Population structuring of the endemic black-cheeked gnateater, *Conopophaga melanops melanops* (Vieillot, 1818) (Aves, Conopophagidae), in the Brazilian Atlantic Forest

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(With 1 figure)

Abstract

Randomly amplified polymorphic DNA (RAPD) markers were used to analyze genetic differentiation among three populations of the endemic Black-cheeked Gnateater (*Conopophaga melanops melanops*) within a larger pristine reminiscent of the Brazilian Atlantic Forest. Analyses of molecular variance (AMOVA) (ϕ_{ST} = 0.13149, P < 0.0001) and the nonparametric test for homogeneity of the molecular variance (HOMOVA) (B = 0.32337; P = 0.0019) showed a statistically significant genetic divergence among the three Black-cheeked Gnateater populations in a continuous transect of 250 km. Some hypothetic explanations for these results are the sedentary nature of the species and the historical isolation of the populations in refuges during the Pleistocene. The present results suggest that the local populations were naturally differentiated along the entire original range before the recent process of massive deforestation.

Keywords: population genetics, suboscines passerines, tropical forest, conservation genetics.

Estruturação populacional do endêmico cuspidor-de-máscara-preta, *Conopophaga melanops melanops* (Vieillot, 1818) (Aves, Conopophagidae), na floresta Atlântica brasileira

Resumo

Marcadores polimórficos de DNA amplificados ao acaso (RAPD) foram utilizados para analisar a diferenciação genética entre três populações do endêmico cuspidor-de-máscara preta (*Conopophaga melanops melanops*) de um amplo remanescente preservado da floresta Atlântica brasileira. Análises de Variância Molecular (AMOVA) (ϕ_{ST} = 0,13149, P < 0,0001) e o teste não paramétrico para a homogeneidade da variância molecular (HOMOVA) (B = 0,32337; P = 0,0019) comprovaram uma divergência genética significativa entre as três populações em um transecto contínuo de 250 km. Algumas explicações hipotéticas para estes resultados são a natureza sedentária da espécie e o isolamento histórico das populações em refúgios durante o Pleistoceno. Os presentes resultados sugerem que as populações locais foram diferenciadas naturalmente ao longo da distribuição original antes do recente processo de intenso desmatamento.

Palavras-chave: genética de populações, passeriformes suboscines, floresta tropical, genética da conservação.

1. Introduction

Determining the genetic structure of populations is essential for testing hypothesis about factors promoting diversification and speciation (McDonald, 2003), as well as for finding appropriate scales for conservation and management (Cegelski et al., 2003). However, studies addressing the genetic structure of Neotropical birds are scarce (Bates, 2000; Höglund and Shorey, 2003; McDonald, 2003; Francisco et al., 2006) and little is known whether the genetic diversity of these species has been lost or not due to habitats destruction.

Bates (2000) demonstrated substantial genetic differentiation among populations of some Amazon forest understory birds in small geographic distances within a continuous forest, and the levels of divergence were greater than those observed for comparable populations of many temperate birds. The sedentary nature of these

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animals may be the primary reason for their diversification, and refuges scenarios could also have contributed (Bates et al., 1999; Bates, 2000).

The Black-cheeked Gnateater *Conopophaga melanops melanops* (Conopophagidae) is a small insectivore suboscine passerine endemic from the Brazilian Atlantic Forest. Its distribution ranges from southeastern Bahia to Santa Catarina (Ridgely and Tudor, 1994) in the coastal lowlands (from 0 to 800 m) (Parker III et al., 1996), where it inhabits forest understory and forest floor (Sick, 1997). Although there are no studies that have evaluated the dispersal pattern of this species, its particular behavior (Alves and Duarte, 1996; Sick, 1997; Alves et al., 2002) and morphological traits, such as long legs and fingers and short wings and tail (Ridgely and Tudor, 1994; Sick, 1997), suggest that Black-cheeked Gnateater is dependent of the forest interior, being relatively sedentary. While the species is still readily found in large forested areas, it is disappearing in many small isolated fragments (Alves and Duarte, 1996; Willis and Oniki, 2003).

The Brazilian Atlantic Forest is one of the five most important hotspots on Earth, presenting not only an exceptional biodiversity and endemism concentration, but also a drastic loss of habitats (Myers et al., 2000). It was originally one of the largest North-South tropical forests in the world, occurring from Rio Grande do Norte (6° S) to Rio Grande do Sul (30° S) States, and was about 200 km wide (Aleixo and Galetti, 1997). Today, most of the forest was logged for sugar cane and coffee plantations (Willis, 1979; Willis and Oniki, 1992), and only 7.5% of its original area remain (Myers et al., 2000) distributed in several small and disconnected fragments and a few large forest tracts (Aleixo and Galetti, 1997). The larger pristine reminiscent is located at São Paulo State, southeastern Brazil, which comprises several contiguous conservation units mostly distributed along the steep coastal slopes and mountain zones of Serra do Mar and Serra de Paranapiacaba regions. It composes a continuum of approximately 600,000 ha (Conselho Nacional da Reserva da Mata Atlântica, 1997), which presumably represent an Atlantic Forest area in which the genetic structure of passerine bird populations could be studied without the interference of habitat fragmentation effects.

The objective of this work was to investigate the spatial distribution of the genetic diversity of Black-cheeked Gnateater along the Atlantic Forest continuum, on a short geographical scale. Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990) analyses were able to demonstrate the occurrence of population structuring in the studied species, indicating that unique local gene complexes can be lost with the increasing deforestation process. If these results are representative of other understory Atlantic Forest passeriforms, they demonstrate the need of establishing protected areas covering as much as possible of the species range in order to achieve genetic variability conservation.

2. Material and Methods

2.1. Study area and birds sampling

This study was carried out at Serra do Mar State Park and Juréia-Itatins Ecological Station. These conservation units are distributed within the areas that compose the larger remaining Atlantic Forest continuum, localized in São Paulo State, southeastern Brazil. Serra do Mar State Park presents 315,390 ha, and Juréia-Itatins Ecologial Station 79,270 ha (Conselho Nacional da Reserva da Mata Atlântica, 1997). Serra do Mar State Park is distributed from northern to central São Paulo State coast, and is subdivided in eight administrative units. Birds were sampled at Picinguaba (23° 20' S and 44° 50' W) and Caraguatatuba (23° 35' S and 45° 23' W) administrative units. Juréia-Itatins Ecological Station (24° 23' S and 47° 01' W) is localized in central São Paulo State coast and is partially connected with Serra do Mar State Park. During the terminal Pleistocene (13,000 to 18,000 years ago), these areas were forest refuges separated from one another by opened and drier vegetation (Viadana, 2002). The linear distance among the study sites varies from 70 km (Picinguaba – Caraguatatuba) to 250 km (Picinguaba – Juréia). All these sample sites are located in the coastal lowlands. Although the higher peaks throughout the study sites are more than 1,100 meters high, they do not interrupt the lowlands that surround them alongside the coast, suggesting that the sampled populations are not currently isolated by geographical barriers.

Fifty-one Black-cheeked Gnateaters were captured from 2003 to 2005. The number of specimens sampled in each locality was: 11 males and nine females in Picinguaba, seven males and four females in Caraguatatuba, and 17 males and three females in Juréia. All birds were caught by a series of mist-nets or lured into a net using playback of song vocalizations. The captured birds were marked with metal rings and rapidly released after the blood sampling.

2.2. Blood collection and DNA extraction

The blood samples were obtained from the vein in the interior of the nail. Before the blood samples were collected, a nail of each specimen was washed with 70% ethanol and cut in the tip. Approximately 10-20 µL of blood were collected and mixed with 10 µL of EDTA (0.5 M), and immediately placed in a 1.5 mL tube with 1 mL of 100% ethanol. After this procedure, the nail was cleaned with 70% ethanol and hemostatic solution to avoid any injury to the captured birds. The samples were stored in –22 °C freezer until the DNA extraction in the laboratory. The DNA extractions followed Lahiri and Schnabel (1993), and the DNA samples were dissolved and stored in lower EDTA concentrations (10 mM Tris-HCl and 1 mM EDTA).

2.3. RAPD-PCR Reactions

A set of six primers (primer 1 [GGTGCGGGAA], primer 2 [GTTTCGCTCC], primer 3 [GTAGACCCGT],

primer 4 [AAGAGCCCGT], primer 5 [AACGCGCAAC], and primer 6 [CCCGTCAGCA]) from GE Healthcare was used to perform RAPD-PCR reactions with the Ready-to-go RAPD Analysis Beads kit (GE Healthcare). The mixture contained thermostable polymerases (AmpliTaq DNA polymerase and Stoffel fragment), dNTPs (0.4 mM of each dNTP), BSA (2.5 µg), buffer [3 mM $MgCl_2$, 30 mM KCl, and 10 mM Tris (pH 8.3)], 25 pmol of a single RAPD primer, 50 ng of DNA, and distilled water, to a total volume of 25 µL. Amplifications were carried out in a PTC-100 thermocycler (MJ Research, Inc.) with the following profile: 1 cycle at 95 °C for 5 minutes followed by 45 cycles of 65 °C for 1 minute, 36 °C for 1 minute, and 72 °C for 2 minutes. Eight microliters of the amplification products were mixed with 1.6 volumes of a loading buffer and run in 1.5% agarose gels in 1 x TBE buffer, with 0.5 µg.mL^{-1} of ethidium bromide. The products were then electrophoretically separated at 90 V for 180 minutes and photographed under UV light with a Kodak EDAS imaging system.

Only clear and unambiguous bands were selected for statistical analyses. To ensure that banding patterns were repeatable, a subset of five individuals was replicated for each primer (always the same individuals) in alternated PCR procedures (see Bouzat, 2001). One negative control (without template DNA) was included in each set of amplifications.

2.4. Statistical analyses

The RAPD gels were analyzed by the presence (1) / absence (0) of each selected (scorable) band. Afterwards, a specific pattern was generated for each specimen, and a binary data matrix was constructed including all of them.

To verify if the number of polymorphic bands was adequate to obtain a good level of precision in the statistical analyses, a bootstrap procedure was utilized to estimate the mean variation coefficient (CV) of the Simple Matching index (5,000 permutations), according to Tivang et al. (1994). This analysis was performed using the software DBOOT (v.1.1) (Coelho, 2000).

The binary data matrix was then submitted to the Euclidean metric distance of Excoffier et al. (1992), using the software AMOVA-PREP (Miller, 1998), and the derived pairwise phenotypic distances matrix was used for the analysis of molecular variance - AMOVA (Excoffier et al., 1992), in WINAMOVA 1.55 (Excoffier, 1992). AMOVA uses the phenotypic distances to describe how the RAPD variance is partitioned in different hierarchical levels (e.g. within and among populations), and tests for significance against the null hypothesis of no population structure (Excoffier et al., 1992; Stewart and Excoffier, 1996). This analysis provides the ϕ_{ST} value, analogous to F_{ST} (Wright, 1951), which is a measure of population subdivision (Zwartjes, 1999), or, in other words, a measure of the degree of genetic differentiation among populations. To test the hypothesis that the observed ϕ_{ST} and component variances values were significantly different from zero and were not generated by chance alone, a nonparametric procedure consisting of 9,999 independent random permutations of individual RAPD patterns across populations was performed (keeping sample sizes constant) to generate the null distributions of the ϕ_{ST} values and the component variances of the AMOVA.

It was also utilized a nonparametric test for homogeneity of molecular variance (HOMOVA) (Stewart and Excoffier, 1996), based on Bartlett's statistic (Bartlett, 1937), to test whether populations have different amounts of RAPD variation (e.g. Stewart and Excoffier, 1996; Lacerda et al., 2001). The HOMOVA tests for statistical differences in intrapopulational molecular variance (heterocedasticity among populations) using the value B of the Bartlett's statistic; B expresses a deviation of population variances from the mean total variance, and its significance is tested by computing the null distribution of Bartlett's statistics utilizing the same procedure used for variance components in the AMOVA (Stewart and Excoffier, 1996). This test was also performed using the software WINAMOVA 1.55 and utilized 9,999 permutations in the significance test.

3. Results

The six RAPD primers used in the PCR amplifications produced 87 distinct bands with the potential to be analyzed. Of this total, 63 were polymorphic (72.41%), generating 51 individually distinct RAPD patterns. The number of polymorphic bands per primer varied from 7 to 15. The size of the bands varied from 300 to 1,500 base pairs (bp). Exclusive RAPD bands were not found within a unique population.

The RAPD-PCR repeatability test revealed that the 63 polymorphic bands were reliable (Figure 1). All the negative controls amplified no bands whatsoever. Also, the bootstrap procedure revealed that the 63 polymorphic bands were enough to obtain a good precision in the genetic analyses (CV < 11%).

AMOVA showed a statistically significant divergence when the three populations were analyzed together (Table 1) and when they were pairwise analyzed (Table 2). Even the Picinguaba-Caraguatatuba pair, distant approximately 70 km, presented a small but significant level of genetic differentiation.

The nonparametric test for homogeneity of the molecular variance (HOMOVA) revealed that the intrapopulational molecular variances were statistically different (heterocedasticity among the three populations: B = 0.32337; P = 0.0019). The same result was observed when the populations were pairwise analyzed: Caraguatatuba–Picinguaba (B = 1.5612; P = 0.0024), Caraguatatuba–Juréia (B = 3.7149; P < 0.00001), Picinguaba–Juréia (B = 4.4882; P < 0.00001), therefore suggesting that the analyzed populations have different amounts of RAPD genetic diversity.

4. Discussion

The significant genetic structuring of Black-cheeked Gnateater, even at a small geographic scale, suggests a
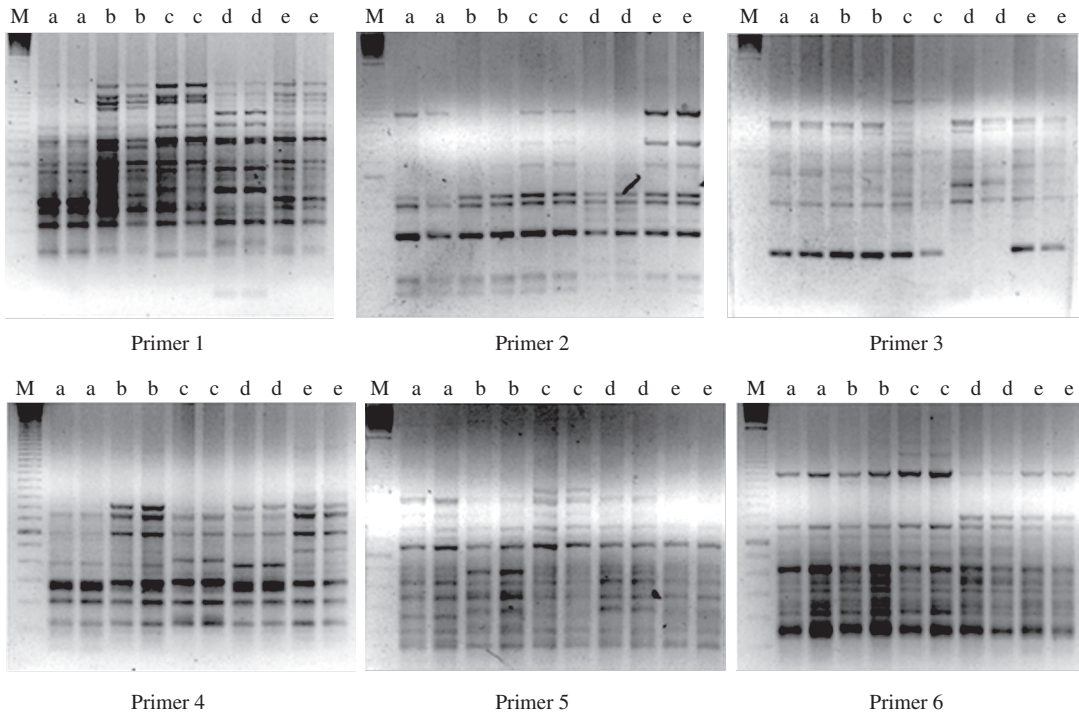
Figure 1. Agarose gels demonstrating the patterns of repeatability of the bands obtained with six RAPD primers (1, 2, 3, 4, 5, 6) using five *Conopophaga melanops melanops* individuals (a, b, c, d, e), in two alternated PCR procedures. M is the molecular weight marker (100 bp ladder).

Table 1. Analysis of molecular variance (AMOVA) in three populations of *Conopophaga melanops melanops* (Picinguaba, Caraguatatuba, Juréia). The P values were calculated using the comparison of the ϕ_{ST} and variance values obtained by 9,999 permutations of the RAPD patterns between populations.

Source of Variation	Degrees of Freedom	Sum of Squares	φ statistic	P
Among population	2	68.375	$\phi_{ST} = 0.13149$	<0.0001
Within populations	48	469.723	$1-\phi_{ST} = 0.86851$	<0.001
Total	50	538.098	-	-

Table 2. The values below the diagonal represent the ϕ_{ST} values between the pairs of *Conopophaga melanops melanops* populations. Above the diagonal are the linear distances (km) between the populations.

	Picinguaba	Caraguatatuba	Juréia
Picinguaba	-	70	180
Caraguatatuba	0.03433**	-	250
Juréia	0.163502***	0.16779***	-

**0.01 < P < 0.0001
***P < 0.0001

sedentary nature, leading to a gene flow that is naturally low. It is in agreement with what would be expected for this species, considering its morphological characteristics. Behavioral aspects seem to be a major explanation for genetic structuring, specially for Picinguaba and Caraguatatuba populations, since they are only 70 km apart and historical records demonstrate that no isolation occurred between these areas in the last glaciation period (Viadana, 2002).

Furthermore, there are no antropic deforestations affecting the connection of these studied sites. During the field work in the Juréia-Itatins Ecological Station, two Black-cheeked Gnateaters were recaptured, one male and one female, that had been marked eight and six years ago, respectively, in the same local geographic position, corroborating the assumption of low vagility, at least for the adults (these recapture data were certificated by CEMAVE/IBAMA).

These results agreed with the data presented by Francisco et al. (2007) that found significant population structuring among five populations of *Chiroxiphia caudata* (Shaw and Nodder, 1793) sampled along a 414 km transect that covers most of this largest Atlantic Forest continuum and includes the areas studied in the present work.

Bates et al. (1999) and Bates (2000) also found substantial levels of genetic differentiation among Amazonian passerine populations in a continuous forest at a local scale (sites 200 km apart), which were explained by the sedentary nature of the studied species. On the other hand, Dantas et al. (2007) did not find significant genetic structuring in the congener *Conopophaga lineata* (Wied, 1831) in semideciduous Atlantic Forest fragments of Minas Gerais State, even when RAPD markers were applied to populations that were more than 600 km apart. Likewise, McDonald (2003) found relatively low levels of genetic differentiation between two populations of Long-tailed Manakin, *Chiroxiphia linearis* (Bonparte, 1838), in Costa Rica, between a mid-elevation (1,300 m) and a sea-level site separated by 115 km, using microsatellite markers. If we consider all these data, it seems that sedentariness levels can vary among Neotropical forest understory birds, and a significant portion of them may present genetic structuring.

Based on the historical records of São Paulo State forest refuges (Viadana, 2002), understory bird populations could have gone through a period of intense isolation in the last glaciation period, which occurred in the terminal Pleistocene (see Viadana, 2002). In the Holocene, these populations would have expanded in a discontinuous fashion from these forest refuges. Although data from Picinguaba and Caraguatatuba indicate that a low natal dispersal may be a major factor causing the genetic structuring in Black cheeked Gnateater, historical isolation and recent deforestations can not be ignored as additional differentiation parameters when comparing Juréia to the other populations. Just because Juréia was an isolated refuge that experienced a period of potential independent evolution in the last glaciation event (Viadana, 2002). Furthermore, although still connected to the tract of forest composed by Picinguaba and Caraguatatuba, the areas surrounding Juréia Ecological Station are highly deforested (Fundação SOS Mata Atlântica and Instituto Nacional de Pesquisas Espaciais, 2002) due to human impacts, which can be reducing the current levels of gene flow.

The present results suggest that the local populations of Black-cheeked Gnateater were naturally differentiated along its entire original range before the recent process of massive deforestation, mainly due to its sedentary behavior. Given the gravity of the current condition of the Atlantic forest (see Myers et al., 2000), it could be supposed that a significant portion of the gene pool of this species may already have been lost with the rampant reduction and fragmentation of this biome. Therefore, the spatial distribution of the genetic variability of the different species should be a key factor to be considered in the development of conservation actions for this biome.

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