Genetic Analysis Reveals Population Structuring and a Bottleneck in the Black-Faced Lion Tamarin (*Leontopithecus caissara*)

M.M. Martins\textsuperscript{a} A.T.A. Nascimento\textsuperscript{b} C. Nali\textsuperscript{b} G.O. Velastin\textsuperscript{b} P.B. Mangini\textsuperscript{b} C.B. Valladares-Padua\textsuperscript{b} P.M. Galetti, Jr.\textsuperscript{a}

\textsuperscript{a}Laboratório de Biodiversidade Molecular e Conservação, Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, e \textsuperscript{b}Instituto de Pesquisas Ecológicas, Nazaré Paulista, Brasil

**Key Words**
- Atlantic forest
- Conservation
- Critically endangered primate
- Black-faced lion tamarin
- *Leontopithecus caissara*
- Microsatellites

**Abstract**

The ability of a population to evolve in a changing environment may be compromised by human-imposed barriers to gene flow. We investigated the population structure and the possible occurrence of a genetic bottleneck in two isolated populations of the black-faced lion tamarin (*Leontopithecus caissara*), a species with very reduced numbers (less than 400) in a very restricted range in the Atlantic Forest of southeast Brazil. We determined the genotypes of 52 individuals across 9 microsatellite loci. We found genetic divergence between the populations, each exhibiting low genetic diversity. Analysis revealed broad- and fine-scale population structuring. Both populations have evidently experienced population reduction and a genetic bottleneck without presenting any apparent detrimental effect. Anyway, measures should be taken to effectively protect the forests where *L. caissara* occurs in order to allow its populations to increase and counteract the eventual effects of genetic impoverishment.

**Introduction**

Habitat destruction and hunting comprise the most serious and pervasive anthropogenic impacts on primate populations in South America [Chapman and Peres, 2001; Estrada, 2009]. Undesirable outcomes derived from human-imposed restric-
tions to populations inhabiting relatively intact forests have, however, been less addressed. One serious form of anthropogenic interference is that which limits or prevents dispersal, such as the construction of highways or reservoirs. Although the surrounding habitat fulfils the requirements of the species, such endeavours create barriers to gene flow, and the disconnected populations can become evolutionarily independent sets. This subdivision is serious for rare species, as post-isolation populations may be smaller than the threshold size for long-term viability.

Small population size may pose a substantial threat to survival due to genetic drawbacks. Small-sized populations may lose genetic diversity through genetic drift [Wright, 1931; Groombridge et al., 2000] and become inbred [Crow and Kimura, 1970] at a faster rate than larger counterparts. Low genetic diversity and inbreeding lead to an increased probability of extinction [Frankham, 1998; Crnokrak and Roff, 1999], as the ability of a population to evolve in a changing environment is reduced. Barriers to gene flow result in genetic structuring, as the divergence between populations tends to broaden out. Reduced populations that have undergone a genetic bottleneck (a loss of alleles) may be more vulnerable to extinction because, for example, if loci associated with disease resistance are harvested, the susceptibility to disease might increase. Bottlenecked populations of the New Zealand robin (*Petroica australis*), for example, have been shown to have an inadequate immune response to parasites [Hale and Briskie, 2007].

Such genetic problems have already been recorded for primates. Small captive and wild primate populations exhibit variable signs of inbreeding or inbreeding depression [Charpentier et al., 2007]. Habitat discontinuity plays an important role in the structuring of primate populations [Grativol et al., 2001; Eriksson et al., 2004; Hayashi and Kawamoto, 2006; Bergl and Vigilant, 2007; Jalil et al., 2008], and genetic bottlenecks, often associated with habitat fragmentation, have been detected for a number of species [Storz et al., 2002a; Goossens et al., 2006; Ruiz-Garcia et al., 2007; Bergl et al., 2008].

There are 4 species of lion tamarin (*Leontopithecus*, family Callitrichidae), all endemic to the Brazilian Atlantic Forest, occupied, colonized and destroyed since the early 17th century and now severely reduced and fragmented [Dean, 1995; Coimbra-Filho and Câmara, 1996; Tabarelli et al., 2005]. All 4 species are threatened and listed as ‘endangered’, and in the case of *Leontopithecus caissara* as ‘critically endangered’, on the IUCN Red List of Threatened Species [IUCN, 2010]. Their conservation has long been a concern [Coimbra-Filho, 1969, 1990; Kleiman and Rylands, 2002; Holst et al., 2006]. The main threat to 3 of the species, *L. rosalia*, *L. chrysopygus* and *L. chrysomelas*, is habitat loss; their forests have been drastically reduced and converted to isolated patches surrounded by agricultural land, cattle pasture and human settlements. The fourth species, the black-faced lion tamarin (*L. caissara*), on the other hand, occupies the last large continuous patch of Atlantic Forest in southeastern Brazil. This region comprises a lowland (<100 m above sea level) evergreen forest on swampy soils [Nascimento and Schmidlin, 2011] unsuitable for development, which has remained relatively undisturbed as a result. The range of *L. caissara*, however, is very small, restricted to a tiny area on the Brazilian coast of the northeast of the state of Paraná and the extreme southeast of the state of São Paulo (fig. 1). In the 1950s, about 40 years prior to its description [Lorini and Persson, 1990], a channel used by native fishermen was widened and extended. This resulted in the total separation of a peninsula occupied by the lion tamarins, creating the island of Su-
Gene flow between the mainland and this newly isolated island population evidently ceased over the last 12 *L. caissara* generations, taking into account the 5-year generation time estimated for lion tamarins [Holst et al., 2006]. The distribution of *L. caissara* today comprises Superagüi Island and two mainland regions: the Ariri and Patos River valleys [Lorini and Persson, 1994; Schmidlin, 2004] (fig. 1).

In the 1990s, the total population of the black-faced lion tamarin was estimated at 260 individuals [Lorini and Persson, 1994]. Line-transect surveys carried out in 2000–2002 indicated about 392 individuals [Nascimento et al., unpubl. data]. The island population and that of Ariri are in a complex of protected areas: Superagüi National Park (33,928 ha, decreed fortuitously in 1989), Lagamar State Park, Taquari Extractive Reserve and Tumba Island Extractive Reserve. Illegal selective logging still occurs in these areas despite their officially protected status. The NGO Insti-
tuto de Pesquisas Ecológicas has been working in the area since 1995, promoting conservation measures and sustainable practices among the local communities and business in order to end the illegal harvesting.

The use of hypervariable molecular markers, i.e. microsatellites, has a multitude of purposes in conservation [Selkoe and Toonen, 2006; Sarre and Georges, 2009]. Genetic analyses are important for the conservation of the black-faced lion tamarin because the major challenge is the management of small and isolated populations. Here, we investigate the genetic structure of 2 L. caissara populations. We also examine whether these 2 populations have experienced a recent genetic bottleneck. As dispersal is impossible between these small populations, we predict an overall loss of genetic diversity and some genetic divergence between them.

Methods

Study Area and Sampling
We sampled lion tamarins from 2 of the 3 existing populations (fig. 1): Ariri and Superagüi. We were unable to sample the population in the Patos River valleys because of its remoteness and the logistical difficulties involved in entering the area. It has still to be surveyed. The mainland sampled population is near the village of Ariri (79°41'32'' S, 72°10'69'' W) in the state of São Paulo. Superagüi Island (79°91'11'' S, 71°82'50'' W) is in the northeastern extreme of the state of Paraná. Both sites are in a tropical-to-subtropical transition, covered by evergreen submontane forest and coastal pioneer formations of the Atlantic Forest, including subxeromorphic or hygrophilic arboreal restinga (coastal forest on sandy soil). Annual rainfall is about 2,000 mm and is evenly distributed throughout the year.

Ecological monitoring of radio-collared black-faced lion tamarins has been carried out at Ariri since 2005 [Nascimento et al., 2011]. The same procedure was used in Superagüi in the 1990s [Valladares-Padua and Prado, 1996] and 2000–2002 [Schmidlin, 2004]. As the genetic sampling was not concurrent with the radio-tagged monitoring, only two groups at Ariri underwent behavioural and genetic sampling. Genetic sampling at Ariri was carried out in two periods (July to October 2003 and April 2008 to February 2009). Samples were taken from the Superagüi population from April to October 2004. The lion tamarins were trapped following the method described by Valladares-Padua [1987]. We searched for a group and followed it until it retired, usually at sunset, to a tree hole. Whenever conditions were favourable, all individuals were removed manually from the hole. A solution of 11 mg/kg of ketamine, 0.06 mg/kg of midazolam and 0.04 mg/kg of atropine sulphate was administered by intramuscular injection. Each animal received a microchip for identification. Sex and age class were recorded based on criteria predefined by one of us (P.B.M.). Blood samples were collected, diluted in 1:1 alcohol (95%) and stored at –20°C. With the exception of group S2, all had their trapping sites (tree holes) plotted with GPS coordinates obtained in situ. The sampling protocol was approved by the International Committee for the Conservation and Management for Lion Tamarins. The Brazilian Environmental Agency (IBAMA) provided the permit (process 11706-1) to trap animals and collect blood samples. Eighteen individuals from 4 groups and 34 individuals from 7 groups were sampled at Ariri and Superagüi Island, respectively (fig. 1). The reason for this imbalance was that the Ariri lion tamarins use tree holes for sleeping sites less frequently than those on Superagüi [Nascimento et al., 2011], limiting the opportunities for low-risk trapping.

Microsatellite Genotyping
Total genomic DNA was extracted from blood samples following a modified phenol-chloroform extraction method [Sambrook et al., 1999]. DNA quantification and degradation were then assessed by electrophoresis on a 1% agarose gel. The amplification of DNA fragments was carried out via polymerase chain reaction (PCR) in a thermal cycler. We tried to use all 19 microsatellites available for Leontopithecus species. From these 19 microsatellites, 17 (89.4 %) pro-
duced robust alleles, of which 9 (47.4%) were polymorphic and 8 (42.1%) monomorphic; the remaining 2 (10.5%) failed to amplify fragments under all tested conditions [Martins and Galetti Jr., 2011]. As such, the 9 polymorphic microsatellite markers used by us were those identified in *L. rosalia* [Grativol et al., 2001], *L. chrysopygus* [Perez-Sweeney et al., 2005] and *L. chrysomelas* [Galbusera and Gillemot, 2008]. One primer of each locus pair was constructed with a M13 tail on the 5’-end, and a fluorescently labelled M13 primer was used in a 3-primer PCR, following a pre-established protocol [Schuelke, 2000]. PCRs were carried out in a 10-μl reaction volume containing: 20 ng of template DNA, 1 μl of each primer, 0.2 mM of dNTP, 1.5 mM of MgCl2 and 1 unit of Taq polymerase (Fermentas). Amplifications were completed in either a Perkin Elmer 2400 thermal cycler or an Eppendorf Gradient Mastercycler, after optimization of published annealing temperatures and profiles. Reaction conditions were: an initial denaturation step of 5 min at 94°C, 30 cycles of 30 s denaturation at 94°C, annealing at 51–61°C for 45 s, extension step for 45 s at 72°C, and finally 10 cycles of 30 s denaturation at 94°C, annealing at 53°C for 45 s, extension for 45 s at 72°C, followed by an extension step of 10 min at 72°C. PCR products were analysed on a MegaBace automatic sequencer, and allele sizes were scored using the programme FRAGMENT PROFILER (version 1.2, Applied Biosystem®). To limit genotyping errors in our data analysis, we re-amplified samples that yielded ambiguous allele peaks, entered genotypes manually onto a Microsoft Excel spreadsheet and checked them all systematically with reference to the original electropherograms.

**Data Analysis**

We used the GENEPOP programme version 3.4 [Raymond and Rousset, 1995] to test for departures from the Hardy-Weinberg equilibrium by an exact test based on the Markov chain method. The same programme was used to investigate linkage disequilibrium (LD) between each pair of loci and measure diversity through expected heterozygosity (Hₑ) and allelic richness. The inbreeding coefficient, as defined by Weir and Cockerham [1984], and average population differentiation (Fₛ) were calculated using the FSTAT programme version 2.9.3 [Goudet, 1995]. Critical values for significance (p = 0.05) were adjusted for simultaneous comparisons with the Bonferroni correction [Rice, 1989] or with the less conservative correction proposed by Benjamini and Yekutieli [2001].

We assessed population structure by using two analytical approaches: (1) classical F statistic analysis of molecular variance (AMOVA) to compare genetic diversity on the following hierarchical levels: between populations, among groups within a population and among individuals within a group; (2) Bayesian clustering methods. We used ARLEQUIN programme version 3.11 [Excoffier and Schneider, 2005] with 1,500 permutations for approach 1 and STRUCTURE programme version 2.2 [Pritchard et al., 2000] plus BAPS programme version 5.2 [Corander and Marttinen, 2006] for approach 2. Unlike F statistic methods, clustering methods do not require previously defined populations. The aim, therefore, is to determine K (the number of clusters of genetically similar individuals) without prior knowledge of the genetic relationships between sampled individuals. We carried out tests in STRUCTURE using 200,000 replicates of a Monte Carlo Markov chain following a 50,000-replicate burn-in. Also, we used ΔK to identify the most likely K, according to the method of Evanno et al. [2005]. Two independent runs were carried out in BAPS: (1) non-spatial clustering (no prior information provided); (2) spatial clustering (coordinates of the trapping sites provided).

The association of Fₘₛ with geographic distance between groups was assessed by the Mantel test using GENALEX programme version 6 [Peakall and Smouse, 2006]. It would be more appropriate to carry out this test using the distance between the centre of the home ranges. However, as the ranges and ranging behaviour were not known for all of the groups, we had no alternative but to use the coordinates of the trapping sites to estimate the distance between groups; a measure which is not ideal, but was the only one we could use. The groups were trapped far from each other. Although unusual for callitrichids, the home ranges of adjacent *L. caissara* groups overlapped very considerably [Nascimento et al., 2011], increasing the chance of gene flow through natal dispersal or extragroup copulations. Distances between tree holes do provide, however, at least a general picture of the spatial distribution of the groups. The S2 group was excluded from the data file on the Mantel test because its coordinates were lost.
To test whether the populations of Ariri and Superagüi had undergone a recent genetic bottleneck, we used the coalescence-based simulation approach described by Cornuet and Luikart [1996] to calculate the $H_E$ under mutation drift equilibrium ($H_{eq}$). The occurrence of a bottleneck in the relatively recent past is expected to produce deviations from this equilibrium, i.e., a heterozygosity excess. Thus, in a bottlenecked population, we should expect $H_E > H_{eq}$. The two-phased mutation model described by Di Rienzo et al. [1994] was used because the mutational dynamics of microsatellites have been best characterized under this modified form of stepwise mutation model [Di Rienzo et al., 1994; Ellegren, 2000]. We used the Wilcoxon signed-rank test implemented in the programme BOTTLENECK version 1.2.02 [Piry et al., 1999], with 0.05 and 0.10 as the proportions of the multistep mutations for each trial. The size distribution of the multistep mutations was characterized by a variance of 12. Because genetic diversity may be lost even in the absence of a demographic bottleneck [Luikart et al., 1998], we tested whether $L. caissara$ populations had experienced a recent population reduction. We calculated the mean of the ratio: number of alleles/range of allele sizes ($M$), as proposed by Garza and Williamson [2001]. According to these authors, bottlenecks are supposed to reduce the number but not necessarily the size range of alleles. Thus, values of $M$ below the critical value 0.68 should be indicative of historical reductions in population size. We used the programme STATISTICA version 5 to generate confidence intervals for $M$ and to test the 9 components of the mean for normality (Kolmogorov-Smirnov test statistics).

Results

Genetic Diversity

The mean $H_E$ across 9 microsatellite loci was 0.42 for the Ariri population and 0.48 for Superagüi (table 1). Allelic richness was low, reaching up to 3.0 in both populations. Genotyping was inconclusive for only 3 individuals (1 locus each) of the 52 sampled lion tamarins, and only the locus Leon15c85 in the Superagüi population exhibited a moderate but significant ($p = 0.015$) deviation from Hardy-Weinberg

Table 1. Expected heterozygosity ($H_E$), observed heterozygosity ($H_O$), allelic richness ($A_R$) and inbreeding coefficient ($F_{IS}$) estimated for microsatellite loci in two populations of $L. caissara$

<table>
<thead>
<tr>
<th>Locus</th>
<th>Ariri</th>
<th>Superagüi Island</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>$H_E$</td>
<td>$H_O$</td>
<td>$A_R$</td>
<td>$F_{IS}$</td>
</tr>
<tr>
<td>Leon2</td>
<td>0.68</td>
<td>0.76</td>
<td>3.00</td>
<td>−0.130</td>
</tr>
<tr>
<td>Leon3c20</td>
<td>0.49</td>
<td>0.67</td>
<td>2.00</td>
<td>−0.378</td>
</tr>
<tr>
<td>Leon15c85</td>
<td>0.29</td>
<td>0.33</td>
<td>2.00</td>
<td>−0.172</td>
</tr>
<tr>
<td>Leon21c75</td>
<td>0.11</td>
<td>0.11</td>
<td>1.99</td>
<td>−0.030</td>
</tr>
<tr>
<td>Leon30c73</td>
<td>0.48</td>
<td>0.65</td>
<td>3.00</td>
<td>−0.354</td>
</tr>
<tr>
<td>Leon31c97</td>
<td>0.43</td>
<td>0.59</td>
<td>2.00</td>
<td>−0.391</td>
</tr>
<tr>
<td>LrP2BH6</td>
<td>0.52</td>
<td>0.72</td>
<td>2.94</td>
<td>−0.399</td>
</tr>
<tr>
<td>Lchµ04</td>
<td>0.36</td>
<td>0.44</td>
<td>2.00</td>
<td>−0.259</td>
</tr>
<tr>
<td>Lchµ07</td>
<td>0.46</td>
<td>0.67</td>
<td>2.00</td>
<td>−0.478</td>
</tr>
<tr>
<td>Mean</td>
<td>0.42</td>
<td>0.55</td>
<td>−0.310</td>
<td></td>
</tr>
</tbody>
</table>

* $p < 0.017$.
equilibrium. Analysis using MICRO-CHECKER version 2.2.3 [Van Oosterhout et al., 2004] suggested the absence of null alleles (p ≤ 0.05) at this locus. In the pooled analysis, departures from Hardy-Weinberg equilibrium were non-significant for both Ariri (p = 0.139) and Superagüi (p = 0.561). Using a Benjamini-Yekutieli-corrected critical value for significance of 0.017, a statistically significant degree of 2-locus LD was indicated for the following pairs: Leon3c20 and Lchμ04 (p = 0.016) and Leon3c20 and Lchμ07 (p = 0.0009) of the Ariri population. LD was also recorded for Leon21c75 and LrP2BH6 (p = 0.006), Leon21c75 and Lchμ04 (p = 0.007), Leon30c73 and Lchμ04 (p = 0.005), and LrP2BH6 and Lchμ04 (p = 0.012) in the Superagüi population. We found no evidence of inbreeding for Ariri or Superagüi (table 1).

Population Structure

The Ariri and Superagüi populations differed significantly (FST = 0.147; p < 0.05) in terms of allelic frequencies. After removing the locus Leon15c85 with deviation from Hardy-Weinberg expectations from the data file, the difference persisted (FST = 0.143; p < 0.05). AMOVA revealed a significant genetic structuring on all three hierarchical levels. The largest percentage of genetic variance (81%) was partitioned among individuals within a group, whereas 13% was partitioned between Ariri and Superagüi, and around 6% accounted for the variance among groups of a population (table 2). The number of population clusters indicated by STRUCTURE and BAPS was 8 and 4, respectively. The non-spatial and spatial analysis using BAPS indicated that the probability of the occurrence of 4 clusters (all Ariri groups, group S1, group S2 and groups S3–S7) was as high as 0.996.

The pairwise linear distance between the 10 trapping sites ranged from 1.155 km (A3 and A4) to 35.398 km (A4 and S3). We found evidence of significant isolation by distance between groups (p = 0.024, R2 = 0.160; fig. 2).

Recent Bottleneck

The Wilcoxon signed-rank test revealed that the proportions of loci with heterozygote excess and deficit were significantly different in the Ariri population (p = 0.024 and 0.013 for the proportions 0.05 and 0.10, respectively) and that of Superagüi (p = 0.006 and 0.004 for the proportions 0.05 and 0.10, respectively), when all 9 loci were considered. As the occurrence of loci in LD may lead to false evidence of a genetic bottleneck [Piry et al., 1999], 1 locus of each pair was removed from the analysis in separate runs. Bottlenecks were recorded, however, at both sites after the removal of each locus (table 3) as well as after the simultaneous removal of 2 loci, each

<table>
<thead>
<tr>
<th>Table 2. AMOVA design and results (average over 9 loci)</th>
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<tbody>
<tr>
<td>Source of variation</td>
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<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Between populations</td>
</tr>
<tr>
<td>Among groups within a population</td>
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<tr>
<td>Among individuals within a group</td>
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</table>
involved in a different LD pair in the Superagüi population (p = 0.019 and 0.011 for the proportions 0.05 and 0.10, respectively).

Mean (±1 SD) estimates of $M$ and 95% confidence intervals recorded for Ariri (0.34 ± 0.17; 0.206–0.466) and Superagüi (0.38 ± 0.18; 0.240–0.523) fell below the threshold value ($M = 0.68$). These results indicate a recent historical reduction in population size on both sides of the channel.

**Table 3.** p values for a genetic bottleneck test at proportions of 0.05 and 0.10 in a multistep mutation model after removal of loci involved in LD of the populations at Ariri and on Superagüi Island

<table>
<thead>
<tr>
<th>Population/removed locus</th>
<th>p for the proportion of</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>Ariri</td>
<td></td>
</tr>
<tr>
<td>Leon3c20</td>
<td>0.037*</td>
</tr>
<tr>
<td>Lchμ04</td>
<td>0.037*</td>
</tr>
<tr>
<td>Lchμ07</td>
<td>0.037*</td>
</tr>
<tr>
<td>Superagüi Island</td>
<td></td>
</tr>
<tr>
<td>Leon21c75</td>
<td>0.009*</td>
</tr>
<tr>
<td>Leon30c73</td>
<td>0.002*</td>
</tr>
<tr>
<td>LrP2BH6</td>
<td>0.013*</td>
</tr>
<tr>
<td>Lchμ04</td>
<td>0.013*</td>
</tr>
</tbody>
</table>

* p ≤ 0.05.
Discussion

Low Genetic Diversity

Assessing lion tamarin DNA from tissues such as hair follicles or buccal cells does not raise the suspicion of genotyping errors. However, blood that derives from precursor haematopoietic cells might raise it, because callitrichids may present blood chimerism [Signer et al., 2000], which potentially makes some homozygous genotypes appear to have heterozygous genotypes. In our study, though, no screened locus exhibited 3 or more alleles for a single heterozygote individual. Had a chimeric pool of cells been included in the analysis, we would expect an excess of heterozygotes relative to the expected heterozygotes, but that was not observed for 8 of the 9 loci. We therefore conclude that chimerism did not affect our assessment.

We found low genetic diversity for the Ariri and Superagüi populations of *L. caissara*. Low genetic diversity has previously been evidenced by investigations on *Leontopithecus* allozymes [Forman et al., 1986] and mitochondrial DNA [Seuánez et al., 2002]. It would seem that low genetic diversity is a trait shared by the entire Callitrichidae family. For microsatellite data, comparisons between populations should ideally be performed with allelic richness, as $H_E$ is sensitive to the sample size. However, allelic richness is rarely reported in genetic studies. Thus, comparing the more often reported mean number of alleles per locus, we have 2.0–3.8 in 4 populations of *L. rosalia* [Grativol et al., 2001], 2.56–2.67 in 2 populations of *L. caissara* (this study) and 3.09 for *Callithrix jacchus* [Nievergelt et al., 2000]. In contrast, the mean number of alleles per locus at 2 sites each for the woolly monkey (*Lagothrix poeppigii*) and the spider monkey (*Ateles belzebuth*), both of the family Atelidae, is 9.4–10.1 and 6.5–6.9, respectively [Di Fiore et al., 2009]. Therefore, the pre-existing amount of genetic diversity in black-faced lion tamarins was probably low and should not be viewed as a consequence of anthropogenic interference to the habitat.

Low genetic diversity in callitrichids has caught the attention of researchers. Pope [1996] suggested that it has its roots in the social system, which includes reproductive suppression by a dominant female. Reproduction is normally restricted to 1 and occasionally 2 lion tamarin females per group [Goldizen, 1990; Dietz et al., 1996]. Furthermore, dominant callitrichid males sire the majority of the offspring born in their group [Nievergelt et al., 2000; Faulkes et al., 2003; Huck et al., 2005]. Field data provide evidence that aggressive behaviour on the part of the dominant individual limits the access of same-sex subordinates to the opposite sex for mating [Abbott et al., 1993; Baker et al., 1993]. Contrary to the prediction, the mean number of alleles per locus in one population of the moustached tamarin (*Saguinus mystax*) was found to be 7.0 [Huck et al., 2005]. Despite the suggestion that a small effective population size induced by variance in reproductive success likely limits an increase in the genetic diversity of callitrichids, the slightly larger measure found for *S. mystax* indicates that social system alone may not be the determinant of genetic diversity.

Despite the expectation of increased inbreeding in small populations, neither population of black-faced lion tamarins was inbred. The absence of inbreeding means a smaller chance of decreasing the population fitness and growth rate, thereby avoiding the negative effect of population size, and attenuating the risk of extinction. However, current limitations regarding the interpretation of the complex association between demographic bottlenecks and consequent genetic outcomes, along with restricted knowledge on how molecular genetic diversity translates into adap-
tive genetic diversity [Bouzat, 2010] prevents us from attaching particular importance to the lack of inbreeding as favouring the retention of genetic diversity in black-faced lion tamarin populations. *L. caissara* appears to challenge the traditional view about the negative impacts of genetic depletion. The mechanisms underlying such an unexpected response in this species should be a matter of further investigation.

**Broad- and Fine-Scale Population Structure**

There is 13% of molecular variance between the two populations sampled, indicating that they are not widely divergent, despite the presence of a barrier to gene flow between them over a dozen generations. The positive significant correlation between genetic and geographical distance strongly suggests an already existing pre-channel difference between the Ariri and Superagüi populations in terms of allelic frequency due to limited long-distance dispersal. Dispersers tend to be more successful when they are familiar with the spatial and temporal distribution of resources in areas where they find themselves. Four of 7 radio-collared male and female *Aotus azarai*, for example, successfully entered groups that shared a common border with their previous territories [Fernandez-Duque, 2009]. Relatedness between *S. mystax* males dropped significantly in territories 590 m or more apart from each other [Huck et al., 2007]. The as much as 96 and 98% overlap in the home range of neighbouring *L. caissara* groups at Superagüi and Ariri, respectively [Nascimento et al., 2011], indicates that individuals have opportunities to assess adjacent territories in terms of the quantity and quality of available resources. Therefore, the groups from the north of Superagüi and the south of Ariri, which are not very distant, could have exchanged members before the enlargement of the channel, provided the forest offered arboreal routes for movement. The already existing distance-mediated genetic difference has likely been enhanced since the widened channel prevented gene flow between the mainland and the island.

There is also a small significant difference (6% of total variance) between groups within a population. This fine-scale structuring is likely created by the mating system. The extended-family group structure of callitrichids [Ferrari and Digby, 1996] and the behavioural opposition of male and female breeder to the mating attempts of subordinates [Abbott et al., 1993; Baker et al., 1993] result in high proportions of full- or half-sibling offspring that probably give rise to the genetic divergence among groups. It would be of great interest to have the results from clustering methods enhancing and unfolding the fine-scale structuring revealed by AMOVA. However, we have reasons to believe that they are unreliable. STRUCTURE indicated a most likely inflated K of 8, which can only be explained by the presence of multiple efficient physical barriers to gene flow among the clusters. However, to our knowledge there is no barrier in the study area other than the channel. Although the results from BAPS (1 cluster at Ariri and 3 on Superagüi) seem more biologically sensible than those from STRUCTURE, the grouping patterns do not. Spatial and non-spatial analyses produced identical outputs: groups more than 30 km apart from each other were joined, while groups separated by less than 2 km were in different clusters. The spatial analysis disregards the effect of geographical distance on genetic divergence in its limitation on the dispersal distance. Similarity between non-spatial and spatial outputs was not expected when considering our significant isolation-by-distance result and what is known of the behaviour of Neotropical primates when dispersing. Meanwhile, by the finding of fine structuring on Superagüi (3 clusters) but not at
Population Structure of Lion Tamarins

Ariri (1 cluster), where average pairwise distance between sampling sites is 11.66 and 2.44 km, respectively, the BAPS results suggest that geographical distance does in fact play a role in population structure.

The fact that clustering methods have been widely used as a complement to the classical Wright’s approach should not be interpreted as a guarantee of accuracy. Despite using two clustering methods, we were unable to determine which lion tamarin groups were more genetically similar. The extremely low polymorphism recorded for microsatellite loci in *L. caissara* may have posed limits to accurate Bayesian assessments. Also, an important reason for the unsuccessful performance of the clustering methods might have been the presence of full siblings. The inclusion of full siblings leads clustering methods to overestimate the number of populations [Anderson and Dunham, 2008]. Because the birth of twins and mate-guarding by the dominant male are pervasive among callitrichids, the probability that most offspring in a group are full siblings is high.

The highest significant difference dictated by AMOVA (81% of total variance) was recorded among group mates. Compelling evidence for a difference at this hierarchical level is difficult to obtain. Maybe dispersers coming from different groups cofounding a new group or extragroup copulations during group encounters are the cause of such a fine structure within groups, despite the opposite homogenizing effect of the reproductive hegemony of a few breeders. We recorded one case of group formation that could possibly contribute to genetic divergence. A young male left his natal group A4, joined an adult female of unknown origin, and together they formed the group A3 just prior to sampling. The couple has bred successfully 3 times since then.

**Recent Bottleneck in Both Populations**

We recorded a genetic bottleneck for both the Ariri and Superagüi populations. The fact that some researchers have failed to find evidence of genetic bottlenecks through shifts in the mutation drift equilibrium despite evidence of population declines in primate populations [Storz et al., 2002b; Milton et al., 2009] could raise the suspicion that our results are an artefact of data analysis. However, besides addressing LD-involved loci pairs, we selected the Wilcoxon signed-rank test, which is regarded as the most robust of all tests implemented in the BOTTLENECK programme [Piry et al., 1999].

Both *L. caissara* populations experienced reduction in size. As stated before, to date there have been only two population estimates, the first in 1994 and the second in 2000–2002. Although the lack of pre-isolation data prevents us from concluding that the enlargement of the channel was the determining factor driving population decline, the moderate genetic divergence between the Ariri and Superagüi populations indicates substantial pre-channel gene flow that characterizes them as a single population. Anyway, the split of this population led to both being smaller than the original, which may have potentially broadened the effect of genetic drift. Whether anthropogenic endeavours other than the isolation of Superagüi contributed to population decline and/or loss of genetic diversity of *L. caissara* is unclear.

Black-faced lion tamarins differ from their congeners regarding habitat disturbance. For example, unlike the black-faced lion tamarins, golden lion tamarins (*L. rosalia*) are suffering multiple environmental threats including habitat loss and animal trafficking, as well as competition with released non-native species. However, *L.*
rosalia have also experienced loss of genetic diversity. Only 6 out of 18 haplotypes have persisted in the remnant wild and captive populations when compared to historical counterparts [Grativol et al., 2008]. This convergent response suggests that molecular markers developed for Leontopithecus, despite exhibiting low diversity, are an effective tool for recording genetic problems. Their suitability paves the way for acting in anticipation of the phenotypic expressions of the genetic drawbacks.

The Black-Faced Lion Tamarin and the Future

We genotyped 52 individuals, which is a highly representative sample size (13.3% of the total population). Thus, this is certainly a comprehensive microsatellite-based population genetic study. Our results revealed that despite their small size and isolated condition, both L. caissara populations have been maintaining not only low genetic diversity without any apparent detrimental effect, but also the capacity to avoid inbreeding. The splitting of a once continuous forest led to the interruption of gene flow. Although moderate divergence between the populations of Ariri and Superagüí has been generated and maintained by limitations in the dispersal distance, it was likely enhanced over the last 12 lion tamarin generations.

Both black-faced lion tamarin populations have experienced reduction in size and have lost genetic diversity. Because population growth contributes to the recovery from bottleneck events, it is of vital importance to provide effective protection to suitable areas within the species’ distribution, since the conservation of the habitats will allow these populations to expand.

We recorded fine-scale structuring in L. caissara populations, but constraints in our microsatellite-based data set prevent us from characterizing it better. We hope that the use of other molecular markers in the future will shed further light on the population structure of L. caissara and, as such, improve our ability to define the appropriate site-specific management actions for this critically endangered species.

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