The fluorescence *in situ* study of highly repeated DNA sequences in domestic horse (*Equus caballus)* and domestic donkey (*Equus asinus)* - Advantages and limits of usefulness in phylogenetic analyses[*]

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ABSTRACT

    The application of chromosome banding and the new molecular cytogenetics techniques, in particular fluorescent *in situ* hybridization (FISH) using chromosome painting probes found early use for understanding the systematic and phylogenetic relationships of species, as well as for providing insights to the possible mechanisms underpinning speciation. Here, we showed an application of primed *in situ* DNA synthesis (PRINS) and fluorescent *in situ* hybridization for comparative detection of the domestic horse (*Equus caballus*) and the domestic donkey (*Equus asinus*) telomere, centromere and nucleolar-organizer region sequences. The number, distribution and kind of highly repeated DNA sequences in across species with regard to their advantage and limit usefulness in phylogenetic analyses are discussed.

KEY WORDS: donkey, horse, telomeres, centromeres, NORs, FISH, PRINS

INTRODUCTION

The standard karyotype of donkey (*Equus asinus*) shows chromosomes arranged according to their relative lengths and the published horse-donkey ZOO-FISH data (Raudsepp et al., 2000). Raudsepp et al. (2000) proposed arranged autosomes into two groups nineteen pairs of meta- and submetacentrics and eleven pairs of acrocentrics. The donkey X chromosome forms 4.67% of the haploid genome which is close to the estimated 5% for the horse (Ohno et al., 1964; Raudsepp et al., 2000). The Y chromosome, an acrocentric chromosome, is the smallest (1.32% of the haploid genome length) (Raudsepp and Chowdhary, 1999). Additional cytogenetic investigation with the FISH using telomeric (TTAGGG)n probe revealed fluorescence signal on both ends of all asine chromosomes and in the terminal parts of EAS6p and EAS30q 8-10 times stronger signals as compared with other chromosomes. Simultaneously, no interstitial telomeric sites in the donkey genome were observed (Raudsepp et al., 2000). In donkey, nucleolar-organizer regions (NORs) with 18S, 5.8S and 28S rRNA genes were detected on eight pairs of acrocentric chromosome (EAS20, -21, -22, -24, -25, -26, -27, -28) (Kopp et al., 1986; Raudsepp et al., 2000).

Karyotypic differences between *Equus caballus* and *Equus asinus* are ascribed to a variety of chromosomal rearrangements like 6 divisions, 10 fusions (e.g., ECA6/EAS19 and EAS22; EAS10 / ECA22 and ECA22), at least 5 pericentric inversions (e.g., EAS 7, 4, 1, X; ECA 7), and 7 centromere repositioning that take place during evolution following divergence from the common ancestor (Gadi and Ryder, 1983; Raudsepp et al., 2001). Furthermore, differences in the number of arms (NF) are also observed between *Equus* species. The donkey NF equals to 52 while, in the domestic horse and Przewalski's horse the NF is 48 (Gadi and Ryder, 1983).

These differences have been attributed mainly to rapid karyotype evolution (Bush et al., 1977; Bradley and Wichman, 1994; Bowling et al., 1997; Carbone et al., 2006).

The aim of present study was to examine the distribution of telomeres, centromeres and nucleolar-organizer regions sequences in donkey by using both PRINS with telomeric-specific oligonucleotide and FISH technique using probe specific to horse centromeres and NORs, respectively.

MATERIAL AND METHODS

Metaphase chromosomes were prepared from blood lymphocytes of a three (2 females and 1 male) donkeys (*Equus asinus*) and three female horse (*Equus caballus)* by following standard procedure:

Oligonucleotide-primed *in situ* DNA synthesis (PRINS) (Cambio Ltd., UK).

The PRINS was applied according to the manufacturer's instruction with minor modifications described by Wnuk et al. (2008).

Preparation of a molecular probe specific to NORs and centromeres

An aliquot of the equine metaphase chromosome culture was spread onto a coverslip and stained with AgI banding technique to identify NORs (Bloom and Goodpasture, 1976). For painting probe acquisition ten copies of the chosen chromosome band of every investigated region (NORs) were dissected with glass microneedles controlled by a micromanipulator attached to an inverted microscope. The horse NORs probes were obtained from chromosome pairs 28 and 31. The horse centromere probes were obtained from the total genomic horse DNA. The dissected fragments were transferred by breaking off the microneedle to a PCR tube containing the collection drop solution. The dissected DNA material as well as genomic horse DNA was amplified in a DOP-PCR using the same degenerate oligonucleotide primers (5´-CCGACTCGAG N6ATGTGG-3´) (DOP PCR) (Telenius et al., 1992). Aliquots of the amplified DNA material were labeled by DOP-PCR with biotin-16-dUTP for FISH experiment. The labeled PCR products were purified using Nick Columns according to the manufacturer's protocol and co-precipitated with 5 µg of salmon sperm DNA.

Preparation of a painting probe specific to horse chromosome 28

The whole chromosome 28 painting probe was obtained by microdissection of horse chromosomes. Probe was labeled with biotin-11-dUTP. Preparation of a molecular probe was according to procedure described by Bugno et al. (2009).

Fluorescence in situ hybridization (FISH)

The biotin-labelled probe in hybridization mix (50% formamide, 10% dextran sulphate, 10% 20 × SSC, 1% Tween 20 and 29% H$_2$O) was applied on chromosome preparations. Briefly, the metaphase spreads were denatured in 70% formamide in saline-sodium citrate buffer (2 × SSC) for 2.5 min at 70°C. The probe was denatured at 70°C for 10 min. The hybridization was carried out in 37°C for three days. Post-hybridization washes were as follows: three times at 50% formamide in 2 × SSC and three times in 2 × SSC at 42°C. Hybridization signals were detected by the avidin-FITC (fluorescein isothiocyanate) and anti-avidin system on propidium iodide stained slides. Microscopic evaluation was

performed under an Opton Axiophot fluorescence microscope equipped with a camera and Lucia software.

RESULTS

The PRINS technique using telomeric-specific oligonucleotide gave fluorescence signals on both ends of all horse and asine chromosomes (Figures 1 A and B). Additionally, terminal parts of EAS6p (red arrow) and EAS30q (blue arrow) showed stronger fluorescence signals as compared with other chromosomes (Figure 1B). It's interesting that the same the telomere - specific oligonucleotide revealed centromeric fluorescence signals on all horse chromosomes (Figure 1A), while none in donkey chromosomes (Figure 1B).
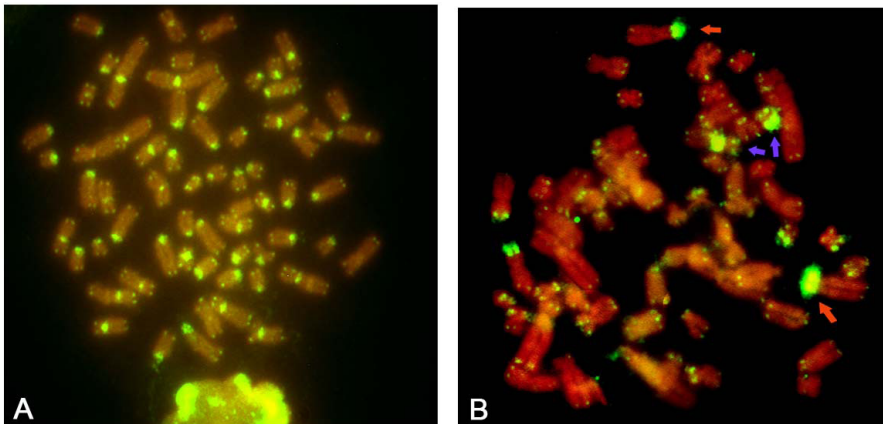

Figure 1. Comparative localization of the telomeric sequences in horse (*Equus caballus*) (A) and donkey (*Equus asinus*) (B) by using PRINS technique. A. Horse metaphase spread shows fluorescence signals in centromeres as well as on the ends of all chromosomes. This finding indicate that some similarity between both repetitive sequences in horse occurs. B. Donkey metaphase spread shows fluorescence signals on the ends of all chromosomes. Arrows indicate exceptionally strong labeled sequences on EAS6p (red arrow) and EAS30q (blue arrow)

Simultaneously, application of the fluorescence *in situ* hybridization with horse centromeric sequences as probes for donkey (*Equus asinus*) chromosomes showed only large centromere blocks on two distinct chromosome pairs (Figure 2).

In horse, fluorescence labeled nucleolar-organizer regions (NORs) were detected on three chromosome pairs identified as ECA1,-28,-31 (Figure 3). In donkey, the NORs-specific probe gave FISH signals on eight pairs of acrocentric chromosome (EAS20, -21, -22, -24, -25, -26, -27, -28) (Figure 4). Variation was

also observed in the signal intensity between individual homologues in a both species.
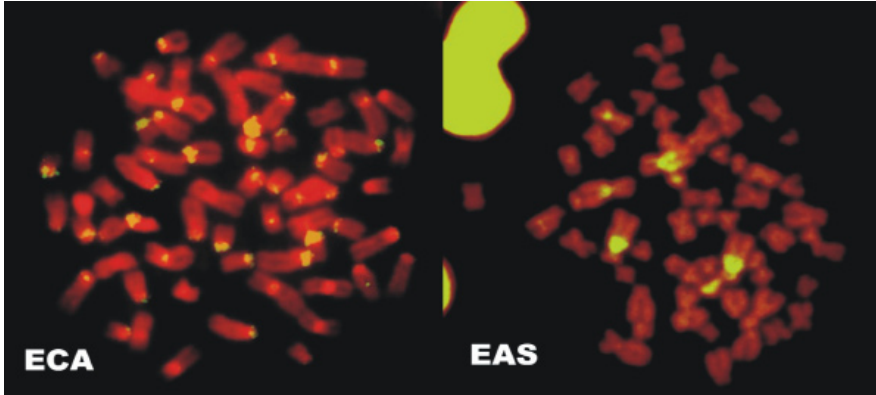

Figure 2. Comparative localization of the centromeric sequences in donkey (*Equus asinus*) by using FISH technique with probes specific to the domestic horse centromeres. ECA - horse (*Equus caballus*) metaphase spread. EAS - donkey (*Equus asinus*) metaphase spread. FISH showed centromeric signals on all horse chromosomes while in donkey showed only large centromere blocks on two distinct chromosome pairs and the absence of signals on the rest chromosomes. This observation suggested that these species have distinct centromeric DNA satellite families
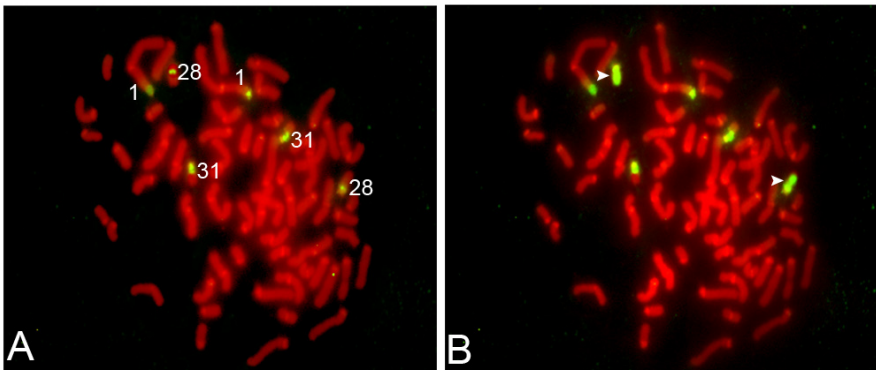

Figure 3. A. horse (*Equus caballus*) metaphase spread after FISH with NORs probe. Distinct signals are seen on three chromosome pairs identified as ECA1,-28,-31. Variation of rDNA signal intensity between different chromosomes is visible. B. FISH with 28 whole chromosome painting probe as well as all NOR loci. Arrowheads indicate positive FISH signals with whole chromosome 28 painting probe
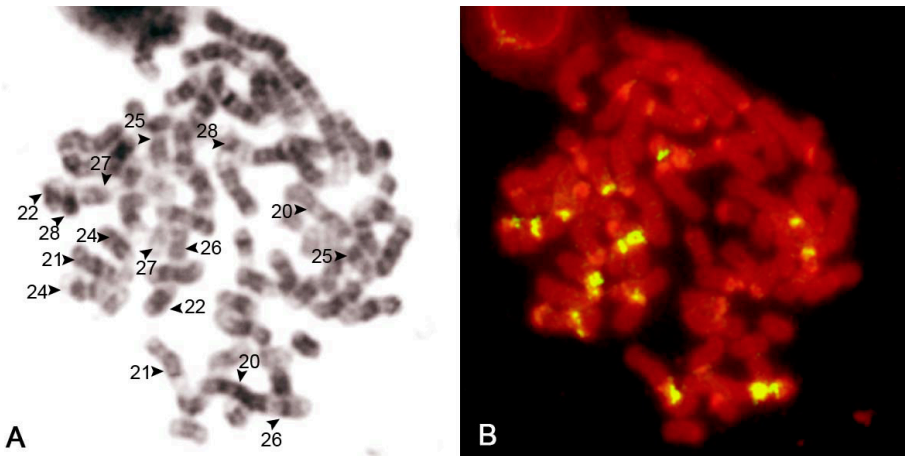
Figure 4. A. Donkey (*Equus asinus*) metaphase spread after GTG technique (A) and FISH technique with probe specific to horse NORs (B). B. Distinct signals are seen on eight pairs of acrocentric chromosome (EAS20, -21, -22, -24, -25, -26, -27, -28). Variation of rDNA signal intensity between different chromosomes is visible

DISCUSSION

The banding techniques are often quite reliable for assessing primary homologues between closely related species and, in these instances, can provide accurate data for studies of genome evolution. In addition, banding techniques allow access to information involving both structural and functional patterns of chromosomes. Nevertheless, banding techniques are limited in their abilities to assess homology within and across species in instances where karyotypes are highly rearranged, making the determination of homology ambiguous at best (Dobigny et al., 2004). The molecular cytogenetic technologies such as PRINS, FISH or GISH can overcome the limitations of conventional banding analyses (de la Sena et al., 1995; Chaudhary et al., 1998; Raudsepp and Chowdhary, 1999; Santani et al., 2002). *In situ* hybridization has provided evidence that homology in banding patterns is significantly related to homology in synteny conservation and gene content (Yang et al., 2003, 2004). Especially, a cytogenetic comparison of distribution and homology of repetitive sequences within and across species can be useful to indentify sister-group relationships among taxa, gives information about the systematic and phylogenetic relationships of species, as well as for providing insights into the possible mechanisms underpinning speciation (Wijers et al., 1993; Bradley and Wichman 1994; de la Sena et al., 1995; Lear, 2001;

Santani et al., 2002; Carbone et al., 2006). Here, we compared localization of three kind of repetitive sequences between two related species using PRINS with telomeric-specific oligonucleotide and FISH with probes specific to horse centromeres and NORs. Application of primed *in situ* DNA synthesis (PRINS) method with the same telomeric oligonucleotide in donkey and horse metaphase spreads gave results that are consistent with previous study carried out on a both species using FISH with telomeric DNA probe and PRINS (Raudsepp et al., 2000; Lear, 2001; Slota et al., 2007; Wnuk et al., 2008). This study confirms also that sites on EAS6p and EAS30q are rather species specific than are subject to polymorphism (Raudsepp et al., 2000). Stronger signals at terminal parts of EAS6p and EAS30q as compared with other donkey chromosomes could be attributed to presence of larger number of telomeric repeats in these chromosome ends or may represent degenerate telomere-like satellite sequences (Murphy et al., 2005). Similar differences in other species have previously been described (Biessmann and Mason, 1992).

Simultaneously, PRINS analysis confirmed also that, as in the horse, no interstitial telomeric sites (ITSs) were detected in the donkey genome (Bowling et al., 1997; Raudsepp et al., 2000; Lear, 2001). The absence of ITSs on the chromosomes of these species, and on the others of *Equids* such as *Equus zebra hartmannae* or *Equus quagga burchelli* may be explained by multiple chromosomal rearrangements or gradual lost of repeated sequences that occurred during evolution following divergence from the common ancestor. Another hypothesis indicated the presence of these sequences in very low copy number due to which they evade detection (Santani et al., 2002).

The presence of fluorescence PRINS centromeric signals on all horse chromosomes but the absence of signals on donkey chromosomes suggested that these species have distinct centromeric DNA satellite families. Furthermore comparative fluorescence *in situ* hybridization with probes specific to the domestic horse centromeres in donkey confirmed this observation. Probably this phenomenon is related to the centromere repositioning (CR) that takes place during rapid evolution for the both species. There is biological phenomenon consisting of the emergence of a new centromere along a chromosome and the inactivation of the old one (Carbone et al., 2006). The inactivation of the old centromere is accompanied by the rapid loss of centromeric satellite DNA and by the dispersal of the pericentromeric duplicons over a relatively wide area (She et al., 2004; Villasante et al., 2007). Observed blocks on two distinct donkey chromosome pairs by comparative FISH are rather large pericentromeric heterochromatin-associated regions than centromeric sequences (Ryder and Hansen, 1979; Belyayev and Raskina, 1998).

Application of comparative hybridization with horse NORs probes in both

species gives similar results as previously described (Derjusheva et al., 1997, 1998; Raudsepp et al., 2000; Slota et al., 2007). Our results did not reveal any additional site in horse or donkey and confirmed variation existence in the signal intensity and frequency between individual chromosome pair in both species. Variation in the intensity and frequency of probe signal observed between chromosomes is common in other *Equids* (Raudsepp et al., 2000; Santani et al., 2002; Slota et al., 2007). It is attributed primarily to the number of ribosomal genes at each locus. Probably, the loss and duplication of rDNA can be induced by unequal crossing over and by non-homologous recombination (hetero-site crossingover) in the germ cells (Guillen et al., 2004). In our opinion the use of NORs (the loci of ribosomal 18S 5.8S and 28S genes) in determining phylogenetic relationships is limited because the conventional silver-staining (AgNOR) method of Bloom and Goodpasture (1976) can not detect inactive NORs, at the same time the method detects other genomic features (Sumner, 1982; Dobigny et al., 2004). On the other hand, the number of repeats may be variable and this is not usually evident even when using hybridization methods. In addition, DNA sequences similar to ribosomal intergenic spacers, but not linked with rDNA loci, have been detected in some genomes (Dobigny et al., 2002), suggesting that molecular investigations may be sometimes misleading in terms of the location of rDNA clusters.

CONCLUSIONS

In conclusion, rDNA hybridization patterns may contribute to valuable additional information on homologues between chromosomal segments, mainly between closely related species. In contrary, comparison study applied between far-related species must be carefully assessed and supported by other molecular analysis.

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