI and 31 Cyt b of camelid sequences available at GeneBank (Table 1) using CLUSTAL-W multiple alignment software [19]. A Median Joining Network was constructed as described in Bandelt et al. [20], using Network 4.1.1.2 (www.fluxus-technology.com).

2.4. Statistical analysis of microsatellite data

Allele frequencies for each marker, determined by direct counting were used to generate the genetic profiles. Genetic diversity was estimated through number of allele per locus (NA), mean number of alleles (MNA), allele size range, observed (h_o), and expected (h_e) heterozygosis at each locus, and average observed (H_o) and expected (H_e) heterozygosis across all loci.

The assignment of casework animals to camelid species was performed using the Bayesian based methods of Pritchard et al. [21] implemented in STRUCTURE software (http://pritch.bsd.uchicago.edu).

Table 1
GenBank access number of the reference sequences used in this study.

Access number	Gene	Species
AY535256–AY535284,Y08812	Cyt b	*Lama guanicoe*
AY535251, AY535252, AY535253	Cyt b	*Lama glama*
AJ566364, AY535249, AY535250, AY839860	Cyt b	*Lama pacos*
AY535254, AY535255	Cyt b	*Vicugna vicugna*
AY126625–AY126631, X56281	Cyt b	*Camelus dromedarius*
AY126618–AY126624, EF076243, EF076246	Cyt b	*Camelus bactrianus*
EU681954, DQ534053, GU117617	COX1	*Lama guanicoe*
AP003426, DQ534054,GU117612	COX1	*Lama glama*
AJ566364, DQ534055, GU117615, GU117616	COX1	*Lama pacos*
DQ534056, GU117613, GU117614	COX1	*Vicugna vicugna*
EU159113	COX1	*Camelus dromedarius*
EF507801, EF507800, EF212038, AP003423, EF507798, EF212037, EF507799	COX1	*Camelus bactrianus*

3. Results

3.1. Mitochondrial DNA

Sequencing Cyt b and COXI gene fragments from casework samples resulted in a total of nine and seven haplotypes, respectively. Since a very few COXI reference sequences were available in the GenBank, six of the seven sequences obtained here were novel sequences.

A Cyt b network tree was constructed using the sequences from the casework samples and the two Old World species, and the four New World camelid species. These data revealed two main clusters clearly separated by 25 mutations, one of them grouping with the Old World camelids species and the other with the New World ones. Moreover, the South American cluster included two subgroups separated by two mutations, one containing all the guanaco haplotypes and the other all the vicuña ones. Respecting the casework samples 18 of the sequences (72%) grouped with the guanaco cluster and the rest (28%) with the vicuña lineage. Similarly, reference sequences of llama and alpaca were also distributed across both groups (Fig. S1a).

In concordance with results observed in Cyt b, the COXI network topology present two clusters split by 11 mutations, one of each corresponding to the Guanaco and the other to Vicuña lineages (Fig. S1b). Casework and domestic camelid reference sequences are both contained in each cluster of the COXI topology.

3.2. Microsatellite data

Amplification of seven loci in all casework samples showed between 5 and 14 alleles per locus, while h_o varied from 0.384 to 0.840 and h_e from 0.688 to 0.858 (Table 2). The average number of alleles was nine and the H_o and H_e were 0.608 and 0.805, respectively. The values from reference samples are shown in Table 3. Comparing both set of data we found that values for casework and llama and guanaco samples are similar. According to these data the casework sample would not corresponded to a mixed population (llamas plus alpacas) because in such a case the genetic diversity values should be higher than those from single reference camelid species. Regarding the vicuña low genetic diversity here observed it may be attributed to isolation and drastic reduction events of these species population.

Allele frequency distributions from the casework population were compared with those reported for guanaco [17,18,22] and vicuña lineages. These comparisons showed that five out of the seven microsatellites here tested exhibited overlapping allele size

Table 2
Microsatellite genetic diversity measures for casework samples in terms of number of alleles (NA), observed heterocigosity (h_o), and expected heterocigosity (h_e).

Locus	NA	h_o	h_e
GLM4	8	0.680	0.826
GLM5	10	0.385	0.820
GLM6	7	0.560	0.777
LAB7	10	0.680	0.857
LAB13	5	0.500	0.689
LAB15	8	0.615	0.811
LAB17	14	0.840	0.859

Table 3
Microsatellite genetic diversity in terms of mean observed heterocigosity (H_o) and expected heterocigosity (H_e), mean number of alleles (MNA). In brackets, sample size (N) is indicated.

Population/species	MNA	H_o	H_e
Casework ($N = 25$)	9.00	0.610	0.805
Llama[a] ($N = 77$)	8.08	0.600	0.770
Guanaco[b] ($N = 133$)	7.39	0.480	0.700
Vicuña ($N = 22$)	5.14	0.640	0.655

[a] Data from Bustamante et al. [22].
[b] Data from Maté et al. [18].

distribution among all camelid species compared (including casework sample). However, at the GLM4 locus, the most frequent llama allele (183 bp) was absent in all casework samples (Fig. 1a). By contrast, the locus LAB13 presented a divergent allelic distribution between both guanaco and vicuña lineages. In guanaco and llama predominated higher allele sizes, while in vicuña lower molecular size alleles were more common. Interestingly, casework samples presented the lower allele size characteristic of vicuña lineage while high allele sizes typical of guanaco/llama were absent (Fig. 1b and c). Although GLM5 allele distribution was partially overlapped across species, five of the largest alleles, usually absent or rare in the camelid species here compared, were observed in the casework samples at high frequency (Fig. 1d and e). These finding might represent alpaca private alleles.

In order to assign casework samples to a particular camelid species, the structure software was run from 2 to 4 K. These analyses showed that for 2 K, more than 95% of casework samples were grouped together with vicuña samples, while llama and guanaco were allocated in the other cluster; for 3 K, still more than 90% of casework samples were assigned together with vicuña, while most

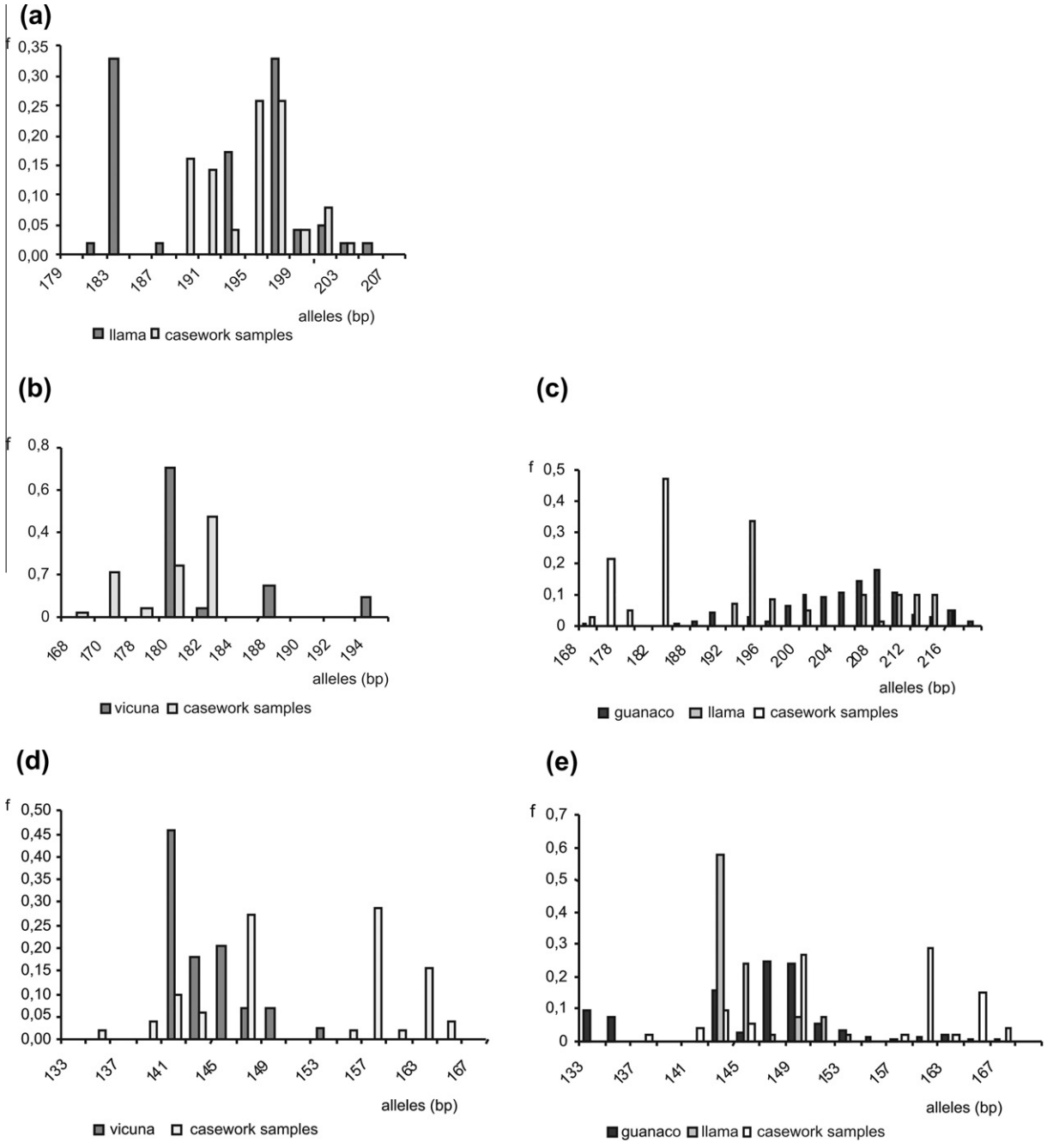

Fig. 1. Allele frequencies distribution for GLM4 (a), and LAB13 (b and c) and GLM5 (d and e) loci in South American camelid species and casework samples.

of llama and guanaco samples were set in separated clusters; for 4 K, samples of each population grouped in separated clusters (Fig. S2). These results are concordant with LAB13, GLM4, and GML5 observations.

4. Discussion

The species determination of a camelid herd alleged by the prosecution to be alpaca but sustained to be llamas by the owner was assessed by the sequencing of two mtDNA fragments and the genotyping of seven microsatellites. Although vicuña and guanaco mitochondrial lineages were detected in the casework sample, most of the individual samples had guanaco mtDNA haplotypes. In fact, these results agree with the findings of Stanley et al. and Kadwell et al. who found that most of the modern day domestic camelids have guanaco haplotypes [10,11] Therefore, our results of mtDNA analysis were inconclusive for the present casework samples and so useless for supporting or rejecting the prosecutor's hypotheses. In contrast, when microsatellite data (nuclear markers) were considered, we found a clearer pattern of species-association. Accordingly, while five of the microsatellite presented an overlapping distribution of allele size ranges, two of them (GLM5 and mainly LAB13) were much more informative and could be used to resolve the casework. LAB13, for instance, exhibits a clear divergent distribution between the guanaco/vicuña lineages. A similar distribution was found by Kadwell et al. [11] for the YWLL46 STR marker.

Worthy to mention, for the case work samples the results of LAB13 marker matched with vicuña allelic distribution suggesting the alpaca origin for these sample. Furthermore, GLM5 marker besides to show some alleles coinciding with the llama profile, some others do not belong to llama or vicuña so they probably represent private alleles of alpaca.

The differences between maternally inherited genomes and nuclear genomic patterns reported here and also found by other authors, may be the consequence of deliberate hybridization performed by the breeders between alpaca males with llama females, to increase body size and fleece weight, followed by subsequent backcrossing with alpaca males to improve the fiber fineness [11,23].

Despite the fact that species identification to resolve South American camelids illegal traffic is complicated because hybridization events occurred between domestic camelid forms, the microsatellites analysis support that the confiscated samples studied here have an alpaca origin or, at least, were alpaca hybrids. The lack of concordance with llama LAB13 allelic profile and the absence of GLM4 high frequency alleles in llamas, allow us to discard these species as source for the casework samples.

To conclude, analysis based on mitochondrial DNA do not allow species identification among South American camelids. However, microsatellites genotyping, especially those that carry private alleles are more useful for species discrimination of these domestic animals. The microsatellite markers GLM5 and LAB13, in addition to those proposed by Sarno et al. [24] and Kadwell et al. [11], could be useful to distinguish among domestic and wild camelids species, and significantly help the resolution of animal trafficking cases. However, in addition to these microsatellites, development and testing of new molecular markers such as single nucleotide polymorphisms (SNPs) to assess domestic camelids identification would be highly desirable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.legalmed.2011.08.001.

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