The complete mitochondrial DNA sequence of the guanaco (Lama guanicoe): Comparative analysis with the vicuña (Vicugna vicugna) genome

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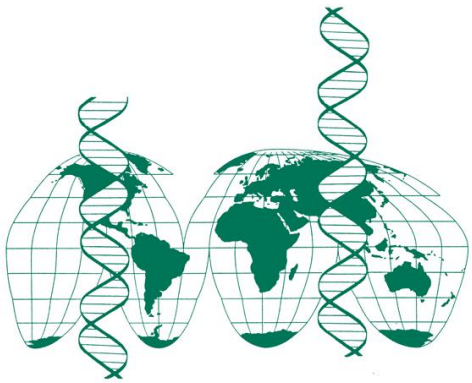

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BRIEF REPORT

The complete mitochondrial DNA sequence of the guanaco (*Lama guanicoe*): comparative analysis with the vicuña (*Vicugna vicugna*) genome

Florencia Di Rocco · Andrés Zambelli · Laura Maté · Lidia Vidal-Rioja

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Abstract South American camelids comprise the guanaco (*Lama guanicoe*) and the vicuña (*Vicugna vicugna*), which are wild species, and the domestic llama (*Lama glama*) and alpaca (*Lama pacos*). This paper presents the first complete mitochondrial (mt) genome of the guanaco and the mt coding sequence of the vicuña. The guanaco mtDNA is 16,649 nt long and its composition and organization are similar to the mitochondrial genome of other mammals. Excluding the control region, comparison of the complete guanaco and vicuña mtDNA showed 4.4% sequence divergence. Nucleotide differences in peptide coding genes varied from 1.9% in ATP6 to 6.4% in Cyt b. These values are compatible with the close relatedness of both species identified by other authors. Based on the differences between the control region sequence here reported and that previously described, we also discuss the occurrence of NUMTs in the genome of South American camelids.

F. Di Rocco (✉) · L. Vidal-Rioja
Instituto Multidisciplinario de Biología Celular (IMBICE), CCT CONICET La Plata-CICPBA, C.C. 403, 1900 La Plata, Argentina
e-mail: genmol@imbice.org.ar

A. Zambelli
Advanta Semillas, Estación Experimental Balcarce, ruta 226 km 60,5, 7620 Balcarce, Argentina

L. Maté
Facultad de Ciencias Veterinarias, UNICEN, C.P. B7000GHG, Tandil, Argentina

Keywords Wild South American camelids · Guanaco · Vicuña · Complete mtDNA · NUMTs

Introduction

Camelids belong to the Order Artiodactyla, Family Camelidae. This group originated in the North American continent around the late Eocene. About 3 million years ago (MYA), Camelid ancestors migrated to Eurasia and South America where they evolved into the Old World camels and New World camelids, respectively (Wheeler 1995). Today, South American Camelids comprise the guanaco (*Lama guanicoe*) and the vicuña (*Vicugna vicugna*), which are wild species, and their domestic relatives the llama (*Lama glama*) and the alpaca (*Lama pacos*) (Franklin 1982).

The common origin of the South American wild camelids and their assignment to different genera has been extensively referenced by Franklin (1982) and Wheeler (1995). However, reports on their molecular differentiation are scarce (Semorile et al. 1994; Stanley et al. 1994; Vidal Rioja et al. 1994; Marín et al. 2007).

Although databases containing complete mitochondrial genomes have rapidly increased and contributed to determine the phylogenetic relationships among a wide range of species, the mitochondrial DNA (mtDNA) of the domestic alpaca is the only complete sequence of a South American camelid so far published (Arnason et al. 2004).

We here report the complete mtDNA sequence and organization of the wild South American camelid guanaco and its comparison with the vicuña mitochondrial coding region. The South American camelid control region previously published by Maté et al. (2004) is also revised.

Materials and methods

DNA extraction, PCR amplification, and sequencing

Blood samples from one *Lama guanicoe* individual from La Esperanza breeding station, Rio Negro province, Argentina, and one *Vicugna vicugna* specimen from Cieneguillas, Jujuy province, Argentina, were obtained by skilled technicians during seasonal shear. Total genomic DNA was isolated from blood samples following the procedure described elsewhere (Bustamante et al. 2002).

Mitochondrial genome sequences were obtained using a PCR-based strategy involving a combination of conventional PCR and long PCR methods in order to amplify overlapping mitochondrial fragments spanning the whole genome. For initial PCR amplification, primers were designed on the alpaca complete mitochondrial sequence determined by Arnason et al. (2004) (GeneBank, AJ566364). For further amplification, primers were designed on guanaco or vicuña newly generated sequences.

PCR reactions were performed with 10 ng genomic DNA diluted in a 50 µl reaction mix with an initial denaturation step at 94°C for 2 min followed by 34 cycles at 94°C for 50 s, 49–54°C annealing temperature for 1 min, 72°C synthesis for 1 min, and a final extension at 72°C for 5 min. Amplification of the control region and the contiguous 6,000 bp fragment at the 3′ end was carried out in one step with the LA PCR kit (Takara, Osaka, Japan) following the procedure advised by the manufacturer. The primer sequences used are listed in Table 1.

PCR products were purified with the QIAquick PCR-purification kit (QIAGENE Inc., Valencia, CA, USA) and bidirectionally sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were analyzed using an Automated 3730 DNA analyzer (Applied Biosystems, Foster City, USA).

Sequence data analysis

The guanaco and vicuña mitochondrial genome sequences have been deposited in GenBank under the accession numbers EU681954 and FJ456892 respectively. The mitochondrial sequences of the two species were determined by manual assembly of the sequenced fragments and aligned using the CLUSTAL W software (Thompson et al. 1994). The protein-coding genes were identified by detection of each corresponding ORF and comparison with nucleotide and amino acid sequences known for other artiodactyls. tRNA genes were identified using the tRNA-scan-SE v1.21 software (Lowe and Eddy 1997). Location and characteristics of repeat motifs were detected by means of the Tandem Repeats Finder v4.0 (Benson 1999).

Nucleotide and amino acid differences as well as the number of transitions (ti) and transversions (tv) were obtained using the MEGA 3.1 software (Kumar et al. 2004). We additionally used the control region (CR) sequences of *Lama guanicoe* (AY856252, AY856166, AY500896, AY500897, AY639017) and *Vicugna vicugna* (AY856320, AY856298, AY500899, DQ270408, DQ270408) available in GenBank in order to examine the CR substitution pattern.

DNA secondary structure prediction for the CR sequences was performed by free energy minimization using the mfold web server http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi (SantaLucia 1998; Zuker 2003).

Results and discussion

Organization and general features of the guanaco mitochondrial genome

The complete mitochondrial sequence of the guanaco is 16,649 nucleotides (nt) long and its L-strand composition is 27.4% T, 26.2% C, 31.7% A, and 14.6% G. Figure 1 shows the organization of the guanaco mitochondrial genome determined in this study. In agreement with the content and arrangement reported for other mammals (Anderson et al. 1981, 1982; Ursing and Arnason 1998), the guanaco genome comprises 13 protein-coding genes, 22 tRNAs, 2 rRNAs, and a control region (CR). As in other vertebrates, the genome organization along the coding region is quite compact, with only twelve 1–6 nt long intergenic spacers. The mtDNA also shows four overlapped reading frames, the longest of which is 43 nt long and includes parts of the ATP6 and ATP8 genes.

The L-strand origin of replication (Ori L) is located between the tRNA-Asn and tRNA-Cys genes. Like in many other vertebrates, Ori L has the potential to fold into a stem-loop secondary structure (Martens and Clayton 1979) which, in the guanaco, is 11–14 nt long.

The 22 tRNA genes range from 59 to 75 nt long. With the exception of tRNA-Ser (AGY), which lacks the entire DHU arm, the other 21 genes can fold into a typical cloverleaf secondary structure. Similarly to other vertebrates (Anderson et al. 1981), guanaco mitochondrial tRNAs contain non-standard base pairing. The acceptor region of the tRNA-Val and tRNA-Met shows an A–G base pair, while that of tRNA-Lys shows a C–U. Moreover, tRNA-Met and tRNA-Lys. respectively contain an U–U and A–C pair in the TψC arm.

All mitochondrial protein-coding genes have either an ATG or ATA (Methionine) start codon, except for ND3, which may have an ATA or the contiguous ATC. The

Table 1 Primers used in this study

Fragment	Length (bp)	Forward sequence	Reverse sequence	Annealing temperature (°C)
MT1	553	GCTCCACTTTCCTTGCTGTC	TCCTTGTTGAGTTCACTCGT	52
MT2	623	GGGCAACCCAAACAAATTAT	GCTGTTGTCACTGTATTGGC	51
MT3	613	TCATACTAATCCCCCTGACCTGACT	TGCAAGCCGTATAGGGGTAT	53
MT4	520	GGCTCCTTAAATTTTCTAAT	CCAGAATTATTGTTCGACTG	51
MT5	999	ACTCCCTGAAGCTACATAGG	GGTCTGAGTGCATATATCAT	49
MT6	901	TGACATTGAATCACAATCCA	TTGTCTTGTAAAGACCTCCT	51
MT7	900	TTTCTACATATCTGCACCCA	TGGTTATCATTACTGGATGG	51
MT8	904	CTCTATTTCCTCTCATTTCTC	GCGTGTAGATAACGAATAAT	49
MT9	922	TGCAAATCATGACAGGACTA	ATTAGGCTTCGTTGTTTGGATGTGT	53
MT10	852	CTAGGAGACCCCGACAACTA	CGGGTTGATGATTTCACG	52
RC	932	AAATATCATTTACCCGCATC	CTGGCACGAAATTTACCAAC	53
MT11	822	GGTTTGATCCCAGCCTTTCTATTAG	GGGGCTTTCGTAAGGGTTTT	55
MT12	856	CTATAGGGAACAAAAGTAAGC	GTCTAAAAAGCTGTACCCTT	50
MT13	900	TGAGAAGATTTGCAAGTAGA	TTGATCGGCAGATCTAAATT	50
MT14	904	GACGAGAAGACCCTATGGAG	AGACAGCTAAGCTGGACATA	52
MT15	905	CCATCCTAGCTCTAACCCTA	GGCCCGATAGCTTATTTAGC	52
MT16	950	GAGGTTTAGCCCTCTTATTTCTAG	TGGGTGCAAATCCAGATAGG	53
MT17	981	TCACAATAACAATCACAATA	CGATCATGATTGGTATAACT	50
COIS1	872	GAACTTGCAATTCAATGTGT	GTGGGAGATTATTCCAAAGC	53
COIS2	856	TCGGCCACCCAGAAGTCTAT	AAAGTCATAGTGGCTATGGG	53
ARNTDS	539	TTGCATCAAAACGAGAAGTT	GGTTATTGATTTCATCCATC	50
ATP86	1,012	CTGCCTCTATATTATAAGCTCA	GTCATTAAAAGGGCTGAGAG	51
CO3	942	TACTTACAATTCTCGAATTTGCCG	TATCCTTTTTCCGGGTCTCT	54
LA12SC1	6,053	GGTTTGATCCCAGCCTTTCTATTA	GTGGGAGATTATTCCAAAGC	68

Fig. 1 Organization of the guanaco mitochondrial genome

analysis of GenBank mtDNA sequences of alpaca and the bactrian (EF212038) and dromedary (NC009849) Old World camels indicates that the ATA start codon has been assigned for the ND3 gene. However, if we consider ATA as the functional start codon, the resulting protein has an Isoleucine insertion at the second position, a feature that is absent in more than 30 eutherian sequences deposited in GenBank. In the camelid species here studied, the ATA sequence includes the three final tRNA-Gly nucleotides. Further comparisons indicated that in most mammalian species, the tRNA-Gly and ND3 genes do not overlap; therefore, the GenBank ATA assignment for the ND3 gene of alpaca and camels possibly represents an incorrect annotation. Contrarily, if ATC is the start codon of the ND3 gene, the deduced protein does not bear insertions and its length is equal to that of other animals. In connection with this, Xu and Arnason (1994) reported an Isoleucine ATC start codon at the ND3 gene of Indian rhinoceros and rodents, the ND2 gene of humans, the ND4L of blue whales, and the ND5 gene of horses, seals and mice. Consequently, we assume that ATC is the most probable ND3 start codon in the camelid species here studied. On the other hand, four (ND1, COIII, ND3, ND4) out of the thirteen coding genes have incomplete TA or T stop codons that could be post-transcriptionally modified by polyadenylation (Ojala et al. 1981).

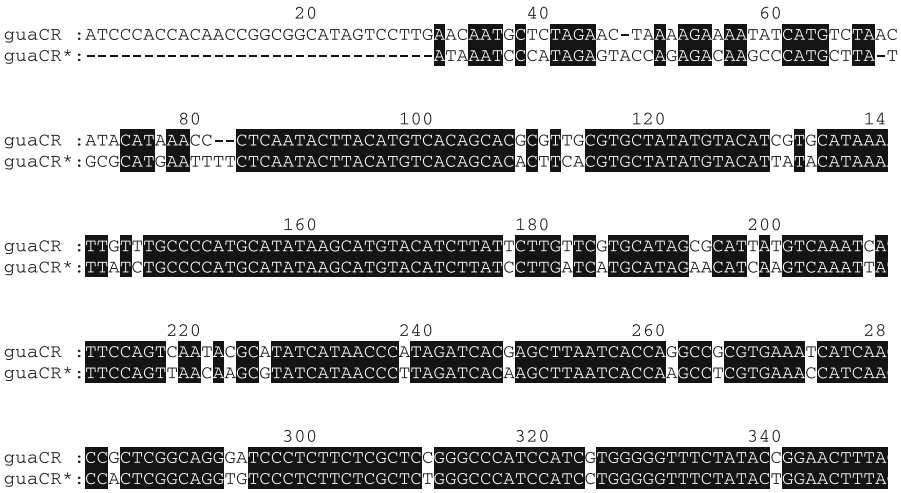

Fig. 2 Alignment of the 5′ Control region end. *guaCR* guanaco control region sequence, *guaCR** guanaco control region- like sequence obtained from the same specimen

In order to determine the complete mtDNA molecule, we used a set of primers producing fragments which overlapped 50–200 bp until the sequence of the circular genome was completed. The guanaco CR thus obtained is 1,215 nt long and shows high similarity (98.6%) to the entire CR reported for alpaca (Arnason et al. 2004) and the partial CR sequences described by Marín et al. (2008) for guanaco. Unexpectedly, the CR is 155 nt longer than the camelid CR previously published by Maté et al. (2004), from which it also differs by a large number of gaps and notorious sequence divergence at both the 5′ (Fig. 2) and 3′ ends. To discard interpopulation or interindividual differences as the cause of the sequence divergence here observed, we performed a new PCR reaction in the same guanaco sample using the primers and conditions reported by Maté et al. (2004). We obtained a sequence (CR*) which differed 18% from the CR sequence described above for the same guanaco individual (See Fig. 2).

In order to distinguish which of these sequences is the mitochondrial CR and which is a possible NUMT, we analyzed their base composition and substitution patterns (ti and tv). The CR followed the general (A+T)>(C+G) pattern reported by Sbisà et al. (1997) for mammals, while the (C+G)>(A+T) inverse ratio was observed for the conserved sequence blocks (CSB) domain of CR*.

Further alignment of the CR here reported with partial CR sequences (513 bp) from guanaco and vicuña from GenBank (data not shown) showed 38 variable sites mainly located at the first 100 bp of the CR. In contrast, the CR* sequence variability was much lower than that of the CR since only 11 polymorphic sites distributed along the complete sequence (1,060 bp) were counted. This observation is consistent with the evolutionary rates of non-coding sequences in the nuclear genome, which are more than ten-fold slower than that of the mitochondrial control region (Zischler et al. 1995). The substitution pattern was clearly different for both sequence sets. While in the CR sequences the ti/tv ratios (R) ranged from 2.5 to 7, evidencing the transition bias expected for mtDNA sequences, CR* transversions were equal to or more frequent than transitions ($R = 0.25–1$).

We also investigated regions that potentially form secondary structures in the CR from mammalian species in South American camelids and observed that most domains can fold into stem-loop stable structures ($dG < 0$) in both the CR and the CR*. In contrast, termination-associated sequence 1 (TAS1) motif which has been functionally associated with the D-loop termination (Madsen et al. 1993) was not able to form this sort of structure in the CR*. Taken together, our findings suggest that the CR sequence corresponds to the guanaco mitochondrial control region and that the CR* probably represents a nuclear copy of mitochondrial origin (NUMT) (Lopez et al. 1994).

Comparison of guanaco and vicuña mitochondrial genomes

The general content and organization of the guanaco and vicuña mitochondrial genomes are similar. We obtained 16,084 nucleotides of the vicuña mitochondrial DNA, encompassing all coding genes and 650 nt of the 5′ portion of the CR. Additional assays were performed using redesigned primer sets and different reaction conditions in order to determine the 3′ portion sequence of this region, but they all resulted in more than one amplification product and ambiguous sequences. These difficulties require further investigation on the nuclear co-amplification of pseudogenes (Zhang and Hewitt 1996), short repeat motifs, homopolymers (Feinstein and Cracraft 2004) or other

Table 2 Nucleotide and amino acid differences between guanaco and vicuña mitochondrial protein-coding genes

Gene	Gene length	nt. differences	aa differences
ATP6	681	13 (1.9)	1 (0.4)
ATP8	204	7 (3.4)	1 (1.5)
ND4L	297	11 (3.7)	0 (0.0)
COIII	784	33 (4.2)	0 (0.0)
COI	1,545	68 (4.4)	0 (0.0)
COII	684	32 (4.7)	0 (0.0)
ND2	1,042	54 (5.2)	7 (2.0)
ND4	1,377	74 (5.4)	2 (0.4)
ND3	350	20 (5.7)	3 (2.6)
ND6	528	31 (5.9)	5 (2.9)
ND1	956	58 (6.1)	0 (0.0)
ND5	1,821	115 (6.3)	10 (1.6)
Cytb	1,140	73 (6.4)	6 (1.6)
Total	11,409	589 (5.2)	35 (0.9)

Percentages are indicated between parentheses

nt nucleotide, *aa* amino acid

artifacts, as possible causes for the CR sequence distortion preventing the sequencing of this region in vicuña.

The alignment of the complete guanaco and vicuña mitochondrial genomes excluding the CR sequence showed 4.4% nucleotide differences. Similar values have been reported for other species from different but closely related genera (Arnason et al.1993).

Total nucleotide substitutions recorded between protein-coding genes were 589, representing 5.2% sequence divergence between the two genomes (Table 2). This percentage is slightly lower than that found by Kadwell et al. (2001) using only the Cyt b gene (5.8–8.9%).

Nucleotide differences between protein coding genes were also analyzed according to codon position and type of substitution. As expected for these genes, transitions at the third position were the most frequent, while transversions were by far less frequent.

Table 2 depicts the comparison between the 13 protein-coding genes. Nucleotide differences varied from 1.9% in ATP6 to 6.4% in Cyt b. In general, genes encoding ATP synthase and Cytochrome c oxidase subunits were more conserved than those encoding Cyt b and the NADH dehydrogenase complex. Differences at the deduced amino acid level ranged from 0.0% for ND4L, COXI, COII, COIII, and ND1 to 2.9% for ND6.

Total nucleotide differences between the tRNA genes from the two species comprised 33 transitions (2.2%) and one transversion.

In addition, 21 (2.2%) and 32 (2%) substitutions were found for the 12S and 16S rRNA genes respectively (Table 3).

Table 3 Nucleotide differences in number and percentage between guanaco and vicuña 12S and 16S rRNA genes

	Length (nt) (gua/vic)	Total	ti	tv	Indels
rRNA12S	967/968	21 (2.2)	19 (2)	1	1
rRNA16S	1,562/1,561	32 (2)	29 (1.8)	2	1
Total	2,529/2,529	53 (2.1)	48 (1.9)	3	2

gua/vic guanaco/vicuña, *ti* transitions, *tv* transversions

Conclusions

Unlike domestic South American camelid species, for which extensive mitochondrial introgression has been reported, there is no evidence that hybridization between guanaco and vicuña may have occurred (Vidal Rioja et al. 1994; Stanley et al. 1994; Kadwell et al. 2001). Thus far, the available information about phylogenetic relationships and genetic differentiation between wild South American camelid species is based on one single mitochondrial gene or a few portions of mitochondrial genes (Stanley et al. 1994; Marín et al. 2007). In this study, we assessed the genetic differentiation between the guanaco and the vicuña using the complete mitochondrial coding sequence.

On the other hand, the currently recognized subspecies of *Lama guanicoe* and *Vicugna vicugna* are still under genetic revision (Wheeler 1995; Sarno et al. 2004; Marín et al. 2008). The finding of a genetic marker that shows sufficient variation to distinguish among recently diverging species is highly desirable. Therefore, the use of a mitogenomic dataset as the one provided here may contribute to further understanding these unresolved questions and others related to the conservation and future management of the camelid species.

Nuclear DNA sequences of mitochondrial origin, also known as NUMTs (Lopez et al. 1994), have been described for a wide variety of eukaryotic species. Herein, we report the presence of mtDNA-like sequences in South American camelid species, probably of nuclear origin. This finding shows important methodological and evolutionary implications. NUMT contamination, if undiscovered or not well characterized, may induce erroneous conclusions in population or phylogenetic studies using mtDNA as a marker. Therefore, caution is essential to avoid NUMTs co-amplification when mtDNA from camelid species is analyzed.

Finally, NUMTs identification may provide the opportunity to gain insight into the differential rate and mode of evolution of their mitochondrial counterpart.

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