ORIGINAL ARTICLE

Vocal variation in relation to genetic structure in an Atlantic forest woodcreeper (*Xiphorhynchus fuscus*): evolutionary and taxonomic implications

Natalia C. García[1] · Luciano N. Naka[2] · Gustavo S. Cabanne[1]

Abstract

The analysis of intra-specific geographical variation is central to the study of the evolutionary and speciation processes, providing insights into the mechanisms leading to current diversity patterns. In the last decades, analyses of within-species variation moved its focus from phenotypic traits to the analysis of variation in genetic markers. Modern phylogeographic studies could be significantly improved by including detailed phenotypic variation analyses, allowing a better understanding of the biological processes driving similar or opposing patterns in phenotype and molecular markers differentiation. Here, we analyze vocal variation within the Lesser Woodcreeper (*Xiphorhynchus fuscus*), an Atlantic forest suboscine passerine, in relation to the genetic lineages previously described for this species. Our results show that two of the genetic clades within this species have their own vocal type, while the other two lineages share a common type. This finding supports the notion that the same events which modeled part of the genetic structure within *X. fuscus* also led to the accumulation of vocal differences. It also provides evidence to support the elevation of the subspecies *atlanticus* to a species-level taxon.

Keywords Bird song · Intra-specific variation · Neotropics · Suboscines · Subspecies

Zusammenfassung

Stimmliche Variation in Relation zur genetischen Struktur bei einer Baumsteigerart (Xiphorhynchus fuscus) atlantischer Wälder und ihre Bedeutung für Evolution und Taxonomie

Für Studien der Evolution und von Artbildungsprozessen ist die Untersuchung intraspezifischer geografischer Variation von zentraler Bedeutung und ermöglicht Rückschlüsse auf die Mechanismen, welche zu den derzeitigen Diversitätsmustern geführt haben. Bei Analysen innerartlicher Variation hat sich während der letzten Jahrzehnte der Schwerpunkt von phänotypischen Merkmalen zur Analyse der Variation genetischer Marker verlagert. Die Einbeziehung detaillierter Analysen phänotypischer Variation wäre ein signifikanter Gewinn für heutige phylogeografische Untersuchungen und würde zu einem besseren Verständnis der biologischen Prozesse führen, welche ähnliche oder entgegengesetzte Muster bei der Differenzierung phänotypischer und molekularer Marker entstehen lassen. Hier untersuchen wir die stimmliche Variation beim Blasskehl-Baumsteiger (*Xiphorhynchus fuscus*), einer suboszinen Singvogelart atlantischer Wälder, in Relation zu den bisher beschriebenen genetischen Abstammungslinien für diese Art. Unsere Ergebnisse zeigen, dass zwei der genetischen Kladen

Communicated by S. Kipper.

✉ Natalia C. García
ngarcia@macn.gov.ar

Extended author information available on the last page of the article

innerhalb der Art ihren eigenen Stimmtyp besitzen, während sich die zwei übrigen Abstammungslinien einen gemeinsamen Typ teilen. Dies bestätigt die Theorie, dass dieselben Ereignisse, welche Teile der genetischen Struktur innerhalb von *X. fuscus* geprägt haben, auch zur Herausbildung stimmlicher Unterschiede geführt haben. Außerdem dient dies als Beleg zur Untermauerung der Erhebung der Unterart *atlanticus* zu einem Taxon auf Artebene.

Introduction

Geographical variation within species has always attracted the attention of evolutionary biologists, mainly because it can provide powerful insights into the speciation process (Endler 1977). With the development of molecular biology techniques, the focus of studies on geographical variation has shifted from the analysis of phenotypic traits to the analysis of variation in genetic markers, which are assumed to evolve in a neutral or almost neutral manner (Winker 2009). In many of these studies, reference to phenotypic variation is through comparisons between genetic lineages and subspecific classification (e.g., Cabanne et al. 2008; Miller et al. 2011; Lougheed et al. 2013).

Subspecies are useful "flags" to detect intra-specific phenotypic geographic variation (Winker 2010; Naka et al. 2012). Most subspecies, however, were described even before the simplest statistical analyses (e.g., *t* test) were developed and available to biologists (Remsen 2005). Thus, because we still do not have a full appreciation of phenotypic variation in many species and subspecies, apparent incongruence of subspecies and genetic clade delimitation does not

necessarily mean incongruence in genetic versus phenotypic variation. While variation in neutral genetic markers mainly provides evidence of the effect of demographic and stochastic processes, phenotype can be the target of different types of selection and drift, and the effects of these latter processes can lead to similar or different patterns in phenotypic and molecular marker differentiation (Zamudio et al. 2016).

To gain a better understanding of the biological process driving differentiation in genetic and phenotype patterns, it is crucial that modern evolutionary studies include detailed analyses of geographic variation in phenotype (e.g., Cabanne et al. 2014; García et al. 2016). Bird vocal phenotype is of particular interest in studies on the speciation process as it may play a key role in the development of reproductively isolation mechanisms among species (see Slabbekoorn and Smith 2002 and references therein). Vocal phenotype may be the only character that allows differentiation in many taxa (Marler 1957; Becker 1982), and it can reflect patterns of genetic divergence better than other phenotypic characters (Rheindt et al. 2008; Greig and Webster 2013).
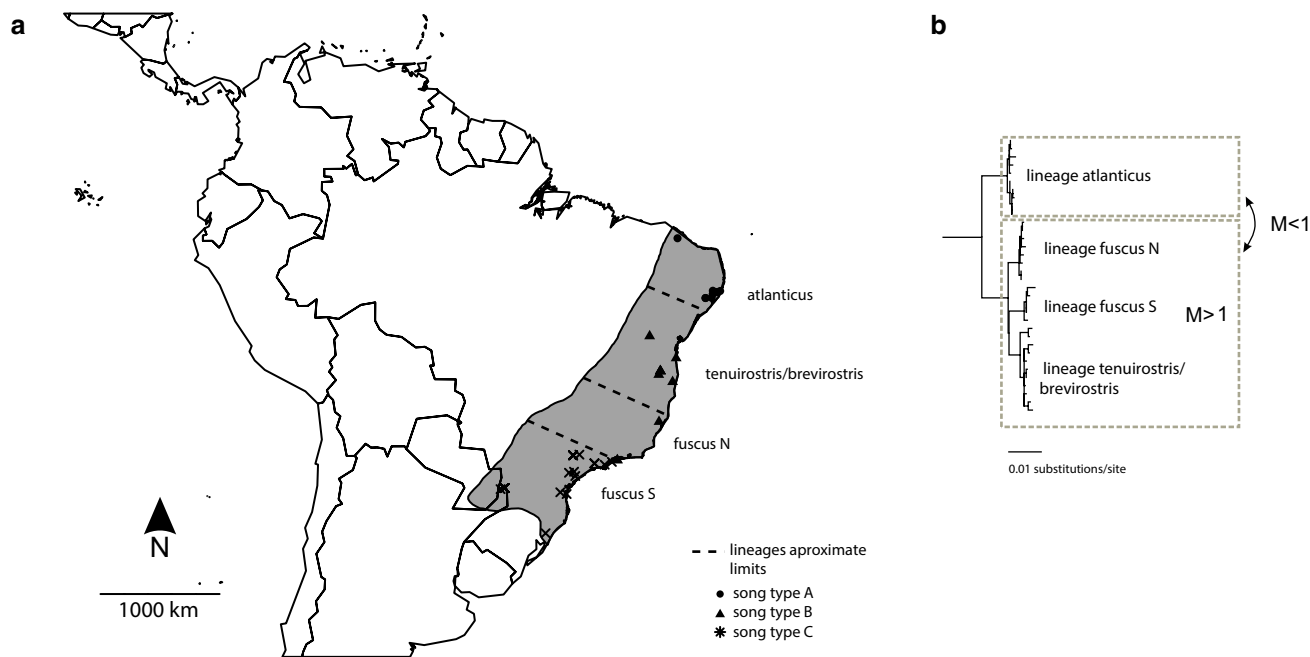

Fig. 1 **a** Distribution of the Lesser Woodcreeper *Xiphorhynchus fuscus* (BirdLife International and NatureServe 2015), with the approximate limits between genetic clades and locality of the recordings analyzed. For recordings that did not have geographical coordinates, these were estimated based on reported recording locality. The map was constructed in R version 3.3.2 (R Core Team 2016) using the "map tools" package (Bivand and Lewin-Koh 2016). **b** Phylogeographic structure (based on mitochondrial and nuclear DNA) and gene flow pattern of the *X. fuscus* lineages (modified from Cabanne et al. 2008). $M > 1$ indicates high gene flow among lineages, $M < 1$ denotes low gene flow. Only lineage atlanticus is genetically isolated from the other lineages according to gene flow rates ($M < 1$)

Here we report our analysis of intra-specific vocal variation in an Atlantic forest passerine, the Lesser Woodcreeper *Xiphorhynchus fuscus,* a species that includes four subspecies (see "Methods" section) that are described based on coloration and size variation (Marantz et al. 2003). A similar number of lineages have been described using neutral genetic data (Cabanne et al. 2008; see Fig. 1). Surprisingly, the phenotypic and genetic variation within this species are not in full agreement, making *X. fuscus* an interesting model to compare different types of variation. We evaluated whether the four genetic lineages within *X. fuscus* accumulated vocal differences as well (either due to drift or selection favoring local adaptation). If this were to be the case, we expected variation in vocalization between the genetic lineages to follow a steep cline, with breaks coinciding with the limits of genetic clades. Alternatively, incongruence in song and genetic variation would indicate the effect of other factors on vocal evolution, such as convergent adaptation or balancing selection (Zamudio et al. 2016). Finally, we used the song dataset to evaluate whether *X. f. atlanticus* shows a degree of vocal differentiation that merits species level recognition, as has been previously suggested based on molecular and morphological data (Cabanne et al. 2008, 2014). Finally, the implications of these results are discussed and taxonomic changes recommended.

Methods

Study model

The Lesser Woodcreeper (family Dendrocolaptidae) is a forest understory bird that is endemic to the Atlantic forests of eastern Brazil (from Ceará to Rio Grande do Sul), eastern Paraguay, and north-eastern Argentina (Marantz et al. 2003). It is a polytypic species with four described subspecies: *atlanticus*, *tenuirostris*, *brevirostris*, and the nominate form. The nominate form, *X. f. fuscus*, inhabits the southern range of the species, from southeastern Brazil to southeastern Paraguay and northeastern Argentina; *X. f. tenuirostris* occurs in coastal eastern Brazil, from central Bahia to the Rio Doce; *X. f. brevirostris* inhabits the interior of the state of Bahia (Marantz et al. 2003). The most distinctive form is *X. f. atlanticus*, endemic of the highly endangered forests of the Brazilian northeast, ranging north to the São Francisco river, from the states of Paraíba to Alagoas and humid patches of forest (locally known as *brejos de altitude*) within the Caatinga dry forest in the state of Ceará (Marantz et al. 2003; Galindo Leal and Câmara 2003).

Some authors consider *X. f. atlanticus* to be a full species (Piacentini 2015, BirdLife International 2017), but others do not (Remsen et al. 2017). Authors that do not recognize this taxon as a full species argue that even though it is highly divergent in terms of phenotype and genetics, further

evidence is required to show that this form is reproductively incompatible with the other populations, as expected by the Biological Species Concept. Specifically, it has been suggested that vocal data could provide such evidence, which has been informative in many other cases of suboscine passerines in the Neotropics (Remsen et al. 2017). With the exception of *X. f. atlanticus,* the three other subspecies are not well supported as independent evolutionary lineages by phenotypic studies, because they are part of a smooth cline of morphology and plumage variation (Cabanne et al. 2014).

Genetically, both mitochondrial and nuclear markers have revealed four well-supported lineages within the species, which include the most divergent northeastern lineage (hereafter referred to as the atlanticus lineage) and a lineage that includes the distributions of both *brevirostris* and *tenuirostris* subspecies (the "tenuirostris/brevistrostris" lineage); the nominate form is separated into a northern and a southern lineage (fuscus N and fuscus S lineages; Cabanne et al. 2007, 2008; see Fig. 1). Genetic studies indicated high levels of gene flow among lineages, except between *X. f. atlanticus* and the others (Cabanne et al. 2008), which supports its status as a species-level taxon.

Acoustic analyses

We analyzed recordings in uncompressed format (WAV files) obtained from digital sound libraries and published sources [Minns et al. 2009; see Electronic Supplementary Material (ESM) Table S1]. To further enhance our dataset we searched the Xeno-canto database (www.xeno-canto.org), but instead of using the recordings available for download in MP3 format, we contacted the respective authors and requested the original files (see ESM Table S1). Since these recordings originated from different sources and were made with different parameters, we standardized them in Gold-Wave v5.70 (GoldWave Inc., St. John's, NL, Canada) by down-sampling from 96 or 48 kHz to 44.1 kHz, reducing to one channel (for two-track recordings), and down-sampling from 24 to 16-bit precision. For each recording, a spectrogram was generated and analyzed using the program Raven, ver. 1.4 (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY; see http://www.birds.cornell.edu/raven), using a 512 fast Fourier transform length with a 50% overlap and a Hann window of 512 samples.

We first visually inspected sonograms to analyze the structure of songs present in the recordings. In addition to simple calls, many species of woodcreepers produce "loud-songs", i.e., a consistently patterned, multiple-note vocalization, typically repeated at regular intervals (Derryberry et al. 2012). We focused our analyses on these type of vocalizations and refer to these hereafter simply as "songs."

We analyzed one song per individual ($n = 42$). On each spectrogram, we selected a song (if there were more than

one) based on sound quality prior to the analyses. On each song, we measured the following parameters: (1) duration of song (SongD), as the time elapsed from the beginning of the first note to the end of the last one; (2) number of notes (NNotes); (3) note rate (NoteR, calculated as NNotes/SongD). We defined a note as a continuous trace in the temporal axis of a spectrogram. Since changes in the note rate are often observed in woodcreepers songs (Derryberry et al. 2012); we also calculated (4) NoteR1, (5) NoteR2, and (6) NoteR3 by dividing each song in three equal parts and calculating the note rate for each part. We also calculated (7) ΔNoteR, as the difference between the highest and lowest of NoteR1, NoteR2, and NoteR3, respectively. We measured (8) maximum (MaxF), (9) minimum (MinF) and (10) emphasized (EmpF) frequencies, and (11) song bandwidth (Band, calculated as MaxF − MinF). The EmpF was obtained using the ''Max Frequency'' option in Raven version 1.4. To ensure consistency across recordings, all measurements were made by the same author (NCG), who was blinded to the geographical origin of the recordings.

Statistical analyses

Based on general song structure, we detected three song types (hereafter referred to as A, B and C). We also requested an external collaborator (unaware of the geographical origin of song recordings) to classify them into A, B or C (A = 10 recordings; B = 11; C = 21; see ESM Table S1). We then performed a stepwise discriminant function analysis (DFA) to see if the acoustic variables we measured could distinguish between these types. We obtained two discriminant functions (DFs) with eigenvalues > 1, and we assessed the effectiveness of the functions we obtained in classifying the recordings. Prior probabilities of group membership were adjusted according to group sizes for classification. We tested the assumption of equality of covariances across groups using the Box's M test. Since Box's M test was significant, we checked that results did not change after re-running the classification using the separate-groups covariance matrix.

As it was not possible to be certain of which genetic clade each recorded bird belongs to (there are no genetic vouchers of recorded birds), we assessed the congruence of geographical distributions of song types and genetic clades in two ways. First, since genetic lineages are distributed across a latitudinal axis, we plotted the scores of each recording for the two DFs we obtained in relation to the latitude of the recordings. This plot allowed us to evaluate if vocal parameters included in the DFs varied according to a continuous or steep cline across latitude by adjusting both a linear and a quadratic function to the relationship between scores for each DF and latitude. If variation follows a steep cline we expect the quadratic function to have

a better goodness of fit to the data than the linear function. We evaluated absolute goodness of fit using the formula $D = \sum \sqrt{(E - O)^2} \div \sqrt{O^2}$, where D is the sum over all cases of absolute deviations between the predicted value according to the adjusted equation (E) and the observed value (O). This also allowed us to examine the geographical distribution of song types in relation to the approximate latitudinal limits of lineages. This approach does not require that we assign recordings to lineages, but neither does it provide statistical significance. Therefore, we took a second approach and linked each recording to the genetic lineage expected at each locality, following Cabanne et al. (2008). We then tested whether differences in DF scores among genetic lineages were significant using a Kruskal–Wallis test, followed by pairwise comparisons using Mann–Whitney U tests, corrected by a Holm–Bonferroni procedure for multiple comparisons. Samples from locations where two genetic lineages overlapped geographically were not considered. As a result, ten recordings were assigned to the atlanticus lineage, eight to the tenuirostris/brevirostris lineage, five to the fuscus N lineage and 13 to the fuscus S lineage. All analyses were performed using SPSS version 15.0 for Windows (IBM Corp., Armonk, NY).

Results

Based on the general structure of the vocalizations of *X. fuscus* across its geographic range we detected two distinct songs (to which we refer as song I and song II). Song I presents three distinct types (A, B and C; see Fig. 2a–c). Type A is characterized by a fast and ascending stuttered trill, with the highest pitched note at the end. Type B presents a lower note rate, with its highest pitch notes at the middle of the song. Type C is similar to A in overall pitch, but with very variable NoteR that falls and rises in pitch at the end. Each type of song I could be emitted alone or as part of a series of repeated songs. Song II was found in recordings from throughout the distribution of *X. fuscus*, with little variation across individuals and lineages. It consisted of a higher pitched note at the beginning, followed by a series of descending notes (Fig. 2d). Some individuals were recorded singing one of song I types (A, B or C), and the common song II. None of the individuals recorded, however, produced more than one type of song I, indicating that individuals of *X. fuscus* can produce two different songs, I and II, but only one type of song I. Therefore, we considered that song type I and II were not homologous and focused our analyses on song I.

After classifying the available recordings of song I into types A, B, or C we measured 11 acoustic parameters on each recording (see Table 1 for summary statistics) and obtained two discriminant functions (DF1 and DF2) that
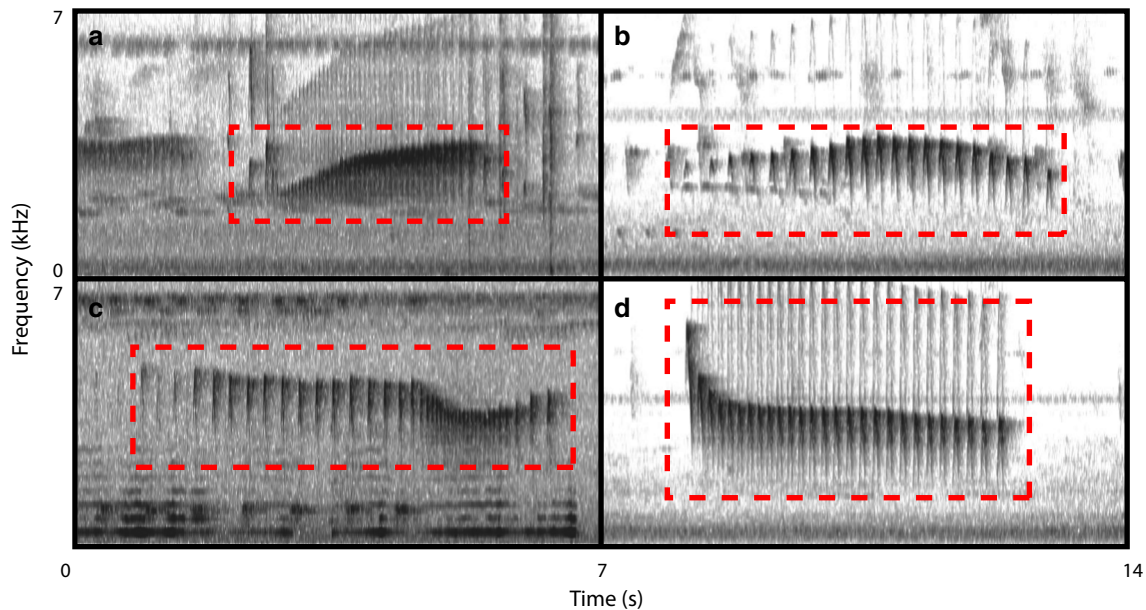
Fig. 2 Spectrograms showing examples of song I types A, B and C (**a**, **b**, **c**, respectively) and song II (**d**). Images were obtained from recordings of the Macaulay Library, Cornell Lab of Ornithology (Ithaca, NY) under catalog numbers 127876 (**a**), 174890 (**b**), 174739 (**c**), and 174928 (**d**). The differences in general shape of each song type were perceived mainly as differences in note rate or song pace. For example, both song I types A (**a**) and C (**c**) start with a slow note rate which then increases significantly, but the slow introduction is much longer in song type C. Type B (**b**), on the other hand, maintains a constant slower pace along the entire song

Table 1 Mean ± standard deviation of the acoustic parameters used for the discriminant function analysis, and correlation coefficients of the variables retained by the stepwise process with each discriminant function

Acoustic variables	Song type			Discriminant functions	
	A (n = 10)	B (n = 11)	C (n = 21)	DF1	DF2
SongD (s)	3.73 ± 0.8	4.23 ± 1.15	4.54 ± 2.16	0.053	0.064[a]
Nnotes	45.4 ± 6.47	18.45 ± 4.48	27.1 ± 9.31	− 0.433[a]	0.103
NoteR (s^{-1})	12.57 ± 2.97	4.45 ± 0.66	6.35 ± 1.29		
NoteR1 (s^{-1})	13.17 ± 5.58	4.63 ± 1.07	4.05 ± 0.63		
NoteR2 (s^{-1})	15.79 ± 2.31	5.09 ± 0.79	7.31 ± 2.77		
NoteR3 (s^{-1})	9.67 ± 3.42	4.4 ± 0.61	8.82 ± 2.11	− 0.215	0.384[a]
ΔNoteR (s^{-1})	7.68 ± 3.05	0.91 ± 0.8	5.45 ± 1.68	− 0.356	0.420[a]
MinF (Hz)	1560.43 ± 378.59	1601.1 ± 313.08	1338.2 ± 448.39		
MaxF (Hz)	4260.13 ± 444.11	3598.32 ± 307.56	4393.01 ± 247.01		
EmpF (Hz)	2699.7 ± 549.76	2074.13 ± 456.8	3054.82 ± 494.67	− 0.136	0.553[a]
Band (Hz)	3626.17 ± 368.7	2929.9 ± 385.98	3798.03 ± 301.97		

SongD, Duration of song; NNotes, number of notes; Note R note rate; NoteR1, NoteR2, NoteR3, note rate in three equal parts of a song; ΔNoteR, difference between the highest and lowest of NoteR1, NoteR2, and NoteR3, respectively, MaxF, MinF, EmpF, maximum, minimum, and emphasized frequency, respectively; Band, song bandwidth. For more details on how these accoustic variables were calculated, see section "Accoustic analysis"

[a]Indicates with which discriminant function (DF1, DF2) correlation is highest

can separate these song types (DF1 + DF2: Wilks' $\lambda = 0.02$, $\chi^2_{10} = 140.6$, $p < 0.001$; DF2: Wilks' $\lambda = 0.22$, $\chi^2_4 = 55.4$, $p < 0.001$; see Fig. 3a). Five acoustic variables were retained by the stepwise DFA procedure. DF1 (representing 72.2% of the total variance) correlated negatively with the NNotes (see Table 1), while DF2 (27.8% of the total variance) correlated positively with EmpF, NRate3, ΔNoteRate, and SongD (see Table 1). DF1 best separates song types A from B and C (with A having the highest NNotes; see Fig. 3a). DF 2 best separates song type B and C (with B having the lowest EmpF, the lowest NRate3, the smallest ΔNoteRate, and also the shortest SongD; see Fig. 3a).
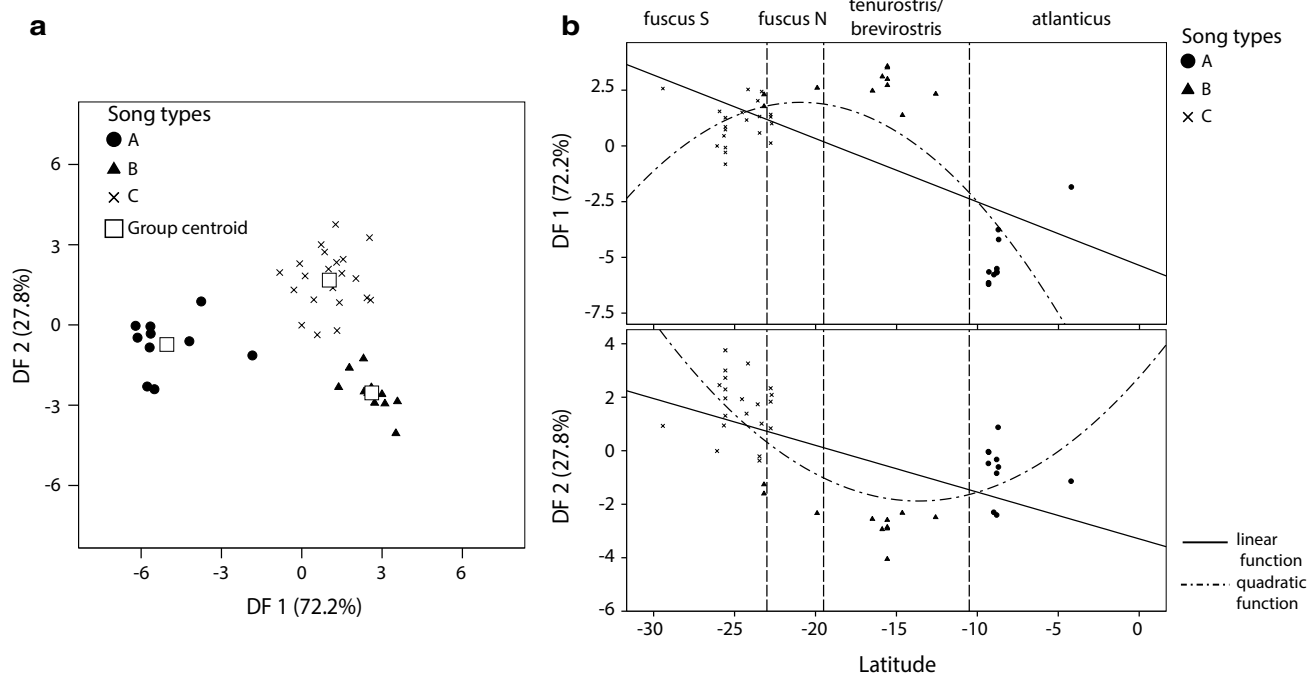
Fig. 3 a Discriminant function 1 (DF1) versus DF2 scores for all individuals, with the percentage of the total variance explained by each function given in parenthesis on its corresponding axis. Individuals are symbol-coded according to the song type they were assigned to, and centroids of each group are shown. **b** Scores of each individual for DF1 and DF2 in relation to the latitude where they were recorded at and genetic lineages. Individuals are also symbol-coded according to the song type they were assigned to

Table 2 Adjustment of linear and quadratic functions for the scores of each individual song for discriminant functions 1 and 2 versus latitude at which the songs were recorded

Statistical variables	DF 1		DF 2	
	Linear	Quadratic	Linear	Quadratic
R^2	0.42	0.6	0.35	0.54
p	< 0.001	< 0.001	< 0.001	< 0.001
D	947	467	247	302

Reclassification of cases using these functions had 100% efficacy when all cases were considered and 97.6% using the jackknife method (where functions are recalculated leaving one case out and then used to classify that case). We then compared the geographic distributions of song types with the approximate limits of genetic lineages (as delimited previously by Cabanne et al. 2008). All individuals assigned to song type A were recorded in northeastern Brazil, in the same area as the atlanticus lineage (Fig. 3b). Most individuals assigned to song type B were distributed along the area corresponding to the tenuirostris/brevirostris and fuscus N lineages, while most song type C recordings were obtained from the geographic range of the fuscus S lineage (Fig. 3b). Both song types B and C were found in the contact zone of lineages fuscus N and fuscus S. Regarding the relationship of DF1 and DF2 with

latitude, both the linear and the quadratic functions adjustments were significant ($p < 0.001$), but the quadratic function had a higher R^2 in both cases (Table 2). However, for DF2 the linear function had a better goodness of fitness (Table 2).

We found significant differences among lineages for the scores of both DF1 (Kruskal–Wallis $H = 28.15$, $p < 0.001$) and DF2 (Kruskal–Wallis $H = 27.92$, $p < 0.001$). The DF1 scores for lineage atlanticus were significantly lower than those for the other three lineages (Fig. 4a; $p < 0.02$ for the three comparisons, which remained significant even after the Holm–Bonferroni correction). The DF1 scores of the lineage fuscus S also differed significantly from those of the remaining lineages (Fig. 4a; $p < 0.02$ for the three comparisons, which remained significant after Holm–Bonferroni correction). The DF2 scores of both tenuirostris/brevirostris and fuscus S lineages differed significantly from those of all other lineages (Fig. 4b; $p < 0.02$ for all comparisons, which remained significant after Holm-Bonferroni correction). Differences between atlanticus and fuscus N lineages were not significant for this function.

Discussion

In this study we have shown that Lesser Woodcreepers produce at least two distinct songs and that one of these songs,
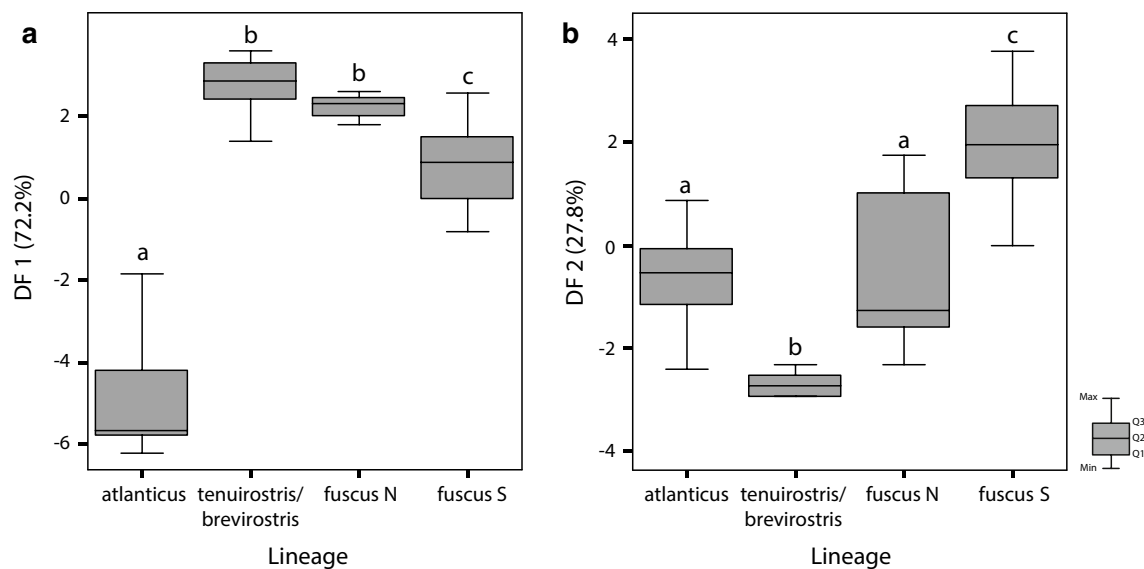
Fig. 4 Box-plots of DF1 (**a**) and DF2 (**b**) scores for each genetic lineage. Groups that differed significantly after Holm–Bonferroni correction of significance levels for the *p* values of Mann–Whitney *U* tests are indicated with different lowercase letters. The mean is shown as a dark horizontal line in boxes, with the quartiles Q1, Q2, Q3

with three discrete types, differs among populations. Two of these song types could be assigned to the atlanticus and fuscus S lineages, respectively, while the third is shared by the tenuirostris/brevirostris and fuscus N lineages. The partial congruence of phylogeographic structure with song variation suggests that similar factors affecting gene flow among populations are also important drivers of song evolution.

Patterns of vocal variation and possible causes

Our analysis revealed that vocal variation showed a latitudinal steep cline pattern, with one break coinciding with the geographic gap between the atlanticus and tenuirostris/brevirostris genetic lineages, and another break coinciding between the fuscus N and fuscus S lineages. Vocalizations can diverge through selective processes but also due to random drift, whether they are innate or learnt (Podos and Warren 2007). Comparisons of variation in acoustic signals with that in genetic markers that vary in a neutral or nearly neutral manner can facilitate a better understanding of the contribution of different factors to signal evolution (Wilkins et al. 2013). The congruence we found between geographic limits of song types and two of the geographic breaks between genetic lineages suggests that the same vicariant/demographic events that led to the evolution of such lineages also promoted the accumulation of vocal differences (see Cabanne et al. 2007, 2008 and the following sections of our discussion).

Matching in phenotype and neutral genetic structure can be due to neutral phenotype divergence or divergent natural/sexual selection that leads to local adaptation (Zamudio et al.

2016). Habitat characteristics can be an important selective factor in vocal evolution, for both species with and without vocal learning (Seddon 2005; García et al. 2015). The Atlantic forest biome contains different forest types where selective forces could differ. However, while song types have a latitudinal distribution, the forests inhabited by *X. fuscus* show greater variation along the longitudinal distribution, from the more humid coastal range to the dryer interior (Cabanne et al. 2014). Therefore, today's forest types do not seem to have affected song variation in the studied species and do not coincide with geographic breaks in their vocalizations.

Differentiation in body size could lead to vocal diversification (Tubaro and Mahler 1998; García et al. 2014) due to the morphological constraint imposed by vocal organs size (Riede and Goller 2014). In *X. fuscus*, body size traits decrease with latitude, with *atlanticus* being the largest subspecies and *fuscus* being the smallest (Cabanne et al. 2014). Contrary to previous studies which suggest that, in general, smaller beaked birds produce more notes than larger ones within the suboscines (Seddon 2005), we have shown that the number of notes (correlated to DF1) also decreases with latitude. In our case, the variation in the number of notes in *X. fuscus* does not seem to be associated to bill size, as expected.

We found that two of the genetic lineages, tenuirostris/brevirostris and fuscus N, share a similar song type. This could be the result of a selective process (e.g., stabilizing selection) maintaining vocal identity among these populations, or due to the high gene flow documented between both populations (Fig. 1; Cabanne et al. 2008). On the other hand,

we have also previously documented high levels of gene flow between fuscus N and fuscus S populations, which have different song types. Therefore, in our case, gene flow does not seem to be a consistent factor to explain song similarity.

The song of *X. fuscus* could have been affected by factors associated to the historical dynamics of their habitat, i.e., by selective and/or stochastic processes, because their characteristics seem to be associated to the long-term dynamism of the Atlantic forest. The Atlantic forest biome can be divided into regions where forests have been relatively stable across the Glacial cycles of the Pleistocene (last two million years) and into regions where forests have been dramatically modified by climatic changes during those same glaciations (Behling 2002; Ledru et al. 2005; Carnaval and Moritz 2008; Carnaval et al. 2014). In the unstable regions, forests have been affected by cycles of advance and regression of grasslands (e.g., in the southern Atlantic forest) and of dry forests (e.g., in northeastern Brazil), where they cycled between continuous to highly fragmented ranges. The stable Atlantic forest is located from the Brazilian state of Bahia to Rio de Janeiro and northern São Paulo (the range of lineages tenuirostris/brevirostris and fuscus N), while unstable regions are the northeastern Atlantic forest (the range of atlanticus), as well as the southern extreme of the biome (range of fuscus S).

According to the acoustic adaptation hypothesis (Morton 1975), the song variation of *X. fuscus* seems to follow the long-term forest stability in each region. The acoustic adaptation hypothesis states that songs in denser and more continuous forested habitats are expected to have a lower note rate and lower frequencies than those in less dense and less continuous forests. The lineages tenuirostris/brevirostris and fuscus N are associated to the most stable region of the biome, and their song (type B; Fig. 2b) presented the lowest overall NoteR (4.45 ± 0.66) and the lowest EmpF (2074.13 ± 456.8), while the other two populations (atlanticus and fuscus S) are associated to unstable forests and their songs (types A and C, respectively) have higher values in both metrics (Table 1). This adaptive interpretation would explain the apparent vocal convergence between atlanticus and fuscus S, two lineages that are among the most genetically distant of the species (Fig. 1).

Stochastic factors linked to demographic fluctuations could have also affected song variation in *X. fuscus*. It is expected that the long-term population effective size of forest organisms is lower in regions with unstable forest (i.e., northeastern Brazil and the southern biome) than in stable forest regions (e.g., Bahia and Espírito Santo). The difference in the effective size may be a consequence of strong demographic fluctuations and bottlenecks that occurred in unstable forests. Low population size could trigger intense genetic drift that could accelerate divergence, especially in

a bird with an innate song, as is the case of *X. fuscus*, and whose genetic constitution has been affected by the forest–demographic fluctuations of the Pleistocene (Cabanne et al. 2008, 2014). A comparative study would allow researchers to confirm if forest instability associated to climate fluctuations affected song variation of Atlantic forest birds, as well as to differentiate whether stochastic (drift) and/or adaptive processes are responsible for the observed variation.

Taxonomic implications

The evidence here presented supports early proposals that *X. f. atlanticus* should be considered a species-level taxon, according to a number of species concepts (see Cabanne et al. 2014). Briefly, the atlanticus lineage can be considered to be a species according to the General Lineage Species Concept (De Queiroz 2007): this population is monophyletic and completely isolated from other conspecific populations (Cabanne et al. 2008), and it can be differentiated by plumage, body size (Cabanne et al. 2014), and vocalizations (this study). The form *atlanticus* can also be recognized as a full species under other species concepts, such as phylogenetic and the biological concepts, because it is monophyletic and genetically isolated (Cracraft 1983; Cabanne et al. 2008). Also, because song divergence is considered to be an important pre-zygotic barrier (Price 2007), our results support the notion of reproductive isolation between atlanticus and its geographically closest lineage tenuirostris/brevirostris (Fig. 1).

Although the southern population of *X. fuscus* (fuscus S) is monophyletic and also presents high levels of song differentiation (Fig. 4), we do not suggest that this population should receive species-level recognition. Levels of genetic divergence are very shallow between fuscus S and fuscus N, and according to previous studies there is extensive gene flow between this and other populations (Cabanne et al. 2008). Additionally, fuscus S is located at the end of a plumage and body size cline (Cabanne et al. 2014), and there is not a steep gap separating its song from neighboring populations (i.e., from fuscus N; Fig. 3b).

The accurate delimitation of species boundaries is a fundamental step—not only for taxonomic purposes, but it is also relevant for studies on macroevolution, biogeography and, particularly, for conservation. The Atlantic forest is one of the most endangered biomes in the Neotropics, and the form *atlanticus* inhabits the Pernambuco Area of Endemism, a thin and highly fragmented stretch of forest restricted to the north of the São Francisco River that represents the most endangered portion of the Atlantic forest (Galindo Leal and Câmara 2003). Therefore, the conclusion that *X. f. atlanticus* should be considered a species-level taxon has important

conservation implications and will require a re-evaluation of the current conservation status of this population.

Compliance with ethical standards

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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Affiliations

Natalia C. García[1] · Luciano N. Naka[2] · Gustavo S. Cabanne[1]

[1] División Ornitología, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia"–CONICET, Av. Ángel Gallardo 470, Buenos Aires, Argentina

[2] Laboratório de Ornitologia, Departamento de Zoologia, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235, Cidade Universitária, Recife, PE, Brazil