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Effects of Forest Fragmentation on Genetic Diversity of the Critically Endangered Primate, the Pied Tamarin (*Saguinus bicolor*): Implications for Conservation

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Abstract

We analyzed DNA at 9 microsatellite loci from hair samples of 73 pied tamarins (*Saguinus bicolor*) located in 3 urban forest fragments and a biological reserve in the city of Manaus, Amazonas, Brazil. The forest fragments had become isolated from the continuous forest 6–15 years prior to the time of sampling. Tests for reduction in population size showed that all groups from the urban forest fragments had undergone genetic bottlenecks. Pied tamarins in this region historically formed one biological population, and the fragments were connected by high levels of gene flow. These results indicate the need to implement a conservation plan that allows for connectivity between the urban fragments, as well as protection from further constriction. Such connectivity could be achieved via the creation and protection of corridors. In addition to the current population trends explained by anthropogenic actions, the species also shows a trend of long-term demographic decline that has resulted in approximately an order of magnitude decrease and began 13 thousand years ago.

Resumen

Se analizaron 73 tamarinos calvo (*Saguinus bicolor*) de tres fragmentos de bosque urbano y una reserva biológica en la ciudad de Manaus, Amazonas. Los frangmentos se aislaron del bosque continuo hace 6 a 15 años antes de la toma de las muestras. Se observó que los grupos de los fragmentos de bosque urbano pasaron por un cuello de botella genético. Históricamente los grupos formaba una sóla población biológica, y las áreas de los fragmentos estaban conectadas por altos niveles de flujo génico. Estos resultados indican la necesidad de implementar un plan de conservación que permita la conexión de los fragmentos urbanos, así como su protección. Tal conectividad podría lograrse a través de la creación y protección de corredores. Además las tendencias actuales de la población es explicada por las acciones antrópicas, las especies a largo plazo también muestran una tendencia de disminución demográfica que ha resultado en un orden de magnitud decreciente y que inció 13 mil años atras.

Subject areas: Conservation genetics and biodiversity
Key words: conservation genetics, forest fragmentation, pied tamarin, sauim-de-coleira, urban forest fragments

The latest IUCN/SSC Primate Specialist Group assessment (http://www.primate-sg.org/) indicates that Brazil is the most species-rich country in the world for this order, with 110 of the 165 Neotropical primate species and approximately one-quarter of the world's primate diversity. Of these 110 species, 40 (36.4% of all Brazilian primates) are threatened with extinction. Causes of these threats are diverse; however, one of the main contributors is habitat destruction and degradation, including habitat fragmentation. Habitat fragmentation causes a series of transformations in ecological processes and functions of the fragments (e.g., Laurance and Bierregaard 1997; Bierregaard et al. 2001; Ferraz et al. 2003; Spielman et al. 2004). It can have genetic consequences for associated species as a result of population size reduction, such as increased genetic drift leading to reduced genetic diversity, fixation of deleterious alleles, and increased pedigree inbreeding and inbreeding depression (Johnson et al. 2004; Martínez-Cruz et al. 2007; Athrey et al. 2011).

Reduced genetic diversity can increase the risk of extinction and decrease the evolutionary potential of populations (Frankham 2005, 2010; O'Grady et al. 2006). Small populations endangered by human actions can have low levels of genetic diversity (Spielman et al. 2004; Garner et al. 2005; DiBattista 2007; Evans and Sheldon 2008). However, low genetic variability in currently threatened species and populations can also be historical, representing the ancestral state, rather than the consequence of recent anthropogenic activities (Johnson et al. 2009). Therefore, it is necessary to understand long-term as well as recent population trends before appropriate conservation measures can be implemented.

As is the case for many Brazilian cities, Manaus—the capital of the Amazon State—has grown in an unplanned and disorderly manner. This growth has resulted in deforestation on the periphery of the city, and the formation of small and degraded urban fragments (Gordo et al. 2013). The remnant fragments have lost many animal and plant species, and have become invaded by alien species (Egler 1992; Gontijo 2008; Marcon et al. 2012; Gordo et al. 2013). Populations of those native species that survived have become isolated with the concomitant effect that gene flow has almost certainly ceased and the potential for pedigree inbreeding has increased.

The pied tamarin (*Saguinus bicolor* Spix, 1823), lives in the forest fragments of the Manaus urban area. *Saguinus bicolor* has the smallest geographic range of any Amazonian primate, not exceeding 7500 km^2 (Röhe 2006). Its distribution is centered on the metropolitan area of Manaus, which has experienced explosive population growth following the establishment of the free trade zone in its present form in 1967 (Castello Branco 1967). Growth has been largely unregulated and unplanned, resulting in ever increasing fragmentation of the remaining forest habitat within the urban matrix. Increased agricultural activities have also impacted the surrounding rural areas. Metropolitan Manaus thus represents one of the "hotspots" of deforestation in the Brazilian Amazon basin (Gordo et al. 2013).

The pied tamarin is currently the most threatened of the callitrichid primates of the Amazon basin (Gordo 2008); it is listed in Appendix I of the Convention on the International Trade of Threatened Species (CITES), and the official list of Brazilian fauna threatened with extinction, published 18 December 2014, lists *S. bicolor* as critically endangered.

As a result of forest fragmentation, the pied tamarin's distribution has been drastically reduced, and is restricted to between the Urubu and Cuieras rivers, and largely within the greater Manaus urban and suburban regions (Figure 1) (Ayres et al. 1980, 1982;

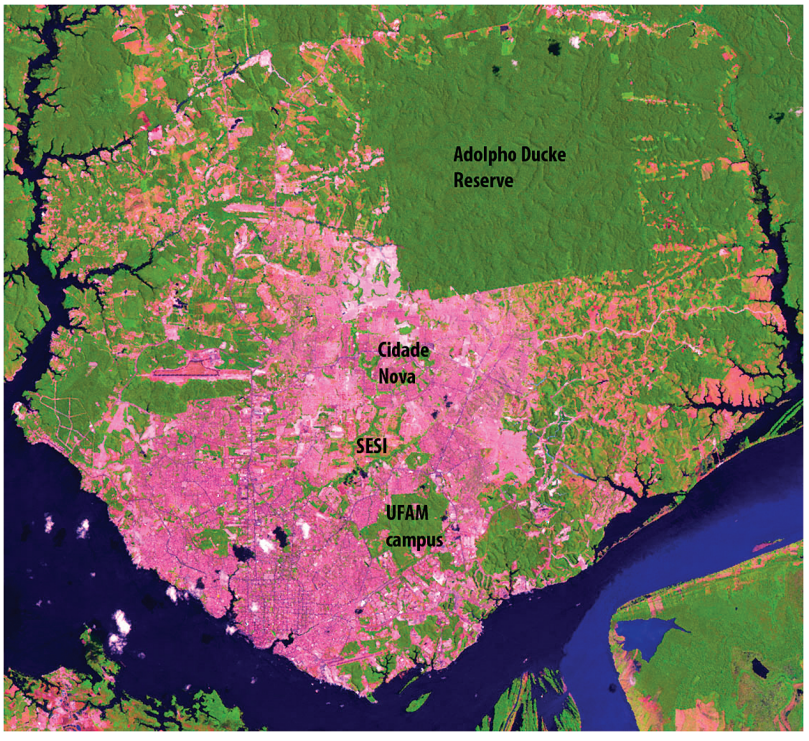

Figure 1. Sampling localities of pied tamarin in Manaus city, Amazonas, Brazil.

Subirá 1998; Röhe 2006). To our knowledge, the pied tamarin is the only nonhuman primate whose principal distribution is within an urban setting. Most of our knowledge on the ecology and distribution of this species prior to forest fragmentation comes from the work of Ayres and Egler (Ayres et al. 1980, 1982; Egler 1986, 1991, 1992, 1993). Environmental education campaigns in the 1990s by the Fundação Vitória Amazônica (www.fva.org.br) and the Federal University of Amazonas (portal.ufam.edu.br) helped to link pied tamarin welfare with conservation of forested areas in the public mind. This renewed conservation interest led to the creation of the *Sauim-de-Coleira* project in 2001, which began to monitor *S. bicolor* in urban forest fragments. Data collected under this project by Röhe (2006) and Gordo (2012) provides the most recent information on the ecology, distribution, and interspecific interactions of *S. bicolor*.

Genetic diversity is one of the criteria used by IUCN for conservation assessment. The loss of genetic diversity not only decreases the potential for future adaptation, it can also lead to increased risk of extinction (Ellstrand and Ellam 1993). Knowledge of the amount and spatiotemporal distribution of genetic diversity in endangered populations are thus indispensable for risk assessment and for the elaboration of management and conservation programs. Therefore, we assessed levels and spatiotemporal distribution of pied tamarin genetic diversity in 3 forest fragments in the Manaus urban area, and in a biological reserve on the periphery of Manaus that is connected to continuous forest. We used 9 microsatellite loci developed by Böhle and Zischler (2002) for the mustached tamarin (*S. mystax*), and tested whether the observed patterns of diversity reflected historical trends, recent anthropogenic influences, or both.

Material and Methods

Sample and Genotyping Collection

Saguinus bicolor individuals were sampled noninvasively in collaboration with *Projeto Sauim-de-Coleira* and coordinated by Marcelo Gordo using a protocol approved under the license SISBIO # 10286-3. Troops of pied tamarins were habituated to tomahawk traps set in specific localities by placing fruit near to, above, or inside the trap. The traps were secured on platforms, and until the moment of capture had open gates, permitting unrestricted access of animals to the fruit. Once trapped, the animals were anesthetized by a qualified technician, and hair samples were plucked from individuals during marking of the animals. Once awake and fully recovered, the animals were released the following day at the point of capture and observed to assure full recovery and normal behavior (Gordo 2012). Hair samples were collected from individuals in the following localities (Figure 1): Campus of the Federal University of Amazonas (UFAM fragment; N = 21), the Cidade Nova district (CNOVA—2 very recently isolated geographically proximate fragments; N = 12), and the Serviço Social da Indústria (SESI fragment; N = 11). The regions had been separated from the continuous forest 15 years, 5–6 years, and 12 years ago, respectively, at the time of sample collection. Hair samples were also collected from individuals captured in the Adolfo Ducke Reserve area (RDUCKE; N = 13). The Adolfo Ducke Reserve is connected to continuous forest on its northern and eastern sides, and it functioned as a control group for the effect of urban fragmentation. All samples were collected between 2000 and 2004. In addition, we analyzed 16 samples obtained from animals run over by cars between the years 1993 and 1999 on the campus of the Federal University of Amazonas. These samples were maintained in alcohol and/or were frozen until processing for analyses.

Total DNA from hair samples was extracted using the Qiagen DNA extraction kit in accordance with the manufacturer's protocol. All samples were screened for variation at 9 pairs of primers developed by Böhle and Zischler (2002) for *S. mystax*; we modified these primers by adding an M13(–21) tail to the 5′ end of the forward primer of each primer pair, thus allowing for dynamic labeling with a fluorescent dye (Schuelke 2000). Each polymerase chain reaction (PCR) contained: 4.4 µL of ddH_2O, 1.0 µL of 10x Buffer (200 mM Tris–HCl, 500 mM KCl, pH 8.4; LGC Biotecnologia, Sao Paulo, Brazil), 0.7 µL of $MgCl_2$ (25 mM), 1.0 µL of reverse primer (2.0 µM), 0.5 µL of forward primer (2.0 µM), 0.5 µL of TET™ labeled M13 label primer (2.0 µM), 0.8 µL of dNTP mix (10 mM dNTP), 0.2 µL of LGC Biotecnologia Taq DNA Polymerase (5 U/µL), and 1 µL of DNA (~ 10 ng). PCRs were run in a Thermo Hybaid thermocycler. An initial denaturation step (94 °C, 1 min) was followed by 25 cycles of 50 s at 94 °C, 50 s at 55 °C, and 1 min at 72 °C; 10 cycles of 40 s at 92 °C, 35 s at 50 °C, and 40 s at 72 °C; and a final extension step of 20 min at 72 °C. PCR products were electrophoresed on a MegaBACE 1000 (GE Healthcare, Sao Paulo, Brazil) and visualized in a MegaBACE 1000 Fragment Profiler v1.2 software (GE Healthcare). Allele sizes were scored against the size standard ET-400 ROX (GE Healthcare).

Data Analysis

Basic population genetic parameters such as the frequencies of observed and expected heterozygosities, Hardy–Weinberg equilibrium (HWE), linkage disequilibrium, and divergence in the allelic and genotype frequencies among the localities were inferred. Significance was adjusted for multiple comparisons using the sequential Bonferroni procedure of Rice (1989). All parameters were inferred in the program ARLEQUIN vers. 3.5.1.2 (Excoffier and Lischer 2010).

Differentiation among fragments was estimated by means of analysis of molecular variance (AMOVA) (Excoffier et al. 1992), which quantifies differentiation among groups of samples defined a priori. We opted to analyze the data using the θ_{ST} (Weir and Cockerham 1984) instead of R_{ST} (Slatkin 1995) metric, as the variance of R_{ST} tends to be much larger than that of θ_{ST} when less than 20 loci are analyzed (Gaggiotti et al. 1999).

Investigating the existence of population structuring without a priori assigning individuals to groups was carried out in the program STRUCTURE version 2.3.3 (Pritchard et al. 2000; Falush et al. 2003) with the goal of assigning individuals to groups, given a specific number of groups (K). We used the "admixture" and "correlated-allelic-frequencies" models. Assignment space was explored with 1 000 000 MCMC chains, preceded by 100 000 MCMC chains discarded as burn-in. Convergence was examined by viewing profiles of posterior probabilities, and values of α. Each analysis was repeated 10 times from a different randomly selected starting point; raw output was processed in Structure Harvester 0.6.92 (Earl and VonHoldt 2012). The 10 independent runs were summarized in the program CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007), and results were visualized in the program DISTRUCT 1.1 (Rosenberg 2004). The most likely number of biological groups (K) was interpreted using Bayes' Rule.

We used to the program IMa2 (Hey and Nielsen 2007) to test if sharing of alleles between fragments was due to ongoing gene flow, or if retained ancestral polymorphism also contributed to observed θ_{ST} patterns of differentiation. With this analysis we specifically tested if observed patterns of differentiation have a historical component (i.e., divergence times significantly greater than 0), or if they

could be accounted for by restricted gene flow only. The 2 models allowing zero and nonzero time divergence between fragments were compared using the hierarchical likelihood ration test within the program IMa2. Solution space was explored using MCMC. We ran 20 parallel chains with dynamic heating, saving every 1000th topology for a total of 300 000 topologies after discarding the first 100 000 topologies as burn-in. All searches were repeated twice to confer convergence. Convergence was examined by viewing profiles of posterior probabilities of estimated parameters. Conversion to demographic parameters was done assuming a generation time of 2 years (range of 18–24 months for females and males, respectively) (Nowak 1999). Convergence, summaries, and plots were generated in the statistical package R (R Development Core Team 2011).

The estimated gene flow was cross-validated in the program MIGRATE 3.2.17 (Beerli 2006; Beerli and Palczewski 2010) using the Bayesian method. We used default search strategy values, except that we ran 20 short chains followed by one long chain. The long chain search was repeated, and convergence was assessed using the Gelman–Rubin criterion (Gelman and Rubin 1992).

Populations that have experienced a recent reduction in effective population size exhibit a loss of genetic variation (Allendorf and Luikart 2006). The number of alleles is reduced more rapidly than the expected heterozygosity (H_E), which is thus greater than the expected heterozygosity of a population at equilibrium (H_{EQ}) (Nei 1987). Allelic loss is also expected to proceed faster than reduction in the range of allele size (Garza and Williamson 2001). Additionally, population decline will result in increase in average time to coalescence for a given allelic diversity (Beaumont 1999). These 3 consequences manifest themselves at slightly different times after population reductions, and thus can be used to investigate reduction onset times (Garza and Williamson 2001). In order to verify if there was a reduction in effective population size in the pied tamarin groups, we used 2 moment-based methods implemented in the programs BOTTLENECK (Piry et al. 1999) and *M*Value (Garza and Williamson 2001). Both programs identify populations that have suffered a recent reduction in effective population size (N_e) or bottleneck effect—via the presence of heterozygosity excess (tested in the program BOTTLENECK), or via reduction in the number of alleles compared to allelic spread (tested in the program *M*value). Expected equilibrium heterozygosity was derived from data simulated under the infinite alleles model (IAM), and the two-phased model (TPM) of mutation, the latter of which is thought to best fit microsatellite data (Piry et al. 1999) and recent bottleneck events (Williamson-Natesan 2005). We ran 10 000 replicates, using TPM composed of 95% SMM (step-wise mutation model) and 5% IAM and a variance of 12 as suggested by the program authors (Piry et al. 1999). Significance was tested via the Wilcoxon signed-rank test. Significance of the *M*Value result was derived from 10 000 simulations assuming stable population, and theta estimated from the data.

To test if there were historical trends in the reduction of effective population size, we also analyzed the data in the program Msvar v1.3 (Beaumont 1999). The program analyzed trends in effective population size over coalescent time. We conducted 5 independent parallel runs sampling every 1000th proposal to collect 20 000 proposals in the MCMC chain in each parallel run. Priors for current and historical population size means and variances were equal, and variances encompassed three orders of magnitude. Prior for mean time of population size change was set at 1000 with variance encompassing time range from 1 000 000 to 0 generations ago. Setting current and historical population size priors equal allowed for testing of demographic size changes using Bayes factors (Beaumont 1999; Girod et al. 2011). The runs were evaluated for convergence, and were pooled to provide an estimate of current and historical effective population size. Convergence was assessed using the Gelman–Rubin criterion (Gelman and Rubin 1992) and the test of alternative hypotheses (population decline vs. stable population-size) as suggested by Beaumont (1999) was tested using Bayes factors. Calculations and plots were performed in the statistical package R (R Development Core Team 2011) using the packages CODA and ggplot2.

Three tests for demographic reductions that result in genetic bottlenecks were implemented because, in theory, they have differential sensitivity to different post bottleneck periods. The 2 moment-based methods are best suited to detect recent population declines (Garza and Williamson 2001; Williamson-Natesan 2005), but are highly sensitive to parametrization (Peery et al. 2012), while the coalescent-based method is best suited for detecting more ancient and historical population declines (Beaumont 1999; Storz and Beaumont 2002). Additionally, a recent simulation study by Girod et al. (2011) suggests that the coalescent approach implemented in MSVar is superior in detecting all population-size changes, and that all 3 methods fail in detecting very recent, and relatively mild population reductions.

Data Archiving

In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary microsatellite genotype data underlying these analyses with Dryad.

Results

Genetic Diversity

The 57 samples of *S. bicolor* collected between 2000 and 2003 were polymorphic for all nine microsatellite loci. Locus SB19 was monomorphic in the Cidade Nova and the recent UFAM sample, but in all cases the 8 remaining loci showed a considerable degree of polymorphism. The total number of alleles detected per polymorphic locus for all the samples varied from 3 (SB2 and SB31) to 24 alleles (SB24) (Table 1). The average number of alleles per locus per population (observed allelic diversity) varied from 3.4 (SESI locality) to 7.5 (UFAM locality). When the 16 samples collected from UFAM between 1993 and 1999 were included, the locus SB19 became polymorphic and allelic diversity increased in the majority of the other loci.

HWE and Linkage Disequilibrium

The average heterozygosity estimated across all loci at each locality varied from 0.544 ± 0.30 (SESI) to 0.644 ± 0.35 (UFAM), and number of alleles per locality varied from 3.4 (SESI) to 7.5 (UFAM) (Table 2). In all localities, HWE was rejected either for locus SB7 or SB8 after sequential Bonferroni correction (P = 0.001). Linkage disequilibrium was observed only in Adolfo Ducke Reserve and in only one of 36 pairwise comparisons.

Analysis of Population Differentiation and Gene Flow

AMOVA indicated significant population differentiation (θ_{ST} = 0.06229, P < 0.001) which was also observed in pairwise population differentiation (Table 3). However, the global test of differentiation among localities (Raymond and Rousset 1995) was non significant (P = 1.00), and there was no association between genetic

Table 1. Genetic variability per locus per populations at 9 microsatellite loci in pied tamarin

Population	SB2	SB7	SB8	SB19	SB24	SB30	SB31	SB37	SB38
UFAM (combined)									
N	3	8	12	2	18	5	4	9	8
H_O	0.649	0.432	0.784	0.027	0.730	0.568	0.730	0.595	0.838
H_E	0.592	0.780	0.891	0.027	0.887	0.559	0.535	0.756	0.804
P	0.480	<0.001	<0.001	1	0.008	0.003	0.061	0.009	0.494
UFAM (historical)									
N	3	7	11	2	14	4	3	6	6
H_O	0.750	0.375	0.750	0.063	0.813	0.563	0.688	0.500	0.813
H_E	0.623	0.806	0.873	0.063	0.907	0.498	0.486	0.754	0.740
P	0.258	<0.001	0.012	1	0.006	0.283	0.153	0.026	0.113
UFAM (current)									
N	3	8	11	1	13	5	4	8	8
H_O	0.571	0.476	0.810	0.000	0.667	0.571	0.762	0.667	0.857
H_E	0.575	0.757	0.900	0.000	0.862	0.609	0.576	0.697	0.823
P	0.768	0.004	<0.001	—	0.021	0.033	0.434	0.386	0.835
RDUCKE									
N	3	6	7	2	7	3	3	7	5
H_O	0.846	0.384	0.846	0.076	0.692	0.615	0.615	0.461	0.846
H_E	0.649	0.876	0.803	0.076	0.855	0.636	0.544	0.735	0.590
P	0.046	<0.001	0.051	1	0.016	0.225	0.187	0.038	0.221
CNOVA									
N	3	2	7	1	10	6	3	7	8
H_O	0.916	0.166	0.583	0.000	0.666	0.916	0.666	0.833	0.750
H_E	0.681	0.471	0.847	0.000	0.869	0.789	0.612	0.793	0.851
P	0.436	0.090	<0.001	—	0.017	0.045	0.174	0.495	0.614
SESI									
N	2	2	9	2	3	3	2	3	5
H_O	0.636	0.636	0.727	0.090	0.818	0.818	0.363	0.454	1.000
H_E	0.541	0.523	0.865	0.090	0.636	0.567	0.312	0.658	0.748
P	0.554	0.581	<0.001	1	0.101	0.225	1	0.129	0.191
All									
N	3	8	17	3	22	6	5	11	9
H_O	0.715	0.368	0.757	0.031	0.715	0.621	0.631	0.621	0.842
H_E	0.619	0.741	0.885	0.041	0.890	0.614	0.495	0.734	0.821
P	0.230	<0.001	<0.001	1	<0.001	<0.001	<0.001	<0.001	<0.001

N = Number of alleles; mono = monomorphic.

Table 2. Parameters of genetic diversity and HWE for fragmented populations of pied tamarin

Population	*N*	Gene diversity over loci	Average no. of alleles	H_O–H_E
UFAM (combined)	37	0.648 ± 0.35	7.7	0.59–0.65
UFAM (historical)	16	0.639 ± 0.35	6.2	0.59–0.64
UFAM (current)	21	0.644 ± 0.35	7.5	0.67–0.72
RDUCKE	13	0.616 ± 0.34	4.7	0.59–0.64
CNOVA	12	0.638 ± 0.35	5.2	0.68–0.73
SESI	11	0.544 ± 0.30	3.4	0.61–0.56
All	73	0.647 ± 0.34	9.3	0.58–0.64

N = Number of alleles.

Table 3. Unidirectional estimates of N_m (below) and F_{ST} (above) based on 9 microsatellite loci in 4 populations of pied tamarin

Population	UFAM	RDUCKE	CNOVA	SESI
UFAM	—	0.03601[a]	0.03601[a]	0.07600[a]
RDUCKE	17.38548	—	0.07177[a]	0.08799[a]
CNOVA	10.64376	6.46696	—	0.10290[a]
SESI	5.86415	5.18241	4.35910	—

[a]Significant value after Bonferroni corrections ($P < 0.008$).

and geographic distances separating the fragments (r = –0.4432; P = 0.75).

Migrate analysis indicated high levels of gene flow among localities (Table 4). Mean geneflow varied from 3.70 to 22.96 effective migrants per generation. IMa2 analyses indicated a similar pattern. The data analyses also indicated that a restricted model with divergence time between localities equal to 0 could not be rejected; thus, the observed population structuring is not due to historical differentiation of localities.

STRUCTURE Analysis

Consistent with the IMa2 results, STRUCTURE found no evidence for more than 1 biological population. The average log posterior probability based on 10 runs for one population was –1976.0 and for 2 populations was –1972.9. Although the probability is slightly higher for 2 populations, it was not significant. Having 3 or 4 biological populations is even less likely than having 1 or 2 populations.

Analyses for Reduction in Population Size

The results obtained from the moment-based analyses of a recent reduction in effective population size (bottleneck effect) are presented in Table 5. BOTTLENECK analyses detected significant deviations in observed heterozygosity from H_E in the 3 urban fragments. Testing for significant reduction in number of alleles implemented in the program *M*Value also indicated that all 3 urban fragments experienced a significant genetic bottleneck. The Adolfo Ducke Reserve locality did not present a significant sign of population reduction; however, it was close to the cutoff value in the *M*value analysis.

Coalescent analyses performed in MSVar showed significant declines in effective population sizes. In all cases population reduction explained the data better than no change in effective population size. In all localities, reduction in effective population size was on the order of a magnitude or more. Most probable time of decline of the species in each locality ranged from from 3.5×10^3 to 10.5×10^3 years before present, while the estimate for the beginning of the decline of the species as a whole was 13.5×10^3 years before present (Table 6).

Table 4. MIGRATE analysis showing pair-wise bidirectional estimates of gene flow (N_m) based on 9 microsatellite loci in 4 populations of pied tamarin

MCMC estimates Ln (*L*) of estimates = –2177.806

Population	Theta [$4N_e$ mu]	$4N_m$ [*x* = receiving population]			
		1, *x*	2, *x*	3, *x*	4, *x*
UFAM	0.60558	—	5.0824	3.6975	7.6423
RDUCKE	0.26483	13.1938	—	4.3549	5.8730
CNOVA	0.57022	7.2064	6.6398	—	7.7522
SESI	0.14244	22.9600	8.8537	4.5103	—

Table 5. Comparisons of tests for reduction in population sizes of pied tamarin

Locality	IAM (Wilcoxon)	TPM (Wilcoxon)	"*M*value" (*P*)
UFAM (combined)	0.01367	0.58984	0.723 (0.016)
UFAM (historical)	0.08203	0.50000	0.677 (0.009)
UFAM (current)	0.02734	0.57813	0.660 (0.003)
RDUCKE	0.17969	0.50000	0.707 (0.055)
CNOVA	0.00391	0.02734	0.707 (0.046)
SESI	0.00977	0.06445	0.578 (<0.001)
All	0.02441	0.63281	0.775 (0.039)

Bottleneck probabilities are calculated under the IAM (Ohta and Kimura 1973) and the TPM (Di Rienzo et al. 1994) of molecular evolution, and are based on heterozygosity excess derived from Wilcoxon signed rank test for mutation-drift equilibrium in *Saguinus bicolor* populations. *M*value represents a ration of allele number to allele size range, and probabilities are based on parametric simulations assuming the TPM.

Discussion

Changes in the Allelic Frequencies and Gene Diversity Indexes

Our results indicate that the genetic diversity levels of the pied tamarin groups in the four forest fragments were not depressed, with observed heterozygosity levels (H_O) varying from 0.544 (SESI) to 0.644 (UFAM), in addition to a considerable average number of alleles found for each microsatellite per population (3.4 in SESI population to 7.5 in UFAM population).

Diversity metrics such as allelic diversity, heterozygosity, and the average number of alleles per locus were comparable among the groups analyzed. However, when we analyzed the pre-2000 samples from the Federal University of Amazonas (16 individuals), we saw increased diversity indexes, and the monomorphic locus SB19 became polymorphic. While some metrics, such as the average number of alleles per locus, are correlated with sample size, others such as allelic diversity and heterozygosity are not, indicating that pre-2000 samples were genetically more diverse. The current population harbors a subset of the diversity of that which originally occurred in the geographic region of the campus of the Federal University of Amazonas. Nevertheless, our results indicate that genetic diversity of the groups still has not been drastically reduced, particularly in comparison with the diversity of the endangered golden lion tamarin (*Leontopithecus rosalia*) from the Atlantic Rainforest of Brazil where Grativol et al. (2001) reported an average of 2.0–3.8 alleles per microsatellite locus per population and observed H_O varying from 0.34 to 0.65.

Gene Flow and Population Structure

Analyses performed in STRUCTURE as well as gene flow analyses performed in the programs MIGRATE and IMa2 indicate that the genetic isolation of the fragments is not a natural demographic phenomenon. Individuals from all the fragments belong to just one historically panmictic population, a biological group characterized by random mating among individuals. The historical gene flow among

Table 6. Comparisons of tests for reduction in population sizes of pied tamarin using a coalescent model implemented in the software MSVar 1.3

Locality	N_e present	N_e present 95% HPD	N_e past	N_e past 95% HPD	Time of population change	Time of population change 95% HPD	Bayes factor (contract vs. expand)
UFAM (combined)	3.239	1.575–5.023	4.896	3.331–6.497	3.856	1.729–5.570	1075.152
UFAM (current)	3.098	1.046–5.020	5.084	3.396–6.652	3.544	1.342–5.593	661.835
RDUCKE	3.273	1.603–4.885	4.945	3.265–6.417	4.018	2.234–5.806	1720.843
CNOVA	3.296	1.299–5.093	4.865	3.316–6.554	3.575	1.525–5.749	214.699
SESI	2.575	0.799–4.285	5.155	3.558–6.744	3.747	1.976–5.468	197891.000
All	3.650	2.016–5.253	4.609	3.226–6.431	4.129	2.147–6.039	146.226

All values are on a Log10 scale. Generation time of 3 years is assumed.

the fragments was also high, and sufficient to prevent differentiation of the areas via genetic drift. Furthermore, any observed differences in allelic frequencies in the fragments are explained by past geneflow, a recurrent process, rather than any historical isolation of the fragments. This conclusion is important for the management and conservation of these fragmented urban groups, and suggest the necessity to implement strategies that will allow for geneflow among the fragments.

These results contrasted with the AMOVA and pair-wise F_{ST} analyses which indicate statistically significant levels of differentiation among the fragments. However, a number of studies have shown that population subdivisions (population structure) may be an artifact caused by a genetic bottleneck which increases differentiation among the populations (Tajima 1989; Templeton 2006). Simplifying, F_{ST} type analyses are based on the distribution of alleles among the studied groups. Any force that will lead to biases in allelic frequencies, such as the relative excess of rare alleles or the loss of alleles may result in the signal of population differentiation. This signal, however, does not reflect the equilibrium condition of the studied group, but rather the effect of the fragmentation itself. As such, the test of global differentiation of Raymond and Rousset (1995) which is not sensitive to small sample sizes or low-frequency alleles did not support differentiation of the fragments ($P > 0.05$) in this study.

Habitat Fragmentation and Bottleneck Effect

The results of the BOTTLENECK analyses provided evidence of significant recent population size reduction in all three urban fragments, but not in the Adolpho Ducke Reserve. The same conclusion can also be drawn from the analyses of the data in the program *M*value which in theory is more sensitive to recent demographic reductions. These findings are important because they clearly demonstrate that the populations that undergo fragmentation are in a genetic disequilibrium. Nevertheless, gene flow and population structure analyses clearly indicated that historical connectivity among populations existed, and that overall genetic diversity has not been significantly affected.

Our analyses indicated that the pied tamarins from the urban fragments are suffering a genetic disequilibrium indicative of a genetic bottleneck, which may be a consequence of the recent habitat fragmentation. The effects of genetic erosion on the viability of small populations that suffer habitat fragmentation are known almost entirely from theory. Due to the speed of their alterations and difficulty in monitoring, the first and most critical stages of the process have not been documented except in a few studies. For example, Srikwan et al. (1996) studied the alterations in the viability of the populations of 3 small mammals isolated in forest fragments in Thailand after the construction of a hydroelectric dam. The survey of the animals marked and recaptured 5, 6, 7, and 8 years after fragmentation and comparisons with undisturbed areas showed that habitat fragmentation led to onset of genetic erosion in the remaining populations of the 3 species: *Maxomys surifer*, *Chiropodomys gliroides*, and *Tupaia glis*. The genetic and demographic responses to the fragmentation were peculiar to each species, reflecting differences in life history and behavior. In *C. gliroides*, the genetic erosion preceded demographic decline.

In addition to suffering the effects of recent fragmentation, *S. bicolor* is also undergoing a long-term population decline. When we collected our samples, the UFAM fragment had been in existence for 15 years, the SESI fragment for 12 years, and the Cidade Nova fragment for 5–6 years. Coalescent analyses performed in MSVar indicated declines in effective population sizes. In all localities, as well as in the species as a whole, declines were an order of magnitude or greater, and began between 3.5 and 5.5 thousand years ago. Long-term decline begining at the Pleistocene/Holocene transition was also observed in the Adolfo Ducke Reserve, which did not present signals of a population bottleneck, and in the species as a whole.

Implications for Conservation

Saguinus bicolor, while not the only organism suffering the effects of habitat fragmentation, has the distinction of being the only primate whose natural distribution occurs in large part within a major metropolitan area. Other primate species, such as the brown howler monkeys (*Alouatta guariba*) are found within urban forest fragments, but these comprise only a small percentage of their distribution. Species such as the common marmoset (*Callitrix jacchus*) are also found within urban settings (for example, in Rio de Janeiro, RJ, and Campinas, SP, Brazil), but in these regions they are introduced and considered invasive.

Thus, effects of urban fragmentation have largely been unstudied in primates. The best-studied urban fauna are birds (Marzluff and Ewing 2008). Avian studies clearly show that urban fragmentation has the largest effect on native fauna since once urbanization occurs, the area rarely reverts back to more natural conditions. Furthermore, the urban matrix is much less like a native landscape than one produced by agriculture, forest clearing, or timber extraction. Many, if not the majority, of the effects of urban fragmentation are demographic, such as increased predation by exotic predators (e.g., cats, dogs, and rats), exposure to diseases and competitors, increase in parasites, and human persecution. However, it is also clear that loss of genetic diversity both decreases the potential for future adaptation and increases the risk of extinction (Ellstrand and Ellam 1993; Frankham 1995a, 2010; Spielman et al. 2004). It is not just the level of genetic diversity, but the loss of genetic diversity that is the problem.

Small populations and those endangered by human actions tend to have low levels of genetic diversity (Garner et al. 2005; DiBattista 2007; Evans and Sheldon 2008). It is generally assumed that low levels of genetic diversity and decrease in genetic diversity are due to human or human-mediated impacts, but they may also be the result of long-term historical trends of population decline (Johnson et al. 2009). Therefore it is necessary to test for both recent anthropogenic and historical population trends before appropriate conservation measures are implemented.

In the case of *S. bicolor*, habitat and population fragmentation—associated with anthropogenic activities—resulted in reduction of genetic diversity and clear signature of a genetic bottleneck. However, there is also evidence of long-term population reduction starting approximately 10 thousand years ago for the species as a whole. This long-term trend is more difficult to interpret. The species is clearly declining, but what are the reasons for this decline? In the earliest studies of this species (Hershkovitz 1977; Ayres et al. 1980, 1982) *S. bicolor* was reported east of the Urubu River as far east as the Uatuma River; however, currently there is no evidence of *S. bicolor* occurring east of the Urubu River (Röhe 2006). There is also evidence of competitive exclusion by the golden-handed tamarin, *S. midas* (Röhe 2006) in areas of sympatry, and it appears that the golden-handed tamarin is expanding its range at the expense of *S. bicolor* (Röhe 2006). Given that the population decline began approximately 10 thousand years ago, it is unlikely to be anthropogenically induced and is not associated with urban growth of the last 40 years. Changes in habitat associated with the transition from the Pleistocene to the Holocene Era, which favor the expansion of

S. midas at the expense of *S. bicolor*, are more likely to have caused the decline.

The current scenario may thus best be interpreted as a recent genetic bottleneck driven by anthropogenic urban habitat fragmentation overlaid on a long-term trend of population decline starting in the early Holocene. Even more important than the loss of genetic diversity, and thus evolutionary potential, is the pied tamarin's increased extinction risk due to pedigree inbreeding and the effects associated with inbreeding depression. The deleterious effects of inbreeding have been well documented in wild populations (e.g., Frankham 1995b; Daniels et al. 2000; Dietz et al. 2000; Flagstad et al. 2003). Additionally, inbreeding depression appears to be greater in wild populations that are in general naturally outbreeding (Crnokrak and Roff 1999), such as those of *S. bicolor*. Thus, inbreeding depression may be sufficiently high to be of biological importance for this species. Most of the forest fragments in Manaus are too small to hold more than 1 or 2 groups; which may include from 4 to 11 individuals, but usually are made up of 6–8 members (Gordo 2012). With no potential for movement of individuals between fragments, and thus no geneflow, the pied tamarins in the Manaus urban fragments are experiencing elevated levels of pedigree inbreeding. Studies have shown that even low levels of gene flow can allow a population suffering from inbreeding depression to recover (Vilà et al. 2003). It is therefore urgent to maintain connectivity between these fragments.

Our results have important implications for the management and conservation of the *S. bicolor* groups occurring in the fragments of Manaus, and in great likelihood for all small native vertebrates found in these fragments as well as fragmented populations in urban settings in other cities. First, it is clear that connectivity among fragments must be maintained. Most of the fragments are very isolated, both from each other and from continuous forest. The home ranges of at least 11 of the 30 groups studied by the Sauim-de-coleira project include major roads which frequently need to be crossed by troop members (Gordo et al. 2013). While it is not known what percentage of animals is run over, it is likely to be large. Gordo (2012) estimated that at least 6–10 animals are run over every year within the Federal University of Amazonas campus, representing 5–10% of the total population of *S. bicolor* of that forest fragment. Where fragments are linked by corridors, the corridors tend to be long and degraded, but they still facilitate movement of individuals (Gordo et al. 2013). The ability to use even degraded corridors may be inherent in the biology of callitrichid primates, many of which seem well adapted to survive and potentially even prosper in human-altered environments due to their small body size, rapid reproductive rates, and ability to exploit resources such as large arthropods and plant gums (Sussman and Kinzey 1984). Thus, even relatively degraded corridors can maintain connectivity between the fragments, creating a metapopulation dynamic, which in turn will minimize the potential for pedigree inbreeding.

Second, while there is evidence that anthropogenically caused forest fragmentation resulted in genetic bottlenecks and loss of genetic diversity within the isolated groups of pied tamarins, there is also strong evidence that demographic decline began in the early Holocene, likely driven by environmental changes associated with the Pleistocene-to-Holocene transition. Reduced genetic diversity or even evidence of demographic declines within a human-altered landscape thus cannot be automatically viewed as human-caused. It is important to keep in mind that different methods used in conservation genetic studies for analyzing demographic trends can have different temporal premises, and thus result in temporally stratified inferences.

Lastly, the impacts of urbanization on native species are poorly studied, but urbanization is ever more prominent and permanent. Educating the urban population about the impacts of fragmentation on native flora and fauna can only improve species conservation (McKinney 2002). An environmentally conscious citizenry who maintains the ecological integrity of urban fragments not only creates a more livable urban environment, it also assures the survival of native fauna and flora within these fragments. With appropriate legislative initiatives, conservation of ecologically functional urban fragments turns into a win-win scenario.

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