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Molecular systematics and biogeography of the Neotropical monkey genus, *Alouatta*

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Abstract

We take advantage of the broad distribution of howler monkeys from Mexico to Argentina to provide a historical biogeographical analysis on a regional scale that encompasses the entire Neotropics. The phylogenetic relationships among 9 of the 10 recognized *Alouatta* species were inferred using three mitochondrial and two nuclear genes. The nuclear gene regions provided no phylogenetic resolution among howler monkey species, and were characterized by very low levels of sequence divergence between *Alouatta* and the *Ateles* outgroup. The mtDNA genes, on the other hand, produced a well-resolved phylogeny, which indicated that the earliest split among howler monkeys separated *cis*- and *trans*-Andean clades. Eight monophyletic mtDNA haplotype clades were identified, representing six named species in South America, including *Alouatta seniculus*, *Alouatta sara*, *Alouatta macconelli*, *Alouatta caraya*, *Alouatta belzebul*, and *Alouatta guariba*, and two in Mesoamerica, *Alouatta pigra* and *Alouatta palliata*. Molecular clock-based estimates of branching times indicated that contemporary howler monkey species originated in the late Miocene and Pliocene, not the Pleistocene. The causes of *Alouatta* diversification were more difficult to pin down, although we posit that the initial *cis*-, *trans*-Andean split in the genus was caused by the late Miocene completion of the northern Andes. Riverine barriers to dispersal and putative forest refuges can neither be discounted nor distinguished as causes of speciation in many cases, and one, the other or both have likely played a role in the diversification of South American howler monkeys. Finally, we estimated the separation of Mesoamerican *A. pigra* and *A. palliata* at 3 Ma, which corresponds to the completion date of the Panama Isthmus promoting a role for this earth history event in the speciation of Central American howler monkeys.

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1. Introduction

At least since Wallace's (1852) introduction of the riverine barrier hypothesis, models seeking to explain the stunning species richness of the Neotropics have garnered considerable attention. More recently, attention has focused on Haffer's (1969) Pleistocene Refuge

Hypothesis, but the high species diversity of the Neotropics, and particularly the Amazon region, has also been posited to result from parapatric divergence due to sharp ecological gradients (Endler, 1977, 1982), floodplain dynamics (Salo et al., 1986), long-term paleoclimatic cycles (Haffer, 1993) or shifts (Bush, 1994), and ecological heterogeneity (Tuomisto et al., 1995). No agreement has been reached regarding the general validity of any of these speciation models, and none are mutually exclusive; thus discriminating among the different processes that may be important in Neotropical

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speciation requires phylogenetic data for more organisms, and experiments capable of falsifying alternative hypotheses (e.g., Patton and Smith, 1992; Patton et al., 1994). Towards these ends we have undertaken a historical biogeographic analysis of howler monkeys,

which provides phylogenetic data for a widespread Neotropical primate, and permits assessment of the roles played by Andean orogeny, the rise of the Panama Isthmus, rivers and forest refuges in the diversification of *Alouatta*.

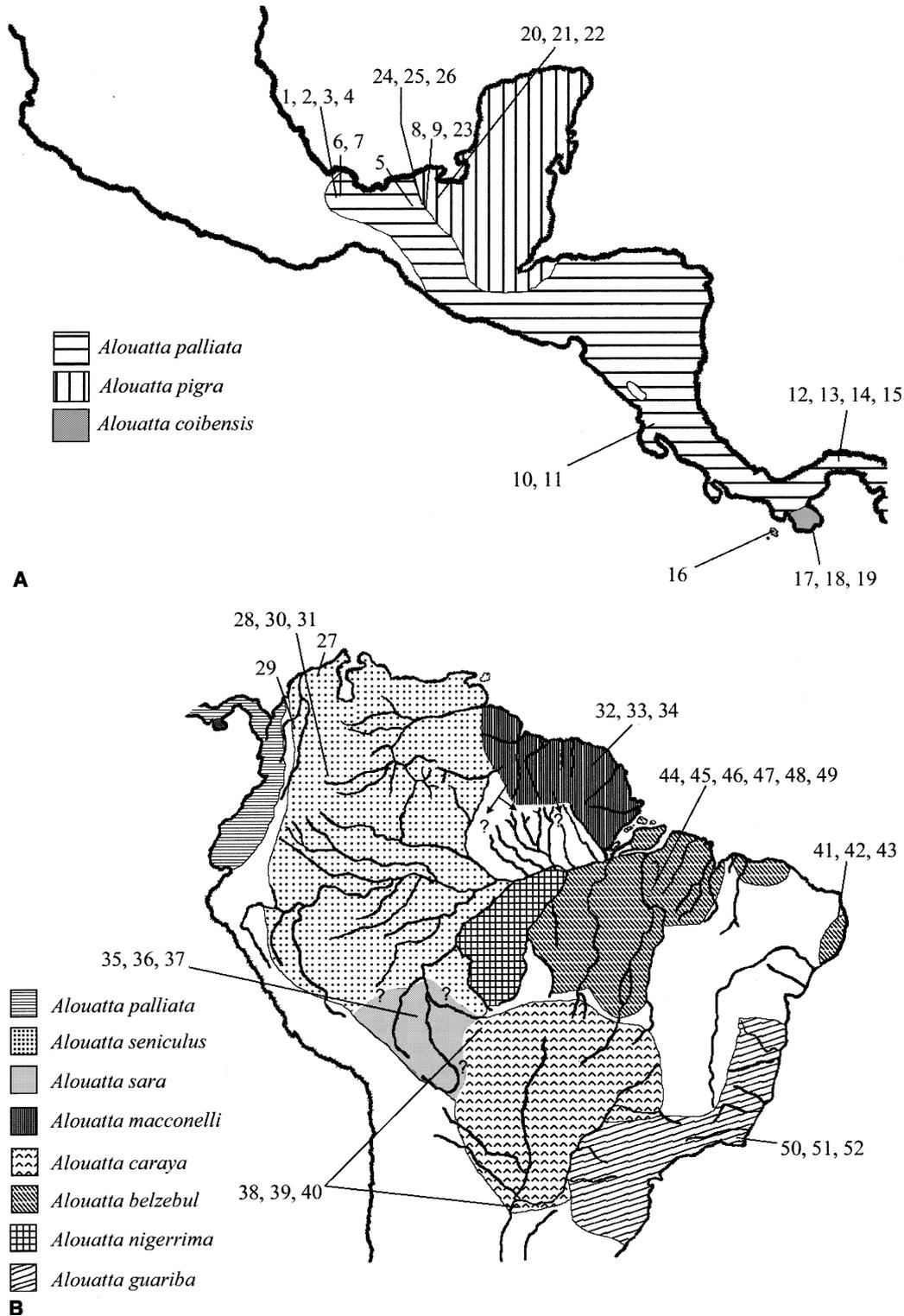


Fig. 1. Distribution of *Alouatta* species and subspecies (modified from Hill, 1962). Numbers show approximate locations for all samples used in this study (see Table 1). (A) Mesoamerican and (B) South American *Alouatta* species (following Groves, 2001).

The distribution of howler monkeys extends from Southern Veracruz State in Mexico, through Central and South America to northern Argentina (Fig. 1). *Alouatta* inhabits the widest range of environments of any Neotropical primate species (Crockett and Eisenberg, 1987), and can be found from sea level to 3200 m, although different species vary in habitat preferences (Crockett, 1998). For example, *Alouatta palliata* and *A. seniculus* occur in habitats ranging from closed-canopy wet evergreen forest to highly seasonal deciduous woodlands and riverine forests, whereas *A. belzebul* is basically a forest species (Crockett, 1998). Most of the species also occur in fragments of forest beside cattle ranches or in agricultural areas (see Crockett, 1998, LCO, pers. obs.), indicating their tolerance to habitat disturbance. Thus only drastic environmental changes or direct perturbation are likely to cause howler monkeys to disappear from an area. As a result, allopatric speciation would presumably take place only across strong barriers to dispersal.

Despite being one of the most studied New World primate genera, the systematic relationships of *Alouatta* species remain unclear (Crockett, 1998; Rylands and Brandon-Jones, 1998; Rylands et al., 2000), and the causes of the evolutionary diversification of howler monkeys is poorly understood. Recent reviews have indicated a lack of consensus regarding the taxonomic status and phylogenetic relationships of *Alouatta* species and geographic populations, pointing to a need for a systematic revision of the genus (Crockett, 1998; Rylands et al., 1995, 2000).

Towards this end we used the mitochondrial cytochrome *b* (cyt *b*) and ATP synthase 8 and 6 genes (ATPase 8/6), and the nuclear-encoded Recombination Activating 1 (RAG1) and Calmodulin (CAL) genes to infer the phylogenetic relationships of nine of the ten *Alouatta* species recognized by Groves (2001). In turn, we used our molecular systematic assessment of *Alouatta* to address the following questions: (1) How does the molecular description of *Alouatta* phylogenetic lineages correspond to described species and subspecies? (2) Are Central and South American howler monkey species reciprocally monophyletic? (3) What are the temporal and spatial patterns of *Alouatta* diversification? (4) Can the speciation history of *Alouatta* be tied to particular geological events, geographical landscape features, and/or particular models for the diversification of Neotropical organisms?

1.1. Systematic background

Hill (1962) proposed that *Alouatta* is comprised of five or six species, but the most recent taxonomic treatments of *Alouatta* recognize 9 or 10 species and up to 19 subspecies (Groves, 2001; Rylands et al., 1995, 2000). Mesoamerican howlers are assigned to three

species mapped in Fig. 1A: (1) *A. palliata* with three subspecies and a *trans*-Andean distribution ranging from Mexico to Ecuador; (2) *A. pigra* restricted to the Caribbean slope from Mexico to northern Honduras; and (3) *A. coibensis* with two subspecies, one found on the Pacific island of Coiba and the second on the adjacent mainland (Azuerro Peninsula, Panama).

South American howlers comprise the remaining 6–7 species (Fig. 1B): (1) *A. seniculus*, with three to six subspecies and a distribution limited by the western cordillera of the Colombian Andes, and covering much of northern South America above the Amazon River; (2) *A. macconelli* (not recognized in the species taxonomy proposed by Rylands et al., 2000) found in Surinam, Guyana, French Guiana, and Brazil, to the north of the lower and middle Amazon; (3) *A. sara*, apparently restricted to the Bolivian Amazon; (4) *A. belzebul*, with three subspecies and a distribution extending south of the main stem of the Amazon River, and east of the Tapajos River to the northern coast of Brazil's Atlantic forest; (5) *A. nigerrima*, also bounded to the north by the Amazon River, to the east and west by the Trombetas and Madeiras rivers, respectively, and to the south by its putatively parapatric boundary with *Alouatta caraya*; (6) *A. guariba*, with two subspecies allopatrically distributed in the northern and southern halves of Brazil's Atlantic forest; and (7) *A. caraya*, restricted to southern Brazil, Paraguay, and northern Argentina.

Our molecular systematic analysis of *Alouatta* includes all the species named above except *A. nigerrima*, and all Mesoamerican howler subspecies ($n = 5$).

2. Materials and methods

2.1. Samples, DNA extraction, amplification, and sequencing

Blood, fecal, or skin samples of 53 howler monkeys were either collected by the authors or obtained through donation (Table 1). In total, samples from 12 distinct *Alouatta* taxa (including species and subspecies) were included in our study. We also included in our analyses samples from two *Ateles* species, representing four subspecies ($n = 10$), *Brachyteles arachnoides* ($n = 2$), and *Cebus capucinus* ($n = 1$) as outgroups. For the most part we follow Groves (2001) treatment for assignment of species names; however, the taxonomic validity of the name *A. macconelli* is uncertain (Rylands and Brandon-Jones, 1998). We used this name to refer to howler monkeys from French Guiana, as proposed by Lima and Seuánez (1991) and Vassart et al. (1996) because they possess karyotypes that are unique to those of *A. seniculus*.

We preserved samples in different buffers according to tissue type: skin and fecal samples were preserved in a

Table 1
Taxa, number of individuals (N), geographical location and donors of samples used in this study

Taxa (N)	Locality	Collector	Reference to figures
<i>Alouatta palliata mexicana</i> (4)	Cascajal del Río, Veracruz, Mexico	INE, UV ^a	1, 2, 3, 4
<i>Alouatta palliata mexicana</i> (1)	Guarda Costa, Tabasco, Mexico	INE, UV ^a	5
<i>Alouatta palliata mexicana</i> (2)	Rancho Camarón, Veracruz, Mexico	INE, UV ^a	6, 7
<i>Alouatta palliata mexicana</i> (2)	CG 1a.secc., Tabasco, Mexico	INE, UV ^a	8, 9
<i>Alouatta palliata palliata</i> (2)	La Pacífica, Costa Rica	K. Glander	10, 11
<i>Alouatta palliata aequatorialis</i> (4)	Barro Colorado Island, Panama	L. Cortés Ortiz	12, 13, 14, 15
<i>Alouatta coibensis coibensis</i> (1)	Coiba Island, Panama	L. Cortés Ortiz	16
<i>Alouatta coibensis trabeata</i> (3)	Río Oria, Azuero, Panama	L. Cortés Ortiz	17, 18, 19
<i>Alouatta pigra</i> (3)	Rancho San Simón, Chiapas, Mexico	INE, UV ^a	20, 21, 22
<i>Alouatta pigra</i> (1)	CG 1a.secc., Tabasco, Mexico	INE, UV ^a	23
<i>Alouatta pigra</i> (3)	CG 2a.secc., Tabasco, Mexico	INE, UV ^a	24, 25, 26
<i>Alouatta seniculus seniculus</i> (3)	Colombia (Cali Zoo)	L. Cortés Ortiz	27, 28, 29
<i>Alouatta seniculus</i> (2)	Colombia	M. Ruiz	30, 31
<i>Alouatta macconelli</i> (3)	French Guiana	M. Ruiz	32, 33, 34
<i>Alouatta sara</i> (3)	Bolivia	N.I. Mundy	35, 36, 37
<i>Alouatta caraya</i> (3)	Santa Cruz, Bolivia	M. Ruiz	38, 39, 40
<i>Alouatta belzebul belzebul</i> (3)	Pacatuba, Paraíba, Brazil	D. Canales	41, 42, 43
<i>Alouatta belzebul belzebul</i> (6)	Tocantins River, Pará, Brazil	I. Sampaio	44, 45, 46, 47, 48, 49
<i>Alouatta guariba</i> (3)	Rio de Janeiro, Brazil	I. Sampaio	50, 51, 52
<i>Ateles geoffroyi vellerosus</i> (3)	Mexico	INE, UV ^a	53, 54, 55
<i>Ateles geoffroyi yucatanensis</i> (3)	Can Cun, Q. Roo, Mexico	INE, UV ^a	56, 57, 58
<i>Ateles geoffroyi yucatanensis</i> (1)	Chetumal, Q. Roo, Mexico	INE, UV ^a	59
<i>Ateles geoffroyi panamensis</i> (1)	Panama (Summit Zoo, Panama)	L. Cortés Ortiz	60
<i>Ateles fusciceps robustus</i> (2)	Panama (Summit Zoo, Panama)	L. Cortés Ortiz	61, 62
<i>Brachyteles arachnoides</i> (3)	Minas Gerais, Brazil	I. Sampaio	63, 64, 65
<i>Cebus capucinus</i> (1)	Panama (Summit Zoo, Panama)	L. Cortés Ortiz	66

Numbers in the last column correspond to numbers in Figs. 3–5.

^a Collected by the group of researchers of the Institute of Neuro-ethology (INE), Universidad Veracruzana (UV): D. Canales-Espinosa, F. García-Orduña, L. Cortés-Ortiz, and E. Rodríguez-Luna.

saturated salt solution (NaCl) of 20% dimethyl sulfoxide and 0.5 M disodium ethylenediaminetetraacetate (EDTA), and blood samples were preserved in Lysis buffer (100 mM Tris/HCl, pH 8, 100 mM EDTA, pH 8, 10 mM NaCl, 0.5% SDS) (Frantzen et al., 1998; Seutin et al., 1991). All samples were stored at -20°C (fecal samples) or at ambient temperature (tissue and blood). Samples, either 100 μl of blood or a small tissue or fecal sample, were digested overnight at 54°C in 500 μl of $2\times$ CTAB buffer solution and 10 μl of 10 mg/ml proteinase K solution. Total genomic DNA was extracted using phenol/chloroform methods (Palumbi et al., 1991; Sambrook et al., 1989), and purified by dialysis rather than ethanol precipitation.

We amplified the entire mitochondrial ATP synthase 8 and 6 genes (ATP8 = 201 bp, and ATP6 = 681 bp), and partial cytochrome *b* gene sequences (cyt *b*, 800 bp). In addition, we amplified 213 bp of the nuclear calmodulin gene (CAL, containing an intron of 160 bp, and 53 bp of the flanking exons 3 and 4 according to the human sequence X52608 in GeneBank) and 1440 bp of the recombination activating gene 1 (RAG1). Double stranded DNA was synthesized in 25 μl reaction volumes under the following conditions: 1–2 μl of DNA solution, $1\times$ PCR buffer (Perkin–Elmer, Forest City, CA), 2 mM of MgCl_2 , 0.8 mM dNTPs, 0.5 μM of each primer, and 0.65 U of Amplitaq polymerase

(Perkin–Elmer, Forest City, CA). Amplification primers and thermocycler conditions for the different genes are shown in Table 2.

Amplification products were electrophoretically separated from unincorporated primers and dNTPs in 1.3% low-melting-agarose gels run in TAE (Tris–acetate low-EDTA buffer, pH 7.8) containing ethidium bromide (1 $\mu\text{g}/\text{ml}$). The single amplification product was cut and heated at 70°C for 5 min, followed by agarose digestion using 1 μl GELase (Epicentre Technologies, Madison, WI) incubated at 45°C for 3 hr. All calmodulin amplification products and a small number of mitochondrial amplifications were cloned using a pGEM vector (Promega) into *E. coli* competent cells; 3–5 clones representing each individual were screened for sequencing. RAG products were sequenced directly without cloning.

Three to five microliters of the purified PCR product was used as a template in a 10 μl cycle sequence reaction volume using the Applied Biosystems (ABI) Taq Dye-Deoxy Terminator Cycle sequencing kit and protocol. Sequencing primers are provided in Table 2. The cycle sequencing product was diluted in 10 μl of distilled–deionized water, purified over Centriscap columns filled with 780 μl of G-50 Sephadex, and then run on an 377 Automated DNA Sequencer following the ABI protocol.

Table 2
Primers and PCR conditions used to amplify different genes in this study

Gene	Primers (E, I) ^a	Sequence	Reference	PCR conditions ^b
Mitochondrial cytochrome <i>b</i>	GLUDG-5' (E)	TGA CTT GAA RAA CCA YCG TTG	Palumbi (1996)	ID 94 °C/3 min, D 94 °C/45 s, A 42 °C/45 s
Mitochondrial cytochrome <i>b</i>	CB3-3' (E)	GGC AAA TAG GAA RTA TCA TTC	Palumbi (1996)	E 72 °C/1 min 30 s, NC = 35, FE 72 °C/3 min
Mitochondrial cytochrome <i>b</i>	CB1-5' (I)	CCA TCC AAC ATC TCA GCA TGA TGA AA	Palumbi (1996)	
Mitochondrial cytochrome <i>b</i>	CB2-3' (I)	CCC TCA GAA TGA TAT TTG TCC TCA	Palumbi (1996)	
Mitochondrial cytochrome <i>b</i>	CB-435L (I)	ATA TCA TTC TGA GGG GCC ACA GT	This paper	
Mitochondrial ATP-synthase 6 and 8	LCO-CO2-L (E)	TAR GCR TGT GWT TGG TGG GTC ATT A	This paper	ID 94 °C/3 min, D 94 °C/45 s, A 50 °C/45 s
Mitochondrial ATP-synthase 6 and 8	LCO-CO3-H (E)	AGC ATT AAC CTT TTA AGT TAA AGA TT	This paper	E 72 °C/1 min 30 s, NC = 30, FE 72 °C/3 min
Mitochondrial ATP-synthase 6 and 8	ATP6-467H (I)	TTG GCT GTT AGT CGT ACG GCT A	This paper	
Mitochondrial ATP-synthase 6 and 8	ATP6-CO3H (I)	TGT GTT TGG TGG GTC ATT A	This paper	
Mitochondrial ATP-synthase 6 and 8	ATP6-640L (I)	GCA CTA GCC GTA CGA CT	This paper	
Calmodulin	CAL1 (E)	GCC GAG CTG CAR GAY ATG ATC AA	Duda and Palumbi (1999)	ID 94 °C/3 min, D 94 °C/30 s, A 50 °C/30 s
Calmodulin	CAL2 (E)	ATG ATG GCA MGN AAA ATG AAG GAC AC	Duda and Palumbi (1999)	E 72 °C/45 s, NC = 40, FE 72 °C/3 min
Recombination activating gene-1	RAG1F (E)	B. Quenouille, unpublished ^c	Unpublished	ID 94 °C/7 min, D 94 °C/45 s
Recombination activating gene-1	RAG9R (E)	B. Quenouille, unpublished ^c	Unpublished	A 58 °C/45 s –0.5° per cycle
Recombination activating gene-1	RAG3F (I)	B. Quenouille, unpublished ^c	Unpublished	E 72 °C/1 min 45 s
Recombination activating gene-1	RAG7R (I)	B. Quenouille, unpublished ^c	Unpublished	NC = 10, D 94 °C/45 s, A 53 °C/45 s
Recombination activating gene-1	RAG3sb (I)	B. Quenouille, unpublished ^c	Unpublished	E 72 °C/1 min 45 s
Recombination activating gene-1	RAG5rb (I)	B. Quenouille, unpublished ^c	Unpublished	NC = 25, FE 72 °C/7 min

^a E, external to the region amplified, used for amplification and sequencing; I, internal, used only for sequencing.

^b ID, Initial denaturation; D, denaturation; A, annealing; E, extension; NC, number of cycles (from D to E); FE, final extension.

^c Primer sequences are available on request to E. Bermingham.

Following gel electrophoresis, chromatograms were edited and aligned using Sequencher 3.1 (Gene Codes Corporation). Nucleotide sequences were visually checked for miscalls due to either bad base spacing or overfluorescence of particular dye nucleotides, reading frame errors, and termination codons.

2.2. Molecular characterization and phylogenetic analysis

We used MacClade 3.07 (Madison and Madison, 1997) to identify different haplotypes for each gene as well as to analyze base composition frequency and determine nucleotide-coding position for protein-coding regions. SeqEd v1.0.3 (Applied Biosystems) was utilized to identify amino acid substitutions. We assessed levels

of saturation for our data set of mtDNA genes by plotting corrected pairwise distances with numbers of transitions and transversions among species (Brown, 1983; Griffiths, 1997).

Phylogenetic analyses were conducted using maximum likelihood (ML), distance (Neighbor Joining, NJ) and maximum parsimony (MP) methods implemented in PAUP 4.0b4a (Swofford, 2000). MODELTEST 3.0 (Posada and Crandall, 1998) was utilized to select the model of nucleotide substitution that best fits our data. We executed a partition homogeneity test (Farris et al., 1994) of mitochondrial combined data using PAUP, to ensure that the data sets for those genes were congruent and could be analyzed together. Distance and MP (heuristic search) analyses were conducted on combined

mitochondrial data for all individuals. We used 1000 bootstrap replicates to assess confidence of the MP and NJ trees. Because of the impracticality of using all individuals to construct a ML tree, we selected two individuals with maximum sequence divergence to represent each of the 8 principal *Alouatta* mtDNA lineages. ML analyses were utilized to construct trees for the combined mitochondrial data and the two nuclear gene sequences (RAG1 and CAL).

2.3. Molecular clock analysis and calibration

To test the constancy of rates of evolution across *Alouatta*, *Ateles*, and *Brachyteles* (Family Atelidae) sequences, we used the two-cluster and branch-length analyses implemented in Lintree (Takezaki et al., 1995). Because outgroups are not included in the Lintree analyses, we used *C. capucinus* as the excluded outgroup, and then compared relative rate constancy for our ingroup (*Alouatta*) and near outgroup (*Ateles* and *Brachyteles*) clades. We also repeated the Lintree analyses using *Ateles fusciceps* as the excluded outgroup, and *Alouatta* as the ingroup. In addition, we used PAUP to calculate the $-\ln L$ values for non-clock and clock-enforced ML trees, and evaluated significance using the Kishino and Hasegawa (1989) approach.

Owing to insufficient fossil data for calibrating an Atelidae molecular clock, we utilized Sanderson's (1997) r8s program to calculate the ages of nodes in an ML mtDNA tree inferred for humans (GenBank: X62996), chimps (GenBank: D38113), and the New World monkeys included in this study. The Sanderson method requires at least one estimated divergence date and we sequentially used a human-chimp split of 7 Ma (Hulsenbeck et al., 2000) and a New World, Old World monkey separation of 40 Ma (Gingerich, 1984; Goodman et al., 1998) to date the split between Atelines and Alouattines. The date estimated in this fashion was very similar to the split estimated for Atelinae and Alouattinae based on nuclear-encoded ϵ -globin and interstitial retinol-binding protein gene (IRBP) intron 1 (Goodman et al., 1998). Thus, with some confidence we used our estimated date of separation between Atelinae and Alouattinae to infer the ages of nodes for *Alouatta*, *Ateles*, and *Brachyteles* using both a clock-enforced ML tree and a local molecular clock approach (Bailey et al., 1992; Schneider et al., 1993).

3. Results

We sequenced a total of 1642 mitochondrial base pairs for 50 *Alouatta* individuals and 12 individuals representing three other Neotropical primate genera (*Ateles*, *Brachyteles*, and *Cebus*) (GenBank Accession Nos. AY065845–AY065907). Much fewer individuals of

Alouatta and *Ateles*, and no individuals of *Cebus* and *Brachyteles* were sequenced for the nuclear-encoded RAG1 ($n = 12$) (GenBank Accession Nos. AY065908–AY065919) and CAL loci ($n = 16$) (GenBank Accession Nos. AY065920–AY065945), because phylogenetic analysis based on preliminary results indicated that these loci showed very low levels of sequence divergence.

3.1. Molecular characterization

Among the taxa sampled, the ATPase 8 and 6 genes overlapped by 40 bp, as compared to the 46 bp overlap observed for chimpanzees, humans, and orangutans. All three mitochondrial genes began with the ATG start codon and terminated with TAA for all individuals, with the exception of the *A. caraya* in which the ATPase 8 gene began with GTG. (The termination codon for cyt *b* was not determined owing to the partial gene sequence obtained). Although nuclear insertions of mtDNA fragments have been reported for primates (Collura and Stewart, 1995; Mundy et al., 2000), three lines of evidence indicate that we have sequenced functional mtDNA genes: (1) the lack of stop codons in the reading frame of our sequences, (2) the lack of ambiguities in our sequences suggesting that only one copy is present per individual; and (3) genes on opposite sides of the mitochondrial genome have similar rates of evolution (Table 3). This evidence would only be compatible with a recent translocation of a mtDNA fragment greater than 8 kb in length which we consider improbable (although see Lopez et al., 1997).

Variable and parsimony informative sites for each gene, by codon position, are shown in Table 3. The estimated mtDNA transition to transversion ratio (ti/tv) among *Alouatta* species was 15.4, versus 10.6 when *Ateles* and *Brachyteles* sequences were included in the analysis. For the 1440 bp RAG1 sequence the seven variable sites observed among *Alouatta* species were transitions, whereas in the 213 bp CAL fragment we observed 13 transitions and two transversions (Table 3). The three *Alouatta* individuals from Mesoamerica sequenced for RAG1 had no substitutions among them, while the 5 RAG1 sequences representing South American howlers had from 0 to 6 substitutions. The maximum level of RAG1 divergence observed between *A. guariba* and *A. belzebul* of six substitutions, compares to 106 changes between the same pair calculated on the basis of the 1642 bp of mitochondrial sequence. The same comparison based on the much smaller CAL fragment yields three substitutions. The CAL intron provided six parsimony informative sites among *Alouatta* species, versus 41 informative sites in the 201 bp ATPase 8 gene.

A partition homogeneity test (Farris et al., 1994) on the combined mtDNA data (three partitions; 1642 bp) indicated that the gene regions did not differ significantly

Table 3
Variable site statistics for each gene with respect to *Alouatta* species and *Alouatta* species and outgroups

		ATPase 8	ATPase 6	cyt <i>b</i>	RAG 1	CAL
<i>Alouatta</i> species	Variable sites	45 (22%)	157 (23%)	134 (17%)	7 (0.48%)	15 (7%)
	First position	15 (33%)	30 (19%)	15 (11%)	1 (14%)	
	Second position	7 (16%)	21 (13%)	7 (5%)	1 (14%)	
	Third position	23 (51%)	106 (68%)	112 (84%)	5 (72%)	
	Informative sites	41 (91%)	145 (92%)	131 (98%)	2 (28%)	6 (40%)
	Amino acid changes	15 (22%)	33 (14%)	15 (5.6%)	2 (0.42%)	
<i>Alouatta</i> and outgroups (<i>Ateles</i> and <i>Brachyteles</i>)	Variable sites	67 (33%)	233 (36%)	227 (28%)	24 (1.6%)	20 (9.4%)
	First position	21 (31%)	58 (25%)	36 (16%)	3 (13%)	
	Second position	14 (21%)	30 (13%)	13 (6%)	2 (8%)	
	Third position	32 (48%)	145 (62%)	178 (78%)	19 (79%)	
	Informative sites	62 (93%)	225 (97%)	220 (97%)	20 (83%)	11 (55%)
	Amino acid changes	27 (40%)	59 (28%)	37 (14%)	4 (0.84%)	

($p = 0.98$). Thus all subsequent mtDNA analyses are based on the combined data. The Tamura–Nei model (TrN; Tamura and Nei, 1993) with gamma (G) distribution was identified by MODELTEST as the best nucleotide substitution model for the *Alouatta* mtDNA combined data, using *Ateles* and *Brachyteles* as outgroups. Nucleotide frequencies were estimated from the data (A: 0.315, C: 0.306, G: 0.102, and T: 0.277), as well as the substitution rates among transitions (A–G: 19.86 and C–T: 22.51). The estimated gamma distribution shape parameter was 0.2818. In turn, we compared the TrN + G tree to one incorporating different substitution rate categories by codon position. The second tree had a significantly better $-\ln L$ score (6489.41 versus 6624.73) and thus we utilized the TrN model with variable codon position substitution rates for our following ML analyses, but TrN + G was used to build the NJ trees.

In the case of RAG1, MODELTEST indicated that the HKY model (Hasegawa et al., 1985) ($I = 0.936$, $ti/tv = 28.85$, $A = 0.271$, $C = 0.216$, $G = 0.265$, and $T = 0.248$) was the best fit to our data. The $-\ln L$ value for the RAG1 tree was 2152.93. The HKY model ($I = 0$, ti/tv ratio = 10.59, $A = 0.192$, $C = 0.341$, $G = 0.276$, and $T = 0.192$) was also the best fit to the CAL data, and so we used HKY model of evolution to construct our tree.

Fig. 2 presents a graphical analysis of uncorrected transition and transversion substitutions plotted against the combined mtDNA data corrected using the TrN + G model of nucleotide substitution. Transversions show no evidence for saturation at any phylogenetic level among the platyrrhine primates studied here. Transitions also show no signal of saturation for comparisons among congeneric species, however, the slope of the relationship between transitions and corrected mtDNA genetic distance decreases for comparisons between the genera indicating some nucleotide saturation at the deeper nodes in the mtDNA tree. No comparable analysis was undertaken for the nuclear genes given the small number of variable sites.

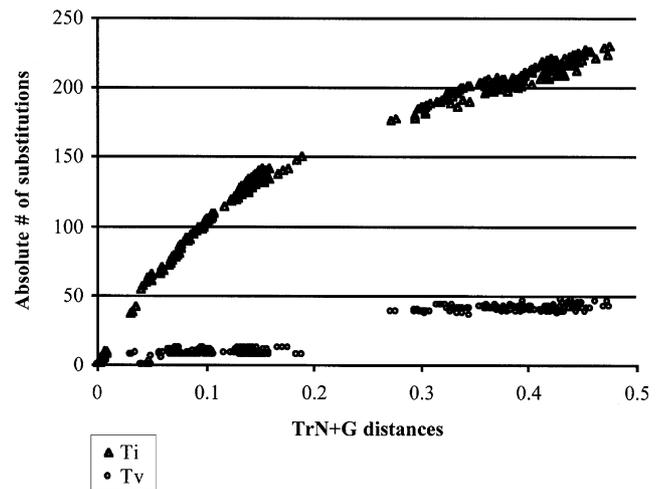


Fig. 2. Saturation assessment for combined mitochondrial data (ATPase 6/8 and cyt *b*). Numbers of transitions (Ti) and transversions (Tv) are plotted against TrN + G pairwise distances for all codon positions.

3.2. Phylogenetic analysis

We observed 28 ATPase 8/6 and 26 cyt *b* haplotypes for the 61 individuals that were sequenced for all three mtDNA genes; the combined data yielded 31 unique mtDNA haplotypes. Neighbor-joining analysis based on TrN + G genetic distances yielded a well-resolved phylogeny for *Alouatta* and outgroup taxa (Fig. 3). A consensus MP cladogram (not shown), based on the two shortest parsimony trees, is fully concordant with the NJ phenogram. Bootstrap values for both the MP and NJ trees are presented in Fig. 3 and show consistently high levels of support for the *Alouatta* phylogenetic relationships detailed below.

Fig. 3 identifies two reciprocally monophyletic clades representing Mesoamerican and South American *Alouatta* species. The average mitochondrial distance between these two major clades is 12.7%. Within the Mesoamerican clade, *A. palliata* and *A. pigra* are well-resolved

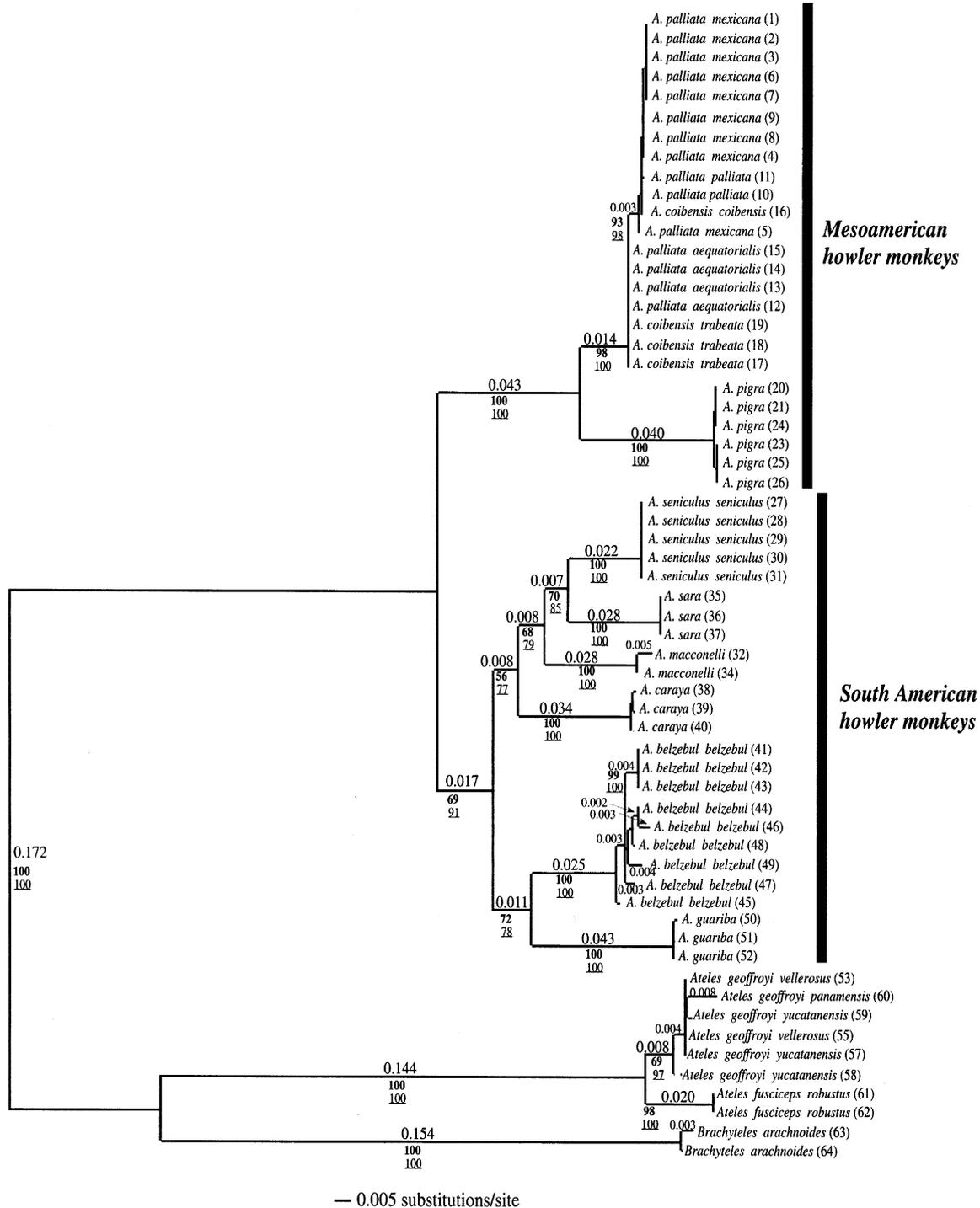


Fig. 3. Neighbor-joining tree of combined mtDNA genes for all individuals used in this study, based on TrN + G distances. The ingroup is constituted of *Alouatta* species and subspecies; the outgroup is composed of *Ateles* and *Brachyteles* individuals. Numbers within brackets correspond to numbers in Table 1 and Fig. 1. Branch length distances are shown on each corresponding branch. Neighbor-joining and maximum parsimony bootstrap values (1000 replications) are shown in bold and underlined respectively under the branches.

sister taxa separated by 5.7% mtDNA sequence divergence. Our mtDNA-based analysis does not distinguish *A. coibensis* as phylogenetically distinct from *A. palliata*, and both the Coiba Island and Azuero Peninsula subspecies carry mtDNA haplotypes that are identical to

sequences observed for *A. palliata* individuals. It is worth noting that *A. coibensis coibensis* and some individuals of *A. p. palliata* and *A. p. mexicana* shared the same mtDNA haplotypes, whereas *Alouatta coibensis trabeata* carried haplotypes only observed in *A. palliata*

in all cases by 100% bootstrap support. Mitochondrial sequence divergence between these six clades ranged from 5.0% to 11.0%. There was weak bootstrap support for clades comprised of *A. belzebul*, and *A. guariba* (NJ: 72%; MP: 78%) and *A. s. seniculus*, *A. macconelli*, *A. sara*, and *A. caraya* (NJ: 56%; MP: 77%) (Fig. 3). The average mtDNA sequence divergence between these two clades was 9.8%, compared to an average distance within clades of 7.2% and 7.1%, respectively. *Alouatta belzebul* showed the highest levels of mtDNA sequence diversity among conspecifics, but maximum divergence between haplotypes did not exceed 0.9%. Samples of this howler species collected from the Tocantins region ($n = 6$) could be distinguished by 12 synapomorphic nucleotide substitutions from the Paraíba samples ($n = 3$), but larger sample sizes would be required to determine if this phylogeographic pattern is phylogenetically significant.

Average mitochondrial divergence was 38.6% between *Alouatta* and *Brachyteles*, 39.2% between *Alouatta* and *Ateles*, and 31.1% between *Ateles* and *Brachyteles*. At the species level, *Ateles geoffroyi* was well differentiated from *A. fusciceps*, but the divergence between them (2.5%) is lower than between any pair of *Alouatta* species.

The RAG1 and CAL gene trees with the lowest $-\ln L$ values are presented in Fig. 4. Maximum-likelihood analysis of both nuclear-encoded genes clearly separated *Alouatta* from *Ateles*, but neither gene provided any meaningful phylogenetic resolution among species. The average genetic distance between *Alouatta* and *Ateles* was 1.6% for RAG1 and 2.7% for CAL, as compared to a mtDNA distance of 39.2%. It is worth noting that in a number of cases we observed more sequence divergence between an individual's CAL alleles, than we observed between species. For example, the two CAL alleles representing *A. pigra* individual 22 are distinguished by

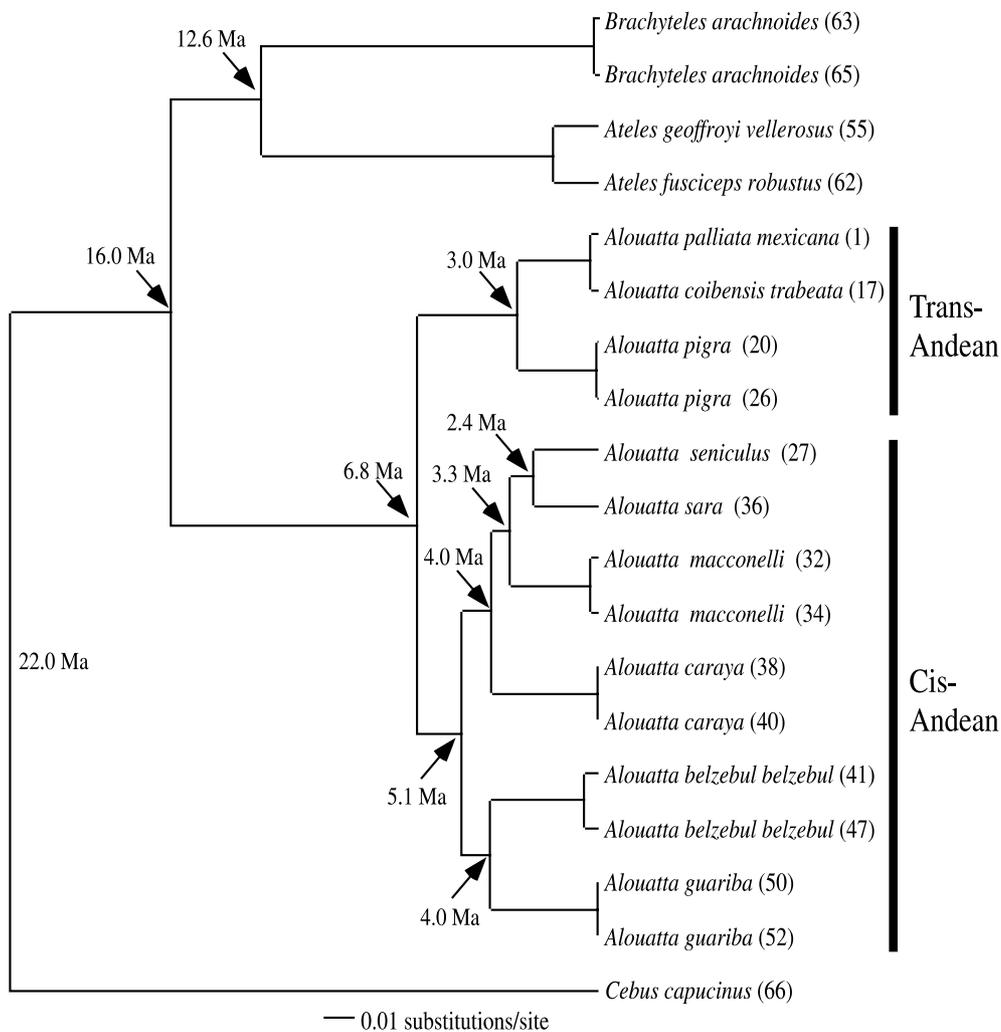


Fig. 5. Maximum likelihood tree of mitochondrial DNA ATPase 8/6 and cyt *b* genes with an enforced molecular clock. Only two individuals representing each clade of Fig. 3 were used. Estimated dates for splitting events of major clades are shown at each node. Numbers within brackets correspond to individuals as they are referred in Table 1.

five substitutions, whereas five different *Alouatta* species share an identical CAL allele.

3.3. Molecular clock

No significant difference was obtained between the $-\ln L$ values for a clock-enforced tree (6540.99; Fig. 5) versus the unconstrained mtDNA-based ML tree (6531.94) ($p = 0.3833$). Furthermore, the branch length and two cluster tests implemented in Lintree (Takezaki et al., 1995) indicated no relative rate differences among the mtDNA sequences representing the Atelinae tested (*Alouatta*, *Ateles*, and *Brachyteles*). There were also no relative rate differences observed when we used *A. fusciceps* as the outgroup, and *Alouatta* as the ingroup.

We calculated a divergence time between Alouattinae and Atelinae of 16 or 17 Ma using the Sanderson (1997) approach, and the chimpanzee–human split or the catarrhine and platyrrhine separation, respectively. The date estimated in this fashion was very similar to the split estimated for Atelinae and Alouattinae based on nuclear-encoded globin genes (Goodman et al., 1998). Moreover, Ruiz-Garcia et al. (in press) estimated a 21 Ma separation between *Cebus* and the Atelinae based on a microsatellite mutation rate of 1.5×10^{-4} averaged across 10 loci. Using our estimate of a 16 Ma split between Atelinae and Alouattinae, we calculate a very similar 22 Ma date of separation between *C. capucinus* and the Atelidae. Thus, with some confidence we used the 16 Ma date of separation between Atelinae and Alouattinae to infer the node ages for *Alouatta*, *Ateles*, and *Brachyteles* using both a clock-enforced ML tree (Fig. 5) and a local molecular clock (Bailey et al., 1992; Schneider et al., 1993). Both approaches yielded very similar estimates of divergence, with the local molecular clock indicating slightly earlier dates of separation at some nodes. For example, we estimate the divergence between *cis*- and *trans*-Andean *Alouatta* species at 6.6–6.8 Ma (Fig. 5), between *A. seniculus* and *A. sara* at 2.3–2.4 Ma, and between *A. belzebul* and *A. guariba* at 3.9–4.0 Ma. *A. palliata* and *A. pigra* divergence time was estimated at 3 Ma with both methods. The diversification of South American species dates to 4.8–5.1 Ma, near the end of the Miocene.

4. Discussion

The phylogeny of *Alouatta* genus reconstructed from mtDNA sequence data is very well resolved and clarifies several of the ambiguities of the systematics of this genus as described below. Phylogenetic relationships among the different species of *Alouatta*, as well as their geographical distribution and age estimates for the origin of these species provide insight of the roles of different causal factors on the diversification of this genus.

4.1. Molecules, information content, and comparative evolution across mtDNA and nuclear genes

Owing to the relatively recent origin of many primate species, mtDNA has been used extensively to reconstruct their phylogenetic relationships (Horovitz and Meyer, 1995; Pastorini et al., 2001; Yoder et al., 1996; Yoder and Yang, 2000). Attention has focused on *cyt b* sequences, but our molecular systematic analysis of *Alouatta* indicates that the ATPase genes are also phylogenetically informative. Mitochondrial ATPase and *cyt b* genes are evolving at similar rates, but some differences can be noted (Table 3). For example, the ATPase genes are somewhat more variable than *cyt b*, a difference that is principally manifest at first and second codon positions. As a result 22% and 14% of the ATPase 8 and 6 changes, respectively, lead to replacement substitutions in comparison to 5.6% of the *cyt b* changes. It should be noted that our *cyt b* sequences comprise only the first 800 bp of the 5' end of the gene, whereas it has been reported that the 3' end of this gene is usually more variable (Palumbi, 1996).

Given the phylogenetic information content of the mitochondrial genes and sequence divergence within and between *Alouatta* species (Fig. 3), we were somewhat surprised that neither RAG1 nor CAL data were capable of resolving the phylogenetic relationships among *Alouatta* species. The nuclear genes could discriminate between *Alouatta* and *Ateles* species, suggesting that these regions might be usefully applied to molecular systematic studies of Atelidae genus-level relationships. Our results are similar to those of other studies in which phylogenetic reconstructions based on nuclear sequence data failed to resolve the relationships of other Neotropical primate species, while phylogenies resolved from mitochondrial sequence data were more robust (Collins and Dubach, 2001; Cropp and Boinski, 2000). Based on the three-times rule (Palumbi et al., 2001), and the ratio of *Alouatta* mtDNA diversity (within species) to divergence (between species), coalescence of nuclear alleles within species would be expected. The failure of nuclear sequence data to resolve the relationships of *Alouatta* species is perplexing, particularly considering that we analyzed sequences from two very different loci—a region of a coding gene that is presumably under strong purifying selection and an intron that probably evolves neutrally. The fact that calmodulin intron sequences from single individuals are, in some cases, more divergent than sequences obtained from different taxa (Fig. 4) indicates that either this locus has not coalesced or that we obtained sequences from more than one locus.

The coalescent times of gene trees can be strongly influenced by social behavior and asymmetric dispersal by the sexes. For example in some primates females are philopatric and males are polygynous, conditions which

would cause greater phylogeographic structuring of matrilineal and changes in the relative coalescent times of mtDNA and nuclear genes that could lead to disagreement between markers regarding evolutionary relationships among populations or species (Hoelzer, 1997). However, both sexes disperse in howler monkeys (Brockett et al., 2000; Clarke et al., 1986; Crockett and Eisenberg, 1987; Crockett and Pope, 1993; Glander, 1992; Pope, 2000; Ostro et al., 2001) and although typically perceived to be polygynous, polyandry has been demonstrated for *A. palliata* (Cortés-Ortiz, 1998; Jones and Cortés-Ortiz, 1998; see also Carpenter, 1934). If polygyny predominates in howler societies, the lack of species-specific allele coalescence in the RAG1 and CAL trees becomes even more surprising. Nonetheless and given the information in hand regarding the social biology of howler monkeys, we conclude that the mtDNA gene tree represents a strong hypothesis of *Alouatta* species relationships.

4.2. Molecular systematics of *Alouatta*

Our mtDNA-based phylogeny for *Alouatta* shows that Central and South American species represent reciprocally monophyletic clades. Moreover, mtDNA haplotypes form 8 clearly defined clades, supported by bootstrap values ranging from 98% to 100%. These clades correspond to the five well recognized species by Hill (1962), *A. palliata*, *A. seniculus*, *A. belzebul*, *A. guariba*, and *A. caraya*; *A. pigra* as recognized by Smith (1970); *A. sara* as recognized by Minezawa et al. (1985) and *A. macconelli* as recognized by Lima and Seuánez (1991) and Vassart et al. (1996).

Our phylogenetic analyses provide strong support for the distinction and sister taxa relationship of *A. palliata* and *A. pigra*. The mitochondrial data thus support the taxonomic view of Mesoamerican howlers that has largely prevailed since Smith (1970) reviewed the status of the subspecies considered by Lawrence (1933), and recognized *A. pigra* as a separate species. Ruiz-García et al. (in press) have also posited an *A. palliata* and *A. pigra* sister species relationship based on 10 microsatellite loci analyzed for a smaller sample of *Alouatta* species.

Frohelich and Frohelich (1987), using dermal ridge patterning on the hands and feet of Central American howlers, proposed that the howlers of Coiba Island and the Azuero Peninsula of Panama are a distinct species, *A. coibensis*, with two subspecies: *A. c. coibensis* and *A. c. trabeata*. The mtDNA data do not support the specific status of either *A. c. coibensis* or *A. c. trabeata*; moreover, both subspecies share mtDNA haplotypes with *A. palliata* individuals. Population studies using more variable genetic markers such as microsatellites may clarify the extent of reproductive isolation among the different named *A. palliata* subspecies, but the mtDNA data in-

dicate that the evolutionary distinctiveness of these taxa is relatively minor.

The lack of evolutionary distinctiveness among named species and subspecies carries throughout the species distribution in Central America. We analyzed 19 individuals ranging from Panama to Mexico and observed a maximum level of mtDNA sequence divergence of 0.5% (Fig. 3). Weak phylogeographic structure was detected between individuals representing howler monkeys collected in Costa Rica and Mexico in comparison to those collected in Panama (excluding Coiba Island). Individuals from Coiba Island ($n = 3$; results not presented for two individuals sequenced only for the ATPase genes) carry the same mtDNA haplotype; it is identical to the dominant ATPase haplotype observed in howler monkeys from Costa Rica and Mexico. We suggest that *Alouatta* colonized Coiba Island via one of the land bridges established during the Pleistocene low sea level stands (Castroviejo, 1997; Colinvaux, 1997). We further conjecture that the minor phylogeographic break separating northern and southern *A. palliata* is located near Panama's Sona peninsula, a filter barrier reported for additional taxa (Bermingham and Martin, 1998).

South American howler monkey species cluster in two clades in the mtDNA phylogeny; the first clade is composed of *A. seniculus*, *A. sara*, *A. macconelli*, and *A. caraya*, and the second comprises *A. belzebul* and *A. guariba*. The mtDNA data are fully consistent with the phylogenetic inferences of Mudry et al. (1994) and Ruiz-García et al. (in press) who indicated a sister relationship between *A. caraya* and *A. seniculus*, and Sampaio et al. (1996) who consider *A. guariba* and *A. belzebul* to be sister taxa. However, the mtDNA tree differs from the relationships proposed by Herskovitz (1949), based on the hyoid bone morphology, and those of Meireles et al. (1999a) based on a globin pseudogene phylogeny. Both authors placed *A. caraya* in a monotypic clade separated from the rest of the South American *Alouatta* species, whereas the mtDNA data support the inclusion of this species in a clade including *A. seniculus*–*A. sara*–*A. macconelli*. It should be noted that the bootstrap support for this clade is not robust (MP: 77%, NJ: 56%), however, constraining *A. caraya* outside all other South American *Alouatta* species provides a significantly worse ML fit to the mtDNA data (SH test, $p = 0.045$, Shimodaira and Hasegawa, 1999).

During the past several decades, systematic attention has also focused on the relationships among subspecies of South American howler monkeys. This is particularly true for the *A. seniculus* group owing to the high levels of allozyme and cytogenetic variation observed across the geographic range of this species (Lima and Seuánez, 1991; Minezawa et al., 1985; Pope, 1992; Sampaio et al., 1996; Stanyon et al., 1995; Vassart et al., 1996). Hill (1962) listed nine subspecies, however, recent cytogenetic and molecular research indicates that *A. seniculus* is a

species complex rather than a single species (e.g. Consigliere et al., 1998). For example, cytogenetic studies support species status for *A. s. arctoidea*, *A. s. sara*, and *A. s. macconelli* (Lima and Seuánez, 1991; Minezawa et al., 1985; Stanyon et al., 1995; Vassart et al., 1996; see Rylands and Brandon-Jones, 1998; for a recent discussion regarding the taxonomy of the *A. seniculus* group). Our mtDNA-based phylogenetic analyses further substantiate the status of *A. sara* and *A. macconelli* as phylogenetically distinct from *A. seniculus* and from one another. Individuals representing each of the three putative species were monophyletic in 100% of the bootstrap trees, and the level of mtDNA sequence divergence among these species is similar in magnitude to the separation observed between *A. palliata* and *A. pigra* (Fig. 3).

In addition to surveying the *A. seniculus* species complex across a portion of its geographic range, we also performed a preliminary phylogeographic assessment of *A. belzebul*. We collected samples ($n = 9$) from two populations (Paraíba and Pará) separated by the dry vegetation of the caatingas (see Fig. 1B). The three Paraíba individuals carried an identical mtDNA haplotype representing a derived lineage in the polyphyletic assemblage of *A. belzebul* mtDNA sequences. The Paraíba howler monkeys were 0.5% diverged from their closest genetic neighbor in the Pará population (Fig. 3). The six Pará *A. belzebul* carried five distinct mtDNA haplotypes with an average sequence divergence of 0.6%. Howler monkeys in the Pará region are also noted for variation in coat color, and morphologically distinctive individuals occur within the same troop (see Armada et al., 1987).

We close our discussion of the molecular systematics and phylogeography of *Alouatta* with a comment regarding its phylogenetic placement in the family Atelidae. Given the absence of *Lagothrix*, the only Atelidae genus missing from our analysis, we can only evaluate the mtDNA data in the light of the following, and opposing, phylogenetic hypotheses. In one view *Ateles*, *Brachyteles*, and *Lagothrix* share a common ancestry independent from *Alouatta* (Ford, 1986; Rosenberger, 1981, 1984; Schneider et al., 1993). Opposing views indicate that *Brachyteles* and *Alouatta* derived from one ancestor while *Ateles* and *Lagothrix* arose from another (Kay, 1990), or that *Lagothrix* and *Alouatta* are sister genera (Dunlap et al., 1985). Molecular studies of the Atelidae based on nuclear DNA sequence data support the first hypothesis (e.g., Meireles et al., 1999b; Schneider et al., 1993, 1996; von Dornum and Ruvolo, 1999), and the mtDNA data also support the phylogenetic separation of *Alouatta* but obviously do not discount the Dunlap et al. hypothesis owing to the absence of *Lagothrix* mtDNA sequence. The mitochondrial genetic distance between *Ateles* and *Brachyteles* is 31%, versus 39% between either of these genera and *Alouatta* suggesting that *Brachyteles* and *Alouatta* are not sister taxa.

4.3. Geographical and chronological formation of *Alouatta* species

The *Alouatta* phylogeny permits strong inference that the diversification of contemporary howler monkey lineages was initiated by Andean vicariance. Application of a molecular clock dates the separation of *cis*-, *trans*-Andean *Alouatta* at 6.8 million years ago (Fig. 5), roughly coincident with the formation of the northern Andes (Lundberg et al., 1998). The subsequent diversification of *cis*-Andean howler monkeys, appears to have preceded that of their *trans*-Andean sister group (Fig. 5), but given the lack of precision estimating mtDNA coalescence this conclusion should be treated cautiously (Edwards and Beerli, 2000). Fossil data indicate that monkeys probably did not colonize Central America prior to the Pliocene completion of the Panama land bridge connecting South America to nuclear Central America (Webb, 1997; Webb and Rancy, 1996), perhaps providing a partial explanation for our hypothesis of later speciation in the *trans*-Andean howlers. Before completion of the Panama land bridge *Alouatta* may have had a very restricted *trans*-Andean distribution and a likelihood of speciation (at least owing to geographical causes) that was perhaps reduced along the narrow Pacific slope of South America relative to the vast Amazon slope.

4.3.1. Diversification of *cis*-Andean howler monkey species

The Pliocene appears to have been a period of active speciation for *cis*-Andean *Alouatta*. Of the six South American species included in our analyses, molecular clock calculations indicate that the youngest species pair split around 2.4 Ma and the oldest species pair separated at 4.0 Ma (Fig. 5). The earliest separation among *cis*-Andean howlers split the lineage leading to *A. guariba* and *A. belzebul*, species which currently inhabit the south bank of the Amazon River and the Atlantic forest of Brazil, from one that would give rise to *A. seniculus*, *A. sara*, *A. macconelli*, and *A. caraya*. The distribution of the second group covers the remainder of the Amazon basin north to coastal Venezuela, west to the Amazon flank of the Andes, and parallel to the Andes south until northern Argentina (Fig. 1A).

Initial diversification among *cis*-Andean howlers (5.1 Ma) is roughly contemporaneous with the formation of the modern Amazon River during the Late Miocene (Lundberg et al., 1998), suggesting that Wallace's (1852) Riverine Barrier hypothesis might provide the causal speciation mechanism. Given the current distribution of *A. macconelli* and *A. belzebul* on the north and south side of the lower Amazon, respectively, geographic isolation on either side of the river may have caused the initial split among *cis*-Andean howler monkeys. Primate communities on opposite sides of Amazonian rivers decrease in species similarity with

increasing river size and distance from headwaters (Ayres and Clutton-Brock, 1992; Peres et al., 1996), implying that the lower reaches of major rivers are considerably more effective barriers to monkey dispersal than upper river reaches (but see Gascon et al., 2000). Although riverine barriers sometimes limit contemporary distributions of species comprising the resultant daughter lineages of *Alouatta* diversification in the cis-Andean region, the origin of all *Alouatta* species is not simply explained by vicariance across these barriers. Patton and co-workers (Patton and Smith, 1992; Patton et al., 1994; Patton and da Silva, 1997; Peres et al., 1996) have demonstrated that definitive tests of Amazonian speciation models are often elusive, and it is certainly the case that our data only permit commentary regarding these models, not tests. Thus, we make no attempt to review all possible causes underlying the formation of each *Alouatta* species, but rather use the mtDNA-based howler monkey phylogeny to draw attention to published remarks regarding the fit of *Alouatta* species to particular speciation models.

Paleogeographic reconstructions (Bigarella and Andrade-Lima, 1982) of the forests of eastern Brazil south of the Amazon suggest that the ancestor to *A. belzebul* and *A. guariba* might have encountered continuous forest in this region (Rylands et al., 1996). The formation of *A. belzebul* and *A. guariba* may have resulted from the subsequent development of the dry caatingas of northeastern Brazil, which separate the eastern Amazon and Atlantic forests, and whose xeric vegetation is inhospitable to monkeys. Under this scenario, movement of *Alouatta* across the dry caatinga regions would have to be postulated to explain the occurrence of *A. belzebul* in the northern extreme of Atlantic forest of Brazil. Bigarella and Andrade-Lima (1982) reported a connection between the Amazon and the Atlantic forest during the Holocene (5000–6000 years ago) suggesting a possible dispersal route during wetter times. The minor phylogeographic differentiation distinguishing the coastal Paraíba population from the interior Pará *A. belzebul* population suggests that dispersal between the eastern Amazon and northern Atlantic forests is periodic.

Among the Northern Amazonian *Alouatta* species included in our analysis, the first split occurred between *A. macconelli* and the ancestor of *A. seniculus* and *A. sara*. *Alouatta macconelli* is restricted to the Guianas and northeastern Brazil, and the limits of its distribution are coincident with areas of endemism for many other species of vertebrates (e.g., Collins and Dubach, 2000; Haffer, 1992; Ron, 2001). Rivers may have played a role in the formation of this species, or at least set limits to its current distribution. Ayres and Clutton-Brock (1992) stated that both large rivers and “fast-running” black water rivers influence primate dispersal, and the distribution of *A. macconelli* is limited by the Amazon to the south and

black water to the northwest (Fig. 1B). The estimated age of divergence between *A. macconelli* and the *A. seniculus*–*A. sara* ancestor (2.9–3.3 Ma) is similar to the age estimated for *Ateles paniscus* (3.3–3.6 Ma, Collins and Dubach, 2000), which has the same distribution as *A. macconelli*. Both species appear too old to be explained by the Pleistocene Refuge model (Haffer, 1969).

The more recent separation of *A. seniculus* and *A. sara* was posited to have resulted from the peripheral isolation of *A. sara* in the Yungus 2 refugium (Minezawa et al., 1985). Brown (1982) proposed this refugium on the basis of butterfly endemism data presented in the context of the general forest refugia model (Haffer, 1969, 1982). We estimated a late Pliocene divergence between *A. seniculus* and *A. sara* (Fig. 5), again too old to be explained by Pleistocene climate changes that Haffer (1969, 1982) initially argued were responsible for the contraction of lowland forest into isolated refuges. More recently Haffer (1997) has suggested that Milankovitch cycles operating across the Cenozoic permit more general application of the refuge model through time, but it seems very unlikely to us that such cycles could have caused the Pliocene bunching of *Alouatta* speciation suggested by our analyses. The Madeiras River forms the eastern boundary of *A. seniculus* and *A. sara*, and the western limit of *A. nigerrima* and could be inferred to have caused the split. Under such a model, *A. nigerrima* should be sister to *A. seniculus* and *A. sara*. If these taxa are not sisters, as suggested by the traditional placement of *A. nigerrima* with *A. belzebul*, our mtDNA data would suggest that the Madeiras River is simply a meeting point of *Alouatta* species that diverged elsewhere. However, a chromosome study (Armada et al., 1987) suggesting a close relationship between *A. nigerrima* and *A. seniculus*, if confirmed through phylogenetic analysis, would permit strong inference that the Madeiras riverine barrier was the underlying cause of speciation.

The current distribution of *A. caraya* is not coincident with any clear geographical barriers; the species is parapatric with *A. sara* to the west, with *A. nigerrima* and *A. belzebul* to the north, and with *A. guariba* to the east (Fig. 1B). The Goia refugium (Brown, 1982) in southeast Matto Grosso, Brazil lies within *A. caraya*'s distribution, but our estimated age (4 Ma; Fig. 5) suggests a Pliocene origin for this species thus discounting the Pleistocene refugium model of speciation presented by Brown for the butterflies of this region (1982).

4.3.2. Evolution of Mesoamerican howler monkey species

The strongly supported sister species relationship of *A. palliata* and *A. pigra* in the mtDNA phylogeny affirms the monophyletic origin of Mesoamerican howler monkeys hypothesized by Smith (1970). The molecular data also permit some evaluation and refinement of the two hypotheses presented by Smith (1970) to account

for the origins of *A. palliata* and *A. pigra*. One Smith model posits a single colonization of Central America from South America followed by the separation of *A. palliata* in the Talamanca region of Costa Rica from *A. pigra* to the north, whereas his second hypothesis recognizes two sequential invasions of Central America by the *trans*-Andean *Alouatta* ancestor in South America.

Our estimated 3 Ma date of separation between *A. palliata* and *A. pigra* permits us to consider the origin of these two species within a temporal context that was unavailable to Smith (1970). It is noteworthy that the Mesoamerican howler monkey split at a time that coincides with the completion of the Panama land bridge (3.1–2.8 Ma, Coates and Obando, 1996); nonetheless the cause of speciation remains obscure. The absence of primate fossils in Central America prior to the Pliocene emergence of the terrestrial corridor linking South America to nuclear Central America provides weak evidence at best (Webb and Perrigo, 1984) that *Alouatta* colonization occurred after land bridge formation. If this was the case, the single colonization hypothesis can be refined in the following manner. Given the current distribution of *A. palliata* and *A. pigra* (Fig. 1A) and the age of the split between these species, the expansion of their ancestor's range to the current northern limit of *Alouatta*'s distribution must have been virtually instantaneous. This is not an unlikely scenario, given the body of fossil-based research documenting the immediate and geographically widespread interchange of mammals immediately following the completion of the Panama land bridge (Stehli and Webb, 1985; Webb, 1997). One can then invoke either allopatric or parapatric speciation models (Endler, 1977; Mayr, 1963) to explain the autochthonous origin of the two Mesoamerican howler monkey species.

Under an allopatric model *trans*-Andean *Alouatta* populations would have been separated by forest loss or reduction in the central Mesoamerica during cold, dry glacial periods (Haffer, 1975; Whitmore and Prance, 1987), with isolated populations persisting in the highlands of Guatemala, Mexico, and Belize and in the Talamanca mountains of Costa Rica. Alternatively, parapatric speciation could be invoked across the ecotone that separates the drier and lower forest of Mexico's Yucatan peninsula occupied by *A. pigra*, from the moist, tall forests inhabited by *A. palliata*. The *A. pigra*/*A. palliata* contact zone lies between the Grijalva and Usumacinta Rivers, holding open the possibility that either or both of these rivers might have acted as barriers to gene flow.

Working against the allopatric speciation model is the lack of consensus (and lack of data) regarding forest loss in central Mesoamerica during glacial periods. Working against the hypothesis of parapatric speciation are the following observations: (1) *A. palliata* and *A. pigra* form mixed troops of howlers in the region of overlap, and

sometimes interbreed suggesting that the contact zone may be secondary and recent; and (2) it is certainly not easy to perceive conditions for parapatric speciation in the current zone of contact that would not apply with equal force elsewhere in the broad distribution of *A. palliata*.

Sequential colonizations of Central America from South American sources imply an allopatric model of speciation, but Smith (1970) did not propose a suitable barrier to dispersal or gene flow. We have proposed models indicating that Pliocene/Pleistocene low sea level stands promoted the likelihood and rate of dispersal of terrestrial and freshwater organisms across the Panama land bridge, whereas high sea level acts as a potential barrier to dispersal (Bermingham and Martin, 1998; Perdices et al., in-press). The low sea level stand at 2.9 Ma (Haq et al., 1987) and the putative development of extensive lowland forest along the extended coastal plain might have promoted the first wave of *Alouatta* expansion into Central America, with dispersal subsequently interrupted or significantly diminished by the high sea level stand extending until 1.6 Ma. Such a scenario is highly speculative, but at least provides a possible cause for the 3 Ma date of separation between *A. palliata* and *A. pigra*. It has been argued that savanna-like vegetation must have existed across much of the isthmus following its rise given the rapid continental interchange of large mammals documented at 2.4 Ma (Webb and Rancy, 1996; but see Colinvaux, 1996). Such vegetation is not conducive to primate dispersal and provides an alternate, perhaps complementary, barrier between the South American *Alouatta* source population and immigrants that colonized Central America in the initial wave of expansion.

The two allopatric scenarios for the origin of *A. palliata* and *A. pigra* carry interesting implications regarding changes in range size, and competitive interactions between the two species. Under both models, *A. palliata* must have increased its range at the expense of *A. pigra*. How much depends on where the boundary between these two species first developed. If the two species sequentially colonized Central America, then *A. pigra*'s distribution has diminished from almost the whole of Central America to the Yucatan Peninsula. Under the single colonization model, *A. pigra*'s range loss would be less but still considerable. The reconstruction of paleogeographic vegetation in Central America is contentious; nonetheless it is probably worth pointing out that the postulated elimination of forests north of Costa Rica through Honduras during the cold and dry phases of Milankovitch cycles (Whitmore and Prance, 1987), may have caused the local extinction of *A. pigra* populations in these areas. Furthermore, Pleistocene climate changes may have isolated *A. pigra* into small forest refugia that have been putatively identified in the south of Mexico, northeast Guatemala,

and Belize (Haffer, 1974, 1975; Toledo, 1982). *Alouatta palliata* may have been confined to the refugia in the Choco region of South America and lower Central America (Haffer, 1975), perhaps recently expanding northward during the warm Holocene interglacial.

Whether the changes in *Alouatta* ranges owe to differential responses to Pleistocene climate fluctuations and the environmental perturbations of man, or to the superior dispersal and/or competitive abilities of *A. palliata* is not immediately obvious. Some insight might come from population genetic studies aimed at inferring the demographic histories of the two species.

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