PATERNITY TEST IN "MANGALARGA-MARCHADOR" EQUINES BY DNA-FINGERPRINTING[1]

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ABSTRACT - GC-rich molecular minisatellite probes isolated from the human genome have presented a poor ability for individualization in horses. In this study new DNA sequences were isolated which could be used in paternity tests in horses. Genomic DNA from "Mangalarga-Marchador" horses was treated with restriction enzymes that preferentially digest non-repetitive sequences, so preserving the structure where mini and microsatellites are located. Four clones (S01, S05, S07 and S09) selected from a genomic library screened with a (TG)n oligonucleotide showed similar hybridization profiles generating bands of DNA-fingerprinting type. Using these probes the individualization power obtained was 10^{-8}, which is 10^5-fold higher than that obtained with M13, another GC-rich type probe. All clones were efficient in parentage detection in crossbreedings and presented a 27 bp consensus sequence, GTTTCATTTATTATTCTTTGGAAGAAA, which was repeated 12, 18, 11 and 21 times in clones S01, S05, S07 and S09, respectively.

Index terms: breeding methods, molecular cloning, progeny testing, horses, identification, genetic polymorphism.

TESTE DE PATERNIDADE EM EQÜINOS MANGALARGA-MARCHADOR PELA TÉCNICA DO DNA-"FINGERPRINTING"

RESUMO - Sondas moleculares de minissatélites CG-ricas isoladas do genoma humano têm apresentado pouca habilidade de individualização em cavalos. Neste trabalho foram isoladas novas seqüências de DNA, que podem ser utilizadas para teste de paternidade em cavalos. DNA genômico de cavalos Mangalarga-Marchador foi tratado com enzimas de restrição que digerem preferencialmente seqüências não-repetitivas, preservando, assim, a estrutura onde os mini e microssatélites estão localizados. Quatro clones (S01, S05, S07 and S09), selecionados a partir de uma livraria genômica com o oligonucleotídeo (TG)n, demonstraram um perfil de hibridização semelhante com bandas do tipo DNA "fingerprinting". Utilizando estas sondas, o poder de individualização obtido foi de 10^{-8}, 10^5 vezes mais elevado do que o obtido com a M13, outra sonda do tipo GC-rica. Todos os clones foram eficientes para a determinação do parentesco em cruzamentos e apresentaram uma seqüência-consensus de 27 pb, GTTTCATTTATTATTCTTTGGAAGAAA, que estava repetida 12, 18, 11 e 21 vezes nos clones S01, S05, S07 e S09, respectivamente.

Termos para indexação: métodos de melhoramento, clonagem molecular, teste de progênie, cavalos, identificação, polimorfismo genético.

INTRODUCTION

Molecular techniques such as DNA-fingerprinting are a powerful tool in the field of genetic analysis in animals. In addition to its use in genetic improvement, this technique has also been used with success in parentage determination. The use of DNA-fingerprinting is superior to serologic tests since it permits accurate exclusion as well as inclusion. This efficacy is a consequence of the strategic localiza-

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tion of minisatellites that may be present flanking or intervening in gene sequences. Minisatellites are genomic sites of high repetitiveness also known as Variable Number of Tandem Repeats. By using a Variable Number of Tandem Repeats DNA sequence as a probe, it is possible to simultaneously determine the genetic differences in a number of genome sites of an animal population (Jeffreys, 1987). The probes most widely used in fingerprinting analysis are portions of hyper-variable GC-rich repetitive genome elements termed minisatellites (Jeffreys et al., 1985a; Bird, 1987; Gardiner-Garden & Frommer, 1987; Pena et al., 1993).

Due to a high degree of endogamy, transgenic minisatellite probes have shown a low analysis power in equines, with a probability of two individuals presenting the same DNAF of 10^{-4} or 1 in a 10,000 individuals (Georges et al., 1988). Determinations of the content of repetitive sequences in equine genome revealed that about 7% are composed of 220 bp minisatellites (Wijers et al., 1993). Recently, different mini and microsatellites have been cloned from equines (Ellegren et al., 1992; Wijers et al., 1993; Sakagami et al., 1994). Using a centrometric probe of a 22 bp consensus sequence, Broad et al. (1995) obtained an individualization power of 10^{-5} which is similar to that achieved by Ellegren et al. (1992) using (TG)n-like sequences that seem to be the most informative ones for horses.

"Mangalarga-Marchador" is a valuable horse breed from the Southern region of the Brazilian State of Minas Gerais. These animals were improved by the end of the last century, as a result of the crosses between different races such as Andaluz, Arabic, Creole and Quarter-horse. Its main features are rusticity and a smooth trot as it keeps three feet on the ground during riding.

In addition to increase accuracy of progeny tests, the replacement of serologic tests by DNA-fingerprinting techniques can lead to advantages in the development of breeding. Cloned DNA probes can be utilized to monitor crossbreedings and libraries of semen and embryos with the objective of eliminating undesirable genotypes through QTLs (quantitative trait loci) identification, and detect deleterious genes and chimerism.

In this report, the isolation of a 27 bp repetitive unit from horse DNA and its utilization in progeny tests in "Mangalarga-Marchador" horses are described.

MATERIAL AND METHODS

Blood collection and preparation of genomic DNA

Blood was collected from prize-winning "Mangalarga-Marchador" animals in Brazilian horse-race competitions and from their offspring. Samples were preserved in EDTA 50 mM and kept at -20°C. Erythrocytes were lysed according to Madsen et al. (1992) and DNA was extracted with phenol and chloroform and precipitated with ethanol (Sambrook et al., 1986).

Construction of equine genomic library

Two different genomic libraries were made from 5 μg of total DNA from three stud horses. The first library was constructed by partially digesting total DNA with *Sau* 3AI to generate fragments ranging from 0.5 to 4 kb which were then ligated to dephosphorylated *Bam* HI-digested Bluescript SKII vector (Stratagene). For the second library, a strategy for exon exclusion and enrichment of fragment containing Variable Number of Tandem Repeats was employed through the digestion of total DNA with enzymes that moderately cleave mammallian genome (non-GC-rich sites). Genomic DNA was extensively digested with *Hind* III (40 U), dephosphorylated, digested once more with *Eco* RI (80 U) and ligated to the Bluescript SKII vector digested with both enzymes.

Ligation, genomic library screening and DNA sequencing

Ligations were performed according to Sambrook et al. (1986), using 1 μg of vector and a molar excess of genomic DNA: 3:1 for *Sau* 3AI and *Hind* III/*Eco* RI libraries. A hundred and twenty ng of the ligation system were utilized to transform competent *E. coli* DH5α prepared according to Sambrook et al. (1986). Transformation efficiency was low, 10^3 to 10^4 clones/μg plasmid (Table 1), when compared to intact vector (10^8). Such a low transformation rate is probably a result of the genotype of the *E. coli* DH5α host (mcr+) that selects against fragments containing methylated sequences such as Variable Number of Tandem Repeats, as previously observed by Rubertis et al. (1993). About 500 recombinant clones from each library were screened for the presence of Variable Number of Tandem Repeats using the synthetic oligonucleotides (TG)n, (CAC)n and (GGAT)n as probes. From the clones tested, a total of 24 hybridized clones from the *Hind* III-*Eco* RI library and 18 from the *Sau* 3AI library were obtained. Only one clone was positive with both (TG)n and (CAC)n probes. The inserts present in plasmids extracted from positive clones were amplified by PCR using univer-

TABLE 1. Screening of the 500 white clones selected in the genomic libraries.

Genomic library	Rate of transformation/ µg vector	Positive clones				Total	(%)
		(TG)n	(CG)n	(CAC)n	(GGAT)n		
Eco RI / Hind III	$1.4x10^{-4}$	14	6	3	1	24	4.8
Sau 3AI	$1.4x10^{-3}$	8	4	5	1	18	3.6

sal and reverse primers or were released by *Pvu* II digestion. All cloned fragments analyzed presented a different molecular weight.

Selected white colonies were transferred to nylon membranes (Amersham N^+) according to the procedures described by Sambrook et al. (1986). The membranes were pre-hybridized for three hours at 65°C in 6X SSC, 5X Denhartd's, 0.5% SDS, 0.05% sodium pyrophosphate (pH 7.0), 0.1% BSA and 3% calf skin protein. Oligonucleotides were labeled with [γ^{32}P]ATP. Hybridization was performed in an oven (Biorad) using hybridization solution without calf skin protein at 38°C. The membranes hybridized with oligonucleotide were washed in 6X SSC, 0.1% SDS at 45°C and those hybridized with M13 in 2X SSC, 0.1% SDS at 60°C.

The positive clones were grown in LB medium and stored in glycerol 50% at -50ºC. Plasmid DNA from recombinant clones was extracted using the boiling-miniprep procedure (Sambrook et al., 1986) and Qiagen columns. DNA sequencing of the selected clones was performed by the automatic method (Applied Biosystems) using M13 universal and reverse primers.

Agarose gel electrophoresis and blotting

Typically, 15 µg of DNA was digested with 80 U of restriction enzyme in a final volume of 200 µL according to manufacturer's recommendation (Pharmacia Biotech). The digests were submitted to electrophoretic separation at a constant voltage of 25 V during 24 hours in 0.8% agarose gel made in 1X TBE (pH 8.0). The gel was then stained with ethidium bromide, photographed, washed twice for 15 minutes in NaOH 0.4 M/1.5 M NaCl followed by two 15 minutes washes in Tris-HCl 0.5 M (pH 7.5)/2.5 M NaCl. The DNA was then transferred to a nylon membrane by blotting with 20X SSC and fixed with U.V. according to Sambrook et al. (1986).

Southern hybridization

In order to find the best restriction enzymes capable of producing a DNA-fingerprinting profile with distinct bands, the DNA of four horses (two males and two females) from different lineages were digested with three different restriction enzymes: *Hae* III, *Hinf* I and *Sau* 3AI, and hybridized with (TG)n as a probe. Hybridization and washing were carried out in a hybridization oven (BioRad). The nylon membranes were washed with 2X SSC and prehybridized in a solution containing casein 1%, 20% of Tris-HCl 0.1 M pH 7.5/NaCl 0.5 M/3% of porcine skin gelatin/ 0.05% of tween 20 (w/v) at 65°C for two hours. Hybridization was performed overnight at 42°C in Dyke's solution (Georges et al., 1988) containing 200 ng of the [α-^{32}P]ATP or [γ-^{32}P]ATP labelled probes (10^9 cpm/µg DNA). After hybridization the membranes were washed twice (5 minutes) with 2X SSC/SDS 0.1% and once (30 minutes) with 1X SSC/SDS 0 1% (42 to 65°C), dried, and exposed against XAR-5 film (Kodak) with intensifier screen for 12 hours or up to four days. For re-hybridization, membranes were first cleaned to remove the probe with 0.1X SSC/SDS 0.1% at 70ºC and then kept in 2X SSC. The membranes were checked for residual radioactivity with a Geiger counter. If the counting was higher than environmental background (> 30 cps), the washing procedure was repeated.

Band scores and statistical analysis

The statistical results were computed manually using the Excel software (Microsoft). To minimize reading errors, two scores were made independently for each gel. Band-sharing (BS) analysis among the individuals was estimated according to Haberfeld et al. (1991) and Bruford et al. (1992). The conventional identity analyses were carried out according to Jeffreys et al. (1985b), Bruford et al. (1992) and Pena et al. (1993) assuming that q can be calculated from the equation $X= 2q - q^2$. Average probability (h) of a band (or a resolvable DNA fragment) being present in the heterozygote state was calculated assuming that $h = 2 (1-q)/(2- q)$ (Gilbert et al., 1991). The mean number of bands per horse was calculated with n = 24 individuals and the results expressed with standard deviation (SD).

Computer analysis of similarity of DNA sequences

Comparisons among DNA sequences and analysis of the repetitive motives of clones and other nucleotide sequences on Genlibrary databases were carried out using the GCG program (Wisconsin), DNA-Strider and Dot Plotter (Don Gilbert, Indiana University).

RESULTS AND DISCUSSION

Equine genomic libraries

The strategy of Variable Number of Tandem Repeats cloning using enzymes that moderately cleave the genome, like *Eco* R1/ *Hind* III, was a success. Without previous fragment size selection, 24 positive clones were obtained by screening 500 white transformants (4.8% efficiency; 2.8% of (TG)n positive- Table 1). This strategy was easier and more efficient than that described by Ellegren et al. (1992), who obtained 30 positive clones in 10,000 screened recombinant clones (0.34% efficiency). As confirmed by DNA sequencing, this strategy also has the advantage of maintaining intact most of the G and C-rich minisatellite sequences, which was not observed in *Sac* II/*Hind* III strategy of Rubertis et al. (1993). This strategy is an alternative way, efficient and more simple for Variable Number of Tandem Repeats cloning, suitable for beginners.

DNA-fingerprinting patterns

The (TG)n-probe hybridized profile obtained with *Hinf* I digested DNA was multilocus kind with distinct bands. In contrast, the *Hae* III profile consisted of a smear showing some distinct bands above 6 kb. A smear was also obtained when DNA digestion was performed with *Sau* 3AI, but it covered all the molecular weight range and showed some strong monomorphic DNA bands. When 13 positive clones from the different libraries were tested, only four clones (S01, S05, S07, S09) showed similar band profiles of DNA-fingerprinting type when genomic DNA was digested with *Hinf* I (three from the *Eco* RI library and one from the *Sau* 3AI library).

When the wash of the Southern membranes was performed at 45°C, the DNA-fingerprinting patterns obtained were of the multilocus kind and showed no background, requiring an X-ray film exposure time no longer than 24 hours (Figs. 1A and 1B). An increase in the stringency temperature to 60-65°C caused a loss of some weak bands like the observed within the cloned probes. These small differences observed in the hybridization profile (appearance/ disappearance of bands) can be explained by the variation of similarity of the consensus sequence between the probes and the targets. The low background obtained with the hybridization procedure used permitted an amplification of the hybridization signal of DNA bands of about 2.2 kb, and consequently more monomorphic bands could be observed in this region (Fig. 1A). M13 probe hybridization allowed the observation of bands varying from 23 to 4 kb. The analysis of DNA bands was difficult near 23 kb and complex below 4 kb (Fig. 1B).

To widen the population analysis, *Hinf* I digested DNA extracted from blood of 36 non-related horses (50% males) and 16 trios were hybridized with the cloned fragments selected from the libraries.

Band scores and statistic analysis

Above the 2.5 kb limit, an average of 13.58 ± 3.3, 7.5 ± 3.3 and 7.6 ± 2.67 bands per individual were observed with the probes selected in this study (Table 2), M13 (Table 3) and oligo (TG)n. The cloned probes presented a similar DNA-fingerprinting pattern and many of these were shared with the oligo (TG)n. However, the oligo (TG)n probe showed more monomorphic bands than polymorphic inherited. From the average population frequency (q) of the resolvable alleles, the probability (h) of a resolvable fragment being in the heterozygote state was calculated, considering that co-migrating bands were always the same alleles. An average h of 0.92 was found when using the library probes, against 0.89 when using M13.

The number of bands and average heterosis of the resolvable fragments using the M13 probe in this study with "Mangalarga-Marchador" horses were the same (n = 7.5 and h = 0.88) found by George et al. (1988) for Belgian halfbreds, but the average probability of a fragment being present in another individual randomly was lower (x = 0.347 against 0.46). The probability of 0.289 (n = 13.58), obtained when using the probes cloned in this study, is a high polymorphic rate compared to 0.51 (n = 13.1) obtained with other horse specific probe cloned by Broad et al.

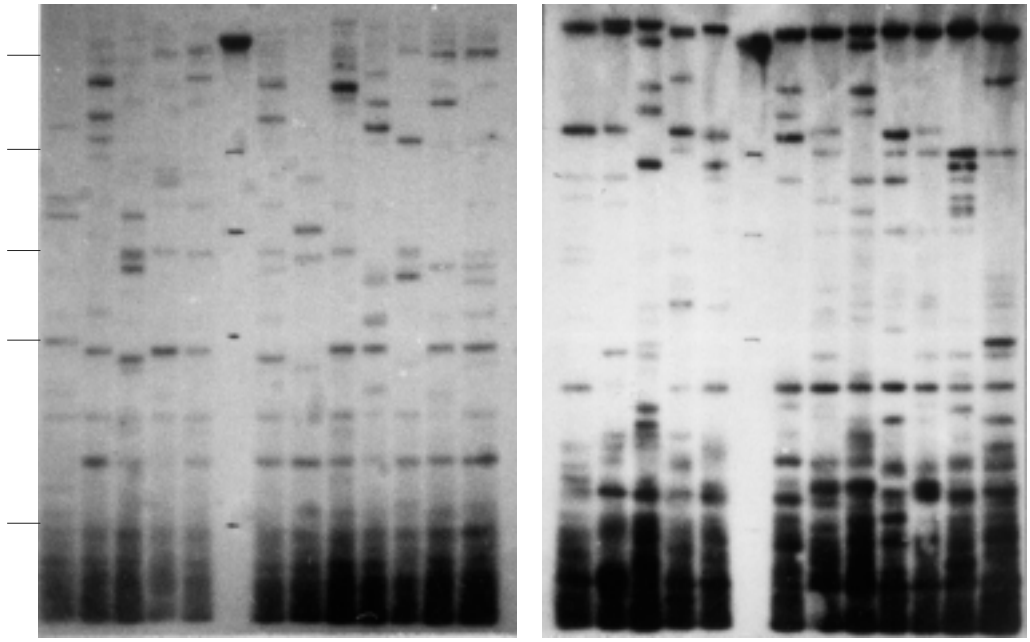

FIG. 1. DNA fingerprinting with *Hinf* I digested DNA of 12 unrelated "Mangalarga-Marchador" equines hybridized with S01 cloned probe (A) and gene-III M13 (B).

TABLE 2. Summary of population analyses using the probe S05[1].

DNA fragments size (kb)	Mean of bands/horse (n)	Band-sharing (x)	Frequency (q)	Number of test bands expected	Heterozygosity (h)
23.1 > b < 9.4	5.5 SD 2.12	0.285	0.154	2.130	0.916
9.4 > b < 6.5	2.25 SD 1.4	0.250	0.134	0.900	0.928
6.4 > b < 4.4	2.25 SD 0.7	0.281	0.152	0.875	0.918
4.3 > b < 2.2	3.67 SD 1.41	0.333	0.183	1.350	0.899
Total	13.58 SD 3.3	0.289	0.156	5.240	0.915

[1] n: mean number of bands scored per horse in 24 individuals; SD: standard deviation; q: frequency of the resolvable maternal alleles; h: the probability of a resolvable fragment being in the heterozygosity state.

TABLE 3. Summary of population analysis of probe M13[1].

DNA fragments size (kb)	Mean of bands/horse (n)	Band-sharing (x)	Frequency (q)	Number of test bands expected	Heterozygosity (h)
23.1 > b < 9.4	3 SD 0.7	0.333	0.183	1.100	0.899
9.4 > b < 6.5	2 SD 1.7	0.333	0.183	0.736	0.899
6.4 > b < 4.4	1.25 SD 1.0	0.250	0.133	0.503	0.928
4.3 > b < 2.2	1.67 SD 0.33	0.555	0.333	0.445	0.800
Total	7.5 SD 3.3	0.347	0.191	2.710	0.894

[1] n = mean number of bands scored per horse in 24 individuals; SD standard deviation; q: frequency of the resolvable maternal alleles; h: the probability of a resolvable fragment being in the heterozygosity state.

(1995). The high potential presented by selected probes in this paper can be related to genetic contents incorporated to "Mangalarga-Marchador", resulting from a diversity of races used as founders. It can also be related to the recent inbreeding developmental state of "Mangalarga-Marchador" horses that have phenotype parameters to be selected and experimentally evaluated by the set of probe/enzyme and the hybridization resolution obtained.

According to Epplen (1988) and Schwaiger et al. (1992), TG-like probes yield more information about horses than any other probe sequence. The average BS values obtained with GC-rich probes for traditional selected races such as Arabian, Thoroughbred, etc. varied from 0.30 to 0.70 against 0.25 to 0.32 with TG-rich probes.

According to Jeffreys et al. (1985b) and to the data presented in Tables 2 and 3, the probability of two non-related individuals presenting the same band pattern was $(0.333)^3$ x $(0.333)^2$ x $(0.25)^{1.25}$ x $(0.55)^{1.67} = 3.89x10^{-3}$ and $(0.285)^{5.5}$ x $(0.25)^{2.25}$ x $(0.281)^{2.25}$ x $(0.333)^{3.67} = 4.5x10^{-8}$ to M13 and cloned probes, respectively. Using the oligonucleotide (TG)n as a probe, this probability was $1.1x10^{-5}$, closer to the value of 10^{-4} to 10^{-8} verified by Ellegren et al. (1992). The probability of finding all the paternal bands in a non-related individual is $1.49x10^{-3}$ for S01 and S05 and 0.056 for M13; and that of not detecting a pair of badly aligned relatives (Bruford et al., 1992) was $7.02x10^{-5}$ and 1.5 x 10^{-2}, respectively. This results in an expected probability of exclusion of a false parentage of 99.993% and 98.5%. Assuming that the expected BS among full siblings can be calculated using the equation $(4+5q-6q^2+q^3)/4(2-q)$ (Jeffreys et al., 1985a; Bruford et al., 1992), the probability of determinating parentage through band profile of a brother of a real father, considering all the father or test bands was 0.62. This was similar to that obtained by Georges et al. (1988) of 0.606 using two accumulatively GC-rich probes (M13 and pUC J).

All bands of crossbreedings could be traced to parental individuals. No mutation or linkage imbalance was observed (Fig. 2). From the 16 crosses analyzed, a minimum of four test bands were obtained in animals showing a high degree of endogamy (PI=250), and a maximum of nine test bands (Fig. 2) in animals presenting a low degree of endogamy (PI = 51442.2),

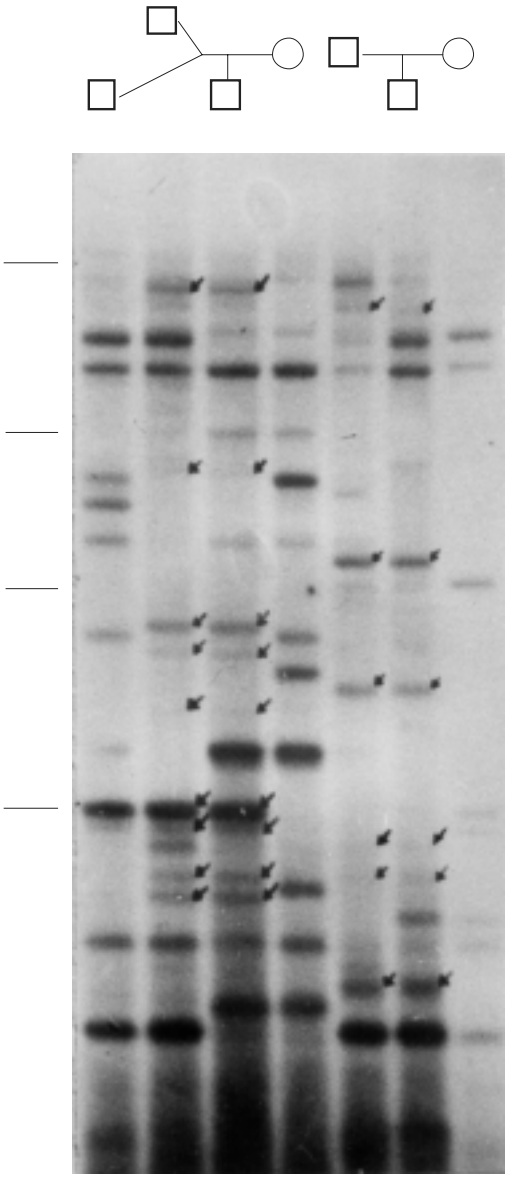

FIG. 2. Two paternity cases resolved by DNA fingerprinting using S05 clone as a hybridization probe to DNA digested with *Hinf* I. Nine bands (arrows) were cleared contributed to the foal (143) by the stallion 141 eliminating the other stallion as the paternal parent. In a second case the foal (132) showed six bands (arrows) contributed by the stallion (130).

that represents a confidence range of 99.6 and 99.998, respectively. According to Pena et al. (1993), the number of parental test bands expected (n.1-(1+q-q^2)) was 2.71 (M13) and 5.24 (S01 and S05).

Characteristics of the cloned sequences

The clones obtained in this study were completely sequenced (except for clone S07, which was partially sequenced) and analyzed. Clones S01, S05, S07 and S09 all showed a tandem repeat of 27 bp, GTTTCATTTATTATTCTTTGGAAGAAA, plus one (TG)n or (CAC)n sequence. A consensus sequence was obtained by aligning the DNA sequences of all four clones. The repeats were identified considering a minimum of 50% homology to that consensus sequence. The number of repeats found in clones S01, S05, S07, and S09 were 12, 18, 11 and 21, respectively (Fig. 3). The entire sequences have been deposited in the GenBank database (accession No. S01 - AF094576; S05 - AF094578; S07 - AF094579; S09 - AF094577).

Surprisingly, when sequences were analyzed, *Alu* I restriction sites were observed with a spacing over 100 bp, except in clone S09, which presented two pairs of these sites spaced by 30 bp and distant 417 bp. This spacing suggests an organization of the *Alu* I sites similar to that observed in human chromosomes, although low levels of nucleotide similarity were found in this experiment.

In this study, polymorphic sequences from "Mangalarga-Marchador" horses were cloned and demonstrated their efficiency in inferring genetic heterosis in these animals. It was also demonstrated that the cloned sequences were superior to the (TG)n and M13 probes in the progeny examination in

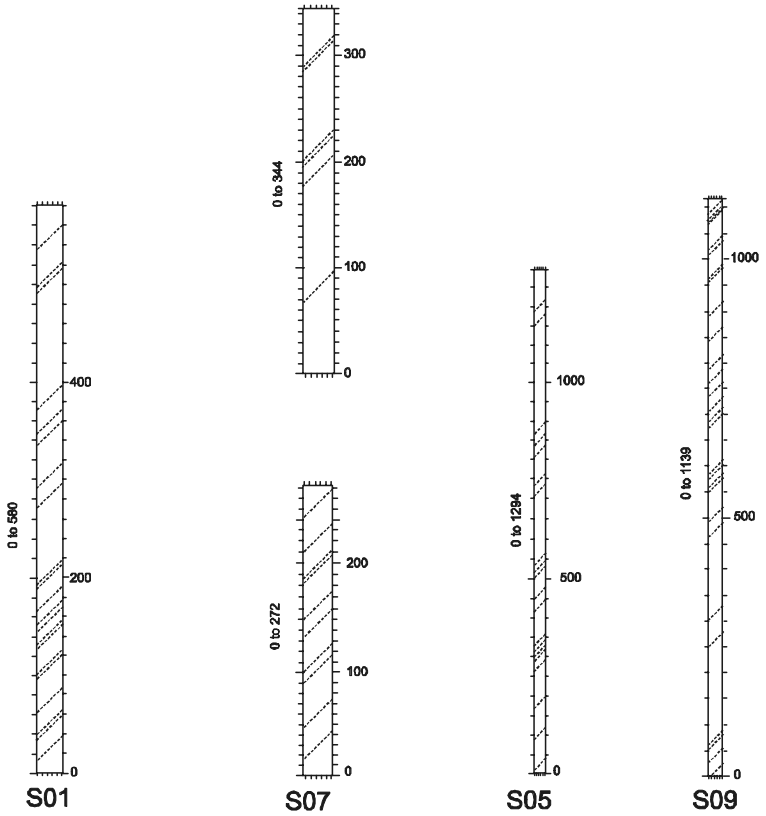

FIG. 3. Dot plot analysis of a number of tandem repeats of the sequenced cloned probes S01, S05, S07, S09, with 14, 18, 11, 21 repeats without bands overlap. S07 repeats were analyzed partially in about 614 bp sequenced.

"Mangalarga-Marchador" horses. These new cloned probes are suitable for investigations of breed affiliations, and will be very useful for breeding strategies. They could be used in genetic mapping and linkage analysis, once the genes associated with the profiles obtained are characterized; and help in quality control programs involving the use of germplasm and in semen banks, by checking chimerism and chromosome integrity.

CONCLUSIONS

1. The equine genome has TG-rich minisatellites sequences.

2. This sequences have high polimorphic index content and are suitable for use in breed affiliation.

3. CG-rich minisatellites sequences have little polymorphism in "Mangalarga-Marchador" equines.

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