**Understanding the relationship between *Alouatta* *ululata* and *Alouatta belzebul* (Primates: Atelidae) BASED ON cytogenetics and molecular Phylogeny**

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**ABSTRACT**

 The genus *Alouatta* Lacépède 1799 comprises a group of neotropical primates distributed from southern Mexico to northern Argentina. Ten species of *Alouatta* occur in Brazil, including *Alouatta belzebul* (Linnaeus, 1766) and *A. ululata* Elliot, 1912; this latter being considered a full species or a junior synonymous of *A*. *belzebul*. In order to clarify the relationship of *A. ululata* with *A. belzebul* and infer their relationships with other *Alouatta* species, karyotypeand mitochondrial DNA data were analyzed. Phylogenetic analyses were carried out with a 801 bp fragment of cytochrome *b* DNA of one of *A*. *ululata* sample and 33 sequences of *A. belzebul, A. caraya, A. fusca, A. nigerrima, A. seniculus,* and *A. macconnelli* available in GenBank, with *Brachyteles arachnoides* as outgroup. The G-band karyotype of a male *A. ululata* showed a diploid number of 49, similar to the one reported for *A. belzebul,* with the same pattern of autosome heteromorphism, apparently resulting from a Y-autosome translocation. Maximum-likelihood and Bayesian analyses and median joining network did not show an internal structure within *A. belzebul* and placed *A. ululata* haplotype within the *A. belzebul* clade. Karyotypic and molecular analyses herein carried out did not allow the separation of *A. ululata* from *A. belzebul*.

**Keywords**: *Alouatta;* karyotype; phylogeny; *MT\_CYB*

**INTRODUCTION**

The genus *Alouatta* Lacépède 1799 comprises a group of large neotropical primates with a wide geographic distribution ranging from southern Mexico to northern Argentina. A region of eastern Amazonia and northeastern Brazil, south of the Amazonas river and above 10º latitude, is inhabited by several *Alouatta* forms . A study of geographic variation of coat color (Bonvicino *et al*. 1989) recognized the single species *A. belzebul* (Linnaeus, 1766) with four subspecies *A. b. belzebul*, *A. b. nigerrima* Lönnberg, 1941, *A. b. discolor* (Spix, 1823) and *A. b. ululata* Elliot, 1912. *A. nigerrima* was raised to species level (Bonvicino *et al*. 2001) while the other forms have been maintained as subspecies. Groves (2001, 2005) synonymized *A. b. discolor* and *A. b. ululata* under *A. belzebul.* Gregorin (2006), in a study of Brazilian species of this genus, raised *A. b. discolor* and *A. b. ululata* to the species level.

The taxonomy of *Alouatta* is mainly based on pelage color (Bonvicino et al. 1989, Gregorin 2006). Previous work classified the pelage of *Alouatta belzebul* in different patterns (Bonvicino *et al*. 1989:141), based on the extent of rufous or yellowish areas over a black background in their dorsal coloration. Based on patterns of pelage color, on absence of records (hiatus of distribution) or on geographic isolation by semi-arid environment, different areas of occurrence were identified, with specimens from areas 1 to 3 identified as *A. b. belzebul* and those of area 4 as *A. b. ululata* (Bonvicino *et al.* 1989, Figure 1). Howlers in areas 1 to 3 inhabit humid tropical forest contrary to howlers of area 4, comprising more mesic regions with higher trees surrounded by semiarid vegetation (Bonvicino *et al*. 1989). Area 1 is the northeastern portion of the Atlantic Forest from Paraíba to Alagoas states, with all specimens with the same pelage pattern; area 2 is the region of east Pará and Maranhão states, with specimens with two pelage pattern; area 3 is the region of the middle/lower Rio Tocantins basin, with highly variable specimens, with five pelage patterns; and area 4 is the region on the northern part of the Maranhão, Piauí and Ceará states, from the Rio Mearim in Maranhão westward to Serra de lbiapaba in Ceará, with specimens with three pelage patterns (Bonvicino *et al*. 1989). Sexual dimorphism is characteristic of *A. b. ululata;* males are black, with rufous areas on limbs, tail and back, while females are yellowish-brown, olive brown or gray.

Figure 1 here.

Some taxonomic consideration was also carried out based on karyotypic data. The genus *Alouatta* shows a wide variability in chromosome number and in their sex chromosome systems (Torres & Ramírez 2003). The karyotypes of *A. belzebul* 2n = 50, XX in females and 2n = 49 in males carrying a Y-autosome translocation was reported by Armada *et al*. (1987) and Lima and Seuánez (1989). The karyotype of *A. ululata,* however, is still unknown.

The different classifications based on morphological data pointed to the need of clarifying the taxonomic status of *Alouatta belzebul* and *A.b. ululata*. In the present study, we report the karyotype of *A. b. ululata* and a molecular phylogeny to understand the relationship between *A. b. ululata* and *A. belzebul*.

**Material and Methods**

*Samples and karyotypic analysis*

One *Alouatta ululata* (CPB71) was captured in Campo Maior, Piauí state, Brazil. Species identifitication were carried out by L. Jerusalinsky based on pelage coloration, and the specimens was housed in the Centro Nacional de Pesquisa e Conservação de Primatas Brasileiros (CPB) belonging to Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio). This specimen showed the tradicional colour of *A. ululata*, with an overall black coloration with rufous hairs on hands, feet and anterior part of dorsum.

 Chromosome preparations were obtained from peripheral blood cultures with 80% RPMI 1640, 20% fetal calf serum, ethidium bromide (5 mg/ml) and colchicine (10-6) for 72 hours at 37°C, following by hypotonic shock with KCl (0.075M) for 30 minutes, pre fixation and fixation with Carnoy (3 methyl alcohol:1 acetic acid). Conventional staining was carried out with 4% Giemsa in 0,1M phosphate buffer. G-banding was carried out by trypsin digestion for 30 seconds followed by Giemsa staining. Chromosomes were paired according to morphology, size and banding patterns and estimates of fundamental number were restricted to autosome pairs. Karyotypic comparisons were carried out with *A. belzebul*.

*DNA extraction and molecular analysis*

 Peripheral blood of *A. ulutata* specimen CPB 71 was collected and DNA was isolated with the phenol-chloroform protocol (Sambrook *et al.* 1989). *Cytochrome b* gene (*MT-CYB*; ca. 801 bp) was PCR amplified with primers L14724 (Irwin *et al.* 1991) and MVZ16 (Silva and Patton 1993), with a pre-denaturation step at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 60 sec, and final extension of 72°C for 2 min. The mitochondrial cytochrome *b* gene was referred as *MT-CYB* following HGNC rules (Eyre *et al*. 2006, HGNC 2009).

 Amplicons were purified with GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Brazil). Sequencing reactions were performed with L14724, cit-alo (Bonvicino *et al*. 2001), AloAotR (Nascimento *et al*. 2007), AloAotF (Nascimento *et al*. 2005). Amplicons were labeled with XL and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and loaded to an ABI PrismTM 3130 platform.

 Sequences were edited and assembled with ChromasPro (version 1.7.5; available at www.technelysium.com.au/chromas.html). Phylogenetic analyses included 26 sequences of *A. belzebul* and from seven other Alouatines deposited in GenBank (Table 1). *Brachyteles arachnoides* (GenBnk JX262672) was used as outgroup.

 Genetic distance estimates were carried out with complete deletion using Kimura’s two parameters, with MEGA (version 5; Tamura *et al.* 2011). The HKY+G was selected as the DNA substitution model with MODELGENERATOR (version 0.85; Keane *et al.* 2006) using the Bayesian information criterion (BIC) for phylogenetic reconstructions.

 Phylogenetic reconstructions based on maximum likelihood (ML) were carried out with PHYML (version 3.0; Guindon *et al*. 2010). Branch support was calculated with the approximate likelihood ratio test (aLRT) with SH-like interpretation, a procedure that is conservative and accurate as bootstrapping but less computationally intensive (Anisimova and Gascuel 2006, Guindon *et al*. 2010). Bayesian analysis (BA) was carried out with MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003). DNAsp 5 was used for haplotype estimates and nucleotide diversity (Librado and Rozas 2009).

 NETWORK (version 4.6.1.1; available at http://www.fluxus-engineering.com) was used for reconstructing a median-joining (MJ) network (Bandelt *et al*. 1999) to evaluate sub-population structure and geographic distribution patterns. MJ was calculated using variable sites only.

**RESULTS**

*Karyotype*

 Karyotypic analysis of a single male *Alouatta ululata* (CPB71) showed2n = 49, The chromosome complement comprised 11 metacentric or submetacentric pairs and 13 acrocentric pairs (Figure 2). G- banding allowed for the identification of all chromosomes pairs (Figure 2).

Figure 2 here

*Molecular phylogeny*

 *Alouatta ululata* together with 26 *A. belzebul* sequences retrieved from GenBank accounted for 16 haplotypes, 11 of which being unique and five were shared by different individuals (Table 1). Haplotype H3 from Pará (Table 1) was the most frequent one. The ML topology was similar to the one obtained by Bayesian analysis (BA; Figure 3), except in relation to the position of *A. fusca*. ML placed *A*. *fusca* as the most basal offshoot, without support, while BA placed *A. caraya* as the most basal offshoot. ML and BA were coincident in grouping (*A. seniculus* (*A. nigerrima, A. macconnelli*)), with low support and *A. belzebul* and *A*. *ululata*, with high support. The *A. ululata* haplotype grouped within the clade of *A. belzebul* (Figure 3).

 MJ analyses (Figure 4) showed a similar result to ML and BA in relation to *A. ululata* and *A*. *belzebul*. Haplotype from *A. ululata* was directly connected with haplotypes from Tocantins (H2). This analysis coincided in showing lack of structure between haplotypes of *A. belzebul* and *A. ululata*.

**DISCUSSION**

*Karyotype*

The *Alouatta ululata* karyotype was similar to the one described for *A. belzebul* by Armada *et al*. (1987) in morphology and the G-band patterns (Figure 2). The Y chromosome of Alouatta ulultata could not be identified with conventional staining but, as in *Alouatta belzebul*, it was apparently translocated to one member of a heteromorphic autosome chromosome pair (No. 17). Conversely, in females, this translocation was not present (Lima and Seuánez 1989). Based on chromosome similarity, *A. belzebul* and *A.* *ululata* are likely to belong to the same species.

Figures 3 and 4 here

*Phylogenetic relationships*

 Molecular analysis showed the close relationship between *A. seniculus*, *A. macconnelli* and *A. nigerrima*, corroborating previous reports based on cytochrome *b* (Bonvicino *et al*. 2001), and mitochondrial and nuclear genes (Cortés-Ortiz *et al*. 2003). The close affinity between *A. fusca* and *A. belzebul,* herein recoveredby BA analysis, was also shown in previous reports based on g1-globin pseudogene (Meireles *et al*. 1999), cytochrome *b* DNA (Bonvicino *et al*. 2001) and several mitochondrial and nuclear genes (Cortés-Ortiz *et al*. 2003). This close relationship, however, was not evident by multicolor, cross-species chromosome painting (de Oliveira *et al*. 2002).

 The *A. belzebul* clade showed a poor resolution with several polytomies. This was probably due to the predominant composition of *A. belzebul* from Pará state (area 2), contrary to *A. ululata* (area 4, H1), and one specimen from the Atlantic Forest (area 1, H5). Specimens from area 3 from the Rio Tocantins basin are extremely polymorphic and likely to be from either bank or from river islands (Bonvicino *et al*. 1989). Samples from area 1, the Atlantic forest, and area 4 (*A. ululata*), are well nested inside the clade, indicating that these forms are conspecific. MJ analyses corroborated ML analysis showing lack of structure between *Alouatta belzebul* and *Alouatta ululata* haplotypes and strongly indicated that cytochrome *b* haplotypes did not provide evidence of their separation. However, this analysis were carried out with a single molecular marker. Incomplete lineage sorting may result in a different *MT-CYB* tree from the species tree.

*Morphology*

 Color pattern of rufous mid dorsum (pattern D4 of Bonvicino et al. 1989) is common to areas 2 in Pará and 4 in Ceará state. Two other patterns also with rufous mid dorsum (D3 and D1) are common to areas 4 in Maranhão and 3 in Pará (Tocantins), while three other patterns of black dorsum (C1, C3 and C4) are unique to area 4 in Ceará and Maranhão states (*ululata*). Since black body may be considered a plesiomorphic character (shared by all areas), we may hypothesize that gene flow took place from west to east. This may also support the hypothesis of conspecificity. As some specimens from area 4 show sexual dimorphism, the significance of this characteristic with respect to reproductive barriers should be investigated.

 Phylogeographic analyses will require a larger number of specimens to show relationships between populations. Special attention should be paid to the basin of the middle/lower course of Rio Tocantins to explain its large howler diversity and to determine whether the remaining populations dispersed from this region.

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Table 1. List of *Cytochrome b* sequences used in phylogenetic analyses, with haplotypes number (H), GenBank accession number (GB no.) and collection locality. Brazilian states (BRA) are Amazonas (AM), Pará (PA), Piauí (PI), Paraíba (PB), Goiás (GO), Mato Grosso (MT), and Santa Catarina (SC). Localities numbers are the same as in figure 1.

|  |  |  |  |
| --- | --- | --- | --- |
| H | **Taxa** | **GB no.** | **Collection site** |
| 1 | *Alouatta ululata* | CPB71 | BRA: PI, Campo Maior (1) |
| 2 | *Alouatta belzebul* | AF289511 | BRA: PA, Tucuruí (31) |
| 3 | *Alouatta belzebul* | DQ387025, AY374344, AY374347, AY374350, AY374353, AY374354 | BRA: PA, Tucuruí (31) |
| 4 | *Alouatta belzebul* | AY374349 | BRA: PA, Tucuruí (31) |
| 5 | *Alouatta belzebul* | DQ387044 | BRA: PB, unknown locality |
| 5 | *Alouatta belzebul* | DQ398008, AF289515, DQ398009 | Brasil, PB, Sapé (10) |
| 6 | *Alouatta belzebul* | DQ387042, AY374351 | BRA: PA, Tucuruí (31) |
| 7 | *Alouatta belzebul* | AY374343, T4-6a1998 | BRA: PA, Tucuruí (31) |
| 8 | *Alouatta belzebul* | AY374352 | BRA: PA, Tucuruí (31) |
| 9 |

*Alouatta belzebul*