mtDNA Diversity in Azara's Owl Monkeys (*Aotus azarai azarai*) of the Argentinean Chaco

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ABSTRACT Owl monkeys (*Aotus spp*.) inhabit much of South America yet represent an enigmatic evolutionary branch among primates. While morphological, cytogenetic, and immunological evidence suggest that owl monkey populations have undergone isolation and diversification since their emergence in the New World, problems with adjacent species ranges, and sample provenance have complicated efforts to characterize genetic variation within the genus. As a result, the phylogeographic history of owl monkey species and subspecies remains unclear, and the extent of genetic diversity at the population level is unknown. To explore these issues, we analyzed mitochondrial DNA (mt DNA) variation in a population of wild Azara's owl monkeys (*Aotus azarai azarai*) living in the Gran Chaco region of Argentina. We sequenced the complete mitochondrial genome from one individual (16,585 base pairs (bp)) and analyzed 1,099 bp of the hypervariable control region (CR) and 696 bp of the cytochrome oxidase II (COII) gene in 117 others. In addition, we sequenced the mitochondrial genome (16,472 bp) of one Nancy Ma's owl monkey (*A. nancymaae*). Based on the whole mtDNA and COII data, we observed an ancient phylogeographic discontinuity among *Aotus* species living north, south, and west of the Amazon River that began more than eight million years ago. Our population analyses identified three major CR lineages and detected a high level of haplotypic diversity within *A. a. azarai*. These data point to a recent expansion of Azara's owl monkeys into the Argentinean Chaco. Overall, we provide a detailed view of owl monkey mtDNA variation at genus, species, and population levels. Am J Phys Anthropol 000:000–000, 2011.

The South American Gran Chaco is comprised of 1,000,000 km^2 of grassland and forests found throughout Argentina, Bolivia, Brazil, and Paraguay. It extends 1,500 km from north to south, and 700 km from east to west (18°–35° S, 57°–66° W, de la Balze et al., 2003). Following the Amazonian rain forest, the Gran Chaco is the second largest biome of the continent (Bertonatti and Corcuera, 2000), yet its ecological development and paleohistory are poorly understood.

Chacoan fauna are characterized by high diversity and low endemism (Porzecanski and Cracraft, 2005). Among the inhabitants of the Chaco are some of the southernmost primates in South America, Azara's owl monkeys (*Aotus azarai azarai*), which may have achieved their present day locations via southward migrations along the Paraná-Paraguay Rivers (Zunino et al., 1985). Other Chacoan primate species are thought to have originated from Amazonian stocks to the north, and eventually populated the region through the continent's waterway corridors (e.g., black howler monkeys, *Alouatta caraya*: Do Nascimento et al., 2007; Zunino et al., 2007). However, little is known about the timing of these Chacoan migrations, or how they may have shaped the genetic diversity in southern owl monkey populations.

Questions concerning owl monkey origins extend to the entire genus. In fact, researchers have only recently begun to agree on the number of extant *Aotus* species (Ford, 1994; Defler and Bueno, 2007; Fernandez-Duque, 2011). When initially described, the genus only included the species *Aotus trivirgatus* (Brumback et al., 1971), although further cytogenetic characterization revealed

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that this taxon had at least three different chromosomal backgrounds ($2n$ = 46–58) (Brumback, 1973, 1974; Ma, 1981, 1983). This diversity led to the current designation of thirteen owl monkey species and subspecies based on karyotypes, pelage coloration, and relative levels of susceptibility to different malaria pathogens (*Plasmodium spp*.) (Hershkovitz, 1983; Ford, 1994; Defler and Bueno, 2003; Di Fiore et al., 2009) (Supporting Information Table S1). However, issues such as the close proximity of species' ranges to one another, hybridism, questionable sample provenance, and the difficulties of tracking nocturnal arboreal primates has meant that few samples have been used to characterize the range of genetic variation within the genus (Ashley and Vaughn, 1995; Defler and Bueno, 2007; Plautz et al., 2009; Menezes et al., 2010, Monsalve and Defler, 2011). This is particularly true with regard to the southernmost owl monkey species *A. azarai*.

In addition, phylogeographic studies of the genus *Aotus* have estimated molecular divergence dates that are not consistent with fossil and cytogenetic evidence. For example, estimates of 3.6 Ma (Ashley and Vaughn, 1995) or 4.7 Ma (Plautz et al., 2009) for the divergence of *Aotus* species do not agree with paleontological evidence like the 11.8–13.5 Ma *Aotus didensis* fossils from La Venta, Colombia (Setoguchi and Rosenberger, 1987; Rosenberger et al., 2009; Takai et al., 2009). They are also not congruent with coalescence dates of ~22 Ma for an *Aotus*-platyrrhine divergence based on nuclear DNA data (Opazo et al., 2006), or ~15 Ma for the emergence of the genus *Aotus* based on whole mitochondrial genomes sequences (Hodgson et al., 2009). If those estimates (3.6–4.7 Ma) for the diversification of the genus were accurate, then they would imply more than 10 million years of lineage stasis before extant owl monkey species began to diverge from one another.

Given these apparent discrepancies, we were interested in exploring further the evolutionary history of *Aotus* through the analyses of molecular genetic data. We hypothesized that the pattern and timing of the radiation of *Aotus* species within South America was more complex and began much earlier than previously postulated. To elucidate the timing and nature of speciation events, and to ascertain the position of our study taxon within the phylogenetic history of *Aotus*, we characterized mitochondrial DNA (mtDNA) variation in a wild population of *A. a. azarai* living in the Argentinean Gran Chaco. We anticipated that the southern species of owl monkeys would be characterized by the accumulation of commonly derived mutations, reflecting the progressive settlement of individuals into regions south of the Amazon River. By contrast, the northern owl monkey taxa would be expected to exhibit greater haplotype diversity as a result of barriers to gene flow caused by geological and hydrological change, or perhaps driven by natural forces selecting for different pathogen regimes.

To test our predictions, we sequenced the entire mitochondrial genome of one Azara's owl monkey (*A. a. azarai*) and one Nancy Ma's owl monkey (*A. nancymaae*). We investigated the phylogenetic origins of *A. a. azarai* by examining the cytochrome *c* oxidase subunit II (COII) gene (Ruvolo et al., 1993; Adkins and Honeycutt, 1994) and compared our data with those from other species within the genus *Aotus*. We further utilized information on the hypervariable control region (CR) to characterize the structure of genetic diversity of the study population. By implementing these three approaches, we are able to describe owl monkey mitochondrial evolution at the genus, species, and population levels.

Abbreviations

AICc	Akaike information criterion correction
BI	Bayesian inference
BIC	Bayesian information criterion
CI	consensus index
COII	cytochrome oxidase II
CR	control region
CSB	conserved sequence blocks
DT	decision theory
ESS	effective sample size
MCMC	Markov Chain Monte Carlo
MJ	median joining
ML	maximum likelihood
mtDNA	mitochondrial DNA
mtTF	mitochondrial transcription factor
RC	rescaled consistency index
RI	retention index
SNP	single nucleotide polymorphisms

MATERIALS AND METHODS

Study area

The Owl Monkey Project (*Proyecto Mirikiná*) studies the Azara's owl monkey population that inhabits the gallery forests along the Pilagá and Guaycolec Rivers (Fernandez-Duque et al., 2001), within the province of Formosa, Argentina (see Fig. 1). Azara's owl monkeys have a species range that extends at least another 180 km south and 300 km west from the study area (Zunino et al., 1985), and there is no evidence suggesting that the study population has been isolated as a result of its geographic location or human activities.

Samples

Since 2001, over 140 individuals have been sampled within the 3 km^2 core study area. Upon capture, each animal was given a physical exam during which hair, blood, or tissue samples (ear punches, skin biopsies) were collected for use in genetic analyses (Fernandez-Duque and Rotundo, 2003). For one individual, the source of DNA was the remains of a placenta and fetus found on the ground in the savannah.

From the collected samples, we characterized mtDNA variation in 118 *A. a. azarai* individuals. Ninety-one of the samples were from individuals who inhabit the core study area. Seven other samples were from individuals captured along the gallery forest as far as 10 km upstream from the core area, while 15 individuals were sampled downstream of this location. One sample came from a male who was captured in the gallery forest along the Monte Lindo River, ~25 km north of the field site. In addition, we collected four samples from captive individuals of unknown geographic origin at the Saenz-Peña Municipal Zoo, located 250 km away from the study area in the city of Saenz-Peña, Chaco Province, Argentina.

For comparative analyses, DNA samples from four putative *Aotus* species and subspecies (*A. nancymaae*, *A. nigriceps*, *A. lemurinus*, and *A. l. grisiemembra*) and six individuals representing three other platyrrhine taxa (two *Callicebus donacophilus*, two *Pithecia pithecia pithecia*, and two *Saimiri sciureus sciureus*) were obtained from the Zoological Society of San Diego. Another five samples of *A. nancymaae* were obtained from individuals

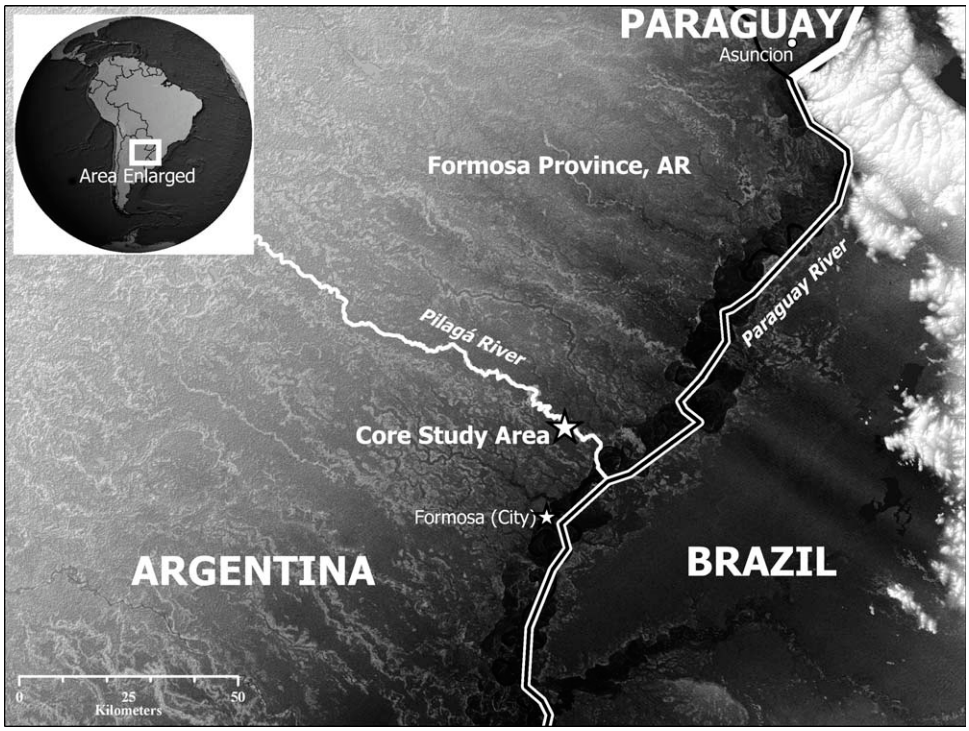

Fig. 1. Study area location in Formosa Province, Argentina. Core study site is located at latitude: 25° 59.4′ South, longitude: 58°, 11.0′ West, and projected using the WGS 1984 coordinate system, geographic panel UTM 21S.

at the DuMond Conservancy for Primates and Tropical Forests (Miami, FL).

DNA was isolated from tissue, blood, and hair roots using QIAamp purification kits (Qiagen), and DNA quantity and quality were assayed on the NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Genetic sequencing

To avoid the amplification of nuclear insertions of mtDNA (numts) in all downstream reactions, the entire mtDNA genome was amplified in two large fragments (LR1 and LR2) for all samples, each ~9 kb in length, with >200 and 1,100 bp of overlap between the ends of the fragments (Raaum et al., 2005; Thalmann et al., 2005; Sterner et al., 2006). Using two pairs of primers designed for human mtDNA (Meyer et al., 2007), we conducted Long Range polymerase chain reactions (LR-PCR) with the Expand Long Range dNTPack (Roche) following the protocol and amplification parameters recommended by the manufacturer.

Next, to determine the unknown sequence of the entire mitochondrial genomes for both *A. a. azarai* and *A. nancymaae*, we designed a panel of 20 overlapping primer pairs to obtain complete mtDNA sequences for samples that had been amplified through LR-PCR. These primers were based on conserved regions shared by *Aotus lemurinus* (FJ785421), *Saguinus oedipus* (FJ785424), and *Aotus trivirgatus* (AY250707). Stretches of consensus (100% shared base identity) extending for ≥20 bases in length were screened for their capacity to function as primers using NetPrimer (Premier BioSoft). Portions of the *A. lemurinus* mitochondrial genome were also directly interrogated using Primer3 (Rozen and Skaletsky, 2000; SourceForge.net). In total, the two methods yielded a pool of 57 pairs of potential primers. After realignment with the *A. lemurinus* sequence, 20 pairs of primers were selected to amplify the mtDNA genome in overlapping fragments ranging from 750 to 1,800 bp in size (Supporting Information Fig. S1, Table 1).

To address questions related to the phylogeographic origin and phylogenetic placement of the study population, we characterized sequence diversity in the COII gene. Using primers redesigned from sequences available in the published literature (Disotell et al., 1992; Ruvolo et al., 1993; Ashley and Vaughn, 1995), we amplified and sequenced the entire COII gene (696 bp in platyrrhines) from LR-PCR products for all samples. Concurrently, for the analysis of population level maternal diversity, we targeted the entire hypervariable CR of the mtDNA molecule. Following the enrichment of mtDNA fragments through LR-PCR, three primer pairs (CR-1 thru CR-3) were used to obtain 1,099 bp of CR sequence for each individual.

PCR cycling parameters were optimized for each primer pair using the Touchgene Gradient thermocycler (Techne), and all subsequent reactions were amplified in GeneAmp 9700 thermocyclers (ABI). Amplified products were visualized on 1% TBE SeaKem agarose (Lonza) via gel electrophoresis. PCR recipes and parameters are detailed in Supporting Information Table S2.

Amplicons were purified by SAP/Exo I digestion (New England BioLabs) and cycle-sequenced using Big Dye™ v3.1 (ABI). Excess dye terminators were removed with the BigDye XTerminator™ purification kit (ABI), and DNA sequences were read on a 3130xl Gene Analyzer (ABI). Read quality of chromatograms was assessed using Sequencing Analysis v5.4 software (ABI), and bidirectional sequences were aligned and assembled using Sequencher v4.9 (Gene Codes) and Geneious Pro

TABLE 1. Aotus-specific primers for amplification and sequencing of the mitochondrial CR and COII gene

Primer name	Genetic region	Oligonucleotide sequence (5′-3′)
LR1-F	Long range fragment 1	GGC TTT CTC AAC TTT TAA AGG ATA
LR1-R	Long range fragment 1	TGT CCT GAT CCA ACA TCG AG
LR2-F	Long range fragment 2	CCG TGC AAA GGT AGC ATA ATC
LR2-R	Long range fragment 2	TTA CTT TTA TTT GGA GTT GCA CCA
01-F	Whole mtDNA	TGA GGA GCG AGT ATC AAG CAC
01-R	Whole mtDNA	GTG ACG GGC GGT GTG TG
02-F	Whole mtDNA	TCG CAG AGT AAG CAG AAG CA
02-R	Whole mtDNA	CTA TGG TGG TGG AGC GTT TT
02-F.2	Whole mtDNA	CGC CAT CTT CAG CAA ACT CC
02-R.2	Whole mtDNA	GGA CAA CCA GCT ATC ACC A
03-F	Whole mtDNA	CTA TGT GGC AAA ATA GTG GG
03-R	Whole mtDNA	CCA TAG GGT CTT CTC GTC TTA
04-F	Whole mtDNA	GGT AGC ATA ATC ACT TGT TCT C
04-R	Whole mtDNA	CTA GGG TTG GGG CAG TTA CA
05-F	Whole mtDNA	CAA TTT CGC AAA GGT CCT AAC
05-R	Whole mtDNA	CTT ATG TTT GGG GTG GAA TGC
06-F	Whole mtDNA	CGA TTC CGA TAC GAC CAA CT
06-R	Whole mtDNA	GGC TTT GAA GGC TCT TGG TC
07-F**	Whole mtDNA	GCA ACC GCA TCC ATA ATT CT
07-R**	Whole mtDNA	CGG CGG GAG AAG TAG ATT G
07-F.2	Whole mtDNA	GCT CCA CAG AAG CAT CCA CT
07-R.2	Whole mtDNA	GAG TAA GCA TTA GAC TGT AAA TC
08-F	Whole mtDNA	GCA TCA ACT GAA CGC AAA TC
08-R	Whole mtDNA	ATG ATT ATA GTG GCT GAT GT
09-F	Whole mtDNA	GCT TCT GAC TTC TAC CCC CAT C
09-R	Whole mtDNA	GGT GTT GCC ATT AAG ATA TA
10-F	Whole mtDNA	CAA CCC TCC CAA TAG AAG CA
10-R	Whole mtDNA	AGT GGG ACA GGT GTT CCT TG
11-F	Whole mtDNA	CTA TGG GCA GCA ACC GTA
11-R	Whole mtDNA	GAG TGG TAG AAT GCT CAG AAG
12-F	Whole mtDNA	CGT TGT CCG AGA GGG TAC AT
12-R	Whole mtDNA	TAA GGG TTG TGT TTT TCG GC
13-F**	Whole mtDNA	CTA TAT CTC TAT CTA CTG ATG AGG
13-R**	Whole mtDNA	GAG TGG GGA TAA GGG TGG TT
13-F.2	Whole mtDNA	TTT CTG ACG GAA TTT ACG GC
13-R.2	Whole mtDNA	CTG TGG CCG TGA ATG TTA TG
14-F**	Whole mtDNA	GCC GAA AAA CAC AAC CCT
14-R**	Whole mtDNA	GTA TGT CAG TGG CCC TCG TT
14-F.2**	Whole mtDNA	GAA TGT GGA TTT GAC CCC AC
14-R.2**	Whole mtDNA	CGT GTG AAT AGG GGT TTT ACA TT
14-F.3	Whole mtDNA	CCT AAC CCT CAC AGC CTG AC
14-R.3	Whole mtDNA	GGC AAG GTT GGC TAG ATT TG
15-F	Whole mtDNA	TAC GAA CGA ATT CAC AGC CG
15-R	Whole mtDNA	ACT GGG GTA GGT CCT TCT ATA GC
16-F	Whole mtDNA	AGC AAT AGC ATG ATT CTT CCT A
16-R	Whole mtDNA	ATT ATG GTG TTT GAG TTG TT
16-F.2	Whole mtDNA	CTC CTT CCC CCT AAT AAG TCT CC
16-R.2	Whole mtDNA	GTT GTT TTG GTT ACT TGT TG
17-F	Whole mtDNA	ACC CCC ACT CAA GCC TAA CT
17-R	Whole mtDNA	GCG GTT GAG GTA TCT GGT GT
17-F.2	Whole mtDNA	ACC AAA ACT AAC AAT ACA AAC TC
17-R.2	Whole mtDNA	GGC GAC GGA GGA GAA GGC
18-F	Whole mtDNA	GTC ATT ATT CCC ACA TGG AC
18-R	Whole mtDNA	GGG TCT TGG CTG GTA GTT CA
19-F	Whole mtDNA	GCC AAA TAT CAT TCT GAG G
19-R	Whole mtDNA	CTG GTT TCA CGG AGG TAG GT
19-F.2	Whole mtDNA	GAG GTG GCT TCT CAG TAG
19-R.2	Whole mtDNA	GAA GTG GGC GGG TTG CTG
20-F	Whole mtDNA	CTC AGC ATT CCC GTA GGT TC
20-R	Whole mtDNA	CTA AGC ATA GTG GGG TAT CTA ATC
LR1-F	Long range fragment 1	GGC TTT CTC AAC TTT TAA AGG ATA
LR1-R	Long range fragment 1	TGT CCT GAT CCA ACA TCG AG
LR2-F	Long range fragment 2	CCG TGC AAA GGT AGC ATA ATC
LR2-R	Long range fragment 2	TTA CTT TTA TTT GGA GTT GCA CCA
CR1 F	CR	TGA ACT ACC AGC CAA GAC CC
CR1 R	CR	CTG GTT TCA CGG AGG TAG GT
CR2 F	CR	CTA CCT CCG TGA AAC CAG CA
CR2 R	CR	TAC GGG AAT GCT GAG GAA AG
CR3 F	CR	CTT TCC TCA GCA TTC CC
CR3 R	CR	TTT TCT GAA GGG TGT GGT TT
tRNA-Asp-F[a,b]	COII	AAC CAT TCA TAA CTT TGT CAA

TABLE 1. (Continued)

Primer name	Genetic region	Oligonucleotide sequence (5′-3′)
tRNA-Lys-R[a,b]	COII	CTC TTA ATC TTT ACT TAA AAG
COII-Seq[a,b]	COII	TTT AGG CGT CCT GGG ATT
COII-F.2	COII	AAT AAT TAC ATA ACT TTG TCA A
COII-R.2	COII	CTC TCG GTC TTT AAC TTA AAA G
COII-int-F	COII	GGC CAT CAA TGA TAC TGA AGC
COII-int-R	COII	TTC ATA GCT TCA GTA TCA TTG ATG G

[a]From the study by Ruvolo et al. (1993).
[b]From the study by Ashley and Vaughn (1995).
All remaining primers designed for current study.
**Primers that failed to work during amplification PCRs. Such primers were re-designed, and are denoted by the suffix –.2 or –.3 at the end of the primer name.

v5.16 (Drummond et al., 2010). All new mtDNA sequences from this study have been deposited in GenBank, and their accession numbers are listed in Table 2.

Phylogenetic analyses

To explore the phylogeographic origin of *A. a. azarai* and its relationship to other owl monkey species, we conducted phylogenetic analyses of COII sequences. Searches in GenBank (NCBI) yielded 23 informative COII sequences representing nine of the fourteen putative species and subspecies usually described when discussing the genus *Aotus* (Supporting Information Table S1). COII sequences for *A. miconax*, *A. hershkovitzi*, and *A. zonalis* were unavailable. Because several of the GenBank sequences lacked the entire 696 bp gene sequence, the COII sequence matrix was pruned to 549 bases (nt positions 16-564) for interspecies analyses. Final COII alignments were also translated (vertebrate mtDNA code) to check for stop codons, the presence of which could indicate the amplification of numts rather than true mtDNA sequences.

The 118 *A. a. azarai* sequences were restricted to the seven unique haplotypes identified in the study population to minimize phylogenetic errors (Zwickl and Hillis, 2002), and then combined with 23 COII sequences from other species of *Aotus* obtained from GenBank, along with the nine non-*azarai* owl monkey sequences generated for this study. The set of 39 *Aotus* COII sequences represented 12 owl monkeys from taxa distributed north of the Amazon River, 11 from the west, and 16 from the south. The six sequences generated from *Callicebus*, *Pithecia*, and *Saimiri* samples were included to obtain a greater range of coalescent points within the platyrrhines, and sequences from single *Macaca*, *Tarsius*, and *Lemur* individuals were used as outgroups.

To select the most appropriate model for our phylogenetic analyses, we ran the program jModelTest v0.1.1 (Guindon and Gascuel, 2003; Felsenstein, 2005; Posada, 2008) using 11 substitutions patterns to survey 88 models of nucleotide substitution (+F base frequencies, rate variation of +I and +G with nCat = 4). The modified Akaike Information Criterion (AICc) setting was implemented because of the small size of comparative nucleotide characters (549), and parallel searches using Bayesian information criterion (BIC) and performance-based decision theory (DT) were conducted. The base tree for our likelihood calculations was optimized for Maximum Likelihood (ML) phylogenetic analysis. All three searches in jModeltest selected the TPMuf1+G model (Kimura, 1981) with a likelihood score (-lnL) of 3370.84. This model was applied to the ML analysis implemented in the phylogenetic program PAUP* 4.0b10 (Swofford, 2002) to maximize the probability of observing the alignment of *Aotus* COII nucleotides. Bootstrap values were estimated based on a set of 10,000 replicates.

Bayesian inference (BI) analysis was undertaken to obtain the most probable set of trees given an evolutionary model and our specific alignment of data, using the software program BEAST v1.5.3 (Drummond and Rambaut, 2007). In addition to BI phylogenetic tree construction, BEAST can estimate coalescent dates using a relaxed lognormal molecular clock that accounts for post-divergence and lineage-specific variations in mutation rate (Drummond et al., 2002, 2006; Ho et al., 2005).

We imported 48 COII sequences (34 *Aotus*, 2 *Callicebus*, 2 *Pithecia*, 2 *Saimiri*, 1 *Macaca*, 1 *Tarsius*, and 1 *Lemur*) into the program BEAUti v1.5.3 to format the run file for BEAST [***AotusCOII.xml***]. This set of taxa was used to provide an adequate evolutionary time depth that encompassed the few known fossil platyrrhines (Table 3). As the TPMuf1+G model selected by jModelTest was unavailable in BEAST, we used the TN93 substitution model with three partitions for codon positions, empirical base frequencies. We implemented a randomly generated starting tree and the Yule Process speciation parameter as the tree prior. We specified the fossil time points as log-normally distributed priors applied to the appropriate taxon designations (Supporting Information Fig. S2). The Markov Chain Monte Carlo (MCMC) search was run with four chains for 10,000,000 generations, with trees sampled every 1,000 generations.

Using the average standard deviation in split frequencies among the four chains (0.01), the level of convergence was assessed (<0.05) and found to denote an acceptable level of post-convergence tree likelihoods that influence the accuracy of our consensus Bayesian tree. The first 1,000 trees were discarded as "burn-in" to remove extraneous pre-convergence probability values (Altekar et al., 2004). We further analyzed the results generated in BEAST in TRACER v.1.5 (Rambaut and Drummond, 2007) to assess the accuracy of the estimations based on the effective sample sizes (ESS) of our data.

Output files from the ML and BI phylogenetic calculations were summarized using TreeAnnotator v1.5.3 (Drummond and Rambaut, 2007) to construct a consensus tree (50%) on the basis of mean node heights and maximum clade credibility values. Summary trees were imported into FigTree v1.2.3 (Drummond and Rambaut, 2007) for visualization. Using MacClade v4.0.8 (Madison and Madison, 2003), the consensus (CI), rescaled consistency (RC), and retention indices (RI) were also assessed.

TABLE 2. CR and COII haplotype definitions for all individuals and species and subspecies analyzed

Taxon	Common name	CR Hg	COII Hg	Freq.	Animal location	GenBank
Aotus azarai azarai	Azara's Owl Monkey	A[a]	AaaØ[a]	26	Wild	XXXX.X
		A[a]	AaaI[a]	1	Wild	XXXX.X
		A1[a]	AaaØ[a]	1	Wild	XXXX.X
		A1a[a]	AaaØ[a]	1	Wild	XXXX.X
		A2[a]	AaaØ[a]	2	Wild	XXXX.X
		A4[a]	AaaØ[a]	1	Wild	XXXX.X
		A5[a]	AaaØ[a]	1	Wild	XXXX.X
		A6[a]	AaaØ[a]	1	Wild	XXXX.X
		B[a]	AaaØ[a]	32	Wild	XXXX.X
		B1[a]	AaaØ[a]	1	Wild	XXXX.X
		B1a[a]	AaaØ[a]	1	Wild	XXXX.X
		B2[a]	AaaØ[a]	1	Wild	XXXX.X
		B3[a]	AaaØ[a]	1	Wild	XXXX.X
		B4[a]	AaaØ[a]	1	Wild	XXXX.X
		B5[a]	AaaØ[a]	1	Wild	XXXX.X
		B6[a]	AaaØ[a]	1	Wild	XXXX.X
		B7[a]	AaaØ[a]	1	Wild	XXXX.X
		B8[a]	AaaØ[a]	1	Wild	XXXX.X
		C[a]	AaaØ[a]	8	Wild	XXXX.X
		C[a]	AaaII[a]	1	Wild	XXXX.X
		C1[a]	AaaØ[a]	13	Wild	XXXX.X
		C1[a]	AaaIII[a]	3	Wild	XXXX.X
		C1a[a]	AaaIII[a]	1	Wild	XXXX.X
		C2[a]	AaaØ[a]	7	Wild	XXXX.X
		C2[a]	AaaIV[a]	1	Wild	XXXX.X
		C2a[a]	AaaØ[a]	1	Wild	XXXX.X
		C2c[a]	AaaØ[a]	1	Wild	XXXX.X
		C3a[a]	AaaØ[a]	1	Wild	XXXX.X
		C3b[a]	AaaV[a]	1	Wild	XXXX.X
		C4[a]	AaaØ[a]	1	Wild	XXXX.X
		C5[a]	AaaØ[a]	1	Wild	XXXX.X
		X[a]	AaaVI[a]	1	Wild	XXXX.X
		Y[a]	AaaØ[a]	1	Sáenz Peña Zoo	XXXX.X
		Z[a]	AaaØ[a]	1	Sáenz Peña Zoo	XXXX.X
Aotus azarai boliviensis	Bolivian Owl Monkey	–	Aab01[b]	1	GenBank	U36846.1
Aotus infulatus	Feline Owl Monkey	–	Aai04[c]	1	GenBank	DQ321662.1
		–	Aai05[c]	1	GenBank	DQ321663.1
		–	Aai09[c]	1	GenBank	DQ321667.1
		–	Aai10[c]	1	GenBank	DQ321668.1
Aotus lemurinus	Grey-bellied Owl Monkey	–	Al001[a]	1	San Diego Zoo	XXXX.X
		–	Al01[b]	1	GenBank	U36845.1
		–	Al02[b]	1	GenBank	U36844.1
		–	Al03[b]	1	GenBank	U36843.1
Aotus lemurinus brumbacki	Brumback's Owl Monkey	–	Alb11[c]	1	GenBank	DQ321669.1
Aotus lemurinus grisiemembra	Grey-handed Owl Monkey	–	Alg002[a]	1	San Diego Zoo	XXXX.X
		–	Alg03[c]	1	GenBank	DQ321661.1
Aotus nancymaae	Nancy Ma's Owl Monkey	–	Ana001[a]	1	San Diego Zoo	XXXX.X
		–	Ana01[c]	1	GenBank	DQ321659.1
		–	Ana02[c]	1	GenBank	DQ321660.1
		–	Ana12113[c]	1	GenBank	AF352255.1
		–	Ana837[d]	1	GenBank	AF352254.1
		–	Ana04[b]	1	GenBank	U36770.1
		–	Ana002[a]	1	DuMond	XXXX.X
		–	Ana003[a]	1	DuMond	XXXX.X
		–	Ana004[a]	1	DuMond	XXXX.X
		–	Ana005[a]	1	DuMond	XXXX.X
		–	Ana006[a]	1	DuMond	XXXX.X
Aotus nigriceps	Black-headed Owl Monkey	–	Ani001[a]	1	San Diego Zoo	XXXX.X
		–	Ani12012[c]	1	GenBank	AF352258.1
		–	Ani718[d]	1	GenBank	AF352256.1
		–	Ani719[d]	1	GenBank	AF352257.1
Aotus trivirgatus	Three-striped Owl Monkey	–	At01[e]	1	GenBank	AY250707.1
Aotus vociferans	Spix's or Noisy Owl Monkey	–	Av07[c]	1	GenBank	DQ321665.1
		–	Av08[c]	1	GenBank	DQ321666.1
		–	Av328[d]	1	GenBank	AF352259.1
		–	Av331[d]	1	GenBank	AF352260.1
Callicebus donacophilus	White-eared Titi Monkey	–	Cd001[a]	1	San Diego Zoo	XXXX.X
		–	Cd002[a]	1	San Diego Zoo	XXXX.X
Saimiri sciureus sciureus	Common Squirrel Monkey	–	Sss001[a]	1	San Diego Zoo	XXXX.X
		–	Sss002[a]	1	San Diego Zoo	XXXX.X

TABLE 2. (Continued)

Taxon	Common name	CR Hg	COII Hg	Freq.	Animal location	GenBank
Pithecia pithecia pithecia	White-faced Saki Monkey	–	Ppp001[a]	1	San Diego Zoo	XXXX.X
		–	Ppp002[a]	1	San Diego Zoo	XXXX.X
Tarsius syrichta	Philippine Tarsier	–	Ts001[f]	1	GenBank	L22784.1
Macaca mulatta	Rhesus Macaque	–	Mm001[g]	1	GenBank	M74005.1
Lemur catta	Ring-tailed Lemur	–	Lc001[f]	1	GenBank	AJ421451.1

Locations: Wild animals sampled in Formosa, Argentina (Lat = 25°, 59.4' South; Long = 58°, 11.0' West).
[a] From this study.
[b] From the study by Ashley and Vaughn (1995).
[c] From the study by Plautz et al. (2009).
[d] From the study by Suarez et al., unpublished.
[e] From the study by Collura et al., unpublished.
[f] From the study by Arnason et al. (2002).
[g] From the study by Disotell et al. (1992).

TABLE 3. Fossil calibration points used to estimate time to most recent common ancestor (TMRCA)

Clade	Fossil	Epoch	Radiometric dates (Ma)	Shape	Calibration mean (Ma)	SD (Ma)	Offset (Ma)
Aotus	*Aotus dindensis*[a]	Miocene	11.8–13.5	Lognormal	−1.0	0.85	6.5
Saimiri	*Neosaimiri*[b]	Miocene	12–15	Lognormal	1.0	1.0	1.0
Platyrrhini	*Branisella bolivana*[c]	Miocene	27	Lognormal	1.0	0.5	22
Platyrrhini + Catarrhini	*Parapithecus grangeri*[d]	Oligocene	36–40	Lognormal	1.0	0.5	30
	Catopithecus browni[e]	Eocene	30–36				
	Proteopithecus sylviae[f]	Eocene	36				
Primates	*Plesiadapiforms*[g]	Paleo/Eocene	62	Lognormal	1.0	0.5	59
	K-T extinction event[g]	Paleocene	65				

[a] From the study by Setoguchi and Rosenberger (1987).
[b] From the study by Hartwig and Meldrum (2002).
[c] From the study by Takai et al. (2000).
[d] From the study by Beard and Wang (2004).
[e] From the study by Simons et al. (1987).
[f] From the study by Takai and Ayana (1996).
[g] From the study by Bloch et al. (2007).

Population genetic analyses

To assess species and population level mtDNA variation, summary statistics, including gene (π) and haplotype (h) diversity, were calculated for both the COII and CR sequences using programs available in Arlequin v3.11 (Excoffier et al., 2005) and DnaSP v4.50 (Rozas et al., 2003). Both data sets were also used to conduct pairwise mismatch analysis (π), and calculate expansion variables (τ), which provide estimates of past population size and dynamics (Rogers and Harpending, 1992). In addition, the neutrality indices Tajima's D (Tajima, 1989a, b) and Fu's F_S (Fu, 1997) were calculated to estimate whether population expansions or contractions had occurred. Transversions (TV) were weighted higher (10) than transitions (TI) (1) in all calculations to account for the differential probability of either occurring across evolutionary time (Excoffier et al., 2005).

Multistate median joining (MJ) networks were generated from both COII and CR sequences using NETWORK v4.5.02 (Bandelt et al., 1999) to investigate the intraspecific phylogenetic relationships among samples based on parsimony (Posada and Crandall, 2001). In the construction of networks with the CR data set, a 1:10 (TI:TV) weighting scheme derived from human CR studies was employed (Bandelt and Parson, 2008). This ratio does not deviate significantly from the 1:9.5 TI:TV ratio previously estimated in primate mtDNA studies (Yoder et al., 1996; Purvis and Bromham, 1997; Yang and Yoder, 1999). In addition to this weighting scheme, the characters at CR nucleotide positions 136, 210, 249, 256, 275, 851, and 927 were down-weighted to reduce the reticulations caused by these hypervariable characters. The caveat inherent to working with character-based networks is that no alternative evolutionary model other than parsimony can be tested.

To explore the demographic history of the study population, pairwise differences among the *A. a. azarai* sequences in both the CR and COII data sets were calculated, and the frequency distributions of observed pairwise mismatches were plotted. For each of the analyses outlined above, the mean number of pairwise differences and raggedness index were calculated (Rogers and Harpending, 1992).

For intraspecific phylogenetic dating, the rho value (ρ), a product of mutation rate (μ), and time (τ), was determined. The rho value reflects the average number of pairwise differences between a set of sequences to a designated root. This value was estimated for all distinct clusters and their sub-branches in the CR data set using NETWORK v4.5.02 (Bandelt et al., 1995, 1999).

The within-population rate of mutation for the *Aotus* mitochondrial CR remains uncertain. Thus, we utilized two different CR mutation rates (ω: changes per site per

million years) to provide high and low estimates of intraspecific genetic coalescence for the study owl monkey population. These two rates were drawn from human (ω: 0.320, Sigurgardóttir et al., 2000) and primate (ω: 0.111, Weinreich, 2000) studies. The pedigree-based human mutation rate translates into 2,843.49 years per CR mutation, whereas the population-based primate mutation rate translates into 8,197.39 years per CR mutation. It should be noted that the 95% confidence intervals and standard errors for coalescent dates do not consider mutation rate errors (Forster et al., 1996).

RESULTS

Whole mtDNA genome sequencing of *A. a. azarai* and *A. nancymaae*

We assessed molecular variation at the genus level by examining whole mitochondrial genome sequences from two previously unexamined *Aotus* species, Azara's and Nancy Ma's owl monkeys. When compared with the mitochondrial genome of *A. lemurinus* (FJ785421, Hodgson et al., 2009) as a reference sequence, we observed a large number of single nucleotide polymorphisms (SNPs) among all three species. *A. a. azarai* and *A. lemurinus* were distinguished from one another by 941 SNPs, *A. a. azarai* from *A. nancymaae* by 985 SNPs, and *A. lemurinus* from *A. nancymaae* by 835 SNPs.

In addition, we observed a striking difference in the sequence composition of the CR of *A. nancymaae* relative to those of *A. a. azarai* and *A. lemurinus* (16,472 bp vs. 16,585 bp in *A. a. azarai* and 16,580 bp in *A. lemurinus*). In *A. nancymaae*, ~13 separate deletions, ranging in size from 2 to 32 bp, shortened the genome by 113 bp, making it the smallest platyrrhine mitochondrial genome present in GenBank (including sequences from Hodgson et al., 2009). These deletions were observed in multiple *A. nancymaae* individuals through direct sequencing, and confirmed through PCR amplification and gel electrophoresis, with CR amplicons from *A. nancymaae* individuals being appreciably smaller than those of other owl monkey taxa (Supporting Information Fig. S3).

Phylogenetic analyses

We noted similar phylogenetic relationships within and among the northern, western, and southern clades of *Aotus* species irrespective of the ML or BI methods employed. The COII phylogeny showed a deep phylogenetic split between *Aotus* species and subspecies living north of the Amazon River and those living south of it (Fig. 2a). The ML bootstrap values associated with this split were high (86 for the first clade of the bifurcation, 97 for the second), and the BI analysis exhibited similarly high posterior probabilities (1.00) for the same clusters of closely related species of *Aotus*.

We estimated the time to the most recent ancestor (TMRCA) at each phylogenetic node (Fig. 2b). The TMRCA estimates varied widely, from 1.78 Ma for *A. a. azarai* (95% HPD: 0.24–3.99 Ma) to 4.68 Ma for *A. nancymaae* (95% HPD: 1.93–8.10 Ma) (Table 4). When dated according to their geographic ranges relative to the Amazon River, the TMRCA of northern species was 7.34 Ma, whereas that of the different southern species was 6.22 Ma. The TMRCA for the genus *Aotus* was 8.95 Ma.

Sequence diversity and population structure in *A. a. azarai*

The mtDNA CR sequence analysis revealed considerable genetic diversity in the Azara's owl monkey study population. Fifty-two polymorphic sites (TI, TV, and insertion–deletion substitutions) were present in 118 individuals, and they defined 30 distinct haplotypes (<5% missing data; Table 5). We also obtained full COII sequences for all 118 individuals, and observed that eight polymorphic nucleotides defined seven unique COII haplotypes. The TI:TV ratio was 4:4 for the eight segregating sites, but transitions were more frequent at the third nucleotide position.

DNA sequences for the COII gene were well conserved when compared with those of the CR (Table 5). The Nei's gene diversity estimate (π) was 0.005 for CR haplotypes, but only 0.001 for the COII haplotypes. Similarly, haplotype diversity estimates yielded values of 0.83 for the CR sequences, and 0.14 for the COII sequences. However, both the CR and COII data sets had modest population expansion values (τ of 5.2 and 3, respectively) and negative Tajima's D and Fu's F_S values.

The network of CR sequences contained three distinct clades, or haplogroups (hg) (Fig. 3a). Each clade consisted of a central, high frequency haplotype, and a number of derivative haplotypes extending from it. The CR network also included three outlier haplotypes that correspond to three animals that appeared to be distantly related to the other individuals within the population. They included a solitary individual captured within the study area (X) and two zoo animals (Y and Z). The intraspecific network generated from the seven unique COII haplotypes was less structured than the one based on CR sequences (Fig. 3b). COII haplotype "AaaØ" represented 93% of the individuals (109 of 118), and the remaining haplotypes were only 1–2 mutational steps away from this founder type.

The mismatch analyses provided details about the demography of southern Azara's owl monkeys. The mismatch distribution for CR sequences of *A. a. azarai* showed a relatively small number of pairwise differences, with the curve being strongly skewed to the left (see Fig. 4). The COII sequences for the same individuals displayed a similar left-skewed mismatch curve, albeit at lower resolution. This limited diversity of COII sequences was reflected in the raggedness index (Table 5), as well as the COII haplotype network (Fig. 3b).

We estimated dates for the primary maternal lineages appearing in the study population using information from the network analyses (Table 6). Of the three major CR haplogroups, hg-A appeared to be the oldest, due to its central location in the network and the presence of two derived haplogroups (hg-B and hg-C) extending from it. We confirmed this impression through the calculation of ρ for each haplogroup relative to its putative ancestral node using two different rates for ω (human: 0.320 vs. primate: 0.111). For hg-B and hg-C, the ancestral node was hg-A, whereas for hg-A, the ancestral node was a median vector (mv) that linked it to the more divergent haplotypes Y and Z that belonged to two zoo animals. The lack of a transitional haplotype directly ancestral to hg-A may have inflated the coalescence estimate for that clade. Even so, these estimates generated coalescence dates of 12,161 years for hg-A, 3,888 years for hg-B, and 3,303 years for hg-C when the faster, pedigree-based human ω is used. The CR haplogroup age estimations

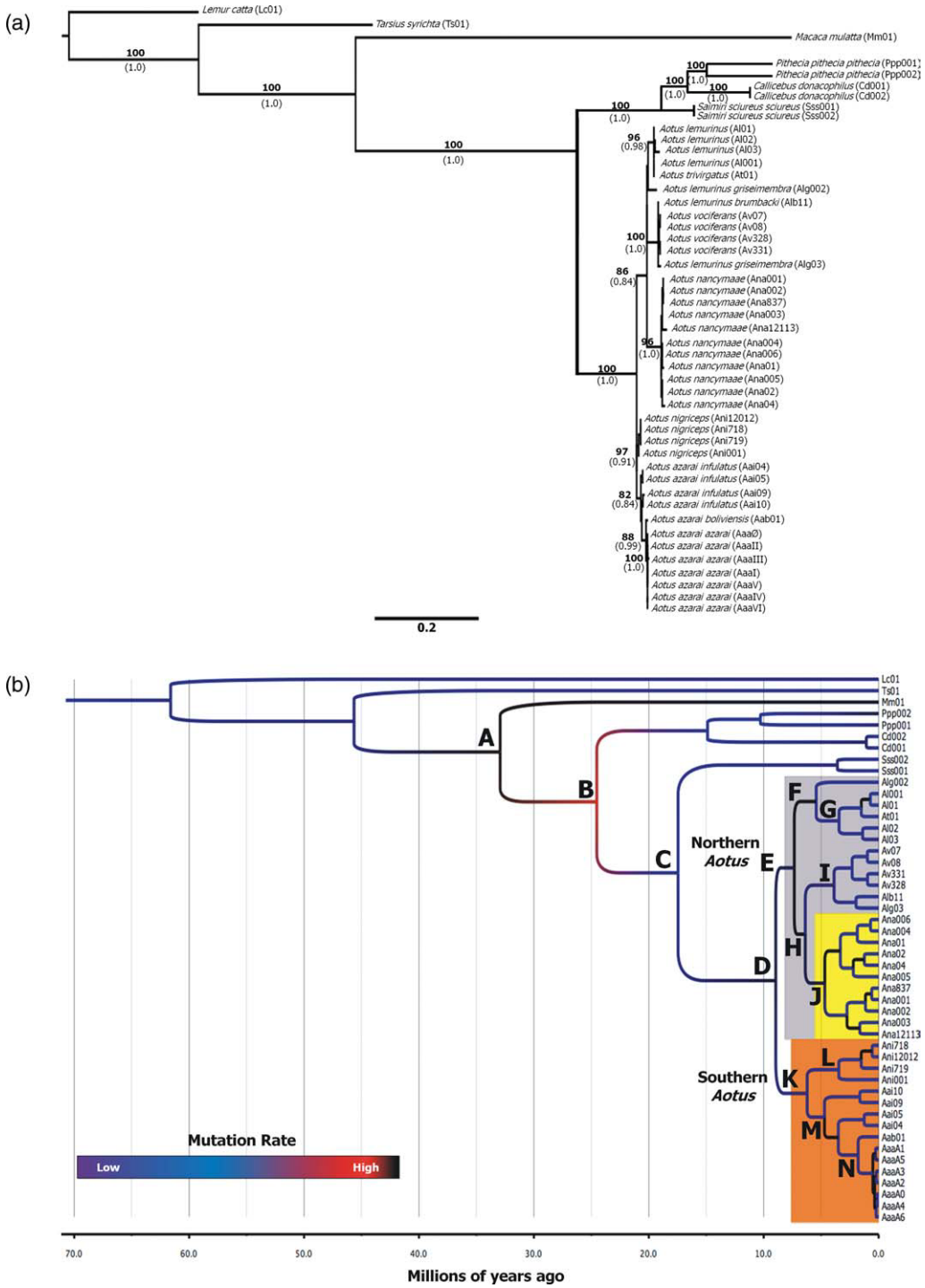

Fig. 2. Panel **a**: A consensus phylogram of COII sequences from the genus *Aotus* constructed using two different tree-building methodologies. Branching patterns and branch lengths are based on the consensus of ML and BI phylogenetic analyses. ML bootstrap values >70% from 10,000 replicates are shown above the branches. Bayesian posterior probabilities are listed below branches in parentheses (BI). The ML phylogram is the consensus of 2,990 trees, with a length of 344 steps, CI = 0.66, RI = 0.82, RC = 0.54. The BI phylogram was based upon four computational chains run in parallel across 10,000,000 generations, with trees sampled every 1,000 generations. Panel **b**: Bayesian chronogram depicting the coalescence times for 48 primate COII sequences. The mutation rate of COII is displayed in a blue (0 = low) to red (0.5 = high) color gradient along branches. Arabic letters located at nodes refer to the splits at which coalescence dates were estimated in Table 4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 4. Molecular dating of COII phylogenetic branches in the genus Aotus

Node[a]	Age[b]	Confidence interval[c]	Notable events
A	32,936,700	30,733,500–35,779,000	Platyrrhine—catarrhine divergence
B	24,528,000	22,704,700–26,750,200	Coalescence of platyrrhines
C	17,452,700	9,904,300–24,412,800	Coalescence of Cebidae, emergence of *Aotus*
D	8,953,100	6,526,500–13,879,400	Coalescence of all extant taxa from genus *Aotus*
E	7,340,500	4,385,800–11,716,700	Coalescence of all "Northern" *Aotus* species
F	5,432,000	††	Divergence of *A. lemurinus* and *A. l. griseimembra*
G	3,440,900	600,900–6,881,900	Coalescence of *A. lemurinus*
H	6,382,100	††	Divergence of *A. vociferans* and *A. nancymaae*
I	2,307,500	410,000–4,799,400	Coalescence of *A. vociferans*
J	4,682,300	1,929,100–8,103,700	Coalescence of *A. nancymaae* ("Western migration")
K	6,215,600	2,777,100–10,516,200	Coalescence of all "Southern" *Aotus* species
L	3,461,800	466,300–7,360,500	Coalescence of *A. nigriceps*
M	4,711,900	1,482,800–8,464,800	Derivation of *A. infulatus* population(s)
N	1,782,200	235,400–3,989,100	Derivation of *A. a. boliviensis*, coalescence of *A. a. azarai*

[a] Node identifications on the Bayesian chronogram in Figure 2a.
[b] All COII dates are rounded to the nearest 100.
[c] 95% HPD confidence intervals listed as: lower bound - upper bound. See Supporting Information Figure S2 for marginal density plots of TMRCA range distributions.
†† Represents a split clade. For split clades and single-frequency taxa, confidence intervals could not be estimated.

TABLE 5. Statistical indices of molecular diversity in Aotus azarai azarai

	A. a. azarai CR	*A. a. azarai* COII
Summary statistics		
Sample size	118	118
Nucleotides (bp)	1,099	696
Polymorphic Sites	52	8
Transitions (TI)	23	4
Transversions (TV)	5	4
Insertion/Deletions	25	0
Haplotypic diversity		
# Haplotypes	30	7
Haplotype Diversity (h)	0.83	0.14
Sequence diversity		
Nei's Gene Diversity (π)	0.005 ± 0.002	0.001 ± 0.001
Tau (τ)	5.2	3
Mean # Pairwise Differences	5.6 ± 2.7	0.8 ± 0.6
Harpending's Raggedness Index	0.01	0.6
p (Harpending's)	0.7	0.6
Selective neutrality		
Tajima's D (1,000 simul.)	−0.4	−1.1
p (D simul < D obs)	ns	0.001
Fu's F_S (1,000 simul.)	−25.2	−3.4
p (sim. $F_S \leq$ obs. F_S)	ns	ns

increase to 35,065 years for hg-A, 11,211 years for hg-B, and 9,523 years for hg-C when the slower, population-based primate ω is applied.

DISCUSSION

Origins of *Aotus*

Through our analyses of the mitochondrial genome, we have characterized the genetic variation of *A. a. azarai* at the genus, species, and population levels. By sequencing the two complete mitochondrial genomes *A. a. azarai* and *A. nancymaae*, we were able to compare total mtDNA diversity with the previously published *A. lemurinus* mitochondrial genome. This comparison reinforced the tripartite distinction of Northern, Southern, and Western owl monkey species, and revealed a surprising series of CR mutations that have occurred in *A. nancymaae* since its split with, and subsequent isolation from *A. lemurinus* and other northern species. Many of the CR polymorphisms specific to *A. nancymaae* were large deletions, the sum of which reduces the CR by 113 bp (Supporting Information Fig. S3). While the deletions do not appear to affect the nucleotide composition of known conserved sequence blocks (CSBs 1-3), 7S DNA loop overlap or mitochondrial transcription factor (mtTF) binding sites, one deletion is situated close to the mitochondrial replication termination site (TAS). Although the functional impact of the *A. nancymaae* deletions is unknown, large CR deletions of this kind have been reported among species of platypus (Gemmell et al., 1996) and subspecies of gorillas (Xu and Arnason, 1996).

Chronology of COII *Aotus* phylogeny

Recent surveys of putative *Aotus* taxa delineated the deep phylogenetic split between northern and southern types, and identified the separation of *A. nancymaae* as the result of a western migratory scenario (Plautz et al., 2009; Menezes et al., 2010). However, these studies estimated the coalescence of all modern *Aotus* lineages at ~4.0 Ma. This estimate permits only a limited amount of time for the emergence of the karyotypic, morphological, and immunological differences that are observed among *Aotus* taxa today. Similarly, a relatively recent north–south owl monkey split would imply an inordinately long period of evolutionary stasis if recent fossil and molecular estimations of 10–12 Ma for the emergence of the genus were accurate (Setoguchi and Rosenberger, 1987; Hodgson et al., 2009; Rosenberger et al., 2009; Takai et al., 2009).

Our analyses point to an older and more complex evolution of the genus (see Fig. 5). Based on the analysis of 39 COII haplotypes from 10 different owl monkey taxa, we estimate that the TMRCA for the genus *Aotus* is 8.95 Ma, a date that is considerably older than the previous coalescent date estimates of 3.6–4.7 Ma (Ashley and Vaughn, 1995; Plautz et al., 2009). This discrepancy may be due to the increased number of putative taxa that were sampled for this study, phylogenetic outgroup rooting, or the use of fossils as calibration time points rather than molecular dating using internal rooting and mutation rates derived from an autosomal

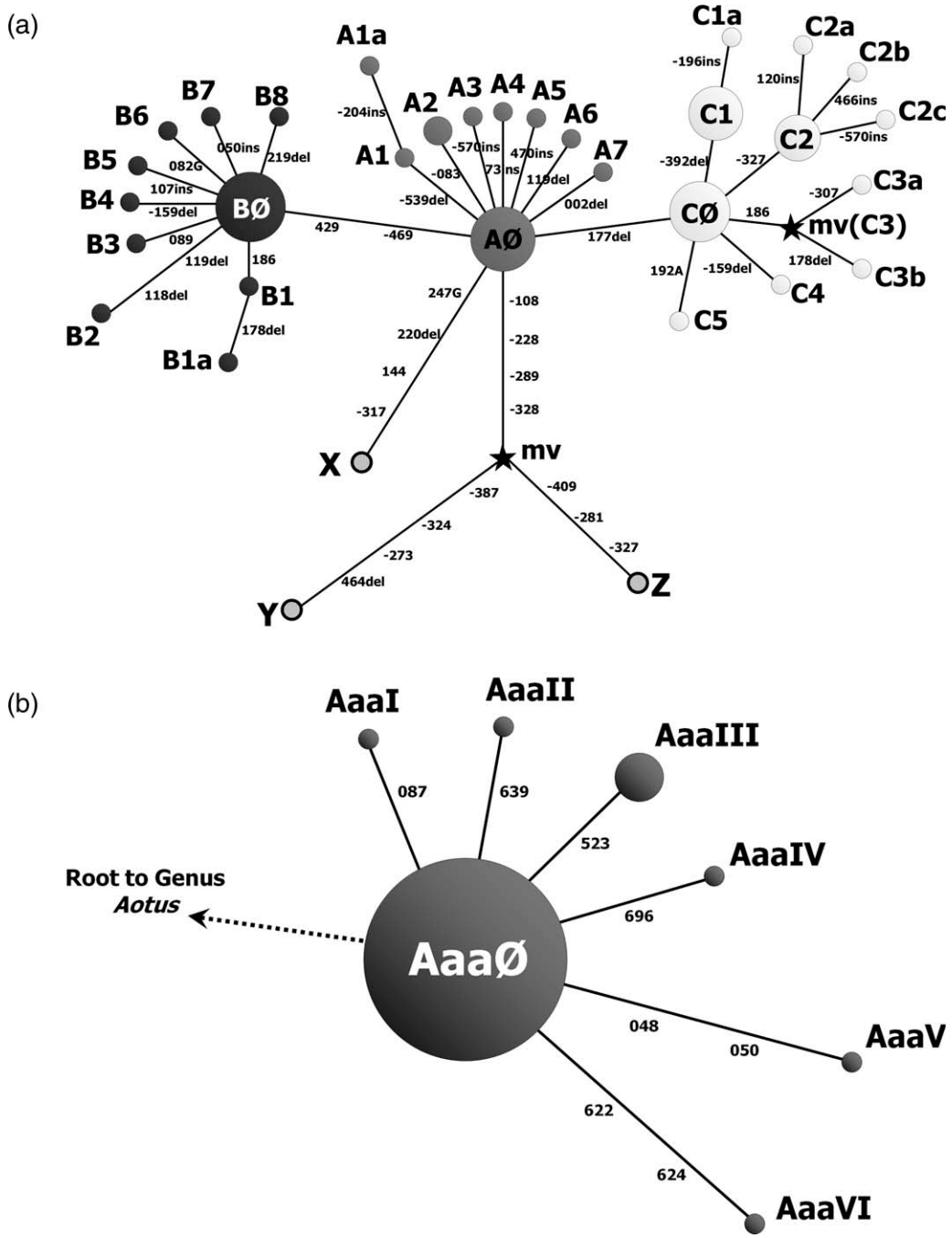

Fig. 3. Panel **a**: Median joining network of *Aotus azarai azarai* CR sequences. The three major clades of CR sequences are designed A, B, and C. Founder haplotypes are given the suffix "Ø," and derivative haplotypes are numbered sequentially from them. The haplotype associated with each *A. a. azarai* sample is indicated in Table 2. Haplotype X represents a solitary individual captured in the study area, while Y and Z represent individuals from the local zoo. Putative intermediate haplotypes are designated as median vectors (mv) in the network. The specific mutations (homologous to human mtDNA positions 579 bases before (denoted [-]) and 509 bases after the "0" point or origin of replication) that define the haplotypes are shown along the branches of the network. Panel **b**: Median joining network of *A. a. azarai* COII sequences. The founder haplotype is given the suffix "Ø" and derivative haplotypes are numbered sequentially from it using Roman numerals to distinguish this network from the relationships defined by the CR data.

locus. Repeated BEAST runs with different parameters (nucleotide substitution models, normalized calibration points) consistently produced time frames similar to the results presented here. In any event, the TMRCA for genetic loci will always predate the actual splitting of populations.

We also observe a deep phylogenetic split between *Aotus* species living north of the Amazon River from those living to the south that may have begun over 8 Ma. Following this split, *A. nancymaae* diverged from other northern groups and became genetically isolated. These clades also correlate with differences in malarial

resistance and pelage coat color observed in species from those geographic regions (Ford, 1994; Defler and Bueno, 2007; Fernandez-Duque, 2011).

The southern "red-neck" clade is comprised of species whose COII sequences cluster on the basis of taxon identity (Fig. 2a, Supporting Information Table S1). Its branches are short and split in a derivative pattern of bifurcations that mimics the same north–south range distribution of those putative taxa. This clinal pattern points to periods of gradual expansion characterized by short episodes of COII sequence divergence among the southern groups. Our coalescence dates also suggest that *A. nigriceps* was the first southern species to diverge from other owl monkey populations beginning some 3.46 Ma.

In support of this interpretation, the majority of southern species are karyotypically identical. All of them possess 49(male)/50(female) karyotypes, with the exception of *A. nigriceps* (51m/52f). These findings suggest that the southern expansion of *Aotus* was gradual (e.g., only one chromosomal fission event and the maintenance of the Y-autosomal fusion event in southern males), with species diversifying steadily in different points in time, not through multiple splits or population bottlenecks (Pieczarka et al., 1993, 1998; Torres et al., 1998). This scenario would fit with the paleogeographic history of the South American continent and the Amazon River, including the formation of the gigantic inland Lago Amazonas by the Andean uplift ~9 Ma and its drainage ~5.0–2.5 Ma, as well as the more recent establishment of southern rivers and the draining of the South American Chaco (Rosenberger et al., 2009).

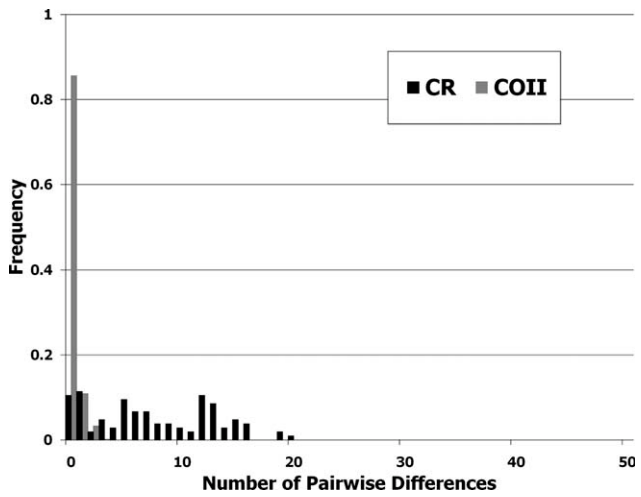

Fig. 4. Pairwise mismatch distributions of *A. a. azarai* CR and COII sequences.

The northern "grey-neck" clade tells a different evolutionary story. Overall, northern owl monkey taxa display phylogenetic relationships characterized by the separation of several clusters, each with shallow bifurcations. Species found north of the Amazon possess a wide range of karyotypes ranging from 46 in *A. vociferans* to 56 in *A. lemurinus* (Defler and Bueno, 2007). Thus, these two patterns of variation may reflect speciation through neutral genetic drift and isolation through paleogeological river formation, allopatric distance, or the possible consequences of karyotype incompatibility.

Alternatively, some external pressure, such as the gradient of severity of malarial parasitism from the tropics to the subtropics could have been involved in the selection of different pathogen regimes. Such a scenario might explain the high levels of allelic diversity at HLA and KIR loci present in different human populations indigenous to the same geographic regions (Belich et al., 1992; Parham et al., 1997, Gendzekhadze et al., 2006). If similar pressures were exerted upon owl monkey populations living in the tropics, then it is possible that northern *Aotus* taxa could have experienced more rapid rates of evolution at immunological loci, as well as structurally across their genomes (Van Valen, 1973).

Our data further suggest that *A. nancymaae* was the first to diverge from the northern clade around 6.38 Ma, once the north–south split had occurred. Its mtDNA lineage is distinguished by 10 characteristic COII nucleotide changes relative to the nearest *Aotus* branch, and it is the only owl monkey taxon to possess a 54(m)/54(f) karyotype. The extent of the molecular distinction of this species suggests the existence of an ancient and temporally pervasive obstacle to gene flow, such as the westward migration of ancestral *A. nancymaae* individuals to western Brazil and eastern Peru, followed by their complete isolation from other owl monkey groups. However, the data and analyses presented in this study do not allow us to distinguish between the effects of population processes and natural selection. Thus, the scenarios described here and above are just a few of the possible interpretations of past events that could have shaped owl monkey evolution.

We also observe that *A. vociferans*, *A. l. griseimembra*, and *A. l. brumbacki* split from *A. lemurinus* and *A. trivirgatus*. This is a curious result, as subspecies *A. l. griseimembra* and *A. l. brumbacki* cluster with the *A. vociferans* clade rather than with the other species of *A. lemurinus*, to which *A. trivirgatus* is closely associated. This branching order may reflect problems with the provenance of these samples or even the accession of numts into databases like GenBank in the place of true mitochondrial sequences, as was suggested by Menezes et al. (2010). Another explanation for these discrepancies is that the longstanding disagreement on species definitions for the genus *Aotus*, coupled with the proximity of

TABLE 6. Molecular dating of CR haplogroup (hg) clades in Aotus azarai azarai

Clade	Ancestral node[a]	Descendant nodes (n)[b]	Age in Mutations (ρ)	SD (σ)	Age (years)	SD (years)	S_E (years)
hg-A	mv	A0–A7	4.28	2.00	19,530	9,140	3,231
hg-B	A0	B0–B8	1.37	0.85	6,240	3,900	1,300
hg-C	A0	C0–C5[c]	1.16	0.67	5,300	2,300	693

All CR dates are rounded to the nearest 10.
[a] The ancestral taxon is the node from which all mutational steps are counted to reach all inclusive descendant nodes in a clade.
[b] Descendant taxa are nodes believed to have arisen from an ancestral node.
[c] mvC3 was included in this haplogroup for coalescence estimates.
SD = Standard Deviation; S_E = Standard Error.

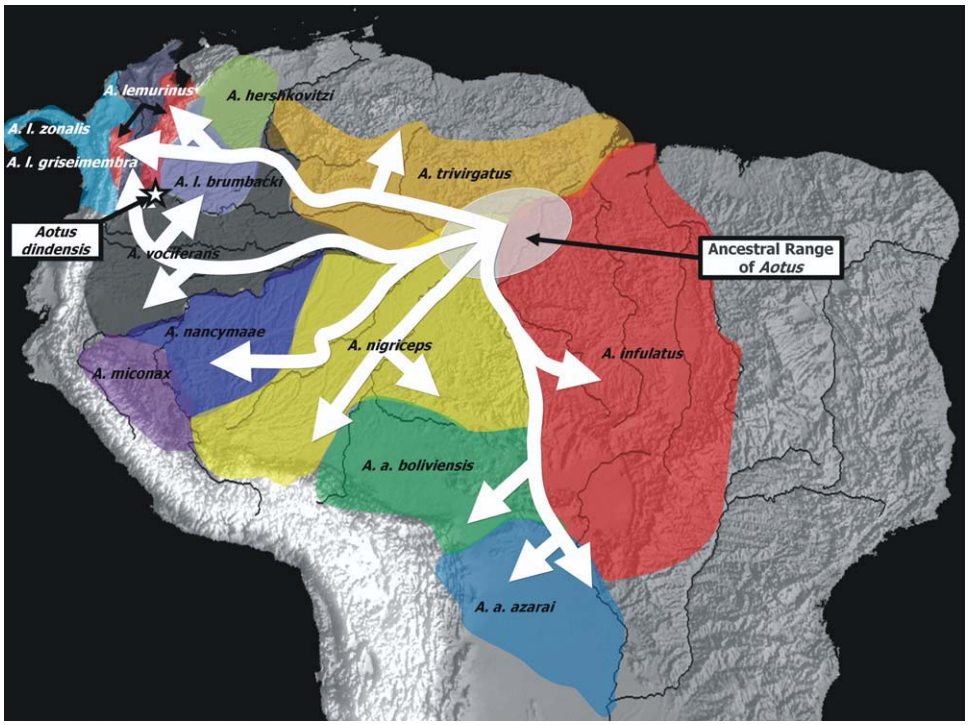

Fig. 5. Hypothetical scenario for the radiation of the genus *Aotus*. The directionality of the population dispersal is posited based on current geographic distributions of owl monkey species and their genetic relatedness as inferred from mtDNA COII sequences and cytogenetic similarities (Supporting Information Table S1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

their ranges to one another, has resulted in the misapplication of species names to samples from the field, or even the incorrect identification of captive animals. Alternatively, a biological influence, such as the hybridism among northern taxa, recently confirmed on the chromosomal level (Monsalve and Defler, 2011), may be responsible to the apparent polyphyly of *A. lemurnius*.

The greater antiquity of northern *Aotus* taxa would have allowed for the many karyotypic changes seen in different owl monkey species. An older time frame for the diversification of the genus (8.95 Ma) would also fit the 11.8–13.5 Ma estimates for the *Aotus dindensis* fossils. In this regard, it has been argued that their features closely resemble those of all modern owl monkey forms, not just of those species that are geographically nearby (Setoguchi and Rosenberger, 1987; Rosenberger et al., 2009; Takai et al., 2009).

mtDNA diversity and phylogeography of *A. a. azarai*

The majority of individuals (115/118) belong to one of three major CR haplogroups or clades. Hg-A is the most diverse of the three and, therefore, potentially the oldest. This interpretation is consistent with ρ coalescence estimates for each of the three haplogroups, and is reinforced by the phylogenetic affinity of hg-A to the most distantly related haplotypes X, Y, and Z. The patterning of the *A. a. azarai* mitochondrial networks, along with the general agreement among different summary statistics (D, F_S, τ, and π), reinforces the possibility that this population has undergone several distinct expansion events in its history, not just a single recent expansion. Even so, it is important to note that the mtDNA genome represents only a single realization of the evolutionary process, and that only the female population history is uncovered by this kind of analysis.

A relatively high degree of CR haplotype sharing among social groups (Fernandez-Duque et al., 2011) limits our ability to reconstruct the colonization processes of the gallery forests along the Pilagá River. However, given that hg-A is the oldest of the three major clades and that it is ubiquitous in most social groups, it is likely to be the ancestral lineage for this population. The relative ages of the derived hg-B and hg-C clades are also consistent with the climatic and geographic processes that drained the southernmost Chacoan forests and flatlands of water some 5,000–7,000 years ago (Iriondo, 1984, 1993). Thus, our haplogroup age estimations suggest the expansion of the population into the Pilagá watershed during or soon after that period. This scenario would also support the hypothesis that both putative *A. azarai* subspecies, *A. a. boliviensis* and *A. a. azarai*, had common origins further north, and only recently moved southward into the newly accessible South American Gran Chaco.

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