Atlantic Forest fragmentation affects the genetic variation distribution pattern in blue manakins, *Chiroxiphia caudata* (Aves, Pipridae)

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Habitat fragmentation is one of the main threats to the biodiversity and one of the main challenges faced by conservation biology. This study assessed the effects of habitat fragmentation on the genetic variability of the blue manakin *Chiroxiphia caudata*, an endemic bird of Atlantic Forest biome. Nine microsatellite loci were used to analyze individuals from five Atlantic Forest areas. Private alleles were found in all areas. F_{st}, D_{est}, Bayesian and Discriminant analysis of principal components (DAPC) indicated that populations are genetically structured, but the distance could not explain the differentiation between areas. The fragmentation and the reduction of gene flow may be acting in order to increase the differentiation between areas. Thus, even a generalist species may be affected by habitat fragmentation. Despite this, the whole complex of fragmented areas in Atlantic Forest appears to play an important role for the blue manakin by sheltering its genetic diversity as a whole.

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Abstract

Habitat fragmentation is one of the main threats to the biodiversity and one of the main challenges faced by conservation biology. This study assessed the effects of habitat fragmentation on the genetic variability of the Blue Manakin *Chiroxiphia caudata*, an endemic bird of Atlantic Forest biome. Nine microsatellite loci were used to analyze individuals from five Atlantic Forest areas. Private alleles were found in all areas. F_{st}, D_{est}, Bayesian and Discriminant analysis of principal components (DAPC) indicated that populations are genetically structured, but the distance could not explain the differentiation between areas. The fragmentation and the reduction of gene flow may be acting in order to increase the differentiation between areas. Thus, even a generalist species may be affected by habitat fragmentation. Despite this, the whole complex of fragmented areas in Atlantic Forest appears to play an important role for the blue manakin by sheltering its genetic diversity as a whole.

Keywords: Pipridae, Population structuring, Conservation biology, Habitat fragmentation

Introduction

Habitat fragmentation stands as one of the main threats to biodiversity and represents an important challenge to conservation biology (Ellis, 2013; Fahrig, 2003; Henle et al., 2004). The fragmentation process may include both habitat loss and the broken apart of habitat into various habitat patches of smaller size (Fahrig, 2003; Wilcove, McLellan & Dobson, 1986). The resulting impacts are (i) the reduction in the total area of the habitat, (ii) the subdivision of the area into various habitat patches, (iii) the decrease in the average size of each habitat patch, and (iv) the isolation of the habitat patches (Fahrig, 2003). Also, habitat patches may become isolated from one another by a matrix of unnatural habitats with different degrees of resistance to species movement (Ewers & Didham, 2006).

A growing number of works have revealed how habitat fragmentation can affect the genetic characteristics of wild populations, being the main consequences the loss of genetic diversity, inbreeding within the patches (Dixo et al., 2009; Haag et al., 2010), and promoting population structuring between patches of habitats (Federman et al., 2014; Haag et al., 2010; Leonardi et al., 2012) even for generalist species (McManus et al. 2015; Saranholi, Chávez-Congrains & Galetti, 2017). These effects are resulted from the increasing of genetic drift and restriction of gene flow in increasingly isolated populations due to habitat fragmentation and, in a long-term, it translates into reduced chances of survival of local populations due to limited adaptability (Frankham, 2005).

The Atlantic Forest (AF) is one of the 25 biodiversity hotspots in the world, characterized by high levels of endemism and by a remarkable loss of natural vegetation (Myers et al. 2000). All these hotspots together covered around 12% of world's surface, but today they represent only

1.4%, while they still host about 567 endemic vertebrates (Galindo-Leal & Câmara, 2003; Myers et al., 2000). The Atlantic Forest is limited to only 11.4% to 16% of its original 150 million hectares, and from the remaining forest, only 9.3% is under protection, mostly in fragmented landscapes (Ribeiro et al., 2009).

Tropical forest understory passerine birds have been demonstrated to be highly sensitive to forest fragmentation due to limited capability to disperse across artificial matrixes of habitats (Bates, 2000; Hansbauer et al., 2008a). Although the ecological and demographic effects of forest fragmentation on these birds have been elucidated in last few years (Moore et al. 2008; Robinson, 1999; Stouffer and Bierregaard Jr., 1995), the genetic impacts of forest fragmentation are still limited to a few empirical evaluations (Cegelski, Waits & Anderson, 2003; Eldridge, Kinnear & Ornus, 2001; Khimoun, et al., 2016; Manel, Berthier & Luikart, 2002).

The Blue Manakin (*Chiroxiphia caudata*), is an Atlantic Forest endemic passerine bird. Piprids in general are known for their attractive colors and their collective prenuptial rituals. They represent one of the most abundant families in sub-tropical forests understory in Central and South Americas (Snow, 2004; Traylor & Fitzpatrick, 1982). The Blue Manakin is known to prefer the innermost areas to borders, but this characteristic does not stop them from moving between patches. Its home range becomes wider in fragmented environments than in non-fragmented ones, probably due to the need of searching for food and shelter, and nesting (Hansbauer et al., 2008a). Males have smaller and more settled home ranges, whilst during pre-reproduction females may increase those areas covering a total of 460 ha, moving between patches that are up to 3.5 km away (Hansbauer et al., 2008b). This species may even be capable to make use of anthropic habitats in various degrees (Hansbauer et al., 2010). Uezu et al. (2005) showed that, within the habitat fragmentation scenario, the Blue Manakin may use forest

92 corridors as their habitat and the distance between patches seem have no negative impact on the
93 abundance of this species, or do not represent high costs for dispersion, at least in short scale. On
94 the other hand, previous analyses of genetic variability and population structuring performed in
95 five populations distributed across a 420 km Atlantic Forest continuum, (Serra de Paranapiacaba
96 and Serra do Mar), demonstrated significant values of genetic differentiation, which were mainly
97 explained by geographical distances between populations. As the areas were devoid of natural or
98 anthropic barriers, behavioral characteristics of the Blue Manakin may be involved in the
99 population structuring (Francisco et al., 2007).

100 The present study aimed to verify how the genetic diversity is distributed in populations of
101 *Chiroxiphia caudata* inhabiting different patches in the fragmented coastal Atlantic Forest. Our
102 hypothesis is that habitat fragmentation can reduce the genetic variation in local populations,
103 while promotes or enhances genetic differentiation between them. Whether any neutral
104 microsatellite signature among populations can be accounted to habitat fragmentation, an
105 isolation by distance model, as view in Francisco et al. (2007), is not expected, and fragmented
106 population will be more divergent. Thus, we also aim to add knowledge on the impacts of habitat
107 fragmentation to the genetic characteristics of tropical forest understory birds.

108

109 **Material and Methods**

110 **Areas of study**

111 Five areas located within the range of the coastal Atlantic Forest (AF) were chosen for this study.
112 Our choice based in sampling small fragments (3 areas), nearest to large urban centers, and two

other sites inside a less modified continuous area of the coastal Atlantic Forest, all of them located in São Paulo State, Brazil.

The three fragmented areas sampled were Reserva do Morro Grande (RMG - 23°43'S, 46°57'W) and Caucaia do Alto (FCA - 23°43'S, 47°01'W), both in the Cotia municipality (SP), and Parque Estadual da Serra da Cantareira (PESC - 23°24'S, 46°35'W), São Paulo (SP). The two areas inside the AF continuous were located about 350 km apart from each other, Parque Estadual de Carlos Botelho (PECB - 24°04′S, 47°57′W), São Miguel Arcanjo (SP), and Estação Ecológica da Boracéia (EEB - 23°37'S, 45°52'W), Salesópolis (SP) (Fig. 1). Based on these, we were able to establish a genetic pattern in fragmented sites, having the continuous area as a control.

Sampling

Specimens from Parque Estadual da Serra da Cantareira (PESC) and Estação Ecológica da Boraceia (EEB) were previously collected and are housed at Museu de Zoologia da Universidade de São Paulo (MZUSP), and the samples are represented by muscle tissues. Blood previously collected or DNA samples of individuals from Reserva do Morro Grande (RMG) and Caucaia do Alto (FCA) were kindly provided by Laboratório de Genética e Evolução Molecular de Aves (LGEMA - USP). Blood samples of individuals from Parque Estadual de Carlos Botelho (PECB) were previously stored at Laboratório de Biodiversidade Molecular e Conservação (LBMC – UFSCar). All samples were collected between 2003 and 2008. A total of 78 samples were obtained; 20 from PECB; 10 from PESC; 18 from EEB; 15 from RMG, and 15 from FCA. The samples collection was conducted according to Brazilian legal requirements and the collection license was provided by Instituto Chico Mendes de Conservação da Biodiversidade (SISBIO - 10013-1).

135

136

137 **DNA extraction and microsatellite PCR**

138 The DNA extraction from blood samples and muscle tissues were performed through the
139 standard Phenol/Chloroform/Isoamyl alcohol protocol (Sambrook, Fritsch & Maniatis,1989).

140 PCR reactions and their amplification conditions for the microsatellite loci were adapted from
141 Francisco, Galetti & Gibbs (2004) and Schuelke (2000). Nine microsatellites (CHIR 1-6, CHIR
142 1-18, CHIR 3-22, CHIR 3-27, CHIR 4-21, CHIR 4-33, CHIR 4-34, CHIR 3-15 and CHIR 2-9)
143 described for *Chiroxiphia caudata* (Francisco, Galetti & Gibbs, 2004) were used. DNA
144 amplification followed a three-primers reaction as described in Schuelke (2000), adding an M13
145 labeled-primer. An M13 tail was added to the 5' end of a specific primer, searching for the best
146 condition to avoid hairpin-loop and changes in annealing temperature. For the CHIR1-16,
147 CHIR3-15, CHIR4-33 and CHIR4-34 loci the M13 tail was added to the forward primer and for
148 CHIR1-18, CHIR2-9, CHIR3-22, CHIR3-27 and CHIR4-21 to the reverse one. The genotyping
149 was carried out using a 3730XL automatic sequencer (Applied Biosystems, USA), and the
150 GENEIOUS 3.4.6 software (Kearse et al., 2012) was used to determine the size of alleles.

151 **Genetic diversity analyses**

152 The presence of null alleles was verified using the MICROCHECKER software (van Oosterhout
153 et al., 2004). The polymorphism information content (PIC) was verified by the CERVUS 3.0.6
154 software (Marshal et al. 1998).

For each population, linkage disequilibrium and departure from HWE were verified using GENEPOP 4.2.2 (Raymond & Rousset, 1995). Number of alleles (N_a), effective number of alleles (N_e), expected (H_e) and observed (H_o) heterozygosity were obtained by GeneAlex (Peakall & Smouse, 2006). Allelic richness (R_a), F_{is} and the likelihood significance of deficit (pL) and excess (pS) of heterozygotes were calculated using the FSTAT software (Goudet, 1995). Sequential Bonferroni correction was employed to reduce errors of multiple comparisons when evaluating statistical significance (Rice, 1989). Recent population bottleneck analysis was performed using BOTTLENECK (Piry, Luikart & Cournet, 1999). Although the infinite allele (IAM) model has been seen more sensitive for detecting population bottleneck (Cristescu et al. 2010; Le Page, Livermore & Cooper, 2000), we also evaluated bottleneck using step wise mutation (SMM) and two-phase (TPM) with 70% SMM, 20% variance, and 1,000 iterations. The Wilcoxon test was used to determine the significance ($p<0.05$).

Population structuring analyses

For testing population structuring among the sampled populations, a Bayesian analysis implemented in the STRUCTURE software (Pritchard, Stephens & Donnelly, 2000) was used to assign individuals to populations, determining the most probable number of populations (K), using admixture models, correlated alleles and without and with prior information of locality. In total, there were 20 independent runs of 200,000 of MCMC interactions, each with a burn-in of 100,000 generations. K values varied between 1 and 6. The most appropriate K value was estimated according to Evanno, Regnault & Goudet et al. (2005), as implemented in STRUCTURE HARVESTER (Earl & vonHoldt, 2012), and the CLUMPAK software (Kopelman et al., 2015) was used to obtain the convergence among the independent runs for each K. The population structuring was also verified by the discriminant analysis of principal

components (DAPC) (Jombart, Devillard & Balloux, 2010) in the Adegenet package (Jombart & Ahmed, 2011) implemented in the R statistical environment (R Core Team, 2017). This statistical method reduces the genetic data to principal components and retains for the analyses the components that minimize the variation within the groups and maximize the variation among then (Jombart, Devillard & Balloux, 2010). F_{st} values and their respective significance values were calculated by FSTAT (Goudet, 1995), and the analysis of molecular variance (AMOVA) was performed using ARLEQUIN (Excoffier, Laval & Schneider, 2005). Isolation by distance was tested by correlating F_{st} values with geographical distances, using the Mantel test, implemented in FSTAT (Goudet, 1995). The D_{est}, an estimator of differentiation that can be more accurate than F_{st} for highly polymorphic markers (Jost, 2008), was also calculate and its statistical significance was evaluated in the R statistical environment (R Core Team, 2017) with the DEMEtics package (Jueterbock et al., 2012). Both DAPC and D_{est} have been considered with high sensibility for detecting population structuring (Jombart et al., 2010; Jost, 2008).

Results

Genetic diversity

All nine microsatellites presented PIC values superior to 0.5, varying between 0.77 and 0.95. A total of 155 alleles were found, varying between 12 and 28 alleles per locus, and a mean number of 17.2 alleles. No significant values for linkage disequilibrium were observed. All sampled populations showed HW deviation for deficit of heterozygotes, as well as significant F_{is} positive values after sequential Bonferroni correction (Table 1). The CHIR3-27 locus contained null alleles in all sampled populations, but the population results remained unchanged when such locus was removed from the analysis.

Although the mean observed heterozygosity appears slightly higher in both sites located in the AF continuous, the mean expected heterozygosity was quite similar among the studied areas. The heterozygote deficit values in the AF continuous were smaller than that found in the three fragmented areas. Private alleles were found in all populations, being seven private alleles for the PECB population, 11 for EEB, 10 for RMG, five for FCA, and four for PESC. These data revealed that each sampled population has specific and unique genetic characteristics. As a result, out of 37 private alleles, approximately 51% were located in the fragmented areas of this study. The effective number of alleles per sampled population ranged from 5.8 to 7.1 and the allelic richness from 8.0 to 8.8 (Table 1). Population bottleneck were found in all populations only using the IAM model (IAM, p = 0.005 - 0.007; SMM, p = 0.410 - 0.715; TPM, p = 0.120 - 0.327).

Population structuring

The isolation by distance (IBD) test indicated that there is a positive correlation between both variables, whilst the distance variation in population differentiation (F_{st}) was only 1.34% (r^2: 0.0134). Therefore, no isolation by distance among the studied populations was observed. The analysis of molecular variance (AMOVA) indicated that 96.5% of the variation occurs within population, and only 3.8% between them.

Obtained pairwise F_{st} values were significant for most pairs of populations. Significant differentiation values were not found only between the RMG and FCA areas (Table 2). When performing the differentiation analysis without CHIR3-27 locus (which presented null alleles), the results remained the same for both numerical and pairwise features that had a significant differentiation.

The pairwise comparisons for D_{est} resulted in values ranging from 0.014 to 0.36 and all D_{est} values were statistically significant ($p<0.05$) (Table 2). The values also indicated lower differentiation values among PECB, EED and PESC (0.14 and 0.22) populations, whilst there were greater divergences when these areas were compared with RMG and FCA populations (0.23 - 0.36, respectively) (Table 2).

The Bayesian analysis without and with geographic information *a priori* indicated the occurrence of population structuring, showing K = 2 according to Evanno et al. (2005) (Fig. 2). RMG and FCA areas were recovered in a single cluster, whereas the other three areas were seen in another cluster. The DAPC analysis also indicated the existence of population structuring within the whole sampled area of Blue Manakins but indicated that each of the five-sampled populations represents a different cluster (Fig. 3), as were also suggested by the D_{est} values found.

Discussion

Our results showed a higher average number of alleles per microsatellite loci (17.2) than found by Francisco et al. (2007), working in different populations located in the continuous forest. Although some methodological bias cannot be discarded, such increased overall number of alleles obtained here can be related to an increase of private alleles. While no significant reduction on the genetic variation was detected here when small fragments were compared with the large continuous area, population structuring related to fragmentation was strongly supported by both DAPC and D_{est} analyses. The recent fragmentation process can be promoting population subdivisions, enhancing the drift genetic effects and restricting gene flow between populations, suggesting that loss of within-population genetic variation can occur in the near future. The

recent bottleneck signature detected here by the IAM model for the five areas may be accounted to the recent process of habitat loss and fragmentation.

The population structuring pattern observed among Blue Manakin populations, even within the AF continuous areas, corroborate previous results that found population structuring among several sites across the AF continuous (Francisco et al., 2007). According to these authors populations up to 100 km apart present no differentiation between them, whilst populations more apart (415 km) were differentiated, and stated that a restricted dispersion behavior of this understory bird could define such differentiation pattern across the AF continuous.

However, differently of this latter study, our results showed no a typical IBD pattern among the populations studied herein. The genetic differentiation between PECB and RMG (apart 112 km) or PESC and FCA (apart 57 km) is higher than between PECB and EEB (222 km). Besides distance, corroborating our initial hypothesis, the fragmentation itself appears to drive this population differentiation, reducing gene flow and facilitating divergent genetic drift between the Blue Manakin populations.

Our analyses also indicated a significant deficit of heterozygotes in the majority of populations analyzed, which may be related to the levels of inbreeding found here. The F_{is} value was significantly high even in the larger area representing the continuous of forest (PECB). However, the F_{is} presented the lower values in the two sampled sites located in the AF large continuous, while the three fragmented areas exhibited the higher F_{is} values, suggesting a higher level of inbreeding in the fragmented areas, probably because the reduction of gene flow between them. In addition to fragmentation, the low dispersion capability of this bird (Hansbauer et al., 2008b) could account to the relatively high level of inbreeding, which could also compromise the genetic variation within the fragmented populations.

In sum, our results indicate that habitat fragmentation can be promoting genetic differentiation within Blue Manakins by isolating populations in small habitat patches, enhancing conditions to a consequent within-population genetic variation reduction, and imposing important challenges for the long-term species conservation.

Conclusions

Our study on *C. caudata* showed that even more generalist species might be genetically affected by habitat fragmentation. The metropolitan areas of São Paulo state include the sites with highest deforestation rates for the Atlantic Forest (Teixeira et al., 2009), and it is necessary to take special care when dealing with populations from these areas. The presence of a large number of private alleles in populations living in fragmented areas suggests that all fragments, including in the metropolitan areas, are important to support the allelic diversity of blue manakins. The loss of individuals belonging to these populations may lead to a loss of private alleles, that will never be found in the other population, consequently leading to a reduction of the whole genetic variability of this species. However, reduction in habitat size and expansion in fragmentation may lead to an increased gene flow reduction as well a reduction in the within-population genetic variation, and a likely reduction in the individuals' adaptability to environmental pressures, increasing the extinction risk. Corridors within patched landscape can avoid unrecoverable reduction of within-population genetic variation of a given species (Christie et al., 2015) and it is here suggested that such action could allow gene flow between blue manakin populations, particularly from those small fragments nearest the AF continuous.

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Table 1(on next page)

Summary of genetic diversity estimates in *C. caudata* based on the nine microsatellite loci.

(N) Number of individuals, mean values for observed (Ho) and expected (He) heterozygosity, significance values for deficit of heterozygotes (Pd), values of F_{is} and probability of F_{IS} values significantly differ from zero (Pf). Significant values are indicated by (*). (Na) represents the total number of alleles per area, (MNa) represents the mean number of alleles per locus, (Ne) the average effective number of alleles, (Pa) the number of private alleles and (AR) mean allelic richness.

Areas	N	Ho	He	F_{IS}	Na	MNa	Ne	Pa	AR
PECB	20	0.74	0.84	0.145*	97	10.7	7.1	7	8.5
EEB	18	0.71	0.83	0.169*	97	10.8	6.8	11	8.6
PESC	10	0.62	0.81	0.280*	72	8.0	5.8	4	8.0
RMG	15	0.66	0.84	0.251*	93	10.3	7.0	10	8.8
FCA	15	0.68	0.81	0.196*	93	10.3	7.3	5	8.8

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Table 2(on next page)

Genetic differentiation pairwise comparisons between populations of *C. caudata*. D_{est} values are given above the diagonal whereas F_{st} values are given bellow the diagonal.

(*) represents significant values of genetic differentiation ($p<0.05$).

1

2

	PECB	EEB	PESC	RMG	FCA
PECB	0	0.18*	0.14*	0.36*	0.23*
EEB	0.076*	0	0.22*	0.27*	0.23*
PESC	0.092*	0.067*	0	0.31*	0.28*
RMG	0.194*	0.145*	0.182*	0	0.18*
FCA	0.114*	0.157*	0.212*	0.107	0

3

Figure 1

The five sampled areas.

Parque Estadual de Carlos Botelho – PECB (37,644.36ha), Caucaia do Alto – FCA (10,000ha), Reserva de Morro Grande – RMG (10,000ha), Parque Estadual da Serra Cantareira – PESC (7,916.52ha) and Estação Biológica Boracéia – EEB (16,450 ha).

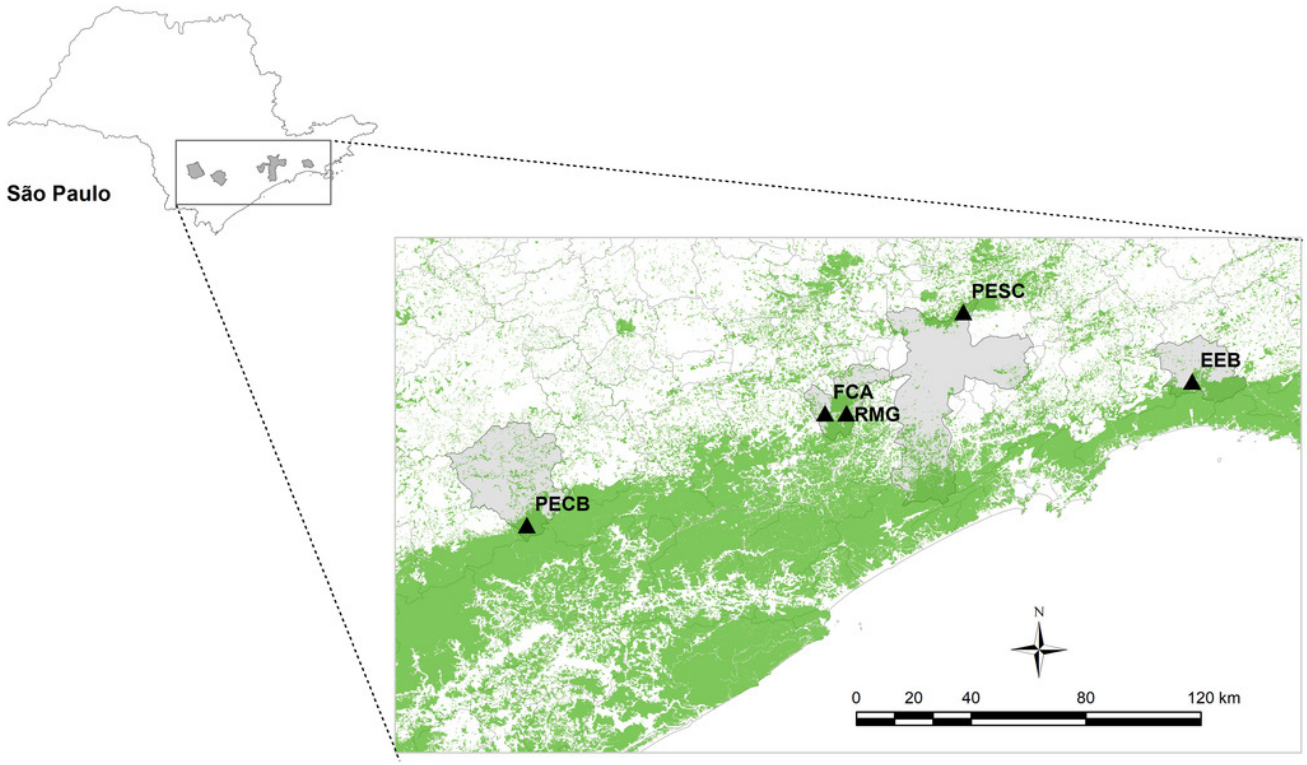

Figure 2

Bar plots of the individual assignments in Bayesian clustering analysis conducted in the STRUCTURE software (Pritchard et al., 2000) for K=2 determined through Delta K (Evanno et al., 2005).

(a) without prior information of population origin; (b) with prior information of population origin.

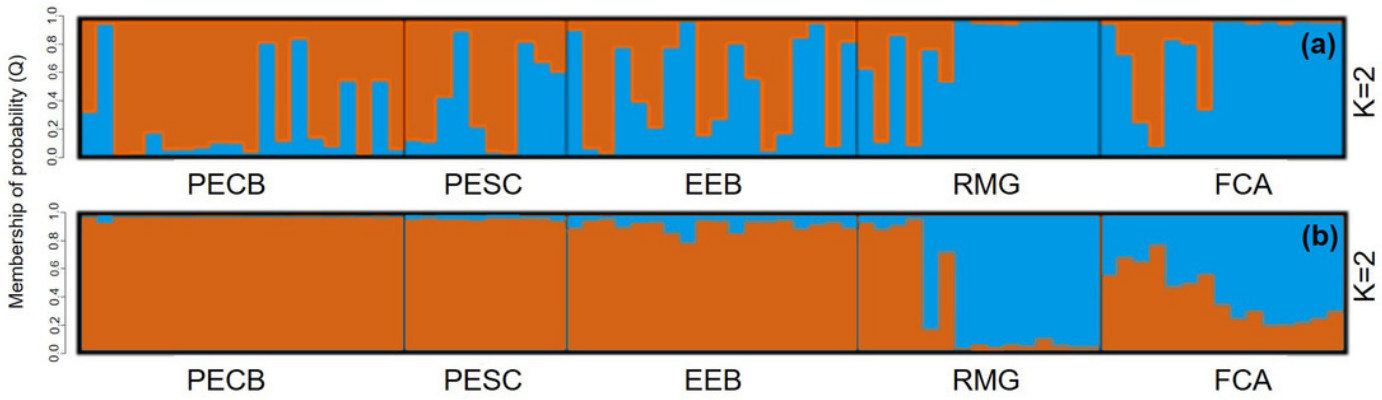

Figure 3

DAPC of blue manakins data. Scatterplot of principal components 1 and 2 with points representing individual genotypes sampled.