Restriction site patterns in the ribosomal DNA of Camelidae

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Abstract

The restriction map of rDNA from South American camelids and the Bactrian camel was analyzed by digestion of high-molecular-weight DNA with endonucleases *Eco*RI, *Bam*HI and the two combined followed by Southern blot hybridization with probes for the 18S and 28S rDNA sequences. We scored a total of 17 restriction sites, six of which were mapped conserved in all the species. The other eleven corresponded to spacer regions and revealed variations between these taxa. The study showed that the two groups differ in the length of the internal transcribed spacer. Also they showed the existence of two regions of fast evolution on the opposite termini of the external spacer. A restriction site present at low frequency in the non-transcribed spacer of guanaco and llama was the only difference encountered within the South American group.

Introduction

Systematics and cytogenetics studies (Franklin, 1982; Larramendy. *et al.*, 1984; Bunch, Foote & Maciulis, 1985; Bianchi *et al.*, 1986) show the four camelids of South America - guanaco (*Lama guanicoe*), alpaca (*Lama pacos*), llama (*Lama glama*) and vicuña (*Vicugna vicugna*) - to be closely related. At the molecular level five fractions of satellite DNA were detected in all four South American (S.A.) camelids (Vidal Rioja *et al.*, 1987). *In situ* hybridization using isolated llama satellites as probes showed that the distribution of these satellite sequences over the chromosomes is rather similar for all four camelids (Vidal-Rioja *et al.*, 1987). Similar restriction patterns have also been obtained with some restriction enzymes (Vidal-Rioja *et al.*, in preparation). These results indicate a fairly well-conserved genome organization of the New World camelids.

The Bactrian camel (*Camelus bactrianus*) and the dromedary (*Camelus dromedarius*) - representatives of Old World camelids - have cytogenetic similarities to their S.A. relatives (Capanna & Civitelli, 1965; Bunch, Foote & Maciulis, 1985) in that they share the same karyotype and C and NOR banding distribution. Their chromosomes may also be matched precisely by G banding. Therefore, Bunch, Footz and Maciulis (1985) inferred that either gene mutations and/or differences in the number of certain DNA sequences may account for some of the divergent evolution observed between Old and New World camelids. Thus far, however, neither genome molecular information on Old World Camelids nor DNA comparisons to their S.A. relatives has been reported.

In mammals, rRNA genes (rDNA) are a multigenic family with hundreds of copies (Long & Dawid, 1980). These copies are tandemly organized and clustered in chromosome locations identified as nucleolar organizing regions (NORs), by means of silver staining (Lau & Arrighi, 1977). Recently, Vidal-Rioja, Larramendy and Semorile (1989) reported an average of six NORs per cell in llama, guanaco and alpaca, whereas in vicuña there were only three. These results were confirmed by *in*

situ hybridization with a probe bearing 18S rRNA sequences. Moreover, in vicuña there were observed variations in both the number of rDNA sites per cell and the amount of ribosomal genes per site. Thus far, no comparable molecular information has been reported for Old World camelids.

Each rDNA repeat unit includes a non-transcribed spacer (NTS) and a coding region which produces 18, 5.8, and 28S rRNAs. The NTS is a useful region for the analysis of interspecific DNA polymorphisms, due to its universal presence, high copy number and rapid alteration (Suzuki, Moriwaki & Nevo, 1987). In a similar fashion, comparative analysis of 28S rRNA genes has been concentrated in the variable regions of these genes (González, Sylvester & Schmickel, 1988).

The aim of this study was to investigate restriction site mapping of rDNA of S.A. and Asiatic camelid species. The patterns obtained were then used to evaluate variations between these taxa (Hillis & Davis, 1986).

Materials and methods

DNA samples

DNA samples from liver and spleen biopsies of specimens of alpaca, guanaco, vicuña and llama were obtained according to Vidal-Rioja *et al.* (1987). For DNA isolation from peripheral blood of the Bactrian camel, the procedure of Kunkel *et al.* (1977) was followed.

DNA restriction and blotting

Five micrograms of DNA from each camelid species and from human (used as procedural control) were single and double digested with the endonucleases *Eco*RI and *Bam*HI (BRL) and then electrophoresed in 0.8% agarose (Maniatis, Fritsch & Sambrook, 1982). λDNA/*Hin*dIII fragments were run in these gels as molecular size standards. After photography the standard was cut out from the gels and the eukaryotic DNA was blotted onto nylon membranes (Z probe, BioRad, Ca.) following Reed (1986).

Probe labelling and hybridization

Two human ribosomal probes were used for hybridization. Probe pR18S, obtained from L. Manuelidis, is a 6 kb *Eco*RI fragment that includes most

of the 18S rRNA gene, the external transcribed spacer (ETS) and a portion of non transcribed spacer (NTS) (Manuelidis & Ward, 1984). Probe pA1, obtained from I. González, is a 7 kb *Eco*RI fragment comprising most of the 28S rRNA gene, the internal spacers (IS) and 200 bp 3' of the 18S rRNA gene (Erickson *et al.*, 1981).

All probes were radiolabeled by nick translation using the Nick Translation Reagent Kit of BRL and dGTP [α-^{32}P] (3000 Ci/mmol, NEN, Dupont).

Hybridizations were carried out in 1.5 × SSPE (20 × SSPE = 3.6M NaCl; 0.2M sodium phosphate buffer pH 7; 0.2M EDTA), 1% SDS (sodium dodecyl sulfate), 0.5% BLOTTO (10 g nonfat dry milk per 100 ml sterile water plus 0.2% sodium azide), 0.1 mg/ml denatured salmon sperm DNA plus probe with a final concentration of 2 × 10^6 cpm/ml at 68°C for 20 h. Afterwards, the membranes were washed with decreasing concentrations of SSC plus 0.1% SDS until a final wash of 0.1% SSC, 1% SDS at 65°C during 30 min. Then, the

Table 1. Ribosomal DNA fragments generated by the endonucleases *Eco*RI and *Bam*HI in the genome of the S.A. camelids and the Bactrian camel.

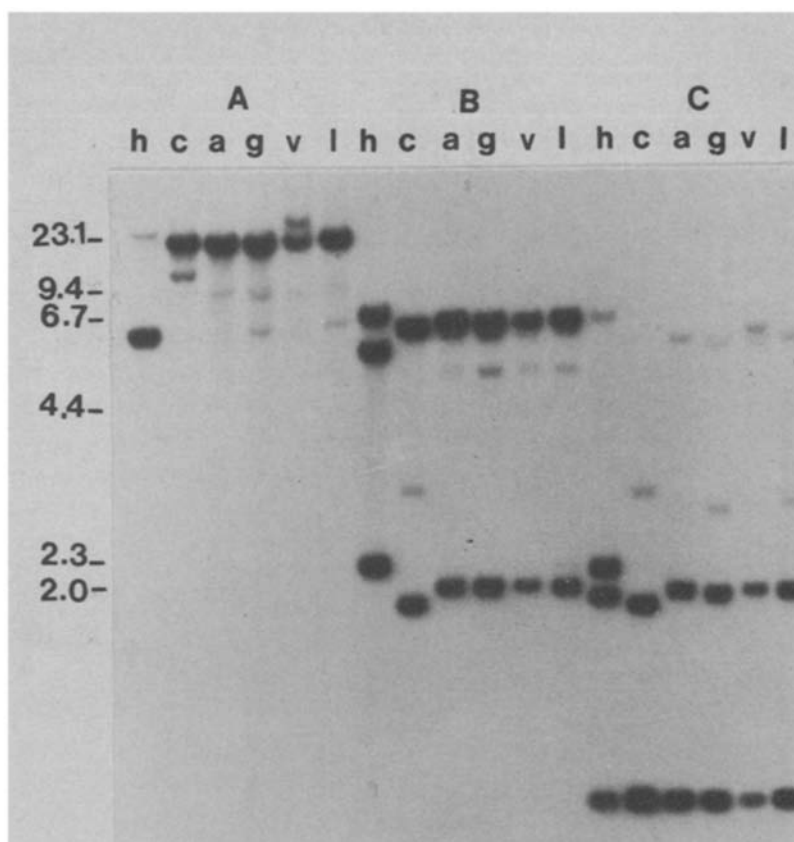| Species	S.A. camelids	Camel
Fragment	Length (kb)	Length (kb)
E_1E_2	15.0	15.0
E_xE_2	–	9.8
E_yE_2	9.1	–
E_zE_2	6.1	–
E_yB_w	0.2	–
B_wE_z	2.8	–
E_zB_y	3.0	–
E_xB_x	–	3.6
B_wB_y	5.8	–
B_xB_z	–	3.2
B_yB_1	2.0	–
B_zB_1	–	1.9
B_1E_2	1.1	1.1
E_2B_2	5.1	4.9
B_2B_3	1.4	1.5
B_3E_3	1.8	1.7
E_3B_p	0.95	–
E_3B_o	–	1.0
E_3B_q	–	1.1
B_3B_o	–	2.7
B_3B_p	2.75	–
B_3B_q	–	2.8
E_2E_3	8.3	8.1

Fig. 1. ³²P-18S rDNA hybridization pattern of human (h), camel (c), alpaca (a), guanaco (g), vicuña (v) and llama (l) DNAs digested with *Eco*RI (A), *Bam*HI (B) and *Eco*RI + *Bam*HI (C). Size markers (kb) obtained by digesting λ DNA with *Hind*III are denoted to the left.

membranes were autoradiographed (Agfa-Gevaert film) at −80°C with intensifying screens for 24-72 h.

A map of the *Eco*RI and *Bam*HI sites in the rDNA unit of camelids was constructed using as reference the sites conserved between human and camelids.

Locations of *Eco*RI and *Bam*HI sites were assessed by averaging the results of four different experiments of restriction and hybridization.

Results

Restriction site analyses of the rDNA unit
Figure 1A shows an *Eco*RI digest of rDNA from New and Old World camelids hybridized with probe pR18S. The human rDNA pattern (used as

procedural control) agrees well with that of Arnheim *et al.* (1980). All camelids have a major band of 15 kb. Minor bands of 9.1 kb and 6.1 kb also appear in the lanes of S.A. camelids. The Bactrian camel has a minor band of 9.8 kb. In each camelid, all these minor bands may derive from a few copies of the ribosomal unit having polymorphisms of the *Eco*RI site on the external spacer. Both major and minor bands produced by cleavage with *Eco*RI and seen in Fig. 1A are plotted in the map of Fig. 3A-B as segments E_1E_2 and E_yE_2, E_zE_2 and E_xE_2, respectively. Table 1 summarizes these data and includes the length of the segment for the two camelid taxa reported here.

Figure 1B shows DNA samples digested with *Bam*HI and probed with 18S rDNA sequences. Under our conditions, the patterns obtained for human DNA agree completely with those described by
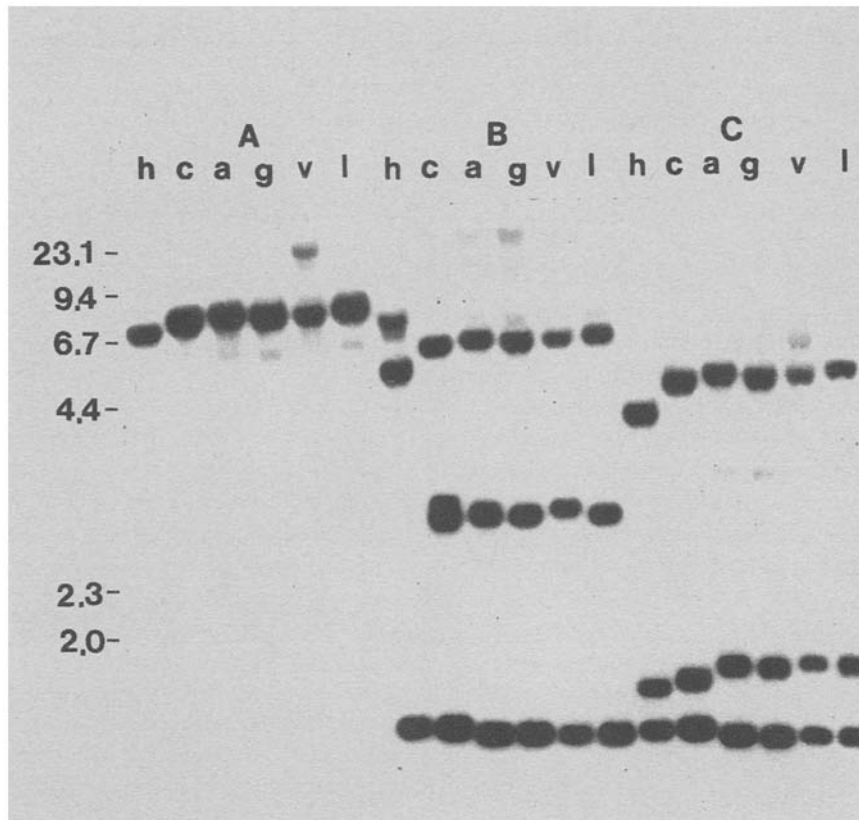
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Fig. 2. ^{32}P-28S rDNA hybridization pattern of human (h), camel (c), alpaca (a), guanaco (g), vicuña (v) and llama (l) DNAs digested with *Eco*RI (A), *Bam*HI (B) and *Eco*RI + *Bam*HI (C). Size markers (kb) obtained by digesting λDNA with *Hin*dIII are denoted to the left.

Wilson *et al.* (1984).

Hybridization of S.A. camelids DNAs shows three major bands of 6.2, 5.8 and 2.0 kb, which in the map correspond to fragments B_1B_2, B_wB_y and B_yB_1 respectively. Light bands detected (present in only few ribosomal units) include one of 5.0 kb found in all S.A. camelids but in different amounts, and another of 4.5 kb, found only in llama and guanaco (Fig. 1B). Due to their polymorphic character, these fragments are not included in the map. However, because the 4.5 kb fragment appears in two different camelids (guanaco and llama), it is considered informative. We conclude that this fragment originates from cuts in B_y and a site between B_w and E_z. In camel the pattern obtained has two prominent bands of 6.0 and 1.9 kb and a minor one of 3.2 kb. In Fig. 3B, these bands are mapped as fragments B_1B_2, B_zB_1 and B_xB_2, respectively. According to these results, fragment B_1B_2 is 200 bp shorter in the camel than in S.A. camelids.

Hybridization of *Eco*RI-*Bam*HI double-digested DNA with pR18S probe results in two major bands in the lanes of each camelid. One extends from the B_1 site within the 18S gene to B_y or B_z into the external spacer and differs between Old and New World camelids by 0.1 kb (Table 1); the other, occurring within the B_1E_2 coding region, has 1.1 kb and seems conserved from human to camelids (Fig. 1C; Fig. 3A-B). Light bands corresponding to fragment B_xB_z in camel, and to E_zB_y in guanaco and llama were detected. Other fragments mapped in Fig. 3 (E_yB_w, B_wE_z and E_xB_x) are beyond the region covered by the probe and consequently not detected by hybridization.

Figure 2 shows the restriction enzyme patterns obtained with probe pA1 containing 28S sequences. Fig. 2A shows the unique band detected with this probe on *Eco*RI digestions in human DNA; this pattern agrees with the one showed by Erickson *et al.* (1981). In camelids this fragment is

slightly different between the Bactrian camel and the S.A. group. The size of this segment flanked by sites E_2E_3 (Fig. 3A-B) is 8.1 kb and 8.3 kb, respectively. The human rDNA digested with BamHI and probed with 28S rDNA sequences produced a pattern totally coincident with others from the literature (Arnheim et al., 1980; Maden et al., 1987). The BamHI portion within the 28S gene in camel DNA (B_2B_3) is slightly larger than that in S.A. camelids (Fig. 2B; Fig. 3A-B; Table 1). On the other hand, the BamHI fragment B_1B_2 appears shorter, in camel (6 kb) than in S.A. camelids (6.2 kb). It is worth noting that this 200 bp size difference among the camelids is similar to the difference observed in the E_2E_3 camelid segment (Fig. 3A-B; Table 1). We suggest that a longer ITS in New World camelids may explain this size variation. In camel, a length polymorphism is expected by the overlap of bands drawn as B_3B_0 and B_3B_q, the two of them different from the single B_3B_p deduced for S.A. camelids. This possibility was assessed with the probe pA^{BE}, a recombinant plasmid having a 1625 bp BamHI-EcoRI human fragment from the 28S gene (Erickson et al., 1981). When this probe was hybridized to camelid DNA digested with BamHI, one band of 2.75 kb was detected in the S.A. camelids, whereas two bands of 2.7 kb and 2.8 kb were identified in the Bactrian camel (data not shown).

Hybridizations of the pA1 probe on BamHI-EcoRI double-digested DNAs (Fig. 2C) highlight size differences between segments B_2B_3 and B_3E_3

(Fig. 3A-B) of the 28S rRNA gene from S.A. camelids and from the camel. In S.A. camelids, these fragments were approximately 1.4 kb and 1.8 kb, respectively, while in the camel they were 1.5 kb and 1.7 kb long (Table 1). Moreover, variants of the EcoRI-BamHI fragment E_2B_2 are also suggested by these assays (Fig. 2C and Fig. 3A-B). Fragments E_3B_0, E_3B_p and E_3B_q, not detected by probe pA1 were, however, deduced for the 3' portion of the rDNA unit.

Discussion

A series of interesting features was revealed by mapping the rDNA of Camelidae using restriction fragment analysis and Southern hybridizations with probes for 18S and 28S rRNA genes. In the 18S rRNA gene, the positioning of EcoRI and BamHI sites had been conserved in the five camelids studied here. These findings agree with the notable evolutionary stability of the 18S rDNA among vertebrates. For example, the divergence between Xenopus and human 18S rRNA gene has been reported to be 2.5% and between human and rodents 0.37% (mouse) to 0.45% (rat) (González et al., 1985), even though these lineages have been separated from one another by 200 MYR and 90 MYR, respectively. Within the 28S rRNA gene the presence of an EcoRI site (E_3) and two BamHI (B_2 and B_3) sites was confirmed for S.A. camelids and the
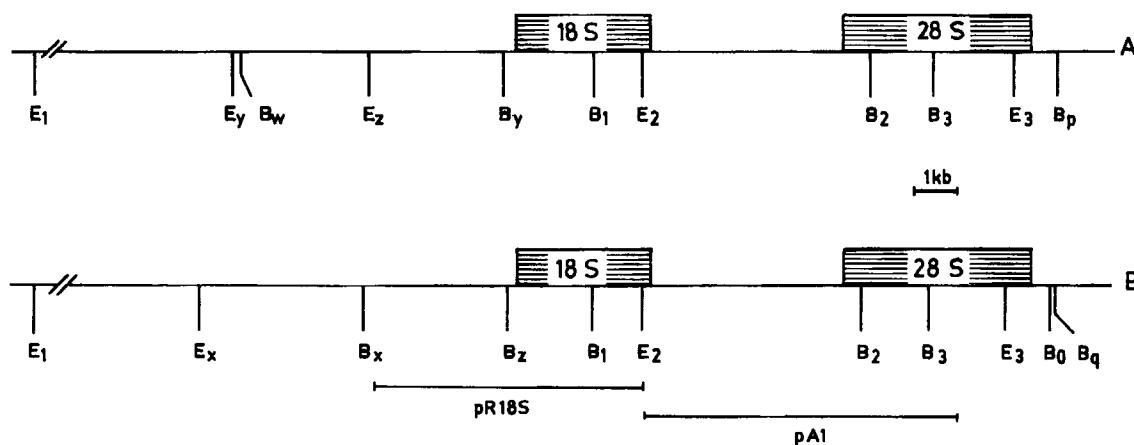

Fig. 3. Restriction map of S.A. camelids (A) and Bactrian camel (B) rDNA unit. E and B indicate restriction sites for EcoRI and BamHI, respectively. Conserved sites within the 18S and 28S genes (boxes) and in the NTS (thin lines) are denoted by the numbers below. Mutated sites in the NTS are represented by subscript letters. pR18S and pA1 represent the length of probes used.

camel. However, the individual segments included between these sites show slight length differences between the two groups of camelids. Accordingly, fragment B_2B_3 in camel is larger than its counterpart in S.A. camelids and B_3E_3 is shorter (Table 1).

The largest subunit of rDNA in several organisms exhibits considerable length variation. This variation generally correlates with the time elapsed since they diverged, and ranges from 2900 bp in prokaryotes to 3392 bp in yeast to 5025 bp in humans (González et al., 1985). These size variations are known to occur by expansions or contractions of 'variable joining sequences' at specific points within the gene. Different terms have been proposed for these variable sequences: divergents or 'D domains' (Hassouna Michot & Bachellerie, 1984), 'expansion segments' (Clark et al., 1984) or 'variable regions' (Chan, Olivera & Wool, 1983). In our results the variations visible in Fig. 2C alter neither the presence of B or E sites nor the total length of the fragment B_2E_3 (Fig. 3A-B). Consequently, it seems reasonable to assume that the differences detected may correspond to insertion/deletion events that are able to expand or contract these zones of the 28S gene. Another mechanism suitable to induce these variations without modifying the length of B_2E_3 would be an inversion that changes the position of B_3. Cloning and comparative sequencing of individual fragments from the two taxa will be necessary to identify the true nature of these assumptions.

The hybridization patterns shown in Figures 1 and 2 provide direct evidence about several ribosomal DNA fragments that differ in length between S.A. camelids and camel. When these fragments are plotted in the maps of Fig. 3, we invariably find that they are fully or partially constituted by sequences of spacer regions. These data indicate extensive heterogeneity mostly of the long non-transcribed spacer among the camelids. This region has been reported to vary among species, individuals, and even among repeats of the ribosomal gene family within individuals (Krystal & Arnheim, 1978). Similar variation was observed in the present study. The E_1E_2 15 kb band detected by the pR18S probe in all camelids was truncated in some of the camel ribosomal repeats by the presence of an E_x site in the external spacer, resulting in the 9.8 kb fragment E_xE_2. In S.A. camelids this truncation occurs in two different positions, giving rise to three classes of

long external spacer: the most common 15 kb fragment, shared with the Bactrian camel, a 9.1 kb fragment containing the E_y site and, a 6.1 kb fragment with the E_z site (Fig. 3A). The lower frequency of the hybridization bands corresponding to fragments E_xE_2, E_yE_2 or E_zE_2 as compared with that of the E_1E_2 segment may be due to a low number of repeats that include these variations.

During BamHI digestions, the segment including the external spacer and the 18S gene of camel is cut into three fragments. The largest one would represent portions of the 18S ribosomal gene extending in the 3' (B_1B_2 fragment) and 5' directions (B_1B_z segment). The 3.2 kb band (Fig. 1B) appears to be a B_xB_z fragment derived from a low number of ribosomal units. In S.A. camelids the 18S probe detects a B_wB_y 5.8 kb segment within some repeats. Moreover, if we assume that in certain of these ribosomal repeats the B_w site is displaced towards the B_y site, then we can explain the 5 kb fragment found in the four camelids and the 4.5 kb fragment found only in llama and guanaco (Fig. 1A). In fact, this last feature is the only difference we were able to observe among the four species of the S.A. taxa.

When hybridized on BamHI digested DNAs, the probe pA_1 provided some information about the 5' end of the long external spacer. In several organisms this region varies even among very closely related species (Brown, Wensink & Jordan, 1972; Arnheim et al., 1980; Suzuki, Moriwaki & Nevo 1987). In the Bactrian camel, this region has two BamHI sites (B_0 and B_q) distributed among different rDNA units, whereas the S.A. camelids have only one site (B_p) within the repeats (Fig. 2B; Fig. 3A-B).

Within Artiodactyla, the rDNA of calf was the first to be isolated and studied in its organization. Meunier-Rotival et al. (1979) reported the size of the rDNA subunit as 33 kb. In camelids, our experiments with the pR18S and pA1 probes covered 26 kb of rDNA sequences, leaving a stretch of the external spacer between the B_p or B_q site in the 3' region of one rDNA unit and the E_1 site in the 5' region of the next unit without characterization. Calf and camelids rDNAs are similar in having restriction fragment length heterogeneity in two regions of the external spacer, one adjacent to the 3' end of the 28S gene and the other about 8 kb 5' from the beginning of the 18S gene. Finally, our data rule out the presence of a BamHI site within

the ITS of camelids similar to the site reported in calf (Meunier-Rotival *et al.*, 1979).

The origin of *Camelidae* may be traced back to 40-45 MYR ago in North America. By the late Miocene, the ancestral camelids diversified into the *Pliauchenia* and *Procamelus* forms, which late in the Pliocene reached South America and Asia, developing the modern taxa *Lama/Vicugna* and *Camelus* (for revisions see Franklin, 1982, and Wheeler, 1991). These data suggest that the two lineages have been evolving separately for the last 5-10 MYR. This separation has resulted in a series of changes among which the restriction enzyme site variation in the rDNA unit was analyzed here. The results document the occurrence of a minimum of ten mutations since the S.A. lineage diverged from its common ancestor with camel. Our data also support the ample conservatism reported previously for the S.A. group. Nevertheless, the finding of one mutation shared by guanaco and llama may suggest a closer relationship between these two camelids. This possibility is being investigated further by analysis of highly repetitive DNA.

Acknowledgements

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