Chromosome Studies in *Alouatta belzebul*

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The chromosome constitution of *Alouatta belzebul* was studied with G-, C-, and silver staining. In ten specimens identified as *Alouatta belzebul*, the chromosome constitution of males (2n = 49) differed from that of females (2n = 50) owing to a Y-autosome translocation. In another single female specimen, probably *Alouatta belzebul nigerrima*, the diploid chromosome number was also 50, though its karyotype was drastically different from that of *Alouatta belzebul belzebul*. Chromosome studies, taken together with phenotypic and field observations, suggest that *Alouatta belzebul belzebul* is phenotypically variable in respect to pelage coloration. This attribute is therefore unreliable for the precise identification of *Alouatta belzebul* sub-species. Conversely, relatively minor phenotypic differences, allowing for the characterization of subspecies within a same species, coexist with unparalleled, drastic karyotypic divergence. These findings clearly question grass morphological attributes as discriminative characteristics of *Alouatta belzebul* subspecies.

Key words: howler monkeys, cytogenetic characterization

INTRODUCTION

The subfamily Alouattinae consists of a single genus, *Alouatta*, comprising six different species: *A. palliata, A. pigra, A. seniculus, A. fuscus, A. belzebul*, and *A. caraya* [Wolfheim, 1983]. This genus has a widespread geographic distribution in the New World, from southern Mexico to the Atlantic coast of Brazil in the southeast [Chiarelli, 1972].

Previous studies of this subfamily are scarce, and several reports are based on the study of chromosomes without banding techniques (see Table 1). The diploid chromosome number in this subfamily has been found to vary from 2n = 43 to 2n = 54, and only a few cases of intraspecific variation have been reported [Koiffmann, 1977, 1982; Yunis et al, 1976].

This paper describes, for the first time, the karyotype of 11 specimens of *Alouatta belzebul*. Our studies demonstrated differences in diploid chromosome number between sexes due to a Y-autosome translocation in the male. Moreover, one of the eleven individuals showed a completely different karyotype, a finding that questions its inclusion in the same species group.

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**TABLE I. Chromosome Number in the Alouattinae**

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</table>

*N = number of specimens studied; M = male; f = female; 2n = diploid chromosome number; ME = metacentric; SM = submetacentric; A = acrocentric; m = microchromosome; t = translocated; ? = unknown.

In Yunis et al [1976], no precise information is supplied on the number of male and female specimens with any particular chromosome constitution. A similar situation was found in the report of Mudry de Pargament et al [1984], in ten specimens of both sexes.
MATERIAL AND METHODS

Description of Specimens

Eleven wild-caught specimens were studied. Ten of these animals (three females and seven males) had been captured in the Tucuruí dam reservoir (49° W and 5° S), on the left margin of the Tocantins river, State of Pará, Eastern Amazonia, Brazil, and were kept in the National Primate Centre (Belém, Pará). These specimens were identified as *Alouatta belzebul* on the basis of their phenotypic characteristics and geographic distribution [see Hill, 1960]. However, pelage coloration was variable between animals. While some specimens showed a general black pelage on the face and body, with characteristic rufous or brownish hairs on their hands, feet, and terminal thirds of their tails, others were completely black. No sexual dimorphism was observed in respect to pelage coloration for any of these two types.

On the basis of pelage coloration, we identified the rufous-handed howlers as *Alouatta belzebul belzebul*, the species holotype. We believe that the wholly black specimens also should be considered as belonging to the same subspecies for the following reasons: 1) The specimens were captured on the left margin of the Tocantins river, in a region where *Alouatta belzebul belzebul* is not sympatric with other *Alouatta belzebul* subspecies [Hill, 1960]. 2) Field studies in the Tucuruí region, where some 1,500 *Alouatta belzebul* specimens were captured and later released to a natural reserve, showed that rufous-handed and wholly black specimens were sympatric and, moreover, were frequently found in the same troops (Schneider & Sampaio, unpublished observations). 3) Wholly black and rufous-handed specimens successfully cross-breed in captivity at the National Primate Centre (Belém, Pará), producing the two types of offspring. Though breeding in captivity cannot be taken as actual proof that these specimens belong to the same subspecies, it seems as if pelage coloration is a simple genetic trait rather than a precise characteristic for subspecies identification.

The 11th specimen studied by us was of unknown origin and had been wild-caught. Its pelage was completely black, and the animal (a female) was more slender than females of the first group. This specimen had been tentatively identified as *Alouatta belzebul nigerrima*, though its precise characterization is unknown.

Cytogenetic Studies

**Mitotic chromosomes.** Whole blood cultures were made in TC 199 with 20% fetal calf serum, 2% PHA (Difco), and heparin (30 IU/ml) for 72 hours at 37° C. G-, C-, and NOR banding was performed following the procedures of Seabright [1971], Sumner [1972], and Goodpasture and Bloom [1975], respectively.

**Meiotic chromosomes.** A testicular biopsy was obtained from one male specimen (*A. b. belzebul*) of proven fertility and kept in the National Primate Centre (Belém, Pará). This material was collected in KCl 0.075 M and later processed as described by Evans et al [1964] for meiotic preparations.
RESULTS
The Chromosome Complement of *Alouatta belzebul belzebul*

The diploid chromosome number in this subspecies was 50, XX in females and 49,X-in males, in whom the Y-chromosome was apparently absent. In the female (see Figs. 1 and 2), 11 autosomal pairs were biarmed, of which three pairs (Nos 2, 10, and probably 11) were metacentric, and 13 pairs were acrocentric. A comparison of male and female karyotypes (see Fig. 3) allowed us to identify the X-chromosome, which was submetacentric with a morphology and G-band pattern similar to that of the human X-chromosome. With C-banding (Fig. 4), constitutive heterochromatin was present in the centromere region of all chromosomes, in the telomeric region of one submetacentric pair, and in the long arm of two acrocentric pairs, where constitutive heterochromatin was intercalar. With NOR staining, the nucleolar organizer regions were present in three chromosome pairs, Nos. 6, 18, and 19 (see Fig. 5).

Sex Chromosome Determination in *Alouatta belzebul belzebul*

The difference between the 49,X - diploid number of males and the 50,XX of females could be due either to a X0/XX system or, alternatively, to a Y-autosome translocation resulting in a X1 X2 Y/X1X1 X2X2 system. To examine these two possibilities, we carefully compared the male and female karyotypes and carried out meiotic studies in the male. A karyotypic comparison between sexes showed that the male had a heteromorphic acrocentric pair (No. 17), in contrast to females, in which this pair was monomorphic. One member of this heteromorphic pair (Fig. 3, arrow) showed a subcentromeric G-positive region that was absent in its smaller counterpart. We propose that this small region corresponds to a translocated Y chromosome. Another heteromorphic pair (No. 9) was also found in the same specimen (see Fig 3), though in this case differences between homologues more likely result from a pericentric inversion than from a translocation.

Meiotic studies clearly confirmed the existence of a translocation and definitively ruled out the possibility of a X0/XX system in this species. An analysis of 213 first meiotic divisions showed that all cells contained 24 elements, comprising 23 bivalents and one trivalent (Fig. 6) This trivalent was formed by the X-chromosome, the Y-carrying chromosome 17, and the normal chromosome 17 (the X1-, Y-, and X2- chromosomes, respectively). The short-arm region of the X1-chromosome was found to pair with the subcentromeric region of one acrocentric chromosome (the Y), the latter being associated to its homologue (the X2) by a long-arm terminal chiasma. A first meiotic disjunction in the heterogametic sex would be expected to result in haploid products with different chromosome numbers: 50% with X1 -X2 (n = 25 chromosomes), and 50% with the Y (n = 24 chromosomes). An analysis of 124 second meiotic metaphases confirmed these expectations: 57 cells contained 25 chromosomes, and 67 cells contained 24 chromosomes (see Fig. 7a,b); the observed deviations from the expected 1:1 ratio were not statistically significant ($\chi^2 = 0.80; p > 0.05$).
The Chromosome Complement of Alouatta belzebul nigerrima

The diploid chromosome number in female *Alouatta belzebul nigerrima* was 50 (Figs. 8, 9), with nine pairs of biarmed autosomes and 15 pairs of acrocentric autosomes. The presumed X-chromosome was submetacentric, with a morphology and G-band pattern similar to the human X-chromosome (Fig. 9). Of the nine pairs of biarmed autosomes, only one (No. 9) was clearly metacentric. One submetacentric pair (No. 8) had two secondary constriction regions in its long arm (Fig. 9a-c). These regions, of variable size, were frequently found in association and were positively stained by silver precipitation (Fig. 9d,e). With C-banding (not shown), constitutive heterochromatin was restricted to the centromere region of all chromosomes.

**DISCUSSION**

The Chromosome Constitution of Alouatta belzebul belzebul

Chromosome studies in *Aloauatta belzebul belzebul* have unequivocally shown that the difference in diploid number between sexes is due to a Y-autosome translocation, ruling out the possibility of a XX/X0 system. Y-autosome translocations have been clearly demonstrated, with mitotic and meiotic studies, in other primate species, such as *Callimico goeldii* [Hsu & Hampton, 1970] and *Aotus trivirgatus* from Bolivia [Ma et al, 1976]. Similar translocations have been proposed to exist in *Alouatta fusca clamitans* [Koiffmann, 1977], *Alouatta palliata* [Ma et al, 1975], *Aotus trivirgatus* from Peru [Ma et al, 1980], and *Cacajao calvus rubicundus* [Dutrillaux et al, 1981, though in these reports, the existence of such translocation was not demonstrated by a meiotic analysis.

Moreover, the existence of a Y-autosome translocation in *Alouatta fusca clamitans* does not seem to be constant in this species [Koiffmann, 1982], a situation comparable to that of *Cacajao calvus rubicundus*, in which the existence of a nontranslocated Y-chromosome also has been reported [Benirschke et al, 1976]. Koiffmann and Saldanha [1974] found two 50,XY male specimens of *Alouatta fusca clamitans* and another two with 2n = 49, each of which presumably lacked a small acrocentric chromosome. In later studies, a third male specimen with 49 chromosome was found to lack a Y-chromosome and showed a heteromorphic submetacentric pair, leading Koiffmann [1977] to propose a Y-autosome translocation. However, Koiffmann [1977] also found another male specimen with 2n = 48,XY carrying autosomal rearrangements.

The Y-autosome translocation reported in *Alouatta palliata* [Ma et al, 1975] appeared to be consistent: among males (7 specimens) the chromosome number was 2n = 53; among females (10 specimens) 2n = 54. In this species, as in *Alouatta belzebul belzebul*, the Y was translocated to the subcentromeric region of an acrocentric chromosome, though in *Alouatta palliata*, the presumed Y-chromosome region was negatively G-banded. In is remarkable, however, that the autosome to which the Y-chromosome was translocated in *Alouatta palliata* had a G-band pattern similar, though not identical, to that of chromosome 17 in *Alouatta belzebul belzebul*. 
What Are the Genetic Implications of a Y-Autosome Translocation in *Alouatta belzebul belzebul*?

The appearance of a de novo Y-autosome translocation, occurring in an originally 2n = 50,XY male, affected chromosome behavior in spermatogenesis and, consequently, the way some genes were transmitted. Crossovers between the autosomal region of the neo-Y and its homologous counterpart No. 17, or the X2, were demonstrated by the presence of a terminal chiasma (see Fig. 6), though it is likely that a partial crossover suppression took place between them. Since the Y-autosome translocation in *Alouatta belzebul belzebul* is a reliable finding (unlike the situation in *Alouatta fusca clamitans*, where the Y-chromosome might or might not be translocated), one cannot precisely estimate the amount of crossover suppression resulting from this translocation. However, we could indirectly estimate it by a comparative analysis of male and female meiosis in *Alouatta belzebul belzebul*, if the number of chiasmata in bivalent 17 (X2-X2) were found to be higher in oocytes than the number of chiasmata between the neo-Y and the X2 in spermatocytes. In should be remarked that such crossover suppression will result in a decrease in the total number of chiasmata in males in respect to females, to a number below expectation. (The total number of chiasmata in XY males is normally less than in XX females because more chiasmata are formed between the two X-chromosomes than between the X and the Y-chromosomes, so that total recombination length is lower in males than in females.)

The genetic consequences of such translocation imply that the male determining genes of the Y-chromosome and the autosomal genes of one chromosome 17 have become syntenic. Furthermore, some of these autosomal genes might be Y-linked, especially those located in close proximity to the inserted Y-chromosome. Thus, pedigree analyses of captive-bred animals might be useful to detect a holandric type of inheritance for any specific marker. Biochemical studies of feral populations of *Alouatta belzebul belzebul* should also take this fact into consideration. Any time a biochemical polymorphism is detected, the incidence of every allele should be compared in each sex to look for the possibility of linkage disequilibria.

Correlation of Subspecies Phenotype and Karyotype

Taxonomic arrangements within the primates, as in other mammalian orders, rely on gross macrostructural attributes rather than on genetic characterizations. Pelage coloration has been of primary importance in the identification of the five *Alouatta belzebul* subspecies described by Hill [1960]: *A.b. belzebul*, *A.b. mexiana*, *A.b. ululata*, *A.b. discolor*, and *A.b. nigerrima*. A close examination of some of our specimens clearly questions the validity of subspecies taxonomy when based on pelage because the ten specimens studied by us showed clear variations, from typical rufous-handed to wholly black. Certain facts suggest that our specimens belong to the same subspecies First, all of them had been captured in a region where *Alouatta belzebul belzebul* is not sympatric with any other *Alouatta belzebul* subspecies. Second, the coexistence of wholly black and rufous-handed specimens had been observed in field studies, even within the same troops. Third, all ten specimens studied by us were karyotypically similar, except for the observed difference between sexes. And fourth, animals of different pelage were observed to cross-breed successfully in captivity. These findings
strongly suggest that these specimen belong to the same subspecies (Alouatta belzebul belzebul) even though their phenotypic characteristics are more variable than previously thought. Additional, more conclusive evidence supporting this possibility has been shown in a population study of some 1,000 Alouatta belzebul belzebul specimens, captured in the Tucurui dam reservoir, in which it was shown that biochemical polymorphisms were in Hardy-Weinberg equilibrium [Barroso et al, 1987]. This finding is good evidence that gene flow occurs naturally in this population in which differences in pelage coloration were observed to be present.

Is Alouatta belzebul belzebul a Karyotypically Uniform Group?

The eleventh specimen studied by us, tentatively identified as Alouatta belzebul belzebul nigerrima, is chromosomally quite distinct from the previous ten. Pairs Nos. 8, 12, 13, and 14 in A.b. nigerrima have no recognizable counterpart in A.b.belzebul. Likewise, chromosome pairs Nos. 4, 6, 10, and 23 in A.b.belzebul have no evident counterpart in A.b. nigerrima. Such karyotypic dissimilarities, taken together with differences in the location and distribution of nucleolar organized and constitutive heterochromatic regions, are obviously more extreme than any intraspecific chromosomal difference previously reported in the Alouattinae (see Table I). For example, in Alouatta fusca clamitans [Koifmann, 1977, 1982], two biarmed chromosomes showed “arm homologues,” owing to probable fusion events that reduced the normal diploid number (2n =50) to 48. Furthermore, in A. seniculus seniculus [Yunis et al, 1976], a pericentric inversion was found to affect, in the homozygous condition, a pair of acrocentric autosomes that had become submetacentric. Contrary to these findings, where intraspecific chromosome variation has not drastically affected the “normal”chromosome constitution of the species under study, our findings point to a considerable amount of chromosome rearrangement within Alouatta belzebul.

Since taxonomic arrangements are based on gross morphological characteristics and not on genetic characterizations (viz, chromosomal, biochemical, or molecular), the above discrepancies are not surprising. Though a morphological characterization might suggest the grouping of karyotypically distinct specimens into one species, chromosome studies clearly question this taxonomic criterion. Further studies at the chromosomal, biochemical, and molecular level are necessary to elucidate this problem.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. G-band chromosomes of female Alouatta belzebul belzebul (pairs 1-15).
Fig. 2. G-band chromosomes of female *Alouatta belzebul belzebul* (pairs 16-24 and X chromosome).

Fig. 3. G-band karyotype of male *Alouatta belzebul belzebul* (2n = 49). Arrow points to Y autosome translocation; the small Y-chromosome is presumably located below the centromere region.

Fig. 4. C-band chromosome of *Alouatta belzebul belzebul* (female) showing centromeric, telomeric, and intercalar heterochromatin (arrows).

Fig. 5. Silver-stained metaphase of *Alouatta belzebul belzebul* (female) showing six nucleolar organizer regions (NORs).

Fig. 6. First meiotic division in a male *Alouatta belzebul belzebul* (2n = 49; see Fig. 3). Note the presence of 24 elements: 23 bivalents and 1 trivalent composed of an association of the X1, the Y, and X2.

Fig. 7. Second meiotic divisions in a male *Alouatta belzebul belzebul* (2n = 49), showing 24 and 25 chromosomes (a and b, respectively).

Fig. 8. G-band chromosomes of female *Alouatta belzebul belzebul nigerrima* (pairs 1-14).

Fig. 9. Top: G-band chromosomes of female *Alouatta belzebul belzebul* (pairs 15-24 and X chromosome). Bottom: Chromosome 8 with G-banding (a-d), and with silver staining (e). Arrows point to variable nucleolar organizer region (NOR). Note association of NORs (d).

REVISADO/IMPRESSO