Kinship and dispersal patterns in *Alouatta caraya* inhabiting continuous and fragmented habitats of Argentina.

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Abstract

Black and gold howler monkeys (*Alouatta caraya*) still inhabit degraded and fragmented forests in southern South America. This fact raises questions regarding the real capacity of the howler's long term survival under such conditions. A detailed molecular study was carried out with the aim of evaluating how the continuous processes of habitat reduction and fragmentation affect the genetic structure of howler monkey populations. Two populations exposed to differences in forest continuity were compared using molecular markers: one inhabiting an undisturbed and continuous forest (CF) and the other, a fragmented forest (FF). FF and CF showed differences in kinship relations and dispersal patterns. The groups in the FF were genetically differentiated; in contrast, there was no differentiation between groups in the CF. Moreover, both males and females disperse in the CF; accordingly, most groups are composed of adult individuals that are not closely related, whereas in the FF, males disperse more than females and groups are composed of closely related adult females. These results suggest that habitat fragmentation modifies the dispersal patterns of black and gold howler monkeys and might reflect a trend towards a reduction in their ability to disperse. This study underscores the use of molecular genetic data as a tool of utmost importance for a better understanding of the social organization and behavioral patterns as well as for conservation purposes.

1. The species

Black and gold howler monkeys (Primates, Platyrrhini, Atelidae) have been intensively studied for decades in a variety of habitats including degraded and fragmented forests throughout their distribution. There is available data of the populations inhabiting northern Argentina on demography, social structure, diet and many behavioral patterns (Rumiz 1990, Rumiz et al. 1986, Zunino and Kowalewski 2008, Kowaleski and Garber 2010). Nowadays, entire fragments of forest are being clear-cut for agriculture and cattle ranching in the southern limit of the geographic range of *Alouatta caraya*, covering the Argentine provinces of Formosa, Misiones, Salta, Corrientes and Chaco (Brown and Zunino 1994, Peres 1997, Zunino et al. 2007). Several studies mention the survival ability of this species in fragmented and impoverished habitats such as those that have suffered selective logging (Bicca-Marques 2003, Zunino et al. 2007, Bicca-Marques et al. 2009). For example Zunino et al. 2007 studied groups inhabiting forests that have been under continuous logging for the past 20 years and found that the ecological density and the number and composition of groups have remained constant. Although *A. caraya* groups still inhabit degraded and fragmented forests, the real capacity of howlers to endure under these levels of habitat degradation in the long-term is still unknown. The ultimate goal of this research was to address this issue.

2. The approach

Molecular genetic methods have been increasingly applied in studies of behavioral ecology and conservation of primates (Fischer et al. 2006, Hernandez et al. 2007, Di Fiore 2009). In particular, the use of microsatellites has allowed to describe primate population characteristics such as mating systems, dispersal patterns and levels of inbreeding that are difficult to detect by observational studies. Microsatellites are short tandem repeats (STR) of genomic DNA sequences dispersed all over the genome which are highly polymorphic since they vary in the number of repetitions of the motive. These markers are codominantly inherited, since the alleles of both parents are detectable in the offspring and therefore, are useful for population genetic studies and kinship determination.

In sharp difference with research carried out on apes, where human-specific polymorphic genetic markers can be employed, most of these markers are not useful in the analysis of more evolutionarily distant species such as Platyrrhines. The underlying reason for this restriction is the lack of homology between human-derived primer sequences and neotropical primate-genomes. Therefore, in order to investigate the population dynamics of black and gold howlers, the first step to accomplish was to detect, isolate and characterize suitable polymorphic genetic markers for this species. Accordingly, prior to this research we identified eleven polymorphic DNA microsatellite markers for the *Alouatta caraya* (Oklander et al. 2007), allowing the opportunity to study kinship relations, and gene flow in this species.

Few data are available on the influence of habitat fragmentation on genetic variation in free-ranging neotropical primates. Loss of genetic variation and inbreeding depression, typically expected outcomes of forest fragmentation, are likely to have highly deleterious implications for the long term viability of animal populations.

In this study we examine by molecular methods how habitat fragmentation impact on the genetic structure of black and gold howler monkeys populations (*Alouatta caraya*) in northern Argentina. For this purpose, fixation indexes (Fst and Fis), kinship relationships between members of each group and paternity/maternity identification of juvenile and subadult individuals were analyzed for each population: one inhabiting an undisturbed continuous forest (CF) and the other a fragmented forest (FF). We expected to find related individuals within the groups, but highly differed among groups in the FF, as consequence of the restrictions in the dispersion caused by habitat fragmentation.

3. The populations studied

Two populations of black and gold howlers located 20 km apart from each other on the boundary of Corrientes and Chaco provinces in Northern Argentina were studied. These two populations inhabit sites that are exposed to similar temperature, precipitation, and photoperiod patterns (Argentina's National Meteorological Service), but display differences in forest continuity. One of the populations included a set of groups living in an undisturbed and continuous forest (CF) located on Brasilera Island that has 292 hectares with no permanent human settlements (27º 18' S, 58º 38' W). The other population studied inhabits a fragmented forest (FF) located in the surroundings of the Corrientes Biological Field Station (27º 30' S, 58º 41' W) (EBCo, MACN-CONICET). Groups studied in the FF are dispersed in forest fragments interrupted by grassland in a total area of 306 hectares. In general, there is only one group associated with each fragment. Individuals from 7 groups in the CF and from 11 groups in the FF were studied (table 1).

3.1. Sampling strategy

A. caraya individuals of both populations are under permanent observation as part of a multidisciplinary research (Oklander et al. 2004, Zunino et al. 2007, Kowalewski et al. 2010, Kowalewski and Garber 2010). Accordingly, captures are frequently required for individual identification. A detailed description of capturing, tagging and sampling are pointed below.

3.1.1. Capture: Black and gold howler monkeys inhabit the top of trees, up to 20 meters high, and rarely come down to ground level. Therefore, one way to capture them is by darting. This procedure was carried out by shooting darts with a Pneu Dart Model 179B Air Pump Rifle, Sheridan. The darts were 1cc Pneu Dart type 'P' with collared needles. A combination of medetomidine hydrochloride (Domitor, Pfizer Corporation) and Ketamine hydrochloride 50 mg/ml (Ketalar, Parke

Davis) was used as anesthetic. Dosage was 150 ug/kg medetomidine hydrochloride combined with 4 mg/kg of Ketamine. When the darted animals were under anesthetic effect and ready to fall, stretched nets were placed under them in an attempt to catch them before they reached the ground. The average anesthesia period was approximately 60 minutes. Once the effect of anesthesia subsided, animals were maintained in a safe place until their complete recovery. Behavioral observations assured this condition prior to animal release into their habitat. All procedures were supervised by veterinaries from the UNNE (Universidad Nacional del Nordeste, Argentina).

3.1.2. Tagging and Identification of Individuals: Ear tagging and ankle color identification bracelet fittings (same material as commercial pet collars) were applied to the narcotized animals.

3.1.3. Sampling: Ear tissue sampling obtained during tagging was used as DNA source. A total set of 43 individuals from 7 groups (table 1) was captured and sampled in the CF in 2005. In addition, tissue samples from 3 monkey corpses found during behavioral studies within the research area in 2004-2005 were gathered and used in the analysis (table 1). In 2004, a set of 50 individuals from 11 social groups (table 1) were captured and sampled for analyses in the FF. Tissue samples from captured individuals and corpses found in the study sites were conserved at room temperature in solid NaCl (Oklander et al. 2004) until DNA extraction in the laboratory. The study complied with current Argentine laws (permissions from the National Resources Board, Subsecretariat of Fauna and Flora, Corrientes Province, Argentina).

4. Genetic Analysis

4.1. Lab procedures

DNA was extracted from ear tissue samples using the standard procedure SDS/ Proteinase K digestion followed by phenol–chloroform organic extraction (Sambrook et al. 1989). Amplifications of 11 microsatellites characterized for *Alouatta caraya* were subsequently performed for each sample as described in Oklander et al. (2007). In all cases, DNA extraction and PCR amplification reactions included negative controls. Allele sizes of PCR products were determined using an ABI 310 Genetic Analyzer and Genescan software version 3.1, using GS-500 TAMRA as size standard (Applied Biosystems, Foster City, USA).

4.2. Statistical Analysis

4.2.1. Fixation indexes

Fixation indexes (F statistics, Wright 1965) were used in order to measure genetic variability within populations. These indexes are based on heterozygosity measures and are used to evaluate the reduction in expected heterozygosity

for random mating at any one level of a population hierarchy relative to another more inclusive level of the hierarchy (Hartl and Clark 2007).

Arlequin 3.0 (Excoffier et al. 2005) and Fstat (Goudet 2001) softwares were used to estimate Fst and Fis indexes within and between each population of *Alouatta caraya* by means of the analysis of variance of allele frequencies -AMOVA (Analysis of Molecular Variance, Weir and Cockerham 1984). These programs use parameters of the statistical F that allow to make inferences even when there are differences in the number of sampled individuals in each subpopulation, or in the subpopulation number within each population. The frequency of null alleles in each locus was also considered by the Cervus software (Marshall et al. 1998).

The statistical Fst is a measure of population genetic structure that allows to estimate gene flow among populations under the hypothesis of neutral alleles (Hartl and Clark 2007). In fragmented habitats, Fst values can estimate the proportion of gene flow among fragments. It is considered that if high gene flow exists among subunits, Fst values should not be significantly different from 0. Therefore, at low rates of gene flow, Fst values are increased. High Fst implies a considerable degree of differentiation among populations or groups (Hedrick 2005). Fst was also used to analyze possible sex bias in dispersal patterns. Dispersions generally happen in juvenile or subadult states (Pusey and Packer 1987). For this reason, only adult individuals were considered for sex-bias analysis. Allele frequencies of individuals of the dispersant sex should be more homogeneous than those of philopatric sex individuals; therefore, the Fst of the philopatric sex in each population is expected to be higher than that of the dispersant sex in each population (Goudet 2002). Fis (inbreeding coefficient) is the proportion of the variance in the subpopulation contained in an individual. High Fis usually implies a high degree of inbreeding (Hartl and Clark, 2007).

4.2.2. Kinship relationships

The Kingroup software (Konovalov et al. 2004) was used for the estimation of kinship relationships among the individuals that compose the groups in both howler populations (table 2). This program calculates kinship coefficients (*r*) based on analysis of maximum likelihood, even evaluating the genetic similarities for progeny with a small number of samples (Goodnight and Queller 1999). Paternity and maternity of juveniles and subadults were also evaluated by Mendelian inheritance between candidate's parents and offspring (table 3). Whenever one allele in all loci analyzed was also detected in the candidate's parent, statistical likelihood analyses were performed in order to obtain the Paternity Index (Edwards, 1972). For this estimation we used the Cervus software (Marshall et al. 1998). Those individuals who achieved paternity indexes higher than 97.0% were considered to be the parents of the juvenile/subadults studied.

5. What the genetic markers tell us about habitat fragmentation

5.1. Fixation indexes

Population analysis of the 11 microsatellites in the FF and the CF suggested many differences between the two populations. AMOVA results in the CF population showed non-significant values in F indexes (Fst=0.050, p=0.173, Fis=-0.012, p=0.627;). In addition, the analyses of Fis values for each group were not statistically significant (table II). Moreover, the analyses of Fst by sex showed similar values (Female Fst=0.056, Male Fst=0.055, p=0.981), indicating that neither females nor males from different groups were genetically differentiated. Non-significant P values mean that Fst values are also similar between sexes.

On the other hand, the AMOVA results for the FF population showed Fst values that suggested significant genetic differentiation among groups (Fst=0.139, AMOVA $p<0.001$). However, significant values were not observed for Fis=-0.153, $p>0.05$. Therefore, these results do not support inbreeding in the FF. Although the Fis analyses of each group were not significant ($p>0.05$), most of them showed higher negative values than those in the CF (table 2). Fst analyses by sex showed significant differences (Female Fst=0.233, Male Fst=0.079, p=0.036) suggesting that females are genetically differentiated between groups, being their differentiation higher than that observed among males from different groups.

5.2. Kinship relationships and paternity/maternity of juvenile and subadult individuals.

Average kinship relationships among adult females and males composing the groups showed similar and relatively low values in the CF (female r=0.144; male r=0.133, p=0.905). These results suggest that there are no differences in kinship relationships among adult males and females in the groups. Average kinship relationships in each group are shown in Table 2. On average, individuals from each group in the CF are related by r=0.118 (not closely related). In the analysis of paternity/maternity of juvenile and subadult individuals of the CF we found a juvenile male individual whose father belonged to a neighbouring group (MJ from group NF, table 3). Additionally, it was found that three out of eight analyzed subadults were living in different groups from those of their parents (MSA group LR, MSA and FSA group X, table 3). These data represent a migration of the analyzed subadults of 37.5%.

In contrast, average kinship relationships among adult females and males showed significant differences in the FF (female r=0.447; male r=0.276, p=0.047). These results indicate that differences exist in female and male grouping in this population. Adult females in the groups are more closely related among each other than males. Females also present higher genetic differences among groups. Average kinship relationships in each group are shown in table 2. Individuals of each group in the FF have an average kinship coefficient of r=0.237. This relationship coefficient is twice higher than that observed in the CF. Furthermore, it was found

that all juvenile and subadult individuals analyzed for paternity and maternity in the FF were in the same group as their progenitors; therefore, they had not dispersed yet (table 3). In addition, we detected an incest case of a female and her son who had fathered an offspring identified as individual H19 (table 3).

6. Diverse genetic landscapes reflect the effects of habitat fragmentation.

Habitat fragmentation may isolate populations and limit dispersal opportunities. By analyzing the genetic variability of the groups of two howler populations with differences in their habitat quality, we were able to discern the strategies used to cope with habitat fragmentation and estimate their long term consequences.

Microsatellite marker analysis allowed to evaluate fixation indexes and kinship relationships and to compare groups of black and gold howlers inhabiting undisturbed and fragmented forests, underscoring many clear differences.

The CF population exhibits a genetic variability that is almost entirely found in all the studied groups, since only 5% (Fst=0.05) of the variation is due to differences among groups. CF analysis by sexes indicates that both sexes disperse in this population. As a result, groups are composed of mature females and males that are not related by close kinship, revealing that these groups are constituted by immigrant individuals. These data are also supported by paternity analyses that confirmed three subadult individuals whose progenitors reside in a different group. Additionally, a case of extra-group paternity was found in the CF, which indicates that, either due to extra-group copulation or male turnover in the groups, there is gene flow among groups. Prior behavioral data on the same groups confirm a high degree of extra-group copulation during potentially fertile periods (Kowalewski 2007). These data would strongly suggest that extra-group fathering might occur. The results of the Fis index are close to 0 in all the groups, showing that neither heterozygosis deficiency nor excess was observed in any of the groups. Summarizing, these results suggest that considerable gene flow exists among groups in the CF population and that there is no indication of inbreeding.

Conversely, the FF population shows significant differences in Fst indicating that the genetic variability in the FF population is distributed among groups and consistent with a recent genetic differentiation among them. On average, only 86% (Fst=0.14) of the total population variability is shared by the groups, suggesting a reduction in the dispersal rate between groups residing in different fragments. Isozyme marker analysis in another howler species (*Alouatta seniculus*) also indicated an influence of habitat fragmentation on gene flow decrease (Pope 1990, 2000). Mature individual analyzed by sexes in the FF indicate dispersal biased towards males. Females that compose groups in the FF are highly related within groups and highly differentiated between groups. The relationship coefficient among them is 0.45, a value expected only among mothers and daughters or sisters. Accordingly, females in this habitat seem to be philopatric. Males showed a relationship coefficient of 0.28. Although this magnitude is significantly smaller

than that for females in this population, it is two fold higher than that found in the CF for both sexes. These results show that males constitute the dispersing sex in this habitat, but their dispersal into other groups occurs half the time less frequently than in the CF.

Paternity analysis in the FF showed that individuals of the same group fathered all studied individuals. Fis analyses of the FF groups mostly showed high negative values. These results usually indicate heterozygosity excess, and therefore absence of inbreeding. But in the FF, mature females are highly related within the groups and their gametes represent a differentiated portion of the gene pool. Gametes from any male coming from other groups might cause heterozigosity 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 in this situation. Another approach to estimate inbreeding is by kinship relationship coefficients. These values averaged 0.24, indicating that most individuals within groups would be closely related (value expected for uncles, cousins, grandsons, half siblings, etc). The increase in philopatric behaviors increases the chances of inbreeding. In FF one case of incest was found, where a female had a son whose father was another of her sons, which indicates consanguineous mating. Hence, inbreeding is occurring in this habitat.

Studies carried out on many taxa have indicated that inbreeding is expensive (Crnokrak and Roff 1999, Foerster et al.2003, Briskie et al. 2004), even to the point that the extinction risk rises significantly (Nieminen et al. 2001). Inbreeding may cause fitness reduction due to a series of genetic factors such as exposing deleterious recessive alleles because of homozygosis increase, heterozygosis decrease, and/or loss of isoenzyme variability (Cmokrak and Roff 1999). Although the inbreeding depression level varies depending on the species, local conditions and reproductive history, in all cases, the costs of inbreeding increase with the relationship coefficient among reproductive couples (Paul and Kuester 2004). The incest case in the FF is a clear example of inbreeding with very high relationship coefficients among progenitors. This fact, plus the high genetic differentiation among groups, and the permanence of juvenile and subadult in their natal groups suggest reduced gene flow in this habitat, revealing that increasing habitat fragmentation may severely limit the howler's ability to disperse.. Altogether, these results also suggest that habitat fragmentation modifies the dispersal patterns of black and gold howler monkeys.

Northeast Argentinean forests are suffering continuous human modifications that may isolate populations and limit dispersal opportunities. If fragmentation and clearing of forest fragments continue as a consequence of uncontrolled deforestation and landscape modification, there will be an isolation of different subsets of howler populations. The increase in isolation levels may be translated into loss of genetic diversity resulting in an inability to respond to selective pressures. Moreover, loss of genetic variability associated to a decrease in the availability of food and a higher risk of diseases could possible lead to local extinction. This study underscores the use of molecular genetic data as a tool of utmost importance for a better understanding of modifications of behavioral patterns produced by

habitat fragmentation, offering a new approach for optimizing wildlife conservation strategies.

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CFGroups	EM (11)	NF (9)	G (10)	LR (11)	MK (7)	VC	X (10)				
Individuals	AM*	AM*	X AM*	X AM*	AM*	AM*	X AM*				
	AM*	AM*	AM*	AM*	AM*	AM*	AM*				
	AM	AF*	AM*	AF*	AF*	AM	AM*				
	AF*	AF*	AF*	AF*	AF*	AF*	AF*				
	AF*	AF*	AF	AF*	SAF*	AF*	AF*				
	AF	SAF	AF	AF*	JF	AF	SAM				
	AF	JM*	AF	SAM*	IM	SAM	SAM*				
	SAM	IM	SAM*	SAF*		SAF*	SAF*				
	JM	IF	SAM*	JF*		JM	JF*				
	JF		SAM	JF*			IM				
	IM		JM*	JF*			IF				
				JM							
FF Groups	CC (7)	CV (6)	EV (9)	H (8)	L1 (5)	L2 (5)	ML (8)	PZ (7)	RS (9)	NN (5)	ZN (6)
Individuals	AM*	AM*	AM*	AM*	AM*	AM*	AM*	AM*	AM*	AM*	AM*
	AF*	AF*	AF*	AM*	AM*	AF*	AM*	AF*	AM*	AF*	AM*
	AF*	AF*	AF*	AF*	AF*	AF*	AF*	AF*	AF*	AF	AF*
	SAF	SAM	AF*	AF	AF*	SAM	AF	SAM*	AF*	JF	AF
	JM	JM	AF	SAM	SAM*	JM	AF	SAF	AF*	IM	SAM*
	JF	IF	SAF	JM*			SAF*	JM*	AF*		JF*
	IF		JF*	JM			JM	JF*	SAM*		
			JM*	IM			JM		SAF*		
			IM						JM*		

Table 1: Composition of 7 groups in the CF and of 11 groups in the FF. A: adults, SA: sub-adults, J: juveniles, I: infants; X: corpses (dead individuals), F: females, M: males, ( ): Number of individuals in the group (not considering dead individuals); *Sampled individuals.

CF Groups	r adult individuals (average)	r adult female dyads	r adult male dyads	FIS
EM	0.230	0.2	0.29	0.094
NF	0.102	0.0/0.0/0.35	0.29	-0.063
GR	0.048	-	0.0/0.01/0.05	-0.014
LR	0.117	0.0/0.0/0.1/ 0.2/0.38/0.64	0.0	0.015
MK	0.142	0.0	0.43	0.056
VC	0.027	0.0	0.0	-0.055
XZ	0.157	0.0	0.0/0.0/0.39	-0.057
Average	0,118	0,144	0,133	
FF Groups				
CC	0.227	0.58	-	-0.036
CV	0.2	0.46	-	-0.018
EV	0.19	0.24/0.35/0.5	-	-0.256
HT	0.49	-	0.72	-0.148
L1	0.12	0.53	0.0	-0.173
L2	0.32	0.66	-	-0.241
ML	0.35	-	0.05	-0.260
PZ	0.317	0.27	-	-0.272
RS	0.24	0.33/0.34/0.39/0.41/0.51/0.67	0.46	-0.176
NN	0.0	-	-	0.188
ZN	0.15	-	0.15	-0.136
Average	0.237	0.447	0.276	

Table 2: Kinship relations and FIS for groups in CF and FF. *r*: Kinship coefficient.

Population	Group	Age/ Sex	Individual	Mother	M Group	Father	F Group	LOD	PI
CF	*NF	JM	ME25	HE20	NF	M17	VN	6,12E+14	99,78
CF	G	JM	Huevo	Orejas	G	Marley	GR	5,60E+14	99,57
CF	LR	JF	Mireya	China	LR	TomX	LR	1,66E+14	99,75
CF	LR	JF	Lila	-	-	Mazzi	LR	5,34E+14	98,36
CF	LR	JF	Milica	China	LR	TomX	LR	3,40E+14	99,92
CF	X	JF	Gordita	Gorda	X	Gatti	XZ	1,89E+14	99,87
CF	G	SAM	Hermoso	-	-	MuertoM	GR	3,43E+14	98,65
CF	**LR	SAM	Julio	Orejas	G	Jose	GR	5,66E+14	99,98
CF	**X	SAM	Alf	Lola	G	Jose	GR	4,91E+14	99,81
CF	**G	SAM	Primo	-	-	M207	XZ	3,92E+14	97,92
CF	MK	SAF	HMuk3	-	-	208Muk	MK	2,03E+14	98,64
CF	VC	SAF	HVec18	HV7	VC	-	-	2,60E+14	98,93
CF	LR	SAF	Migui	Chile	LR	Mazzi	LR	4,79E+14	99,95
CF	X	SAF	Gorda	-	-	M207	XZ	2,09E+14	98,46
FF	EV	JF	H25	H12	EV	M3	EV	3,14E+14	98,10
FF	EV	JM	M21	H10	EV	M3	EV	9,18E+14	99,02
FF	H	JM	M1	-	-	M15	HT	3,40E+14	97,60
FF	PZ	JM	M36	H35	PZ	M32	PZ	3,42E+14	97,33
FF	***PZ	JF	H19	H35	PZ	M33	PZ	8,14E+14	98,67
FF	RS	JM	M24	H17	RS	M12	RS	6,22E+14	99,02
FF	ZN	JF	H1	H15	ZN	M16	ZN	6,63E+14	98,34
FF	PZ	SAM	M33	H35	PZ	M32	PZ	7,08E+14	98,79
FF	ML	SAF	H6	-	-	M6	ML	5,15E+14	98,59
FF	L1	SAM	M14	H21	L1	-	-	3,83E+14	98,22
FF	RS	SAF	H4	-	-	M12	RS	4,88E+14	97,03
FF	RS	SAM	M8	H17	RS	M12	RS	8,45E+14	99,00
FF	ZN	SAM	M7	H15	-	M16	-	3,62E+14	98,97

Table 3: Juveniles and subadults maternity and paternity. Includes: Group, Age, Sex, Mother's Group (M Group), Father's Group (F Group), LOD, and Paternity index (PI) values. *extragroup paternity, **dispersant subadults, ***incest case