Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: Simona Ceraulo, Francesca Dumas (2022). Mapping CAP-A satellite DNAs by FISH in *Sapajus cay paraguay* and *S. macrocephalus* (Platyrrhini, Primates). *Caryologia* 75(4): 87-92. doi: 10.36253/caryologia-1939

Received: September 15, 2022

Accepted: December 24, 2022

Published: April 28, 2023

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Mapping CAP-A satellite DNAs by FISH in *Sapajus cay paraguay* and *S. macrocephalus* (Platyrrhini, Primates)

Simona Ceraulo, Francesca Dumas*

Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, 90100, Palermo, Italy
*Corresponding author. E-mail: francesca.dumas@unipa.it

Abstract. Satellite DNAs such as Cap-A sequences are potentially informative taxonomic and phylogenetic markers useful for characterizing primate genomes. They have also been used as cytogenetic markers facilitating species identification in many taxa. The aim of this work is to map Cap-A sequences by FISH (fluorescent *in situ* hybridization) on two Platyrrhini (Primates) species genomes, *Sapajus cay paraguay* and *S. macrocephalus*, in order to study their distribution pattern on chromosomes. The Cap-A probes showed bright signals with almost the same interstitial pattern of distribution in correspondence with C and CMA3 rich regions on six pairs of chromosomes in both *Sapajus* species. An additional pair was detected on *S. macrocephalus*. The analysis of the results, compared with previous literature data on other phylogenetically close New World species, shows that Cap-A satellite sequences have a genus-specific pattern, but with slight species-specific patterns that are useful as phylogenetic and taxonomic markers.

Keywords: heterochromatin, karyotype, genome, New World monkeys.

INTRODUCTION

Apart from coding regions (about 2%), the human genome includes highly repetitive sequences (about 98%) which are usually underestimated in genome analyses due to their complexity; these sequences are known as the dark matter of the genome (Ahmad et al. 2020) and consist of satellite DNA (satDNA), defined as tandemly arranged repeats that represent a considerable proportion of the heterochromatic portion of chromosomes in the eukaryotic genome. satDNA, at first seen as serving no useful purpose, is now known to be associated with genome function, chromosome evolution, speciation, and diversity, comprising different kind of elements such as satellite DNAs, SINEs (Short Interspersed Nuclear Elements), LINE retrotransposons (Long Interspersed Nuclear Elements), and rDNA repeats (Ahmad et al. 2020, Ceraulo et al. 2021 a, Dumas et al. 2022).

Thus, satDNAs are potentially informative cytogenetic markers which can be used to study karyotype evolution and address taxonomic issues. They

display high evolutionary rates and consist of tandem repeats organized in the type of large arrays (up to Mb size) typically associated with chromosome landmarks such as centromeres, telomeres, and heterochromatic regions (Ahmed 2020). They evolve by mechanisms of gene conversion, and unequal crossing-over which are involved in what is known as concerted evolution (Sander Lower et al. 2018). satDNAs have high intraspecific sequence homogeneity and interspecific differences, making satDNAs potential taxonomic markers and, in some cases, allowing their use for phylogenetic inference. Furthermore, satDNAs have been used as cytogenetic markers facilitating species identification in many taxa (Prakhongcheep et al. 2013 a,b, Cacheux, et al. 2018).

Among repetitive sequences, Cap-A is a satDNA that has been analyzed in many mammals through molecular comparative sequence analysis (Valeri et al. 2018), and their history has been reconstructed in mammals. This analysis led researchers to show that a Cap-A like sequence is present as a single monomer in most eutherians such as Chiroptera, some Eulipotyphla, and some Rodentia, and also in *Homo sapiens*. Indeed, in *H. sapiens*, it is only a sequence within the intron of the NOS1AP (nitric oxide synthase 1 adaptor protein) gene. No Cap-A like sequence was found among Marsupialia or Monotremata, the sister clades to Eutheria, presumably due to the occurrence of low copy numbers or because the sequence has diverged (Valeri et al. 2018, Valeri et al. 2020). On the other hand, Cap-A duplication and expansion have been shown in New World monkeys (NWMs); this amplification may be explained by a mechanism in which the Cap-A intronic segment was transferred to heterochromatic regions in the ancestral Platyrrhini genome followed by a hyper-expansion through unequal crossing (Valeri et al. 2020).

Comparative cytogenetics using different kinds of repetitive sequence probes mapped by fluorescence *in situ* hybridization (FISH) on chromosomes led us to study sequence pattern distribution among species allowing genomic comparison (Ceraulo et al. 2021 b, c). Cap-A sequence probes have been mapped by FISH in many taxa, including Primates (Valeri et al. 2018, Valeri et al. 2020), in order to study their distribution pattern. This work permits researchers to show that Cap-A is an abundant satDNA in Platyrrhini with a high accumulation in blocks in some genomes. In particular, Cap-A has been found in representatives of the three Platyrrhini families (Cebidae, with the exception of Callitrichines, Atelidae and Pitheciidae, with the exception of the *Callicebus* genus), with genome abundance ranging from less than 1% up to 5%, and chromosome localization which is always associated with non-centromeric constitutive heterochromatin (Valeri et al. 2018, 2020). Furthermore, intragenus research analyzing Cap-A distribution on four *Saimiri* species has also been performed (Valeri et al. 2020) showing slight pattern differences between species.

The fact that Cap-A is present across Platyrrhini led researchers to show its utility as a marker for chromosome and genome evolution studies in NWMs (Valeri et al. 2018). This is especially important because of the extinction to an alarmingly large number of NWM species due to rapid habitat loss.

The Cap-A sequence was first described in the tufted capuchin monkey *Sapajus apella* (previously classified as *Cebus apella*); Cap-A was identified after digestion of genomic DNA with restriction enzymes and with DNA–DNA hybridization (Malfoy et al. 1986, Fanning et al. 1993).

Thus, in order to extend previous studies using Cap-A as a marker in Platyrrhines, two additional *Sapajus* species, *S. cay paraguay* and *S. macrocephalus* (Cebidae), were analyzed, mapping the Cap-A probe by FISH. This study will help clarify *Sapajus* chromosome evolution and add potentially useful data for taxonomic, systematics, and conservation issues.

Indeed, the cytogenetic information about *Sapajus* is poor, whereas more species from the phylogenetically close *Cebus* have been analyzed (Garcia et al. 2002). The number of specimens karyotypically analyzed is low, and most samples have not been studied, especially from the recently recognized *Sapajus* genus. Karyotypes among the two taxa are very similar, and these species have been hypothesized to be distinguishable for the non-centromeric heterochromatin block of some chromosomes, with a differential chromosomal position in each of them (Mudry, 1990, Garcia et al. 2002).

MATERIAL AND METHODS

Peripheral blood from male samples of *S. cay paraguay* and *S. macrocephalus* (Cebidae) was collected from primates at the ISTC-CNR of Rome, in accordance with international and institutional ethics rules. Metaphases were obtained from lymphoblast cell cultures in RPMI culture medium, following standardized protocols. Cell harvesting was performed after 3 h incubation with colcemid 10 µL (10 µg/mL Gibco), followed by hypotonic treatments of 0.075 M KCl for 20 min at 37 °C, following standard protocols (Dumas et al. 2022). Metaphases of the analyzed species were stained pre- and post-FISH using chromomycin A3 (CMA3) and 4',6-diamidino-2-phenylindole (DAPI)

staining, according to a recent protocol (Lemskaya et al. 2018), with some adjustments. CMA3 staining of GC-rich regions and DAPI staining of AT-rich regions were useful for identifying chromosomes and preferential insertion sites of Cap-A sequences.

DAPI images were inverted with a photo editing program (Adobe Photoshop C 2022 V23.3.2); inverted gray bands generally correspond to dark G-bands or light R bands; the DAPI inverted karyotypes for the *S. cay paraguay* and *S. macrocephalus* species were compared with previously published banded karyotypes of the phylogenetically close species *S. apella* and *Cebus capucinus* (Garcia et al. 2002, Milioto et al. 2022).

Human DNA extraction from lymphoblast cell lines was performed using the Pure Link DNA kit (Invitrogen), according to the basic DNA extraction protocol. Cap-A was amplified by Polymerase Chain Reaction (PCR) from human DNA; the following universal set of primers, developed for the PCR of CAP-1 repeats in Primates, were used: (Cap-A F: ACTTCCTCACTGACCTGTCTT; Cap-A R:GGGCTGATGCTTAATGTAGCA).

Genomic DNA was amplified in 50 µL PCR-reactions: five units of Taq GOLD DNA Polymerase (Invitrogen), the template DNA, 500 nM of each primer, 200 µM each of dATP, dCTP, dTTP, and dGTP in 10 mM TRIS-HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl. PCR reactions were performed using an Applied Biosystems SimplyAmp (Thermo Fisher Scientific) with the following cycling parameters: 30 cycles each of 94 °C, 60 s; 55 °C, 60 s; 72 °C, 60 s, following a 3 min denaturation at 94 and with a final elongation step of 72 °C, 10 min. A bright band of about 1500 pb was visualized on 1% agarose gel. The PCR products were directly labeled through Nick Translation using 11-dUTP-Fluorescein (green) (Invitrogen).

FISH was performed following previously described protocols (Dumas and Sineo 2014, Dumas et al. 2015) using Cap-A probes obtained by PCR as previous described (Valeri et al. 2018, 2020). The hybridization mix consisted of 2.5 ng/L of probe, 50% formamide, 10% dextran sulfate, and 2xSSC, with an incubation time of 18 h at 37 C. Detection was performed at medium stringency, with washing at low temperatures (45 °C) and at high saline buffer concentration of SSC 0.1 Tween, 15 min, PBS 1min.

C banding was done sequentially post-FISH through a protocol which included denaturation with formamide (Fernàndez et al. 2002).

After FISH, the metaphases were analyzed under a Zeiss Axio2 epifluorescence microscope. Images were captured using a coupled Zeiss digital camera. At least ten metaphase spreads were analyzed for each sample.

RESULTS

Sequential staining, banding, and FISH mapping were performed for the two *Sapajus* species. The inverted DAPI karyotypes of *S. cay paraguay* and *S. macrocephalus* were almost the same as those of the other congeneric species previously published (Garcia et al. 2002, Milioto et al. 2022), with both species having the diploid number 2n = 54; for the karyotype reconstruction, we followed a previous publication (Garcia et al. 2002), with ten pairs of meta/submetacentric chromosomes in *S. cay paraguay* (pairs 1–10), eight pairs in *S. macrocephalus* (1–7, 9), and fourteen and sixteen acrocentric chromosomes, respectively, thus differing over chromosome pairs 8 and 10, which are subtelocentric in the former and acrocentric in the latter. DAPI/CMA3 staining was helpful for identifying chromosomes and preferential sites of Cap- A insertion (Fig. 1, 2).

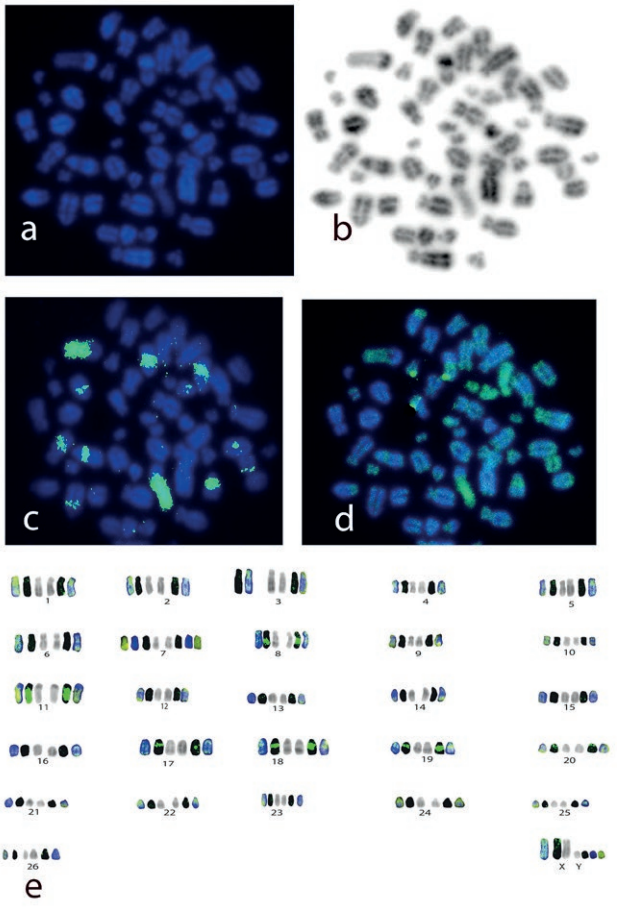

Figure 1. Metaphases of *S. cay paraguay* in DAPI (a), in DAPI inverted (b), Cap-A probe mapping (c), CMA3/DAPI stains (d), the reconstructed karyotype of *S. cay paraguay* from the metaphase in a) after sequential CMA3/ DAPI, DAPI inverted, FISH with Cap-A probes (e).

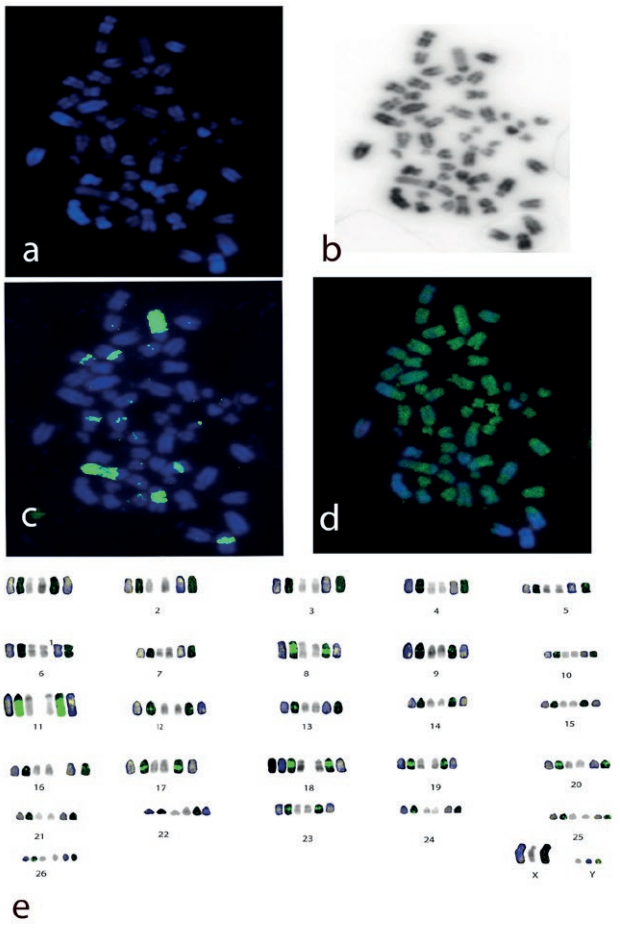

Figure 2. Metaphases of *S. macrocephalus* in DAPI (a), in DAPI inverted (b), Cap-A probe mapping (c), CMA3/DAPI stains (d), the reconstructed karyotype of *S. macrocephalus* from the metaphase in a) after sequential CMA3/ DAPI, DAPI inverted, FISH with Cap-A probes (e).

Cap-A probe mapping revealed bright signals on the metaphases of the two species analyzed, with a similar accumulation pattern and slight differences (Fig. 1, 2): twelve signals were on six chromosome pairs: 5 acrocentric and a subtelocentric chromosome pairs, respectively pairs 11, 17-20 and 8. Additional signals were found on acrocentric chromosome pairs 23 in *S. macrocephalus*, for a total of fourteen (Fig. 2).

The post-FISH C banding pattern obtained was compared with previously published C banding of phylogenetically close species such as *S. apella* (Dumas et al. 2022). Chromosomes pairs with evident C bands were: 8, 11, 18-20; other C bands were at the centromeres of acrocentric chromosomes (Fig. 3), as in the previously analyzed *Sapajus* species.

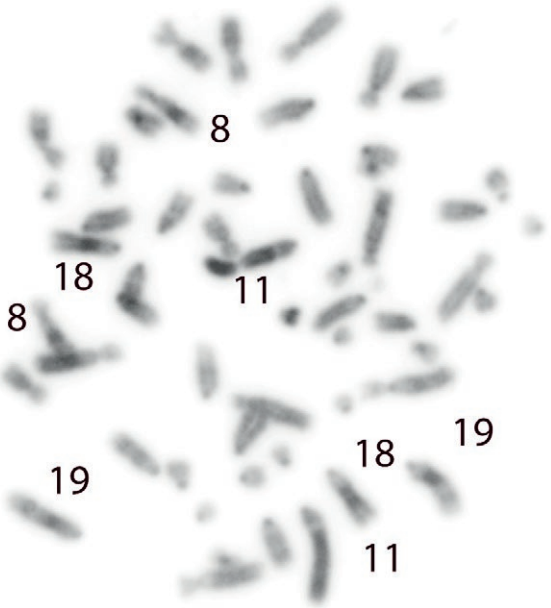

Figure 3. Metaphases of *S. cay paraguay* with C bands. Example of representative chromosome pairs with evident bands are indicated with numbers.

DISCUSSION

Cap-A satDNA have been mapped in many Mammals, including in Primates; previous works have shown that Cap-A is highly amplified in NWMs, localized at non-centromeric positions, with different patterns in the different Platyrrhini species (Valeri et al. 2018, 2020). To extend Cap-A distribution analysis to more primate samples, we used FISH to map Cap-A probes in two species of the genus *Sapajus*. In the two species, chromosome pairs having these signals were identified as 8, 11, 17-20, (Fig. 1, 2); an additional signal was detected on chromosome pair 23 in *S. macrocephalus* (Fig 2); the probe signals fall on CMA3 rich regions in correspondence to the big interstitial C bands (Fig. 3).

Our results for *S. cay paraguay* and *S. macrocephalus* were compared with previous Cap-A mapping data on other platyrrhine species (*Sapajus xanthosternos*, *Saimiri boliviensis, Aotus infulatus, Alouatta guariba, Lagotrix Lagotricha, brachyteles hypoxanthus, Callicebus nigrifrons, Chiropotes satanas, Pithecia irrorata*, *S. boliviensis, S. sciureus, S. vanzolinii* and *S. ustus*) (Valeri et al. 2018, 2020).

The analysis of the results compared with the one from the previous species of the same genus *S. xanthosternos* permitted us to hypothesize that the same chromosome pairs would have these signals. Indeed, in *S. xanthosternos*, six pairs showed signals plus an addtional

on a single chromosome, for a total of thirteen signals. Whereas twelve signals were detected on chromosome pairs in *S. cay paraguay*: on acrocentric chromosome pairs 11, 17-20, and on the subtelocentric chromosome pair 8; moreover, additional signals were found on the acrocentric chromosome pair 23 in *S. macrocephalus*, with a total of fourteen Cap-A signals.

However, one pair seems to have a different Cap-A pattern; indeed, in the previously analyzed *S. xanthosternos* species, the Cap-A probe signal covers both arms, almost all the q and the p arm, while in *S. cay paraguay* and *S. macrocephalus* all the Cap-A probe signals cover just part of the q arm. This difference could presumably be due to an intrachromosomal rearrangement such as an inversion that has amplified and dislocated the sequences differently (presumably on the subtelocentric/acro chromosome pair 8).

Analyzing our results in relation to all the previous available data from different taxa (Valeri et al. 2018, 2020), it is possible to underline that Cap-A localization has high interspecific repeat homogeneity within a genus; indeed, the *Saimiri* species have almost the same chromosomes harboring the Cap-A sequences, with slight differences (Valeri et al. 2020), as it also occurs in the species from the *Sapajus* genus as shown above.

Cap-A probe signals are abundant, around fourteen or fifteen, among *Saimiri* species and are, in the distal regions of the short arms, and in the interstitial heterochromatin of five to seven chromosome pairs. Among *Saimiri* species, signals are on the same chromosome pairs, while others are additional or absent in same specimens. This slightly different location of the Cap-A probe found between the *Samiri* species is particularly evident, especially on chromosomes involved in rearrangements, such as chromosomes 5 and 15. These differences in the Cap-A hybridization pattern in squirrel monkeys has been reported in captivity and in nature; for this reason, it has been hypothesized that Cap-A mapping patterns may be useful in revealing the origin of chromosome sets in hybrids more precisely than chromosome morphology or banding patterns (Valeri et al. 2020).

The link between Cap-A distribution and rearrangements in *Saimiri* is in agreement with the different Cap-A position shown on subtelocentric chromosome between the *Sapajus* species analyzed here and the previously analyzed *S. xanthosternos* (Valeri et al. 2018). Furthermore, *Saimiri* species also show additional chromosomes with Cap-A signals, just as it occurs on *S. microcephalus* in our study. Thus, it can be confirmed the hypothesis that new acquisition of Cap-A occurs; it is presumably due to unequal crossing-over and concerted evolution as previous suggested (Sander Lower et al. 2018).

We observed a slight, variable chromosomal localization of Cap-A signals among the species of the *Sapajus* genus, thus we hypothesized that these differences can be used as taxonomic markers for species identification, in agreement with what was previously shown among *Saimiri* species. This evidence is in agreement with the hypothesis that satDNA sequences, in general, can be used as cytogenetic markers facilitating species identification in many taxa (Prakhongcheep et al. 2013 a,b, Cacheux, et al. 2018).

Furthermore, through the classic cytogenetic approach, detecting heterochromatin with differential chromosomal position has already been hypothesized as distinguishing species (Mudry, 1990, Garcia et al. 2002). The correspondence of the Cap-A signal with heterochromatin block extends the hypothesis regarding the possibility of distinguishing species not only by C bands but also through the Cap-A pattern.

In conclusion, this work demonstrates the presence of Cap-A satellite sequences on chromosomes of *Sapajus* genomes, with a genus specific pattern interstitially in correspondence with C and CMA3 rich regions, but with slight species-specific patterns that can be useful as phylogenetic and taxonomic markers.

ACKNOWLEDGEMENTS

We are grateful to Elsa Addessi, Serena Gastaldi, and Arianna Manciocco for providing the *Sapajus* blood samples from primates of the ISTC-CNR of Rome, Italy.

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