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Population Genetic Structure of an Endangered Endemic Primate (*Leontopithecus chrysomelas*) in a Highly Fragmented Atlantic Coastal Rain Forest

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Keywords
Agroforest · Barriers to gene flow · Conservation · Endangered primate · Genetic differentiation · Lion tamarins

Abstract

This study evaluated the genetic structure of wild populations of the endangered primate, *Leontopithecus chrysomelas*. We tested the assumption that populations of *L. chrysomelas*, given their larger population size and a higher degree of habitat continuity, would have higher genetic diversity and less genetic structuring than other lion tamarins. We used 11 microsatellites and 122 hair samples from different locations to assess their genetic diversity and genetic structure, and to make inferences about the isolation by distance. The overall expected heterozygosity (0.51 ± 0.03) and the average number of alleles (3.6 ± 0.2) were relatively low, as is the case in other endangered lion tamarins. Genetic clustering analyses indicated two main clusters, whereas the statistical analyses based on genotype similarities and F_{st} suggested further substructure. A Mantel test showed that only 34% of this genetic differentiation was explained by the linear dis-

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tance. In addition to linear distance, structural differences in the landscape, physical barriers and behavioural factors may be causing significant genetic structuring. Overall, this study suggests that these populations have a relatively low genetic diversity and a relatively high population genetic structure, putting in question whether the presence of agroforest systems (known locally as *cabruca*) is enough to fully re-establish functional landscape connectivity.
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Introduction

Deforestation often causes primate populations to become smaller and isolated [Ferreira da Silva and Bruford, 2017]. The degree of habitat disturbance and the properties of the landscape influence primate dispersal and gene flow, consequently affecting genetic diversity and population density, and ultimately extinction probabilities [Arroyo-Rodríguez and Mandujano, 2009]. Lion tamarins, *Leontopithecus* spp., are an example of primates that rarely travel on the ground, especially in open areas [Coimbra-Filho and Mittermeier, 1973]. Limited connectivity of forest habitat in a landscape comprising forest and non-forest can hence constrain lion tamarin dispersal [Raboy et al., 2010]. Given the high degree of fragmentation in the Atlantic Coastal Rain Forest [Ribeiro et al., 2009], we expect populations of this endangered species to have limited gene flow and thus a relatively high degree of genetic structure and low retention of within-population genetic diversity over time [Moraes et al., 2017].

Of the 4 species of lion tamarins, the golden-headed lion tamarin (GHLT), *Leontopithecus chrysomelas* (Kuhl 1820), has the highest population estimates – 6,000–15,000 individuals [Pinto and Rylands, 1997] – and largest current geographic distribution; however, its remaining forest habitat is extremely fragmented [Coimbra-Filho and Mittermeier, 1973]. The degree of habitat fragmentation is not uniformly distributed across its range. The eastern portion of the GHLT distribution consists of relatively continuous forest in various stages of degradation, connected by shade-cocoa agroforest referred to as *cabruca*, in which some of the forest canopy is retained [Pinto and Rylands, 1997; Raboy et al., 2010]. This area includes Una Biological Reserve (REBIO), situated within the largest forest patch in the GHLT distribution and considered to be the only patch adequate to hold a viable population with zero probability of extinction and retention of 98% of the genetic diversity over 100 years [Holst et al., 2006; Zeigler et al., 2010]. *Cabruca* is the dominant forest type in this region and is thought to play an important role in GHLT conservation because it functions as a viable habitat for survival and reproduction, and presumably permits dispersal and gene flow [Raboy et al., 2004; Oliveira et al., 2011]. By contrast, the western portion of the species' geographic distribution comprises semi-deciduous forest and is more highly fragmented than the evergreen forest in the eastern region. The non-forest matrix in this region is largely dominated by cattle pasture [Pinto and Rylands, 1997; Guidorizzi, 2008; Raboy et al., 2010].

The objectives of this study were to assess the genetic diversity of wild GHLTs in different habitats present within its distribution in order to calculate the degree of population genetic structure and to use these results to infer the level of gene flow in relation to geographic distance. For this, we used microsatellite markers that were simple, short and neutral repetitive sequences of genomic DNA with a high rate of

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mutation and polymorphism and therefore suitable for studies focusing on recent population genetic structure [Goldstein and Schlotterer, 1999]. We expected that, given their larger population size and higher degree of habitat continuity, GHLT populations, particularly those in the eastern region, would manifest higher genetic diversity and less population genetic structuring than other species of lion tamarins, whose populations are significantly smaller (less than 3,000 individuals) [Holst et al., 2006]. We also examined the assumption used in conservation planning for GHLTs that the presence of *cabruca* in the eastern region results in relatively continuous suitable habitat and that, as a result, the predominant factor influencing population genetic similarity will be the distance between populations.

Materials and Methods

Study Area and Sample Collection

We collected samples between 2003 and 2009 from GHLTs in Una Biological Reserve (referred to as REBIO), and in privately owned lands in the eastern and western regions of the species, geographic distribution in southern Bahia, Brazil (Fig. 1). The 18,715-ha federal Biological Reserve [ICMBio, 2015] consists of relatively pristine forests on the eastern side (known locally as Maruim) and degraded forests on the western side (known locally as Piedade) [Holst et al., 2006]. The privately owned lands in the eastern portion of the species distribution included: (1) *cabruca* in the Ilhéus municipality (sampled at 3 different farms), and a mosaic of forest types in both (2) Ararauna (Una municipality) and (3) Teimoso (Jussari municipality) farms.

The structure of *cabruca* is similar to that of mature forests, but with a lower density of trees in the overstory, and an understory replaced by cocoa trees. Forest mosaics are characterized by a combination of 2 or 3 types of vegetation: *cabruca*, mature forest, and secondary regrowth [Raboy et al., 2004; Oliveira et al., 2011]. In the western portion of the species' distribution, all samples were collected from GHLTs in a 450-ha privately owned farm called Barro Branco, situated in a forest fragment that has been isolated for nearly 50 years. This fragment was composed of semi-deciduous secondary forest in various stages of regeneration surrounded by cattle pasture and held an estimated 32 GHLTs. In the western portion of the species' distribution the forest remnants, such as Barro Branco, are smaller than those found in the east and have suffered intensely from edge effects caused by selective logging and cattle grazing [Guidorizzi, 2008].

Hair samples of individually marked GHLT individuals were pulled directly from 122 animals distributed in 5 geographic groups: REBIO (81), Ilhéus (18), Ararauna (9), and Teimoso (7) in the eastern portion, and Barro Branco (7) in the western portion of the species distribution. These groups were captured at 6-month intervals as part of the ongoing ecological studies to take biometric measures, monitor group compositions and change the radio-collars used to track and monitor the groups [Raboy and Dietz, 2004; Oliveira et al., 2011; Catenacci et al., 2016]. Focal groups were prebaited in high-use areas for approximately 1 week and captured using Tomahawk live traps. Subsequently, they were brought to an onsite laboratory for processing and released the next day early in the morning at the site of capture. Capture protocols, detailed in Dietz et al. [1996] and Catenacci et al. [2016], were approved by the Brazilian authorities (IBAMA, ICMBio, SISBIO). Within REBIO, we collected samples from social groups in two separate parts of the reserve, 9 from Maruim and 6 from Piedade. We did not observe lion tamarins dispersing between the two portions of the Reserve, which were separated by approximately 13 km. We sampled 3 social groups in Ilhéus: Almada and Bonfim groups, which were neighbouring groups with an estimated 80% territory overlap, and Santa Rita which was located 3 km away from the other 2 groups [Oliveira et al., 2011]. We sampled 2 social groups in Barro Branco, and 1 group each in Ararauna and Teimoso. All hair samples were stored in paper bags and preserved in boxes containing silica at the DNA Collection of Wild Fauna and Flora certified as a bona fide depository by the Genetic Heritage Management Council (Conselho de Gestão do Patrimônio Genético) of the Brazilian Ministry of the Environment.

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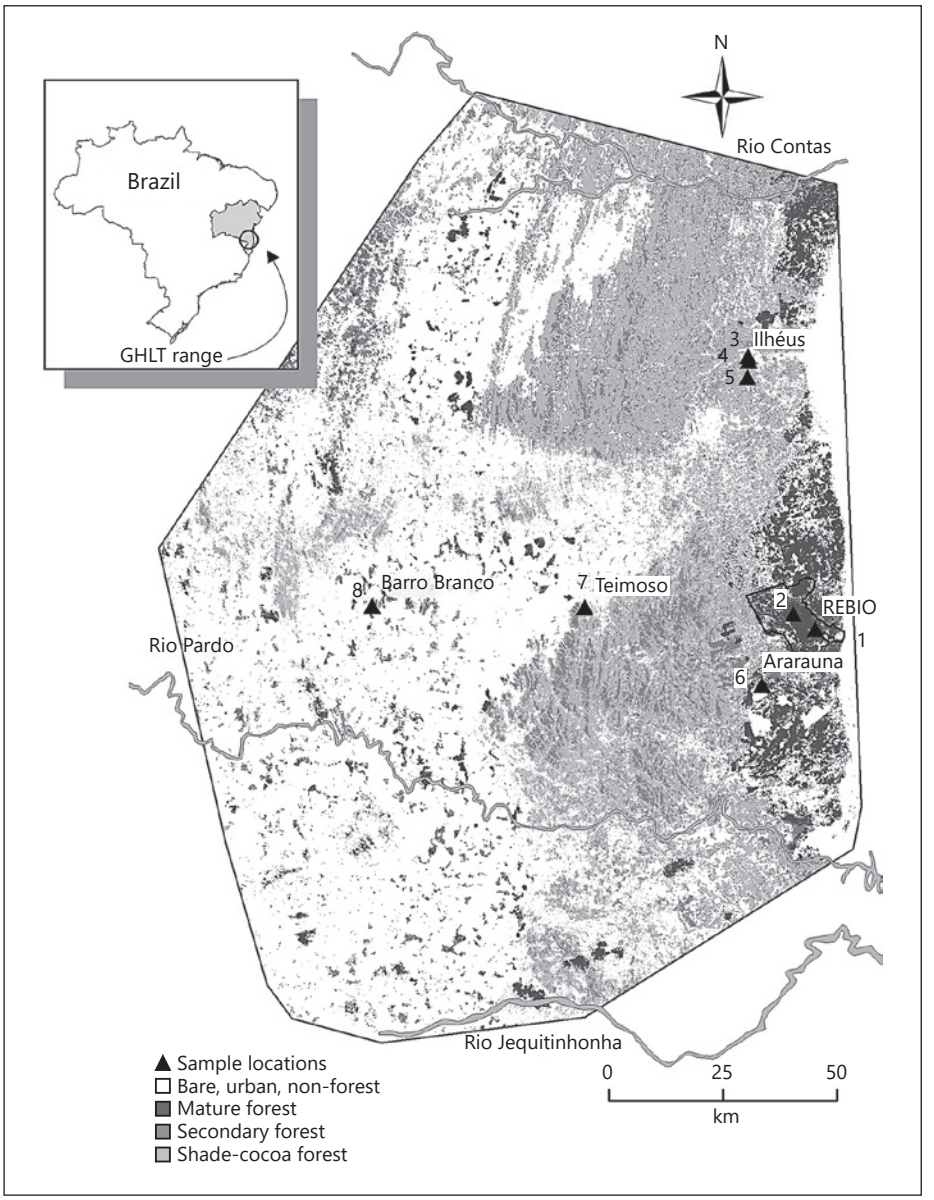

Fig. 1. Geographic distribution of *L. chrysomelas* in Bahia State (Brazil) and sampling locations (triangles): 1 and 2 = Maruim and Piedade, both in Una Biological Reserve (REBIO; black polygon) (15°11'54" S, 39°03′35" W); 3, 4 and 5 = Bonfim, Almada and Santa Rita farms (14°40'01" S, 39°11'44" W; 14°39'28" S, 39°11'49" W; 14°41'56" S, 39°11'50" W), all three in Ilhéus municipality; 6 = Arariauna (15°18'29" S, 39°10'07" W); 7 = Teimoso farm (15°09'16" S, 39°31'47" W); 8 = Barro Branco (15°08'25" S, 39°57'21" W). Land cover categories were reclassified from Landau et al. [2003].

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Table 1. PCR cycle conditions, based on Perez-Sweeney et al. [2005] and Galbusera and Gillemot [2008]

Primer	Genbank	Repeat	Repeat motif	Size, bp	Initial temp.	Cycles	Temperature			Final temp.
							denaturation	*annealing*	*extension*	
Leon2	AY706915	DI	$(CA)_{18}(CG)(CA)_3$	207	95°C 15 min	35×	94°C 1 min	55°C 1 min	72°C 1 min	72°C 5 min
Leon21	AY706922	DI	$(GT)_{19}(NA)_1(GT)_5$	285	95°C 15 min	35×	94°C 1 min	61.8°C 1 min	72°C 1 min	72°C 5 min
Leon27	AY706925	DI	$(CA)_{11}$	195	95°C 15 min	35×	94°C 1 min	58°C 1 min	72°C 1 min	72°C 5 min
Leon30	AY706927	DI	$(TC)_{25}(AA)(TC)(TG)_{16}$	256	95°C 15 min	35×	94°C 1 min	58°C 1 min	72°C 1 min	72°C 5 min
Lchμ1[a]	DQ979343	TETRA	$(TTTA)_8(TTT)(ATTT)(TT)$	214	94°C 5 min	30×	94°C 30 s	56°C 45 s	72°C 45 s	72°C 10 min
Lchμ3[a]	DQ979345	TETRA	$(GATA)_{16}$	339	94°C 5 min	30×	94°C 30 s	60°C 45 s	72°C 45 s	72°C 10 min
Lchμ4[a]	DQ979346	TETRA	$(GATA)_{14}$	409	94°C 5 min	30×	94°C 30 s	56°C 45 s	72°C 45 s	72°C 10 min
Lchμ5[a]	DQ979348	TETRA	$(GAAG)_{21}$	298	94°C 5 min	30×	94°C 30 s	58°C 45 s	72°C 45 s	72°C 10 min
Lchμ6[a]	DQ979349	DI	$(AC)_3(CA)_3(CC)(AC)_8$	187	94°C 5 min	30×	94°C 30 s	56°C 45 s	72°C 45 s	72°C 10 min
Lchμ8[a]	EF583690	DI	$(TG)_{23}$	238	94°C 5 min	30×	94°C 30 s	58°C 45 s	72°C 45 s	72°C 10 min
Lchμ9[a]	EF583691	DI	$(CA)_{19}$	429	94°C 5 min	30×	94°C 30 s	58°C 45 s	72°C 45 s	72°C 10 min

[a] Primer with M13 based on Schuelke's [2000] protocol. There were 10 additional cycles with the same conditions, except for the annealing temperature of 53°C, to anneal the labelled M13 primer.

Laboratory Procedures

The laboratory procedures were performed between the years 2009 and 2010. DNA was extracted from 122 hair samples using DNeasy blood and tissue kits (Qiagen) following the manufacturer's protocol. We used multiple hairs (about 15 hairs) to extract 200 μL of eluate per individual. Eleven microsatellites were amplified, and all analysed individuals had less than 35% of missing loci. To estimate the genotyping error rate, we also re-amplified approximately 5% randomly chosen samples.

PCR reactions were carried out for each locus separately in a 10-μL volume, and products from 1 to 4 loci were diluted and pooled together, based on yield, size range and fluorescent dye, for genotyping. DNA amplifications were performed in the thermocycler TC412 (Techn), and profiles of the amplification reactions can be seen in Table 1. Seven polymorphic loci isolated from *L. chrysomelas* were used – Lchμ1, Lchμ3, Lchμ4, Lchμ5, Lchμ6, Lchμ8 and Lchμ9 [Galbusera and Gillemot, 2008]. These primers were constructed with M13 tails and used in combination with an M13 primer that had the complementary sequence but was dye-labelled, following the protocol established by Schuelke [2000]. These PCR reactions contained: ≤10 ng of DNA, 0.2 μM of the reverse and M13-fluorescent primers, 0.0135 μM of the M13-tailed forward primer, 1 unit of platinum *Taq* DNA polymerase (Invitrogen), 1× PCR buffer (Invitrogen); 1.5 mM $MgCl_2$ (Invitrogen), 100 μM of each dNTP (Amersham Biosciences), and 1% trehalose for the elimination of spurious bands. Microsatellite alleles were separated and visualized on the ABI 3500 automatic sequencer using GS 600Liz size standard. GeneMapper 4.1 software (Applied Biosystems) was used to assign allele sizes to individuals.

Four microsatellite markers isolated from *Leontopithecus chrysopygus* were also used: Leon2, Leon21, Leon27 and Leon30 [Perez-Sweeney et al., 2005]. The PCRs were performed con-

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taining: ≤10 ng of DNA; 0.5 μM of reverse primer and 0.5 μM of forward primer marked with dye labelled on its 5′ end, and the HotStar Taq Master Mix Kit in the concentration of 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of dNTP and 2.5 units of HotStart Taq (Qiagen). In addition, 1 mg/mL of bovine serum albumin was used to improve amplification. Microsatellite genotyping was performed in the MegaBACE 1000 automatic sequencer and the ET-ROX 550 size standard (GE Healthcare). The fragments were identified through the Genetic Profile 2.2 software (Amersham Biosciences 2.2).

Data Analyses

Sample Sets

Our genetic analyses considered two sample sets: one ("total individuals")in which we included all individuals, related and unrelated, and another ("unrelated individuals") in which we excluded closely related individuals to increase the accuracy of genetic structure analysis [Rodríguez-Ramilo and Wang, 2012]. We tested our data in these two ways following recommendations that researchers should consider the effects of monogamy and family organization on the population structure [Anderson and Dunham, 2008]. The social system of lion tamarins consists of family groups with cooperative breeding and a typically monogamous mating system [Baker et al., 1993, 2002]. Furthermore, the clustering analyses performed by STRUCTURE can be influenced by the presence of highly related individuals in the samples [Rodríguez-Ramilo and Wang, 2012]. Our samples were obtained from social groups, which might bias our results. However, withdrawing related individuals from the analyses also influences the results of STRUCTURE by reducing the sample size in each region [Kalinowski, 2011]. Therefore, these two data sets – total individuals and unrelated individuals – were considered in genetic analyses. However, because these two sets had similar results, for the majority of the subsequent analyses we showed only the results of the total sample set.

We inferred the pairwise relationships using the maximum likelihood estimator (ML) implemented in the ML-relate software that can account for null alleles [Kalinowski et al., 2006]. The relationship (half-siblings, full siblings, parents-offspring and unrelated) was estimated for each pair of individuals in a social group while taking into account the loci containing null alleles. Because the relationships are strongly affected by sampling error, we also performed likelihood ratio tests using 100 simulations to pairs of individuals who are more likely to be half-siblings, full siblings or parents-offspring (putative relationships) versus unrelated (alternative relationships). Only 1 individual from each significantly related pair indicated by ML ($p < 0.05$) was removed in order to maximize the sample number within each social group. To prioritize the probable parents (adult individuals) in the sample set, individuals' age group [following Baker et al., 1993, 2002] and observational data were also used (apart from molecular data) when identifying the (un)related individuals. These removals were performed manually in order to keep in the unrelated sample set all those individuals that were unrelated to any other individual within their social group through pairwise comparisons. Thus, a total of 90 samples were considered *unrelated individuals*: Ilhéus (10), Ararauna (7), Teimoso (5), REBIO (63) and Barro Branco (5).

Population Genetic Tests

We estimated the error rate as the ratio between observed number of allelic differences and total number of allelic comparisons [Bonin et al., 2004]. We also performed Hardy-Weinberg equilibrium tests in GENALEX version 6 [Peakall and Smouse, 2006]. The *p* values for all tests were corrected for multiple comparisons using the sequential Bonferroni procedure – *p* interval: $0.0045 < \alpha < 0.0500$ [Rice, 1989].

Standard Genetic Indices

We obtained indices of genetic diversity in 4 genetic groups assigned on the basis of some genetic structure analyses (see Results): Ilhéus, Teimoso, REBIO-Ararauna (constituted by REBIO and Ararauna geographic groups together) and Barro Branco. We used GENALEX6 [Peakall and Smouse, 2006] to estimate allele frequencies, number of alleles (N_a), observed heterozygosity (H_o), and unbiased expected heterozygosity (H_e) per locus and population. We calculated allelic richness and the private allelic richness using HP-Rare [Kalinowski, 2004, 2005].

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The mean number and standard deviation for genetic indices per locus and population were also reported. After testing the data normality using Shapiro's test, we tested the significant differences between pairwise comparisons of all means using an ANOVA test (*F* and *p* values reported) and the Kruskal-Wallis test (χ^2 and *p* values reported) to parametric and non-parametric data, respectively. These analyses were performed in R 3.2.2 software [R Core Team, 2016].

Because pairwise comparisons of the mean of all genetic indices using the total samples versus unrelated samples showed no statistically significant differences, we showed only the results of the total individuals set. This is also a good indication that relatedness is not an issue to estimate the genetic indices [as in Peterman et al., 2016; Moraes et al., 2017]. Deviation from mutation-drift equilibrium in REBIO Una (having a large enough sample size, also for the unrelated set) was also tested with BOTTLENECK 1.2.02 (10,000 replications) [Piry et al., 1999]. As the mutation model underlying our microsatellite markers was unknown, data were analysed under the 2-phase model (assuming 95% single-step mutations) as well as the stepwise mutation model.

Population Genetic Structure

We tested for population genetic structuring using the STRUCTURE 2.3 software [Pritchard et al., 2000]. STRUCTURE uses a Markov chain Monte Carlo procedure to estimate the posterior probability that the data fit the hypothesis of K clusters (Pr [X/K]). We estimated the number of clusters using 10 independent runs for K = 1–8, a Markov chain Monte Carlo procedure of 1,000,000 steps and a period of burn-in of 500,000 steps. Initially, the program was run without a priori information from the source population, using the models of admixture and correlated allele frequencies. As multiple methods for the estimation of K are recommended [Evanno et al., 2005; Kalinowski, 2011], we therefore determined the number of genetic groups using: (1) the optimal value of the posterior probability (K [Pr [X/K]]) given as LnP(K) [Pritchard et al., 2000] and (2) the modal value of ΔK, a measure of the second-order rate of change in the likelihood of K [Evanno et al., 2005]. The results of these analyses were generated using STRUCTURE HARVESTER [Earl and von Holdt, 2012].

We also investigated the degree of genetic differentiation among the 5 main geographic groups using factorial correspondence analysis (FCA) performed in GENETIX [Belkhir et al., 1996–2004], and the values of global and pairwise F_{st} measures [Wright, 1978] performed in GENALEX6 [Peakall and Smouse, 2006]. The F_{st} results were generated using a significance level of 0.05 and 9,999 permutations. In order to test for isolation by distance [Rousset, 1997], we used GENALEX to perform the Mantel test [Mantel, 1967] with 9,999 random permutations. The relationship between matrices of F_{st} and linear distances in kilometres (16–98 km) was tested between the 5 main geographic groups.

Results

Population Genetic Tests

In total, 162 alleles (approx. 5%) selected randomly were attempted for re-amplification. However, 2 alleles (1 locus for 1 individual) could not be typed for both amplifications, and another 16 alleles could be typed for only 1 amplification, but not for the other. Among the effectively re-amplified 144 alleles in 5 loci, we found 4 allelic drop-outs, corresponding to an error rate of 2.8%. When separating the samples by their geographic groups, REBIO presented significant deviations from Hardy-Weinberg equilibrium in 4 loci, but only when Piedade was included in the analyses.

Standard Genetic Indices

The mean number of alleles per locus and population was 3.6 ± 0.2, and the mean allelic richness was 1.5 ± 0.07. The overall H_o was 0.5 ± 0.04 and was not significantly different from the overall H_e of 0.51 ± 0.03. Most overall pairwise comparisons be-

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Table 2. Genetic diversity at 11 microsatellites in the clusters (based on FCA results) of *L. chrysomelas* distributed in south-eastern Bahia, Brazil

	Ilhéus						Teimoso						REBIO-Arara una						Barro Branco					
	n	N_a	AR	PR	H_o	H_e	*n*	N_a	AR	PR	H_o	H_e	*n*	N_a	AR	PR	H_o	H_e	*n*	N_a	AR	PR	H_o	H_e
Leon2	15	4	1.7	0.75	0.7	0.7	7	2	1.4	0.65	0.4	0.4	70	7	1.8	0.47	0.7	0.7	5	3	1.6	0.47	0.8	0.6
Leon30	15	4	1.8	0.82	0.6	0.8	7	2	1.5	0.85	0.7	0.5	85	6	1.6	0.53	0.6	0.6	7	4	1.6	1.35	0.6	0.6
Leon21	16	3	1.3	0.23	0.3	0.3	7	1	1.0	0.00	0.0	0.0	81	5	1.6	0.42	0.6	0.6	7	3	1.7	0.98	0.7	0.7
Leon27	16	3	1.5	0.00	0.3	0.5	7	2	1.1	0.08	0.1	0.1	82	4	1.4	0.01	0.3	0.4	1	2	2.0	0.68	1.0	1.0
Lchμ4	18	2	1.2	0.50	0.2	0.2	7	2	1.1	0.05	0.1	0.1	86	4	1.5	0.10	0.4	0.5	7	4	1.8	1.18	0.7	0.8
Lchμ8	18	5	1.6	0.20	0.8	0.6	7	3	1.6	0.71	0.7	0.6	84	7	1.5	0.33	0.4	0.4	7	4	1.8	0.74	0.7	0.8
Lchμ9	18	4	1.6	0.66	0.7	0.6	7	2	1.5	1.02	0.0	0.5	86	5	1.4	0.13	0.4	0.4	7	2	1.1	0.03	0.1	0.1
Lchμ1	17	3	1.6	0.13	0.6	0.6	7	2	1.4	0.44	0.6	0.4	87	3	1.5	0.14	0.5	0.5	7	2	1.4	0.02	0.4	0.4
Lchμ3	17	3	1.6	0.59	0.6	0.6	7	3	1.6	0.59	0.9	0.6	88	5	1.8	1.15	0.6	0.8	7	4	1.8	0.45	0.7	0.8
Lchμ5	17	5	1.8	0.57	0.6	0.8	7	3	1.6	0.33	0.7	0.6	86	7	1.8	0.81	0.4	0.8	7	5	1.8	0.93	0.4	0.8
Lchμ6	17	2	1.3	1.03	0.2	0.3	7	3	1.4	0.34	0.1	0.4	86	5	1.6	0.42	0.5	0.6	6	6	1.8	0.57	0.7	0.8
Mean	17	3.5	1.5	0.5	0.5	0.5	7.0	2.3	1.4	0.5	0.4	0.4	84	5.3	1.6	0.4	0.5	0.6	6.2	3.5	1.7	0.7	0.6	0.7
SD	0.3	0.3	0.2	0.3	0.1	0.1	0.0	0.2	0.2	0.3	0.1	0.1	0.5	0.4	0.1	0.3	0.1	0.0	0.6	0.4	0.2	0.4	0.1	0.1

n, sample size; N_a, observed allele number; AR, allelic richness; PR, private allelic richness; H_o, observed heterozygosity; H_e, unbiased expected heterozygosity; SD, standard deviation.

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Table 3. STRUCTURE results of *L. chrysomelas* using two sample sets: total and unrelated individuals

K	Mean LnP(K)		SD		ΔK	
	total	unrelated	total	unrelated	total	unrelated
1	–3,171	–2,119	0.44	0.22	–	–
2	**–2,910**	**–1,982**	**0.64**	**1.96**	**201.73**	**44.90**
3	–2,779	–1,933	30.70	7.33	0.76	3.36
4	–2,671	–1,909	32.40	6.62	1.18	2.31
5	–2,602	–1,870	1.58	5.37	9.84	2.61
6	–2,548	–1,844	2.49	12.91	3.41	1.04
7	–2,486	–1,805	0.77	5.85	42.00	12.65
8	–2,456	–1,840	10.80	10.71	–	–

The highest estimates for K are shown in bold. The LnP(K) and standard deviation (SD) were used according to Pritchard et al. [2000], and ΔK according to Evanno et al. [2005].

tween sites showed no significant differences ($p > 0.05$) with a particular exception for the average number of alleles N_a ($p \leq 0.01$), likely due to differences in sample sizes. As such, we only found significant differences in the allelic richness when we compared Teimoso and REBIO-Ararauna ($F = 6.41$, $p = 0.02$), and Teimoso and Barro Branco ($F = 9.13$, $p = 0.007$). Also, when we compared the H_e of Teimoso and that of REBIO-Ararauna ($F = 6.41$, $p = 0.02$), and Teimoso and Barro Branco ($F = 9.13$, $p = 0.007$), we found significant differences (Table 2). Given that variations in the N_a and H_e averaged over populations for each locus, using 7 specific markers for the target species ($N_a = 2.5–5.0$; $H_e = 0.40–0.70$) and 4 specific markers for *L. chrysopygus* ($N_a = 2.8–4.0$; $H_e = 0.38–0.64$), were similar to each other, we considered that our data did not show ascertainment bias. Finally, for both mutation models (2-phase model and stepwise mutation model) we found no evidence (Wilcoxon test: $p > 0.05$; mode-shift test: normal L-shaped frequency distribution) for bottlenecks (heterozygosity excess) in REBIO Una.

Population Genetic Structure

In our tests for genetic clustering, the probability value with the lowest variance occurred when K = 2, indicating population subdivision. Estimates of K using ΔK also presented the highest value when K = 2 (Table 3). When K = 2, REBIO is in a cluster with Ararauna (REBIO-Ararauna) and Ilhéus, Teimoso and Barro Branco are in another cluster (Ilhéus-Teimoso-Barro Branco). In contrast, the FCA showed a grouping between REBIO and Ararauna and some degree of differentiation among other remaining areas (Fig. 2). Comparisons between pairs of populations (F_{st}) showed moderate to high differentiation among all remaining areas (Table 4); although when we considered unrelated individuals, REBIO and Ararauna presented no significant differentiation ($F_{st} = 0.02$, $p = 0.24$). Global F_{st} measures confirmed this significant and high genetic differentiation among populations ($F_{st} = 0.21$, $p = 0.0001$). In addition, Ilhéus, Teimoso, REBIO-Ararauna, and Barro Branco showed private alleles

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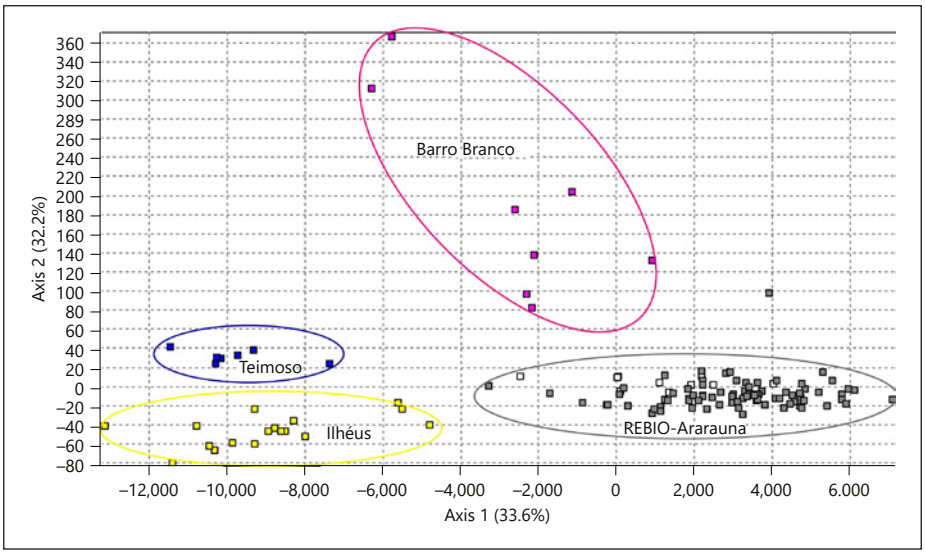

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Fig. 2. The clusters of *L. chrysomelas* individuals distributed in southern Bahia, Brazil, using factorial correspondence analysis and total individuals sampled. Each individual is represented by a point, and genetic clusters are highlighted by the polygons: Barro Branco, Ilhéus, REBIO-Araraua and Teimoso. Note that individuals from Araraua geographical group are shown in white, mainly hidden by grey dots that represent individuals from REBIO geographical group.

with some differentiation among groups. The Mantel test done on the total individuals showed a significant correlation between the matrices of geographic distance and genetic differentiation among groups ($r^2 = 0.34$, $p = 0.008$). However, the correlation was lower and non-significant when the test was done on the unrelated individuals ($r^2 = 0.14$, $p = 0.07$).

Discussion

Genetic Diversity

Our results indicate that GHLT populations may be showing signals of relatively low genetic diversity, within the range observed for other lion tamarins and similar to the captive GHLT populations (Table 5) – although differences in analyses and sampling between these studies should not be ignored. Genetic studies with lion tamarins differ in the number of samples, populations and microsatellite loci, and in the type (skin, blood or hair) and origin (wild life or captivity) of samples, making it difficult to compare them. Considering these differences, the mean expected heterozygosity for GHLT was similar to that observed for *Leontopithecus rosalia* [Grativol et al., 2001; Moraes et al., 2017] and not much higher than the values reported for *Leontopithecus caissara* [Martins and Galetti, 2011; Martins et al., 2012] and *L. chrysopygus* [Ayala-Burbano et al., 2017]. The average number of alleles was relatively high for

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Table 4. Pairwise linear distance, F_{st} and its significance (p) based on 9,999 permutations for 122 GHLTs distributed in south-eastern Bahia, Brazil

	Linear distance, km	F_{st}	p value
REBIO × Ararauna	16	0.14	0.000
Teimoso × Ararauna	42	0.41	0.030
Teimoso × REBIO	45	0.28	0.000
Teimoso × Barro Branco	46	0.37	0.005
Ilhéus × REBIO	61	0.17	0.000
Ilhéus × Teimoso	65	0.32	0.033
Ilhéus × Ararauna	71	0.31	0.001
Barro Branco × Ararauna	87	0.28	0.020
Barro Branco × REBIO	96	0.17	0.000
Barro Branco × Ilhéus	98	0.25	0.000

Table 5. Genetic diversity in all 4 *Leontopithecus* species

Species	Samples	Loci	Pops	N_a	H_e	Mantel test (r^2)	Reference
L. chrysomelas (wild)	84	11	1	5.3	0.57	0.34*	This study
L. chrysomelas (captive, Brazil)	104	16	2	4.8	0.6	–	Orefice [2015]
L. chrysomelas (captive, Europe)	29	9	1	3.6	0.51	–	Galbusera and Gillemot [2008]
L. caissara (wild)	42	9	2	2.6	0.45	0.16	Martins et al. [2012]
L. chrysopygus (wild)	10	15	1	2.0	0.40	–	Ayala-Burbano et al. [2017]
L. chrysopygus (captive, Brazil)	37	15	3	2.3	0.46	–	Ayala-Burbano et al. [2017]
L. rosalia (wild)	57	5	4	2.8	0.54	0.92	Grativol et al. [2001]
L. rosalia (wild)	239	14	6	3.7	0.56	–	Moraes et al. [2017]

Number of samples, loci, and populations (pops) analysed; N_a, mean number of alleles; H_e, mean expected heterozygosity; r^2, significant isolation by distance obtained in each study cited. The asterisk (*) indicates a significant isolation by distance for all *L. chrysomelas* samples (122) and populations (4) in this study. N_a and H_e estimate for REBIO-Ararauna (the largest sample; 84 individuals) only are reported, in order to avoid bias due to small sample sizes in this study. As such, values for *L. chrysopygus* from the wild might be underestimated but are reported by lack of alternatives.

GHLTs in the wild, but this might be (partially) due to differences in sample sizes (for which this parameter is relatively sensitive). Moreover, the average number of alleles per subpopulation of GHLTs was similar to the subpopulation variation observed for *L. rosalia* – 2.0 ± 0.4–3.8 ± 0.3 [Grativol et al., 2001] and 2.9 ± 0.7–5.0 ± 1.8 [Moraes et al., 2017]. Although our sampling and laboratory procedures follow previous studies using hair samples captured from lion tamarins [Orefice, 2015; Ayala-Burba-

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no et al., 2017; Moraes et al., 2017], these results may also be influenced by genotyping error.

Our results show that the genetic diversity of the Barro Branco population was comparable to that of the other studied populations, although GHLT populations in the western region are smaller, more isolated by a matrix of non-forest, with high rates of mortality [Guidorizzi, 2008; Raboy et al., 2010], and had a small sample size. In fact, Barro Branco had the highest average of all genetic diversity indexes (including many private alleles), except for the number of alleles. It should be noted that these results are based on neutral nuclear markers (microsatellites), which are less sensitive to bottleneck effects than mitochondrial haplotypes. The highest average of the nuclear genetic diversity indexes in Barro Branco might be an indication of a recent bottleneck, resulting in a small population that still had not had time for nuclear allele fixation through genetic drift [Birky et al., 1983]. This genetic contribution of the western population to the overall genetic diversity of the species warrants the need for further research (using other genetic markers and more populations) to understand the ecological processes affecting the demography and, consequently, the genetics of this population. GHLT populations in the western region may represent an important gene pool for this species – particularly in future scenarios of climate change [Meyer et al., 2014] – but this should be investigated further.

The genetic diversity results also indicate the importance of ex situ populations for the conservation of the GHLT (Table 5). Results found in this study showed similar estimates of genetic diversity with those found in captive GHLT populations [Galbusera and Gillemot, 2008; Orefice, 2015], even though the captive population is based on a relatively small number of founders (around 40). In addition, these similar genetic diversities among captive populations and our results were found even using different types of samples: skin (n = 29, loci = 9, N_a = 3.67, H_e = 0.59 [Galbusera and Gillemot, 2008]), blood (n = 55, loci = 16, N_a = 5.1, H_e = 0.65) and hair (n = 49, loci = 16, N_a = 4.6, H_e = 0.55) [Orefice, 2015]. The importance of genetically diverse captive populations for the conservation of lion tamarin species with markedly smaller wild populations has also been documented [Ayala-Burbano et al., 2017; Moraes et al., 2017]. In the case of *L. rosalia*, the captive population contributed greatly to the wild population by means of a reintroduction programme [Kierulff et al., 2012; Moraes et al., 2017].

Population Genetic Structure

Our results indicate a genetic structuring of GHLT populations. The analyses of genetic structure using Bayesian model-based clustering (i.e., STRUCTURE) showed differentiation into 2 clusters. FCA and F_{st} analysis further differentiate the data into 4–5 discrete clusters. Both results are informative for conservation planning. When the microsatellite data were split into 2 clusters, the first cluster included REBIO-Ararauna. The similarity is probably related to the proximity between the areas: they are separated by 16–20 km of relatively continuous rain forest [Oliveira et al., 2011]. The second cluster included samples from Ilhéus, Teimoso and Barro Branco. These 3 geographic groups are relatively distant from each other (>40 km), and their genetic similarities could be due to historical connections. Again, it should be noted that mtDNA is more sensitive to bottleneck effects than nuclear genes, and that gene flow sufficient to maintain nuclear panmixia may allow differentiation of mitochondrial lineages in different local demes. However, there is no additional evidence for this.

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Furthermore, genetic structure results could also be influenced by sample size and/or genotyping errors [Pompanon et al., 2005; Kalinowski, 2011; Meirmans, 2015].

Even though the genetic structure is clear, as indicated by the STRUCTURE analyses (K = 2), alternative scenarios of more structure were suggested when considering FCA and F_{st} results. These results indicate that REBIO-Ararauna forms a discrete cluster that differs from the other geographic groups as much as the other geographic groups differ among themselves – Ilhéus, Teimoso and Barro Branco. These are important considerations for conservation, because the relative continuity of the landscape currently provided by the *cabruca*-dominated matrix may not be enough for the maintenance of gene flow, particularly for long distances such as observed between these patches (≥20 km). A landscape genetic study showed that *L. rosalia* may be able to disperse up to 8 km if the landscape is functionally connected [Moraes et al., 2018].

One issue embedded within the concept of habitat fragmentation is the importance of geographical distance as a metric of genetic isolation among populations [Gibbs, 2001; Fahrig, 2003]. In our case, this holds true when considering the cluster REBIO-Ararauna. Nevertheless, population genetic simulations have shown that gene flow in lion tamarins is expected to be limited by distance and by other factors such as the type of non-forest matrix and barriers, leading to a strong genetic differentiation among groups over time [Di Fiore and Valencia, 2014]. In this study, the Mantel test estimated that only 34% of the variance in genetic distance among GHLT populations can be explained by geographic distance alone. On the other hand, the correlation between the GHLT populations was not significant when considering only unrelated individuals within each population. Thus, isolation by distance is a partial explanation for the variance in genetic distance given an incomplete sampling. Other factors such as physical barriers (e.g., open areas and roads) and behaviour may help explain additional variance in the differentiation among GHLTs, similar to observations for *L. rosalia* [Di Fiore and Valencia, 2014; Moraes et al., 2018].

Implications for Conservation

This study suggests that GHLT populations have a relatively low genetic diversity similar to *L. rosalia* [Grativol et al., 2001; Moraes et al., 2017] and *L. chrysopygus* [Martins and Galetti, 2011; Martins et al., 2012]. However, GHLTs have a larger overall population size and geographic distribution compared with other lion tamarins, which might imply a relatively secure conservation status [Holst et al., 2006]. Furthermore, GHLTs showed a process of population genetic structuring, and linear distance did not explain the genetic distance between sites when including unrelated individuals. These findings differ from the expected, because until now we believed that the relative continuity of the landscape in the eastern region from the GHLT geographic distribution, enabled by *cabrucas*, was sufficient to maintain a panmictic population (or a simple isolation-by-distance situation) and a high genetic diversity [Holst et al., 2006]. Apart from geographic distance, there appear to be additional, unknown barriers or resistance (physical and/or behavioural) to gene flow within these apparently continuous forest mosaics [as also observed by Radespiel et al., 2008].

Although our results indicate a genetic structuring process of GHLT populations in the eastern region, the Ilhéus population (residing in the *cabruca* habitat) retained a genetic diversity similar to that in the REBIO-Ararauna cluster, which includes the largest protected conservation area for this species. Furthermore, field studies showed

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that GHLTs reproduce and persist well in *cabruca*, to the point of maintaining a high population density [Oliveira et al., 2011]. The importance of *cabruca* for species conservation has been reported, since it offers resources similar to those found in native forests [Raboy et al., 2004; Oliveira et al., 2010]. However, whether functional connectivity is fully maintained based on preservation of *cabruca* without additional conservation intervention requires further evaluation. Other gaps in landscape connectivity (e.g., open areas and roads), which vary according to species behaviour and biology [Zeigler et al., 2013; Tischendorf and Fahrig, 2000], must be identified and addressed in conjunction with the continued preservation of *cabruca* and other agroforestry systems.

Another consideration for conservation is the importance of both eastern and western populations in terms of region-specific and overall genetic diversity. To the east, the population of GHLTs in Una Biological Reserve represents an important genetic population (e.g., no evidence for a recent genetic bottleneck was found in our study). The REBIO Una and surrounding forests have been considered a potential source population for the conservation of GHLTs because of the likelihood that the population will maintain genetic diversity over time [Holst et al., 2006; Zeigler et al., 2010], and that they are located in a climatically suitable region for GHLT persistence [Meyer et al., 2014; Guy et al., 2016]. However, the maintenance of a viable population of GHLTs in the REBIO Una can be compromised if the process of deforestation in its surroundings continues [Zeigler et al., 2013].

Regarding the western portion of the species' range, previous studies suggested that neglecting western populations may have negative implications for the conservation of the species [Guidorizzi, 2008; Zeigler et al., 2010, 2013]. Future actions should investigate the adaptive genetic variation of populations of GHLTs in this region, make inferences about the species' population structure and propose possible management options to preserve its genetic diversity. For other vertebrates, this region was considered a climate refuge from the late Pleistocene that holds greater and more stable genetic diversity [Carnaval et al., 2009]. Furthermore, given its potential genetic adaptation to drier environments, the genetic variation in the western populations might constitute a valuable resource allowing the GHLT to be able to adapt to climate changes or to longer periods of droughts.

Our results may serve as a baseline for future assessments and conservation plans. Recent research has shown that the situation for GHLTs has worsened especially in the western portion of its geographic distribution [Raboy et al., 2010; Zeigler et al., 2010; Meyer et al., 2014]. Only 36% of the habitat within the GHLT distribution area (and study area) is suitable for the species' persistence [Guy et al., 2016]. To the east, rapid loss of their natural habitat, the conversion of *cabruca* to other agricultural crops or pastures threaten GHLTs [Pinto and Rylands, 1997; Holst et al., 2006; Raboy et al., 2010]. Additionally, urban expansion of the cities of Ilhéus and Una has turned the region into one of the largest urban centres in southern Bahia. This development, with accompanying highways, presents potential physical barriers to dispersal. These modifications in the habitat and a social system with difficult acceptance of dispersers could be contributing to the population structuring and viability of GHLTs [Di Fiore and Valencia, 2014] and, therefore, should be assessed and considered during the elaboration and implementation of conservation programmes for the species.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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