Centromeric enrichment of LINE-1 retrotransposon in two species of South American monkeys *Alouatta belzebul* and *Ateles nancymaae* (Platyrrhini, Primates)

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Abstract. LINE-1 sequences have been linked to genome evolution, plasticity and speciation; however, despite their importance, their chromosomal distribution is poorly known in primates. In this perspective, we used fluorescence *in situ* hybridization (FISH) to map LINE-1 probes onto two representative platyrrhine species, *Aotus nancymaae* (Cebidae) and *Alouatta belzebul* (Atelidae), both characterized with highly rearranged karyotypes, in order to investigate their chromosomal distribution and role and to better characterize the two genomes. We found centromeric enrichment of LINE-1 sequences on all biarmed and acrocentric chromosomes co-localized with heterochromatin C-positive bands. This distribution led us to hypothesize that LINE 1 sequences may have a role in the centromere architecture and karyotype organization of platyrrhine genomes.

Keyword: transposable elements, C-banding, molecular cytogenetics probes, genome evolution.

INTRODUCTION

Through classic and molecular cytogenetics, many primates have been shown to have variable karyotypes; many kinds of probes have been mapped, including single locus probes (Dumas and Sineo 2010, 2012), and Bacterial Artificial Chromosomes (BAC) (Dumas and Sineo 2014; Dumas *et al.* 2015) and whole chromosome paints have been used (Dumas *et al.* 2007; Dumas *et al.* 2012), showing a high rate of intrachromosomal and intrachromosomal rearrangements. In particular, among Platyrrhini (New World primates) living in tropical and neotropical regions, the genera *Alouatta* (howler monkeys) (Cebidae) and *Aotus* (owl monkey) (Cebidae) have very derived karyotypes. Originally, one or only a few species were recognized in these two genera: in *Aotus* there was just one, while later up to eleven species were described, with many of them showing different karyomorphs and having diploid numbers ranging between 2n=46 to 56; *Alouatta* went from five rec-

ognized species up to 15, with diploid numbers ranging between 2n=43 to 58. Furthermore, both species show an extra sex chromosome system due to a translocation between an autosome and the Y chromosome. Among the two genera, chromosome painting has been applied to two *Aotus* species, *Aotus nancymaae*, and *Aotus lemurinus greisemebra* (Stanyon *et al.* 2004; Stanyon *et al.* 2011) and six *Alouatta* species, including *Alouatta belzebul* (Consigliere *et al.* 1996, 1997; de Oliveria *et al.* 2002), showing high genome variability. BAC-FISH has also been performed on *Aotus* and *Alouatta* showing intrachromosomal rearrangements (Dumas *et al.* 2015; Scardino *et al.* 2020a). Although, these species have been studied through molecular cytogenetics with different kinds of probes, repetitive sequences have been poorly studied and, among them, only rDNA and Telomeric probes have been mapped often (Mazzoleni *et al.* 2017; 2018, Ceraulo *et al.* 2021a). The study of these sequence probes' distribution can help locate useful cytogenetic markers for evolutionary and phylogenetic studies.

Repetitive elements have been extensively investigated in order to clarify their possible role in genome evolution and organization (Ahmed and Liang 2012; Biscotti *et al.* 2015; Dumas *et al.* 2016; Mazzoleni *et al.* 2017, 2018; Milioto *et al.* 2019; Paço, *et al.* 2019; Scardino *et al.* 2020b). In primates, repetitive sequences constitute about 50% of their genome and are linked to chromosome evolution (Mathews *et al.* 2003; Jurka 2007; Xing *et al.* 2007; Kvikstad and Makova 2010). A class of repetitive sequences called Long Interspersed Elements of the family 1 (LINE-1) are retrotransposable; the biological roles of this repetitive DNA fraction have been linked to many mechanisms implicated in the genome structure, evolution and disease (Zhu *et al.* 2011; Paço *et al.* 2019). In addition, their involvement in genome architecture such as in DNA packaging, centromere stability and plasticity, gene expression, and epigenetic mechanisms has been shown (Kim and Han 2015; Klein and O'Neill 2018; Ahmed *et al.* 2020). These sequences have also been supposed to be promoters of genomic evolutionary changes and of biological diversity among vertebrates, with an important role in speciation (Böhne *et al.* 2008; Belyayev 2014, Klein and O'Neill 2018). With advances in DNA technologies, the approaches useful for identifying them have changed, with the main approaches being the use of restriction enzyme digestion of DNA, *in situ* hybridization and bioinformatic analysis of DNA sequencing data. In mammals and primates, LINE-1 were studied through different approaches including the use of restriction enzymes (Seuanez *et al.*, 1989) or whole genome screening in simians (Ohshima *et al.* 2003). In humans and anthropoids,

LINE-1 sequence comparisons have been made (Ovchinnikov *et al.* 2001, 2002, Mathews *et al.* 2003) showing that LINE-1 amplification may change rapidly during primate evolution giving different families with variable forms Among New World monkeys, high LINE activity has also been shown in the *Saimiri* and *Saguinus* lineages (Callitrichini), and reduced activity has been found in the *Ateles* lineage (Boissinot *et al.* 2004, Sookdeo *et al.* 2018). So far, however, the distribution of these repetitive sequences through FISH with LINE-1 probes has been studied in few platyrrhine species, belonging to the Callithricini subfamily of the Cebidae family (Serfaty *et al.* 2017, Ceraulo *et al.* 2021b).

The objective of this study was to overcome this lack and analyze the distribution of these LINE-1 sequences by FISH onto two more representative platyrrhine genomes, *Aotus nancymaae* (Atelidae) and *Alouatta belzebul* (Cebidae), with the aim of contributing to the understanding of their role and dynamics as well as the evolution of these highly derived groups of species.

MATERIALS AND METHODS

Following the standard protocol (Scardino *et al.* 2020b), metaphases were obtained from primary fibroblast cell line cultures for *Aotus nancymaae* and *Alouatta belzebul*.

L1 Probe preparation

DNA extraction from the cell culture pellet derived from the fibroblast cell line was done according to the basic DNA extraction protocol from Invitrogen. LINE-1 retrotransposon was amplified through polymerase chain reaction (PCR) using the following primers: L1R, 5'-ATTCTRTTC CAT TGG TCT A-3' and L1F 5'-CCA TGC TCATSGAT TGG -3' (Waters *et al.* 2004).

200 ng of genomic DNA was amplified in 50 μl reactions in an Applied Biosystems PCR SimpliAmp thermal cycler (Thermo Fisher Scientific): five units of Taq DNA Polymerase were incubated together with 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. Cycling parameters were 30 cycles of 94°C, 30 s; 52.5°C, 30 s; 72°C, 30 s, following a 2 min denaturation at 94°C.

Products were visualized on 1% agarose gel. The PCR amplification products were labelled through nick translation using 11-dUTP-Fluorescein.

FISH, karyotyping and chromosome staining

Fluorescence *in situ* hybridization (FISH) was performed following previously described protocols (Scardino *et al.* 2020a, b; Milioto *et al.* 2019; Vizzini *et al.* 2021); C-banding was done sequentially, post-FISH, according to a protocol which includes denaturation with formamide (Fernandez *et al.* 2002). The karyotype was reconstructed using both G-banding and inverted DAPI banding, in agreement with previous published karyotypes: *Aotus nancymaae* (Stanyon *et al.* 2004, 2011; Ruiz-Herrera *et al.* 2005; Dumas *et al.* 2016) and *Alouatta belzebul* (Consigliere *et al.* 1998; de Oliveira *et al.* 2002; Stanyon *et al.* 2011). DAPI images were inverted with a photo editing program (Adobe Photoshop); inverted gray bands correspond to dark G bands.

The chromosomes with the LINE-1 probe signals were identified using inverted DAPI. After FISH, the metaphases were analyzed under a Zeiss Axio2 epifluorescence microscope. Images were captured using a coupled Zeiss digital camera, and the chromosomes were classified according to the nomenclature proposed by Levan *et al.* (1964).

RESULTS

Metaphases of the two analyzed species, obtained through cell culture and chromosome harvesting, were stained post-FISH using DAPI.

The species studied here have the diploid number of 2n=50 and 2n=54, respectively, in *Alouatta belzebul* and *Aotus nancymaae* (Fig 1, 2). The former has 11 pairs of metacentric and submetacentric chromosomes (1-11) and 13 acrocentric chromosomes pairs (12-24) plus XY; the second species has 18 pairs of metacentric and submetacentric chromosomes (1-18) plus the XX, and 7 acrocentric/subacrocentric chromosome pairs (19-26); C-banding showed signals at the centromeric position of both biarmed and acrocentric chromosomes pairs (Fig 1, 2) with peculiar amplified C bands on the bigger subtelocentric chromosomes, in agreement with previous analysis (Torres *et al.* 1998).

In all the analyzed species, the LINE-1 probe mapping revealed bright signals at the centromeric position of almost all chromosomes, with a variable signal amplification. On subtelocentric chromosomes, signals were very bright on the p arms (Fig. 1, 2). Chromosome X was rich in LINE-1 at the centromere. We did not find L1 signals in areas away from centromeres. We speculated that L1 elements should be in a lower copy number in chromosome regions far from centromeres, below the

detection efficiency of FISH, or our probe was not able to hybridize the variable or degraded L1 elements.

DISCUSSION

In general, many mammalian species have deposition of this LINE-1 element in euchromatic regions in G-positive bands (Parish *et al.* 2002; Waters *et al.* 2004), while in other few species it occurs in heterochromatic regions, especially in the centromeric region (Waters *et al.* 2004).

However, the pattern of LINE-1 distribution at the centromere is not a common phenomenon among the mammalian genome (Waters *et al.* 2004; Dobigny *et al.* 2004, 2006; Acosta *et al.* 2008; Vieira-da- Silva *et al.* 2016; de Sotero-Caio *et al.* 2017); indeed, these elements are not often incorporated at major core centromeres, with the exception of the X chromosome euchromatic regions where they are usually abundant along the chromosomal length (Waters *et al.* 2004; Acosta *et al.* 2008). On the other hand, massive accumulations of repetitive elements at the centromeres was previously shown in many other mammals, such as bats and rodents (Sotero-Caio *et al.* 2017; Paço *et al.* 2015; Paço *et al.* 2019), and in some primates (Carbone *et al.* 2012; Serfaty *et al.* 2017, Ceraulo *et al.*, 2021b). In particular, among primates species, LINE-1 have been previously identified through FISH into platyrrhine genomes, in *Saguinus midas* and *Saguinus bicolor* (Serfaty *et al.* 2017), in *Saguinus mystax*, *Leontocebus fuscicollis*, *Leontopithecus rosalia* (Ceraulo *et al.*, 2021b) (tamarins of the Cebidae family).

This result is in agreement with sequence data analysis that showed active LINE on Cebidae species (Boissinot *et al.* 2004) and, more recently, also in Atelidae (Sookdeo *et al.* 2018). At the beginning, from an analysis of just *Ateles paniscus* (Boissinot *et al.* 2004), the extinction of LINE 1 in Atelidae was proposed, but a larger phylogenetic sampling permitted researchers to show their presence (Sookdeo *et al.* 2018).

In our work, we found LINE-1 elements by FISH in the two species analyzed of both the Cebidae and Atelidae families, at centromeric position in agreement with previous cytogenetic molecular data (Serfaty *et al.* 2017, Ceraulo *et al.*, 2021b) and supporting also previous molecular data (Sookdeo *et al.* 2018). LINE-1 probes displayed a non-random distribution by accumulating primarily in CMA3 positive bands at centromeres or pericentromeric regions, co-localizing with C-positive heterochromatin bands (Fig 1, 2); the co-localization of LINE-1 with C-positive bands was previously identified

Alouatta belzebul
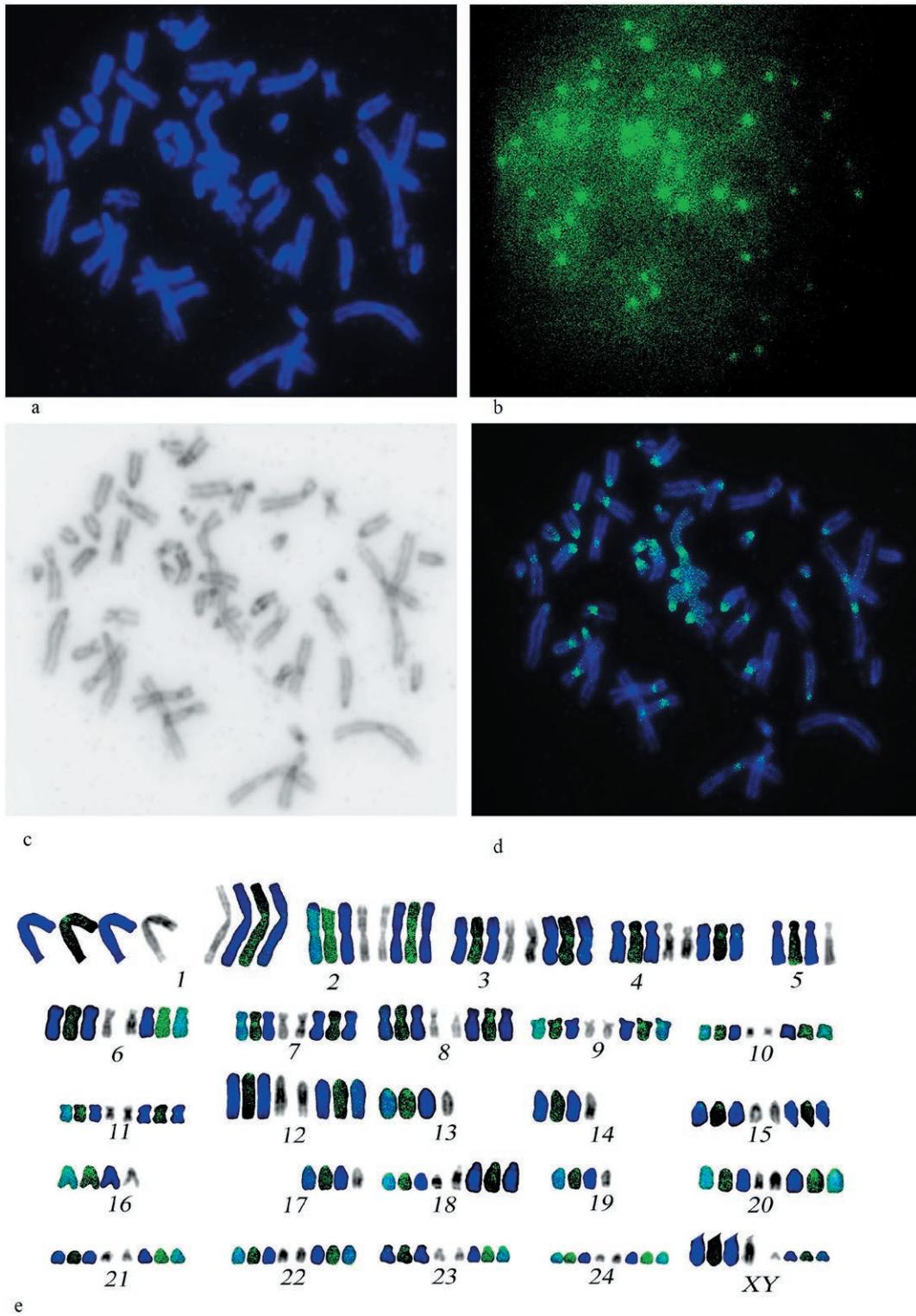
Figure 1. Examples of *Alouatta belzebul* metaphases in DAPI blue (a), FISH with LINE-1 probe in green (b), sequential C-inverted banding (c), DAPI and LINE-1 overlap (d); the reconstructed karyotype from another metaphase of the species after sequential staining and probe mapping (e).

Aotus nancymae
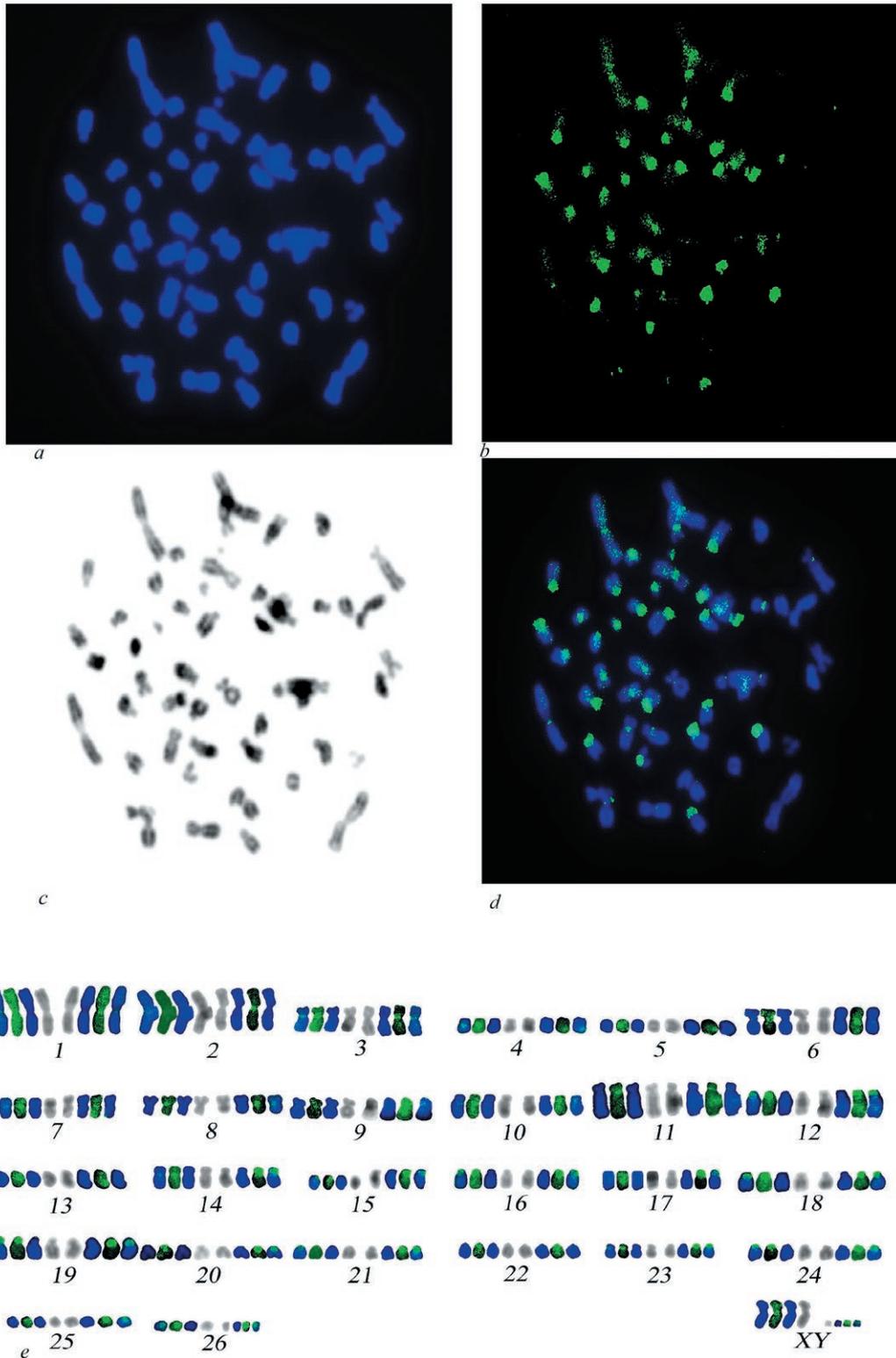
Figure 2. Examples of *Aotus nancymaae* metaphases in DAPI blue (a), FISH with LINE-1 probe in green (b), and G-inverted banding (c), DAPI and LINE-1 in overlap (d); the reconstructed karyotype from the same metaphase after sequential staining and probe mapping (e).

not only in primates but also in other taxa (Kapitonov *et al.* 1998; Serfaty *et al.* 2017). The finding of the centromeric enrichment of LINEs in all analyzed platyrrhine species permitted us to hypothesize that this accumulation might have occurred in the common ancestor of all Platyrrhini, contributing to their current karyotype features. These very intense, amplified and bright signals at centromeres possibly indicate that LINE-1, together with the alpha satellite DNA, are presumably responsible for the architecture of almost all biarmed and acrocentric chromosomes in Platyrrhini. Traditionally, alpha-satellite DNA has been identified as the main DNA component of primate centromeres; it consists of multiple repeat units forming a larger repeat unit, and the larger units are repeated tandemly (Koga *et al.* 2014), with exception of marmosets where the larger repeat unit is not present (Cellamare *et al.* 2009). The presence of LINE-1 at the centromere position is not a surprise; indeed, in the pericentromeric region of the human genome, for example, in addition to satellite DNA, additional elements, mainly retrotransposon elements, have also been shown (Ahmed *et al.* 2020). Moreover, the presence of different and diverse sequences at the centromere have also been shown in the platyrrhine genome, indicating that this region has very variable components in New World monkeys (Valeri *et al.* 2021). Furthermore, although transposable elements and satellite DNA present differences in their structure, genomic organization, spreading mechanisms and evolutionary dynamics, several studies have highlighting their relatedness, with transitions from transposable elements to alpha-satellite DNA, and vice versa, through a process known as the DNA remodeling mechanism (Mestrovich *et al.* 2015; Paco *et al.* 2019). The possible link between transposable elements and alpha-satellite DNA, could be show in the LINE signal amplification at the centromere position of subtelocentric/acrocentric chromosome p arms, localizing with heterochromatin C-positive bands in Ateles chromosomes; these regions are the same where often it is found telomeric signal probes and rDNA probes amplification in many primates, as previous works have demonstrated (Mazzoleni *et al.* 2017, 2018, Ceraulo *et al.*, 2021a); thus, this observation presumably indicates that these LINE-1, alpha-satellite and other repetitive sequences could be involved to this DNA remodeling mechanism. However, considering that LINE have also been linked with chromosomal rearrangements (Böhne *et al.* 2008; de Sotero-Caio *et al.* 2017, Klein and O'Neill 2018), we should take into consideration that these sequences could have had a role in the process leading to the increased rates of chromosomal evolution responsible for the highly rearranged karyotypes of many taxa; for example, the link between

evolutionary reshufflings and LI accumulation have been hypothesized in other platyhirrine species *Saguinus mystax*, *Leontocebus fuscicollis*, *Leontopithecus rosalia* (Ceraulo *et al.*, 2021b) and from data obtained in rodent genera, where the most derived species display a higher level of LINE-1 accumulation on both autosomal and sex chromosomes then the more conserved ones (with accumulation usually only in the sex chromosomes) (Dobigny *et al.* 2004; Rebuzzini *et al.* 2009; Vieira- Dasilva *et al.* 2016).

In order to clarify this hypothesis regarding the link between evolutionary reshufflings and LI accumulation, more samples should be analyzed in the future in a comparative perspective considering derived and conserved primate taxa.

CONCLUSION

Chromosomal studies through FISH mapping onto the analyzed species' genomes permitted the localization of the LINE sequences at the centromere position of biarmed and acrocentric chromosomes, co-localizing with C-positive bands. In a comparative perspective, the presence of these sequences at centromeres in many platyrrhine species led us to propose that LINE-1 could have had a role in the architecture and organization of the present features of platyrrhine karyotypes. In the future, studies on more samples at the species and population level could help in understanding their origin, evolutionary dynamics and function in the karyotypes of primates.

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