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Molecular Phylogenetics of *Aotus* (Platyrrhini, Cebidae)

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Abstract The accurate identification of taxa of *Aotus* is essential for 1) the development of precise biomedical assays, 2) the determination of potential illegal traffic of this genus, and 3) conservation. Although many studies have contributed to what we know about the phylogenetics of *Aotus*, none used a sufficiently large number of samples to clarify its complexity. To address this need, we sequenced 696 base pairs of the mitochondrial cytochrome-oxidase II gene (*mtCOII*) in 69 specimens of 7 taxa of *Aotus*. We also analyzed 8 microsatellite loci in 136 individuals of 6 taxa. In contrast to previous studies, we sampled only wild individuals and have a precise geographical origin for each one. The mtDNA results showed that: 1) the northern gray-necked group of *Aotus* is genetically more homogeneous than the polyphyletic red-necked group of *Aotus*; 2) the ancestors of *Aotus vociferans* seem to be the original species candidate for the current *Aotus*; 3)

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Aotus azarae azarae and *A. a. boliviensis* are the most differentiated taxa, likely a result of extreme genetic drift during stasipatric speciation; 4) the first genetic splits found among taxa of *Aotus* occurred during the Pliocene (or even Miocene) while the most recent ones happened during the Pleistocene, when forest refugia may have played an important role in speciation. The mean number of microsatellite alleles was 3–5.33 alleles per locus. We found some private alleles that could be useful in helping to identify illegal trade, although a larger sample size is needed to ensure that these alleles are really private to the relevant taxa. These new findings increase our understanding of the phylogeny of *Aotus* and the level of genetic diversity within different taxa of *Aotus*.

Keywords *Aotus* · Colombia · Microsatellites · *mtCOII* gene · Perú · Phylogenetic analyses

Introduction

Aotus is an established nonhuman primate model for the study of malaria and other diseases. It has also been highly coveted by illegal traffickers in Colombia since 1970 (Hernández-Camacho and Cooper 1976; Maldonado *et al*. 2009). These demands can negatively affect the distribution and abundance of taxa of *Aotus* in their natural habitats. To protect this genus it is necessary to characterize the different taxa accurately and to determine the geographical origins of illegally traded animals. Unfortunately, the exact phylogeny of *Aotus* is in question.

Until 1983, primatologists suggested that the genus *Aotus* included only one species, *Aotus trivirgatus*, containing diverse allopatric subspecies (Hernández-Camacho and Cooper 1976; Hershkovitz 1949). However, Hershkovitz (1983) described 9 allopatric species split into 2 groups. One was a primitive gray-necked group north of the Amazon River that contained 4 species (*Aotus lemurinus* [with 2 subspecies, *A. l. lemurinus* and *A. l. griseimembra*], *A. brumbacki*, *A. trivirgatus,* and *A. vociferans*). The second was a red-necked group of 5 species (*Aotus nancymaae, A. miconax, A. nigriceps, A. infulatus,* and *A. azarae* with 2 subspecies, *A. azarae azarae* and *A. a. boliviensis*) located mostly south of the Amazon River. Ford (1994) questioned the accuracy of Hershkovitz's classification and concluded that there were only 7 species: *Aotus trivirgatus*, *A. vociferans* (which included *A. lemurinus* and *A. brumbacki*), *A. nancymaae*, *A. miconax* (although these last 2 could be a single species), *A. nigriceps*, *A. azarae* (including only *A. a. azarae*), and *A. infulatus* (including *infulatus* and *azarae boliviensis*). Adding greater complexity to the taxonomy of *Aotus*, Groves (2001) listed 4 subspecies of *Aotus lemurinus* (*A. lemurinus lemurinus*, *A. l. griseimembra*, *A. l. zonalis,* and *A. l. brumbacki*) in his influential book. He also listed *Aotus hershkovitzi, A. trivirgatus,* and *A. vociferans* in the gray-necked group, and *A. miconax, A. nancymaae, A. nigriceps,* and *A. azarae* (with 3 subspecies: *A. azarae azarae*, *A. a. boliviensis,* and *A. a. infulatus*) in the red-necked group. Groves (2001) accepted *brumbacki* as a form of *lemurinus* but noted that all the taxa were differentiated from *vociferans*, whereas *infulatus, azarae,* and *boliviensis* formed a complex with overlapping characteristics, close to *Aotus nigriceps* but specifically distinct. We show the geographic distributions of the various taxa in Fig. 1.

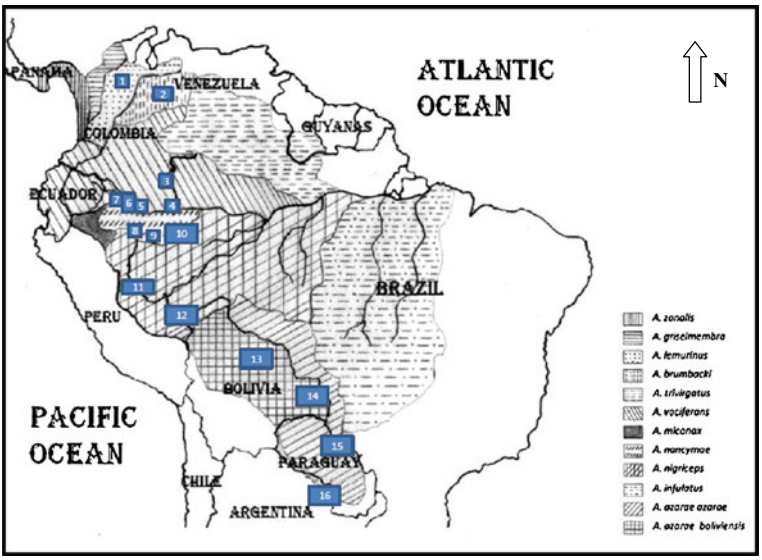

Fig. 1 Geographic distribution of the different taxa of *Aotus*. Numbers indicate the geographical origins of those individuals sampled in the wild. 1=Sucre Department, Colombia (*A. griseimembra*); 2=San Martín, Meta Department, Colombia (*A. brumbacki*); 3=La Chorrera, Iguaraná-Paraná River, Colombia (*A. vociferans*); 4=Amazon River from Puerto Nariño to San Juan de Atacuarí, Colombia (*A. vociferans*); 5=Nanay River, Perú (*A. vociferans*); 6=Tamboryacu River, Perú (*A. vociferans*); 7=Santa Maria, Perú (*A. vociferans*); 8=Tahuayo, Perú (*A. nancymaae*); 9=Quebrada Yanuyacu, Perú (*A. nancymaae*); 10=Carmen, Yavarí River, Perú (*A. nancymaae*); 11=Tarapoto, San Martin Department, Perú (*A. nigriceps*); 12=Yarinacocha, Ucayali Department, Perú (*A. nigriceps*); 13=San Javier-Ibiató, Cerrado Province, Beni Department, Bolivia (*A. azarae boliviensis*); 14=Santa Cruz Department, Bolivia (*A. azarae boliviensis*); 15=Paraguay River, Paraguay (*A. azarae azarae*); 16=Negro River, Formosa, Argentina (*A. azarae azarae*).

Karyotype studies do not necessarily support the above taxonomies. For example, Defler *et al.* (2001) showed that *Aotus hershkovitzi* is not differentiated from *A. lemurinus lemurinus* and that the taxon described by Hershkovitz as *A. l. lemurinus* was in fact *A. l. zonalis*. Defler and Bueno (2007) studied a specimen karyotyped by Torres *et al.* (1998), as $2n=50$, and sampled in the Quindio region of Colombia, and defined this as a new species called *Aotus jorgehernandezi*. Ma (1981) compared the karyotypes of *Aotus nigriceps* and *A. azarae boliviensis* and showed that a fusion of the Y chromosome occurred with the short arm of a medium-size subtelocentric autosome in both taxa. G-banding data demonstrated that this autosome was the same in both taxa (Ma 1981; Ma *et al.* 1980), although the species differ by 3 autosomic rearrangements. Similarly, Pieczarka and Nagamachi (1988) and Pieczarka *et al.* (1993) found that the karyotypes of *Aotus infulatus* and *A. azarae boliviensis* suggested that these are the same species. A particularly relevant karyotype speciation model in the case of *Aotus* is the stasipatric speciation model (Bush 1981; White 1968, 1978), which predicts fixation of new mutant homokaryotypes and the generation of reproductive barriers in only a few generations (theoretically 2 or 3) if the effective population size is extremely small with endogamous mating, as is the case in *Aotus* (White 1978). This could produce very different genetic profiles in neighboring populations of *Aotus* (Hershkovitz

1983). For example, Hershkovitz (1983) showed that the southern-most *Aotus* species, *A. azarae boliviensis* and *A. a. azarae*, are derived from northern *Aotus* species. This suggests that these subspecies may have very different genetic profiles from the population or species sources.

The nature of the ancestral *Aotus* is also debated. Both *Aotus vociferans* ($2n$=46, 47, 48) and *A. griseimembra* ($2n$=52, 53, 54) have a chromosomal polymorphism, unrelated to sex, suggesting that these may be the 2 most ancient species of the current *Aotus* (Defler and Bueno 2007). However, Ma *et al.* (1985) suggested that *Aotus vociferans* is derived from *A. brumbacki* by a single fusion event or that *A. brumbacki* is an intermediate form between *A. vociferans* and *A. griseimembra*. Ma (1981) and Galbreath (1983) also suggested that the ancestral *Aotus* had a karyoype of $2n$=54. Only 2 taxa of *Aotus* have a $2n$=54: *Aotus nancymaae* and 1 of the karyotypes of *A. griseimembra*. In addition, Defler and Bueno (2007) suggested that the ancestor of *Aotus lemurinus*, from the Colombian Andean mountains, was the original *Aotus* species. However, this seems highly improbable because it is a very specialized species adapted to high altitudes (up to 3200 m.a.s.l.), and its high chromosome number ($2n$=58) could help it to adapt selectively and increase its capacity for gene recombination (Martin 1990).

Only 3 studies have employed DNA markers in an attempt to resolve some of the confusing phylogenetic aspects of *Aotus*. The first 2 used the mitochondrial cytochrome-oxidase subunit II gene (*mtCOII*; Ashley and Vaughn 1995; Plautz *et al.* 2009), which has proved to be important in resolving phylogenetic and phylogeographic questions within diverse neotropical primate genera, including *Cebus*, *Saimiri*, *Lagothrix*, *Ateles, Alouatta,* and *Saguinus* (Ascunce *et al.* 2003; Collins and Dubach 2000; Ruiz-García and Pinedo-Castro 2010; Ruiz-García *et al.* 2010a). The third study analyzed 5 mitochondrial genes and 1 Y-linked gene (SRY; Menezes *et al.* 2010). However, all these studies analyzed a small number of specimens (6, 12, and 18 respectively).

We use *mtCOII* sequences for a large sample of *Aotus* to test several hypotheses regarding the phylogeny of *Aotus*. First, we hypothesize that the *Aotus* gray-necked group is more genetically homogeneous than the red-necked group (following Ashley and Vaughn 1995 and Plautz *et al.* 2009). Second, we test whether an ancestor of the current gray-necked group is the ancestor of all living taxa of *Aotus*, as suggested by morphological, karyotype, and molecular data from previous studies (Ashley and Vaughn 1995; Hershkovitz 1983). Third, Hershkovitz (1983) claimed that the southernmost *Aotus* species could be the most derived ones and those where stasipatric speciation could be greater. Thus, we hypothesize that taxa *Aotus* affected by stasipatric speciation (*A. azarae boliviensis* and *A. a. azarae*) will show a higher degree of genetic differentiation than other Neotropical primate taxa, because genetic drift is extreme in this speciation model. Fourth, we hypothesize that temporal split processes within *Aotus* occurred in the Miocene–Pliocene and in the Pleistocene, and are similar to those found in other Neotropical primates (Ashley and Vaughn 1995; Plautz *et al.* 2009; Ruiz-García and Pinedo-Castro 2010). Finally, we also test whether microsatellite markers show a high degree of gene diversity for this genus, as reported for other Neotropical primates (*Allouatta*, *Ateles*, *Cebus* and others: Ruiz-García 2005; Ruiz-García *et al.* 2006; 2007) and whether private alleles (those unique to a population or species) exist in the sample.

Materials and Methods

Sampling Procedures

We analyzed 696 base pairs (bp) of the *mtCOII* gene in 69 individuals of *Aotus*. We give the geographical origin of each sample in Table IA. We also sequenced 9 samples from 6 different Neotropical primate species as outgroups: 2 *Cebus albifrons* (from the Napo and Ucayali Rivers, Perú), 2 *Cebus apella* (from the Amazonas and Guainia Departments, Colombia), 2 *Cebus capucinus* (from the Cauca and Córdoba Departments, Colombia), 1 *Ateles paniscus* from the northern Brazilian Amazon, 1 *Ateles chamek* from the Mamoré River in Bolivia, and 1 *Ateles geoffroyi* sampled from the Petén area in Guatemala.

We also analyzed 136 *Aotus* for 8 DNA microsatellites to determine the mean allele number per locus and to identify possible private alleles that characterize each taxon of *Aotus*. We give the sample sizes and geographical origins of these animals in Table IB.

A licensed veterinarian (H. Gálvez) collected blood samples (0.2–2 ml) from the Peruvian *Aotus nancymaee* and *A. vociferans* for both *mtCOII* gene and for microsatellites. M. Ruiz-García visited various indigenous communities living along major rivers in the region and sampled the remaining individuals and species. He requested permission to collect biological materials from either carcasses or live individuals that were already present in the community. These samples consisted of muscle, integument, or teeth from hunted individuals that were discarded during the cooking process or hairs with bulbs plucked from live pets. He visited communities only once and all samples were donated voluntarily. No financial or other inducement was offered for supplying specimens. These sampling procedures complied with all relevant Colombian, Peruvian, Bolivian, and Paraguayan laws. We also obtained approval from each indigenous community where we collected samples.

Molecular Analyses

MtCOII *Gene* We extracted DNA from blood, taken in vacutainers, with disodic EDTA, and extracted DNA from muscle and skin with phenol–chloroform (Sambrook *et al.* 1989). We obtained DNA from hair with follicles and from teeth using 10% Chelex resin (Walsh *et al.* 1991). We used the primers L6955 (5′-AACCATTTCATAACTTTGTCAA-3′) and H7766 (5′-CTCTTAATCTTTAACT TAAAAG-3′) to amplify via polymerase chain reaction (PCR) the mitochondrial *COII* gene, located in the lysine and asparagine tRNAs (Ashley and Vaughn 1995; Collins and Dubach 2000). We performed each PCR in a 50-μl volume with reaction mixtures including 4 μl of 10× buffer, 3 mM $MgCl_2$, 2 mM dNTPs, 1 μM of each primer, 2 units of *Taq* DNA polymerase, 13.5 μl of H_2O, and 2 μl of DNA. PCR reactions were performed in a Geneamp PCR system 9600 (PerkinElmer) and in a Bio-Rad thermocycler. We used the following temperatures and cycles: 95°C for 5 min, 35 cycles of 45 s at 95°C, 30 s at 50°C, 30 s at 72°C, and a final extension time for 5 min at 72°C. We used the molecular weight marker ϕX174 DNA digested with *Hin*dIII and *Hin*fI to check all the amplifications, including positive and negative controls, in 2% agarose gels. We purified the amplified samples with membrane-binding spin columns (Qiagen), directly sequenced the double-stranded

Table I Description of the taxa of *Aotus*, sample sizes, and geographic origins for (A) *mtCOII* sequences (69 individuals) and for (B) 8 microsatellite loci (136 individuals)

Species	SS	I	GO
A. *mtCOII* sequences			
Aotus vociferans	33	6	Santa Maria and Tamboryacu rivers (Napo River), Perú
		3	Nanay River, Perú
		22	Puerto Nariño to San Juan de Atacuarí (Colombian Amazon River), Colombia
		1	La Chorrera, Igaraná-Para River, Colombia
		1	Decomissed at Bogota, Colombia
A. nancymaae	15	5	Tahuayo River, Perú
		5	Quebrada Yanayacu, Perú
		5	Carmen at the Yavarí River, Perú
A. griseimembra	9	2	Sucre Department, Colombia
		7	decomissed at Bogota, Colombia
A. brumbacki	4	1	San Martín, Meta Department, Colombia
		3	decomissed at Bogota, Colombia
A. nigriceps	2	1	Tarapoto, San Martin Department, Perú
		1	Yarinacocha, Ucayali Department, Perú
A. azarae azarae	3	1	Paraguay River, Paraguay
		2	Rio Negro, Formosa, Argentina
A. azarae boliviensis	3	1	San Javier, Beni Department, Bolivia
		1	Santa Cruz Department, Bolivia
		1	Cali laboratory, Colombia
B. Microsatellite loci			
A. griseimembra	47	2	Monpós, Bolivar Department, Colombia
		4	Achí, Cauca River, Bolivar, Colombia
		2	Los Colorados, Bolivar, Colombia
		4	Magangué, Bolivar, Colombia
		5	Zaragoza, Antioquia Department, Colombia
		6	Caucasia, Antioquia Department, Colombia
		11	Colosó, Sucre Department, Colombia
		5	Tolú, Sucre Department, Colombia
		5	San Vicente de Chuachí, Santander, Colombia
A. vociferans	27	1	La Chorrera, Igaraná-Paraná River, Colombia
		15	Puerto Nariño to San Juan de Atacuarí Colombian Amazon River, Colombia; Guaviare River, Vaupes Department, Colombia; Puerto Asis, Putumayo Department, Colombia
		4	Santa Maria and Tamboryacu rivers (Napo River), Perú
		4	Nanay River, Perú
A. nancymaae	32	16	Yahumá, Amazon River, Perú
		4	Tahuayo River, Perú
		5	Quebrada Yanuyacu, Perú
		5	Carmen at the Yavarí River, Perú

Table I (continued)

Species	SS	I	GO
		1	Aramazá, Brazil
A. brumbacki	11	7	Meta Department, Colombia
		4	Tuparro National Park, Vichada Department
A. zonalis	10	5	Los Katios National Park, Chocó, Colombia
		2	Truandó River, Chocó, Colombia
		2	Acandí, Chocó, Colombia
		1	Rio Sucio, Chocó, Colombia
A. a. boliviensis	9	4	Chimoré River, Carrasco Province, Cochabamba Department, Bolivia
		4	San Javier-Ibiató, Cercado Province, Beni, Bolivia
		1	Santa Cruz Department, Bolivia

SS=sample sizes; GO=geographical origins; I=number of individuals

DNA in a 377A (ABI) automated DNA sequencer, sequenced the samples in both directions, and then repeated the sequencing of each sample to ensure accuracy.

The use of the *mtCOII* gene (and of mitochondrial coding regions) for inferring primate phylogeny can be problematic because of the rapid rate of molecular evolution at mitochondrial loci. There is also a saturation problem regarding a phylogenetic informative signal at the third position within codons at the intergeneric level. However, the gene can be informative at the intrageneric level among some New World taxa (Ascunce *et al*. 2003). Nevertheless, we cannot exclude the possibility that some sequences obtained in this study represent mitochondrial DNA fragments inserted into the nuclear genome (numts) rather than true mtDNA (Chung and Steiper 2008). We note, however, that all amino acid translations of the sequences obtained showed the presence of initial start and terminal stop codons and the absence of premature stop codons. The sequences were deposited at GenBank (Bankit: 1393688–1406216).

Microsatellites

We used 8 microsatellite markers (AP74, D5S111, D5S117, D6S260, D8S165, D14S51, D17S804, and PEPC3). The AP74 marker was designed for *Alouatta palliata* and PEPC3 for *Cebus apella*, while the remaining markers were designed for humans (Ellesworth and Hoelzer 1998). These microsatellites have been successfully used in other Neotropical primates such as *Alouatta*, *Ateles, Lagothrix, Cebus, Saimiri,* and *Saguinus* (Ruiz-García 2005; Ruiz-García *et al.* 2006; 2007).

Our final PCR volume and reagent concentrations for the DNA extraction from blood were 25 μl, with 3 μl of 3 mM $MgCl_2$, 2.5 μl of buffer 10×, 1 μl of 0.04 mM dNTP, 1 μl of each primer (forward and reverse; 4 pmol), 13.5 μl of H_2O, 2 μl of DNA, and 1 *Taq* polymerase unit per reaction (1 μl). For the PCR reactions with hair, the overall volume was 50 μl, with 20 μl of DNA and twofold amounts of $MgCl_2$, buffer, dNTPs, primers, and *Taq* polymerase.

We performed all PCR reactions in a PerkinElmer Geneamp PCR System 9600 thermocycler for 5 min at 95°C, 30 1-min cycles at 95°C, 1 min at the most accurate annealing temperature (57°C for AP40, 50°C for AP68, and 52°C for the remaining markers), 1 minute at 72°C, and 5 min at 72°C. We kept amplification products at 4°C until they were used in a denatured 6% polyacrylamide gels in a Hoefer SQ3 sequencer vertical chamber. Depending upon the size of the markers analyzed, and the presence of 35 W as a constant, we stained the gels with $AgNO_3$ (silver nitrate) after 2–3 h of migration. We used the molecular markers *Hin*fI and ϕ174 (cut with *Hin*dIII).

We repeated the PCR reactions 3 times for DNA extracted from hair. Thus, allelic dropout was highly improbable, but we cannot completely exclude the existence of null alleles, which could increase the number of false homozygous genotypes. Nevertheless, it is improbable that all loci were affected in the same way.

Data Analyses

mtDNA We aligned mtCOII sequences manually and using the DNA Alignment program (Fluxus Technology Ltd.). We used the FindModel program to determine the most probable evolutionary model for the sequence set of *Aotus* among 28 models.

We used the number of polymorphic sites (S), the haplotypic diversity (H_d), the nucleotide diversity (π), the mean number of nucleotide differences (k), and the θ statistic by sequence to determine genetic diversity within groups. We used the following tests to measure genetic heterogeneity and to determine possible gene flow estimates: H_{ST}, K_{ST}, K_{ST}*, Z, and Z* (Hudson *et al.* 1992a, b); *Snn* (Hudson 2000); χ^2 (on the haplotypic frequencies with permutation tests of 10,000 replicates); G_{ST} (from the haplotypic frequencies), γ_{ST}, $N_{ST,}$ F_{ST} (Hudson *et al.* 1992a; from the nucleotide sequences), K_{xy} (mean proportion of nucleotide differences between taxa), and D_a (net number of nucleotide substitutions per site among taxa).

Following Ashley and Vaughn (1995), we used a transition–transversion rate ranging from 5:1 to 15:1 to obtain genetic distance trees. We used different genetic distances based on the Kimura 2 and Tamura 3 parameters and the Log-Det method (Kimura 1980; Nei and Kumar 2000; Tamura 1992). We included uniform and different heterogeneity patterns among lineages and rates among sites. We used the tree algorithm for the neighbor-joining procedure (Saitou and Nei 1987). We also used the methods of maximum composite likelihood (with and without different gamma rates and heterogeneous patterns; Tamura *et al.* 2004), maximum likelihood, and maximum parsimony (with close-neighbor-interchange with search level 1 and random addition with 100 replicates). These analyses were performed with Mega 4.1 and PAUP*4.0b8. We also used a general time reversible model (GTR) to perform a Bayesian analysis using BEAST v. 1.4.8 (Drummond and Rambaut 2007). This was selected as the preferred model by the FindModel Program. It has nucleotide substitution with the gamma distributed rate varying among sites, and 9 rate categories (GTR+G). We ran 2 separate sets of analyses, assuming a coalescence-based model and a relaxed molecular clock with an uncorrelated log-normal rate of distribution (Drummond *et al.* 2006). We combined results from the 2 independent runs (50,000,000 generations with the first 5,000,000 discarded as burn-in and parameter values sampled every 100 generations). We checked the effective sample

size for parameter estimates and convergence using Tracer version 1.4 (Rambaut and Drummond 2007). We also estimated the lower and upper 95 % highest posterior densities (HPD) for the essential parameters with the same program. We estimated the final tree in TreeAnnotator v 1.4.5 and visualized in FigTree v. 1.2.2.

Finally, we used Network 4.2.0.1 to apply the median joining network (MJ) (Bandelt *et al.* 1999) (Fluxus Technology Ltd.) to estimate divergence times among the haplotypes. Once the haplotype network was constructed, we estimated the ρ statistic (Morral *et al.* 1994) and calculated the standard deviation (σ) (Saillard *et al.* 2000). The ρ statistic is an unbiased and independent of past demographic events that could have influenced the shape of a given evolutionary tree. Ruvolo *et al*. (1991) determined a mutation rate of 0.85% per million years per Hominoidea lineage, which represents 1 mutation each 199,402 yr. Using this mutation rate, Ashley and Vaughn (1995) determined a temporal separation of 3.6 MYA between *Aotus nancymaae* and the clade conformed by *A. griseimembra* and *A. azarae boliviensis*. This mutation rate is almost identical to that determined by Ruiz-García and Pinedo-Castro (2010) for *Lagothrix*. These authors used Adkins and Honeycutt's (1994) data to estimate a 26.7% sequence divergence average between *Lagothrix lagotricha* and various cercopithecoid genera. Harrison (1987) determined a temporal split of 35 million yr between the platyrrhine and catarrhine primates. Taking into account a sequence divergence of 26.7% and a time split event of 35 MYA, gives an estimation of one mutation every 191,000 yr at the *mtCOII* gene for *Lagothrix*, which is almost identical to that determined for *Aotus*. Therefore, we used one mutation each 195,000 yr which is the average of 199,000 and 191,000 yr.

Microsatellites

We determined the mean number of microsatellite alleles per species and locus. We also determined the number of possible private alleles for each microsatellite.

Results

mtDNA Aotus vociferans had more haplotypes than other species and the highest number of segregating sites (Table II). It also had a high mean number of differences and nucleotide diversity, with only *Aotus azarae boliviensis* presenting slightly higher values. However, we had only 3 sequences for *Aotus azarae boliviensis*. We had a similar number of sequences for *Aotus nancymaae* and *A. griseimembra* as for *A. vociferans*, but their gene diversities were clearly lower than those of *A. vociferans*. All the genetic differentiation statistics were significant for the taxa of *Aotus* taxa studied (Table III).

Overall, the genetic heterogeneity statistics were high or very high (Table III), suggesting that there are large genetic differences among the taxa *Aotus* we compared and that they are all full species. The lowest pairwise values were for *Aotus vociferans* and any other taxon, and for *A. griseimembra-A.brumbacki* (Table IV).

FindModel selected the Hasegawa–Kishino–Yano evolutionary model (HKY; Hasegawa *et al.* 1985) whereas Akaike information criterion (AIC; Akaike 1974)

Table II Genetic diversity statistics for 6 *Aotus* species, and for the overall genus *Aotus*, based on *mtCOII* gene sequences

Species	NS	NSS	NH	H_d	K	π
Aotus griseimembra	9	0	1	0	0	0
A. brumbacki	4	1	2	0.5000	0.500	0.00085
A. vociferans	32	101	16	0.8851	13.185	0.02235
A. nancymae	15	19	10	0.9333	4.705	0.00797
A. nigriceps	2	6	2	1.0000	6.000	0.01017
A. a. boliviensis	3	24	3	1.0000	16.000	0.02712
Overall *Aotus*	65	252	34	0.9505	33.412	0.05663

NS=number of sequences; NSS=number of segregating sites; NH=number of haplotypes; H_d=haplotype diversity; K=average number of differences; π=nucleotide diversity

selected γ (AIC=8260.91). However, the maximum likelihood ratio suggested that the general time reversible (GTR) plus γ (LnL=−4123.57) was the most probable evolutionary model for the sequences for *Aotus*. Whenever possible, we incorporated this evolutionary model into phylogenetic analyses.

The genetic distance, maximum likelihood, and preferred maximum parsimony trees gave identical results that were only slightly different from the bootstrap results. The neighbor-joining tree with the Kimura 2P genetic distance (Fig. 2) suggests that *Aotus vociferans* constitutes a cluster with the exception of 1 individual. Within this cluster we found 3 subclusters, although their bootstrap percentages were rather low. One of these subclusters contained 17 *Aotus vociferans* sampled in the area of Puerto Nariño and San Juan de Atacuarí (Colombian Amazon). A second subcluster included 9 *Aotus vociferans*, 5 of which had the same Colombian origin. One of these individuals came from Bogotá and the remaining 3 were from the Nanay River (Peruvian Amazon). The third subcluster included 3 individuals from the Tamboryacu River, near Santa Clotilde, and 3 from the Santa Maria River. Both rivers are tributaries of the Napo River in the Peruvian Amazon.

The cluster most related to *Aotus vociferans* consisted of 2 closely related but differentiated taxa: *Aotus brumbacki* and *A. griseimembra*. The next cluster was

Table III Genetic heterogeneity statistics and gene flow estimates among all the *Aotus* species sequenced for the *mtCOII* gene

Statistics		Probability	Statistics		Gene flow (*Nm*)
χ^2	325 df=165	0.0001*			
H_{ST}	0.19235	0.0001*	γ_{ST}	0.75958	0.16
K_{ST}	0.74468	0.0001*	N_{ST}	0.91834	0.04
K_{ST*}	0.47314	0.0001*	F_{ST}	0.90447	0.05
Z	438.75	0.0001*			
Z*	5.5159	0.0001*			
Smm	1	0.0001*			

* = statistically significant

Table IV Genetic heterogeneity statistics between *Aotus* species pairs

Species 1	Species 2	Kxy	G_{ST}	γ_{ST}	N_{ST}	F_{ST}	D_a
Aotus griseimembra	*A. nancymae*	23.33	0.301	0.783	0.901	0.899	0.036
	A. nigriceps	25.00	0.468	0.927	0.883	0.880	0.037
	A. vociferans	22.16	0.223	0.351	0.699	0.702	0.026
	A. brumbacki	8.25	0.676	0.967	0.970	0.970	0.014
	A. a. boliviensis	164.00	0.437	0.957	0.960	0.951	0.264
A. nancymae	*A. nigriceps*	30.03	0.082	0.564	0.827	0.822	0.042
	A. vociferans	29.98	0.044	0.479	0.704	0.702	0.036
	A. brumbacki	24.52	0.121	0.675	0.896	0.894	0.037
	A. a. boliviensis	161.07	0.051	0.887	0.947	0.936	0.256
A. nigriceps	*A. vociferans*	29.66	0.099	0.165	0.679	0.677	0.034
	A. brumbacki	24.25	0.158	0.889	0.869	0.866	0.036
	A. a. boliviensis	161.00	0.125	0.907	0.943	0.932	0.254
A. vociferans	*A. brumbacki*	22.09	0.103	0.212	0.688	0.690	0.026
	A. a. boliviensis	164.59	0.067	0.655	0.927	0.911	0.254
A. brumbacki	*A. a. boliviensis*	167.08	0.148	0.943	0.960	0.950	0.269

composed of *Aotus nancymaae*. We detected 2 subclusters in this ensemble. The first subgroup was composed of all specimens sampled at the Tahuayo River (5) and 2 individuals of the quebrada Yanayacu, while the second contained 3 individuals sampled at the quebrada Yanayacu and 5 individuals sampled at the Yavarí River (Carmen). Comparatively, *Aotus nancymaae* is more related to some species of the gray-necked group, such as *A. vociferans, A. brumbacki,* and *A. griseimembra*, than to other *Aotus* species of the red-necked group. The next robust clade contained 2 individuals sampled in the Peruvian Amazon that had geographical and physical characteristics typical of *Aotus nigriceps* (Yarinacocha, Ucayali Department and Tarapoto, San Martín Department). Thus, *Aotus nancymaae* and *A. nigriceps* are discernible molecularly. The remaining taxa of *Aotus* of the red-necked group were very different from all of the other taxa of *Aotus* analyzed. These divergent *Aotus* are composed of 2 clearly defined clusters. The first cluster was made up of 3 individuals of *Aotus azarae azarae* from Paraguay and Argentina. The second was made up of 3 individuals of *Aotus azarae boliviensis* and 1 *A. vociferans* from La Chorrera, Igará-Paraná River within the Colombian Amazon.

The best Bayesian tree (highest log clade credibility=−119.661; Fig. 3) had the most divergent *Aotus* lineage. This contained *Aotus azarae azarae*, *A. azarae boliviensis,* and the rare *A. vociferans* haplotype from the Igará-Parana River, with a posterior probability (p) of 1. The next most divergent clade was constituted by *Aotus nigriceps* (p=1). The main cluster was constituted by 2 well defined lineages. One was made up of *Aotus brumbacki-A. griseimembra* (p=1) and *A. vociferans* (p= 0.985) while the other was composed of all the *A. nancymaae* haplotypes (p=0.935). We found 3 well delimited lineages within *Aotus vociferans* (p=1, 0.587, and 0.888) and 2 within *A. nancymaae* (Fig. 3, p=0.504, and 0.987). Most clades showed a mean posterior probability density >90% and are therefore statistically significant.

Fig. 2 Neighbor-joining tree with the Kimura 2p genetic distance applied to 69 *mtCOII* gene sequences of different taxa of *Aotus* and 9 outgroups (*Ateles paniscus, Ateles geoffroyi, Ateles chamek, Cebus capucinus, Cebus albifrons* and *Cebus apella*). Numbers in the nodes are bootstrap percentages.

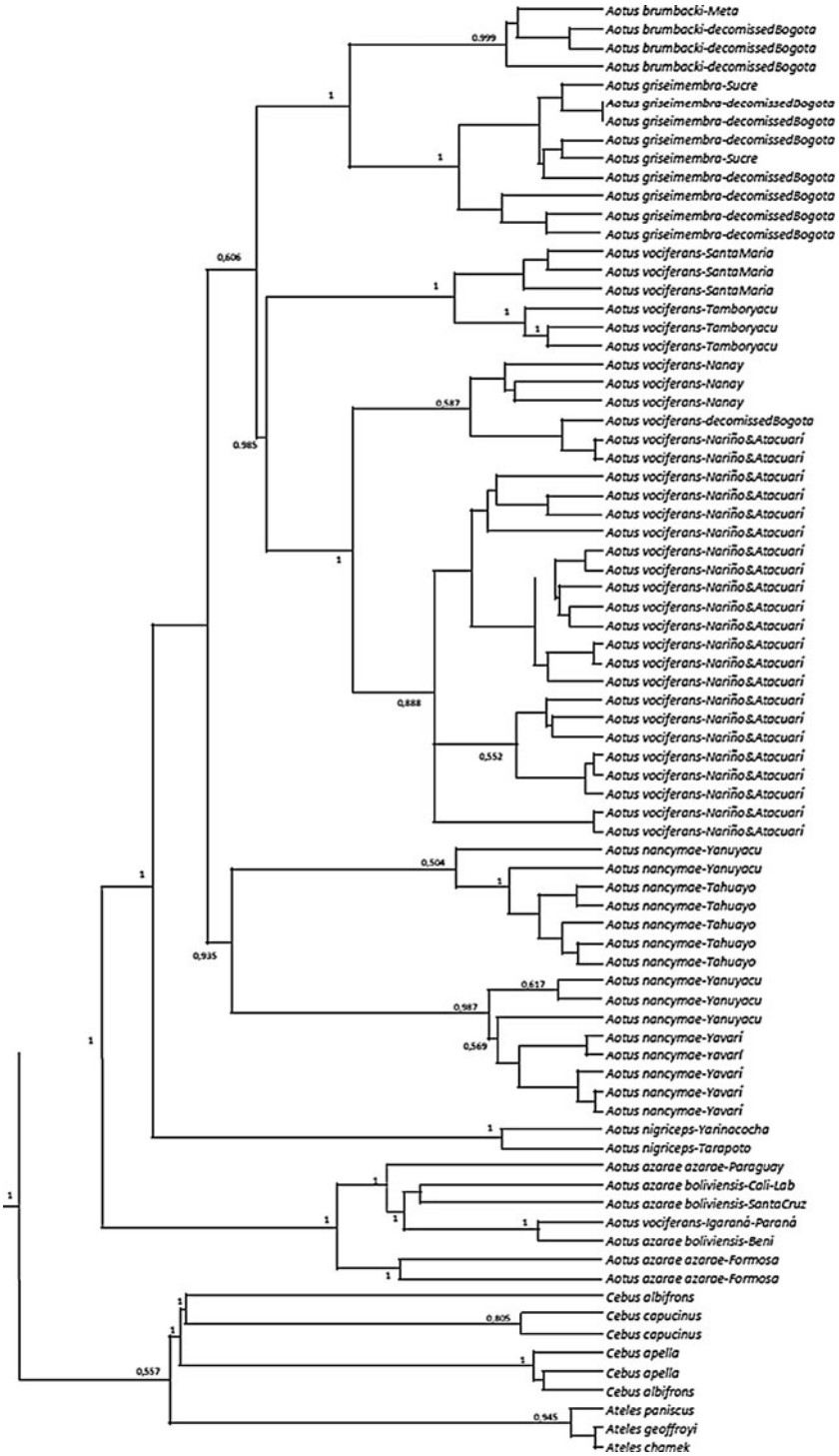

Fig. 3 Bayesian tree applied to 69 *mtCOII* gene sequences of different taxa of *Aotus* and 9 outgroups (*Ateles paniscus, Ateles geoffroyi, Ateles chamek, Cebus capucinus, Cebus albifrons*, and *Cebus apella*).

The median joining network (Fig. 4) showed results to those of the previous analyses with the haplotypes of *Aotus vociferans*, *A. nancymaae*, *A. griseimembra,* and *A. brumbacki* more or less related to each other. The other taxa of *Aotus* were progressively differentiated from this group, first *Aotus nigriceps* and second *A. azarae azarae*. The third group, composed of *Aotus azarae boliviensis* and *Aotus vociferans* from the Igará-Paraná River, was highly differentiated from the first.

Using the ρ statistic, we estimated that the main *Aotus vociferans* haplotype diverged from the main haplotypes of *A. nigriceps* at 0.74±0.04 MYA, from *A. griseimembra* at 0.97±0.05 MYA, from *A. nancymaae* at 1.93±0.07 MYA, from *A. azarae azarae* at 2.1±0.11 MYA, from *A. brumbacki* at 2.17±0.12 MYA, and from *A. azarae boliviensis* at 4.44±0.23 MYA. This main haplotype of *Aotus vociferans* showed a separation from other *A. vociferans* haplotypes 57,000–598,000 yr ago. The main haplotype of *Aotus nancymaae* showed higher split times from the other taxa of *Aotus* than did *A. vociferans* (from *A. griseimembra*, 3.44±0.21 MYA; from *A. brumbacki*, 2.7±0.17 MYA; from *A. nigriceps*, 2.34±0.13 MYA; from *A. azarae azarae*, 6±1.1 MYA; and from *A. azarae boliviensis*, 12.5±1.2 MYA). The 2 main haplotypes of *Aotus nancymaae* diverged around 0.5 MYA. The unique haplotype of *Aotus griseimembra* had the following temporal divergences: 0.4±0.03 MYA for *A.*

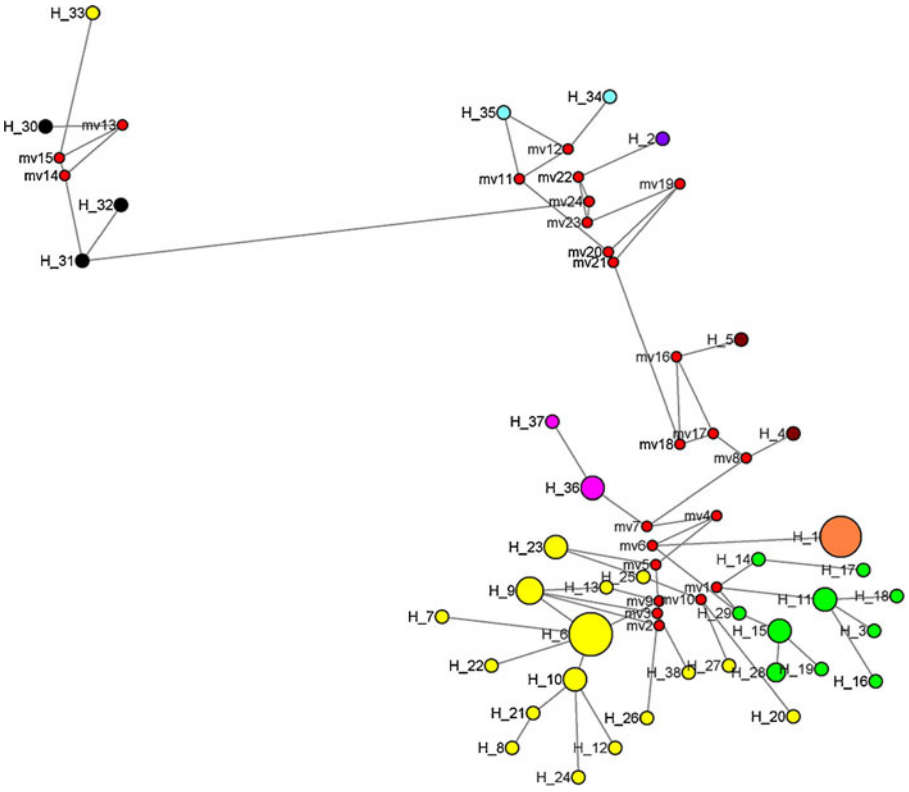

Fig. 4 Median-joining network. Haplotypes of *Aotus* are represented in different colors: yellow=*Aotus vociferans*; green=*A. nancymaae*; orange=*A. griseimembra*; pink=*A. brumbacki*; brown=*A. nigriceps*; dark blue=*A. azarae azarae,* Paraguay; light blue=*A. azarae azarae,* Formosa, Argentina; black=*A. azarae boliviensis*; red=intermediate haplotypes not found.

brumbacki, 0.6±0.05 MYA for *A. nigriceps*, 2±0.15 MYA for *A. azarae azarae,* and 4.6±0.34 MYA for *A. azarae boliviensis*. The 2 haplotypes of *Aotus brumbacki* separated 50,000±7000 yr ago. The 2 haplotypes of *Aotus nigriceps* separated 0.6±0.2 MYA. Finally, the most divergent haplotypes of *Aotus azarae boliviensis* split 2.3±0.9 MYA. The temporal splits among the taxa of *Aotus* with the Bayesian tree were very similar (assuming a divergence between *Cebus* and *Aotus* of 20 MYA) to those determined from the median joining network.

Microsatellites

We found diverse alleles at all 8 microsatellite loci (Table V). *Aotus griseimembra* presented the highest mean allele number, followed by *A. vociferans*, while *A. azarae boliviensis* had the lowest number. The mean number of alleles was similar for the 8 microsatellites across the taxa of *Aotus* studied. We observed 13 private alleles at AP74, 3 at D5S11 (in *Aotus griseimembra*, *A. brumbacki*, and *A. zonalis*), 5 at D5S117 (in *A. griseimembra*, *A. vociferans*, and *A. brumbacki*), 13 at D6S260 (in *A. vociferans*, *A nancymaae*, *A. brumbacki,* and *A. zonalis*), 8 at D8S165 (in *A. griseimembra*, *A. vociferans,* and *A. azarae boliviensis*), 9 at D14S51 (in *A. griseimembra*, *A. vociferans*, *A. nancymaae*, *A. brumbacki*, and *A. azarae boliviensis*), 3 at D17S804 (in *A. griseimembra* and *A. nancymaae*), and 5 at PEPC3 (*A. griseimembra*, *A. nancymaae*, *A. brumbacki*, and *A. azarae boliviensis*). However, these private alleles may be due to the small sample sizes.

Discussion

Our *mtCOII* data support all 4 hypotheses we tested regarding the phylogeny of *Aotus,* whereas our microsatellite data suggest that the genetic diversity within *Aotus* is comparable to that of other Neotropical primate genera (Ruiz-García *et al.* 2007).

In support of our first hypothesis, we found that the gray-necked group (*Aotus griseimembra, A. brumbacki,* and *A. vociferans*) *sensu* (Hershkovitz 1983) is more genetically cohesive than the red-necked group (*A. nancymaae, A. nigriceps, A. azarae azarae,* and *A. azarae boliviensis*). Some of our analyses even indicated that *Aotus nancymaae* and *A. nigriceps* were more related to gray-necked species than to *A. azarae azarae* and *A. a. boliviensis*. This supports the suggestion that *Aotus nancymaae* and *A. a. boliviensis* are too different to be in the same group (Ashley and Vaughn 1995). Our findings also agree with Plautz *et al.* (2009) and Menezes *et al.* (2010), in that *Aotus nancymaae* was the red-necked species most related to the gray-necked ones. This suggests that Hershkovitz's red-necked group (Hershkovitz 1983) is artificial. Further, *Aotus azarae azarae* and *A. a. boliviensis* are clearly differentiated forms based on the *mtCOII* gene and they are extremely divergent with regard to other *Aotus*.

Because our study did not include *Aotus infulatus*, we were unable to compare the genetics of this species with other taxa. However, if *Aotus azarae azarae* and *A. infulatus* are related, as suggested by Plautz *et al.* (2009), this would imply that *A. a. azarae* and *A. a. boliviensis* are different species, while *A. a. azarae* and *A. infulatus* represent 2 subspecies of the same species. An important difference between our

Table V Microsatellite alleles found at 8 microsatellite loci in the *Aotus* species studied

Species	AP74	D5S111	D5S117	D6S260	D8S165	D14S51	D17S804	PEPC3	average
Aotus griseimembra	**150, 154**	**147**, 157	**137**, 139	179, 195	131, 135	127, 135	163, 165	**257**, 263	
	156, 158	161, 163	141, 143		137, 139	139, 141	169, 171	265, 267	
	160, 162	165, 167	145, **149**		141, **143**	**143, 145**	173, **175**	**275**	
	164	169, 171			**145**, 147	**147**	**179**		
		173, 175			149				
No. of alleles	7	10	6	2	9	7	7	5	6.63±2.45
A. vociferans	**157**, 159	163, 165	141, 143	163, 167	139, 147	**151, 157**	161, 163	261, 265	
	161, 163	169,177	**163, 165**	169, **173**	**163, 165**		165, 169	267	
	165, 167		167	**175, 177**	**179**				
	169			**181, 185**					
				187, 195					
No. of alleles	7	4	5	10	5	2	4	3	5±2.51
A. nancymae	159, 161	163, 165	141, 143	**159, 161**	131, 135	**129**, 133	161, 163	**259**, 261	
	167, 169	169, 177	163, 165	**165**, 167	137, 141		165, 167	263, 265	
	171		167	169, 179	149, 153		173, **183**	267	
				189					
No. of alleles	5	4	5	7	6	2	6	5	4.75±2.12
A. brumbacki	161, 163	**153**, 157	**157**, 167	**155, 157**	135, 139	127, 131	165, 173	**273**	
	165, **179**	165, 169		159, 167		137, 141	177		
		171, 173		**171**		**169**			
		177							
No. of alleles	4	7	2	5	2	5	3	1	3.63±1.99
A. zonalis	**145**	167, 175	—	**153**	135,153	131, 137	165, 167	-	

Table V (continued)

Species	AP74	D5S111	D5S117	D6S260	D8S165	D14S51	D17S804	PEPC3	average
		177, **179**				139	171, 173 177		
No. of alleles	1	4	—	1	2	3	5	—k	2.67±1.63
A. a. boliviensis	**153, 155**	171	139	163	**123, 125** **133**	**123**, 133 **135**, 137	165, 169	**255**, 265	
No. of alleles	2	1	1	1	3	4	2	2	2±1.07
Average	4.33±2.5	5.33±3.08	3±2.35	4.33±3.67	4.5±2.74	3.83 ±1.94	4.5±1.87	3.2±1.79	

Bold numbers indicate private alleles

findings and those of Plautz *et al.* (2009) is the relationship of *Aotus nigriceps* to other taxa of *Aotus*. The individual that Plautz *et al.* sequenced came from the general area of the Juruá River in Brazil, but they did not provide a precise location. It was very different from other individuals of *Aotus*, just as we found with our *Aotus azarae boliviensis* data. Based on our findings, we suggest that this individual is not *Aotus nigriceps*, but is probably *A. azarae boliviensis*. If so, the geographical distribution of this taxon is much broader than previously reported. An alternative hypothesis is that there are different molecular lineages or even different species within *Aotus nigriceps*. This would be supported if there are different secondary endemism foci in the Huallaga and Ucayali rivers (in the case of the *Aotus nigriceps* we analyzed) and in the Juruá, Madeira, or Purús rivers in Brazil (in the case of Plautz *et al.* 2009). However, neither our data nor karyological data (Galbreath 1983; Ma 1981; Ma *et al.* 1980; Pieczarka and Nagamachi 1988; Pieczarka *et al.* 1993), support the suggestion of Plautz *et al.* (2009) that *Aotus nigriceps* is similar to the ancestral *Aotus*. In addition, our molecular data do not support Ford's hypothesis that *Aotus vociferans* is a unique species north of the Amazon River (Ford 1994). In contrast, our analyses support the notion that *Aotus griseimembra, A. brumbacki,* and *A. vociferans* are closely related (gray-necked group), with each taxon having high bootstrap percentages. We observed no haplotype mixture among these taxa, and thus found no evidence that *Aotus vociferans* should be labeled as a unique species north of the Amazon River. Ford (1994) also suggested that *Aotus azarae* should not exist. However, our data showed high genetic divergence of *Aotus azarae azarae* and *A. a. boliviensis* from the other taxa of *Aotus*, including *A. nigriceps*.

In terms of the ancestral *Aotus* species, our data permit some interesting insights. We can exclude the ancestor of *Aotus griseimembra* as the original form. All *Aotus griseimembra* sequences displayed a unique haplotype, whereas *A. vociferans* showed 16 different haplotypes. This suggests that *Aotus vociferans* had more time to diversify genetically than *A. griseimembra*. If chromosome evolution occurred via fission rather than fusion events, as traditionally thought (Defler and Bueno 2007), then this suggests that the ancestor of *Aotus vociferans* was the original form of the current taxa of *Aotus*, as *A. vociferans* has the lowest number of chromosomes of all *Aotus* species. Moreover, our molecular findings do not support the notion that *Aotus vociferans* derived from *A. brumbacki* as suggested by Ma *et al.* (1985). Of the 2 potential candidates for the origin of the *Aotus* species from the upper Amazon, *A. nancymaae* and *A. vociferans*, we found that genetic diversity is lower in *A. nancymaae*, and that it seems to have undergone more genetic drift than *A. vociferans*. This implies that it is more probable that the ancestor of *Aotus vociferans* could be the original form of the current *Aotus* than the ancestor of *A. nancymaae*. Thus, we propose that the ancestor of the current *Aotus vociferans* could be the origin of all the other taxa of *Aotus*. This species is distributed in the western Amazon, an area that has proven to be the origin and point of dispersion of other Neotropical mammals, including primates. For example, *Saimiri boliviensis* and *S. sciureus macrodon*, from the western Amazon, are the ancestors of the other *Saimiri* species (Lavergne *et al.* 2010); *Cebus apella macrodon*, from the western Amazon, may be the ancestor of the other taxa of *C. apella* (Ruiz-García and Castillo 2010); and *Lagothrix lagotricha poeppigii*, from the Peruvian Amazon, may be the ancestor of the other taxa of *Lagothrix* (Ruiz-García and Pinedo-Castro 2010). We also

previously showed that this area has the highest levels of genetic diversity at the mitochondrial *NADH5* gene for jaguars and consider this site to be the possible point of origin of this species (Ruiz-García *et al.* 2011). Similarly, the original *mtCyt-b* haplotypes of the lowland tapir (*Tapirus terrestris*) derived from the western and upper Amazon, and from there radiated to the rest of South America (Ruiz-García *et al.* 2010c; de Thoisy *et al*. 2010).

In support of our third hypothesis, the large differences between *Aotus azarae azarae* and *A. a. boliviensis* when compared to the other *Aotus* analyzed suggest that southern *Aotus* forms and, possibly, some northern haplotypes of *Aotus* have been affected by extreme levels of genetic drift. This supports the stasipatric speciation model for the formation of some of the southern *Aotus* species (Bush 1981; White 1968, 1978). One *Aotus vociferans* sampled from the Colombian Amazon (Igará-Paraná River) was more similar to *A. azarae boliviensis* haplotypes than to other *A. vociferans* haplotypes. This could mean that *Aotus azarae boliviensis* originated from a rare *A. vociferans* haplotype. If so, some populations of *Aotus vociferans* may have played an important role in the evolution of southern *Aotus* species. Our findings are in agreement with those of Hershkovitz, who suggested that the red-necked group derived from the gray-necked group (Hershkovitz 1977, 1983). Hershkovitz (1983) also stated that *Aotus azarae boliviensis* (and *A. infulatus*) have an interscapular whorl, found in only 1 species of the gray-necked group: *A. vociferans*. Our molecular findings complement this morphological evidence. If *Aotus vociferans* is ancestral to *A. azarae boliviensis*, then this morphological trait would have arisen only once. Further, species of the red necked-group have the slow glyoxalase I allele whereas *Aotus griseimembra* and *A. vociferans* have the fast allele (Ma *et al.* 1982). Nevertheless, *Aotus azarae boliviensis* presents both allele forms, which could support a connection between *A. vociferans* and *A. a. boliviensis*. There is one gray-necked *Aotus* enclave (*A. vociferans*) in the territory of a southern red-necked *Aotus* (*A. nigriceps*) in Ayapá Lake and Purús River areas (in Redemcao) (Lönnberg 1941). Thus, there is genetic, morphological, and geographical evidence for the important role of *Aotus vociferans* in the appearance of southern populations of *Aotus*.

Our fourth hypothesis was that the temporal split processes within *Aotus* occurred in the Miocene–Pliocene and in the Pleistocene, and are similar to those found in other Neotropical primates. Ashley and Vaughn (1995) estimated the temporal separation of *Aotus nancymaae* and *A. griseimembra* at 3.6 MYA using the mutation rate of Ruvolo *et al*. (1991). We obtained a very similar temporal separation of 3.44 MYA (Pliocene) between the same 2 taxa of *Aotus* taxa. In contrast, Barroso *et al.* (1997) determined a temporal separation of 2.7 MYA between *Aotus nancymaae* and *A. azarae azarae* whereas our temporal split between these taxa was around 6 MYA. Two species (*Aotus vociferans* and *A. griseimembra*) showed the most conservative temporal splits with regard to the other taxa of *Aotus* analyzed. Other taxa showed higher (and less probable) temporal divergences. It is likely that the taxa most affected by genetic drift during the stasipatric speciation process showed the greatest skewed and unrealistic temporal divergence splits. This is observable in *Aotus nancymaae*, *A. brumbacki*, *A. nigriceps,* and especially in *A. azarae azarae* and *A. a. boliviensis*. In contrast, this phenomenon is not observed in *Aotus vociferans* or in *A. griseimembra*. The most differentiated taxon, *Aotus a. boliviensis*, was closely

related to a very differentiated haplotype of *A. vociferans* (from the Igará-Paraná River, Colombian Amazon) in all our phylogenetic analyses. *Aotus vociferans* presented the highest number of haplotypes and the highest nucleotide diversity of the taxa for which we obtained a large number of sequences. In addition, the pattern homogeneity test showed that *Aotus azarae boliviensis* presented different evolutionary rates for *mtCOII* with regard to the other taxa of *Aotus*, with the exception of some individuals of *A. vociferans*. This suggests that *Aotus azarae azarae* is more related to other taxa of *Aotus* than to *A. a. boliviensis*.

Taking *Aotus vociferans* or *A. griseimembra* as the original species of the genus, we can correlate the temporal splits with important geologic and climatologic events. The most differentiated taxon, *Aotus azarae boliviensis*, diverged from the 2 possible original taxa around 4.4–4.6 MYA. This corresponds to the lower Pliocene and to the formation of the Amazon River during the Late Miocene, 5.0 MYA (Lundberg *et al.* 1998). Menezes *et al.* (2010) determined an identical time split for the original diversification of the current taxa of *Aotus* (4.62 MYA). Therefore, the ancestor of the current *Aotus azarae boliviensis* could have come from an uncommon *A. vociferans* haplotype at the northern bank of the Amazon River. *Aotus azarae azarae* and *A. nancymaae* (both on the southern bank) may have originated from an *A. vociferans* ancestral stock (northern bank) around 2 MYA. The appearance of these 2 taxa could correlate with the beginning of the Pleistocene around 1.6–2 MYA (Rendford 2002) and changes in the western Amazon. *Aotus vociferans* and *A. griseimembra* are the 2 possible ancestral taxa that separated around 1 MYA. This coincides with the Pre-Pastonian glacial period at 0.80–1.30 MYA, characterized as the highest glacial peak of the first Quaternary glaciation (Nebraska-Günz), when a dry period coincided with large fragmented forests. *Aotus nigriceps* diverged more recently around 0.6–0.75 MYA, regardless of whether the ancestors of *A. vociferans* or *A. griseimembra* were its ancestral stock. The highest glacial peak of the second glaciation (Kansas-Mindel) occurred around 0.65 MYA when a dry period again produced new fragmented forests. It is interesting to note that *Aotus nancymaae* and *A. nigriceps* coinhabit the east bank of the Purus River, south of the Amazon River (Ford 1994). The 2 species are similar, but they represent 2 different and independent speciation events, probably from *Aotus vociferans.* Finally, *Aotus brumbacki* probably separated from *Aotus griseimembra* around 0.4 MYA. This separation may have been produced by other maximum glacial peaks of the Kansas-Mindel glaciation (0.45 MYA) and by another secondary peak of this glacial period, 0.35 MYA. Within the species that presented different haplotypes, the temporal divergence among the main haplotypes ranged from 0.057 (*Aotus brumbacki*) to 0.6 MYA (*A. vociferans*). The high glacial peak of the second glaciation (Kansas-Mindel, 0.65 MYA) could be responsible for the haplotype separation within *Aotus vociferans, A. nancymaae,* and *A. nigriceps*. The last glacial period (Wisconsin-Würm) developed from 120,000 to 10,000 yr ago, with a maximum glacial peak 18,000 yr ago and may have generated the divergence between the haplotypes of *Aotus brumbacki*. Therefore, although the first separation events in *Aotus* were in the Pliocene, the Pleistocene forest refugia invoked by Haffer (1969, 1982) may be responsible for a major portion of the speciation events in *Aotus*. Thus, our results partially disagree with the conclusions of Ashley and Vaughn (1995), but agree with those of Ma (1981) that a large fraction of the evolution in *Aotus* was induced by the

climatic changes effected by Milankovitch's cycles. These cycles operated across the Quaternary, with cold and dry phases, and generated refuges in the Amazon (Haffer 1997; Whitmore and Prance 1987).

Finally, our microsatellite results showed a high degree of polymorphism and gene diversity in *Aotus* as we previously found in other Neotropical primates (Ruiz-García 2005; Ruiz-García *et al.* 2006; 2007). We also found an elevated potential number of private alleles within the species studied. Microsatellites could help to identify specimens of *Aotus* that were obtained illegally and that have unknown geographic origins. However, because the microsatellites employed were designed for other species (*Alouatta palliata*, *Homo sapiens,* and *Cebus apella*), the amplicons should be sequenced for each taxon of *Aotus* to confirm that the number of repetitions (and motifs) were the same in each taxon studied (Lau *et al.* 2004). Although we sequenced multiple alleles in diverse taxa of *Aotus* (*A. vociferans* and *A. nancymaae*), we did not sequence all alleles for all the microsatellites analyzed, and may not have detected all private alleles. In addition, some sample sizes of several taxa of *Aotus* were small and it is possible that by increasing the sample size, the number of private alleles would decline.

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