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What do we know about the heterochromatin of capuchin monkeys (*Cebus*: Platyrrhini)?

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Cytogenetic studies in *Cebus* spp. have highlighted a peculiar genomic feature: the presence of conspicuous regions of extracentromeric heterochromatin distributed throughout the karyotype. This study investigates the distribution, variability and composition of heterochromatin and their possible implications for genome dynamics. We performed a molecular cytogenetic analysis in 253 individuals from seven of the 12 currently recognized species. The proportion of heterochromatin was related to genome size and ranked, from smallest to largest, from *C. xanthosternos* to *C. albifrons*. Interspecies comparative genome hybridization analyses suggested that the differences among species are not related to heterochromatin content but to changes in the Y chromosome. The pattern revealed by DAPI/CMA_3 staining showed that *Cebus* heterochromatic DNA has a GC-rich composition. The distribution frequencies of heteromorphisms and polymorphisms were not randomly distributed, because a distinguishable pattern could be recognized for each group. *Cebus cay* and *C. nigritus* had a higher level of heterochromatin variability than previously reported. In conclusion, the wide variability among species of the genus *Cebus* is mostly due to the repetitive DNA fraction of its genome.

ADDITIONAL KEYWORDS: *Cebus* – GC content – genome dynamics – genome size – heterochromatin – iCGH – molecular cytogenetics – Neotropical primates – polymorphisms.

INTRODUCTION

The focus of our interest is the organization and functioning of the primate genome. There are only a few studies on the genome of capuchin monkeys (genus *Cebus*), one of the most complex groups of Neotropical primates. From a chromosomal and genomic point of view, they are distinguished from the other Neotropical primates by having the most conserved karyotype, a larger amount of euchromatic genome shared with humans, and large and conspicuous heterochromatic regions distributed throughout the karyotype. The last trait is related to the total number of chromosome pairs with extracentromeric heterochromatic blocks, as well as block size, frequency and composition (different motifs and number of repeats) in each chromosome pair (Mudry de Pargament, Labal de Vinuesa & Brieux de Salum, 1985; Matayoshi, Seuánez & Nasazzi, 1987; Mudry 1990; Ponsà *et al.*, 1995; Nieves, 2007). In addition, extracentromeric heterochromatin is genus-specific, although intraspecific and interspecific variability have been described (Martinez *et al.*, 2004; Nieves *et al.*, 2008, 2011).

Constitutive heterochromatin is found in telomeres, centromeres and chromosomal arms, where it plays a major role in genome stability and spatial organization (Dillon, 2004; Saksouk, Simboeck & Déjardin, 2015). Models of nuclear architecture

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describe the nucleus as a highly compartmentalized organelle (Foster & Bridger, 2005; Bártová & Kozubek, 2006). Comparisons of chromatin arrangement in the nucleus among species thus provide valuable information for the study of evolutionarily conserved and functionally relevant structures of the nuclear architecture (Neusser *et al.*, 2007). In addition, detection of quantitative variations in the constitutive heterochromatin of both plant and animal species suggests that it may be involved in mechanisms leading to speciation (Scherthan *et al.*, 1989; Garagna *et al.*, 1995; Zuckerkandl & Hennig, 1995; Guerra, 2000; Redi *et al.*, 2001; Avramova, 2002).

The origin and maintenance of heterochromatin remain of debate. One area of debate concerns a karyological peculiarity of *Cebus* monkeys: why does the genome of some species contain so much heterochromatic DNA over their life history? The proportion of heterochromatin in the karyotype of *Cebus* species shows a geographical gradient, decreasing from west to east and from north to south. Moreover, species living north of the Amazon River have a higher proportion of heterochromatin than those to the south (Martinez *et al.*, 2002; Nieves, 2007). *Cebus nigritus* and *C. cay* from northern Argentina and *C. nigritus* from Rio Grande do Sul in Brazil are found at the southernmost distribution limit of the genus. Phenotypically, they differ mainly in pelage colour, which varies from brown, to dark brown to black (Cabrera, 1957). These phylogenetically closely related species are well-suited to illustrate the particular features of the *Cebus* genome: *C. cay* has an extracentromeric heterochromatin block representing 75% of the q arm of chromosome 11, but this is absent in *C. nigritus* (Matayoshi *et al.*, 1987; Mudry *et al.*, 1991). Moreover, an exhaustive karyological revision of these two species revealed that the former has more genomic structural rearrangements (heterochromatin polymorphisms, inversions, translocations) than the latter (Nieves & Mudry, 2016, and references therein).

The present study investigates some of the issues relating to the distribution, variability and composition of the heterochromatin in *Cebus* spp. and their possible implications for genome dynamics. Within this framework, we estimated genome size (GS) using Feulgen image analysis densitometry, analysed heterochromatin variability by C-banding staining, determined frequencies and of polymorphisms characterized nucleotide composition by DAPI/CMA_3 staining, and assessed quantitative differences in DNA content using interspecies comparative genome hybridization (iCGH). On the basis of the results obtained, we propose a new role for heterochromatin in the *Cebus* genome.

MATERIALS AND METHODS

We studied 253 adult (both sexes) *C. albifrons*, *C. apella*, *C. cay*, *C. libidinosus*, *C. nigritus*, *C. olivaceus* and *C. xanthosternos*. Samples were collected from individuals caught in the wild or living in captivity throughout the geographical distribution of the genus. Most of the captive monkeys had been confiscated from illegal trade or kept as pets – of unknown origin – and later donated to a zoo (Table 1). The material analysed was processed at the laboratory 'Grupo de Investigación en Biología Evolutiva' (GIBE) over the last 35 years for different research projects. The methods described below were therefore applied to different individuals.

KARYOLOGICAL CHARACTERIZATION

After each animal was anaesthetized intramuscularly with ketamine chloride (20 mg/kg), a heparinized blood sample (3–5 mL) was obtained from the femoral vein under aseptic conditions. Samples were cultured and chromosomes were subjected to G- and C-banding protocols (Steinberg *et al.*, 2014). Species status was confirmed by analysing and comparing G- and C-banded metaphases with previously published karyotypes of *C. albifrons* (García *et al.*, 1976), *C. apella* (García *et al.*, 1978), *C. capucinus* (Dutrillaux, Couturier & Viegas-Pequignot, 1978), *C. paraguayanus* = *C. cay* (Matayoshi *et al.*, 1986), *C. xanthosternos* (Seuánez *et al.*, 1986), *C. nigritus* (Mudry *et al.*, 1991), *C. nigrivitattus* = *C. olivaceus* (Martinez, Aguilera & Ferreira, 1999) and *C. libidinosus* (Amaral *et al.*, 2008).

In addition, we used the C-banding images to determine the percentage of the karyotype corresponding to heterochromatin (% Het) per individual and species. This analysis was performed using MicroMeasure v.3.3 (Colorado State University, USA).

ANALYSIS OF HETEROCHROMATIN VARIABILITY

We analysed all 243 individuals of *C. cay* and *C. nigritus* (Table 2) to investigate the genome of species in marginal areas of the genus distribution. Variability was determined in terms of heteromorphisms and polymorphisms, by counting the number of extracentromeric heterochromatin bands (C+) on all metaphases from each animal. We focused on chromosome pairs 4, 6, 11, 12, 13, 17 and 19, i.e. those presenting extracentromeric heterochromatin blocks.

Table 1. Information on the *Cebus* specimens used in this study by species, captivity institution, capture site in the wild, geographical origin and biological source of the material analysed

Species ($2n$)	Institution*	Site of capture in the wild*	Geographical origin	*N*
Cebus albifrons (52, 54)	ZC; URRAS		Mexico; Colombia	5
Cebus apella (54)	URRAS; CNP		Colombia; Brazil	2
Cebus cay (54)	Zoological Gardens		Unknown	80
	CAPRIM		Paraguay	22
		Locality of Encarnación	SE Paraguay	99
	PUCCH	Province of Salta	NW Argentina	3
			Peru	4
			Brazil	2
			Born in captivity	2
	EFA		Bolivia	1
			Salta, Argentina	6
			Unknown	1
Cebus libidinosus (54)	CNP		Brazil	1
Cebus nigritus (54)	PEEP		Misiones, Argentina	11
		PNI in Province of Misiones	NE Argentina	1
		Province of Misiones	NE Argentina	3
		Province of Misiones	NE Argentina	2
	ZBA		Unknown	5
	PUCCH		Brazil	1
Cebus olivaceus (52)	CNP		Brazil	1
Cebus xanthosternos (54)	CNP		Brazil	1
Total number of *Cebus* individuals analysed				**253**

*ZC, Zoológico de Chapultepec, Dirección General de Zoológicos de la Ciudad de México, México; URRAS, Unidad De Rescate y Rehabilitación De Animales Silvestres, Bogotá, Colombia; CNP, Centro Nacional De Primatas, Belém, Brazil; Zoological Gardens, Institutions in Argentina: ZBA, ECAS, PEEP, REHM, RSP, JZCB, Escobar, ZCTES; CAPRIM, Centro Argentino de Primates; PUCCH, Pontificia Universidad Católica de Chile; EFA, Estación de Fauna Autóctona; PEEP, Parque Ecológico El Puma; PNI, Parque Nacional Iguazú; ZBA, Jardín Zoológico de Buenos Aires, Argentina.
$2n$ = chromosomal number; *N*, number of individuals analysed. NE, northeast; NW, northwest; SE, southeast.

The absence/presence of a C+ band on one or both homologues and differences in heterochromatin block size between homologues were considered heteromorphisms, while translocations and inversions of C+ bands were considered polymorphisms.

We also determined if the variability (as defined above) was randomly distributed or if it displayed a geographical distribution; this could also help to identify the uncertain geographical origin of individuals from zoos. For this analysis, we compared all *C. cay* and *C. nigritus* individuals of known and unknown geographical origin (referred to as wild and geographically unknown, respectively). Finally, we calculated the frequencies of polymorphisms and heteromorphisms for each chromosome pair having extracentromeric heterochromatin.

Estimation of genome size

We used Feulgen image analysis densitometry to assess GS. Air-dried blood smears were prepared from individuals of all the species except *C. apella* (for which fresh blood was unavailable); data on the GS of *C. apella* can be found at http://www.genomesize.com/. Smears were stored in the dark prior to staining. The Feulgen reaction was run as described by Hardie, Gregory & Hebert (2002). At least 30 nuclei were measured per individual sample and integrated optical densities were converted to GS in picograms using *Sus scrofa domesticus* (2.91 pg) and *Gallus domesticus* (1.25 pg) as standards. The standards used in calculating GS were of the same tissue type as the respective sample. The stained nuclei were photographed under a Leica DM2500 microscope equipped with a digital camera. GS was measured using the Bioquant image analysis software (V8.40.20; Bioquant Life Science). The procedures involving the Feulgen reaction, photography, nuclei measurements and GS estimation were performed in collaboration with the laboratory of Dr T. Ryan Gregory at the University of Guelph, Canada.

DAPI/CMA_3 staining

Chromosomal preparations from the seven species studied were incubated in methanol at room temperature for 2 h. Slides were then allowed to dry, washed with McIlvaine buffer and incubated in 75 μL DAPI

Table 2. Number of specimens of *Cebus cay* and *Cebus nigritus* of different geographical origins, showing C-band polymorphisms per analysed chromosome pair

Chromosome pair	Pol/Het	*Cebus cay*							*Cebus nigritus*			*N*
		Paraguay	NW Argentina (Salta)	Peru	Bolivia	Unknown	Brazil	Born in captivity	NE Argentina (Misiones)	Brazil	Unknown	
4	Pol	0	0	0	0	0	0	0	0	0	0	
	Het	70	0	0	0	7	0	0	0	0	0	
6	Pol	0	0	0	0	0	0	0	0	0	0	
	Het	81	9	4	1	80	2	1	11	1	2	
11	Pol	0	0	0	0	0	0	0	0	0	0	
	Het	11	0	0	0	3	0	0	17	1	5	
12	Pol	11	0	0	0	4*	0	0	0	0	2*	
	Het	7	0	0	0	6	0	0	0	0	0	
13	Pol	0	1*	0	0	1*	0	0	2*	1*	3*	
	Het	47	0	0	0	14	2	0	0	0	0	
17	Pol	0	0	0	0	0	0	0	0	0	0	
	Het	110	5	4	1	76	0	2*	12	1	5	
19	Pol	0	0	0	0	0	0	0	0	0	0	
	Het	121	9	0	1	79	0	0	13	0	5	
N		121	9	4	1	81	2	2	17	1	5	**243**

The absence/presence of a C+ band on one or both homologues and differences in heterochromatin block size between homologues were considered heteromorphisms (Het), while translocations and inversions of C+ bands were considered polymorphisms (Pol). An asterisk indicates the presence of a paracentric inversion. *N*, number of individuals analysed.

solution (0.01 mg/mL) in a wet chamber in the dark for 20 min. The preparations were washed successively with distilled water, McIlvaine buffer and distilled water. This was followed by incubation in 50 µL CMA_3 solution (0.6 mg/mL McIlvaine buffer) in a wet chamber in the dark for 1.5 h, followed by a further wash in distilled water. The samples were mounted and observed under a Leica DMLB fluorescence microscope. Black and white chromosome images were obtained with a Leica DFC 340 FX camera. Two images per metaphase were obtained at 1000× magnification using appropriate filters. The black and white images corresponding to each filter were stored separately and subsequently pseudo-coloured. Image processing was performed with Image Pro-Plus 4.5 (Media Cybernetics Inc.).

Interspecies comparative genome hybridization

iCGH experiments were conducted for three species, in pairs, following Fantini *et al.* (2016): *C. nigritus* vs. *C. xanthosternos* and *C. nigritus* vs. *C. olivaceus.* These two species pairs were selected because they show extreme values for GS and heterochromatin content: *C. nigritus* and *C. xanthosternos* have a comparatively lower heterochromatin content and smaller GS, while *C. olivaceus* has a comparatively higher heterochromatin content and larger GS. Total genomic DNA for iCGH experiments was extracted from the blood samples using a DNA extraction kit (Qiagen) and labelling was conducted following a modified nick translation protocol with FITC-dUTP (green) or rhodamine-dUTP (red). In total, 100 ng of each genomic probe was used in pairs (one probe was marked in green and the other in red) in 2.5 µL of hybridization mixture. The probe mixture was denatured at 70 °C for 7 min and then kept at 37 °C for 90 min for co-hybridization. Freshly prepared slides with chromosomal spreads were pre-incubated at 37 °C for 1 h, submerged in 2× saline sodium citrate (SSC) for 30 min and finally denatured in 0.1 M NaOH/70% ethanol at room temperature for 4 min, and then sequentially dehydrated in an ethanol series of 70, 90 and 100%. Hybridization was conducted in a wet chamber at 45 °C overnight. Post-hybridization washes followed standard protocols with 0.4× SSC/0.3% Tween 20 at 70 °C and 2× SSC/0.1% Tween 20 at room temperature. Slides were counterstained with DAPI and analysed with a Leica DMLB fluorescence microscope. Chromosome images were obtained with a Leica DFC 340 FX camera. Three images per metaphase were visualized using appropriate filters. Image processing was performed with Image Pro-Plus 4.5 (Media Cybernetics Inc.).

RESULTS

KARYOLOGICAL CHARACTERIZATION AND ANALYSIS OF HETEROCHROMATIN VARIABILITY

All specimens were karyologically characterized and taxonomically identified to the species level. Based on G-C banding patterns of each species, metaphases with species-specific C-banding patterns are presented in Figure 1. The individuals examined showed centromeric C+ bands on all chromosomes of the complement. For chromosomal pairs presenting extracentromeric heterochromatin blocks,

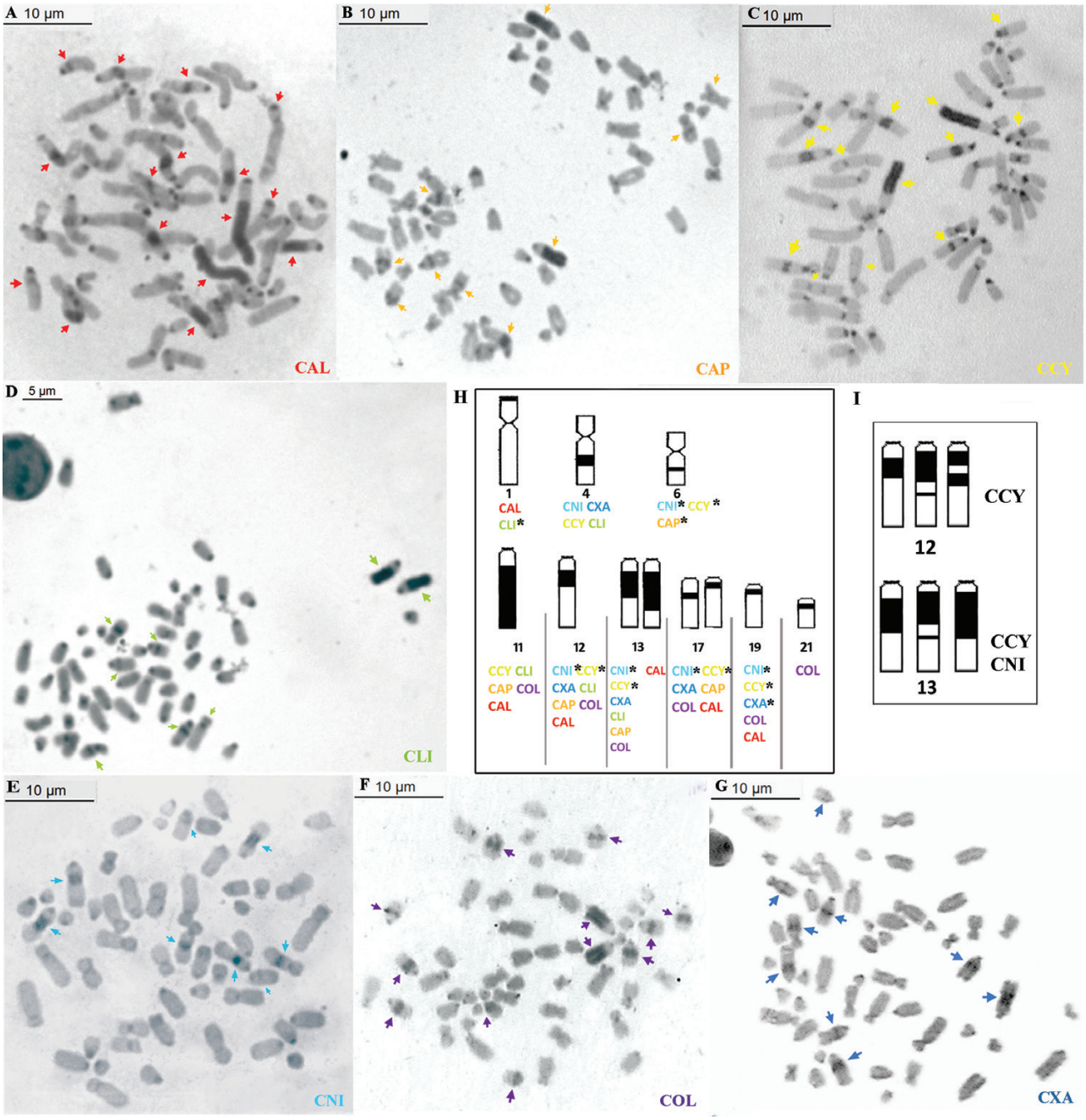

Figure 1. C-banding patterns *Cebus* species. A, *C. albifrons* (CAL, red); B, *C. apella* (CAP, orange); C, *C. cay* (CCY, yellow); D, *C. libidinosus* (CLI, green); E, *C. nigritus* (CNI, cyan), F, *C. olivaceus* (COL, purple); G, *C. xanthosternos* (CXA, blue). H, schematic representation of chromosomal pairs with extracentromeric heterochromatic blocks and heteromorphisms in the species studied. Species with polymorphic heterochromatic blocks are indicated by an asterisk. I, schematic representation of the two paracentric inversion variants observed in the heterochromatic blocks of chromosome pairs 12 and 13 of *C. cay* and *C. nigritus*.

we observed a large proportion of polymorphisms, although the localization of the blocks coincided in most of the species (Fig. 1H, I). We also observed C+ bands in the telomere of the p arm of pair 1 of *C. albifrons* and *C. libidinosus* and C+ bands in the q arm of *C. olivaceus*.

In chromosome pairs with extracentromeric C+ bands or blocks in the 243 individuals of *C. nigritus* and *C. cay*, only *C. nigritus* had a heterochromatic block in both homologues of chromosome pair 4, while this was absent in both homologues of chromosome pair 11 (N = 23; Fig. 2A). All individuals of *C. cay* showed variability in the number of homologues of the same pair with or without the heterochromatic block (e.g. 14 of the 220 individuals were heteromorphic for pair 11; Fig. 2A). In addition, there were differences in the frequency of each type of heteromorphism. In both *C. nigritus and C. cay* the most variable heteromorphic chromosome pairs were 6, 17 and 19, but at different frequencies. In *C. nigritus* the highest frequencies were observed for pairs 17 (31%) and 6 (24%), and in *C. cay* for pairs 6 (34%) and 17 (29%). Chromosome pair 19 showed a similar percentage of heteromorphism in the two species (22 and 20% for *C. cay* and *C. nigritus*, respectively; Fig. 2B). The analysis of polymorphisms revealed that paracentric inversions were the only chromosomal rearrangement observed (illustrated in Fig. 1I). We detected two paracentric inversions: one involving two bands of different width in pair 13 of *C. nigritus* and in pairs 12 and 13 of *C. cay*, and the other involving two bands of the same width in pair 12 of *C. cay*. Overall, these two variants were present in 6% of pair 12 and 1% of pair 13 in *C. cay*, while in *C. nigritus* they were found in 20% of chromosome pair 13 (Fig. 2B).

We also checked for an association between polymorphisms and geographical origin of the individuals. No clearly distinguishable heterochromatin pattern was observed between wild and captive specimens of *C. cay* and *C. nigritus* (Fig. 2C–E).

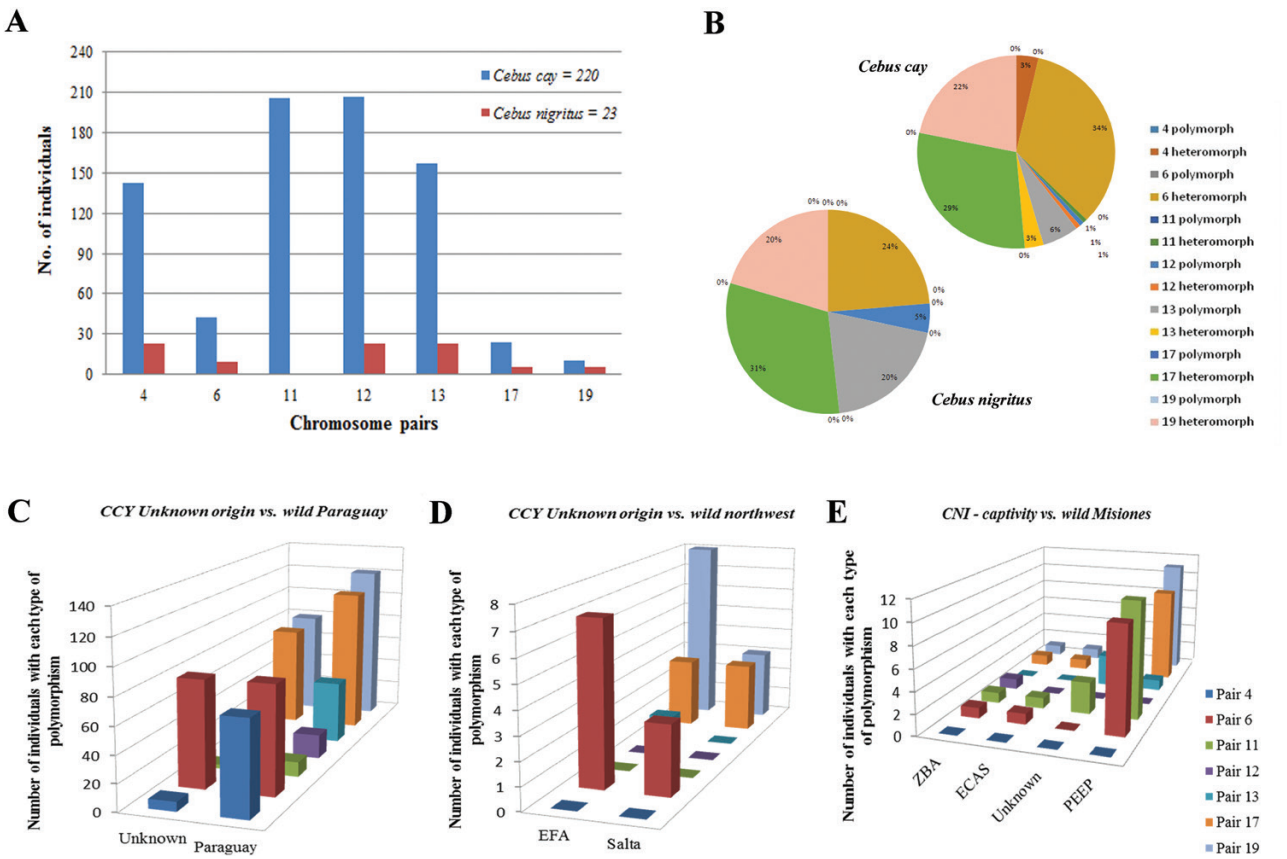

Figure 2. Analysis of extracentromeric heterochromatin variability in *Cebus cay* and *Cebus nigritus* (N = 243). A, number of individuals with heterochromatic blocks present in both homologues of the studied pairs. B, percentage of polymorphisms and heteromorphisms for each studied chromosome pair. C–E, comparison of the distribution patterns of heterochromatin polymorphisms between specimens taken from individuals held in captivity and in the wild.

Estimates of GS and proportion of heterochromatin

The substantial differences in size and amount of heterochromatic blocks among *Cebus* species underline the importance of quantifying the proportion of heterochromatin in the karyotype. Based on C-banding results, we obtained the following heterochromatin contents: *C. xanthosternos* (5.27 ± 0.66), *C. nigritus* (5.6 ± 0.77), *C. cay* (8.47 ± 0.92), *C. libidinosus* (8.57 ± 0.48), *C. apella* (9.31 ± 0.0006), *C. olivaceus* (10.47 ± 0.61) and *C. albifrons* (12.5 ± 1.09). The amount of heterochromatin also correlated with GS, from smallest to largest ($r = 0.776$, $P = 0.047$): *C. xanthosternos*, *C. nigritus*, *C. cay*, *C. libidinosus*, *C. olivaceus* and *C. albifrons* (Fig. 3). Published data on the GS of *C. apella* are not up to date, and it is doubtful whether they in fact from this species. This may explain why the correlation was not statistically significant. This species was thus excluded from the analysis.

Heterochromatin composition

The pattern of DAPI/CMA_3 staining was similar in the *Cebus* species studied: DAPI (+) signals showed no consistent pattern while CMA_3 (+) bands coincided with the heterochromatic blocks and were stained bright yellow (Fig. 4). This pattern indicates that *Cebus* has GC-rich extracentromeric heterochromatin.

Analysis of quantitative DNA differences (iCGH)

In the iCGH experiments, no differential signals were detected in the autosomal chromosome complement in the comparison either between *C. nigritus* and *C. xanthosternos* (Fig. 5A, B) or between *C. nigritus* and *C. olivaceus* (Fig. 5C, D). Although the heterochromatic regions of their genomes were therefore distinct, image overlapping and further analysis showed no quantitative differences between the studied genomes. The only distinct positive hybridization signal was localized on the Y chromosome when comparing *C. nigritus* with *C. olivaceus* (Fig. 5C). In particular, the Y chromosome of *C. nigritus* had more non-heterochromatic DNA than that of *C. olivaceus*. This differential hybridization signal was only detected in the metaphases of *C. nigritus*, while the Y chromosome showed no signal in the metaphases of *C. olivaceus* (Fig. 5D).

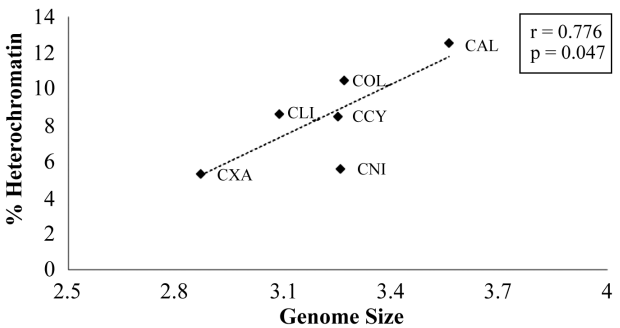

Figure 3. Correlation between heterochromatin content (%) and genome size in *Cebus* species.

DISCUSSION

Genomic studies of the genus *Cebus* have been somewhat fragmentary and have focused on a particular topic of interest in a limited number of species. Cytogenetics has provided additional data and novel approaches to our knowledge and understanding of *Cebus* genome dynamics, mainly for species at the southernmost distribution limit of the genus. Our study of *C. cay* and *C. nigritus* is the first to include: (1) a large number of individuals from numerous and diverse geographical origins; and (2) simultaneous analyses of heterochromatic variability, heterochromatin rearrangements, and frequency and size of heterochromatic blocks.

In previous studies, the heteromorphism index, calculated as the difference in block size between homologues of each chromosome pair, was analysed for pairs 4, 11, 12, 13 and 17 (with C+ band in both homologues) of 11 individuals of *C. cay* (formerly *C. apella paraguayanus*) from Argentina and Paraguay (Mudry de Pargament *et al.*, 1985; Mudry de Pargament & Labal de Vinuesa, 1988). The authors concluded that pairs 17 and 13 were the first and second most heteromorphic, respectively, in more than 50% of the monkeys from the two countries. However, they considered only block size and not the presence or absence of the band as a heteromorphism. Matayoshi *et al.* (1987) qualitatively analysed the frequency and size of the C+ bands in the chromosomal pairs of *C. cay* and *C. nigritus* mentioned above. They found that pairs 11 and 13 showed wide variability in block size between species and that the heterochromatin blocks were always present in the studied pairs, in agreement with Mudry de Pargament *et al.* (1985) and Mudry de Pargament & Labal de Vinuesa (1988). Compared with the studies mentioned above, our analysis based on over 200 individuals indicated that the heterochromatic variability observed for the southern populations is the highest so far published. We found that all chromosome pairs with heterochromatin blocks are heteromorphic, albeit at different frequency. Previous studies of *C. cay* found that chromosome pairs 13 and

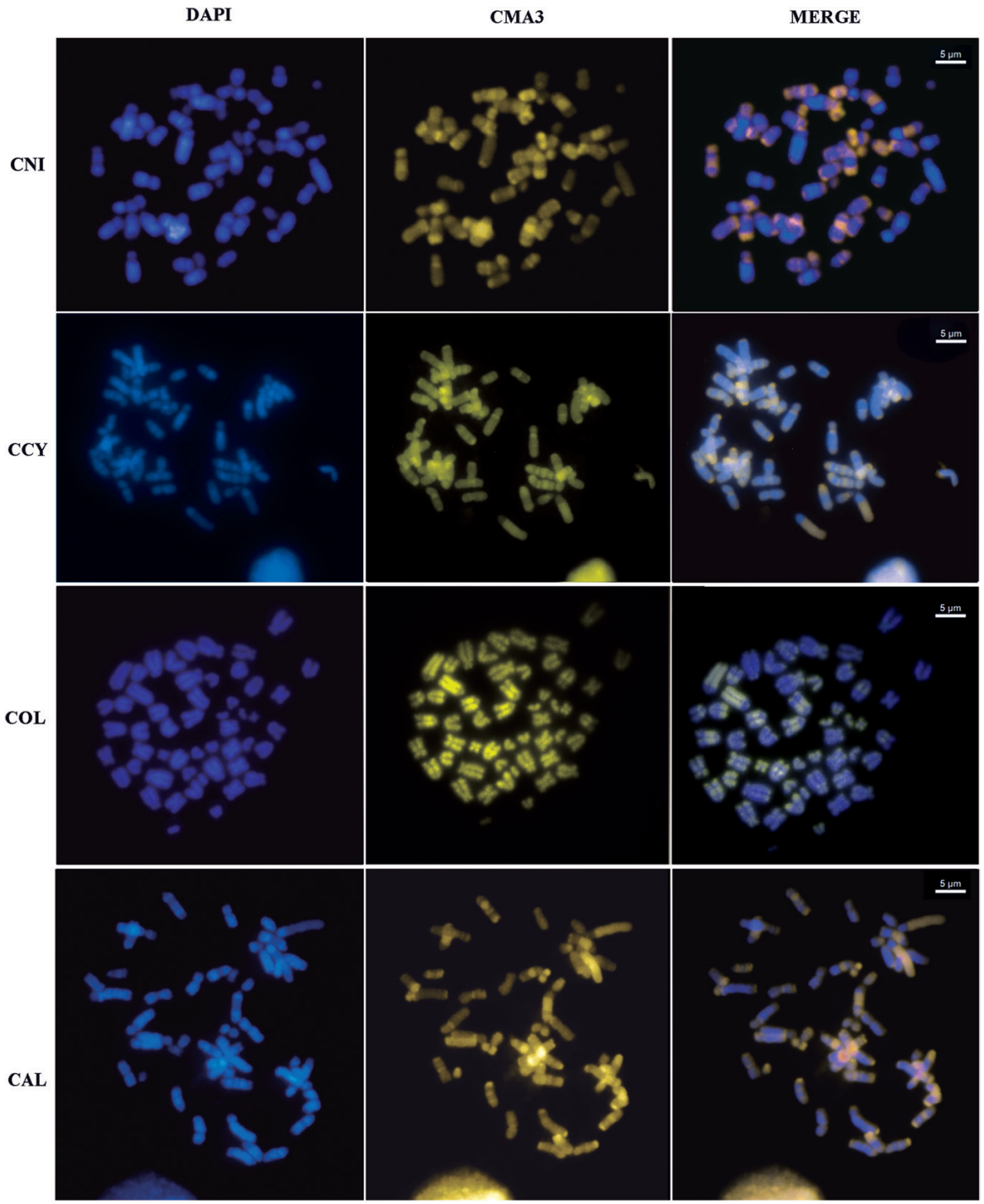

Figure 4. DAPI/CMA_3 banding patterns in *Cebus* species: *C. nigritus* (CNI), *C. cay* (CCY), *C. olivaceus* (COL) and *C. albifrons* (CAL). The heterochromatic blocks characteristic for each species are identified as intensely stained CMA_3 (+) bands (yellow).

17 were the most heteromorphic and that this species was more polymorphic than *C. nigritus* for all chromosome pairs. This was confirmed by our results, which also showed that all chromosome pairs in *C. cay* were highly polymorphic (Fig. 2A, B). This chromosomal characteristic of *C. cay* may have been noticeable due to the relatively larger sample size used in our work. Analysis of variability in the C-band patterns of

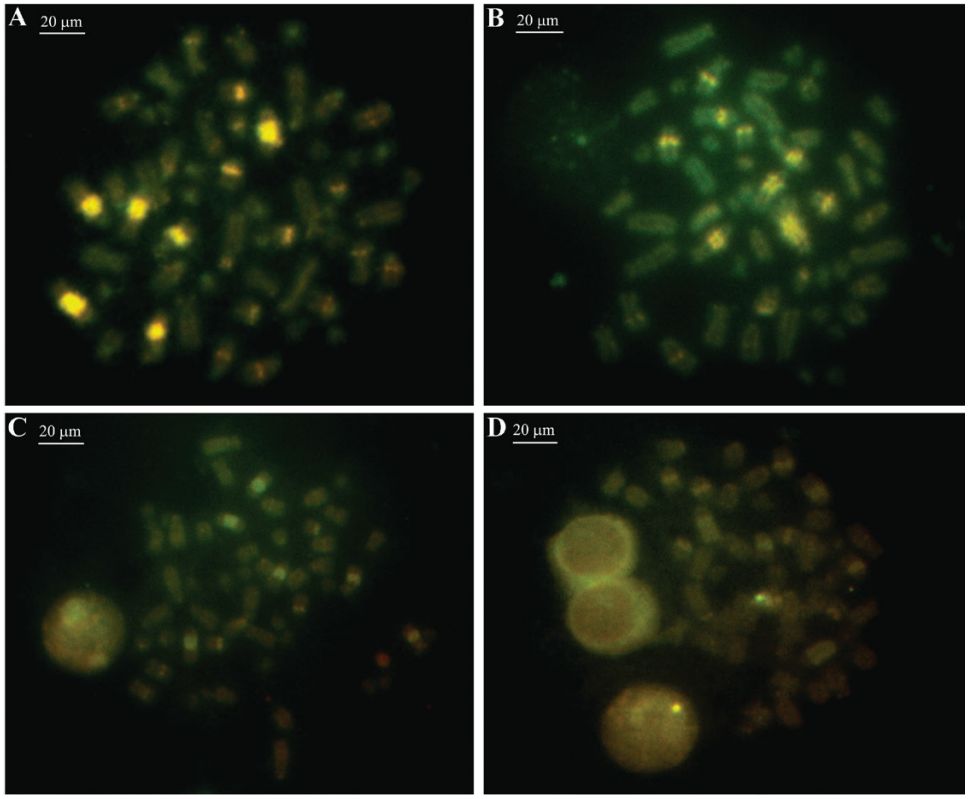

Figure 5. iCGH analyses between genomes with low C-values (CNI and CXA) and between genomes with low (CNI) and high (COL) C-values. A, CNI (FITC, green) vs. CXA (rhodamine, red): genomic probes hybridized onto CNI chromosomes. B, CNI (FITC, green) vs. CXA (rhodamine, red): genomic probes hybridized onto CXA chromosomes. C, CNI (rhodamine, red) vs. COL (FITC, green): genomic probes hybridized onto CNI chromosomes. D, CNI (rhodamine, red) vs. COL (FITC, green): genomic probes hybridized onto COL chromosomes. Abbreviations: CAL, *C. albifrons*; CNI, *C. nigritus*; COL, *C. olivaceus*; CXA, *C. xanthosternos*.

C. cay and *C. nigritus* allowed us to confirm a species-specific heterochromatin pattern, whereas there was only a weak relationship between geographical origin and heterochromatin pattern at the intraspecific level; the use of similar samples and sufficiently large sample sizes for the geographical origin data sets may have helped to corroborate a possible correlation (Fig. 2C–E). On the other hand, the frequencies of heteromorphisms and polymorphisms were not randomly distributed, and a clear pattern could be recognized for each group. In the context of structural chromosomal rearrangements, we found two paracentric inversions involving the heterochromatin of regions 12q and 13q of chromosome pairs 12 and 13, respectively (Fig. 1I). A paracentric inversion causing two heterochromatic bands has been previously reported only for chromosome 12q in 39% of *C. cay* individuals from Paraguay, these bands being of the same width (Martinez *et al.*, 2004). In the present study, we found that in *C. cay* and *C. nigritus* this polymorphism appeared either as two bands of equal width or as two bands of different width. Our analyses show that these marginal species have a higher level of heterochromatin variability than previously described.

When we analysed the heterochromatin content in the genome of all the studied species – expressed as its percentage in the karyotype – we observed that *C. olivaceus* and *C. albifrons* had the largest number of heterochromatic blocks and the largest proportion of heterochromatin (*c*. 10%). Moreover, the proportion of heterochromatin between *C. libidinosus* and *C. cay* and between *C. xanthosternos* and *C. nigritus* was very similar and corresponded to an intermediate and low number of blocks, respectively. These results agree with GS estimates (Fig. 3), but they only partially explain the observed heterochromatic diversity within the genus, suggesting a role of heterochromatin in genome dynamics. Regarding quantitative differences in DNA leading to the divergence of *Cebus* species, our results from the iCGH experiments suggest that they might have entailed changes only in the Y chromosome, where regions of relative DNA gain are present in *C. nigritus* but absent from *C. olivaceus*. These regions of DNA gain were not quantitatively revealed

when comparing *C. nigritus* with *C. xanthosternos*, the latter being phylogenetically more closely related to *C. nigritus* than to *C. olivaceus*. We previously compared the genomes of *C. cay* and *C. nigritus* using iCGH and found that the former had more repetitive DNA while the latter had more coding DNA (Nieves, Mühlmann & Mudry, 2010). When we analysed the genome of a captive-born female hybrid of these species to determine their relative contribution, we observed that the genome contained more repetitive sequences than those of the parental species (Fantini, Mudry & Nieves, 2011). In these comparisons, DNA gain was not detected on chromosome Y of either *C. nigritus* or *C. cay*. Although *Cebus* spp. genomes vary in DNA amount (between 2.87 and 3.98 pg), which is directly related to the proportion of heterochromatin, the variation in DNA amount seems to be scattered throughout the genome so that quantitative genomic differences at the chromosomal level were only detected in the Y chromosome. Overall, our results suggest that genomic variability is higher between species living at the southern limit of the distribution of the genus (*C. cay* and *C. nigritus*) – which are robust species of the genus – than between 'gracile and robust' species, in accordance with the proposals of Amaral *et al.* (2008) and Ruíz-Garcia, Castillo & Luengas-Villamil (2016).

We have explored the characteristics of *Cebus* heterochromatin with different cytogenetic tools. However, our primary goal was to investigate not only the distribution, variability and composition of the heterochromatin but also their possible implications for genome dynamics. In mammals GC content is correlated with potentially relevant genomic features such as GS variation, the distribution of transposable elements and, from a functional point of view, gene density, recombination rate and expression level (Eyre-Walker & Hurst, 2001; Kudla *et al.*, 2006). A high GC content in the heterochromatic portion of the genome has been proposed to confer high stability to large genomes or to provide protection against chemical damage, because most chemical mutagens have a higher affinity for GC- than for AT-base pairs (Vinogradov, 1998). Previous genotoxicity studies of the effects of nitroimidazole derivatives in the peripheral blood of *C. cay* have revealed that damage is concentrated in heterochromatin-bearing chromosomes rather than being randomly distributed in the genome (Mudry *et al.*, 2011). We found that in *Cebus* the heterochromatin is GC-rich and chromosomes with heterochromatin are damaged by nitroimidazole derivatives. These results suggest that, in *Cebus*, the heterochromatin has a specific function and support the hypothesis that larger genomes with GC-rich heterochromatic DNA are more stable and protected from potentially damaging agents.

Why then does the genome of some species contain so much heterochromatic DNA over their life history? Karyological similarities reported for the gracile and robust capuchins together with our results support the hypothesis that heterochromatin would have appeared in the genome before the divergence of the two major lineages. Subsequently, differential heterochromatic amplification may have occurred among the species of the genus, with *C. cay* and *C. nigritus* – at its southernmost distribution limit – showing markedly increased heterochromatin polymorphisms and variability, as expected for marginal species (Coyne & Orr, 2004). The large variability in heterochromatic polymorphisms, GS and chromatin composition described herein are consistent with this assumption and with previous molecular and morphometric studies that showed substantial differences in mitochondrial DNA and in cranial shape and size among different populations of *Cebus* at the southern distribution limit (Martinez *et al.*, 2005; Casado *et al.*, 2010; Hassel *et al.*, 2013; Aristide *et al.*, 2015).

Constitutive heterochromatin is considered an important component of eukaryotic genomes because of the essential role it plays in nuclear architecture, DNA repair and genome stability, among other functions (Strom *et al.*, 2017). Recent studies have proposed that heterochromatic domains form via phase separation, and mature into a structure that includes liquid and stable compartments. This model and, in particular, liquid properties of the domain can be used to explain unusual heterochromatin behaviours, such as the association between distal heterochromatic islands and the main domain observed in *Drosophila*. Such theories provide other, testable hypotheses about the regulation of heterochromatin functions (Strom *et al.*, 2017).

Phase separation is emerging as a general mechanism for genome organization. The combination of our knowledge of the structural properties and variability of the heterochromatin in *Cebus* spp., together with a three-dimensional analysis of the genomic structure and its interactions with other molecules, will provide a better understanding of the principles governing the nuclear architecture of heterochromatin (Klosin & Hyman 2017). The wide heterochromatin variability among species of the genus *Cebus* is mostly due to the repetitive DNA composition of its genome. Our cytogenetic approach regarding the *Cebus* has thus emphasized the importance of including heterochromatin characterization in phylogenetic analyses and provided evidence of its major role in genome architecture.

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