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Hybridization Between Neotropical Primates with Contrasting Sexual Dichromatism

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Abstract Hybridization is relatively well documented among Old World primates, but poorly investigated among New World monkeys. We investigated hybridization between the sexually dichromatic howlers *Alouatta caraya* and *Alouatta guariba clamitans*, whose lineages diverged *ca.* 5 million years ago. These taxa show allopatric distributions with a few recently discovered narrow contact zones. We collected 169 individual fecal samples of *A. caraya* and *A. g. clamitans* within ($N = 121$) and outside ($N = 48$) two contact zones in southern Brazil. We used mtDNA and Y-chromosome (SRY gene) sequences, and three diagnostic microsatellite loci to investigate their parental origin. We found 33 individuals (27%) with evidence of hybrid origin in the contact zones. Thirteen individuals presented mtDNA of *A. caraya* origin and Y-chromosome of *A. g. clamitans* origin and eight individuals have the opposite combination of markers. We assigned the hybrid origin of the remaining 12 individuals based on the discrepancy between their uniparental markers and microsatellite data, or a combination of diagnostic alleles of both species. This is the first

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evidence of hybridization between *A. caraya* and *A. g. clamitans*. We hypothesize that this hybridization is bidirectional, ancient, and geographically wider than previously thought. The confirmation of hybridization in our contact zones provides evidence that interspecific differences in male coat color do not function as prezygotic barriers. We concluded that sexual selection by means of mate recognition is a weak explanation for the evolution of sexual dichromatism in these species. Finally, our system highlights the great complexity of this mechanism and its potential for the evolution and diversification of primates.

Keywords *Alouatta caraya* · *Alouatta guariba clamitans* · Contact (hybrid) zone · Mate recognition · Noninvasive sampling

Natural hybridization is a widespread phenomenon in eukaryotes that seems to be more common in mammals than previously recognized (Arnold and Meyer 2006; Shurtliff 2013). Among primates, there is plenty of evidence of hybridization between congeneric strepsirrhines, haplorrhines and catharrhines species (*Cercopithecus*, *Eulemur*, *Macaca*, *Papio*, *Tarsius*, *Hylobates*, and *Homo*) (Arnold and Meyer 2006; Evans *et al*. 2001; Green *et al*. 2010; Merker *et al*. 2009; Noda *et al*. 2001; Phillips-Conroy and Jolly 1986; Samuels and Altmann 1986; Struhsaker *et al*. 1988; Tosi *et al*. 2002, 2003; Watanabe and Matsumura 1991; Wyner *et al*. 2002) and reports involving taxa belonging to different genera (*Theropithecus* and *Papio*, *Hylobates* and *Symphalangus*) (Dunbar and Dunbar 1974; Myers and Shafer 1979). In the New World, hybridization has been inferred from phenotype (Aguiar *et al*. 2008; Passamani *et al*. 1997) or confirmed by molecular data (Cortés-Ortiz *et al*. 2007; Kelaita and Cortés-Ortiz 2013; Malukiewicz *et al*. 2015; Silva *et al*. 1992) in a few species of *Alouatta*, *Callithrix*, *Cebus*, *Saguinus*, and *Saimiri* (Arnold and Meyer 2006; Cortés-Ortiz *et al*. 2007).

The nine currently recognized species of *Alouatta* (howlers) are allopatric through most of their ranges from southern Mexico to southern Brazil, but a few historically secondary contact zones have been reported (Cortés-Ortiz *et al*. 2015a, b). Molecular data have been used to confirm hybridization between *Alouatta palliata* and *Alouatta pigra* in a contact zone in the northern limit of the distribution of the genus in Mexico (Cortés-Ortiz *et al*. 2007; Kelaita and Cortés-Ortiz 2013). Hybridization between these species appears to be unidirectional. While a cross between a male *A. palliata* and a female *A. pigra* produces viable offspring, the opposite cross does not. This pattern is consistent with Haldane's rule, according to which the heterogametic sex (males in mammals) is more likely to be inviable or sterile (Cortés-Ortiz *et al*. 2007, 2015a). The patterns of genetic variation found in hybrid/backcrossed individuals in Mexico suggest that the directionality results from chromosomal, cytonuclear, or genomic incompatibilities (Cortés-Ortiz *et al*. 2015a; Kelaita and Cortés-Ortiz 2013). Except for this single area, reports of hybridization in howlers are suggestive but not conclusive (Cortés-Ortiz *et al*. 2007, 2015a). The oldest known suggestion of mixed groups in howlers dates back to the nineteenth century when a female *Alouatta caraya* and a male *Alouatta guariba clamitans* were found living together (Isabelle 1983). The first mention of hybrids between these taxa comes from a morphological study based on museum specimens collected on the upper Paraná River in the 1940s, in which some individuals presented a mosaic pelage coloration pattern (Lorini and Persson 1990). Although putative

hybrids between *A. caraya* and *A. g. clamitans* have been reported (Aguiar *et al*. 2007, 2008, 2014; Bicca-Marques *et al*. 2008; Dias *et al*. 2015; Gregorin 2006), there has been no genetic confirmation that they hybridize in the wild.

Contact zones between *Alouatta caraya* and *Alouatta guariba clamitans* have been reported in northeastern Argentina and southern Brazil (Agostini *et al*. 2008; Aguiar *et al*. 2007, 2008, 2014; Bicca-Marques *et al*. 2008; Gregorin 2006; Holzmann *et al*. 2015). The home ranges of syntopic monospecific groups can overlap extensively and mixed-species troops can arise (Agostini *et al*. 2010; Aguiar *et al*. 2008). The adults of these taxa are similar in size and appearance, and have both evolved biphasic sexual dichromatism in which male coat color changes during development (Bicca-Marques and Calegaro-Marques 1998; Van Belle and Bicca-Marques 2015). Therefore, there are four adult color phenotypes. In adult *A. caraya* males are fully black and adult females are yellowish or bridled-tawny, whereas adult *A. g. clamitans* individuals are reddish and dark brown, respectively (Bicca-Marques and Calegaro-Marques 1998; Gregorin 2006). The distinction in coat coloration within and between species enables the detection of intermediate and mosaic color patterns in contact zones that have been inferred to be hybrids (Agostini *et al*. 2008; Aguiar *et al*. 2007, 2008, 2014; Bicca-Marques *et al*. 2008). The observation of births of hybrid offspring of both sexes in captive howlers, from a male *A. caraya* and a female *A. g. clamitans* (Jesus *et al*. 2010), supports the link between intermediate/mosaic phenotypes and hybridization between these taxa. The survival of the male hybrid for 1.5 yr. is evidence of the viability of the heterogametic sex for this pair of parents. However, it died before reaching sexual maturity, so we do not know if it was fertile.

Alouatta caraya and *Alouatta guariba clamitans* are not sister species and belong to divergent clades that separated up to 5.1 million yr. ago (Cortés-Ortiz *et al*. 2003; Martins *et al*. 2011; Steinberg *et al*. 2014). Their contrasting sexual dichromatism is thought to have evolved independently because their sister taxa and other congeners do not show this trait. The first hypothesis developed to explain the evolution of sexual dichromatism in these taxa related coat color to sexual differences in energetic and thermal requirements (Thorington *et al*. 1979). However, studies of the thermoregulatory behavior of free-ranging *A. caraya* (Bicca-Marques and Calegaro-Marques 1998) and *A. g. clamitans* (Bicca-Marques and Azevedo 2004) found no support for this hypothesis. Alternative hypotheses focused on sociosexual selective pressures, namely signaling male fitness (e.g., fighting ability, maturity, health status; Crockett 1987; Darwin 1871; see also Bicca-Marques and Calegaro-Marques 1998) or sexual identity recognition (Crockett 1987; Paterson 1985; see also Bicca-Marques and Calegaro-Marques 1998). Whereas the first of these hypotheses relates to female mate choice or to the assessment of the strength of potential sexual competitors, the latter relates to the mate recognition concept. Irrespective of the selective advantage(s) that led to dichromatism, both hypotheses imply intraspecific recognition of potential sexual partners based on coat color. The standard trichromacy characteristic of *Alouatta* spp. enables them to physiologically distinguish both intra- and interspecific variations in adult color phenotypes (Kainz *et al*. 1998).

We investigated hybridization between the sexually dichromatic howlers *Alouatta caraya* and *Alouatta guariba clamitans* using uni- and biparental genetic markers assessed in biological samples collected in areas of sympatry and allopatry between these species. We also tested the hypothesis that *A. caraya* and *A. g. clamitans* avoid crossbreeding by

recognizing their distinctly colored adults of each sex. If such mate recognition is the driving force for the evolution of sexual dichromatism in these taxa, then we predict the absence of hybrids in contact zones.

Methods

Study Areas

We conducted population surveys in two contact zones between *Alouatta caraya* and *Alouatta guariba clamitans* in Brazil (Fig. 1). Both regions are highly deforested and fragmented. The first study site is a single 150-ha, old growth, highly disturbed fragment of seasonal semideciduous forest in a matrix of pasture that is contiguous to the riparian forest of the left bank of the upper Paraná River (23°22′52.3″S, 53°45′39.6″W) in Icaraíma municipality, Paraná State (Aguiar *et al.* 2008). We surveyed a few additional small fragments in this region, but found no howlers. The second contact zone is a region located in the ecotone between the Atlantic forest and temperate grassland (29°35′0.0″S, 54°59′0.0″W) on the border of São Francisco de Assis and São

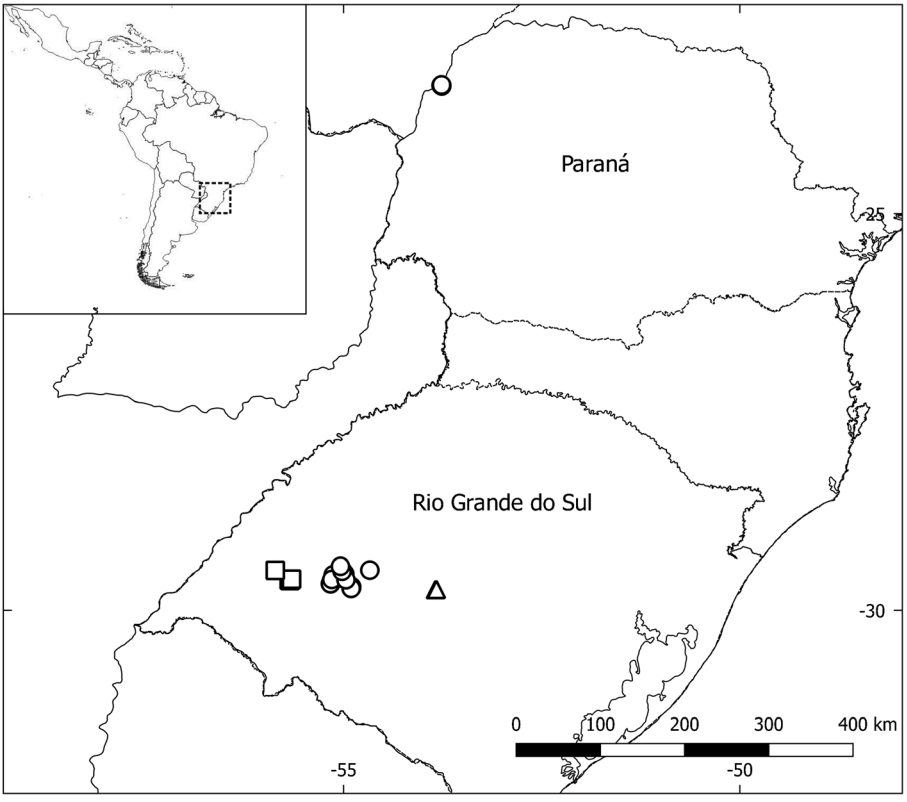

Fig. 1 Location of sampling sites in contact zones between *Alouatta caraya* and *Alouatta guariba clamitans* in the states of Paraná and Rio Grande do Sul, southern Brazil. Contact zones are indicated by open circles. Parental populations of *A. caraya* and *A. g. clamitans* are indicated by open squares and triangles, respectively.

Vicente do Sul municipalities, Rio Grande do Sul State (Bicca-Marques *et al*. 2008; Dias *et al*. 2015; Silva 2010). It is composed of several relatively small forest fragments spread over an undulating terrain dominated by rocky hills partially covered with grasslands, gallery forests, and secondary forests in different stages of regeneration (Bicca-Marques *et al*. 2008). These fragments are in a matrix of pasture and, in some cases, commercial pine tree plantations or annual cultures (e.g., soybean). They are 3–26 km away from each other. We also collected samples in presumed parental populations of both species in Alegrete (*A. caraya*) and Santa Maria (*A. g. clamitans*) municipalities, Rio Grande do Sul (Fig. 1). These parental populations are located *ca.* 80 km and 130 km from our studied contact zone, respectively.

Molecular Data Acquisition and Analysis

We surveyed at least 42 wild groups of howlers in contact zones in Paraná ($N = 8$) and Rio Grande do Sul ($N = 34$). Most groups ($N = 33$) were phenotypically monospecific (23 *Alouatta caraya* and 10 *Alouatta guariba clamitans*). Nine groups (five in Paraná and four in Rio Grande do Sul, corresponding to 63% and 12% of the surveyed groups in each area, respectively) were a mix of apparently purebred individuals of both species and/or apparently hybrid individuals. Nevertheless, our sampling was limited to a single 150-ha fragment in Paraná. We also surveyed at least five groups in each parental population in Rio Grande do Sul (Fig. 1).

We collected 169 individual fecal samples, 121 in the contact zones of Paraná ($N = 19$) and Rio Grande do Sul ($N = 102$), and the remaining 48 in the putative parental populations of *Alouatta caraya* ($N = 30$) and *Alouatta g. clamitans* ($N = 18$) in Rio Grande do Sul. We collected fecal samples immediately after defecation in most cases ($N = 164$) or while still fresh in latrines ($N = 5$) (Gilbert 1997). In the latter situation, we collected only one or two spatially separated samples from each latrine to minimize the risk of sampling feces from the same individual repeatedly. We stored samples in 50-mL tubes filled with *ca*. 25 mL of 70% ethanol at ambient temperature for a few hours to a few days in the field and then kept them frozen in the laboratory until extraction.

We extracted genomic DNA from 100 to 200 mg of feces using the QIAamp DNA Stool Mini Kit (QIAGEN), following the manufacturer's protocol. We used negative controls within specialized workstations in an ancient DNA-type room to reduce the possibility of extract contamination (Surridge *et al*. 2002). We eluted these fecal sample extracts into a final 100 μL that we stored at −20°C for up to 24 mo before further processing.

To identify the maternal and paternal lineages of putative hybrids, we first tested for fixed (diagnostic) differences in the mtDNA control region and the Y-chromosome SRY gene of *Alouatta caraya* and *Alouatta guariba clamitans*. First, we used all available control region sequences from both species in GenBank, which consisted of an unpublished mtDNA genome (KY202428) for *A. g. clamitans* and 39 sequences of *A. caraya* (from Ascunce *et al*. 2003, 2007) and its whole mtDNA genome (Finstermeier *et al*. 2013). Although the aforementioned sequences clearly showed that the control regions of the mtDNA of the two species are quite different, they were limited in number and range, so we tested whether the differences were valid for a larger number of individuals in a broader geographical range using 263 sequences from both species from an ongoing phylogeographic study (Bonatto *et al*., unpubl. data). These sequences came from individuals from multiple locations in the distribution of each species, all ≥200 km away from our two study regions.

We used the primers How-S7 and How-RA1 (Ascunce *et al*. 2003) to amplify a fragment of *ca*. 713 bp of the mitochondrial control region via polymerase chain reaction (PCR). We used a volume of 20 μL containing 1 μL of genomic DNA, 0.6 μL of 1.5 mM of $MgCl_2$, 2 μL of 0.2 mM dNTPs, 2 μL (1×) of 10× buffer, 2 μL of a 0.2 μM of each primer, 0.1 μL of Platinum Taq DNA Polymerase 5 U/μL (Invitrogen), 4 μL (1 M) of 5 M betaine, and 6.3 μL of ddH_2O. After an initial denaturation period of 3 min at 94°C, amplifications proceeded with five cycles of denaturation at 94°C for 45 s, primer annealing at 55–50°C (in a touchdown approach decreasing 1°C in each cycle) for 45 s, and primer extension at 72°C for 90 s, followed by 35 cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 90 s. We used a final extension step at 72°C for 5 min.

No sequence was available for the Y-chromosome of these species. Thus, we amplified and sequenced *ca*. 782 bp of the SRY gene using primers described by Moreira (2002) using DNA from blood samples of 13 males (5 *Alouatta caraya* and 8 *Alouatta guariba clamitans*) collected in locations >500 km away from our study regions (as for mtDNA). We found five fixed SRY differences between our taxa. However, the amplification success of the fecal samples with these primers was low (<10%), probably owing to its large fragment size. We therefore used these sequences to design a new set of primers (SRY-F2–5′-AAAGTAACAACGAATTTGGTAGAA-3′ and SRY-R2: 5′- ATCACGAG ACCACACAAGG-3′) that amplified a shorter fragment (*ca*. 300 bp) of the SRY gene, and contained two of the initial five interspecific polymorphisms. We performed PCRs as described previously with the following changes. After an initial denaturation for 4 min at 94°C, amplifications proceeded with 7 touchdown cycles at 94°C for 45 s, primer annealing at 60–53°C for 45 s, and primer extension at 72°C for 90 s, followed by 33 cycles at 94°C for 45 s, 53°C for 45 s, and 72°C for 90 s. We used a final extension at 72°C for 10 min. We amplified each sample twice to check for consistency of results.

We visually inspected allele size and quality of the PCR products of both uniparental markers in 1% agarose gel. We considered the reactions successful when DNA intensity was >30 ng/μL. Then, we purified sample products following the FasTap protocol. Samples were sequenced at Macrogen Inc. We inspected all sequences from both uniparental markers visually in Chromas Lite 2.1 (Technelysium Pty Ltd.). We aligned the sequences using the MUSCLE algorithm, implemented in AliView (Larsson 2014). Whenever necessary, we checked alignments visually and corrected them manually.

We also genotyped samples with three biparental microsatellite loci to help identify F1 hybrids. We initially tested eight microsatellite loci used previously in our laboratory for an ongoing population genetics study of both species (Bonatto *et al*., unpubl. data), five of which (AC14, AC17, D8S165, D17S804, 157 used for *Alouatta caraya* by Oklander *et al*. 2007) did not produce diagnostic alleles, or had reduced amplification efficiency because of the difficulties associated with fecal samples. The three microsatellite loci that we used (Ab7, Ab10, Ab17) were originally described for *Alouatta belzebul* (Gonçalves *et al*. 2004). Using these autosomal loci we found diagnostic alleles for the Rio Grande do Sul parental populations with a reasonable amplification success. Different alleles at these loci seem to be fixed in *A. caraya* and *Alouatta guariba clamitans*, allowing us to assign hybrids between these species with a reasonable level of confidence (see Results and Tables I and II for descriptions of loci and alleles). The simple assignment of hybrids is possible using only three or four diagnostic microsatellite markers (Boecklen and Howard 1997; Cortés-Ortiz *et al*. 2007). We conducted microsatellite PCR reactions with 0.2 mM of dNTP, 0.02 μM of forward primer, 0.2 μM of reverse primer, 1.5 mM of $MgCl_2$, 1 M of Betaine, 0.2% Triton,

Table I Characteristics of three microsatellite loci sampled from 111 *Alouatta caraya* and *Alouatta guariba clamitans* individuals

Locus	Number of alleles	Allele range size (bp)	Missing data (%)
Ab7	3	200–212	18
Ab10	3	210–214	19
Ab17	12	210–260	14

1× buffer, and 0.25 U of Platinum Taq DNA Polymerase (Invitrogen) in a final volume of 10 μL containing 3–5 μL genomic DNA. Following an initial denaturation period of 3 min at 94°C, amplifications of Ab7 proceeded with eight touchdown cycles at 94°C (45 s), 65–58°C (45 s), and 72°C (90 s), and 37 cycles at 94°C (45 s), 58°C (45 s), 72°C (90 s), and a final extension at 72°C (30 min). We followed a similar protocol to amplify Ab10 and Ab17, with differences in temperature and number of touchdown cycles, which included five cycles at 65–60°C (45 s), and 11 cycles at 60–50°C (45 s), respectively.

Fluorescently labeled primers were used to genotype the loci in an automated sequencer at Macrogen Inc. We reanalyzed all samples twice or three times per locus and found 100% concordance among replicates. We scored allele sizes using Peak Scanner Software 2 (Applied Biosystems). Both extraction and amplification reactions included negative controls.

Data Availability Sry sequences are available in the following links.
https://www.ncbi.nlm.nih.gov/nuccore/MF289493
https://www.ncbi.nlm.nih.gov/nuccore/MF289494

Ethical Note

All research reported here complied with the appropriate national and institutional ethical guidelines and adhered to Brazilian legal requirements and to the Code of Best Practices for Field Primatology (https://www.asp.org/resources/docs/Code%20of_Best_Practices%20Oct%202,014.pdf). We declare no competing interests.

Table II Allele size (bp) of three diagnostic microsatellite loci in parental populations of *Alouatta caraya* and *Alouatta guariba clamitans*

Locus	*A. caraya* (*N*)	*A. g. clamitans* (*N*)
Ab7	200 (14)	210, 212 (13)
Ab10	212 (10)	214 (13)
Ab17	236, 240, 248, 252, 260 (14)	214, 242 (14)

The number of individuals with each allele is shown in parentheses

Results

Alouatta caraya and *Alouatta guariba clamitans* have divergent mtDNA sequences, including 46 diagnostic differences in the segment we studied (Table III). Of the 146 sequences obtained from both study regions, 101 (69%) were assigned to *A. caraya*, whereas the remaining 45 (31%) were assigned to *A. g. clamitans*. All 28 individuals from outside the contact zones had mtDNA haplotypes of the expected species (19 *A. caraya* and 9 *A. g. clamitans*) given their sampling location and phenotype (when available). Most of the 118 sequences from the contact zones were assigned to *A. caraya* ($N = 82$, 69%) and the rest to *A. g. clamitans* ($N = 36$, 31%).

Considering only the SRY sequences from individuals from regions outside our study regions, we identified two nucleotide sites that distinguish between *Alouatta caraya* and *Alouatta guariba clamitans* (Table IV, GenBank accession nos. MF289493–MF289494). Of the 79 SRY sequences from our study areas, 49 (62%) were assigned to *A. caraya*, whereas the remaining 30 (38%) were assigned to *A. g. clamitans*. Eleven (61%) of the 18 SRY sequences from the Alegrete and Santa Maria (Rio Grande do Sul) parental zones were assigned to *A. caraya.* The remaining seven sequences (39%) were assigned to *A. g. clamitans*. Although most of the SRY sequences found in the parental populations corresponded to the expected species, two individuals in the *A. caraya* population and three individuals in the *A. g. clamitans* population had the SRY sequence attributed to the other species. However, the mtDNA and autosomal microsatellites of these individuals were diagnostic of the expected parental species. Thirty-eight (62%) of the 61 Y-chromosome samples from the contact zones were assigned to *A. caraya*, while the remaining 23 (38%) were assigned to *A. g. clamitans*.

We successfully genotyped the three polymorphic loci containing diagnostic alleles for each species for 111 individuals from both parental populations and contact zones, with 14–19% missing data (Table I). We found no evidence of shared alleles for these loci between the two species in the putative parental populations (Table II). We found microsatellite evidence of hybridization in at least one locus (Table V) in 20 of 82 samples (24%) from the contact zones, but we could not determine the generation of interspecific mating. However, individuals from both parental populations presented only alleles diagnostic of the respective species, suggesting absence of hybridization in recent generations.

In total, 33 of the 121 individuals (27%) from the contact zones (7 of 19 individuals [37%] from Paraná and 26 of 102 individuals [26%] from Rio Grande do Sul) showed evidence of hybridization based on uniparental markers and/or biparental microsatellites. Six males were likely F1 hybrids because they had a combination of mtDNA and SRY haplotypes of both parental species and were interspecific heterozygous at all analyzed loci. Three putative females were also likely F1 hybrids because all their genotyped microsatellite loci were interspecific heterozygous. Three males with mtDNA from one species and SRY from the other are also putative hybrids, although the absence of microsatellite data prevents their assignment to any specific hybrid class. Some individuals are likely multigenerational backcrossed hybrids because they showed a single interspecific heterozygous locus or homozygous loci from different parental species or discordant mtDNA and SRY but monospecific autosomal loci.

In summary, 13 males showed *Alouatta caraya* mtDNA and *Alouatta guariba clamitans* Y-chromosome, and 8 males showed *A. g. clamitans* mtDNA and *A. caraya* Y-chromosome. We assigned 12 additional putative hybrids based on the

Table III Diagnostic polymorphisms in *Alouatta caraya* (AC) and *Alouatta guariba clamitans* (AG) based on 146 sequences of a fragment of the mitochondrial control region

Position/ species	1 5 4 5 8	1 5 4 6 9	1 5 4 8 0	1 5 5 6 5	1 5 5 8 5	1 5 6 1 3	1 5 6 1 8	1 5 6 2 2	1 5 6 2 6	1 5 6 2 7	1 5 6 3 4	1 5 6 4 0	1 5 6 4 4	1 5 6 5 5	1 5 6 6 2	1 5 6 6 2	1 5 6 6 3	1 5 6 7 4	1 5 6 8 4	1 5 6 8 5	1 5 6 9 6	1 5 6 9 1	1 5 7 2 8	1 5 7 3 7	1 5 7 3 1	1 5 7 4 4	1 5 8 0 2	1 5 8 0 3	1 5 8 0 4	1 5 8 1 9	1 5 8 1 0	1 5 8 1 1	1 5 8 1 2	1 5 8 1 6	1 5 8 1 7	1 5 8 2 8	1 5 8 2 4	1 5 8 4 6	1 5 8 4 7	1 5 8 4 8	1 5 8 5 9	1 5 8 5 2	1 5 8 5 8	1 5 9 0 0	1 5 9 1 4	1 5 9 4 6
AC	C	T	C	C	A	A	T	C	C	A	G	A	T	G	A	A	C	A	G	T	G	G	A	T	G	C	A	A	G	C	A	G	C	C	G	G	T	C	A	G	G	T	G	C	T	T
AG	T	C	T	T	G	G	C	T	A	G	A	T	C	C	C	C	A	T	C	C	A	A	C	C	A	T	G	C	A	T	T	C	T	A	A	A	C	T	T	T	A	C	A	T	C	C

Position based on the complete sequence of *A. caraya* KC757384 extracted from GenBank

Table IV Diagnostic polymorphisms in *Alouatta* based on a fragment of the SRY gene

Position/Species	1	1	1	1	1	2
	5	5	6	7	9	0
	4	6	8	1	0	5
A. palliata	T	A	T	A	G	T
A. pigra	T	A	T	A	G	T
A. caraya	C	A	A	A	A	C
A. g. clamitans	C	G	A	C	A	C

Alouatta pigra (accession no. DQ875680) and *Alouatta palliata* (DQ875675) were extracted from GenBank. Position is based on the *Alouatta palliata* sample

discrepancy between their uniparental markers and microsatellites, or on a combination of diagnostic microsatellite alleles of both species (Table V).

Discussion

We provided the first genetic evidence of bidirectional hybridization between *Alouatta caraya* and *Alouatta guariba clamitans*. Both types of cross between these taxa (male *A. caraya* × female *A. g. clamitans* and male *A. g. clamitans* × female *A. caraya*) seemed to produce viable F1 hybrids as hypothesized based on field and captive data (Agostini *et al.* 2008; Cortés-Ortiz *et al.* 2015a; Jesus *et al.* 2010). Our hybrid system seems to be more complex than that reported between the northern congeners *Alouatta palliata* and *Alouatta pigra*. Only female F1 offspring resulting from female *A. pigra* and male *A. palliata* is capable of producing viable and fertile F2 offspring when crossing with a pure *A. pigra*, a pure *A. palliata*, or a backcrossed male with SRY of *A. pigra*. This system is compatible with the full operation of Haldane's rule (Cortés-Ortiz *et al.* 2007). Some of our results, such as males with Y-chromosome from one species and mtDNA and all autosomal alleles from the other (Table V), suggest that at least some F1 male hybrids may be fertile in our *A. caraya* × *A. g. clamitans* system. Additional data on fertility and autosomal loci are needed to better assess whether Haldane's rule also operates in our system. The occurrence of individuals with varying genotype patterns with alleles/haplotypes belonging to both species (Table V) is compatible with the presence of multigenerational hybrids (e.g., F2, backcrosses). Again, information from a larger set of loci is needed to fully investigate the matings that produced these patterns.

Although hybridization may positively influence speciation and evolution (Arnold 1997), it is more likely to have negative effects if its causes are anthropogenic (Seehausen *et al.* 2008). We could not assess whether the hybridization of our study species resulted from natural causes or from anthropogenic forest loss and fragmentation. However, the occurrence of individuals with monospecific microsatellite alleles and with a combination of mtDNA and Y-chromosome haplotypes of both species in one of our parental populations ≥80 km away from the nearest known contact zone points to an older hybridization process, over many generations, in which contraction and expansion of forest due to natural factors may have brought the two species

Table V Information on putative hybrids between *Alouatta caraya* (ac) and *Alouatta guariba clamitans* (ag) in contact zones in Paraná and Rio Grande do Sul in southern Brazil based on mtDNA, Y-chromosome (SRY), and three microsatellite loci

ID	Sex[a]	mtDNA	SRY	Ab7	Ab10	Ab17
Rio Grande do Sul						
g2–2	**?**	**ac**	**ag**	**ac/ag**	**ac/ag**	**ac/ag**
g4–8	?	ag	ac	ag	ag	ag
g5–10	**F**	**ag**	**–**	**ac/ag**	**ac/ag**	**ac/ag**
g5–11	**M**	**ac**	**ag**	**ac/ag**	**–**	**ac/ag**
g6–12	M	ag	ac	ag	ag	ag
g8–16	**M**	**ag**	**ac**	**ac/ag**	**–**	**ac/ag**
g8–18	F	ag	–	ac/ag	ac/ag	ag
g10–23	**?**	**ag**	**ac**	**ac/ag**	**ac/ag**	**ac/ag**
g10–24	?	ag	ac	ac	ag	ac/ag
g12–29	**M**	**ac**	**ag**	**ac/ag**	**ac/ag**	**ac/ag**
ac-3	M	ac	ag	ac	ac	ac
ac-5	M	ac	ag	–	ac	–
ac-7	?	ac	ac	ac/ag	ag	ac
ac-8	?	ac	ag	–	ac	–
ac-10	?	ac	ag	ac	ac	ac
ac-14	M	ac	ag	ac	ac	ac
ac-16	?	ac	ag	–	ac	–
ac-20	?	ac	ag	–	ac	–
ac-13_3	M	ag	ac	–	–	–
1	**?**	**ag**	**ac**	**ac/ag**	**ac/ag**	**–**
9	M	ag	ag	ag	ag	ac/ag
10	?	ac	ag	–	ac	ac/ag
13	?	ag	ac	–	–	–
17	?	ac	ag	ac	ag	ac
19	?	ag	–	–	–	ac/ag
76	M	ac	ag	–	–	–
Paraná						
1-a	**?**	**ac**	**–**	**ac/ag**	**–**	**ac/ag**
4-a	F	–	–	ag	ac	ac
5-a	M	ag	ag	ac	ag	ag
12-a	?	ag	–	ac	ag	ag
14-a	F	ac	–	ag	ac	ac
21-a	**F**	**–**	**–**	**ac/ag**	**ac/ag**	**–**
32-a	?	ag	ag	ac	ac	ac

Dash denotes missing data. Bold identifies putative F1 hybrids

[a] Male and female assignments are based on direct observations of howlers from which we collected fecal samples; a question mark denotes that we did not observe the individual directly or we did not note its sex. We posteriorly assigned the sex of some individuals based on the presence of the SRY gene (e.g., g2–2, g4–8, g10–23)

together (Bicca-Marques *et al.* 2008). It is also uncertain if the acceleration of habitat (forest) loss and fragmentation occurring in these contact zones will increase or reduce the hybridization between these species. Arboreal primates depend on large, or at least highly connected, tracts of forest to maintain gene flow and for populations to persist long term. Although howlers can cope well with habitat spatial restriction at the individual level, long-term species viability is uncertain because small isolated populations face higher risks of deterministic (e.g., disease, hunting) or stochastic (e.g., genetic drift, biased sex ratios, extreme climatic events) effects resulting from forest loss and fragmentation (Arroyo-Rodriguez and Dias 2010; Bicca-Marques 2003). Therefore, the negative long-term effects of forest fragmentation may either result in population extirpations that decrease the probability of interspecific contact and hybridization in the fragmented landscape of the study regions, or force isolated and small mixed-species groups to hybridize as their only breeding option.

Finally, our findings suggest that sexual selection via mate recognition is a weak explanation for the evolution of sexual dichromatism in these taxa. The confirmation of hybridization in our contact zones provides evidence that the striking interspecific differences in male coat color do not function as prezygotic barriers, as hypothesized by Aguiar *et al.* (2007). Indeed, heterospecific mating appears to be common whenever *Alouatta caraya* and *Alouatta guariba clamitans* live in syntopy, as shown by the proportion of hybrid individuals in the study populations in our contact zones (26–37%), and the proportion of interspecific copulations in the forest fragment surveyed in Paraná (16%; Aguiar 2010). Females of both species do not avoid mating with males of the other species, despite being physiologically capable of distinguishing the coat colorations of conspecific and heterospecific males (Kainz *et al.* 1998). Hybridization between these taxa in the wild may indicate that even if sexual dichromatism evolved for long-distance sex recognition (Bicca-Marques and Calegaro-Marques 1998), this trait is not an effective behavioral or prezygotic barrier to hybridization in contact zones (Moynihan 1968). Future studies should evaluate color variation in these dichromatic howlers as a surrogate for fitness (signaling fighting ability and/or aggressiveness, health, maturity, or resource occupancy: Van Belle and Bicca-Marques 2015).

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