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Genetic Consequences of Habitat Fragmentation in Black-and-Gold Howler (*Alouatta caraya*) Populations from Northern Argentina

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Abstract Human-induced habitat fragmentation might seriously affect behavioural patterns and the survival of species whose ecological requirements strongly depend on specific environmental conditions. We compared the genetic structure and dispersal patterns of 2 populations of *Alouatta caraya* (Plathyrrhini, Atelidae) to understand how habitat reduction and fragmentation affect gene flow in this species. We sampled individuals from 7 groups living in continuous forest (CF, n=46, 22 males and 24 females), and 11 groups that inhabit a fragmented forest (FF, n=50, 24 males and 26 females). FST values based on 11 microsatellite loci showed a recent genetic differentiation among groups in the FF. In contrast, the CF showed no differentiation among groups. Further, FST values between sexes, as well as kinship relationships, also exhibited differences between habitats. In the CF, both males and females disperse, leading to nondifferentiated groups composed of adults that are not close relatives. Conversely, in the FF, some groups are differentiated, males disperse more than females, and groups are composed of closely related adult females. Our results suggest that habitat fragmentation modifies the dispersal patterns of black-and-gold howlers. These differences between habitats may reflect a reduced gene flow, providing genetic evidence that suggests that habitat fragmentation severely limits the howler's ability to disperse. An increasing level of isolation due to uncontrolled deforestation may cause similar loss of genetic diversity on other arboreal primates, and nonprimates that depend on

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forest continuity to disperse, reducing their abilities to cope with environmental changes.

Keywords *Alouatta caraya* · Dispersal · Fragments · Microsatellite DNA

Introduction

Demographic variables such as group size, density, composition of social units, and male and female differential dispersal affect the genetic structure of primate populations (Bergl and Vigilant 2007; Hammond *et al.* 2006; Hoelzer *et al.* 2004). Habitat fragmentation may isolate populations, limiting dispersal opportunities or increasing its associated costs, thus favoring individual philopatric behaviours (Sterck 1998). Philopatry, however, increases the likelihood of inbreeding (Heshel and Paige 1995; Tomiuk *et al.* 1998). Dispersal of one or both sexes may function as a mechanism to maintain genetic variability and avoid inbreeding (Field and Guatelli-Steinberg 2003; Pusey and Wolf 1996). In mammalian species, wherein female dispersal is assumed to be an inbreeding avoidance strategy, both sexes often leave their natal groups when they reach sexual maturity (*Alouatta seniculus*: Clutton-Brock 1989; *Alouatta caraya*: Rumiz 1990; *Saccopteryx bilineata*: Nagy *et al.* 2007). Estimating sex-biased dispersal by direct observation is not always feasible, especially in animals with long life spans or long-distance dispersal such as nonhuman primates (Hammond *et al.* 2006). Genetic methods such as microsatellite analyses represent a valuable analytical tool for species management and conservation, because they allow researchers to determine genetic variability and population dynamics, as well as to perform paternity tests, interindividual relatedness evaluation, and assessments of effective population size (Altmann *et al.* 1996; Charpentier *et al.* 2007; Constable *et al.* 2001; Gagneux *et al.* 1999; Gerloff *et al.* 1999; Hughes 1998; Keller and Waller 2002; Leigh *et al.* 2008; Vigilant *et al.* 2001). These methods also provide important insights about dispersal patterns at varying geographic scales, which enable evaluation of how dispersal might have affected historical gene flow (Prugnolle and de Meeûs 2002).

The existence of a negative synergistic effect associated with lack of genetic variability and environmental changes has been documented in several species (Heshel and Paige 1995; Pray *et al.* 1994). The consequences of inbreeding and outbreeding on reproductive success or fitness are some of the major concerns in conservation biology in species suffering from habitat degradation, including reduction, fragmentation, or other processes. Small and isolated populations can lose genetic variation within short periods of time, i.e. plants (Young *et al.* 2000), fish (Sato and Harada 2008); birds (Bouzat *et al.* 1998); mammals (Mitrovski *et al.* 2008). This decline in variation may also lead to a population with reduced abilities to withstand environmental changes and diseases, promoting a population's local extinction (Keller and Waller 2002). Disease and parasite resistance have been related to genetic diversity in a range of animal species, i.e., fish (Hedrick *et al.* 2001; Lively *et al.* 1990) and sheep (Coltman *et al.* 1999).

Howlers are considered a colonizing, highly plastic, and resilient species that are able to endure moderate changes in the habitat (Bicca-Marques 2003; Crockett 1998;

Crockett and Eisenberg 1987; Estrada *et al.* 2006; Zunino *et al.* 2007). Habitat characteristics and the degree of human alteration influence the demographic conditions of several *Alouatta* species (Chapman and Balcomb 1998; Chapman and Chapman 2000; Crockett 1996, 1998; Kowalewski and Zunino 1999; Pope 1998; van Belle and Estrada 2007). For example, van Belle and Estrada (2007) found that the mean population density of *Alouatta pigra* in fragmented forests increases, whereas the mean number of adult males decreases compared with that in protected forests, suggesting that adult males are possibly more vulnerable to habitat saturation or isolation.

Black-and-gold howlers (*Alouatta caraya*) inhabit diverse habitats, which include flooded, riparian, and semideciduous forests, and present a wide distribution range covering northern Argentina, central and southwestern Brazil, Paraguay, and eastern Bolivia (Hirsch *et al.* 2002). In northern Argentina, black-and-gold howler populations exhibit remarkable differences in population density and social structure in their distribution area (Brown and Zunino 1994; Kowalewski and Zunino 2004; Zunino *et al.* 2001). For example, on the island system along the Parana River, human impact is very low and forests remain well preserved, with higher densities of howlers than on the mainland, where the habitat is usually fragmented and altered by human activity (Kowalewski and Zunino 2004; Rumiz 1990; Thorington *et al.* 1984; Zunino and Bravo 1996; Zunino *et al.* 2001). Therefore, *Alouatta caraya* offers an interesting analytical biological model based on its ability to colonize different types of forests and to survive in fragmented and modified habitats. In addition, a set of suitable genetic markers for this species is now available (Oklander *et al.* 2007). The use of these new markers overcomes the restrictions imposed by previous studies based on allozyme and chromosomal polymorphisms that failed to reveal geographic variation patterns in *Alouatta caraya* (Mudry de Pargament *et al.* 1998; Szapkievich and Mudry 2003).

We aimed to study the effects of anthropic habitat degradation on the genetic structure of black-and-gold howlers by exploring if habitat fragmentation reduces the dispersal capacity of individuals, and thus the gene flow among groups. For this purpose we compare the genetic structure and dispersal patterns of 2 black-and-gold howler populations inhabiting 2 different habitats: a forest that has suffered fragmentation and selective logging for *ca.* 30 yr (fragmented forest [FF]) and a continuous forest (CF) habitat that has not been affected by severe anthropic modifications (Zunino *et al.* 2007). These populations present differences that allow us to test different hypotheses on dispersal patterns and gene flow (Table I). For example, in the FF, only 1 group inhabits each fragment, and fragments are separated from one another by grassland extensions. Therefore, individuals dispersing from their natal groups must travel long distances on the ground, risking predation or other sources of mortality before reaching another fragment (Kowalewski 2007).

If habitat fragmentation reduces the dispersal capacity of individuals, we expect to find a lower replacement of individuals in the fragmented habitat leading to: 1) genetically differentiated groups, 2) each group composed of more closely related individuals than in the continuous habitat, and 3) as a result of close kinship relations among adults in the fragmented habitat, higher values of endogamy than in the continuous habitat. Our findings shed light on differences in dispersal patterns in howlers depending on different ecological and demographic conditions.

Table I Main differences between the 2 study sites

Characteristics	Continuous forest (CF)	Fragmented forest (FF)	Source
Ecological conditions	Flooded forest with <diversity; <richness; continual forest	Semideciduous forest with >diversity; >richness; forest in patches in a matrix of grasses	1–5
	Low human impact; forests well preserved	High human intervention. Continuous deforestation and logging	3
	No predation	Low level of predation	4
Demographic conditions	Higher proportion of multimale groups	Higher proportion of unimale groups	3, 5
	High overlap of groups' home ranges	No overlap of group's home range; groups are isolated in each fragment	3
	Regular intergroup encounters and extragroup copulation	Intergroup encounters and extragroup copulation rare	4
	High density	Low density	1–5
	Births across the year	Birth seasonality	3
	No reported cases of infanticide	2 confirmed cases of infanticide reported	6, 7
	Males and females disperse	Males disperse	8, Present study
	Groups composed of adults that are not closely related	Groups composed of closely related adult females	8, Present study

References: 1: Rumiz *et al.* 1986; 2: Rumiz 1990; 3: Kowalewski and Zunino 2004; 4: Kowalewski 2007, 5: Zunino *et al.* 2007; 6: Zunino *et al.* 1986; 7: Peker *et al.* 2006; 8: Oklander 2007

Materials and Methods

Study Sites

We studied 2 populations of black-and-gold howlers at 2 nearby sites of northern Argentina located 20 km apart. Although these 2 sites are exposed to similar temperature, precipitation, and photoperiod patterns (Argentine National Meteorological Service), there are differences with regard to anthropogenic impacts on the forests.

CF population: This population inhabits Brasilera Island near the confluence of the Paraná and Paraguay rivers (27° 18′S, 58° 38′W; Fig. 1). This island has an area of 292 ha with no permanent human settlements. Howler groups on the island live in a continuous forest, and their home ranges usually overlap≤60% (Kowalewski 2007). We studied 7 groups, composed on average of 9.6±1.4 (range 7–11) individuals, with an average of 5.3±1.1 adults per group (range 4–7). The average number of adult males per group was 2.1±0.7 (range 1–3), and that of adult females was 3.1 ± 0.9 (range 2–4). The sex ratio was 1.5 adult female per adult male (Table II).

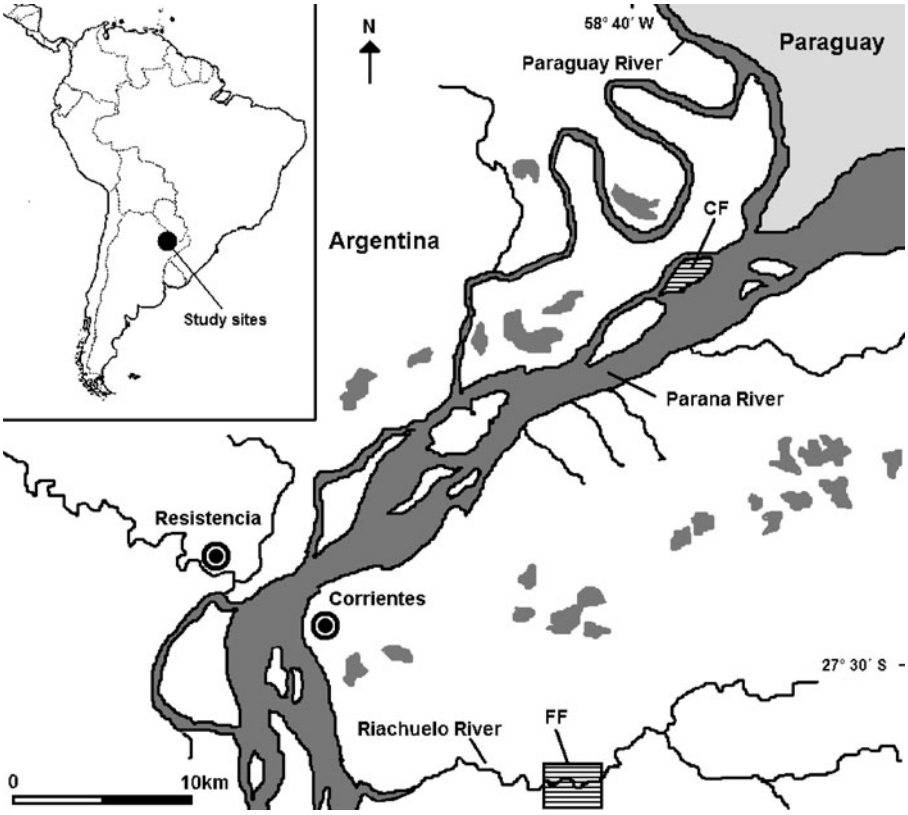

Fig. 1 Location of study sites. Striped areas indicate the study areas. Continuous forest, Brasilera Island, Chaco province (27° 18′S, 58° 38′W); fragmented forest, NW Corrientes province (27° 30′S, 58° 41′W).

FF population: This population is located in the surroundings of the Corrientes Biological Field Station (EBCo, MACN-CONICET; 27° 30′S, 58° 41′W; Fig. 1). The howler groups we studied occupy scattered fragments interrupted by grassland in a total area of 306 ha, and there is generally only 1 group associated with each fragment. We studied 11 groups, composed on average of 6.8±1.5 (range 5–9) individuals, with an average of 3.9±1.0 adults per group (range 3–6). Five of 11 groups contained >1 adult male. The average number of adult males per group was 1.7±0.5 (range 1–2), and that of adult females was 2.4±0.8 (range 2–4). The sex ratio was 1.5 adult female per adult male (Table II).

Data are available on the habitat, demography, and behavior of groups of *Alouatta caraya* from the FF site since 1980 (Rumiz 1990; Rumiz *et al.* 1986; Zunino *et al.* 2007). Since the 1960s, when cotton crops dominated the landscape of this site, the fragmentation of the gallery forest has been continuous and intense. Today, this site is under continuous deforestation for cattle ranching and pastures (Zunino and Kowalewski 2008). Remaining forest fragments have become considerably smaller in size, and the forest corridors along the rivers and streams have been interrupted, thus diminishing the effective connectivity among fragments.

The number of adult individuals per group did not differ significantly among the populations we analyzed (Kolmogorov-Smirnov 2-sample test, $p>0.10$).

Table II Composition of the groups and detail of individuals sampled within each group

Group code	No. of A M	No. of A M sampled	No. of A F	No. of AF sampled	No. of SA M	No. of SA M sampled	No. of SA F	No. of SA F sampled	No. of JM	No. of JM sampled	No. of J F	No. of J F sampled	No. of infants
(a) Groups in continuous forest													
EM	3	2	4	2	1	0	0	0	1	0	1	0	1
NF	2	2	3	3	0	0	1	0	1	1	0	0	2
G	3 (1[a])	3	4	1	3	2	0	0	1	1	0	0	0
LR	2 (1[a])	2	4	4	1	1	1	1	1	0	3	3	0
MK	2	2	2	2	0	0	1	1	0	0	1	0	1
VC	3	2	3	2	1	0	1	1	1	0	0	0	0
X	3 (1[a])	3	2	2	2	1	1	1	0	0	1	1	2
(b) Groups in fragmented forest													
CC	1	1	2	2	0	0	1	0	1	0	1	0	1
CV	1	1	2	2	1	0	0	0	1	0	0	0	1
EV	1	1	4	3	0	0	1	0	1	1	1	1	1
H	2	2	2	1	1	0	0	0	2	1	0	0	1
L1	2	2	2	2	1	1	0	0	0	0	0	0	0
L2	1	1	2	2	1	0	0	0	1	0	0	0	0
ML	2	2	3	1	0	0	1	1	2	0	0	0	0
PZ	1	1	2	2	1	1	1	0	1	1	1	1	0
RS	2	2	4	4	1	1	1	1	1	1	0	0	0
NN	1	1	2	1	0	0	0	0	0	0	1	0	1
ZN	2	2	2	1	1	1	0	0	0	0	1	1	0

F=females; M=males; A=adults; SA=subadults; J=juveniles; I=infants

[a] Corpses

Focal Subjects

We captured and tagged adult (A), subadult (SA), and juvenile (J) individuals from both populations to identify them and obtain DNA samples. We released the subjects after recovery in the same place of capture. A description of the procedure is provided in Oklander *et al.* (2007). We removed a small fragment of epithelial ear tissue for DNA extraction. We preserved the tissue samples from captured individuals and corpses found in the study sites at room temperature in solid NaCl (Oklander *et al.* 2004) until DNA extraction in the laboratory. In November 2004, we captured 52 randomly selected individuals in the FF. Of those individuals, we identified 50 as belonging to 11 groups (24 males and 26 females; 37A, 6SA, 7 J). In June 2005, we captured 43 randomly selected individuals from 7 groups in the CF. In addition, we gathered tissue samples from 3 monkey corpses found during behavioral studies (Kowalewski 2007; Oklander 2007) and used them in the analysis. In total, we obtained 46 genotypes from the CF (22 males and 24 females; 32A, 8SA, 6 J; Table II). Our study complied with current Argentine laws (permissions from the National Resources Board, Subsecretariat of Fauna and Flora, Corrientes Province, Argentina).

DNA Extraction and Microsatellite Amplification

We extracted DNA from the ear tissue samples using standard procedure sodium dodecyl sulfate (SDS)/Proteinase K digestion followed by phenol-chloroform organic extraction (Sambrook *et al.* 1989). We genotyped each sample with 11 microsatellites previously characterized for *Alouatta caraya* (Oklander *et al.* 2007). We performed polymerase chain reaction (PCR) amplifications in a final volume of 25 μl using 5–10 ng of DNA template. We included negative controls in all DNA extraction and PCR amplification reactions. We determined allele sizes of PCR products via an ABI 310 Genetic Analyzer and Genescan version 3.1, using GS-500 TAMRA as size standard (Applied Biosystems, Foster City, CA).

Statistical Analysis

We determined allele frequencies, observed and expected heterozygosity, number of alleles, and Hardy-Weinberg equilibrium for both populations of *Alouatta caraya*, using Arlequin 3.0 (Excoffier *et al.* 2005) and FSTAT v.2.9 (Goudet 2001, Online resource 1). We estimated exclusionary power for parentage analysis and frequency of null alleles in each locus via Cervus (Marshall *et al.* 1998). We estimated Wright's statistics FST and FIS (Wright 1965, 1969) for comparisons between and within the CF and the FF. We also used Arlequin and FSTAT to estimate these parameters by means of the analysis of variance of allele frequencies: AMOVA (analysis of molecular variance; Weir and Cockerham 1984). We used a locus-by-locus AMOVA analysis that is suggested when there are uncertain data at some loci in some individuals (Langergraber *et al.* 2007). FST is frequently used as a measurement of population subdivision (population structure), and it provides an appropriate method to estimate gene flow among populations under the hypothesis of neutral alleles (Hartl and Clark 2007). In fragmented habitats, FST values can be used to estimate the proportion of gene flow. It is typically accepted that FST values

of *ca.* 0.15 indicate significant genetic differentiation among subpopulations or groups (Frankham *et al.* 2002; Hedrick 2005). FIS is the reduction in heterozygosity in an inbred individual, relative to the heterozygosity expected by random mating in the population to which the inbred individual belongs. Generally high FIS implies a considerable degree of inbreeding (Hartl and Clark 2007).

We used FST to analyze possible sex bias in dispersal patterns (Goudet *et al.* 2002) using FSTAT v.2.9. We considered only adult individuals for this analysis. Natal dispersal—emigration from the group in which an individual was born—generally occurs during the juvenile or subadult states (Pusey and Packer 1987). Secondary dispersal after an individual remains in a non-natal group may also occur (Pusey and Packer 1987). When analyzing adult individuals, we evaluated both those that had already gone through natal or secondary dispersal, as well as those that had remained in the group until maturity. Allele frequencies of individuals of the dispersant sex should be more homogeneous than those of the philopatric sex individuals; therefore, the FST of the philopatric sex is expected to be higher than that of the dispersant sex in each population (Goudet 2001).

We calculated 2 sets of geographical distances via Google Earth 4.3 for the population inhabiting the FF: 1) the shortest straight line distances among forest fragments and 2) the shortest straight line distance from each forest fragment to the river. We calculated the genetic distance between groups, as both group pairwise FSTs and Slatkin's linearized genetic distances between FF groups, via Arlequin 3.0 (*op. cit*). We analyzed possible correlations between geographic and genetic distances via a Mantel test. We represented the Slatkin's linearized genetic distance results graphically via multidimensional scaling (MDS) in XLSTAT 2008.5.02. In all cases we tested significance using 2000 permutations.

Kinship Relationships

We used Kingroup (Konovalov *et al.* 2004) to estimate kinship relations. This program calculates kinship coefficients (r) based on analysis of maximum likelihood, evaluating possible relatedness (Goodnight and Queller 1999). We calculated pairwise kinship coefficients (r) for all adults sampled in each group, as well as average r for each and both sexes within groups. In the CF, we considered 7 groups for all calculations, except for the adult females in group G, wherein we sampled only 1 adult female. In the FF, we considered 11 groups for kinship relations among adults, but only 5 groups for kinship relations among adult males, and 8 groups for adult females because we didn't have same-sex dyads for all groups (Table II). We also used kinship relationships to investigate sex bias in dispersal patterns (Goudet *et al.* 2002) via the FSTAT v.2.9 (Goudet 2001). We used 2-sided significance tests based on 2000 randomizations.

Paternity/Maternity Testing

We analyzed paternities and maternities of juvenile (J) and subadult individuals (SA) from the groups studied. We considered all adult and subadult individuals sampled as possible mothers and fathers in each population. We investigated the paternities and maternities of 14 individuals from the CF: 6 J (2 males and 4 females) and 8 SA

(4 males and 4 females); and 13 individuals from the FF: 7 J (4 males and 3 females) and 6 SA (4 males and 2 females) (Table II and Online Resource 3). We evaluated Mendelian inheritance among candidate parents and offspring. Whenever 1 allele in all loci analyzed was also detected in the candidate's parent, we performed statistical likelihood analyses to obtain the paternity index (PI; Edwards 1972). We estimated LOD, delta, trust level, and PI values in each case via Cervus (Marshall *et al.* 1998). We considered individuals that achieved PIs >97.0% to be the parents of the juveniles and subadults. Assigned paternities always had complete matching of parents and offspring genotypes (Online Resource 3).

Results

Interpopulation Analysis

FST values between the CF and FF populations are significantly different from 0 (FST=0.101; p<0.001), indicating significant genetic differentiation. Neither the number of alleles per locus (FF: 4.73±2.57; CF: 5.36±2.66; t=0.6, df=20, p=0.57) nor the expected heterozygosity (FF: 0.50±0.19; CF: 0.57±0.20) (t=0.8 df=20 p= 0.44) is significantly different between the 2 populations (Online Resource 1).

Population Analysis in the CF

The results of the AMOVA in the CF population revealed no significant genetic differentiation among groups (FST=0.050, p=0.173) and no indication of endogamy (FIS=–0.012, p=0.627). The FIS index was close to 0 in all the groups (confidence interval 99% bootstrap −0.050/0.021), indicating that groups show neither heterozygosis deficiency nor excess (Table III). Further, the analyses of

Table III FIS values and kinship coefficients (r) for groups analyzed in the CF (Brasilera Island)

	Groups						
	EM	NF	G	LR	MK	VC	X
No. of individuals analyzed	4	6	7	11	5	5	8
No. of adult individuals analyzed	4	5	4	6	4	4	5
FIS	0.094	−0.063	−0.014	0.015	0.056	−0.055	−0.057
p value	0.284	0.807	0.601	0.404	0.321	0.792	0.736
Average r adult individuals (range)	0.230 (0–0.76)	0.102 (0–0.35)	0.048 (0–0.23)	0.117 (0–0.64)	0.142 (0–0.43)	0.027 (0–0.16)	0.157 (0–0.75)
r adult female dyads	0.2	0.0/0.0/ 0.35	–	0.0/0.0/0,1/0.2/ 0.38/0.64	0.0	0.0	0.0
r adult male dyads	0.29	0.29	0.0/0.01/ 0.05	0	0.43	0.0	0.0/0.0/ 0.39

FST and r by sex with adult individuals showed similar values (female FST= 0.056, male FST=0.055, p=0.981; female r=0.144; male r=0.133, p=0.905), suggesting that both sexes disperse. Kinship relationships among all adults, females, and males composing the groups showed similar and relatively low values (Table III). Likelihood analysis showed that 5 of 7 groups contained 1 male-female dyad that was related as siblings or cousins (5 dyads from 35 male-female dyads analyzed). Two of 6 groups we analyzed for female kinship relations contained 1 dyad of related females as sisters or half-sisters, while 4 had unrelated female pairs (2 related dyads from 13 female-female dyads analyzed). Two of 7 groups we analyzed for male kinship relations contained 1 dyad of males related as brothers or half-brothers, while 5 had unrelated or not closely related male pairs (2 related dyads from 11 male-male dyads analyzed) (Table III, Online Resource 2). On average, adult individuals from each group in the CF are related by r=0,118; thus most adult individuals in each group are not closely related (Table III, Online Resource 2).

Population Analysis in the FF

The results of the AMOVA in the FF population revealed significant genetic differentiation among groups (FST=0.139, p<0.001). However, FIS was not statistically significant (FIS=–0.153, p>0.05), although the majority of FIS values showed higher negative values than those of the CF (confidence interval 99% bootstrap −0.240/−0.065; Table IV).

FST and r differed significantly between the 2 sexes (female FST=0.233, male FST=0.079, p=0.036, female r=0.447; male r=0.276, p=0.047), suggesting differences in dispersal behavior between females and males in this population. Average kinship relations among adults that compose the groups showed higher values than in the CF (Table IV). Likelihood analysis showed that 3 of 11 groups contained 1 male-female dyad related as siblings or parent-offspring (3 dyads from 29 male-female dyads analyzed). Six of 7 groups analyzed for female kinship relations contained ≥1 dyads of closely related females (mother-daughter or sisters; 12 dyads from 15 female-female dyads analyzed). Two of 5 groups analyzed for male kinship relations contained one dyad of males related as brothers or father-son, while 3 had unrelated or not closely related male pairs (2 dyads from 5 male-male dyads analyzed; Table IV, Online Resource 2). Thus adult females in groups were more related to each other than males were, and exhibited higher genetic differences among groups, suggesting that females in this habitat were philopatric. Individuals from each group in the FF had an average kinship coefficient of r=0.237, 2 times higher than that observed in the CF.

Genetic differences between groups did not correlate with geographical distances among fragments (Table V; Mantel test, regression coefficient=−0.005, p=0.605). Group pairwise FSTs showed that 4 groups (PZ, RS, H, ZN) differed significantly from all other groups (10/10), or from all but 1 group (9/10; Table VI). We refer to these 4 groups as the most genetically differentiated ones (Fig. 2). Three of these 4 groups resided in the forest fragments most distant from the riparian forest (Table V). The rest of the groups also showed significant differences but only with 3–6 groups

Table IV FIS values and kinship coefficients (r) for groups analyzed in the FF (EBCo)

	Groups										
	CC	CV	EV	H	L1	L2	ML	PZ	RS	NN	ZN
No. of individuals analyzed	3	3	6	4	5	3	4	6	9	2	5
No. of adult individuals analyzed	3	3	4	3	4	3	3	3	6	2	3
FIS	–0.036	−0.018	−0.256	−0.148	−0.173	−0.241	−0.260	−0.272	−0.176	0.188	−0.136
p value	0.661	0.618	0.990	0.903	0.943	1.000	0.989	0.995	0.990	0.225	0.932
Average r adult individuals (range)	0.227 (0–0.36)	0.2 (0.01–0.46)	0.19 (0–0.5)	0.49 (0.24–0.72)	0.12 (0–0.53)	0.32 (0.07–0.66)	0.35 (0.05–0.68)	0.317 (0–0.68)	0.24 (0–0.67)	0.0	0.15 (0.14–0.15)
r adult female dyads	0.58	0.46	0.24/0.35/0.5	–	0.53	0.66	–	0.27	0.33/0.34/ 0.39/0.41/ 0.51/0.67	–	–
r adult male dyads	–	–	–	0.72	0.0	–	0.05	–	0.46	–	0.15

Table V Shortest straight line distances in meters among fragments obtained by means of Google Earth

	CV	EV	H	L1	L2	ML	PZ	RS	NN	ZN
CC	630	1058	2737	2686	2260	3073	2831	1470	2012	2406
	CV	1594	1889	1878	1490	2260	2001	1338	1404	1493
		EV	3257	3683	3325	4032	3736	630	3304	3005
			H	1074	1214	1170	918	2050	1842	151
				L1	280	186	116	2855	860	698
					L2	700	544	2626	512	789
						ML	227	3247	890	759
							PZ	3005	1030	646
								RS	2925	2299
									NN	1878
82	311	236	590	0	0	165	0	1654	66	865

Last row: Shortest straight line distances in meters from each forest fragment to the river

(Table VI). These results showed that the groups in FF had different levels of genetic differentiation. Multidimensional scaling (MDS) representation of linearized Slatkin's genetic distances shows that PZ, RS, and H groups are clearly separated from the others (Fig. 3).

Paternity/Maternity Testing

Paternity exclusion power in the CF was 0.925, when both parents were unknown and 0.993 when 1 parent was known. Paternity exclusion power in the FF was 0.859, when both parents were unknown and 0.983 when 1 parent was known. We identified 13 fathers and 9 mothers for 14 individuals analyzed in the CF, and 12 fathers and 10 mothers for 13 individuals analyzed in the FF (Online Resource 3). In CF we found a juvenile male (ME25 from group NF, Table II) whose father belonged to a neighboring group (V), suggesting that there was gene flow among groups, either as a result of extragroup copulation or male turnover in the groups.

In addition, we found that 3 of 8 subadults (37.5%) were living in different groups from those of their parents (male SA group LR, male SA and female SA group X, Table II). Paternity and maternity analysis in the FF habitat showed that adult individuals of any given group fathered all studied subadults and juveniles of that group. Further, we detected an apparent incest case between a female and her son, which had fathered an offspring identified as individual H19. As the assigned father of this offspring (H19) is a SA (M33), we had also assigned its parents (H35 and M32). We tested M32 as the possible progenitor of H19 and found 1 exclusion. Therefore, we can reject the hypothesis of M33 as an older sibling from the same father. Moreover, we sampled all males in that group, so there is a very low probability (only extragroup copulation) of not having sampled the actual father (Online Resource 3).

Table VI Group pairwise FSTs significance in the FF

	CV	EV	H	L1	L2	ML	PZ	RS	NN	ZN
CC	0.309+ −0.009	0.033+−0.004*	0.031+−0.005*	0.165+−0.010	0.184+−0.009	0.098+−0.007	0.009+−0.002*	0.004+−0.002*	0.297+−0.008	0.016+−0.002*
	CV	0.085+−0.006	0.027+−0.004*	0.348+−0.011	0.385+−0.011	0.191+−0.010	0.012+−0.003*	0.046+−0.004*	0.116+−0.007	0.098+−0.007
		EV	0.009+−0.002*	0.327+−0.011	0.183+−0.007	0.011+−0.002*	0.002+−0.001*	0.003+−0.001*	0,059+−0,008	0.011+−0.002*
			H	0.032+−0.004*	0.023+−0.003*	0.006+−0.002*	0.005+−0.002*	0.004+−0.001*	0.076+−0.005	0.006+−0.002*
				L1	0.508+−0.012	0.072+−0.007	0.002+−0.001*	0.044+−0.004*	0.279+−0.010	0.036+−0.004*
					L2	0,059+−0,006	0.008+−0.002*	0.005+−0.002*	0.104+−0.005	0.051+−0.005*
						ML	0.003+−0.002*	0.014+−0.003*	0,057+−0,005	0.020+−0.003*
							PZ	0.000+−0.000*	0.040+−0.004*	0.002+−0.001*
								RS	0.018+−0.003*	0.005+−0.001*
									NN	0.046+−0.006*
5/10	3/10	6/10	9/10	4/10	4/10	5/10	10/10	10/10	3/10	9/10

p-values with 2000 permutations. *Significant values ($p<0.05$). Last row: rate of significant differences with other groups

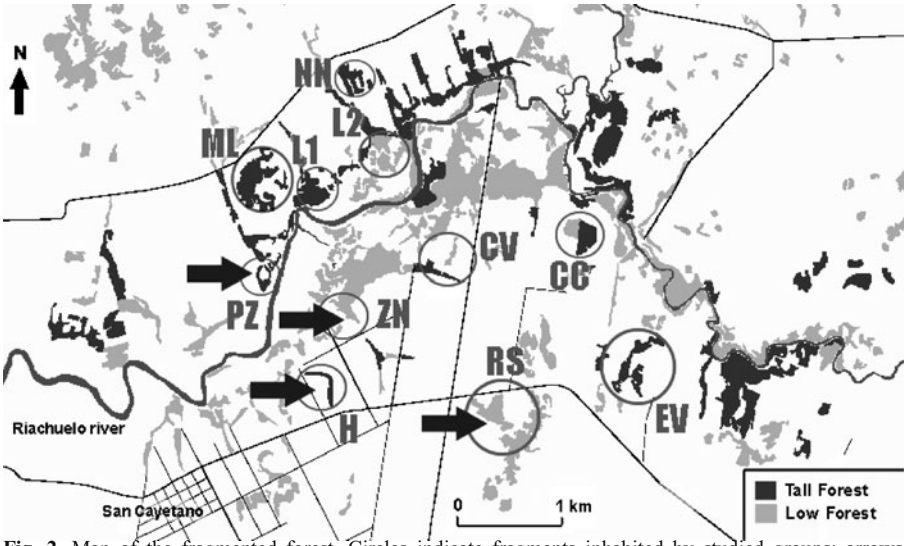

Fig. 2 Map of the fragmented forest. Circles indicate fragments inhabited by studied groups; arrows indicate fragments inhabited by studied groups with most genetic differences.

Discussion

Our comparisons between CF and FF indicate that black-and-gold howler populations exhibit different genetic structure depending on habitat. Within the CF population, the groups share most of their genetic variability and are composed of adult individuals that are not closely related. Groups included within the FF, however, have a statistically significant genetic differentiation and are composed of closely related females. Consistent with prediction 1), we found significant differences in FST, indicating that genetic variability in the FF population is distributed among groups, and that reveals a recent genetic differentiation among them. Genetic differences observed among FF groups did not correlate with

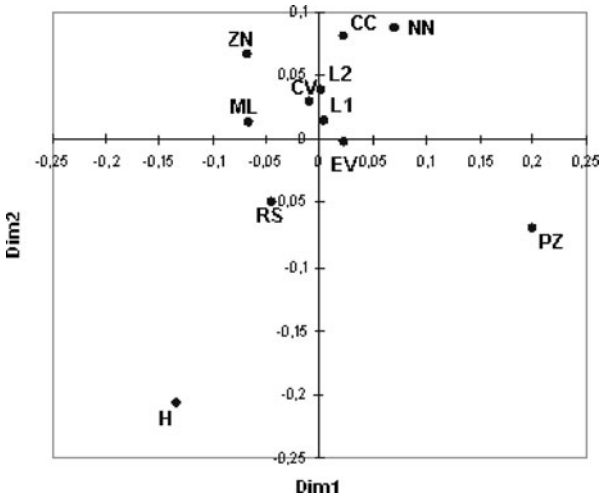

Fig. 3 Graphical representation of Slatkin's linearized genetic distances between FF groups using multidimensional scaling (MDS). Kruskal stress (1)=0.189. Dim=dimensions.

geographical distances among fragments, but most groups that showed significant genetic differences with other groups were located in the most distant fragments from the riparian forest. Group PZ inhabits a fragment of riparian forest and also shows genetic differentiation with respect to all the other fragments we studied. This is possibly related to the anthropogenic fragmentation of the forest due to logging and river shore crumbling as a consequence of the deforestation. In this regard, discontinuities along the riparian forest could also cause group isolation by interrupting natural fauna corridors. These results suggest a reduction in the dispersal rate in the fragments isolated from gallery forests along the rivers and are consistent with the hypothesis that suggests that the island system and the riparian forests are biological corridors for *Alouatta caraya* (Brown and Zunino 1994). Analysis based on isozyme markers in another howler species (*Alouatta seniculus)* also indicated an influence of habitat fragmentation on gene flow decrease (Pope 1990, 2000).

Consistent with prediction 2), our analysis of group composition in the CF indicated that most groups were composed of mature females and males that are not closely related, revealing that both sexes disperse in this population. As a result, groups are generally formed by immigrant individuals. In contrast, the relationship coefficient between adult individuals in the FF indicated that most individuals in each group were related. In addition, the FST analysis and relationship coefficients of mature individuals discriminated by sex in the FF indicated differences in kinship relationships among the adult males and the adult females in the groups. Adult males of the groups within the FF were significantly less related than females. However, the relationship coefficient among males in FF was 2 times higher than the one found in the CF for both sexes.

Females that compose groups in the FF are highly related within the groups and highly differentiated among them, suggesting the formation of matrilines. In areas with isolated fragments, dispersers may incur significant mortality costs when crossing unfavorable habitats to reach suitable patches (Lawson Handley and Perrin 2007). Our molecular analyses provide a general picture of the genetic variability that individuals of a group share at one time, but do not allow us to determine whether individuals leave their groups but fail to enter into others (unsuccessful dispersal). FF females may leave their groups, but the genetic results suggest that they do not join others. However, there is no reason to believe that females have a higher mortality rate than males during dispersal, so we suggest that females remain in their natal groups.

Our results suggest that males constitute the dispersing sex in the FF population, but their dispersal into other groups occurs 50% less frequently than when males join groups in the CF. Male-biased dispersal in the FF may be explained by 3 nonmutually exclusive factors: inbreeding avoidance, local resource competition, and local mate competition (Favre *et al.* 1997). By any of these factors, it would be more costly for a male to remain in a group than for a female, especially in a highly dimorphic species (Packer 1979). The lower rate of male dispersal in the FF may be due to the higher dispersal costs associated with the isolation of forest fragments. Alternatively, there may be higher competition between males for reproductive positions in the FF (Calegaro-Marques and Bicca-Marques 1996), because most of the groups are unimale (Table I). Unimale groups

and male-male social intolerance are characteristic of the FF, unlike the CF, where multimale groups predominate and males are tolerant of one another within groups (Kowalewski 2007; Oklander 2007).

Our data do not completely support prediction 3) that suggests that endogamy values would be higher in the continuous habitat, because we found nonsignificant FIS values in both populations. However, for the CF population, we found FIS indexes close to 0 for all groups, suggesting that considerable gene flow exists among groups, with no indication of endogamy. Conversely, in the FF population, the FIS analyses within groups mostly showed high negative values. These results generally indicate heterozygosity excess, and hence, an absence of inbreeding. But in the FF, mature females are highly related within groups; therefore, their gametes represent a differentiated portion of the gene pool. Gametes from a male coming from any other group might cause heterozygosity excess in the first offspring generation when joining those of these females (Chesser 1991; Pope 1992), which makes potential inbreeding situations extremely difficult to detect by the FIS index. For this reason, we estimated endogamy levels and gene flow with our resulting data from paternity and maternity analyses. Paternity analyses in CF show three individuals with parents in other groups, representing a dispersal of 37.5% of the subadults, and a son sired by a male living in another group. As we had no behavioral data from these groups at the time of this individual conception, we cannot confirm that this juvenile was fathered by an extragroup male or a former male resident of the group that had later dispersed to a neighboring group. However, behavioral data from the same groups obtained during 2003–2004 show a high degree of extra-group copulation (32%) with males from neighboring groups or solitary males (Kowalewski 2007), which would suggest that extragroup paternity may occur. Nevertheless, this result indicates that gene flow, either due to extragroup copulation or male turnover in the groups, occurs among groups in the CF. Meanwhile, in the FF, we found that all the subadult and juvenile individuals we analyzed for paternity/maternity analysis were in the same group as their progenitors; therefore, they had not dispersed yet. We also found one apparent case of incest, which is a clear example of endogamy with very high relationship coefficients among progenitors.

Altogether, our results suggest the existence of higher dispersal costs in the fragmented forest due to environmental restrictions caused by the isolation of forest fragments. The high genetic differentiation among groups plus the apparent incest event in the FF suggest reduced gene flow, revealing that increasing habitat fragmentation may severely limit the howler's ability to disperse among groups. These results also suggest that different ecological situations, such as habitat fragmentation, may modify the dispersal patterns of black-and-gold howlers. If fragmentation and clearing of forest fragments continue as a consequence of uncontrolled deforestation and landscape modification, mainly for soy bean and other crop monocultures and/or cattle ranching, the isolation of different subsets of several animal species populations will increase. Other arboreal primates and nonprimates that depend on forest continuity to disperse are likely to show similar anthropic-induced effects on genetic diversity, possibly resulting in an inability to respond to challenging selective pressures.

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