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Heterochromatin and chromosome evolution: a FISH probe of *Cebus apella paraguayanus* (Primate: Platyrrhini) developed by chromosome microdissection

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ABSTRACT. Neotropical Primate karyotypes are highly variable, particularly in the heterochromatic regions, not only regarding the amount of heterochromatin, but also the composition. G and C banding and FISH techniques provide useful information to characterize interspecific relationships. We used chromosome microdissection to develop a FISH probe of the chromosome 11 heterochromatic block (11qHe+) of *Cebus apella paraguayanus* (CAPp). Fragments of the 11qHe+ microdissected from fibroblast cell culture were collected in a PCR tube, amplified by degenerate oligonucleotide primer-PCR and subsequently labeled. The specificity of the FISH probe was confirmed in metaphases of some Ceboidea species. Signals were located in the He+ of chromosomes 4, 11, 12, 13, and 19 of CAPp and in the He+ of chromosomes 4, 12 and 13 of *C. a.*

nigritus (CAPn); no signals were observed when other Ceboidea species were analyzed. We propose that the heterochromatin observed in CAPp and CAPn is specific for these species. We consider this *C. apella* heterochromatin identity as a possible key for the interpretation of chromosomal evolution in these Ceboidea.

Key words: Heterochromatin, Chromosome evolution, Micro-FISH, *Cebus apella*

INTRODUCTION

In a framework of chromosomal evolution, molecular cytogenetic techniques, such as fluorescence *in situ* hybridization (FISH), allow accurate inferences about the mechanisms that underlie chromosomal evolution (Wienberg and Stanyon, 1997; Mudry et al., 2001; Neusser et al., 2001; Redi et al., 2001). In addition to FISH, chromosome microdissection permits us to synthesize probes from specific chromosome regions providing informative markers for the differential identification of the rearrangements involved in evolutionary processes (micro-FISH probes) (Guan et al., 1992; Meltzer et al., 1992; Mühlmann-Díaz et al., 1995; Behrens et al., 1997; Taguchi et al., 2003).

New World Primates (Neotropical Primates, Platyrrhini) have a great variability of karyotypes, including not only the modal number but also the genomic rearrangements among species of the same genus. Nevertheless, comparative cytogenetic studies (C, G, NOR banding techniques, and FISH analyses) have shown that euchromatic regions are mostly conserved in the Ceboidea (Dutrillaux and Couturier, 1981; Medeiros et al., 1997; Wienberg and Stanyon, 1998; García Haro et al., 2002; Barros et al., 2003). Interestingly, heterochromatic regions observed in these mammalian chromosomes are highly variable in quantity, quality and location (Matayoshi et al., 1987; Mudry de Pargament and Slavutzky, 1987; Mudry de Pargament and Labal de Vinuesa,1988; García Haro et al., 2003).

Nevertheless, C-banding techniques and restriction enzyme digestion have demonstrated that there are different kinds of heterochromatin, with a great variability, among Platyrrhini species (Ma and Jones, 1975; Seuanez et al., 1983; Mudry et al., 1994; Pieczarka et al., 2001; García Haro et al., 2003). Among them, *Cebus*, considered the genus with the most ancestral karyotype, shows the greatest amount of constitutive heterochromatin in all of the reported karyotypes (Dutrillaux, 1979; García et al., 1983; Clemente et al., 1990; Ferrucci et al., 1995; García Haro et al., 2003). In *Cebus apella* (CAP), this heterochromatin shows two different highly repeated DNA sequences: CAP *A*, in interstitial and telomeric regions, and CAP *B*, in the centromeric region, with considerable intra- and interspecific variability (Mudry et al., 1985; Clemente et al., 1987; Ferrucci et al., 1995; Ponsá et al., 1995; García Haro, 2001).

Cebus apella paraguayanus (CAPp) and *C. a. nigritus* (CAPn) karyotypes are an interesting example. Their genomes differ only in the heterochromatic block of the #11 pair, which is absent in CAPn and present in CAPp, defining the subspecies (Mudry, 1990; Mudry et al., 1991).

The purpose of the present study was to obtain a FISH probe of a heterochromatic segment of chromosome 11 of CAPp by chromosome microdissection in order to analyze the possible role of heterochromatin variability in the *Cebus* speciation process.

MATERIAL AND METHODS

Samples and chromosome preparations

Peripheral blood samples from four individuals of CAPp and one CAPn were employed: two males and one female from the Zoological Park of Buenos Aires (JZBA), one male from the Biological Station of Corrientes (EBCo), and one male from the Center of Subtropical Ecological Research (CIES) - Administracion de Parques Nacionales Iguazú, Misiones, Argentina, respectively. One female of *Saimiri boliviensis* (SBO) and one female of *Aotus azarae*, both from the Córdoba City Zoo, Argentina. Among Atelidae, one male of *Ateles chamek* from the Córdoba City Zoo. Cell cultures were performed following conventional methods (Buckton and Evans, 1973, modified). Additionally, a 1-cm^3 biopsy from sub-epidermal tissue of a male CAPp was supplied by the JZBA to establish a fibroblast cell line. This tissue culture was conducted following conventional methods to improve the efficiency of the probe development (Nieves et al., 2004). Metaphase spreads for chromosome microdissection were prepared on 24 x 40-mm coverslips and G-band technique was performed (Seabright, 1971, modified). For FISH, metaphase spreads were prepared on 24 x 50-mm slide glasses. Chromosome spreads of a male *Homo sapiens* (HSA) were analyzed by FISH as a positive control.

Microdissection of the 11qHe+ of CAPp

Coverslips with cell spreads were placed on an inverted microscope (Nikon Phase Contrast EL WD 0.3). Heterochromatic blocks of chromosome pair 11 of CAPp (11qHe+) were microdissected with a glass needle attached to a mechanical micromanipulator (Eppendorf 5171). Needles were produced with different diameters, depending on the chromosome condensation, from glass capillaries using a pipette puller (Narishige PC-10). Nine scraped chromosomes were placed into a PCR tube by breaking each tip at the bottom of the tube. Degenerate oligonucleotide primer (DOP)-PCR was performed for the amplification of the microdissected DNA (Telenius et al., 1992). An Eppendorf thermal cycler was used for all the PCR protocols.

DOP-PCR, specific amplification and probe labeling

Amplification of the microdissected material was carried out with a slightly modified protocol (Mühlmann-Diaz et al., 2001). Five microliters of water was added to the collected material and heated for 10 min at 95°C. Subsequently, 20 µL of a PCR reaction solution was added, consisting of 2X buffer = 100 mM KCl, 20 mM Tris-HCl, 0.2% Triton-X, 3 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM DOP primer = 5´CCGACTCGAGNNNNNNATGTGG3´ and 2 units of Taq polymerase. PCR-RAMP cycles were as follows: 10 cycles at 94°C for 1.5 min and at 30°C for 3 min, ramping to 72°C over 4 min. This ramp cycle was followed by 30 cycles of regular PCR cycles: 94°C for 1.5 min, 56°C for 1.5 min, and 72°C for 1.5 min. Products were checked by electrophoresis on 2% agarose gel.

Amplification of 3 µL of the first DNA product was conducted in a final volume of 50 µL containing 1X buffer Taq polymerase (2 mM $MgCl_2$, 200 µM dNTP), 3 µL DOP primer (100 µM) and 2 units Taq polymerase, and then exposed to thirty cycles of 90°C for 1.5 min, 56°C for 1.5 min and 72°C for 1.5 min. Each PCR product was confirmed by electrophoresis on 2% agarose gel before continuing with the probe development. Two microliters of the final PCR products was labeled with 1 µL Spectrum Orange-dUTP (50 nM Vysis) by a regular PCR reaction, as described above. Labeled products were precipitated overnight with 2/3 volume $AcNH_4$, 10 µg Herring Sperm and three times final volume of pure ethanol. The air-dried pellet was resuspended in millipore-filtered (MQ) water.

Fluorescence *in situ* hybridization and image analysis

FISH was performed with 1 µL of the 11qHe+ probe dissolved in 4 µL of hybridization mix (50% formamide, 20% Dextran sulfate in 2X SSC). The mixture was denatured at 85°C for 5 min and stored at 50°C before being applied to the metaphase spreads. Slides with chromosomal spreads were denatured in 70% formamide/2X SSC, at 68°C for 2 min, followed by dehydration in ice-cold ethanol (70, 90 and 100% sequentially for 1 min each). Hybridization was conducted in a wet chamber at 37°C for 24 h. Post-hybridization washes followed standard protocols at 40°C and counterstained with DAPI (Schweizer, 1976). Slides were analyzed with an Olympus BX-51 fluorescence microscope and images were acquired with an Optronics camera.

RESULTS

Primary DOP-PCR products from the nine dissected chromosomes were observed as a 400- to 600-bp band by electrophoresis on 2% agarose gel, along with the specific PCR products. The final labeled product was observed as a 300- to 500-bp band (Figure 1).

Probe specificity

The specificity of the probe was demonstrated by FISH in metaphases of CAPp. We found eight signals: the entire heterochromatic region of pair 11 and the extracentromeric heterochromatic blocks of the chromosome pairs 4, 12 and 13 previously characterized with C-banding (Figure 2a). One individual showed 10 signals: the whole heterochromatic region of pair 11 and the extracentromeric heterochromatic blocks of the pairs 4, 12, 13, and 19 previously characterized by C-banding (data not shown). DAPI staining confirmed the identity of the heterochromatic blocks of all pairs in which the signal of the 11qHe+ was observed. The positive control performed with the HSA X probe (in green) onto metaphases of the same CAPp individuals confirmed the conservation of the human sexual chromosome, observed in most of mammals, and also in these primates (Figure 2a). A different pattern was observed when metaphases of CAPn were analyzed; we found only six signals; these were the heterochromatic blocks of chromosomes 4, 12 and 13, previously characterized by C-banding (Ponsá et al., 1995) (Figure 2b). These results are concordant with previous research that determined chromosome 19 as the most polymorphic pair of the karyotype, including presence/absence of the band and length variability, analyzed by C-banding (Mudry de Pargament and Labal de Vinuesa, 1988).

Analysis of the conservation of the CAPp 11qHe+ onto metaphases of two other *C.*

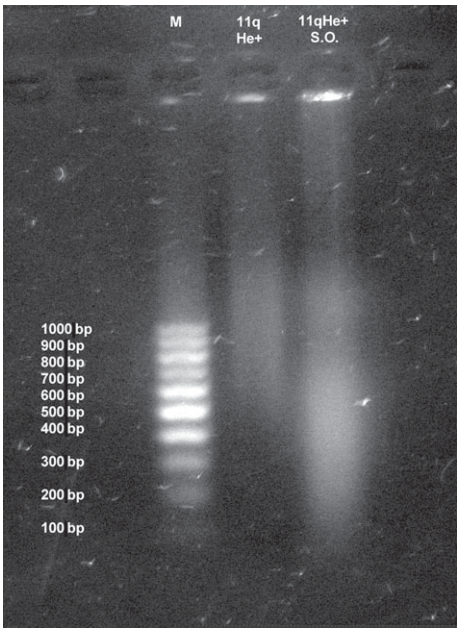

Figure 1. Agarose gel (2%) showing: to the left, the molecular weight marker (M); at the center, a 400- to 600-bp band, corresponding to the nine microdissected segments 11qHe+ of *Cebus apella paraguayanus*. To the right the 300- to 500-bp band, corresponding to the 11qHe+ probe labeled with Spectrum Orange (S.O.).

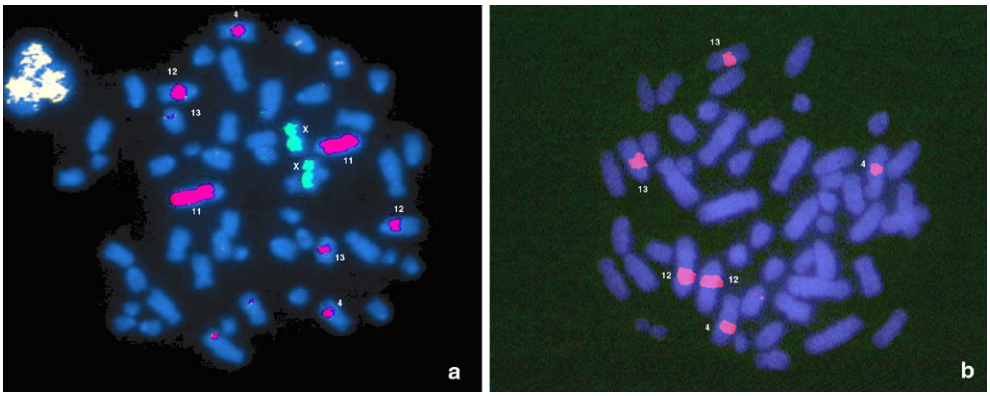

Figure 2. a. Metaphase of a *Cebus apella paraguayanus* female showing the 11qHe+ probe in red and the human X in green. **b.** Metaphase of a *Cebus apella nigritus* male showing the 11qHe+ probe in red in chromosomes 4, 12 and 13.

apella subspecies (*C. a. apella* and *C. a. xantosthernos*) showed positive signals in four to six pairs that presented heterochromatic blocks, previously characterized by C-banding (Matayoshi et al., 1987; Martinez et al., 1999; and Nieves M, de Oliveira EHC, Amaral PJS, Nagamachi C et al., unpublished results).

Conservation of CAPp heterochromatin in Ceboidea

When metaphases of a male HSA were studied with the 11qHe+ probe, no signals were observed, whereas a signal was observed with the control HSA X probe, demonstrating the specificity of the heterochromatic blocks (Figure 3a).

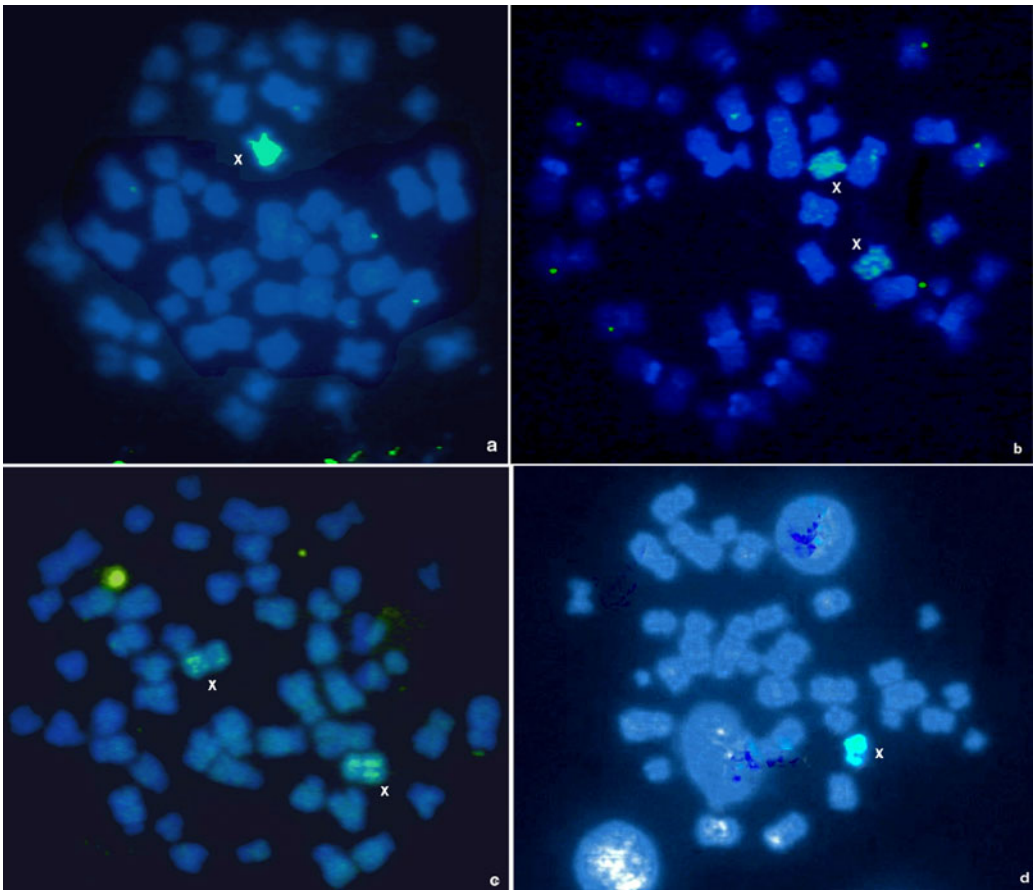

Figure 3. a. Metaphase of a *Homo sapiens* male with the human X in green. **b.** Metaphase of a *Saimiri boliviensis* female showing the two X chromosomes in green. **c.** Metaphase of an *Aotus azarae* female, showing the two X chromosomes in green. **d.** Metaphase of an *Ateles chamek* male, showing the X chromosome in green.

The analysis of three other Ceboidea genera using the 11qHe+ probe showed the same result in all of the samples illustrated in Figure 3b, c and d, as follows: 3b) A female of SBO: no signals for 11qHe+ were observed, whereas two signals for the X using HSA probe appeared in green, corroborating the conservation of the HSA X chromosome in SBO. 3c) A female of *Aotus azarae*; as in SBO, only the two signals for the X with HSA X probe were observed. No signals were observed for the 11qHe+ probe of CAPp. 3d) Only the green signal corresponding to the X with the HSA probe was observed in a male of *Ateles chamek*.

DISCUSSION

Quantitative variation of heterochromatin within and among species can be due to several mechanisms: multiple replication, unequal exchange, amplification, accumulation, and deletion (John, 1988; Garagna et al., 1995). In this sense, there is a growing body of evidence about added heterochromatin fixed as a polymorphism without phenotypic expression (e.g., Ma and Jones, 1975; Pardue and Hennig, 1990; Wichman et al., 1991; Baimai, 1998; Redi et al., 2001).

Comparative studies of banded chromosomes in primates have shown that the organization of euchromatic DNA is very conservative. This is not the case with constitutive heterochromatin (García et al., 1983). New World Primates (Platyrrhini) karyotypes are still evolving, and despite their conserved euchromatin, many chromosomal rearrangements have been reported in the last 20 years, due to the speciation process (Dutrillaux, 1979; Clemente et al., 1990; Medeiros et al., 1997; de Oliveira et al., 2002).

Specificity of CAP heterochromatin

Among the Ceboidea, CAP shows the most varied C-banding pattern when we consider only presence/absence of a specific band in any member of the pair (Pieczarka, 1995; Borrell, 1995; Martinez et al., 1999). Variation in extracentromeric heterochromatin has been reported for other *Cebus* species, but in *C. apella* drastic variation in the amount of heterochromatic region of pair 11, allows subspecific differentiation (Dutrillaux et al., 1978; Mudry et al., 1985, 1991; Matayoshi et al., 1987; Ponsá et al., 1995).

It is well known that when a C-band pattern is analyzed, the absence of a C-band does not indicate a lack of heterochromatin, as is evident from mice with fewer than 100 copies of the long-range repeat in chromosome 1 (Redi et al., 2001). Meanwhile, the number of repeats rather than sequence specificity determines the staining properties of heterochromatic DNA.

In this sense, in order to determine if the 11qHe+ of CAPp is different from the remaining CAP heterochromatin, we developed the #11qHe+ FISH probe. The first and most important finding was that the probe hybridized with all of the heterochromatic blocks, discarding the hypothesis of a specific sequence for #11q of CAPp. Nevertheless, we found that there is only one type of heterochromatin present in both CAPp and CAPn genomes: the one microdissected from the 11qHe+, with a different number of amplifications, and different from the one observed in the centromeres (Seuanez et al., 1983; Ferrucci et al., 1995; García Haro et al., 2003). Moreover, when two other CAP subspecies were analyzed with the probe, positive signals were obtained in the chromosome pairs when extracentromeric heterochromatin was present.

Finally, no positive signals were obtained when metaphases of other Ceboidea genera were analyzed. Taken together, these findings permit us to propose a species-specific heterochromatin identity for CAP.

Further characterization of the heterochromatic blocks present in CAPp karyotype would be useful. Sequencing the developed 11qHe+ probe and searching for homologies with already known sequences will permit accurate inferences as well as a better interpretation about the process of chromosomal evolution in *Cebus*.

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