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Qualitative and quantitative analysis of the genomes and chromosomes of spider monkeys (Primates: Atelidae)

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Heterochromatin distribution and chromosomal rearrangements have been proposed as the main sources of karyotype differences among species of Neotropical primates. This variability suggests that there could be differences at other smaller-scale levels of DNA organization as well. In particular, quantitative differences between genomes result from gains and losses of individual DNA segments, and may result in varying genome sizes (*C*-values) among species. In this work, we studied the genomes of 23 individuals from four species in the genus *Ateles* (Primates: Platyrrhini): *A. chamek*, *A. paniscus*, *A. belzebuth*, and *A. geoffroyi*. We analyzed genome size and its relationship with the presence of chromosomal rearrangements and patterns of heterochromatin distribution. The *C*-value presented in this work for *Ateles chamek* is the first estimate for this species (3.09 ± 0.23 pg), whereas our estimates for *A. belzebuth* (2.88 ± 0.06 pg) and *A. geoffroyi* (3.19 ± 0.24 pg) differed from those previously published. Fluorescent in situ hybridization (FISH) and interspecies comparativegenomic hybridization (iCGH) analyses revealed that differences in genome size among species relate to localized blocks in both heterochromatic and euchromatic regions, the latter of which appear to be genetically unstable. There were also quantitative differences in Y chromosome content. It remains to be seen whether the chromosomal characteristics of *Ateles* here discussed are common to platyrrhine monkeys, but it is clear that these monkeys exhibit some intriguing genomic features worthy of additional exploration. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **118**, 752–762.

KEYWORDS: *C*-value – genome – heterochromatin – interspecies comparative genomic hybridization – primates – repetitive DNA.

INTRODUCTION

The genomes of related species can be compared from a variety of qualitative and quantitative perspectives, ranging from individual DNA sequences to chromosome morphology to bulk properties of the entire genome such as size, base composition, or repeat content (e.g. Elliott & Gregory, 2015a, b). Qualitative characters include chromosome form, the distribution of heterochromatin blocks, the presence/absence of certain genes, or the conservation of syntenic associations, among others. By contrast, quantitative differences such as variability in genome size (*C*-value, or haploid DNA content) result from gains or losses of DNA segments in the genome.

Although intraspecific variation in genome size appears to be minor in most cases, large differences in nuclear DNA content are often seen among closely related taxa (Ronchetti *et al.*, 1993; Boulesteix, Weiss & Biémont, 2006). Most of the diversity in genome size has been attributed to quantitative differences in the repetitive fraction of the genome, in particular the abundance of transposable elements (Manfredi Romanini, 1985; Redi *et al.*, 2001; Redi & Capanna,

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2012; Elliott & Gregory, 2015a, b). The presence of transposons and other repetitive DNA can lead to ectopic recombination resulting in mutations and chromosomal rearrangements (Kidwell & Lisch, 2002; Belyayev, 2014). As these processes should be more common in genomes that contain many transposable elements, large genomes should hold higher rates of rearrangement and mutation (Kraaijeveld, 2010).

Repetitive DNA sequences may be conspicuous in specific regions of the karyotype and recognized as heterochromatin by differential staining methods. A fundamental question in the study of genome-size evolution that remains unresolved is whether different repetitive DNA components vary together in a correlated fashion when comparing closely related species. Heterochromatin has been pointed out as one of the possible factors involved in karyological diversification among several taxa of vertebrates (Capanna, Civitelli & Cristaldi, 1977; Reig, 1984; Capanna, 1995; Avramova, 2002). Within Primates, Platyrrhini present the greatest proportion of extracentromeric heterochromatin among all described species (reviewed by Seuánez, Bonvicino & Moreira, 2005). This noticeable cytogenetic feature is conspicuous in a few genera incuding *Cebus*, *Ateles*, *Saimiri*, *Callithrix* and *Aotus*. Among them spider monkeys (Atelidae, *Ateles* É. Geoffroy, 1806) exhibit karyological features of particular interest in the study of genome like heterochromatic variation and chromosomal rearrangements (Fig. 1), but remains unclear the relationship between them and genome size in each species.

The range of genome size variation among primates is small compared with many other vertebrates. Published primate genome size estimates range just over two-fold, from 2.26 pg in *Callicebus torquatus* (Callithricidae) to 5.26 pg in *Tarsius syrichta* (Prosimii). This is less than half of the overall variability seen among mammals, which in turn is a relatively constrained group (especially as compared to amphibians, which vary 120-fold) (Gregory 2005; Morand & Ricklefs 2005; Rylands & Mittermeier 2009). Also previous genome size estimations (www.genomesize.com) need to be revised and updated. It must be noted that most of these primate *C*-value estimations were performed during the 70's & 80's (Manfredi Romanini, 1972; Pellicciari *et al.*, 1982), and that since then the taxonomy of primate species has been updated significantly (Rylands, Mittermeier & Silva, 2012), as have the methods used for DNA quantification. The advent of Feulgen image analysis densitometry (Hardie, Gregory & Hebert, 2002), in particular, is of particular relevance when it comes to studying rare or endangered species from whom sample collection is difficult to perform.

In this context, the aims of our work are to estimate *C*-value of some *Ateles* species and to analyze its relationship with the presence of chromosomal rearrangements and the proportion of heterochromatin in each species and thereby to identify and localize, at the chromosomal level, qualitative and quantitative differences among their genomes. Our hypothesis is that there is genome size differences between *Ateles* species that mirror the chromosomal variability found among them. Taking into account the known chromosomal characteristics of *Ateles* species and that during cytogenetic reorganizations amplification of transposable elements often occurs we expect to find more differences in *C*-value between species with great differences in heterochromatin proportion on their karyotypes, or with differences in the presence/absence of chromosomal rearrangements. Conversely, we hypothesized that those *C*-value differences are chromosomally conspicuous and can be detected in interspecies comparative genomic hybridization experiments (iCGH). Previous work in other primate species have shown that these regions are of particular interest and are involved in genome size changes (Neusser *et al.*, 2005; Nieves, Mühlmann & Mudry, 2010; Fantini, Mudry & Nieves, 2011). In this regard our expectation is that *Ateles* species with bigger genome size would show regions of relative DNA gain compared to *Ateles* species with smaller genome size, and that those gains are located specially in heterochromatic regions and chromosomes involved in rearrangements.

MATERIAL AND METHODS

Samples and chromosome preparations

Metaphase spreads for *in situ* hybridization experiments (iCGH & FISH) were prepared from phytohemagglutinin stimulated lymphocytes or fibroblasts in culture from 23 adult individuals of *Ateles chamek* (ACH), *A. geoffroyi* (AGE), *A. belzebuth* (ABE) and *A. paniscus* (APA) (Table 1). For this work we have sampled both wild born and captive individuals, from different regions of South and Central America, in an effort to add taxonomical accurate information to the already registered *C*-values. Standard procedures of hypotonic treatment and methanol/acetic acid fixation (3:1, v/v) were used. For each specimen classical cytogenetic characterization was done following modified standard protocols (Steinberg *et al.*, 2014). The taxonomic designation (at species level) of each specimen was conducted by cytogenetic means – analysis of ten metaphases with each technique (conventional staining, G- and C-bands) and comparison to published *Ateles* sp. karyological patterns (Pieczarka, Nagamachi & Barros, 1989; Morescalchi *et al.*, 1997; De Oliveira *et al.*, 2005) – and completed with phenotypic and geographic origin (when known) information.

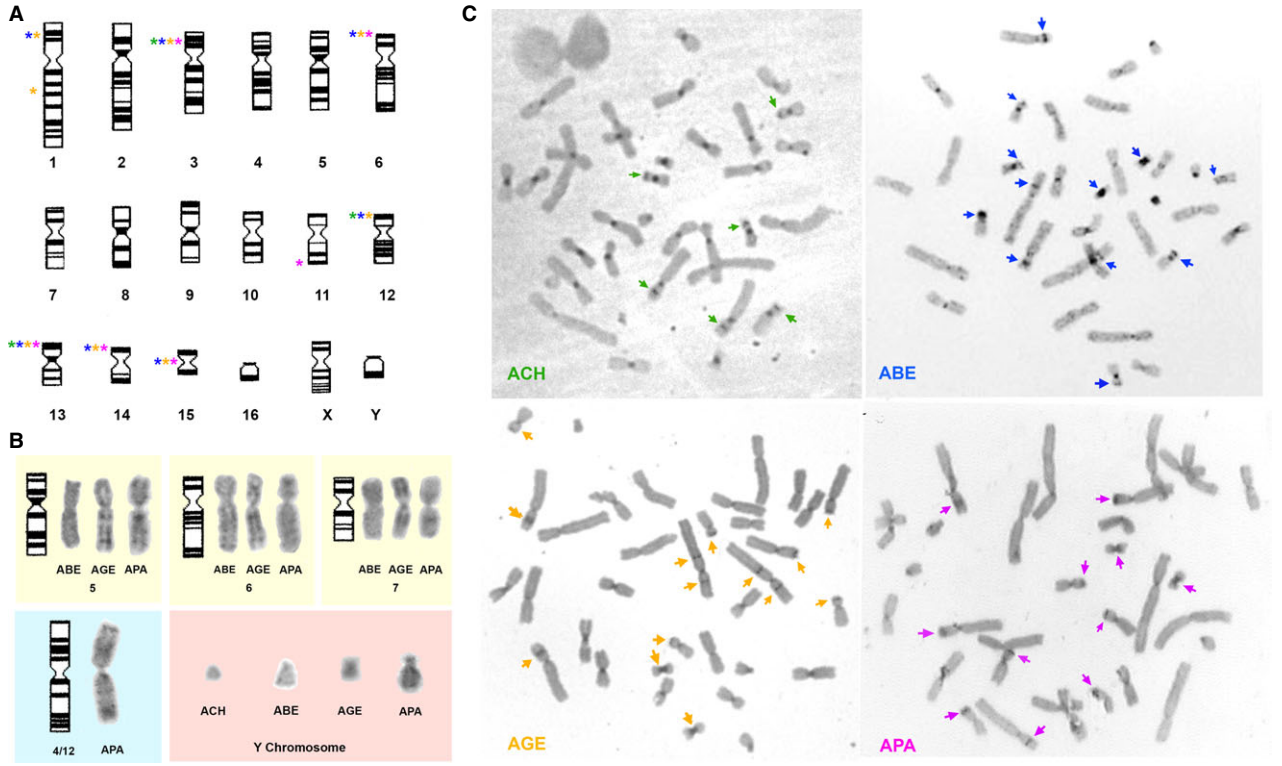

Figure 1. Chromosomal variability in *Ateles*. A, G-band ideogram of *Ateles chamek* and specific chromosomal location of C-band regions of ACH (green asterisks), ABE (blue asterisks), AGE (orange asterisk) and APA (pink asterisks). B, chromosomal variants found in *Ateles* due to inversions (yellow boxes), translocations (blue box) and changes in size and morphology of Y chromosome (red box). C, metaphases showing C-banding pattern of ACH (green arrows), ABE (blue arrows), AGE (orange arrows) and APA (pink arrows). Modified from Nieves *et al.* (2005).

Genome size

To assess genome size, we used Feulgen image analysis densitometry (Hardie *et al.*, 2002). Air-dried blood smears were prepared from each individual sampled and were stored in the dark prior to staining. The Feulgen reaction was run as described before in Hardie *et al.* (2002) including post-fixation overnight in MFA (85 methanol: 10 formalin: 5 acetic acid), hydrolysis in 5 N HCl for 2 h at room temperature, 2 h staining in freshly prepared Schiff reagent, and a series of metabisulfite and distilled water rinses. Representative Feulgen-stained nuclei from three species of monkeys are shown in Figure 2. A minimum of 30 lymphocyte nuclei was measured per individual sample and integrated optical densities were converted to genome size in picograms using erythrocytes of *Gallus domesticus* (1C = 1.25 pg) as the internal standard, which were stained in the same run as the unknowns.

For data analysis and comparison, a two-way nested analysis of variance (ANOVA) was run using InfoStat, 2013 version (InfoStat Group, FCA National University of Córdoba, Argentina). After measurements were done, species were arranged into groups according to their *C*-value. Comparisons by iCGH were made between each possible maximum and minimum *C*-value *Ateles* species pair.

Probe labelling, interspecies comparative genomic hybridization and FISH

Total genomic DNA for iCGH experiments was extracted (QIAGEN) from the blood samples and labelling was conducted following a modified nick translation protocol in the presence of FITC-dUTP or Rhodamine-dUTP (Table 1). Whole chromosome paints from each human chromosome (HSA WCP) (Coriell Institute, NJ, USA) were also labelled using the same nick translation protocol, in order to perform FISH experiments of synteny analysis in ACH chromosomes. Briefly, 1.5 μg of the template DNA were mixed with 1.3 μL of digestion mix [containing 10× polymerase buffer and DNase (0.05 U μL^{-1})] in a polymerase chain reaction (PCR) tube for DNA digestion. Incubation was performed for 10 min at 15 °C. The enzyme was inactivated at 90 °C for

Table 1. *Ateless* species, biological material and techniques applied on this work

Species	Individuals	Starting material	Genome size	Probe label	iCGH	FISH
ACH (*Ateles chamek*)	6♀ + 5♂	Fresh blood sample	This work	Rhodamine (red) & FITC (green)	This work ACHxAPA ACHxAGE	This work
APA (*A. paniscus*)	2♂	Frozen blood sample; fibroblast in culture	Manfredi Romanini (1972)	Rhodamine (red) & FITC (green)	This work APAxACH APAxABE	de Oliveira *et al.* (2005)
AGE (*A. geoffroyi*)	5♀ + 4♂	Fresh blood sample	This work; Manfredi Romanini (1972)	Rhodamine (red) & FITC (green)	This work AGExACH AGExABE	Morescalchi *et al.* (1997)
ABE (*A. belzebuth*)	1♂	Fresh blood sample	This work; Manfredi Romanini (1972)	Rhodamine (red)	This work ABExAGE	de Oliveira *et al.* (2005)

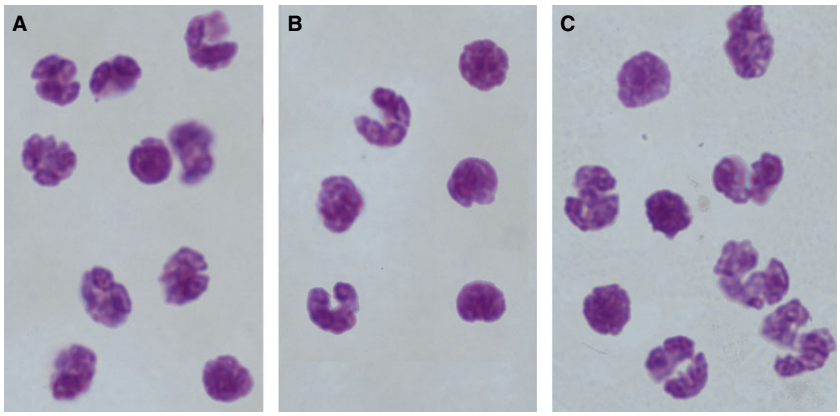

Figure 2. Representative Feulgen-stained lymphocyte nuclei from three species of monkeys. A, *Ateles geoffroyi* (1C = 3.19 pg), (B) *A. belzebuth* (1C = 2.88 pg), (C) *A. chamek* (1C = 3.09 pg).

10 min. For the labelling and polymerization phase, the digested DNA was mixed with polymerization mix [containing dNTP (20 nM), labelled nucleotide (10 μM), polymerase (1 U μL^{-1}) and Milli-Q (MQ) water]. Incubation was performed for 30 min at 37 °C. The enzyme was inactivated at 94 °C for 10 min.

Every iCGH or HSA WCP probe pair was mixed in 2.5-μL hybridization mixture containing 30% formamide, 30% polyethylene glycol, 10% 20× SSC, 28% NaI and 2% Tween. For iCGH, one hundred nanograms of each genomic probe were used in pairs (ACH vs. APA; ACH vs. AGE; APA vs. ABE, AGE vs. ABE) in each case. The probe mixture was denatured at 70 °C for 7 min. iCGH probes were then kept at 37 °C for 90 min for cohybridization. Freshly prepared slides with chromosomal spreads were pre-incubated 1 h at 37 °C, then submerged in 2× SSC 30 min and finally denatured in 0.1 M NaOH/70% ethanol at room temperature for 4 min, followed by dehydration in an ethanol series (70, 90 and 100% sequentially; 2 min each). Hybridization was conducted in a wet chamber at 45 °C over night. 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 analyzed with a Leica DMLB fluorescence microscope. Chromosome images were obtained with a Leica DFC 340 FX camera. A total of 15 metaphases were photographed for each FISH or iCGH experiment. Three images per metaphase were obtained using DAPI, FITC and

Rhodamine filters. Metaphases were arranged according to previously described karyotypes using Photoshop CS (Adobe). Image processing was performed with Image Pro-Plus 4.5 (Media Cybernetics Inc.).

In the iCGH experiments, for each chromosome a line profile of fluorescence intensity vs. chromosomal length was obtained. We calculated the red: green fluorescence intensity (r/g) ratio for each chromosomal pair of at least four metaphases from each CGH. Only if both genomes have the same DNA content in a particular region the ratio will be around one (r/g = 1). Cut-off values were empirically determined comparing ACH genomic probes hybridized with each other on its own chromosomes.

RESULTS

The estimated genome sizes of the *Ateles* species studied here were 3.09 ± 0.23 pg for *A.* chamek, 2.88 ± 0.06 pg for *A. belzebuth*, and 3.19 ± 0.24 pg for *A. geoffroyi* (Table 2). These values are significantly different from each other (two-way nested ANOVA between species, $P = 0.0034$). They also differed from previous estimates performed in the 1970s using older densitometric methods (Table 2). A relatively small amount of variability was recorded among individual samples within each species, which could reflect either real intraspecific differences (e.g. sex chromosomes and/or variability in repetitive DNA) and/or measurement error.

The results do not include data from APA because no fresh blood sample was available to perform the estimation. We decided to use the APA *C*-value from the literature in order to design the rest of the experiments. According to our results we arbitrarily divide the species into two groups – minimum and maximum genomes – based on a gradient from lowest to highest *C*-value, in order to perform the comparisons between species (Table 2).

Table 2. *Ateles C*-value from this and previous works

Species	*C*-value ± SD (pg)	Previous *C*-value (pg)	Group
ABE	2.88 ± 0.06**	3.63	MIN
ACH	3.09 ± 0.23**	N/A	MIN
AGE	3.19 ± 0.24**	3.25	MAX
APA	N/A	3.47	MAX

MIN, minimum genome size; MAX, maximum genome size.

Previous *C*-values were taken from Manfredi Romanini (1972).

**Significant *C*-values between species, two-way nested ANOVA, $P = 0.0034$. 'Group' refers to an arbitrary classification made for the purposes of the comparisons.

For the iCGH experiments we previously determined cut-off values using hybridization of the same ACH genome on itself. Results showed that chromosomal regions without quantitative differences display a red: green ratio between 1.4 and 0.8.

When comparing distinct *Ateles* genomes with each other we found that *C*-value differences between species are undoubtedly recognizable at the chromosomal level and lie on euchromatic regions and heterochromatic blocks. Both minimum *C*-value species, ACH and ABE, showed no specific regions of gain when compared to maximum *C*-value species, APA or AGE (Figs 3, 4A–C). On the contrary, both species with larger genome size showed relative gains in particular chromosomal regions, mostly heterochromatic regions but also euchromatic ones (red signals in Fig. 3; green signals in Fig. 4). Interestingly, we also found quantitative differences located in Y chromosomes of these *Ateles* species. In Figure 3B (arrow) chromosome Y on APA was highly marked with a red signal meaning a salient quantitative difference with ACH's Y chromosome. In AGExABE we observed an evident red signal on Y chromosome compatible with a relative DNA gain of ABE, despite ABE having a smaller genome size (Fig. 4A, B, arrow; Table 2). Also, in APAxABE we observed positive signals located on Y chromosome. In this case two distinct signals were very noticeable: a red one next to the centromere, where ABE genome has a relative DNA gain compared to APA; and a bigger green one, compatible with an APA relative DNA gain (Fig. 4C, arrow).

Each pairwise comparison between species with genome size differences led to particular results that are worth mentioning. When comparing ACH genome against APA, most of chromosomes showed a red:green ratio around 1, all of them compatible with no quantitative differences – no DNA gains or losses – between species. Also no chromosomal region with a red:green ratio < 0.8 (ACH relative DNA gain) was found (Fig. 3A, B). By contrast, conspicuous signals of APA gained DNA were revealed both on ACH and APA chromosomes. Specifically, on ACH metaphases positive signals with a red: green ratio higher than 1.4 localized on heterochromatic blocks in chromosomes 3 and 12, telomeric regions of chromosomes 7 and 16, and in the fully euchromatic chromosome 13 (Fig. 3A). Plus, on APA chromosomes, positive signals were also found on heterochromatic blocks in chromosomes 3, 6 and 11, nucleolar organizer region in chromosome 8 and telomeric regions of chromosomes 5, 13 and 16 (Fig. 3B). All these signals revealing a clear APA relative DNA gain in those regions. No gain or loss signal was detected in APA

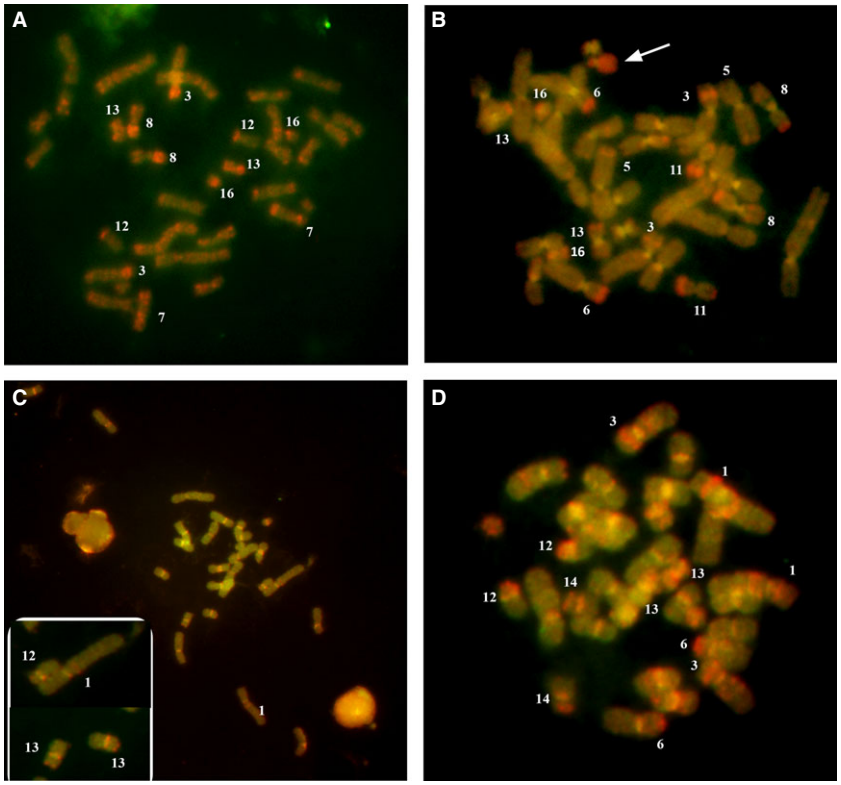

Figure 3. Interspecies comparative genomic hybridization between a minimum *C*-value genome (ACH) and two different ones maximum *C*-value genome (APA and AGE). A, ACH (FITC, green) vs. APA (Rhodamine, red) genomic probes hybridized onto ACH chromosomes. B, ACH (FITC, green) vs. APA (Rhodamine, red) genomic probes hybridized onto APA chromosomes. Arrow indicates Y chromosome. C, ACH (FITC, green) vs. AGE (Rhodamine, red) genomic probes hybridized onto ACH chromosomes (incomplete metaphase). Box detail shows chromosomes 1 and 12 (up) and pair 13 (down) (D). ACH (FITC, green) vs. AGE (Rhodamine, red) genomic probes hybridized onto AGE chromosomes. In each case numbers identifies chromosome pair.

rearranged chromosome 4/12. We also compare ACH genome against AGE, the other species with high *C*-value (Fig. 3C, D). We found similar results as in the comparison with APA. Briefly, we did not detect any region compatible with an ACH DNA excess and small red signals were found on ACH chromosomes 1, 12 and 13 (Fig. 3C, box) and in AGE chromosomes 1, 3, 6, 12, 13, 14 and Y evidencing a relative excessive amount of AGE DNA (Fig. 3D). Except for the signals on AGE Y chromosome and ACH chromosome 1, the signals detected on this comparison lay on heterochromatic regions.

When we compared the other species pair of minimum–maximum *C*-value, ABE and AGE, we found that AGE only showed DNA gains in heterochromatic regions compared to ABE (Fig. 4A, B). When the comparison between ABE and APA was made, almost the same pattern of signals was detected (Fig. 4C). Positive green signals ($r/g < 0.8$) of APA genomic probe where observed on heterochromatic regions in APA chromosomes 3, 6, 11, 13 and 14, and also in euchromatic regions in APA chromosomes 8 and 9. No signals were observed in APA rearranged chromosome 4/12.

In order to better characterize the chromosomal similarities and differences among these four species, we also analyzed the karyotype of ACH by chromosome painting using human WCP probes (Fig. 5A–F). All human sets of chromosome-specific probes hybridized to ACH metaphases except for the human Y chromosome. We could confirm the presence of important syntenies, even those located on chromosomal regions involved in regions of DNA gain unveiled by the iCGH such as human 15/22 in ACH chromosome 3, 2/16 in ACH chromosome 6, 19/20 in ACH chromosome 8, 2/10 in ACH chromosome 13, and human chromosomes, 12, 13 and 17 in ACH chromosomes 2, 12, and 14 respectively (Fig. 5A–F).

DISCUSSION

Most previous studies of *Ateles* genome have taken a qualitative perspective (Kunkel, Heltne &

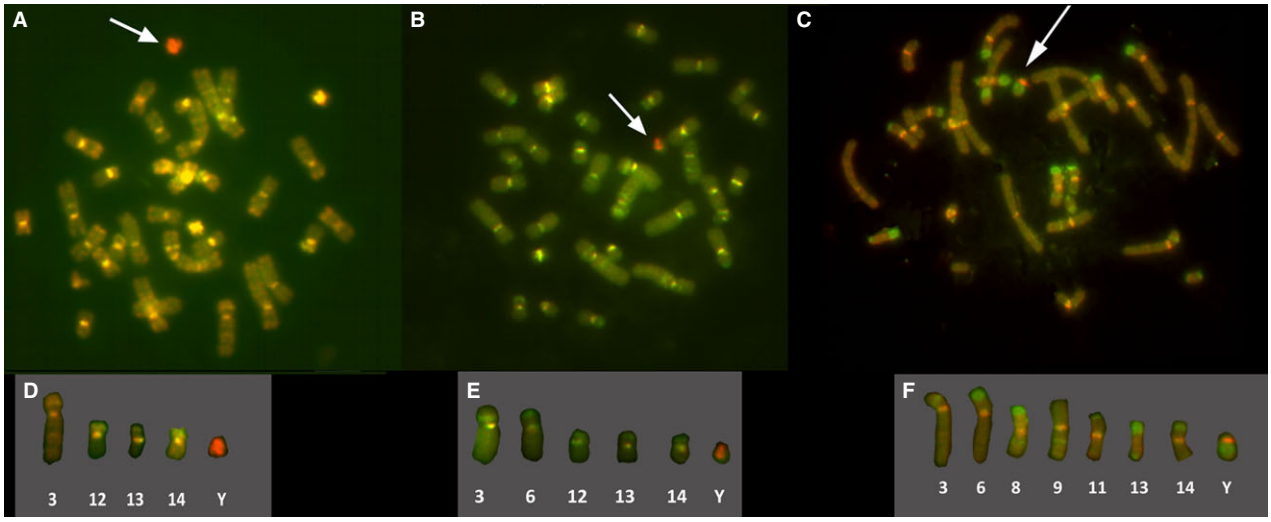

Figure 4. Interspecies comparatives genomic hybridization between a minimum *Ateles C*-value genome (ABE) and two different ones maximum *C*-value genomes (APA and AGE) (A–C); main signals in each metaphase show differences between genomes (D–F). A, AGE (FITC, green) vs. ABE (Rhodamine, red) genomic probes hybridized onto ABE chromosomes. B, AGE (FITC, green) vs. ABE (Rhodamine, red) genomic probes hybridized onto AGE chromosomes. C, APA (FITC, green) vs. ABE (Rhodamine, red) genomic probes hybridized onto APA chromosomes. D, ABE chromosomes 3, 12, 13, 14 and Y with positive signals. E, AGE chromosomes 3, 6, 12, 13, 14 and Y with positive signals. F, APA chromosomes 3, 6, 8, 9, 11, 13, 14 and Y with positive signals. Arrows show Y chromosome in each metaphase.

Borgaonkar, 1980; de Boer & de Bruijn, 1990; Herzog *et al.*, 1992; Seuánez, Alves & O'Brien, 1994; Medeiros *et al.*, 1997; Collins & Dubach, 2001; García *et al.*, 2002; Nieves *et al.*, 2005). Karyologically, the differences among species have been characterized by chromosomal rearrangements and their resulting variants (Fig. 1B). In the present work we have described new genomic similarities and differences between *Ateles* species, based in large part on quantitative properties.

The estimates of genome size presented here differ from those published more than 30–40 years ago by Manfredi Romanini (1972). There are a number of possible explanations for this discrepancy, including differences in measurement methods or mismatched species identifications. Indeed, many aspects of primate taxonomy have been updated since the early 1970s, and without voucher specimens it is impossible to confirm that the same species were analyzed in both studies. Some recent comparisons have shed light on the relationship between genome size and content across a broad scale (Elliott & Gregory, 2015a, b), but much work remains to be done in examining such patterns on the scale of closely related species (Redi & Capanna, 2012).

In the present study, the regions identified by iCGH were, in part, heterochromatic ones, and those species showing evident regions of relative DNA gain also had the highest *C*-values. The iCGH analysis carried out showed that the genome size differences are not only detectable at the chromosomal level, but lie in different regions of the genome, not only heterochromatic regions. Among species with small genomes, *A. chamek* showed no regions of DNA gain when compared to the larger genome species, *A. geoffroyi* and *A. paniscus*. Homology analysis by FISH of the karyotype of *A. chamek* showed no qualitative differences with the karyotypes of *A. belzebuth*, *A. geoffroyi* and *A. paniscus* (Morescalchi *et al.*, 1997; De Oliveira *et al.*, 2005) revealing that differentiation between the genomes of these species at the chromosomal level is mostly quantitative. Moreover, as shown by genomic comparisons, smaller *C*-value genomes like that of ACH seem to be completely included and represented in the large ones, indicating that larger genomes in *Ateles* result from the addition of particular DNA sequences rather than major changes in basic composition. There is, however, evidence that chromosomal rearrangements are relevant to genome evolution in *Ateles*, as the largest genome size difference is found between ACH and APA, both with noticeable differences in the presence of chromosomal rearrangements (variants of chromosomes 5, 6 and 7 and 4/12 chromosome fusion, Fig. 1) (Medeiros *et al.*, 1997; Nieves *et al.*, 2005) and at the extremes of total heterochromatin amount. These results agree with our hypothesis' predictions.

Conversely, the differences we observed in Y chromosomes did not always correspond to genome size differences between species. The Y chromosomes of

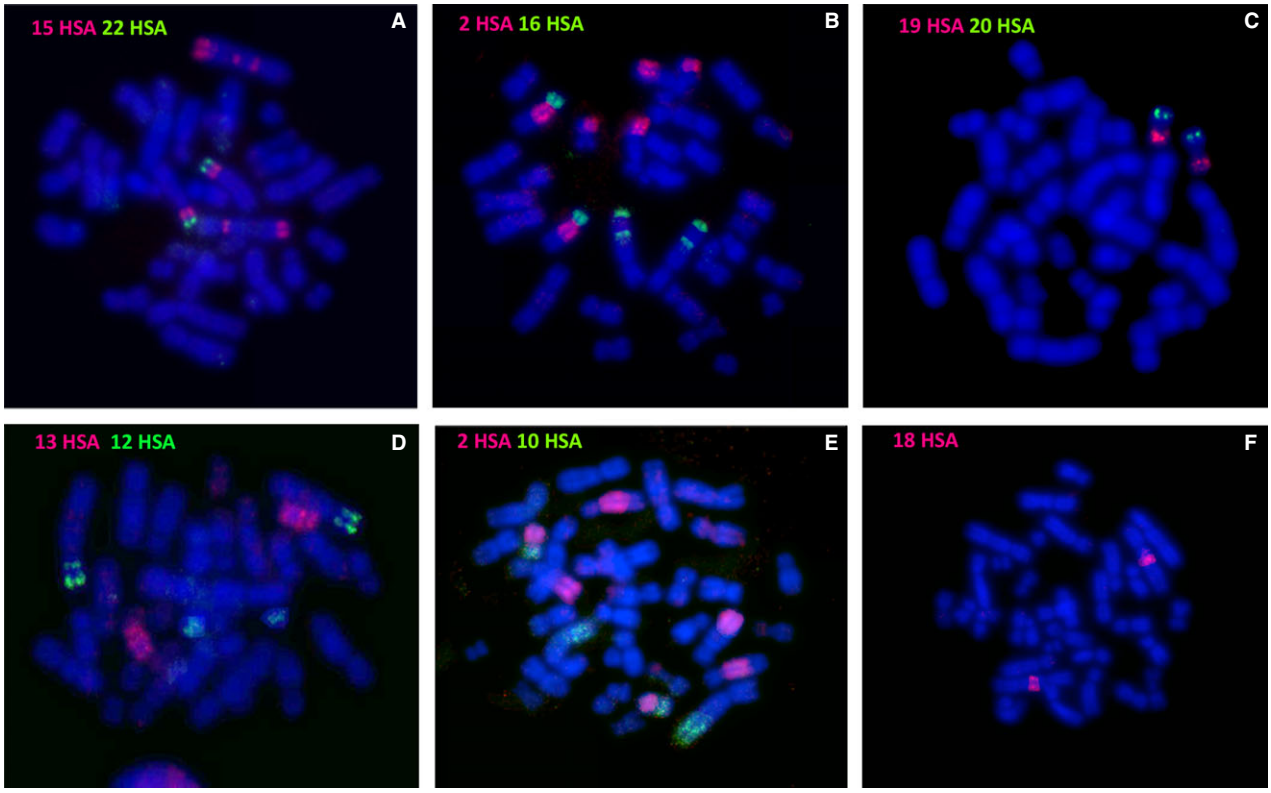

Figure 5. FISH using human whole chromosome painting probes on *A. chamek* chromosomes. (A) Hybridization signals of human 15 (red) and 22 (green) probes in *A. chamek* chromosome arm 3p (synteny) and chromosome 2 pericentromeric and q terminal region (15 HSA, red). (B) Hybridization signals of human 2 (red) and 16 (green) probes in *A. chamek* chromosomes 6 (synteny), 1 (16 HSA, green), 3 and 13 (2 HSA, red). (C) Hybridization signals of human 19 (red) and 20 (green) probes in *A. chamek* chromosome 8. (D) Hybridization signals of human 13 (red) probe in *A. chamek* chromosome 12; and human 12 (green) probe in *A. chamek* chromosomes 2p and 16. (E) Hybridization signals of human 2 (red) and 10 (green) probes in *A. chamek* chromosome 13 (synteny), 1 (10 HSA, green), 3 and 6 (2 HSA, red). (F) Hybridization signal of human 18 (red) probe in *A. chamek* chromosome 1.

Old World Primates are often highly heterochromatic, have a large amount of repetitive and ampliconic DNA, and harbour almost no genes, making them notoriously difficult to sequence and analyze (Bachtrog, 2013). In platyrrhine monkeys, the Y chromosome is very peculiar, showing polymorphisms in size and morphology, autosomal translocations, and an absence of homology with great apes' Y chromosome (Solari, 1994; Mudry *et al.*, 1998). So, our work represents an interesting approach to its understanding. A notable qualitative polymorphism in Y chromosomes has been described in *Ateles* karyotypes before, although none of them involves heterochromatin (Nieves *et al.*, 2005 and references therein). Taking into account our results, most of the variation found on *Ateles* Y chromosomes seems to include only changes in size and morphology rather than in composition (Fig. 1, red box), which would explain the remarkably high fluorescence intensity observed in the Y chromosome. In this regard, Gifalli-Iughetti & Koiffmann (2009) showed by chromosome microdissection that *Ateles* Y chromosomes are homologous to that of *Brachyteles arachnoides,* another species from the family Atelidae. The authors suggested that *Ateles* Y chromosomes appear to contain additional DNA as a consequence of the chromosome rearrangements experienced by this genus, an idea similar to our hypothesis about the entire genome. For the moment, the composition of the DNA gained on the Y chromosome remains unclear.

Some chromosomal regions identified by iCGH showed evidence that qualitative and quantitative differences might be correlated. For example pair 6, which presents different chromosomal variants and heterochromatin amount in each species, or pair 12, involved in a chromosomal fusion, show a differential amount of DNA in most of the comparisons we have made. A possible explanation for these differences, although speculative, may relate to lineage-specific

expansion of transposable elements, which in turn could be related to species-specific genomic rearrangements (Kidwell & Lisch, 2002; Böhne *et al.*, 2008). Future work should tell us more in this regard.

In this work we also have found quantitative differences in particular euchromatic areas: Y chromosome, nucleolar organizer region, APA chromosomes 5, 8, 9, 16, and ACH chromosomes 13 and 1. Specifically, the peculiarities of the APA genome show that this is a species with an accumulation of chromosomal autapomorphies with a counterpart in genome size. Some of these euchromatic regions involved in genome size variation – as for example pairs 8 and 9 of APA – also appeared to be genetically unstable. In a preliminary study using a sister chromatid exchange technique, APA showed a remarkably high number of exchanges in those regions (M. Nieves, unpubl. data). Genomic quantitative differences in euchromatic regions have been observed in the comparative genomic analysis between other species, although they are few records. One of the most relevant examples for the purposes of our study is the comparison with that between the genomes of *C. nigritus* and *C. cay* (Nieves *et al.*, 2010). According to it, DNA differences involve different genomic regions, being preferentially repetitive type in *C. cay* and repetitive coding or dispersed in *C. nigritus*. Another example is the comparison between the genome of the dog (Carnivora, *Canis lupus*) and the two-toed sloth Linnaeus (Xenarthra, *Choloepus didactylus*) on the chromosomes of the Taiwan pangolin (Pholidota, *Manis pentadactyla pentadactyla*) (Yu *et al.*, 2012). The pangolin genome was more similar to that of the dog than to the sloth, implying a closer genetic relationship between Pholidota and Carnivora than between Pholidota and Xenarthra. Since the iCGH technique is genome-wide, has chromosome-level resolution, and does not need full genome sequencing, it has considerable potential in systematics and other fields, including conservation biology.

Finally, it is almost obvious to think that the effect of repetitive DNA in genome size is manifested through the state of condensation of chromatin whose extreme case is heterochromatin. However, estimating the amount and degree of chromatin condensation is complex. An approach of this kind was made by Vinogradov (2005) by analyzing the genomes of different species of vertebrates under an adaptive paradigm for the variation of genome size. He suggested that the degree of chromatin condensation serve to the fine adjustment of the nucleoskeletal and/or buffer function of DNA. Conclusions such as this are only affordable in studies that conduct analysis of a large number of species, rather than a single genus with several species from a single Order as in this work. However the analysis of the variables analyzed here is one of the first focused on Neotropical primates with a significant proportion of heterochromatin in the karyotype, and perhaps serve as a continuation of an issue that was addressed in other species.

In conclusion, the present work has shown that spider monkeys exhibit karyological features of significant interest in the study of genome evolution. By examining closely related congeners, it is possible to investigate the factors that account for differences in genome size on a small scale. In the case of *Ateles* monkeys, these differences relate to more than just heterochromatin gain or loss, but do not appear to be associated with major changes in composition. Future work could expand on this study to explore other platyrrhine monkeys that exhibit similar chromosomal properties, thereby providing further insights into primate genome structure and evolution.

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