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**Effects of fragmentation and hybridization on geographical patterns of  
song variation in the endangered Yellow Cardinal *Gubernatrix cristata***

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The song learning process may lead to small-scale geographical variation in vocalizations of oscine birds. This variation can be further enhanced by the effects of habitat fragmentation or hybridization. Populations of the endangered Yellow Cardinal *Gubernatrix cristata* are now patchily distributed in the southern South American thorny shrubland forests and are small as a consequence of the pressure exerted by increased habitat transformation and illegal trade.

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We study the Yellow Cardinal's vocalizations throughout its distribution and assess how habitat characteristics and fragmentation, as well as hybridization with the Common Diuca *Diuca diuca*, have affected song patterns of different populations. We expected to find song differences among populations and songs to be more similar to those of the Common Diuca in areas where hybridization occurs. Multivariate analyses revealed significant differences in song between the four populations studied and confirmed that songs are more similar to those of Common Diuca where hybridization takes place. These results, in conjunction with genetic studies, can help to establish management units that preserve genetic and cultural variation in this endangered species.

**Keywords:** cultural units, oscines, vocalizations, hybrids

The song of passerine birds is a conspicuous display shaped mostly by sexual selection in the context of male-male competition or mate attraction (Catchpole & Slater 2008) and is an essential trait in social interactions of birds (Odom *et al.* 2014). Songbirds acquire their specific song patterns by social learning and this is probably one of the best studied examples of a cultural trait in non-human animals (Slater 1986). This non-genetic transmission of vocal traits across generations can be a powerful source of variation due to copying inaccuracies (Dawkins 1976, Mundinger 1980, Lynch 1996). Imitative vocal learning enables the ready generation and rapid transmission of novel patterns of vocal structure (Slater 1989, Slabbekoorn & Smith 2002) leading to differences in song among individuals and among populations of the same species (Catchpole & Slater 2008).

Geographical variation in bird songs evolves through the dynamic interplay of song learning mechanisms and isolation and is influenced by the effects of dispersal behaviour, cultural drift, genetic drift, cultural selection, natural selection and sexual selection (Podos *et*

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al. 2004, Podos & Warren 2007). Cultural drift can lead to the random fixation of a few song types or note types in isolated populations (Lynch & Baker 1994, Grant & Grant 1996, Baker *et al.* 2001, Koetz *et al.* 2007) over short evolutionary timescales (Baker *et al.* 2003, Edwards *et al.* 2005). This process is important in conservation biology as large contiguous populations become increasingly reduced to small isolated populations through habitat loss and fragmentation, potentially having an impact on culturally transmitted traits (Laiolo & Tella 2007, Caro & Sherman 2012, Jamieson & Lacy 2012). Bird songs, in particular, become impoverished as a result of disruption of cultural transmission (Laiolo & Tella 2005, 2006, Parker *et al.* 2010, 2012).

Incorporating the study of cultural attributes in a conservation context has been recently suggested as a novel aspect of the diversity and status of animal populations (Slabbekoorn & Smith 2002, Laiolo & Jovani 2007, Wildermuth *et al.* 2013). It has been proposed that animal culture should be integrated into conservation biology (Whitehead *et al.* 2004) and that ‘culturally significant units’ should be preserved in small and endangered populations (Ryan 2006). The songs of small populations isolated by habitat fragmentation have been shown to correlate with population viability (Laiolo 2008, Laiolo *et al.* 2008), with smaller populations, that are more susceptible to extinction, presenting lower variability in their songs.

In the context of rapid landscape change and deep concern about diversity loss, understanding how landscape fragmentation affects and structures natural populations is important for improving ecological knowledge and defining optimum strategies for conserving threatened species (Pearman & Wilbur 1990, Diniz-Filho & Telles 2002, Manel *et al.* 2003). We analyze the geographical song variation of the Yellow Cardinal *Gubernatrix cristata* (Thraupidae), a passerine endemic to southern South America, which at present is

categorized by IUCN as Endangered (BirdLife International 2016). In the past, this species was widely distributed in the thorny deciduous shrubland forests of central Argentina (Espinal region), most of Uruguay and parts of southern Brazil (Ridgely & Tudor 1994; Fig. 1). However, for over a century there has been a continuous removal of individuals, mainly males, to commercialize them as cage birds (Pessino & Tittarelli 2006, BirdLife International 2016). This, in addition to the conversion of their forest habitat to agriculture and cattle pasture, has caused a marked decline in range and population size. Now the species' distribution is discontinuous, with the main populations found at the extremes of the original range (Collar *et al.* 1992, BirdLife International 2016; Fig. 1). In Brazil, Yellow Cardinals are now very rare (Fontana *et al.* 2003, Machado *et al.* 2008).

A further consequence of rarity is that hybrids of Yellow Cardinals with the Common Diuca *Diuca diuca minor* have been found in the southern part of the Cardinal's range, probably as a consequence of the lack of Cardinal males due to illegal trade (Bertonatti & López-Guerra 2007). We therefore analyzed Yellow Cardinal vocalizations throughout the species' range and assessed how habitat characteristics and fragmentation, as well as hybridization with the Common Diuca, affected song patterns of different populations. We expected to find song differences among populations because, in the presence of natural selection shaping vocalizations, songs should show features that are better transmitted in each particular environment (Morton 1975). If song geographical variation is a consequence of cultural drift we expect to find random loss of vocal variants in different populations, that is then intensified by habitat fragmentation. We also expected to find songs more similar to those of the Common Diuca in areas where hybridization occurs.

## METHODS

### *Song recordings*

Recordings were obtained during four breeding seasons (September –January 2011–2014) in four different populations covering most of the known distribution of the Yellow Cardinal in Argentina and Uruguay: 1) Corrientes (C); 2) San Luis (SL); Rio Negro (RN); and 4) Uruguay (U; Fig. 1). The possibility of repeated recording of the same male was minimal since most birds were previously ringed. The songs of Common Diucas were recorded in the southern part of the Yellow Cardinal’s distribution (La Pampa and Rio Negro provinces) where hybridization events have been registered (Bertonatti & López Guerra 1997; Fig. 1). Songs of Diucas from other areas (n = 3) were obtained from the Cornell Lab of Ornithology’s Macaulay Library collection (catalog numbers 135815, 20607 and 20605). All recordings were made within close proximity to singing birds (5–10 m) using a Zoom ZOH4NK H4n Handy Recorder. The dataset included 3–8 high quality songs per individual of 30 Yellow Cardinal males and 18 Common Diucas. Some songs were discarded because of poor recording quality but a total of 245 songs were analyzed. We digitalized songs with the Sound Analysis software package “RavenPro” version 1.4 (Charif *et al.* 2007) and visualized spectrograms with a ‘Hamming’ window type, a frame size of 256 samples, and a bandwidth of 224 Hz. For the colour scheme we chose the ‘grayscale’ option, and brightness and contrast values were set at 50% in all cases to standardize the measurements.

### *Song analysis*

Songs were characterized as a series of elements (notes) emitted successively with short intervals and followed by an interval of silence longer than the song. A note was defined as any continuous trace in the temporal axis of the spectrogram. We identified different note

types in Yellow Cardinal songs which were visually classified on the basis of ascending or descending frequency modulations (Fig. 2).

On each song spectrogram we also measured 14 acoustic variables (Fig. 2). These variables can be classified into three categories: 1) frequency-, 2) time-, and 3) structure-related variables. We measured: 1) five frequency variables (measured in Hz): maximum and minimum frequencies of the song (SFMAX and SFMIN), the bandwidth of each note ( $SBAND = SFMAX - SFMIN$ ) from which we extracted maxSBAND, corresponding to the note with the maximal value of SBAND, and minSBAND corresponding to the note with the minimal value of SBAND, and emphasized (i.e. peak) frequency (SFEMF, corresponding to the frequency with the highest amplitude in the song); 2) six temporal variables (measured in ms): song duration (SD), maximum note length (maxNL: length of the note with maximum duration), minimum note length (minNL: length of the note with minimum duration), maximum inter-note interval (maxInI: maximum silence duration between the end of a note and the beginning of the next one), minimum inter-note interval (minInI: minimum silence duration between the end of a note and the beginning of the next one) and sound proportion (SP, measured as the difference of the duration of the song with the duration of all inter-note intervals); and 3) three structural variables: number of notes (NN), proportion of simple notes (PSN: defined as ascending or descending notes – see below Yellow Cardinal Repertoire in Fig. 2) and proportion of initially ascending notes (PIA). When harmonics were present we measured the frequency band with the highest energy. Values for the acoustic variables were averaged across multiple songs (3–8) for each bird.

### *Statistical analyses*

Because multiple variables were measured on each song, we used multivariate analyses to compare patterns of variation between different locations. We first checked the data to meet the assumptions of subsequent analyses (MANOVA and Discriminant Function Analysis): we looked for outliers and lack of normality and univariate homoscedasticity both graphically and analytically and tested for independence by examining the association between variables using Pearson correlation coefficients and principal component analysis (PCA). We discarded correlated variables keeping only those with higher loading values in the principal components. Thus, we only used variables that were independent for further analyses.

To test for differences in song between Yellow Cardinal populations we conducted a multivariate analysis of variance (MANOVA) with the independent measures as response variables and locality as the explanatory variable. To determine whether songs could be grouped by populations (i.e. localities) based on the independent acoustic variables we carried out a discriminant analysis.

Finally, we assessed whether there was cultural introgression among songs of Yellow Cardinals and Common Diucas in areas where hybridization events were recorded. We applied a discriminant function analysis adding 18 Common Diuca samples as a new group. All multivariate analyses were performed with Infostat (Di Rienzo *et al.* 2013).

## **RESULTS**

We analyzed the songs of 30 Yellow Cardinals belonging to four different populations: 10 individuals from Corrientes (C), 7 from Rio Negro (RN), 4 from San Luis (SL) and 9 from Uruguay (U).

We identified five different note types in Yellow Cardinal songs (Fig. 2). Song elements were relatively simple with no, one, or three shifts in frequency modulation.

Correlation of acoustic variables was high and significant for SFMAX, SFMIN and SFEMF, maxINI and minINI, and for SD and SP. Structural variables were not correlated among each other but showed moderate correlation ( $0.2 < r < 0.4$ ,  $P < 0.05$ ) with frequency variables. The highest correlation was between SFMAX and SFEMF ( $r = 0.80$ ,  $P < 0.0001$ ). Seven acoustic variables were eliminated based on their correlation coefficients and their weight on the PCA axes. Thus, the final data set for the MANOVA and discriminant analyses consisted of the following variables: two frequency (SFEMF, minSBAND), three temporal (SP, maxNL and minInI) and two structural variables (NN and PSN).

The MANOVA showed significant song variation between the four populations (Pillai's Trace  $F_{21,66} = 2.85$ ;  $P = 0.0006$ ). Hotelling comparisons showed that all populations differed from one another except from SL, which only differed from U but not from the other two Argentinian populations (Table 1).

The first two axes of the discriminant analysis explained 95% of the differences between populations and the discriminant function correctly assigned 87% of the individuals to their population of origin (4 errors in 30 cases, Table 2). This indicates that songs of each Yellow Cardinal population are characteristic and different to songs of other populations. The most important variables that allowed this separation were SFEMF, minSBAND, PSN and minINI.

Songs of Common Diucas are much more elaborate and complex than Yellow Cardinal songs. At least 25 different note types were identified in 72 songs recorded from 18 individuals. The discriminant function correctly assigned 87% of the individuals to their population of origin (6 errors in 48 cases, with only one error between species assigning a Common Diuca song to the Yellow Cardinal population from RN). The biplot of the first two



axes of the discriminant analysis showed a closer proximity of the songs of RN Yellow Cardinals to those of Common Diucas (Fig. 3), indicating that the songs of this population are the more similar ones. Diuca songs from areas without Yellow Cardinals ( $n = 3$ ) fall within the polygon containing Common Diuca songs in the discriminant analysis (Fig. 3).

## DISCUSSION

This is one of the few studies of song variation in endangered oscine birds (Valderrama *et al.* 2007, Laiolo *et al.* 2008) and is the first analysis of geographical song variation in the endangered Yellow Cardinal. The results of this study show that Yellow Cardinal populations are differentiated in their acoustic characteristics. Emphasized frequency is the variable that better distinguishes between individuals from different sites with cardinals from Uruguay showing the highest frequencies in their songs (mean = 3789.96 Hz) and birds from San Luis the lowest value (mean = 2467.08 Hz). Populations also differ in number of notes, sound proportion, proportion of simple notes, minimum inter-note interval and minimum bandwidth. Although songs from San Luis were not statistically different from the other two Argentinian populations (Corrientes and Rio Negro), they were all correctly assigned to this population when performing the discriminant analysis. This indicates that songs of each Yellow Cardinal population are characteristic and that individuals can be allocated by their vocalizations.

Geographical differences in song might arise as a consequence of natural selection or sexual selection and the effects of drift (Podos & Warren 2007). This latter process leads to the random loss of vocal variants (Slabbekoorn & Smith 2002), establishing a few song types or note types in isolated populations (Baker *et al.* 2001). In fragmented landscapes, the effects of drift become more pronounced (Laiolo & Tella 2007). Thus, the vocal differentiation pattern found in this species might be related to the random loss of vocal

variants in separate populations. Alternatively, songs might be adapted to different environments. The acoustic adaptation hypothesis states that songs are adapted to be optimally transmitted in a particular habitat (Morton 1975). Yellow Cardinals are distributed along the deciduous shrubland habitat (the *Espinal*). For this habitat, three different phytogeographic provinces have been identified: (1) Chaqueña, including the San Luis population, (2) del Espinal, including Corrientes, and (3) del Monte, present in Rio Negro, the southern part of the distribution (Cabrera 1976). These regions show differences in vegetation cover and forest density that might act as selection pressures on song transmission, Chaqueña being the most enclosed area and Espinal the most open. In open areas, higher frequencies and narrower frequency ranges are favoured (Morton 1975, Boncoraglio & Saino 2007). This tendency is observed for frequencies of Yellow Cardinal songs (Corrientes showing the highest value of SFEMF in Argentina) but not for bandwidth (SL showing the lowest value). Thus, natural selection or plasticity in song production might be partially favouring certain song features in specific habitats.

Finally, vocal differences of the southern population might be influenced by the hybridization with the Common Diuca. We observed that songs were more similar to those of Diucas where hybridization takes place. Although Diuca songs are more complex than Yellow Cardinal songs (Diuca repertoire = 25 note types versus cardinal repertoire = 5 note types), frequency and temporal song features of the southern Cardinal population are the most similar to those of Diucas. In an area where conspecific males were scarce, the choice of Common Diuca males by Yellow Cardinal females could be related to the similarity of song (Grant & Grant 1997, Qvarnstrom *et al.* 2006) and cultural introgression might be affecting cardinal vocalizations in this region. An alternative explanation is that convergent evolution to the same environment is driving the characteristics of both songs (Wiley 1991). However, we analyzed the songs of Diucas belonging to different parts of its distribution (Fig. 3), that

corresponded to very different habitats, and did not find that these songs fell outside the range of the song features of Common Diucas overlapping with Yellow Cardinals.

We do not know if the song differences found in this study are biologically meaningful to Yellow Cardinals. In a series of playback studies, Ratcliffe and Grant (1985) and Grant and Grant (2002) found that territorial male *Geospiza* finches responded more strongly to local song types than they did to conspecific song types from other sites. Greater responses to local songs were attributed to geographic isolation and a resulting lack of familiarity with foreign song types (Ratcliffe & Grant 1985). In general, territorial males respond most strongly to familiar dialects, followed by foreign dialects, and heterospecific song (Slabbekoorn & Smith 2002). In future, playback experiments should be carried out to test the responses of territorial males at one site to playback of both local songs and songs recorded at a different site, as well as to songs of Common Diucas.

The main goal of most conservation programs is to maintain diversity, which is mainly measured at genetic level (Earnhardt *et al.* 2001). However, it has been suggested that animal behaviour should also be integrated in conservation biology with the aim of preserving ‘culturally significant units’ (Whitehead *et al.* 2004, Ryan 2006). In this study, we show that cultural units based on song characters can be identified for the Yellow Cardinal. Previous studies have found diverse patterns of correlation of vocal characters with genetic variation (MacDougall-Shackleton & MacDougall-Shackleton 2001, Ellers & Slabbekoorn 2003, Wright *et al.* 2005, Nicholls *et al.* 2006, Saranathan *et al.* 2007). For our study species, we found that the ‘cultural significant units’ that we identified in this work are also genetically distinct (M. Domínguez, unpublished results). We recommend that the geographic patterns of variation determined for the Yellow Cardinal are taken into account in conservation planning for this endangered species.

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**Table 1.** Hotelling comparisons performed with MANOVA results. Means with common letters are not significantly different ( $P > 0.05$ ). U: Uruguay, C: Corrientes, SL: San Luis, RN: Rio Negro.

| Population \ Variables | SFEMF (Hz) | minSBAND (Hz) | NN   | PSN  | SP (ms) | maxNL (ms) | minInI (ms) | n  |    |
|------------------------|------------|---------------|------|------|---------|------------|-------------|----|----|
| U                      | 3789.96    | 2263.41       | 7.44 | 0.47 | 1.58    | 0.25       | 0.14        | 9  | A  |
| C                      | 2922.40    | 1656.92       | 6.38 | 0.35 | 0.79    | 0.14       | 0.13        | 10 | B  |
| SL                     | 2467.08    | 1377.27       | 5.85 | 0.35 | 0.70    | 0.16       | 0.22        | 4  | BC |
| RN                     | 2797.43    | 1733.98       | 6.08 | 0.13 | 1.11    | 0.18       | 0.21        | 7  | C  |

**Table 2.** Classification of songs to sampling populations based on discriminant analysis. U: Uruguay, C: Corrientes, SL: San Luis, RN: Rio Negro.

| Population | U | C  | SL | RN | Total | Error (%) |
|------------|---|----|----|----|-------|-----------|
| U          | 6 | 3  | 0  | 0  | 9     | 33.33     |
| C          | 0 | 9  | 0  | 1  | 10    | 10        |
| SL         | 0 | 0  | 4  | 0  | 4     | 0         |
| RN         | 0 | 0  | 0  | 7  | 7     | 0         |
| Total      | 6 | 12 | 4  | 8  | 30    | 13.33     |

Figure Legends

**Figure 1.** Map showing the historic distribution of Yellow Cardinals (dark grey area) in shrubland forests of South America and song examples of four remaining populations included in this study. The hybrid zone is delimited within the white dotted circle. U: Uruguay, C: Corrientes, SL: San Luis, RN: Rio Negro.

**Figure 2.** Song spectrogram showing the Yellow Cardinal's repertoire with the five different note types (from A to E) and variables measured on the song: maximum (SFMAX) and minimum frequencies (SFMIN), emphasized frequency (SEMF), bandwidth of the note (BAND), song duration (SD), note length (NL), inter-note interval (InI), number of notes (NN), simple notes (C and D) and initially ascending notes (B, C and E).

**Figure 3.** Discriminant analysis with Common Diuca samples. Individual songs are plotted against the first two canonical axes and variables defining these axes are shown: NN (number of notes), SP (sound proportion), PSN (proportion of simple notes), minInI (minimum internote interval), maxNL (maximum note length), minSBAND (minimum bandwidth) and SFEMF (song emphasized frequency). Polygons include Common Diuca songs (squares) and songs of Yellow Cardinals from each geographic area: Uruguay (pentagons), Corrientes (stars), San Luis (black circles) and Rio Negro (triangles). Grey circles highlight Diuca songs from areas where yellow cardinals are absent.

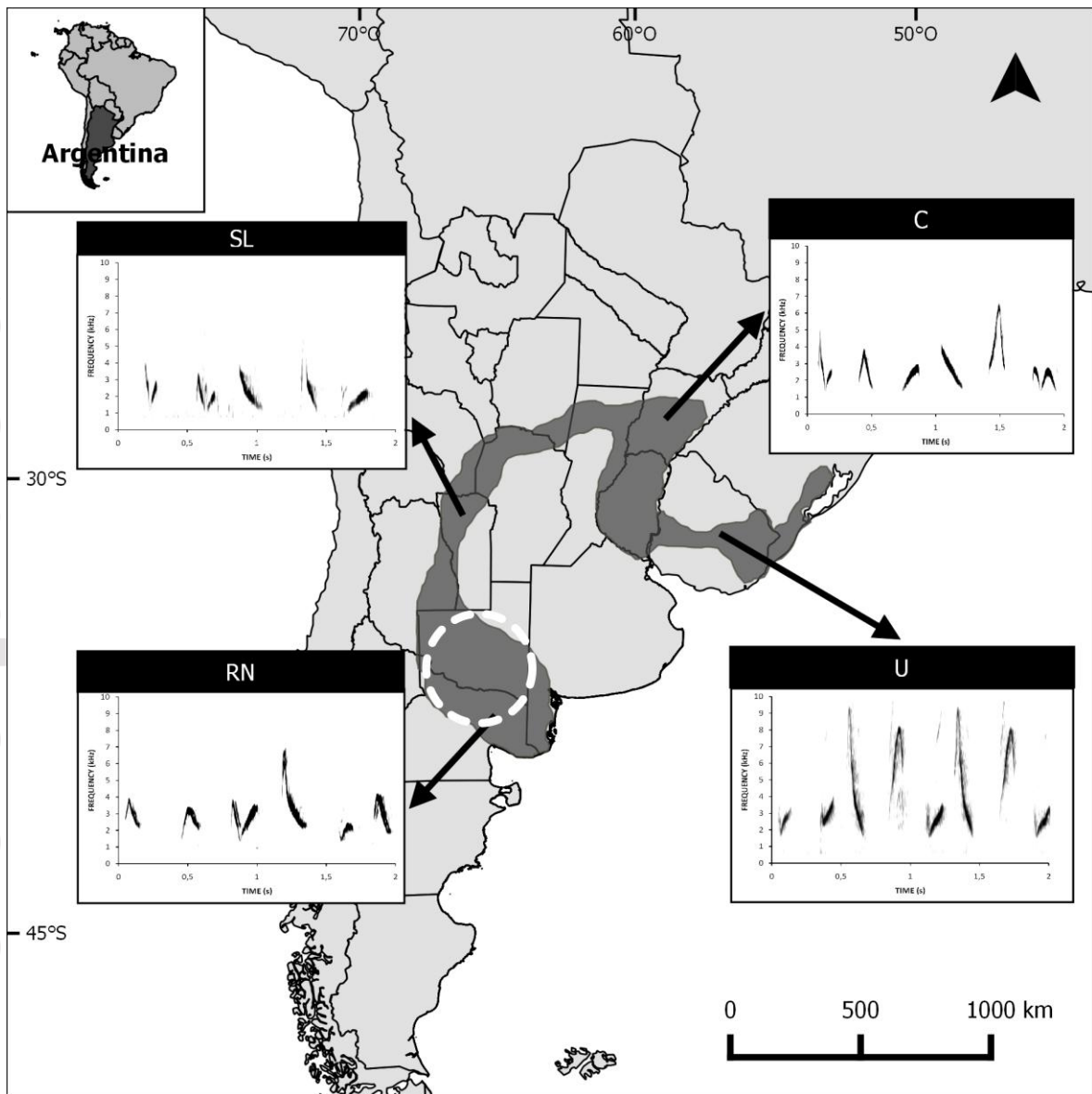


Figure 1

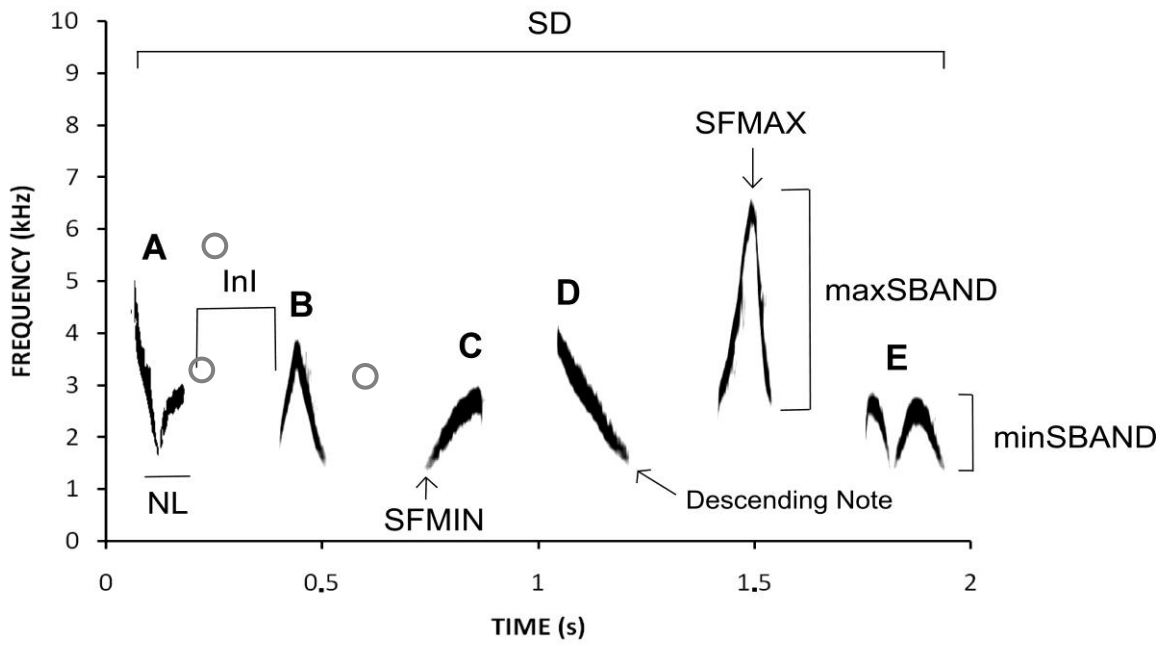


Figure 2

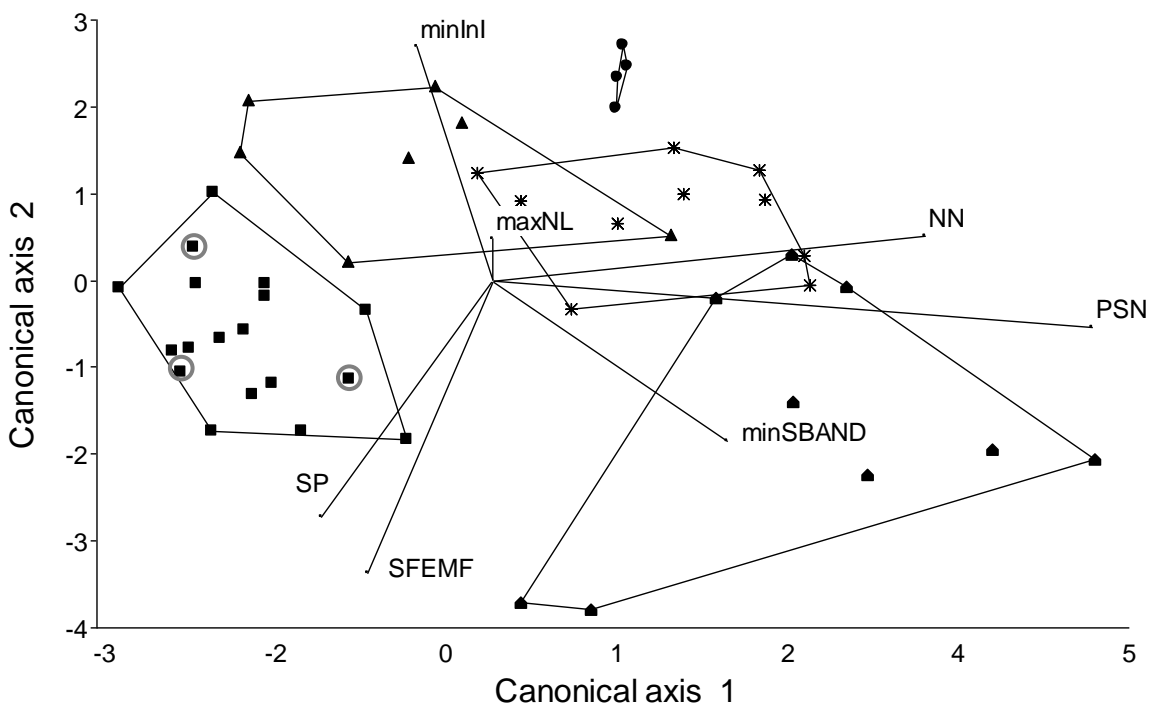


Figure 3