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ORIGINAL ARTICLE

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Molecular phylogenetics of howler monkeys (*Alouatta*, Platyrrhini)

A comparison with karyotypic data

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Abstract Molecular phylogenetic analyses of seven Brazilian *Alouatta* species, based on cytochrome *b* DNA sequence data were carried out. Parsimony and neighbor joining topologies grouped *Alouatta belzebul* and *A. fusca* as sister groups in one clade while another, well-supported clade contained *A. seniculus* as the most basal offshoot, followed by *A. nigerrima* as a sister lineage of *A. macconnelli*/*A. stramineus*. Estimates of inter-specific sequence divergence were generally low, and estimates of the time of divergence indicated that the main *Alouatta* lineages emerged during a short evolutionary interval. A comparison with karyotypic data confirmed the molecular topology showing a closer relationship between *A. macconnelli* and *A. stramineus* in respect to *A. nigerrima*. It also showed that the XX/XY sex chromosome system was maintained in several lineages while the $X_1X_2Y/X_1X_1X_2X_2$ system appeared independently at least three times during the radiation of howler monkeys. Moreover, the $X_1X_2Y_1Y_2/X_1X_1X_2X_2$ system might have appeared once or, alternatively, twice and independently.

Introduction

Alouatta is the most geographically widespread neotropical primate genus, the distribution of which extends from southern Mexico through Central America down to southern Brazil and northern Argentina. The most recent checklist recognized only four species in Brazil (Groves 1993) while a recent review raised the number of Brazilian species to nine: *Alouatta caraya*, *A. fusca*, *A. belzebul*, *A. discolor*, *A. ululata*, *A. seniculus*, *A. stramineus*, *A. puruensis* and *A. nigerrima* (Gregorim 1995). Bonvicino et al. (1995), however, recognized *A. macconnelli* as a valid species in addition to the nine reported by Gregorim (1995).

Morphologic, karyologic and molecular studies were coincident in showing that *Alouatta* is a monophyletic genus (Hershkovitz 1949; Oliveira 1996; Meireles et al. 1999) although intrageneric arrangements were not coincident. Morphologic studies based on the anatomical characteristics of the hyoid bone indicated three species groups: *A. seniculus* (with *A. seniculus*, *A. belzebul* and *A. fusca*) and two monotypic groups, *A. caraya* and *A. palliatta* (Hershkovitz 1949). Conversely, another study (Gregorim 1995), based on cranial and pelage coloration, resulted in a phylogenetic arrangement someway different from the previous groups: ((((*A. seniculus*, *A. stramineus*, *A. puruensis*, *A. nigerrima*) (*A. ululata*, *A. belzebul*, *A. discolor*)) *A. fusca*) *A. caraya*).

Karyotypic analyses succeeded in demonstrating that the genus *Alouatta* is very diverse, with drastic differences in the chromosome complement between species, populations or karyomorphotypes of uncertain taxonomic status (Armada et al. 1987), representing discrete limits between howler monkey populations. This was the case for *A. seniculus*, *A. fusca* and *A. belzebul* (*sensu* Groves 1993), which were shown to be species complexes rather than single, well-defined taxa (Armada et al. 1987; Oliveira 1996). In fact, karyologic studies based on diploid and fundamental number and G-band patterns indicated that several morphotypes, previously ranked as subspecies, were likely to be valid species (Lima and Seuánez 1991; Oliveira 1996). This was the case for *A. seniculus* (=*A. seniculus seniculus*), *A. stramineus* (=*A. seniculus stramineus*), *A. nigerrima* (=*A. belzebul nigerrima*), *A. belzebul* (=*A. belzebul belzebul*) and *A. macconnelli* (=*A. seniculus macconnelli*). Few reports, however, have traced phylogenetic pathways to establish the evolutionary relations of these taxa based on karyotypic analyses (Armada et al. 1987; Oliveira 1996).

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In Memoriam W. Beermann

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Molecular studies have established phylogenetic relationships between neotropical primate genera (Schneider et al. 1993, 1996; Canavez et al. 1999a) and these studies were coincident in showing that *Alouatta* was the most basal offshoot of an Atelid clade that included *Ateles*, *Brachyteles* and *Lagothrix*. Few reports, however, have established intrageneric phylogenetic topologies in neotropical primates (see Tagliaro et al. 1997; Canavez et al. 1999b) and especially in the genus *Alouatta* (Figueiredo et al. 1998; Meireles et al. 1999).

Here we present a molecular phylogenetic analysis of seven Brazilian *Alouatta* species based on DNA sequence data of the mitochondrial cytochrome *b* gene. Phylogenic relationships and times of divergence between clades, species limits, and karyotypic data are discussed.

Materials and methods

DNA was isolated (Smith et al. 1987; Sambrook et al. 1989) from blood or liver samples of 22 howler monkeys, one *Brachyteles arachnoides* and one *Callicebus personatus* (Table 1). Cytochrome *b* was amplified with primers citb1 (5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′) and citb2 (5′-AACTGCAGTCATCTCCGGTTTACAAGAC-3′) under the following conditions: five cycles of 94°C (1 min), 46°C (1 min) and 72°C (90 s) followed by five cycles of 94°C (1 min), 48°C (1 min) and 72°C (90 s); ten cycles of 94°C (1 min), 49°C (1 min) and 72°C (90 s), and 15 cycles of 94°C (1 min), 50°C (1 min) and 72°C (90 s). The light strand (ca. 1014 bp) was cycle-sequenced with BigDye terminator according to the manufacturer's instructions (Perkin-Elmer) using sequencing primers citb1 and internal primer cit-alo (5′-ATAGCCACAGCATTCATAGGC-3′). Sequencing reactions were run in an ABI Prism 377 automatic DNA sequencer.

Sequences were aligned with CLUSTALW (Thompson et al. 1994). P-distance estimates were used for constructing neighbor joining trees, with bootstrap values from 1000 replicates, using MEGA 3.01 (Kumar et al. 1993). This program was also used for estimating transition/transversion ratios. Parsimony analysis (PAUP 3.1.1; Swofford 1993) was carried out by branch-and-bound search, with bootstrap values calculated by heuristic search from 1000 replicates. Decay index was estimated according to Bremer (1988).

The rate of divergence of cytochrome *b* was calibrated at 12.9 million years before present (MYBP), corresponding to the splitting of *Alouatta*/*Brachyteles* and at 14.3 MYBP, corresponding to the splitting of *Alouatta*/*Callicebus* (Goodman 1996). The calibrated rate and p-distance values were used for estimating the time of divergence of the *Alouatta* lineages as hypothesized on the neighbor joining tree.

Results

Cytochrome *b* sequence data of the specimens studied herein were deposited in GenBank (Table 1). In all cases, transition/transversion ratios were high and stop codons or deletions were not detected, indicating that the presence of nuclear, mitochondrial DNA-derived pseudogenes was unlikely.

Sequence data showed that the three *A. belzebul* from Paraíba State were identical to one another as were the two *A. seniculus* from Barcelos islands, the two *A. mac-*

Table 1 Species, GenBank Accession number, identification number and geographic origin of specimens

Species (GenBank Accession number)	Specimen number	Locality (in Brazil)
A. caraya (AF289516)	Ac 406	Rio Quilombo, Manso Dam reservoir, Chapada dos Guimarães (15°27′ S, 55°44′ W), Mato Grosso State
A. caraya (AF289519)	Ac 592, Ac 621	Rio Casca, Manso Dam reservoir, Chapada dos Guimarães, Mato Grosso State
A. caraya (AF289517)	Ac 05	Unknown
A. caraya (AF289518)	MN 36077, MN 36080	Serra da Mesa Dam reservoir, Goiás State
A. seniculus (AF289982)	MN 59014, MN 61638	Candiru and Maniva islands, Rio Negro, Barcelos (00°58′ S, 62°55′ W), Amazonas State
A. stramineus (AF289983)	MN 61639	Igarapé do Limão (00°09′ N, 63°15′ W), tributary of right bank of Rio Aracá, tributary of left bank Rio Negro, Barcelos, Amazonas State
A. macconnelli (AF289984)	Jari 2094, Jari 2097	Rio Jari, Amazonas State
A. nigerrima (AF289985)		Unknown
A. belzebul (AF289514)	Ab 689	Tucuruí Dam reservoir (03°45′ S, 49°40′ W), Pará State
A. belzebul (AF289511)	Ab 1030	Tucuruí Dam reservoir, Pará State
A. belzebul (AF289512)	Ab 1033	Tucuruí Dam reservoir, Pará State
A. belzebul (AF289513)	Ab 1088	Tucuruí Dam reservoir, Pará State
A. belzebul (AF289515)	Pb 01, Pb 02, Pb 03	Pacatuba Farm, Sapé (07°05′ S, 35°13′ W), Paraíba State
A. fusca clamitans (AF289986)	CPRJ 815, CPRJ 853	Unknown
A. fusca (AF289987)	ZOO-SP 17089	Unknown
Brachyteles arachnoides (AF289989)	CPRJ 1091	Unknown
Callicebus personatus (AF 289988)	CPRJ 1627	Unknown

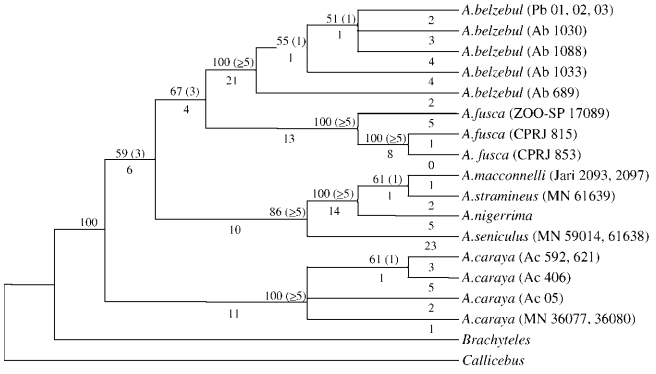

Fig. 1 Parsimony tree showing phylogenetic relationships of *Alouatta* species (branch-and-bound search algorithm). *Numbers* above nodes represent bootstrap values estimated by heuristic search with 1000 replicates. Estimates of Bremer's index are shown in *parentheses*. Number of synapomorphies or autopomorphies is shown below each branch. Consistency index=0.78; tree length=431 steps

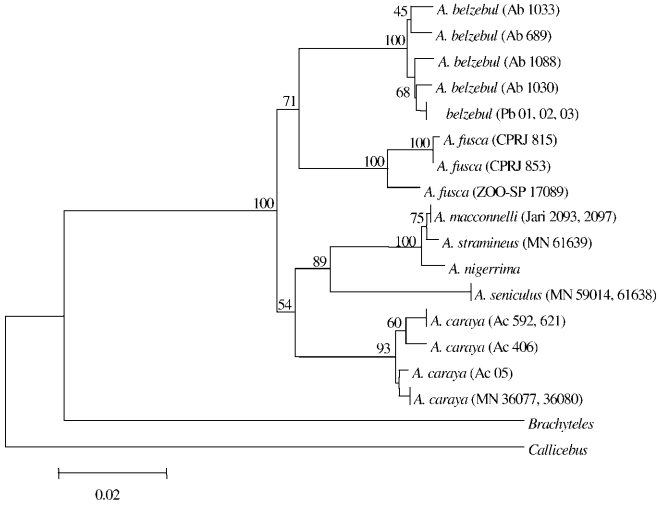

Fig. 2 Neighbor joining tree. *Numbers* near nodes are bootstrap values estimated with 1000 replicates

connelli from the Jarí River and the two *A. caraya* from Serra da Mesa. Conversely, inter-individual variation was observed in *A. belzebul* from Tucuruí and *A. caraya* from Manso, a reason why individual haplotypes were included in phylogenetic analyses. A single, most parsimonious tree with 431 steps, and a mostly congruent neighbor joining tree are shown in Figs. 1 and 2, respectively. Both topologies grouped *A. belzebul* and *A. fusca* as sister branches of a clade, and, in another clade, *A. seniculus* was the most basal offshoot followed by *A. nigerrima* as a sister lineage of a most derived *A. macconnelli*/*A. stramineus* clade. The position of the (((*A. macconnelli*, *A. stramineus*), *A. nigerrima*), *A. seniculus*) clade was unclear because it was weakly grouped with *A. caraya* with neighbor joining (bootstrap=54%) or with the (*A. belzebul*, *A. fusca*) clade with parsimony (bootstrap=59%). A paraphyletic arrangement was observed in the *A. belzebul* clade because some haplotypes from Tucuruí grouped with haplotypes from Paraíba.

A comparison of sequence data showed low divergence values between intra- and inter-specific haplotypes (Table 2). In both cases where inter-individual variation was observed (*A. belzebul* from Tucuruí, and *A. caraya* from Manso), divergence between sympatric haplotypes was equal to or lower than 1%. Very low distance values (mean=0.006) were observed between *A. macconnelli*, *A. stramineus* and *A. nigerrima*; these values were lower than divergence estimates within *A. caraya* and *A. belzebul*.

Considering 12.9 MYBP as the time of divergence of *Alouatta*/*Brachyteles* and 0.152 as the average distance between these genera, the rate of divergence of cytochrome *b* DNA sequences equalled 1.17% per million year (pMY). A similar rate (1.22% pMY) was estimated between *Alouatta*/*Callicebus*, considering 14.3 MYBP as their time of divergence and 0.174 as the average distance between these genera. These estimates were very close to the overall rate of cytochrome *b* DNA sequence divergence of 1% pMY (Brown 1985). Table 3 summarizes the average sequence divergence and the estimated time of divergence of the main *Alouatta* clades.

Discussion

Neighbor joining and parsimony grouped *A. belzebul* and *A. fusca* as sister branches in one clade while, in a separate clade, *A. seniculus* was the most basal offshoot, followed by *A. nigerrima* as sister lineage of the most derived *A. macconnelli* and *A. stramineus*. The grouping of *A. belzebul* and *A. fusca* as sister branches was also reported with γ^1-globin pseudogene sequence data, with *A. caraya* as the most basal offshoot followed by *A. seniculus* and, subsequently, by the most derived *A. belzebul*/*A. fusca* clade (Meireles et al. 1999). An identical topology for these four species was evident with parsimony analysis (Fig. 1) although relationships within the (*A. seniculus* (*A. nigerrima* (*A. macconnelli*, *A. stramineus*))) clade was ambiguous because of the different arrangements observed with neighbor joining and parsimony topologies and by low bootstrap estimates.

The close estimates of the time of divergence of the two most basal *Alouatta* lineages in the neighbor joining tree (Fig. 2) indicated that they separated from one another in a short time span. Meireles et al. (1999), calibrating the rate of divergence at the *Alouatta*/*Cebus* separation at 21 MYBP, estimated that *A. belzebul* separated from *A. fusca* 1.0 MYBP. With a rate of divergence of 1.17% pMY, calibrated at the time of *Alouatta*/*Brachyteles* separation, cytochrome *b* data pointed to a much earlier time of *A. belzebul*/*A. fusca* divergence (4.0 MYBP). These differences might be due to a low γ^1-globin pseudogene variability, resulting in distance values more than ten times lower than with cytochrome *b*. Moreover, our estimates showed that divergence times of the main basal lineages were very close to one another:

Table 2 P-distances between haplotypes

		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
A. belzebul	1. Ab 1030	0.010	0.007	0.010	0.006	0.049	0.047	0.043	0.054	0.055	0.056	0.061	0.052	0.051	0.047	0.045	0.142	0.165
	2. Ab 1033		0.010	0.009	0.009	0.050	0.049	0.044	0.053	0.054	0.055	0.061	0.053	0.052	0.051	0.049	0.145	0.169
	3. Ab 1088			0.010	0.006	0.051	0.050	0.045	0.054	0.055	0.056	0.060	0.054	0.053	0.050	0.047	0.141	0.162
	4. Ab 689				0.009	0.047	0.046	0.042	0.051	0.052	0.053	0.061	0.051	0.050	0.046	0.044	0.141	0.164
	5. Pb 01, Pb 02, Pb 03					0.047	0.046	0.042	0.053	0.054	0.055	0.061	0.053	0.052	0.049	0.046	0.140	0.164
A. fusca	6. CPRJ 815						0.001	0.016	0.049	0.052	0.051	0.063	0.055	0.054	0.051	0.053	0.160	0.167
	7. CPRJ 853							0.015	0.050	0.053	0.052	0.062	0.056	0.055	0.052	0.054	0.159	0.166
	8. ZOO-SP 17089								0.050	0.051	0.050	0.060	0.054	0.053	0.050	0.050	0.155	0.160
A. macconnelli	9. Jari 2094, 2097									0.003	0.007	0.046	0.049	0.047	0.044	0.046	0.157	0.168
A. stramineus	10. MN 61639										0.008	0.047	0.050	0.049	0.045	0.047	0.158	0.171
A. nigerrima	11.											0.049	0.051	0.050	0.046	0.049	0.159	0.173
A. seniculus	12. MN 61638, MN 59014												0.056	0.054	0.052	0.054	0.152	0.169
A. caraya	13. Ac 592													0.008	0.007	0.009	0.145	0.173
	14. Ac 406														0.008	0.010	0.147	0.171
	15. Ac 05															0.005	0.147	0.170
	16. MN 36077, MN 36080																0.147	0.170
Brachyteles	17.																	0.186
Callicebus	18.																	

Table 3 Average divergence and time of divergence between groups according to neighbor joining tree (rate of divergence=1.17% pMY)

Divergence	Average distance	Divergence (MYBP)
A. macconnelli from *A. stramineus*	0.003	0.3
A. macconnelli and *A. stramineus* from *A. nigerrima*	0.008	0.6
Between *A. belzebul* haplotypes	0.009	0.8
Between *A. caraya* haplotypes	0.008	0.6
A. macconnelli, *A. stramineus* and *A. nigerrima* from *A. seniculus*	0.045	3.8
A. belzebul from *A. fusca*	0.047	4.0
A. macconnelli, *A. stramineus*, *A. nigerrima*, and *A. seniculus* from *A. caraya*	0.049	4.2
A. macconnelli, *A. stramineus*, *A. nigerrima*, *A. seniculus*, and *A. caraya* from *A. belzebul* and *A. fusca*	0.055	4.7

4.7 MYBP at the splitting of the two main groups (Fig. 2; Table 3), and 4.2 MYBP at the node between *A. caraya* and the branch leading to the common ancestor of *A. seniculus*, *A. nigerrima*, *A. stramineus* and *A. macconnelli*. Within this latter clade, *A. seniculus* probably emerged some 3.8 MYBP.

Estimates of inter-specific sequence divergence were generally low (Table 2), especially in the ((*A. macconnelli*, *A. stramineus*) *A. nigerrima*) clade, with values similar to or lower than within *A. caraya* and *A. belzebul*. The low level of divergence between *A. macconnelli*, *A. stramineus* and *A. nigerrima* indicated that these species diverged very recently. Because DNA sequences are unlikely to have coalesced during the short time-span encompassed between emergence of *A. nigerrima* and separation of *A. macconnelli* from *A. stramineus*, molecular analyses may not provide accurate reconstructions of branching patterns. This limitation and retention of ancestral polymorphisms might explain why cytochrome oxidase II DNA data failed to show *A. stramineus* and *A. macconnelli* as different monophyletic taxa (Figueiredo et al. 1998).

Karyotypic analyses might thus be illuminating for determining ambiguous phylogenetic branchings. These studies (Armada et al. 1987; Lima and Seuánez 1991) showed that *A. macconnelli* and *A. stramineus*, although sharing the same diploid number (47, 48 or 49) and sex chromosome system ($X_1X_2Y_1Y_2/X_1X_1X_2X_2$), differed in two chromosome pairs as a result of a translocation. This is why genetic introgression between them was considered unlikely and they were considered valid species (Bonvicino et al. 1995). On the other hand, *A. nigerrima* showed 2*n*=50 in the female, with 9 pairs of biarmed autosomes against 11 pairs in *A. macconnelli* and *A. stramineus*, indicating that these two latter species were more similar to one another than they were to *A. nigerrima*. These findings were coincident with our cytochrome *b* topology and were therefore useful in reinforcing branching patterns resulting from low distance estimates.

In view of the fact that *Alouatta* is a karyotypically diverse genus, molecular phylogenies might also be useful for understanding chromosome evolution in this taxon. The three above-mentioned species are karyotypically different from *A. seniculus* (2*n*=43–45) in which an XY/XX sex chromosome system was reported (Yunis et al. 1976) and confirmed in one animal analyzed herein (2*n*=44). Similarly, the XY/XX sex chromosome system is present in *A. caraya* (2*n*=52; Mudry et al. 1990; Oliveira 1996).

Conversely, *A. belzebul* (*sensu* Gregorim 1995) showed a diploid number of $50,X_1X_1X_2X_2$ in the female and $49,X_1X_2Y$ in the male (Armada et al. 1987), while *A. fusca* showed different karyomorphic groups: 2*n*=52,XY/52,XX; 2*n*=48 or 50 with an XY/XX sex chromosome system; $2n=49,X_1X_2Y/50,X_1X_1X_2X_2$; and $2n=45,X_1X_2Y/46,X_1X_1X_2X_2$ (Oliveira et al. 1995, 1998), indicating that *A. fusca* must be a species complex. Comparisons of G-band karyotypes showed that three autosome pairs in *A. fusca* with $2n=49,X_1X_2Y/50,X_1X_1X_2X_2$ lacked inter-specific homologs in populations with $2n=45,X_1X_2Y/46,X_1X_1X_2X_2$ and that their de novo sex chromosomes (the neo-Y and X_2 chromosomes) were derived from different autosomes (Oliveira 1996). This was good evidence that Y-autosome translocations originating the $X_1X_2Y/X_1X_1X_2X_2$ sex chromosome system must have occurred independently in two *A. fusca* populations and also in *A. belzebul*. Conversely, our molecular topologies indicated that the XY/XX sex chromosome system was independently maintained in *A. fusca* species complex, *A. caraya* and *A. seniculus*.

The $X_1X_2Y_1Y_2/X_1X_1X_2X_2$ sex chromosome system was also found in *A. sara* and *A. arctoidea*; chromosome painting demonstrated that these species shared identical Y_1, Y_2, and X_2 chromosomes (Stanyon et al. 1995) but we do not know, however, whether they are the same as the Y_1, Y_2, and X_2 of *A. macconnelli*/*A. stramineus*. If this were the case, the rearrangements that originated this sex chromosome system must have occurred in the common ancestor of these species. Alternatively, these rearrangements could have occurred independently (and involving different autosomes) in two separate ancestors, one of *A. macconnelli* and *A. stramineus* and another, of *A. sara* and *A. arctoidea*.

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