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Atlantic forest fragmentation and genetic diversity of an isolated population of the Blue-manakin, *Chiroxiphia caudata* (Pipridae), assessed by microsatellite analyses

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RESUMO. **Fragmentação da mata Atlântica e variabilidade genética de uma população isolada do Tangará-dançarino, *Chiroxiphia caudata* (Pipridae), verificada através de análise de microssatélites**. A fragmentação dos habitats restringe o fluxo gênico, resultando na perda de variação genética e depressão por endocruzamento. A Mata Atlântica tem passado por extensas perdas de habitat desde a colonização européia a cinco séculos atrás, e muitas populações de aves estão sendo extintas. Análises de variabilidade genética podem ser importantes para a determinação da viabilidade das populações a longo prazo e para direcionar planos de manejo. Neste trabalho, foi analisada a variabilidade genética do Tangará-dançarino, *Chiroxiphia caudata* (Pipridae), em um fragmento de 112 ha, isolado há 73 anos, e em uma área controle de 10.000 ha, utilizando-se *loci* ortólogos de microssatélites. Três dos nove *loci* testados foram polimórficos. A população do fragmento não apresentou déficit significativo de heterozigose. Embora a diversidade genética, estimada através da heterozigose esperada e riqueza alélica, tenha sido menor no fragmento, as diferenças não foram significativas. Isto sugere que um fragmento com este tamanho possa ser suficiente para manter a variabilidade da população, ou então, 73 anos não foi tempo suficiente para haver perda significativa de variabilidade genética. No entanto, alelos mais raros podem ter sido perdidos, o que é esperado em populações pequenas e isoladas. Uma grande divergência genética foi encontrada entre as duas populações. Apenas estudos futuros dentro de áreas contínuas permitirão verificar se esta diferenciação foi causada pela fragmentação ou se é um resultado da estruturação populacional natural da espécie.
Palavras-chave: Aves, fragmentação florestal, variabilidade genética.

ABSTRACT. Habitat fragmentation is predicted to restrict gene flow, which can result in the loss of genetic variation and inbreeding depression. The Brazilian Atlantic forest has experienced extensive loss of habitats since European settlement five centuries ago, and many bird populations and species are vanishing. Genetic variability analysis in fragmented populations could be important in determining their long-term viability and for guiding management plans. Here we analyzed genetic diversity of a small understory bird, the Blue-manakins *Chiroxiphia caudata* (Pipridae), from an Atlantic forest fragment (112 ha) isolated 73 years ago, and from a 10,000 ha continuous forest tract (control), using orthologous microsatellite loci. Three of the nine loci tested were polymorphic. No statistically significant heterozygote loss was detected for the fragment population. Although genetic diversity, which was estimated by expected heterozygosity and allelic richness, has been lower in the fragment population in relation to the control, it was not statistically significant, suggesting that this 112 ha fragment can be sufficient to maintain a blue-manakin population large enough to avoid stochastic effects, such as inbreeding and/or genetic drift. Alternatively, it is possible that 73 years of isolation did not accumulate sufficient generations for these effects to be detected. However, some alleles have been likely lost, specially the rare ones, what is expected from genetic drift for such a small and isolated population. A high genetic differentiation was detected between populations by comparing both allelic and genotypic distributions. Only future studies in continuous areas are likely to answer if such a structure was caused by the isolation resulted from the forest fragmentation or by natural population structure.
Key words: Birds, forest fragmentation, genetic variability, manakins.

Habitat fragmentation is predicted to restrict gene flow, which can result in the loss of genetic variation and inbreeding depression (Storfer 1999). These effects can be minimized by immigration, which may reduce inbreeding and introduce well-adapted individuals with high fitness (Lande and Barrowclough 1987, Slatkin 1987, Storfer 1999). However, species differ in their dispersal ability and will therefore react differently to isolation (Ouborg 1993, Paradis *et al*. 1998). The available data suggest that birds inhabiting Neotropical forest undergrowth present limited ability to colonize patches separated by open areas when compared to birds of temperate and open habitats (Willis 1974, Stouffer and Bierregaard Jr. 1995, Bates 2000), in the manner that for some species, populations with reduced number of individuals remain trapped by isolation in small forest fragments, being more exposed to chance events (Stouffer and Bierregaard Jr. 1995, Develey and Stouffer 2001).

Although forest fragmentation has been pointed out as one of the most important causes of local extinction of Neotropical birds (Willis 1974, 1979, Terborgh and Winter 1980, Willis and Oniki 1992, Stouffer and Bierregaard Jr. 1995, Robinson 1999), studies have focused primarily on environmental and demographic aspects, being restrict to reports on what species persist or disappear under different conditions of fragmentation (Willis 1974, 1979, Karr 1982, Stouffer and Bierregaard Jr. 1995, Robinson 1999). However, extinction in the Atlantic forest avifauna has been considered time-lagged (Brooks and Balmford 1996), and genetic variability in fragmented popu-

lations could be important in determine their long-term viability (Shaffer 1981, 1987, Gilpin and Soulé 1986, Storfer 1999, Hudson *et al*. 2000).

The Brazilian Atlantic forest is one of the five most important hotspots in the planet, presenting not only an exceptional biodiversity and endemism concentration, but also an exceptional loss of habitats (Myers *et al*. 2000). This ecosystem has been extensively exploited since European settlements five centuries ago. Most of the forest was logged for sugar cane and coffee plantations (Willis 1979, Willis and Oniki 1992, Aleixo and Galetti 1997) and currently, only 7.5% of its original 1 million km^2 area remains (Myers *et al*. 2000) distributed in several small disconnected fragments and a few large forest tracts (SOS Mata Atlântica and INPE 1992).

Although some of these fragments are able to maintain a significant number of small-sized species (Willis 1979, Aleixo and Vielliard 1995, Cândido Jr. 2000), nothing is known about insularization effects on genetic variability of remaining populations, and how prone these populations are to suffer inbreeding depression and disappear in the future.

Manakins (Pipridae) are small frugivorous non-migratory birds found exclusively in the Neotropics. They are widely distributed in tropical and subtropical forests and woodlands undergrowth, and are rarely seen in open areas. They are well known for their lek-mating behavior and striking males sexual plumage, while females are green dull-colored (Snow 1976, Ridgely and Tudor 1994, Sick 1997, Brumfield and Braun 2001).

The Blue-manakin, *Chiroxiphia caudata*, is a small species (23.4g) distributed in southeast Brazil (south Bahia, Minas Gerais and south Goiás to Rio Grande do Sul), eastern Paraguay (west to Canendiyu and Paraguarí) and northeastern Argentina (Misiones and Corrientes). Like the other representatives within the genus, it is known by perform a highly specialized cooperative courtship display, in which two to six males exhibit to only one female (Foster 1981, Willis and Oniki 1988, Ridgely and Tudor 1994, McDonald and Potts 1994). This species is one of the most common understory birds found in highly disturbed fragments in the Atlantic forest of Brazil (Willis 1979, Aleixo and Vielliard 1995) and may have an ecological advantage upon more specialized bird species.

The purpose of this paper was to assess the effects of forest fragmentation on the genetic variability of Blue-manakin, using orthologous microsatellite loci. We hypothesized that genetic variability would be lower in a 112 ha fragment, rather than in a 10,000 ha area.

MATERIAL AND METHODS

Study areas. The genetic variability of Blue-manakins from a 112 ha semideciduous Atlantic forest fragment located in São Carlos, southeast Brazil (21º 58' S, 47º 51' W), was analyzed. This patch is surrounded by pasture and sugar-cane plantations, and the region is completely disturbed, remaining only a few small cerrado and Atlantic forest disconnected fragments. The deforestation in the region and the establishment of the current boundaries occurred in 1929, when local farmers had in the logging their principal economic activity (Primavesi *et al*. 1999). The exact historical record of isolation process and the large distance in relation to source areas (tens of kilometers) make of this fragment suitable for studies regarding to isolation and heterozygosity.

In order to compare, we also analyzed the genetic variability of Blue-manakins from Morro Grande reserve, which was adopted as a control area. This is a 10,000 ha continuous Atlantic forest tract, located in Cotia (23º 30' S, 46º 50' W), western from the metropolitan region of São Paulo, south-east Brazil. These study areas are about 185 km apart.

Sample collection and DNA isolation. Birds were captured using mist nets (12 x 2.5 m) in 1999 and 2000, and marked with metal rings before release. A 10µL blood sample was collected from each animal by cutting nail tip. A volume of EDTA, approximately three times the blood volume, was added to the samples, which were stored in freezer at –20ºC. DNA extraction was according to Lahiri and Nurnberger (1992).

Cross-specific microsatellite loci and PCR amplification. We used the Dpµ01, Dpµ03, Dpµ05, Dpµ15 and Dpµ16 primers described for Yellow warbler, *Dendroica petechia* (Emberizidae, Parulinae) (Dawson *et al*. 1997), and LTMR6, LTMR8, LTMR15 and SJR133 described for Long-tailed manakin, *Chiroxiphia linearis* (Pipridae) (MacDonald and Potts 1994) (Table 1). PCR reactions were performed in PTC-100™ MJ-Research thermocycler in 10µL volume, containing 150 ng DNA, 0.2 mM dNTPs, 1x thermo buffer, 50 ng of each primer, 3mM $MgCl_2$ and 1U Taq polymerase. Thermocycler was programmed for 30 cycles, following an initial denaturing step at 94ºC for 3 min. First 10 cycles consisted of 30 s at 94ºC, 30 s at 55ºC and 30 s at 72ºC. The remaining 20 cycles consisted of 30 s at 94ºC, 30 s at 60ºC and 30 s at 72ºC.

Amplification products were resolved on 12% polyacrylamide denaturing gels containing 8 M urea. Gels were run at 47 W for 4.5–7 h. Results were visualized by silver staining (Comincini *et al*. 1995). Success of orthologous loci amplification was determined by the visualization of one product about the same size as the observed for the original species (see Dawson *et al*. 1997). Alleles sizing was visually performed using 10 bp ladder.

Data analysis. Within-population, deficiency and/or excess of heterozygotes were calculated by comparing the expected (H_E) and observed (H_O) levels of heterozygosity, using score test (U test) (Rousset and Raymond 1995), as implemented in the software GENEPOP v. 3.3 (Raymond and Rousset 1995a); and by calculating inbreeding coefficient (F_{IS}) (Weir and Cockerham 1984), using the software FSTAT v. 2.9.3 (Goudet 1995). Departures from Hardy-Weinberg equilibrium (HWE) were calculated using exact test (Raymond and Rousset 1995b).

Table 1. Characterization of nine orthologous microsatellite loci used to assess genetic structure of a fragmented and a continuous population of Blue manakin, *Chiroxiphia caudata*. Product size in base pairs (bp), number of alleles and mean allele frequency for polymorphic loci (mean ± SD) are presented.

Locus	Primer sequence	Product size (bp)	Nº. alleles (nº. individuals)	Mean frequency
*Dp*µ01	F-TGGATTCACACCCCAAAATT R-AGAAGTATATAGTGCCGCTTGC	160	1 (15)	-
*Dp*µ03	F-GAATTACCCATTATTGGATCC R-AGCAGCAAAACAAACCAG	170	1 (17)	-
*Dp*µ05	F-GGTCTGTGCTCTGTATGG R-TCTGAATATTGAACAGCCTA	118	1 (15)	-
*Dp*µ15	F-GGCTGCAAACTCATTATCTC R-ATTGAGTCTGTCAGGTCCAG	162	1 (10)	-
*Dp*µ16	F-ACAGCAAGGTCAGAATTAAA R-AACTGTTGTGTCTGAGCCT	158	1 (20)	-
LTMR6	F-GCCATGCCACAGGAGTGAGTC R-AGTCATCTCCATCAAGGGCAT	202	1 (17)	-
LTMR8	F-AATGACACCCCACATTCACTG R-TGCCCAAATAGCAAAGGAACC	144-154	6 (38)	0.166±0.197
LTMR15	F-CATTATTCCATAGTGCAAAGC R-AACAGGTGCATCACTAAGCAG	182-216	13 (36)	0.076±0.089
SJR133	F-CATGCTTCATGGCTCAGTTCA R-TGTGGGCAAGTGTGGGTGTAT	134-150	5 (22)	0.199±0.170

The prediction that genetic diversity is lower in the fragment population was tested by comparing its expected heterozygosity and allelic richness (EI Mousadik and Petit 1996) to the values obtained for the control population. Allelic richness is a measure of the number of alleles independent of the sample sizes, which was calculated using FSTAT. These values were obtained for each locus separately and the value means were compared using non-parametric Mann-Whitney U test, according to the software BioEstat 2.0 (Ayres *et al*. 2000).

Genetic differentiation between the pair of populations was estimated by calculating fixation index (F_{ST}) (Weir and Cockerham 1984), in FSTAT. The significance of F_{ST} was obtained by testing: if allelic distribution is identical across populations, using Fisher exact test (Probability-test) (Raymond and Rousset 1995b), as implemented in GENEPOP; and if genotypic distribution is identical across populations, using the log-likelihood (G) based exact test (Goudet *et al*. 1996), performed in FSTAT. Values were obtained for each locus separately and overall.

Table 2. Probability values for significance of departure for Hardy-Weinberg equilibrium, using Probability-test.

	Population	
Locus	Fragment	Control
LTMR8	0.233	0.059
LTMR15	0.702	**0.013**
SJR133	0.738	0.220
All loci	0.646	**0.008**

RESULTS

Although all of the primers here tested have successfully amplified a product presenting the expected size, only loci LTMR8, LTMR15 and SJR133 were polymorphic (Table 1). These three loci presented dinucleotidic repetitive arrays and seem to be reliable markers for population studies on Blue-manakin.

Hardy-Weinberg equilibrium and Genetic variability. There were no evidences for deficiency of heterozygotes in the fragment population, since all of the polymorphic loci were in HWE equilibrium (Table 2) and no deficit or excess of heterozygotes was detected by F_{IS}. Although locus LTMR8 has presented a significant value for score test, which compared deficit of H_O in relation to H_E, it was very close to significance, and the overall value, pooled across all loci was not statistically significant (Table 3). Although fragment sample size is small, it seems to be representative of this population. This assumption become evident when the number of captured birds is plotted against the capture efforts (time of net exposure) (Figure 1).

For the control population, locus LTMR15 was not in HWE equilibrium (Table 2), and presented an extremely significant deficit of heterozygotes, detected by comparing expected and observed levels of heterozygosity. F_{IS} was positive and have also differed significantly from zero for locus LTMR15. Significant departure from HWE equilibrium and loss of heterozygotes (comparing H_O and H_E) was also detected for control population when all of the loci were pooled, probably because of the influence of locus LTMR15 (Table 3).

Genetic diversity estimated by H_E and allelic richness (Table 3) was not significantly lower in the fragment in relation to the control population (Man-Whitney test: U = 2.0; $P = 0.275$ and U = 2.0; $P = 0.275$, respectively).

Population differentiation. The two populations analyzed were genetically differentiated. Exact test used to compare allelic distribution indicated significant differentiation between populations when the loci were separately analyzed and when results across all loci were pooled (Table 4). When genotypic distribution was considered, only locus SJR133 did not present significant differentiation. Nevertheless, the value obtained was very close to significance, and the overall value was also significant. Furthermore, some specific alleles for each population have been found for all of the three loci analyzed.

DISCUSSION

Orthologous loci amplification. The success of cross-species microsatellite loci amplification obtained here suggests that primer-binding sites can be conserved even in distantly related species, as elsewhere reported (Rico *et al*. 1996, Dawson *et al*. 1997, Primmer and Ellegren 1998).

However, it has been argued that levels of polymorphism in orthologous loci seem to decay with increasing phylogenetic distance (Rico *et al*. 1996, Harr *et al*. 2000). The main mechanism leading to microsatellite loci decay is the accumulation of point mutations in the repetitive array, which may decrease slippage rate, resulting in monomorphic loci (Primmer and Ellegren 1998, Harr *et al*. 2000, Kruglyak *et al*. 2000). On the other hand, some loci are recognized to maintain variation even in distantly related species (Primmer and Ellegren 1998).

Available data indicate high probabilities of polymorphism when cross-species primers are applied in closely related species (Rico *et al*. 1996, Bouzat *et al*. 1998, Petren *et al*. 1999). In the present study, polymorphism was only obtained when cross-specific loci were applied within the same genus (*Chiroxiphia*). However, locus SJR133, presented by *C. linearis*, has been previously isolated from a genomic library of Florida scrub jay (MacDonald and Potts 1994). Furthermore, locus *Dp*μ03, which were monomorphic for *C. caudata*, was highly polymorphic when applied for Rufous gnateater, *Conopophaga lineata* (Conopophagidae) (pers. obs.). Although the relationship between phylogenetic distance and orthologous microsatellite loci variation remains controversial, our results support the hypothesis that microsatellite evolution may differ substantially between particular loci (Primmer and Ellegren 1998).

Genetic difference. Both allelic and genotypic frequencies indicated that fragment and control populations were genetically differentiated. Geographic patterns of genetic diversity can be generated by various mechanisms, such as (1) clinal variations of neutral or adaptive traits; (2) historical isolation of subdivided populations; (3) secondary contacts among formerly isolated and divergent populations or (4) divergent selective pressures along species distribution. In the case of new founded or small isolated populations genetic drift and

Table 3. Number of individuals analyzed for each orthologous microsatellite locus (*N*), allelic richness (*AR*), expected (H_E) and observed (H_O) heterozygosity, and inbreeding coefficient (F_{IS}) for a fragmented and a continuous Blue-manakin population.

Locus	*N*	*AR*	H_E	H_O	P_d	P_e	F_{IS}	P_l	P_s
Fragment									
LTMR8	16	4.117	0.558	0.375	**0.044**	0.965	0.336	0.066	0.975
LTMR15	16	5.483	0.771	0.875	0.754	0.281	-0.141	0.941	0.216
SJR133	10	3.0	0.570	0.500	0.433	0.784	0.135	0.441	0.750
All loci					0.277	0.759	0.083	0.275	0.758
Control									
LTMR8	22	4.223	0.640	0.772	0.943	0.104	-0.212	0.983	0.083
LTMR15	20	8.411	0.870	0.650	**0.000**	1.000	0.258	**0.008**	1.000
SJR133	12	4.833	0.757	0.666	0.127	0.881	0.124	0.291	0.925
All loci					**0.012**	0.976	0.082	0.083	0.925

P_d is the value for significance of difference between H_O and H_E (heterozygotes deficit) and P_e the value for significance for heterozygotes excess. The probability that F_{IS} value differ significantly from zero was calculated by randomization method (based on 3,000 randomizations and level of significance at 5%) . P_l is the proportion of randomizations that gave a larger F_{IS} than the observed, and P_s is the proportion of randomizations that gave a smaller F_{IS} than observed.

Table 4. Genetic differentiation between a fragmented and a continuous population of Blue-manakin, detected by fixation index (F_{ST}).

Locus	F_{ST}	P_a	P_g	S. A.	U. A.
LTMR8	0.117	**0.009**	**0.044**	4	1/1
LTMR15	0.026	**0.004**	**0.022**	4	2/7
SJR133	0.069	**0.026**	0.058	3	0/2
All loci	0.068	**0.0001**	**0.001**		

P_a is the value of significance for population differentiation tested using Probability test (allelic distribution), and P_g is the value of significance for population differentiation using log-likelihood G test (genotypic distribution) (based on 1,000 randomizations). S.A. is the number of shared alleles between the two populations and U.A. are the number of unique alleles of each population (Fragment/Control).

inbreeding may be the principal mechanisms leading to genetic divergence (Simberloff and Cox 1987, Slatkin 1987, Smith *et al*. 1997, Barton and Hewitt 1999, Kark *et al*. 1999).

The fact that the fragment population is in HWE equilibrium, not presenting a statistically significant deficiency of heterozygotes, suggests that inbreeding is not the main mechanism responsible for such genetic differentiation. However, founding events and drift may not be excluded. Alleles could be arisen or lost independently in isolated and divergent populations, although the presence of alleles unique to each population could occur because of the small sample sizes. Different authors have found higher genetic differences among populations of Neotropical forest species than in temperate ones, which have been attributed to sedentariness (Brawn et al. 1996, Brumfield and Capparella 1996, Bates 2000). However, only large geographical-scale studies could answer whether the divergence found is due to natural population structuring along Blue-manakin distribution and whether interruption of gene flow, caused by forest fragmentation, can be leading to additional genetic differentiation.

Genetic variability and conservation. The absence of heterozygote deficits, the maintenance of HWE equilibrium, as well as the not significant loss of genetic diversity compared to the control population, suggest that the studied fragment presents sufficient area to maintain a Blue-manakin population large enough to avoid drastic stochastic effects, such as inbreeding, or 73 years of isolation was not sufficient time for genetic stochastic effects to be statistically detected (although basic data on longevity are not available for most of Neotropical birds).

Manakins and other small-sized understory fruit-eating birds have usually high abundances in small forest patches (Snow 1976, Aleixo and Vielliard 1995, Cândido Jr. 2000), which may reflect high adaptation to local disturbance, and can also contribute to the maintenance of the within-population genetic variability even in small isolated fragments.

Most of the Neotropical understory birds are not able to colonize isolated forest patches and dispersal is assumed to be low (Willis 1974, 1979, Stouffer and Bierregaard Jr. 1995, Greenberg and Gradwohl 1997, Bates 2000). Sugar cane and other crops are a true barrier for manakins and other small forest dweller birds. The Blue-manakin is restricted to forest habitats, and has low propensity to cover open areas (Sick 1997). The study site is completely disturbed and the few small Atlantic forest remnants are tens of kilometers apart one another. Furthermore, this area is at least 150 km away from the remaining large forested areas, which are well preserved in the mountain ranges along Atlantic coastal regions. Then, if some immigration occurs, it is an improbable factor contributing to additional genetic variability.

Although not statistically significant, values obtained for both H_E and allelic richness were lower in the fragment population. Given that the obtained sample must be representative of the population (see Figure 1), some alleles have been likely lost, specially the rare ones, what is expected from genetic drift for such a small and isolated population (Fuerst and Maruyama 1986, Leberg 1992).

The unexpected loss of heterozygotes detected for locus LTMR15 in the control population was probably caused by null allele and/or allele dropout effects in this locus specifically for this population, since loci LTMR8 and SJR133 were in Hardy-Weinberg equilibrium and did not present heterozygote deficit.

One of the major objective in analyze genetic variability in disturbed ecosystems is to determine minimum area requirements to maintain long-term viable populations (Haig 1998). The genetic variability of fragmented populations has been rarely assessed in Neotropical birds (Bates 2000), and this is the first study to report data on Atlantic forest species. Forest fragmentation has been considered the main cause of bird population decline in Atlantic forest (Willis 1979, Aleixo and Galetti 1997). In a near future a large number of species and populations are expected to disappear if not submitted to management (Brooks and Balmford 1996, Aleixo and Galleti 1997). Genetic variability analysis will be pivotal in determining minimum viable populations and minimum area requirements, as well as providing information on genetic distance of populations, which could be important to guide possible translocation plans and corridors establishment (Gilpin and Soulé 1986, Storfer 1999). By now, however, it is not possible to conclude that other fragments even about the same size of the studied area could maintain significant levels of genetic variability in Blue-manakin populations, since populations inhabiting different fragments can experience bottlenecks and/or founder events with variable intensities (Bouzat *et al*. 1998, Tarr *et al*. 1998).

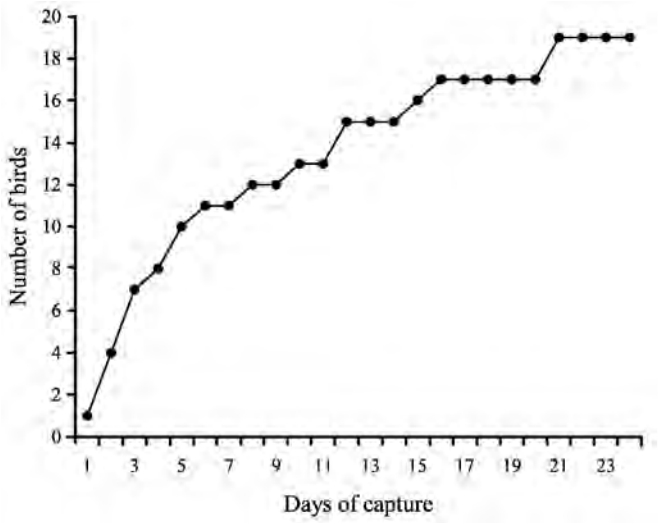

Figure 1. Cumulative number of Blue-manakins captured in a 112 ha Atlantic forest fragment. Each capture day sums eight hours of capture using eight 12 x 2.5 m mist nets. The capture rate significantly changed after 16 days of capture.

The Blue-manakin is not currently endangered in the Atlantic forest, but this species is a component of the important guild of Atlantic forest understory seed dispersers bird, and its absence in some forest fragments could contribute to reduced habitat resilience and affect the population of some plants which they disperse (Foster 1987, Galetti and Pizo 1996, Pizo 1997).

The magnitude of the habitat fragmentation effects on the genetic diversity of Atlantic forest birds may be better understood only when the genetic variability and structuring of non-fragmented populations to be known, which will be achieved with studies on a more broad geographical scale, including populations distributed within the remaining continuous areas.

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