

Comparative Molecular Cytogenetics and Chromosome Evolution

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Genome Research**Phylogenetic inferences of Atelinae (Platyrrhini) based on multi-directional chromosome painting in *Brachyteles arachnoides*, *Ateles paniscus paniscus* and *Ateles b. marginatus***E.H.C. de Oliveira,^{a,b,c,*} M. Neusser,^{a,*} J.C. Pieczarka,^b C. Nagamachi,^b I.J. Sbalqueiro^c and S. Müller^a^aInstitut für Anthropologie und Humangenetik, Department Biologie II, Ludwig-Maximilians-Universität, Munich (Germany);^bLaboratório de Citogenética, Depto de Genética, Universidade Federal do Pará, Belém, PA (Brazil);^cLaboratório de Citogenética Animal, Depto de Genética, Universidade Federal do Paraná, Curitiba, PR (Brazil)

Abstract. We performed multi-directional chromosome painting in a comparative cytogenetic study of the three Atelinae species *Brachyteles arachnoides*, *Ateles paniscus paniscus* and *Ateles belzebuth marginatus*, in order to reconstruct phylogenetic relationships within this Platyrrhini subfamily. Comparative chromosome maps between these species were established by multi-color fluorescence in situ hybridization (FISH) employing human, *Saguinus oedipus* and *Lagothrix lagothricha* chromosome-specific probes. The three species included in this study and four previously analyzed species from all four Atelinae genera were subjected to a phylogenetic analysis on the

basis of a data matrix comprised of 82 discrete chromosome characters. The results confirmed that Atelinae represent a monophyletic clade with a putative ancestral karyotype of $2n = 62$ chromosomes. Phylogenetic analysis revealed an evolutionary branching sequence {*Alouatta* {*Brachyteles* {*Lagothrix* and *Ateles*}}} in Atelinae and {*Ateles belzebuth marginatus* {*Ateles paniscus paniscus* {*Ateles belzebuth hybridus* and *Ateles geoffroyi*}}} in genus *Ateles*. The chromosomal data support a re-evaluation of the taxonomic status of *Ateles b. hybridus*.

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The subfamily Atelinae (Atelidae, Platyrrhini) comprises the four genera *Brachyteles* (woolly spider monkeys), *Lagothrix* (woolly monkeys), *Ateles* (spider monkeys) and *Alouatta* (howler monkeys) and is accepted as a monophyletic clade by most authors. The relationship of *Brachyteles* with the other genera of Atelinae (Atelidae, primates) is still controversial; however it is considered to be the sister group of *Lagothrix* in most of the recently proposed phylogenies based on molecular data (Schneider et al., 1993; Canavez et al., 1999; Meireles et al., 1999; von Dornun and Ruvolo, 1999), as well as G-banded chromosome analysis (Péquignot et al., 1985). DNA sequence

analysis grouped *Brachyteles* and *Lagothrix* closest to each other, followed by *Ateles* and finally *Alouatta* (Schneider et al., 1993, 1996; Harada et al., 1995; Porter et al., 1997a, b; Meireles et al., 1999). Morphological studies have led to a variety of different arrangements of the four Atelinae genera. Ford (1986) presented a trichotomy involving *Ateles*, *Brachyteles* and *Lagothrix*, with *Alouatta* as sister group to this clade, while Rosenberger et al. (1992) grouped *Brachyteles* and *Ateles* closest to each other, followed by *Lagothrix* and then *Alouatta*. Kay (1990) identified two sister subclades, one formed by *Alouatta* and *Brachyteles* and the second grouping *Lagothrix* and *Ateles*.

The taxonomy within genus *Ateles* is not yet fully resolved. According to Froehlich et al. (1991) the genus is comprised of three species and 8 subspecies, whereas Collins and Dubach (2000) listed four species (Table 1). The latter investigators per-

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Table 1. Taxa of the genus *Ateles* recognized by Collins and Dubach (2000)

Species	2n	G-banding/chr. nomenclature	Cross species FISH
<i>A. paniscus paniscus</i>	32	Pieczarka et al (1989)	this study
<i>A. belzebuth belzebuth</i>	34	Medeiros et al. (1997)	---
<i>A. belzebuth marginatus</i>	34	Medeiros et al. (1997)	this study
<i>A. belzebuth chamek</i> ^a	34	Medeiros et al. (1997)	---
<i>A. hybridus</i> ^b	34	Medeiros et al. (1997)	Garcia et al. (2002)
<i>A. geoffroyi</i>	34	Kunkel et al. (1980)	Morescalchi et al. (1997)

^a Formerly classified as *A. paniscus chamek* (Kellogg and Goldman, 1944).
^b Formerly classified as *A. belzebuth hybridus* (Froehlich et al., 1991).

formed mitochondrial COII and D-loop sequence comparison and placed *A. p. paniscus* as the most basal, followed by *A. b. hybridus* with *A. b. marginatus* and *A. geoffroyi* as the last to diverge.

The genera *Brachyteles* and *Lagothrix* share the highest diploid chromosome number found among New World monkeys (2n = 62; Dutrillaux et al., 1980; Péquignot et al., 1985). Based on R-banding patterns of these genera, Péquignot et al. (1985) suggested that the karyotype of *Brachyteles* is more ancestral compared to that of *Lagothrix*. According to these investigators, the differences observed between their karyotypes are due to the occurrence of three pericentric inversions or centromeric shifts, a reciprocal translocation or insertion and the presence of additional heterochromatin segments in *Lagothrix*. Dutrillaux et al. (1980) placed *Lagothrix* as the sister group of *Ateles* in a study that included these two genera and *Brachyteles*, but no species of genus *Alouatta*. In contrast to *Brachyteles* and *Lagothrix*, *Ateles* has one of the lowest chromosome numbers among Platyrrhini monkeys, which ranges from 32 in *A. p. paniscus* to 34 in all the other analyzed *Ateles* species (Kunkel et al., 1980; de Boer and Bruijn, 1990; Medeiros et al., 1997) (Table 1). Based on banding patterns, Medeiros et al. (1997) proposed a phylogeny involving the different species of *Ateles*, placing the karyotype of *A. p. chamek* as the most basal, followed by *A. p. paniscus*, and *A. geoffroyi* as the most derived. In addition, the investigators made assumptions about the biogeography and radiation of the group, suggesting that speciation in *Ateles* was strongly influenced by the quaternary refuge formation. The results of the analysis of mitochondrial DNA sequence variation (Collins and Dubach, 2000), however, placed *A. p. paniscus* as the most basal species of this genus.

In recent years, some Atelinae species were analyzed with human chromosome painting probes. These included *Lagothrix lagothricha* (Stanyon et al., 2001), *Ateles geoffroyi* (Morescalchi et al., 1997), *Ateles belzebuth hybridus* (Garcia et al., 2002) and five *Alouatta* species (Consigliere et al., 1996, 1998; de Oliveira et al., 2002). The findings showed that the majority of associations of human homologous segments found in Neotropical primates so far are also observed in Atelinae: 3a/21, 5a/7a, 10a/16a, 8a/18, 2b/16b, 14/15a₁. In addition, Atelinae exclusively share derived fissions of chromosome 1, 4, 5 and 15 homologs that resulted in chromosome forms 1a₁, 1a₂, 4a, association of 4b/15a₂, 4c, inversion 7a/5a/7a and 5b, indicating that this group is of monophyletic origin.

With the aim of reconstructing the phylogenetic relationship between the four Atelinae genera and to obtain a deeper

insight into chromosome evolution in genus *Ateles*, we analyzed the karyotypes of *B. arachnoides*, *Ateles p. paniscus* and *Ateles b. marginatus* by multi-directional chromosome painting, employing human, *Saguinus oedipus* and *L. lagothricha* chromosome-specific probes. The integration of our results with previous publications on Atelinae comparative molecular cytogenetics allowed us to use chromosomal data in a phylogenetic analysis comprising all the genera of Atelinae, as well as to propose a chromosomal phylogeny for Atelinae including four *Ateles* species.

Materials and methods

Cell samples

Chromosome preparations for in situ hybridization experiments were obtained from whole-blood cultures of one individual of *B. arachnoides* (BAR), kept at Centro de Primatologia do Rio de Janeiro (Rio de Janeiro, Brazil), one male and one female *L. lagothricha*, one female *A. b. marginatus*, kept at Centro Nacional de Primatas (Ananindeua, PA, Brazil) and one individual of *A. p. paniscus*, kept at Parque Zoológico do Museu Emilio Goeldi (Belém, PA, Brazil). The diploid numbers found in all the specimens were as described before (Koiffmann, 1977; Dutrillaux et al., 1980; Péquignot et al., 1985; Medeiros et al., 1997).

G-banding, probe composition, in situ hybridization and detection

G-banding was performed according to Garcia et al. (2000). Human and New World monkey chromosome-specific painting probes were previously described in Stanyon et al. (2001) and Müller et al. (2001), respectively. Multi-color probe sets H1–H4 comprised all 24 human and S1–S4 all 24 *S. oedipus* chromosome-specific probes. Probe set L1 was composed of those *L. lagothricha* chromosome paint probes that provided more detailed subchromosomal information than those derived from *S. oedipus* homologs (Neusser et al., 2001, de Oliveira et al., 2002). All probe sets were labeled by DOP-PCR in Boolean combinations in the presence of Biotin-dUTP, Digoxigenin-dUTP (Roche) and TAMRA-dUTP (Applied Biosystems/PE). Hybridization in situ and detection were carried out exactly as described in Neusser et al. (2001) and de Oliveira et al. (2002).

Microscopy and image analysis

Metaphases were analyzed with a cooled CCD camera (Photometrics C250/A equipped with a KAF1400 chip) coupled to a Zeiss Axiophot microscope. Merging of chromosome images was performed using the software Adobe Photoshop version 3.0. Chromosomes were identified by computer-enhanced DAPI banding (SmartCapture VP 1.4, Digital Scientific, Cambridge, UK).

Maximum parsimony analysis (PAUP)

Table 1 summarizes the *Ateles* species included in this phylogenetic analysis. A data matrix was established (Table 2 and 3), based on the presence or absence of discrete chromosomal homology characters in the three species *B. arachnoides*, *A. p. paniscus* and *A. b. marginatus*, included in our study, in four previously published Atelinae species and in the phylogenetic outgroup *Cebus apella*. These characters (1) comprised 76 conserved syntenic seg-

Table 2. Summary of characters (homologous chromosomes and syntenic association of homologous chromosome segments) that were used to assemble the binary data matrix (i = inversion, f = fission)

1. 1a ₁	2. 1a ₁ /1a ₂	3. 1a ₁ /1c	4. 1a ₁ /4b	5. 1a ₁ /6	6. 1a ₁ /14
7. 1a ₂	8. 1a ₂ /5b	9. 1a ₂ /7b	10. 1b	11. 1b/2b	12. 1c
13. 1c/1b	14. 1c/6	15. 1c/6 i	16. 2a	17. 2a/2b	18. 2a/3b
19. 2a/10b	20. 2a/20	21. 2b	22. (2b/16b) ₁	23. (2b/16b) f	24. (2b/16b) ₂
25. 3a/3b	26. 3a/6	27. 3a/21	28. 3b	29. 3b/3c	30. (3b/15b)
31. 3c	32. 3c/7b	33. 3c/13b	34. 3c/15b	35. 4a	36. 4a/4b
37. 4b	38. 4b/4c	39. 4b/15a ₂	40. 4c	41. 4c/7a	42. 4c i
43. 4c/16b	44. 5a/5b	45. 5a/7a	46. (5a/7a) _i	47. 5b i	48. 5b
49. 5b/7a	50. 5b/8b	51. 5b/12	52. 7a/7b	53. 7b	54. 8a/8b
55. 8a/16a	56. 8a/18	57. 8b i	58. 8b	59. 8b/12	60. 9/18
61. 9/22	62. 10a/10b	63. (10a/16a) ₁	64. (10a/16a) i	65. 10b	66. 12 i
67. 12/15a ₁	68. 13 i	69. 14	70. 14/15a ₁	71. 15a ₁ /15a ₂	72. 15a ₁ /15b
73. 15a ₂ /15b	74. 15b	75. 15b/22	76. 16a/16b	77. 16b	78. 18
79. 19/20	80. 20 i	81. 21	82. Y/15b		

Bold characters were derived by comparative G-banding analysis. The nomenclature of chromosome segments is following Neusser et al. (2001) and de Oliveira et al. (2002).

ments detectable by chromosome painting and associations thereof and (2) six characters obtained by G-banding pattern comparison (Table 2). The character nomenclature for human homologous segments detectable with human, *S. oedipus* and *L. lagothericha* probes paint probes followed Neusser et al. (2001) and de Oliveira et al. (2002): 1a₁ (1p21→pter), 1a₂ (1p12→p21), 1c (q21→q31), 1b (q32→qter), 2a (q12→qter), 2b (pter→q12), 3b (p24→pter, p12→p21, q12→q13, q27→qter), 3c (p21→p24, q13→q26), 3a (p12), 4a (4q31.3→qter), 4b (4q23→q31.2), 4c (4pter→q22), 5a (5q31.3→qter), 5b (5pter→q31.2), 6, 7a (p22, q11, q21), 7b (p11→p21, q11→q21, q22→qter), 8a (8p), 8b (8q), 9, 10a (10q), 10b (10p), 11, 12, 13a (q14→qter), 13b (q11→q13), 14, 15a₁ (q11→q24), 15a₂ (15q13→q21.2), 15b (q11→13, q25→qter), 16a (16p), 16b (16q), 17, 18, 19, 20, 21, 22, X and Y.

The data was subjected to maximum parsimony analysis (PAUP 4.0 software; Phylogenetic Analysis Using Parsimony, Swofford 1998), using the exhaustive search option. All characters had the same weight, based on the premise that chromosome rearrangements occur by equal chance. The relative stability of nodes was assessed by bootstrap estimates based on 1000 iterations. Each bootstrap replicate involved a heuristic parsimony search with 10 random taxon additions and tree-bisection-reconnection branch swapping. Two different PAUP analyses were performed: one of them including only characters based on FISH data, while the second included characters derived both by FISH and G-banding comparison.

Results

All human, *S. oedipus* and *L. lagothericha* sets of chromosome-specific probes delivered reproducible results when hybridized to metaphases of *B. arachnoides* (BAR), *A. p. paniscus* (APP) and *A. b. marginatus* (ABM), except for the human Y-chromosome probe. Centromeric and heterochromatic chromosome regions were not hybridized by any of the paint probes, except for *L. lagothericha* chromosome-specific probes in *B. arachnoides*, where centromeric regions were weakly hybridized. Chromosome identification was performed on the basis of DAPI banding. In each experiment a minimum of 10 cells were examined. Figure 1A–F illustrates representative FISH experiments with these probe sets.

Ateles belzebuth marginatus

The diploid number of *A. b. marginatus* was 34, with a submetacentric X chromosome. Figure 2A illustrates the G banding patterns, following the chromosome nomenclature proposed by Medeiros et al. (1997), together with the homology of

human and *S. oedipus* chromosomes. *S. oedipus* chromosome specific probes produced 44 signals: Only chromosome 11 was entirely conserved between *S. oedipus* and *A. b. marginatus*. All other chromosomes were rearranged and showed associations of *S. oedipus* homologs 1/3/15, 12/11/16/7/11, 1/9/17/9, 19/13/16, 6/18, 10/20, 16/8/21, 5/2, 7/6, 4/8, 2/1 and 22/9.

Human painting probes produced 52 signals, with only three conserved syntenies on separate chromosomes: HSA 11, 13, 17 and X. Human chromosome 9 also remained conserved but was involved in a Robertsonian fusion. Association of human homologous segments 9/18/8a/16a/10a/16a, 12/15a₁/14/1a₁/4b/15a₂, 22/15b/3b/2a, 3c/7b/1a₂, 5b/8b, 16b/2b/1b, 1a₁/6/1c, 19/20, 4c/7a/5a/7a, 21/3a/6 and 10b/2a were observed.

Ateles paniscus paniscus

Hybridization of human, *S. oedipus* and *L. lagothericha* chromosome-specific probes to *A. p. paniscus* chromosomes (2n = 32, the chromosome nomenclature follows Pieczarka et al., 1989) revealed identical hybridization patterns for most chromosomes. Compared to *A. b. marginatus*, *A. p. paniscus* chromosome 4 showed association of human homologs 13a/13b/3c/7b/1a₂ and can be derived from *A. b. marginatus* chromosomes 4 and 12 by a tandem fusion (Fig. 2B). *A. p. paniscus* chromosome 7 (association 1a₁/6/1c/6/1c) differs from *A. b. marginatus* chromosome 7 by a pericentric inversion (Fig. 2C). A detailed comparative G-banding analysis between both *Ateles* species revealed no further intra-chromosomal rearrangements.

Brachyteles arachnoides

The G-banded karyotype of *B. arachnoides* with the homology maps between this species, human, *S. oedipus* and *L. lagothericha* chromosomes is shown in Fig. 2D. Chromosomes were ordered according to the nomenclature proposed by Péquignot et al. (1985). Human painting probes revealed 41 conserved homologous segments. *S. oedipus* probes produced 34 signals: Sixteen autosomal syntenic groups (5, 7–13, 15–19, 21 and 22), as well as the X and Y chromosome are conserved in *B. arachnoides*. The remaining chromosomes were homologous to more than one chromosome of *Brachyteles*. The *S. oedipus* Y chromosome specific probe weakly hybridized to the Y chromo-

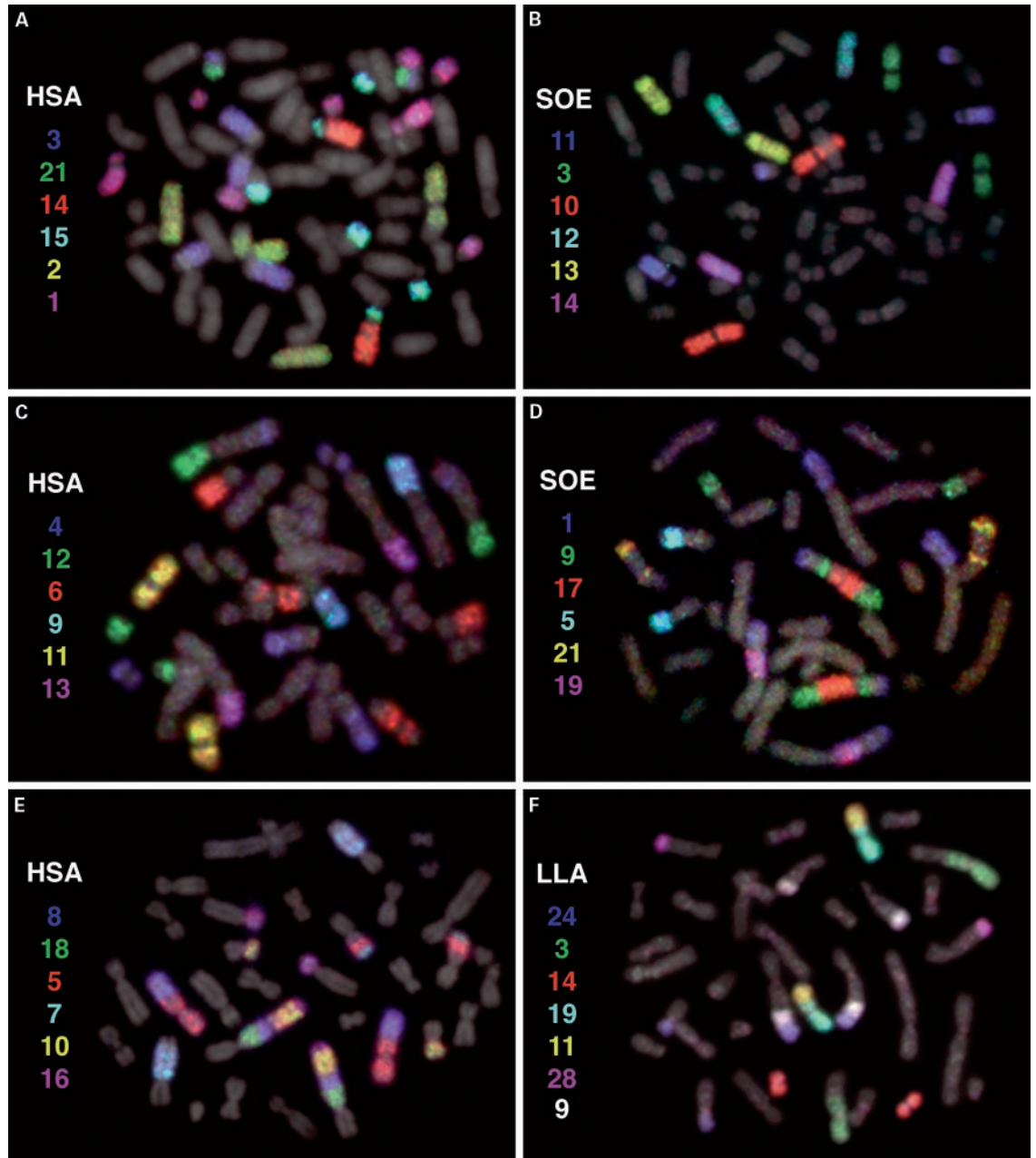


Fig. 1. Representative multi-color FISH experiments with human (HSA), *S. oedipus* (SOE) and *L. lagothericha* (LLA) painting probe sets to **(A)** and **(B)** *B. arachnoides*, **(C)** and **(D)** *A. p. paniscus*, **(E)** and **(F)** *A. belzebuth marginatus* metaphases. To visualize the hybridization pattern and chromosomal counter stain simultaneously, an overlay of both images is shown. Beside each metaphase the respective probe composition and false color assignment is given.

some of *Brachyteles*, confirming that this chromosome is the smallest of the complement.

Human homologous syntenic groups corresponding to chromosome pairs 6, 9, 11, 12, 13, 17, 19, 20, 22 were conserved as separate chromosomes in *B. arachnoides*. The associations of human 2b/16b, 10a/16a, 8a/18, 5/7a, 14/15a and 3a/21 homologs frequently observed in New World monkeys were present. Furthermore, as observed in *L. lagothericha* and genus *Alouatta*, human chromosomes 1 and 4 are fragmented in four and three

different chromosome segments, respectively, the chromosome 4b homolog being translocated to chromosome 15a homologous material. The hybridization results were identical to those previously obtained in *L. lagothericha* (Stanyon et al., 2001), with the exception of an inversion leading to a chromosome form homologous to human chromosomes 10/16/10/16. Reinvestigation of *L. lagothericha* with the respective human probes, however, revealed the presence of this inversion also in the *L. lagothericha* individual studied (data not shown).

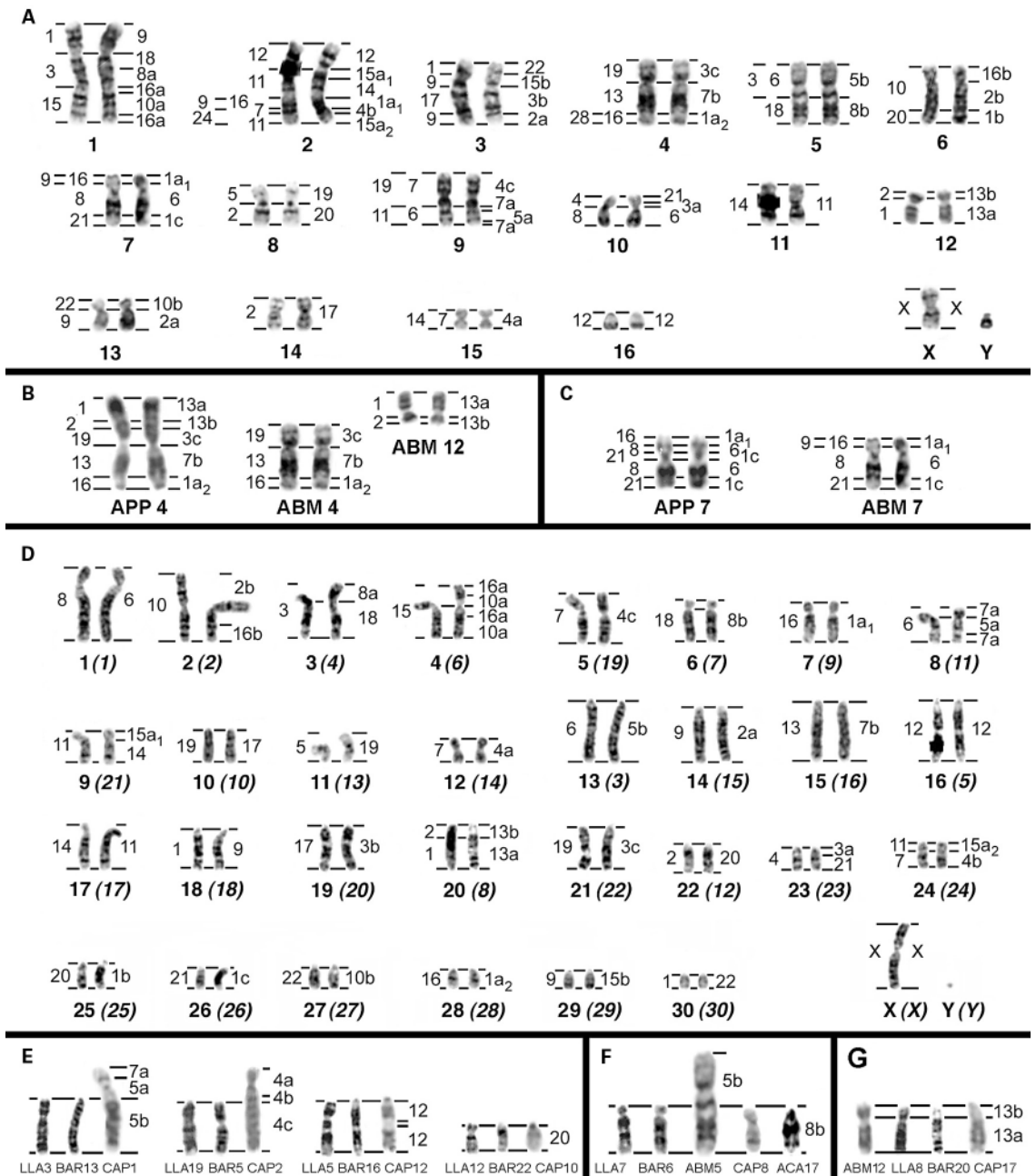


Fig. 2. (A) G-banded karyotype of *A. belzebuth marginatus* together with the assignment of human, *S. oedipus* and *L. lagothericha* chromosome-specific painting probes. Chromosome numbering (below each chromosome) follows citations given in Table 1. Numbers beside chromosomes indicate human (right), *S. oedipus* (left) and *L. lagothericha* (far left) homologous chromosome regions, horizontal bars the borders of homologous regions. **(B)** and **(C)** Chromosomal differences between *A. belzebuth marginatus* (ABM) and *A. p. paniscus* (APP) detected by chromosome painting: **(B)** a tandem fusion and **(C)** a pericentric inversion. **(D)** G-banded karyotype of *B. arachnoides*,

together with the assignment of human (right) and *S. oedipus* (left) homologous chromosome regions. Below each chromosome the numbering proposed by Pequignot et al. (1985) and in brackets the chromosome homology with *L. lagothericha*, following the nomenclature used by Stanyon et al. (2001) is given. **(E–G)** Chromosomal differences between *B. arachnoides* (BAR), *L. lagothericha* (LLA), *A. belzebuth marginatus* (ABM), *Alouatta caraya* (ACA) and *Cebus apella* (CAP) detected by G-banding, which were informative for the reconstruction of chromosomal phylogenies (see text for details).

Comparative G-banding analysis

Comparative G-banding analysis (Fig. 2E–G) revealed five intra-chromosomal rearrangements between *B. arachnoides* and *L. lagothericha* not detectable by chromosome painting. A comparison with chromosome forms found in *C. apella*, *Alouatta caraya* and *Ateles b. marginatus* that were used as out-

group species to determine ancestral states, was performed. The results indicated that *B. arachnoides* chromosomes 13, 16 and 22 conserved Atelinae ancestral chromosome forms, from which *L. lagothericha* homologs may be derived by pericentric inversions, whereas *B. arachnoides* chromosome 5 showed a derived inversion (Fig. 2E). Furthermore, *B. arachnoides* chro-

Table 3. Binary character matrix used in the parsimony analysis, comprising 82 characters (0 = absent, 1 = present)

char	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36c	37	38	39	40	41
CAP	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0	1	1	1	1	0	1	0
BAR	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0	1	0	1	0	1	1	0
LLA	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0	1	0	1	0	1	1	0
AGE	1	0	0	1	1	1	1	0	1	1	1	1	0	1	1	1	0	1	1	0	1	1	0	1	0	1	1	1	0	1	1	1	0	0	1	0	1	0	1	1	1
APP	1	0	0	1	1	1	1	0	1	1	1	1	0	1	1	1	0	1	1	0	1	1	0	0	0	1	1	1	0	1	1	1	1	0	1	0	1	0	1	1	1
ABM	1	0	0	1	1	1	1	0	1	1	1	1	0	1	0	1	0	1	1	0	1	1	0	0	0	1	1	1	0	1	1	1	0	0	1	0	1	0	1	1	1
ABH	1	0	0	1	1	1	1	0	1	1	1	1	0	1	1	1	0	1	1	0	1	1	0	1	0	1	1	1	0	1	1	1	0	0	1	0	1	0	1	1	1
ACA	1	0	0	0	0	0	1	1	0	1	0	1	0	0	0	1	0	0	0	1	1	1	1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	0	1	1	0
HSA	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0

char	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82
CAP	0	0	1	1	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0
BAR	1	0	0	1	1	0	1	0	0	0	1	0	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0
LLA	0	0	0	1	1	1	1	0	0	0	0	1	0	0	1	1	1	0	0	0	0	1	1	1	1	0	1	0	1	0	0	0	1	0	0	1	0	0	1	0	0
AGE	0	0	0	1	1	0	1	0	1	1	0	1	0	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	1	0	0	0	1	1	0	1	0	1	0	0	
APP	0	0	0	1	1	0	1	0	1	0	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	1	1	0	1	0	0	0	1	1	0	1	0	1	0	1	
ABM	0	0	0	1	1	0	1	0	1	0	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	1	1	0	1	0	0	0	1	1	0	1	0	1	0	1	
ABH	0	0	0	1	1	0	1	0	1	0	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	1	1	0	1	0	0	0	1	1	0	1	0	1	0	0	
ACA	0	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1	0	0	1	0	1	1	1	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	
HSA	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	0	0	1	0	1	0	0	

Bold characters were derived by comparative G-banding analysis.

mosome 6 and its *L. lagothericha* homolog appear to share an inversion with *A. b. marginatus* chromosome 5 not observed in *C. apella* or *A. caraya* (Fig. 2F). Finally, *B. arachnoides* chromosome 20 and its *C. apella* homolog probably share the acrocentric chromosome form, from which the submetacentric homologs of *L. lagothericha* and *A. b. marginatus* are derived by an inversion (Fig. 2G).

Phylogenetic analysis

In a first analysis, homologous chromosomes and chromosome segments were identified by multi-directional chromosome painting in seven Atelinae species (four *Ateles* species, *B. arachnoides*, *L. lagothericha* and *A. caraya*, the latter considered to have conserved the majority of *Alouatta* ancestral chromosome forms) and in the phylogenetic outgroup *C. apella*. This data was translated into numerical characters according to Table 2. The resulting binary data matrix with all species included in this study is given in Table 3. Maximum parsimony analysis resulted in only one most parsimonious tree (76 steps long, consistency index = 1.00; retention index = 1.00, homoplasy index = 0.00); genus *Ateles* formed a monophyletic group with *A. b. marginatus* as the most basal clade, followed by *A. p. paniscus* and finally a clade formed by *A. geoffroyi* and *A. b. hybridus*. Phylogenetic relationships between genera *Ateles*, *Brachyteles*, *Lagothrix* and *Alouatta* could not be resolved. This tree is illustrated in Fig. 3A.

A second analysis, including both the chromosome painting and the six characters derived from G-banding data (Tables 1 and 2), resulted in a tree with 82 steps (Fig. 3B). Both the consistency and retention indexes were 1.00. Within genus *Ateles*, both trees as well as the bootstrap values at each node were identical. Considering all four Atelinae genera, however, *Alouatta* appears as the most basal clade, followed by *Brachyteles* and finally *Ateles* and *Lagothrix*.

Discussion

Most classifications of New World monkeys, based on morphological and molecular studies, agree with the monophyly of Atelinae, comprising *Brachyteles*, *Lagothrix*, *Ateles* and *Alouatta*. There is, however no consensus yet concerning phylogenetic relationships between Atelinae genera, nor within genus *Ateles*. Here we report on a comparative molecular cytogenetic study including members from all four Atelinae genera that included four species from genus *Ateles*. For this purpose we analyzed *A. b. marginatus*, *A. p. paniscus* and *B. arachnoides* by multi-directional chromosome painting. In the attempt to reconstruct chromosomal phylogenies we further included previously published data on *A. geoffroyi* (Morescalchi et al., 1997), *A. b. hybridus* (Garcia et al., 2002), *L. lagothericha* (Stanyon et al., 2001) and *A. caraya* (de Oliveira et al., 2002) using *C. apella* (Garcia et al., 2000, de Oliveira et al., 2002) as an outgroup.

Evolutionary chromosome rearrangements can be considered to be “rare genomic changes” (see Rokas and Holland, 2000, for review) with low levels of convergence. We attempted to reconstruct the succession of chromosomal rearrangements that led from the putative Platyrrhini ancestor (2n = 54 chromosomes, Neusser et al., 2001) to extant Atelinae species, in order to be able to distinguish between ancestral and derived chromosome forms. Shared derived traits would indicate a closer phylogenetic relationship between species as compared to species that do not share this synapomorphism. In a second approach, we subjected the chromosomal characters observed to a maximum parsimony analysis (PAUP).

All species included in our study shared derived chromosome forms 14/15a₁, 7a/5a/7a, 5b, 4a, 4b/15a₂, 4c, 1a₁ and 1a₂, further supporting the proposed Atelinae ancestral karyotype with 2n = 62 chromosomes (de Oliveira et al., 2002) and hence Atelinae monophyly (Fig. 3A).

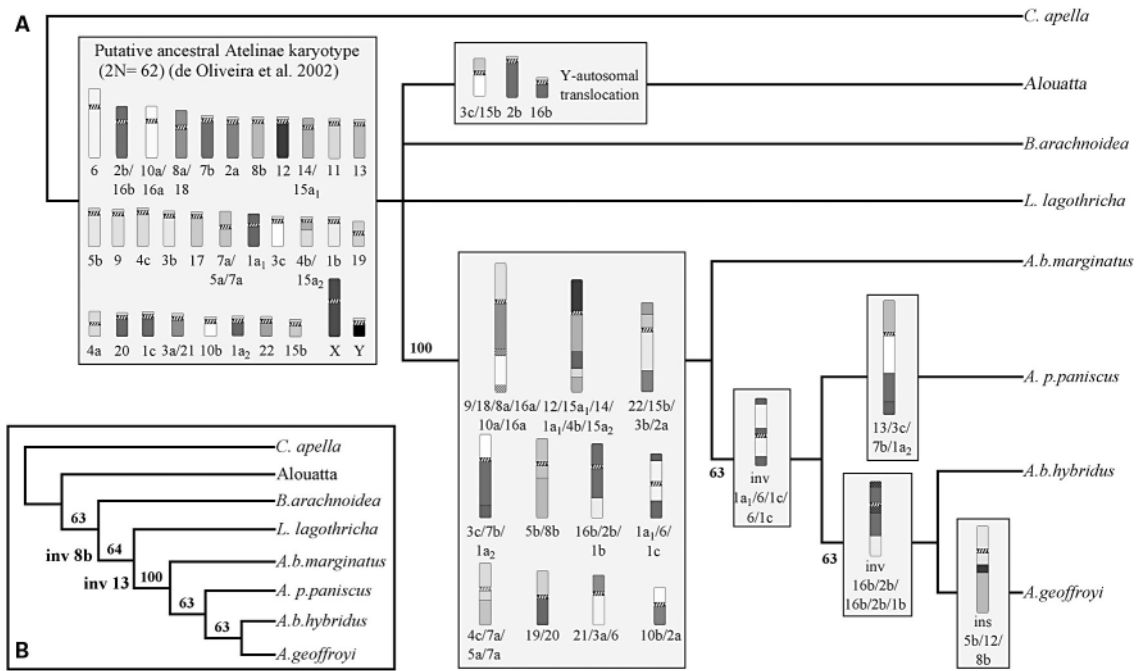


Fig. 3. Reconstruction of chromosomal phylogenies in Atelinae by a maximum parsimony approach (PAUP): **(A)** when employing only multi-directional chromosome painting data, a polytomy of all four genera was observed, while the branching sequence within genus *Ateles* was unequivocal. **(B)** This polytomy was resolved by the introduction of additional characters derived by comparative G-banding analysis, namely the inversions of the human chromosome 8b and 13 homologues (see text for details). Bold numbers indicate bootstrap values. The chromosome nomenclature followed Neusser et al. (2001) and refers to homology with human chromosomes.

Concerning genus *Ateles*, 17 tandem or Robertsonian type fusions and three fissions are necessary to derive the putative ancestral *Ateles* karyotype conserved in *A. b. marginatus* ($2n = 34$) from the ancestral Atelidae karyotype. Only *A. b. marginatus* chromosomes 11, 12, 14 and 15 remain conserved, compared to the ancestral Atelidae karyotype. In the assumed last common ancestor of *Ateles p. paniscus*, *A. geoffroyi* and *A. b. hybridus* a pericentric inversion in chromosome form 1a₁/6/1c, leading to 1a₁/6/1c/6/1c (APP 7, ABH 7 and AGE 7) took place. *A. geoffroyi* and *A. b. hybridus* exclusively share the derived pericentric inversion 16b/2b/16b/2b/1b on ABH 6 and AGE 6, whereas in *A. p. paniscus* a species-specific tandem fusion occurred, forming APP 4 (Fig. 2B) with the association of 13/3c/7b/1a₂. In *A. geoffroyi* a species specific insertion of human chromosome 12 homologous material occurred into AGE 5. This interpretation is in agreement with the hypothesis published by Medeiros et al. (1997), based on comparative G-banding analysis. However, it is discordant with the proposal of Collins and Dubach (2000) based on mitochondrial DNA variation, which placed *A. p. paniscus* as the most basal in comparison with *A. b. marginatus* and *A. geoffroyi*. Nevertheless, the cytogenetic evidence strongly argues for a reclassification of *A. belzebuth hybridus*, since its karyotype shares no synapomorphies exclusively with *A. b. marginatus*, but instead the derived inversion of chromosome 6 with *A. geoffroyi*.

When comparing putative ancestral karyotypes of *Ateles* and *Alouatta*, no shared derived chromosome forms between any of the extant species were observed, despite the occurrence

of numerous chromosome rearrangements in both phylogenetic lines. The attempt to establish chromosomal phylogenies between the four genera employing chromosome painting characters alone, however, was hampered by the fact that both *B. arachnoidea* and *L. lagothericha* conserved the ancestral Atelinae karyotype. This polytomy could be resolved by the introduction of additional chromosomal traits derived from comparative G-banding analysis. The derived inversion shared by *B. arachnoidea* chromosome 6, *L. lagothericha* 7 and *A. b. marginatus* 5 may constitute a synapomorphism absent both in *Alouatta* and *Cebus* and argues for a close link between these three genera to the exclusion of genus *Alouatta* (Fig. 2F). Furthermore, considering that *B. arachnoidea* chromosome 20 and its *C. apella* homolog share the ancestral acrocentric chromosome form, whereas the *L. lagothericha* and *Ateles b. marginatus* homologs are derived by a synapomorphic inversion (Fig. 2G), genera *Ateles* and *Lagotherich* would share a last common ancestor. Dutrillaux et al. (1986) introduced a similar hypothesis on the basis of comparative R-banding analysis; however, under the assumption that *Lagotherich* and *Brachyteles* would differ by three inversions and one translocation. The investigators further argued that *Ateles* and *Lagotherich* shared two characters not found in *Brachyteles*, whereas our results revealed only one derived chromosome form shared by *Ateles* and *Lagotherich* to the exclusion of *Brachyteles* and *Cebus* (Fig. 2E–G).

Alouatta as the sister group of the clade comprising *Brachyteles*, *Lagotherich* and *Ateles* agrees with previous views based on morphological (Rosenberger, 1992) and molecular data

(Schneider et al., 1993, 1996; Meireles et al., 1999). The grouping of *Ateles* and *Lagothrix*, however, is controversial. Kay (1990) argued in favor, Ford (1986) presented a trichotomy of *Brachyteles*, *Lagothrix* and *Ateles*, while Rosenberger (1992) grouped *Brachyteles* and *Ateles* together, with *Lagothrix* as sister-group. Schneider et al. (1993, 1996) and Meireles et al. (1999) grouped *Lagothrix* and *Brachyteles*, with *Ateles* as a sister-group.

In conclusion, the cladistic reconstruction based on the identification of ancestral versus derived chromosome forms and the PAUP analysis resulted in the same most parsimonious phylogenetic tree: a polytomy of the four Atelinae genera when considering only chromosome painting data and a branching sequence {*Alouatta* {*Brachyteles* {*Lagothrix* and *Ateles*}}, when additionally introducing characters from intra-chromosomal rearrangements that were only detected by G-banding. These results on the one hand emphasize the importance of intra-

chromosomal rearrangements as cladistic markers, which to date have not yet been exploited to their full extent. On the other hand, these observations pinpoint the limitation of the multi-directional painting approach presented here. Despite an increased subregional definition, compared to the use of human painting probes alone, some inversions could only be visualized by a varying centromeric index. In this respect, classical banding analysis remains an important comparative cytogenetics tool, in particular in combination with the secure establishment of gross chromosomal homologies by chromosome painting. Nevertheless, the delineation of evolutionary intra-chromosomal rearrangements with subregional probes such as BACs can be considered to be superior. A detailed molecular cytogenetic reinvestigation of the two key inversions that occurred during Atelinae karyotype evolution promises to substantiate the current interpretation of Atelinae chromosomal phylogeny.

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