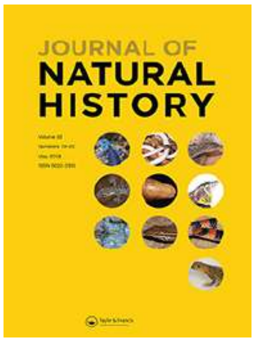

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Tegmina-size variation in a Neotropical cricket with implications on spectral song properties

Raysa Martins Lima[a], Karl-L. Schuchmann[b,c,d], Ana Silvia Tissiani[c], Lorena Andrade Nunes[e], Olaf Jahn[b,c], Todor D. Ganchev[c,f], Marcos Gonçalves Lhano[a] and Marinêz I. Marques[c,d]

[a]Centro de Ciências Agrárias, Ambientais e Biológicas (CCAAB), Universidade Federal do Recôncavo da Bahia, Cruz das Almas, Brazil; [b]Department of Vertebrates (Ornithology), Zoological Research Museum Alexander Koenig, Bonn, Germany; [c]Instituto Nacional de Ciência e Tecnologia em Áreas Úmidas, Universidade Federal de Mato Grosso, Cuiabá, Brazil; [d]Departamento de Biologia e Zoologia, Universidade Federal de Mato Grosso, Cuiabá, Brazil; [e]Programa de Pós-Graduação em Enfermagem e Saúde, Universidade Estadual do Sudoeste da Bahia, Jequie, Brazil; [f]Department of Electronics, Technical University of Varna, Varna, Bulgaria

This study evaluates the relationship between shape and size of tegmen, harp, mirror, and spectral range of calling song frequency of a Neotropical cricket subpopulation (*Lerneca inalata beripocone*. In addition, we compare intraspecific morphological divergence and calling song properties between individuals from different sites of the Pantanal of Poconé, Mato Grosso, Brazil. Regression analysis showed that the dominant and maximum calling song frequencies were negatively correlated with tegmen size, i.e. frequencies are either lower or higher depending on the corresponding size variation in resonance structures of the forewings. Canonical variable analysis demonstrated marked intraspecific differences in morphometric characters between localities of a *L. inalata* subpopulation c. 35 km apart (SESC-Pantanal Advanced Research Base and Pouso Alegre Farm, Mato Grosso, Brazil). *Lerneca inalata beripocone* at SESC had larger forewings than conspecifics from Pouso Alegre Farm. These morphological variations of wing properties related to reproductive behaviours were interpreted as fitness parameters, likely shaped by restricted gene flow during temporal habitat isolation episodes. Such isolation patterns occur in the Pantanal wetlands for several months during the annual hydrological cycle.

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Introduction

During reproduction, male crickets produce complex species-specific calling songs (Alexander 1962; Montealegre et al. 2009) for which several morphologically differentiated regions relate to differences in song parameters and to the physical nature of sound production (Walker 1962; Nocke 1971; Koch et al. 1988; Desutter-Grandcolas 1998; Bennet-Clark 1999, 2003). Crickets produce their songs by superimposing their tegmina (right over the left forewing) and rubbing the file teeth of the ventral side of the upper tegmen against the plectrum (scraper) of the opposite wing to produce vibrations of

CONTACT Marinêz I. Marques marinez513@gmail.com

both tegmina (David et al. 2003). The harp is the main resonator in the wing, and as such determines the frequency of the sound, while the mirror serves as a secondary resonator (Elliott and Koch 1985; Koch et al. 1988; Bennet-Clark and Bailey 2002). The speed of file and spectrum movements against each other is triggered by a mechanism in which the resonance of the forewing regulates the velocity at which the teeth of the file are caught and released by the plectrum (Elliott and Koch 1985; Koch et al. 1988; Prestwich et al. 2000; Bennet-Clark and Bailey 2002).

The dominant frequency (lowest harmonic frequency, generally in the range of 3–8 kHz) is one of the song components considered important in orthopteran species recognition (Morris 2008).

Klingenberg et al. (2010) argued that the geometry of different parts of the tegmen and their positions are subject to selection via acoustic performance. This finding is in concordance with Webb and Roff (1992) who described three relationships between song and forewing morphology in *Gryllus firmus*: (a) a significant relationship was found between the size of the stridulatory file and note length, (b) sound intensity is associated with resonance areas, and (c) no significant correlation exists between dominant frequency and length of the stridulation file.

Besides being an important tool for the analysis of sound frequency in crickets, (Klingenberg et al. 2010), harp shape and size have also been identified as important taxonomic characters (Gorochov 2007, 2014).

Geometric morphometrics is an adequate technique to analyse shape and size variations in organisms, combining geometric shape with multivariate statistics (Klingenberg and Monteiro 2005). By this procedure, data are generated from anatomical structures defined as homologous. Such allometries in geometric morphometrics can be applied to morphological characters, utilizing multivariate analyses, canonical methods, Procrustes and Mahalanobis distances (overview Bookstein 1991; Klingenberg et al. 2010).

Morphometric analyses have also been applied to study patterns of geographic variation and intraspecific differences in insects (Allegrucci et al. 1987; Diniz-Filho et al. 2000; Aytekin et al. 2007; Thorpe 2008; Nunes et al. 2012; Lima et al. 2014), including grasshoppers (Song 2009; Bidau et al. 2012).

In particular, by allowing the insertion of diverse anatomical features, the wings of insects can reveal a range of information (Grodnitsky 1999). A genetic-quantitative analysis using cricket wing characters concluded that these are structures that undergo little variation caused by environmental influence (Brown 1999), thus being a character with a high heritability (Nunes et al. 2008). However, in various other studies intraspecific morphological differences in tegmina variation in crickets and their evolutionary potential, contradict the hypothesis of the genetic stability of forewing characters (e.g. Klingenberg et al. 2010).

In this study, we report variation of tegmen and harp shape and size of a subpopulation of *Lerneca inalata beripocone* from the Pantanal wetlands of Brazil within a small distribution range (Lima et al. 2016). Furthermore, we verify the impact of these morphological structures on sound frequencies and compare intraspecific sound variations of individuals collected from the two localities in the northern Pantanal. We hypothesize that the observed variations in morphological structures are a result of the hydrological inundation cycle causing an annual habitat isolation of 4–6 months, possibly leading to restricted gene flow within the subpopulation.

Material and methods

Project, study area, and sampling

This study is part of the National Institute of Science and Technology of Wetlands Program (INAU/UFMT/CNPq). Research was conducted under permission of the Brazilian Ministério do Meio Ambiente (MMA), Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), Sistema de Autorização e Informação em Biodiversidade (SISBIO, permit nos. 39095-4 and 29890-2).

Our study was carried out at seven cerrado sites in the Pantanal of Poconé, Mato Grosso, Brazil: (A) within the vicinity of the SESC-Pantanal Advanced Research Base (−16.49879 S, −56.41309 W; 119–131 m asl, one site) and (B) Pouso Alegre Farm (−16.50303º S, −56.74533º W; 115–126 m asl, six sites). In the following, these locations are referred to as SESC and PA.

Fieldwork at PA was conducted between September and November 2013 and at SESC in July 2013. *Lerneca i. beripocone* is an uncommon cricket species in the northern Pantanal and only known from the *terra typica* in the Pantanal of Poconé. A total of 58 specimens were collected in dense understorey and soil cover vegetation of semideciduous forests.

Audio data collection

Audio recordings of calling songs were obtained from 41 males (38 from PA, three from SESC). Due to tegmen scrapes during collecting only 35 males were considered for the morphometric correlations of tegmen and frequency features. From one PA study site, FE1 no calling song audios were collected. Therefore our acoustic data refers to six study sites.

Data collection was performed in two shifts: 08:00–12:00 h and 18:00–22:00 h. Stridulations were recorded with a Zoom H4N^{TM}(Tokyo, Japan) and a Sony PCM-D50^{TM} (Tokyo, Japan), before capturing the individuals. Environmental temperatures during recordings were fairly stable (Table 1).

The audio stridulations of collected specimens were deposited at the INAU Pantanal BioData Center (IPBC/LETA/UFMT), with individual identification codes (species, georeferenced location, date, and time).

Table 1. Study sites (geographical coordinates see Material and methods), study periods, mean temperature (°C), number of stridulating males of *Lerneca inalata beripocone*.

				Mean frequency (kHz) ±SD		
Study site	Study period*	Mean temperature ºC	Number of males	MNF	MXF	DF
SESC-Pantanal Advanced Research Base						
Flooded forest edge	J/2013	23.2	3	4.1 ± 0.1	5.2 ± 0.6	4.7 ± 0.3
Pouso Alegre Farm						
Flooded forest 1	S-O/2013	25.0	10	4.0 ± 0.2	5.5 ± 0.3	4.7 ± 0.1
Flooded forest 2	S-N/2013	28.4	10	4.1 ± 0.3	5.8 ± 0.3	4.9 ± 0.2
Non-flooded forest	S-O/2013	29.6	6	4.3 ± 0.2	6.0 ± 0.2	5.2 ± 0.1
Flooded forest 3	S-N/2013	27.5	5	4.0 ± 0.2	5.7 ± 0.6	4.8 ± 0.3
Forest edge	S-N/2013	28.6	1	4.4	5.5	4.9

*J = July, S = September, O = October, N = November.
Mean frequency: MNF = minimum, MXF = maximum, DF = dominant.

Morphological data collection

The left tegmen of each *L. i. beripocone* individual was removed to measure the shape in order to differentiate and group individuals. After removal, tegmina were mounted between a slide and cover slip and photographed (Zeiss SteREO Discovery V20^{TM}, Oberkochen, Germany). Tegmina were measured in TPSDig2 ^{TM} (Rohlf 2006) based on 16 landmarks to capture the shape and centroid size, and distributed in the main areas involved in sound production (Figure 1 and Table 2).

We applied the centroid size as a commonly used morphometrical estimator, representing the generalized size of structures such as tegmen, harp, and mirror. The centroid size equals the square root of the sum of the square distances of landmarks from a set with a previously defined centroid (Kendall 1981; Bookstein 1986, 1990, 1991).

The stridulatory file, which corresponds to the second cubital vein (Cu2), anterior to the node (convergence region of veins from the basal area), correlates to landmarks 2 and 6. The harp is composed of diagonal veins (Di), first cubital vein (Cu1), second cubital vein (Cu2), which forms the boundary of the harp with the basal area, and by cross-veins, represented by landmarks 3 to 5, 7, and 9 to 11, similar in shape to a right triangle (Ragge 1955; Simmons and Ritchie 1996). The mirror, delimited by the vein Cu1 parallel to the median vein (M), which also forms the boundary of the chordal area and harp in the most distal part of the apical area, corresponds to landmarks 10 to 16. The scraper, located in the anterior region of the node, corresponds to landmarks 1 and 8 (Figure 1).

The terminology for the tegmina regions and veins is adopted from Ragge (1955, 1965); sound description follows Zefa (2006).

Individuals analysed are stored in the Entomology Section of Zoological Collection (CEMT), Bioscience Institute, Federal University of Mato Grosso.

Organization and analyses of datasets

The analyses are divided into two sections. Analyses in section 1 were carried out using datasets of tegmen shape and size, and frequency of calling song of the individuals from all study sites. In section 2, the collection sites and tegmen shape were treated as fixed parameters in the analyses, i.e. tegmina were used as vectors by measuring shape and size in order to differentiate individuals.

Analysis of tegmen shape and size and bioacoustics. Tegmen shape was analysed with MANOVA and principal component analysis (PCA); and tegmen size with ANOVA, applying a Tukey post-hoc analysis, to verify means that are significantly different from each other. These data were related to the morphological and bioacoustics variables (frequency of calling song: minimum, maximum, dominant) of 35 individuals, without distinction of the collection site (Klingenberg et al. 2002). A generalized Procrustes analysis was generated from Cartesian coordinates and applied to PCA to identify the similarities and differences between the wing shapes of individuals.

Regression analyses were performed to evaluate the correlation between the variables shape and size, and the frequency covariates (maximum, minimum, dominant) using the software MorphoJ ^{TM} (Klingenberg 2011).

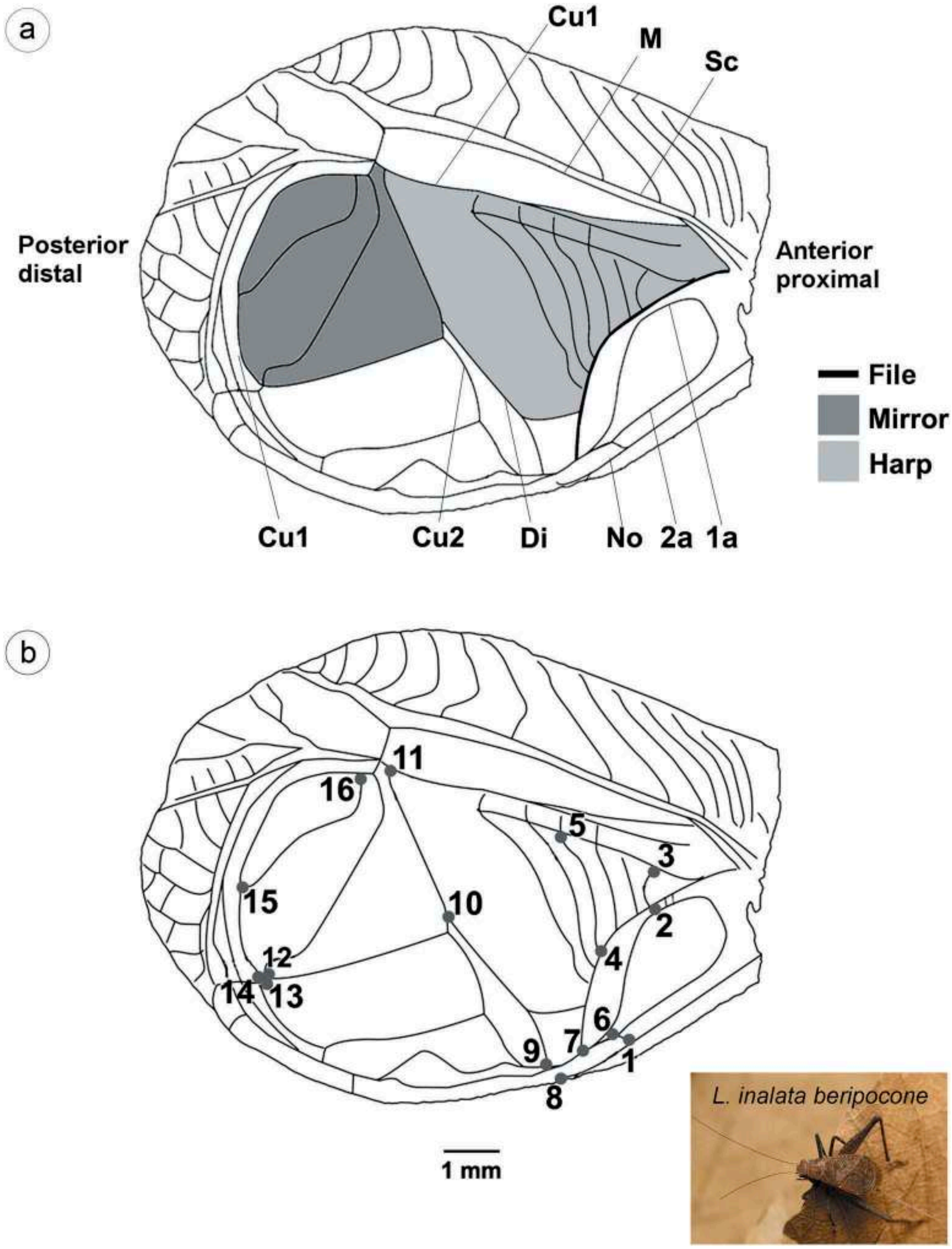

Figure 1. *Lerneca inalata beripocone*: Left tegmen, morphology (a) and landmarks (b) used for morphometric analyses. Vein abbreviations: Cu1 = cubit 1, M = media, Sc = subcostal, 1a = first anal, 2a = second anal, No = node, Di = diagonal, Cu2 = cubit 2.

Subpopulation analysis. The collection sites and tegmina shape were considered as fixed factors in the analysis of 58 individuals. Canonical variable analysis (CVA) (10,000 bootstrap replicates) was employed to estimate the variation in wing shape in

Table 2. Study sites (geographical coordinates see Material and methods), number of *Lerneca inalata beripocone* males and associated mean centroid size for left tegmen, harp, and mirror.

		Mean centroid size (mm)*		
Study site	Number of males	Tegmen	Harp	Mirror
SESC-Pantanal Advanced Research Base				
Flooded forest edge	3	14.22	6.81	6.06
Pouso Alegre Farm				
Flooded forest 1	10	13.48	6.49	5.77
Flooded forest 2	10	13.38	6.44	5.80
Non-flooded forest	6	12.63	6.11	5.52
Flooded forest 3	5	13.24	6.37	5.64
Forest edge	1	13.02	6.17	5.57

*The centroid size equals the square root of the sum of the squared distances of landmarks.

individuals from different localities, as was the grouping analysis UPGMA (unweighted pair group method with arithmetic mean) to evaluate possible morphometric patterns.

Since the recordings for each individual varied in length, with a minimum period of 40 s, three song phrases, 10 s each were extracted (up to 87–120 notes of each individual were analysed) and used for statistical analyses combining *L. i. beripocone* geomorphometric data and bioacoustics parameters.

Calling songs were analysed using the Avisoft SasLab Lite software (Avisoft Bioacoustics, Glienicke, Germany). Spectrograms were computed with fast Fourier transform (FFT) algorithm (length 256 points, Blackman-Harris windowing, and window overlap of 50% (Orsini et al. 2017). To illustrate site specific sound frequency variation patterns, examples of characteristic individual calling songs from four selected study sites were analysed according to amplitude (dB), instantaneous frequency (kHz, based on signal zero crossings), and relative intensity (dB). For this purpose, four selected syllables of one echeme were selected (Figure 2). Zero crossings were detected after bandpass filtering of the audio signal with a Butterworth filter of order four and cut-off frequencies (1000, 7000) Hz.

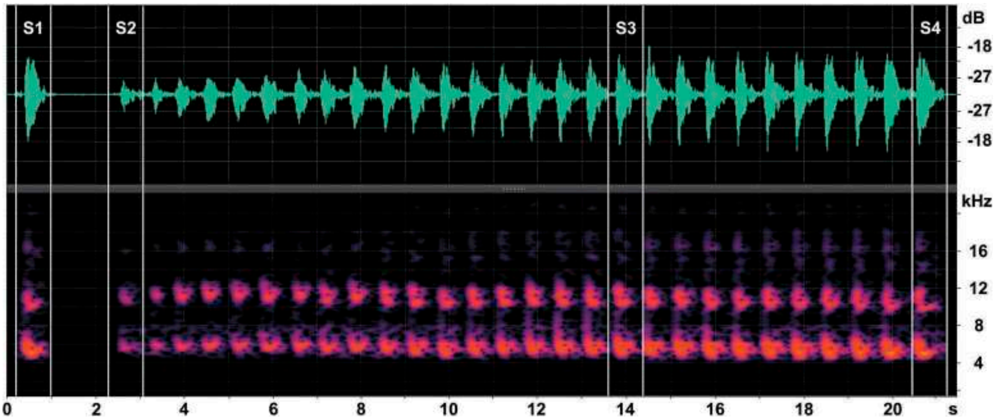

Figure 2. *Lerneca inalata beripocone*: Oscillogram of a calling song echeme (top, vertical axis shows frequency components in dB, horizontal axis time in seconds) and spectrogram (below, vertical axis indicates frequencies in kHz, horizontal axis time in seconds) of a representative male from Pouso Alegre (site flooded forest 1, FF1). Example: framed syllables (S1–S4) selected for further frequency analyses.

Three sound frequency variables (kHz) were quantified: minimum (MNF), maximum (MXF), and dominant (DF). In all recordings, the spectral peaks of *L. i. beripocone* sound emissions were in the range (25, 35 dB) above the noise floor. Dominant frequency and zero crossings were computed with MATLAB (MatWorks Inc., Natick, MA, USA).

For a general comparison of the two geographical study sites (SESC and PA), we calculated the mean amplitude frequency based on minimum and maximum calling song frequencies.

Results

Analysis of geomorphometrics and calling song

The PCA showed that most of the variation in tegmen shape (37.8%) is explained by the first two principal components (PCs). The changes in shape for the first two PCs consisted of relative variations of structures in some tegmen regions. PC1 and PC2 were associated with a change particularly in the veins delimiting the harp (landmarks 3, 4, and 5), while landmark 15 refers to the vein that divides the mirror (Figure 3). The

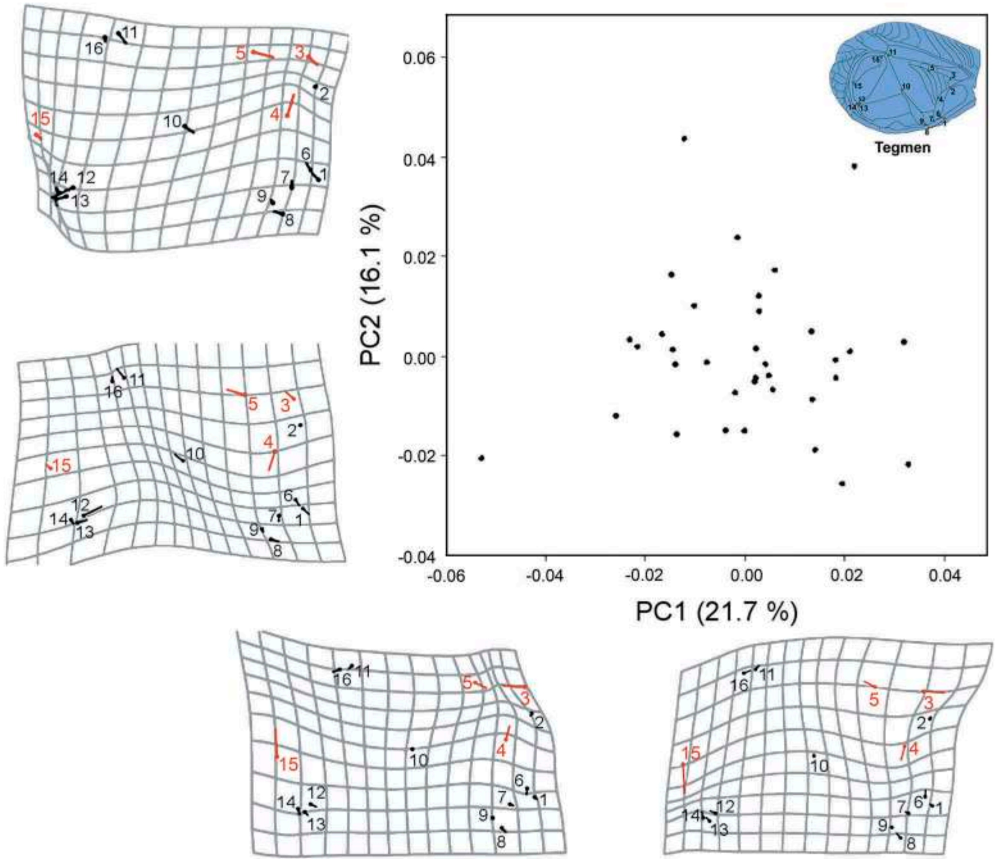

Figure 3. Thin-plate spline using 16 landmarks to depict left wing shape variation in *Lerneca inalata beripocone* along each PC axis. Red landmarks explain most of the tegmen shape variation.

other PCs are similarly composed of changes in different tegmen regions and not located in a single part.

The PCA, using only the landmarks from 3 to 5, 7, and 9 to 11 representing the harp, showed that most of the variation in the shape of this structure is concentrated in the first two PCs, explaining 64.4% of the variation. The thin-plate splines (Figure 4) show that shape changes in the first two PCs consisted of relative changes and deformations, especially in landmarks 3, 4, and 5, located in the node region, in vein 1a and the vein parallel to Cu1 (Figure 1).

The PCA for the mirror region, landmarks 10 to 16, indicate that the first two PCs explain 65.5% of the total available variation in the shape. According to the thin-plate splines (Figure 5), the shape changes verified for the first two PCs consist of relative changes and deformations, especially in vector 6, landmark 15, and the vein that divides the speculum.

When harp and mirror were analysed separately, shape variation became more evident than considering the tegmen as a whole. In other words, the landmarks located on the posterior of the mirror anterior to the harp explain more of the variation, according to the thin-plate splines shown in Figures 3–5.

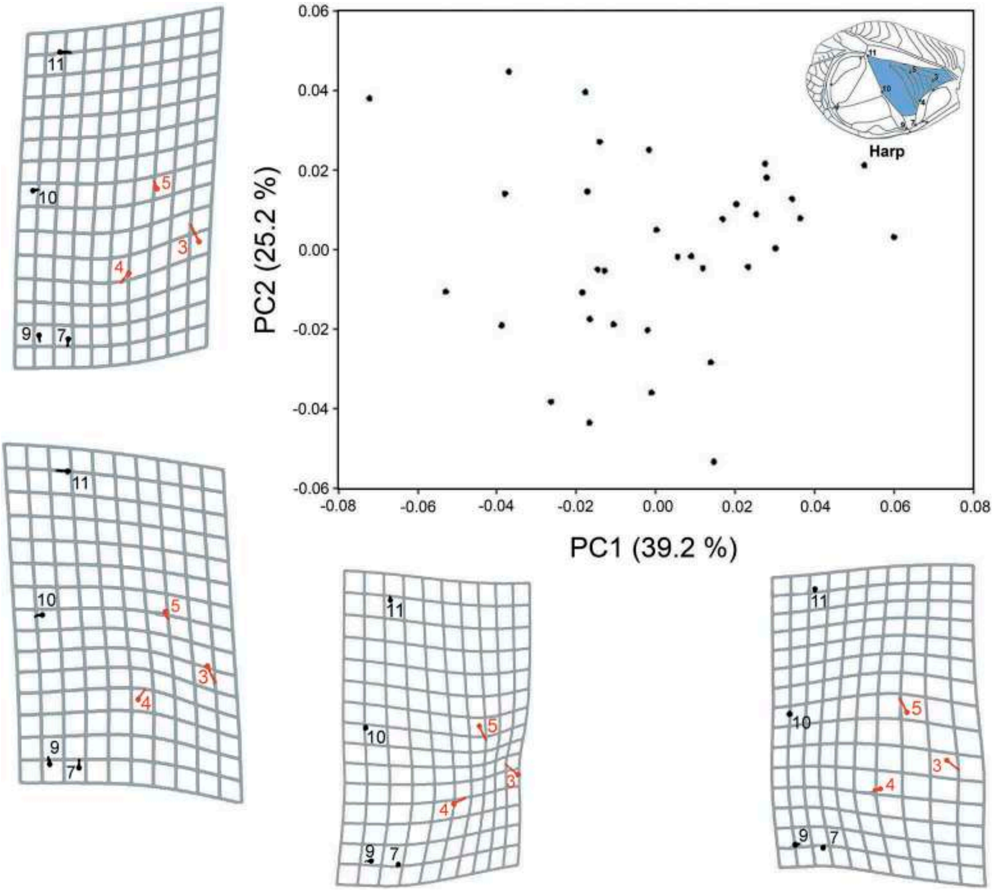

Figure 4. Thin-plate spline using seven landmarks to depict harp shape variation in *Lerneca inalata beripocone* along each PC axis. Red landmarks explain most of the tegmen shape variation.

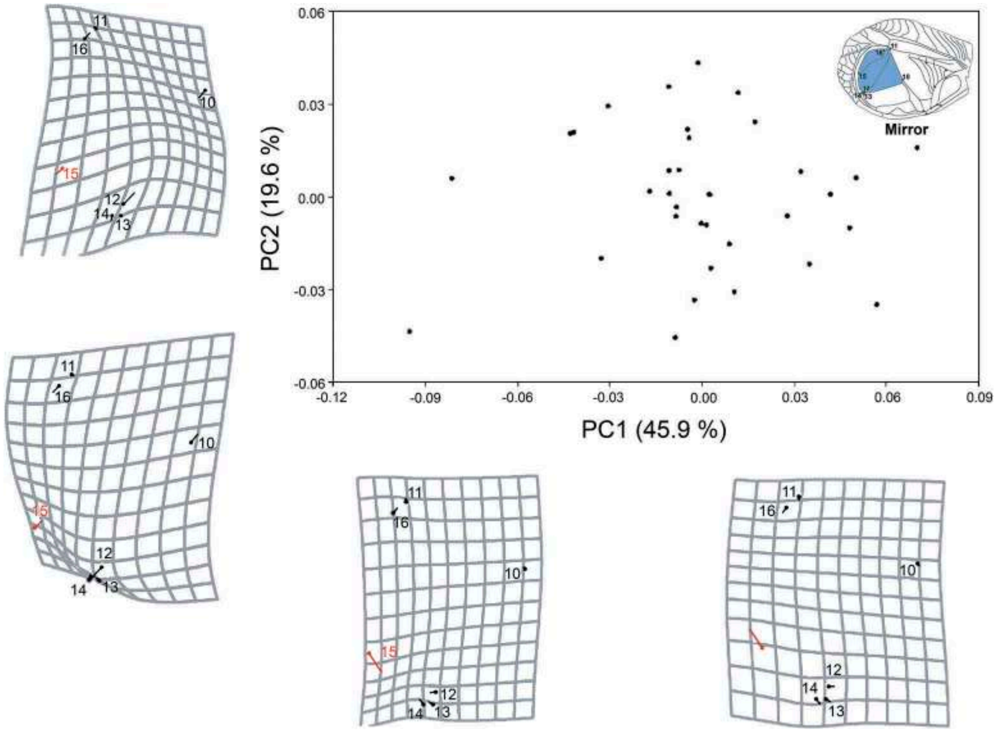

Figure 5. Thin-plate spline using seven landmarks to depict mirror shape variation in *Lerneca inalata beripocone* along each PC axis. Red landmarks explain most of the tegmen shape variation.

Calling song MXF means differed between SESC and PA, with 5.0 kHz at SESC and 5.8 kHz at PA, corresponding to a mean amplitude (MXF-MNF) frequency of 0.9 kHz at SESC and 1.6 kHz at PA.

Regression analysis indicated that the maximum and dominant frequencies are determined by wing size, evaluating the tegmen as a whole and the harp individually (Table 3). Taking the tegmen structure as a whole, there is a negative correlation between size and maximum frequency, and between size and dominant frequency, with high significance and error margins of less than 5%, indicating collinearity between these effects. No significance was observed when mirror morphological matrices were compared with shape, size and song frequencies.

Subpopulation analysis

The CVA showed that most of the tegmen shape variation of *L. i. beripocone* is explained by the first two dimensions, with a gradual decrease in the eigenvalues. The first three canonical variables explained 83% of the variation in the tegmen shape when individuals of all studied areas were treated cumulatively. The result is shown in the grouping analysis (UPGMA), performed on the average tegmina shape from both study sites. Tegmina shape of pooled individuals from PA varied between each collecting site but more so when compared with those from SESC (Figure 6). This is indirectly supported when compared to characteristic calling song patterns (oscillogram amplitude dB s^{-1}) of

Table 3. Regression analysis comparing matrices of shape and size using measurements of the complete left tegmen, harp, and mirror with matrices of song frequencies of *Lerneca inalata beripocone*.

Morphological structure	Compared matrices	Likelihood
Tegmen	Size vs. shape	0.8469^{NS}
	Size vs. MNF	0.1048^{NS}
	Size vs. MXF	0.0180*
	Size vs. DF	0.0097**
	Shape vs. MNF	0.7765^{NS}
	Shape vs. MXF	0.2039^{NS}
	Shape vs. DF	0.6202^{NS}
Harp	Size vs. shape	0.5516^{NS}
	Size vs. MNF	0.2897^{NS}
	Size vs. MXF	0.0159*
	Size vs. DF	0.0296*
	Shape vs. MNF	0.7295^{NS}
	Shape vs. MXF	0.8296^{NS}
	Shape vs. DF	0.8460^{NS}
Mirror	Size vs. shape	0.5071^{NS}
	Size vs. MNF	0.6360^{NS}
	Size vs. MXF	0.0946^{NS}
	Size vs. DF	0.1057^{NS}
	Shape vs. MNF	0.5864^{NS}
	Shape vs. MXF	0.5908^{NS}
	Shape vs. DF	0.6353^{NS}

Values marked *are significant ($p < 0.05$), **($p < 0.01$), NS = not significant.
MNF = minimum, MXF = maximum, DF = dominant.

individuals from corresponding study sites (examples shown in Figure 7). Actually, the calling song of the *Lerneca* subpopulation shows high site-specificity in syllable pulses and composition with modulation in frequency and intensity as exemplarily shown for four different syllables of one echeme shown in Figure 8. When DF and syllable duration of calling songs were compared for the different study sites (examples in Figure 9) similar variations of intraspecific frequencies were observed, also confirming such study site specificity for these parameters.

Discussion

In this study, we characterize variations of geometric size and shape of the forewings (tegmen) of males from a Neotropical cricket subpopulation of *Lerneca inalata* to analyse morphological implications on spectral calling song variations. Several studies demonstrate a link between wing morphology of stridulatory organs and the calling song structure. It is therefore reasonable to predict that the shape of cricket tegmina has been influenced by sexual selection on acoustic properties (Simmons and Ritchie 1996; Montealegre 2009; Pitchers et al. 2014). This relationship between morphological features and acoustical structure in many Orthoptera species is supported by the negative correlation between frequency and body size (Scheuber et al. 2003; Montealegre 2009). Our regression analyses confirm such an inverse relationship between tegmen size and MXF and between tegmen size and DF at an intraspecific level of the calling song in *L. i. beripocone*, indicating close collinearity ($p < 0.02$) between these parameters. Hence, the larger the male size, the lower is the maximum frequency of his calling song. Furthermore, significant results were obtained when comparing harp size with MXF and DF ($p < 0.03$).

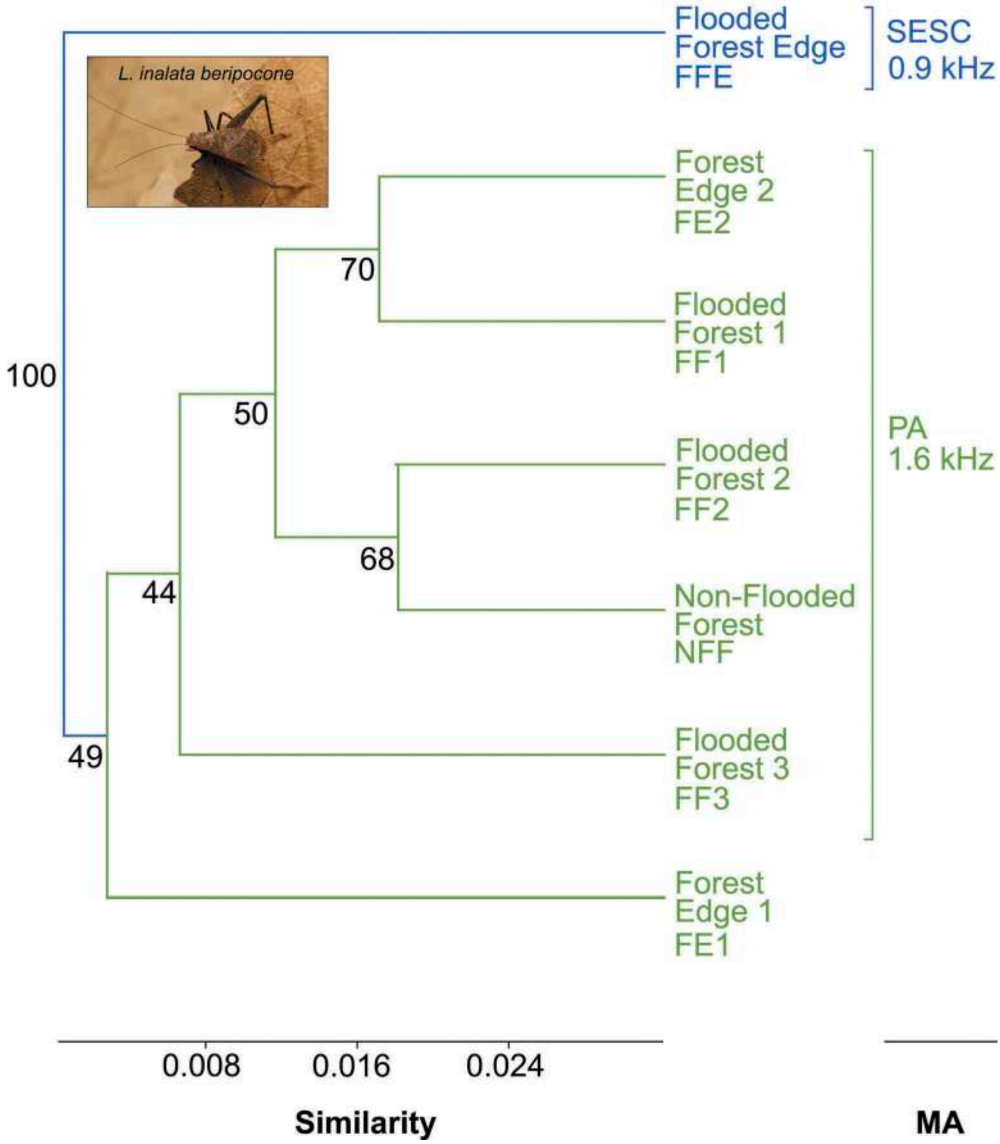

Figure 6. UPGMA dendrogram based on wing shape with 10,000 bootstrap replicates (left), of the subpopulation of *Lerneca inalata beripocone*; mean amplitude (MA, right) of frequency of calling song (right) from two locations in the Pantanal of Poconé, MT, Brazil. SESC (FFE = flooded forest edge), PA (FF1, FF2, FF3 = flooded forest; NFF = non-flooded forest; FE1, FE2 = forest edge).

Such a negative correlation between harp area and DF has also been described in *Gryllus campestris* by Klingenberg et al. (2010), Simmons (1995), and Simmons and Ritchie (1996), and in many species of katydids, including *Cyphoderris monstrosa* (Mason 1996), *Requena verticalis* (Bailey and Yeoh 1988), and *Tettigonia cantans*) (Latimer and Sippel 1987). Because of these findings, it has been widely accepted that female crickets can evaluate male body size by the carrier frequency characteristics (frequency of maximum amplitude). Meanwhile, a large body of literature reports no

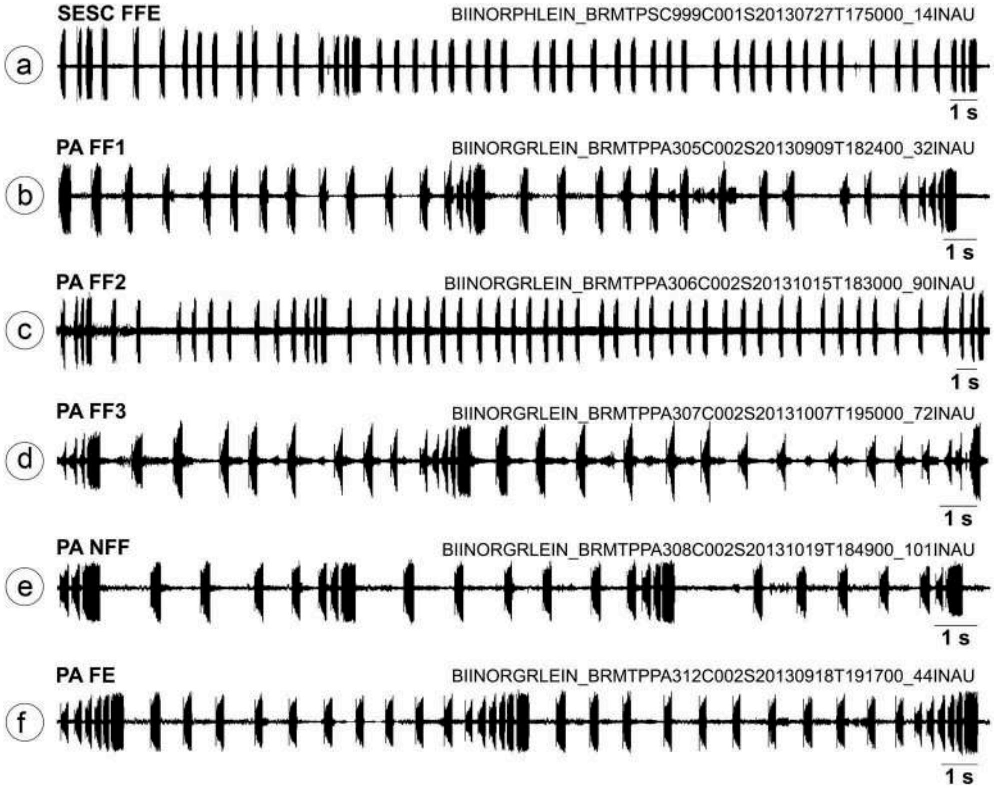

Figure 7. *Lerneca inalata beripocone*: Examples of calling song patterns (oscillograms, frequency components in dB/seconds) from six Pantanal study sites. (a) SESC, flooded forest edge, FFE; (b) Pouso Alegre, flooded forest 1, PA FF1; (c) Pouso Alegre, flooded forest 2, PA FF2; (d) Pouso Alegre, flooded forest 3, PA FF3; (e) Pouso Alegre, non-flooded forest, PA NFF; (f) Pouso Alegre, forest edge, PA FE. BIINORGRLEIN codes refer to individual recordings.

such correlation in calling song frequency and male size (review Verburgt and Ferguson 2010). In a more recent study Pitchers et al. (2014) showed for the Australian (allo) species complex of the black field cricket (*Teleogryllus commodus*) across the vast species range of c. 6000 km that this relationship is variable among populations.

According to our UPGMA analysis (Figure 6) from the subpopulation of *L. i. beripocone*, the formation of two different tegmen-size groups was noticeable, clearly separating SESC individuals (35 km apart) from those collected at PA sites. However, at the PA site we observed a considerable morphometric intraspecific variation in tegmen shape and size of harp and mirror between individuals of different study sites separated by short distances of 90 to 1050 m.

Intraspecific variations of morphological characters and calling song properties are poorly documented in the literature and exist only for *Gryllus campestris* (Simmons and Ritchie 1996; Scheuber et al. 2003; Jacot et al. 2004). According to our knowledge, this study is the only one for a tropical cricket based on intraspecific morphometrics related to tegmen size and spectral song properties. Examples of characteristic frequency component variations in syllable amplitude (dB),

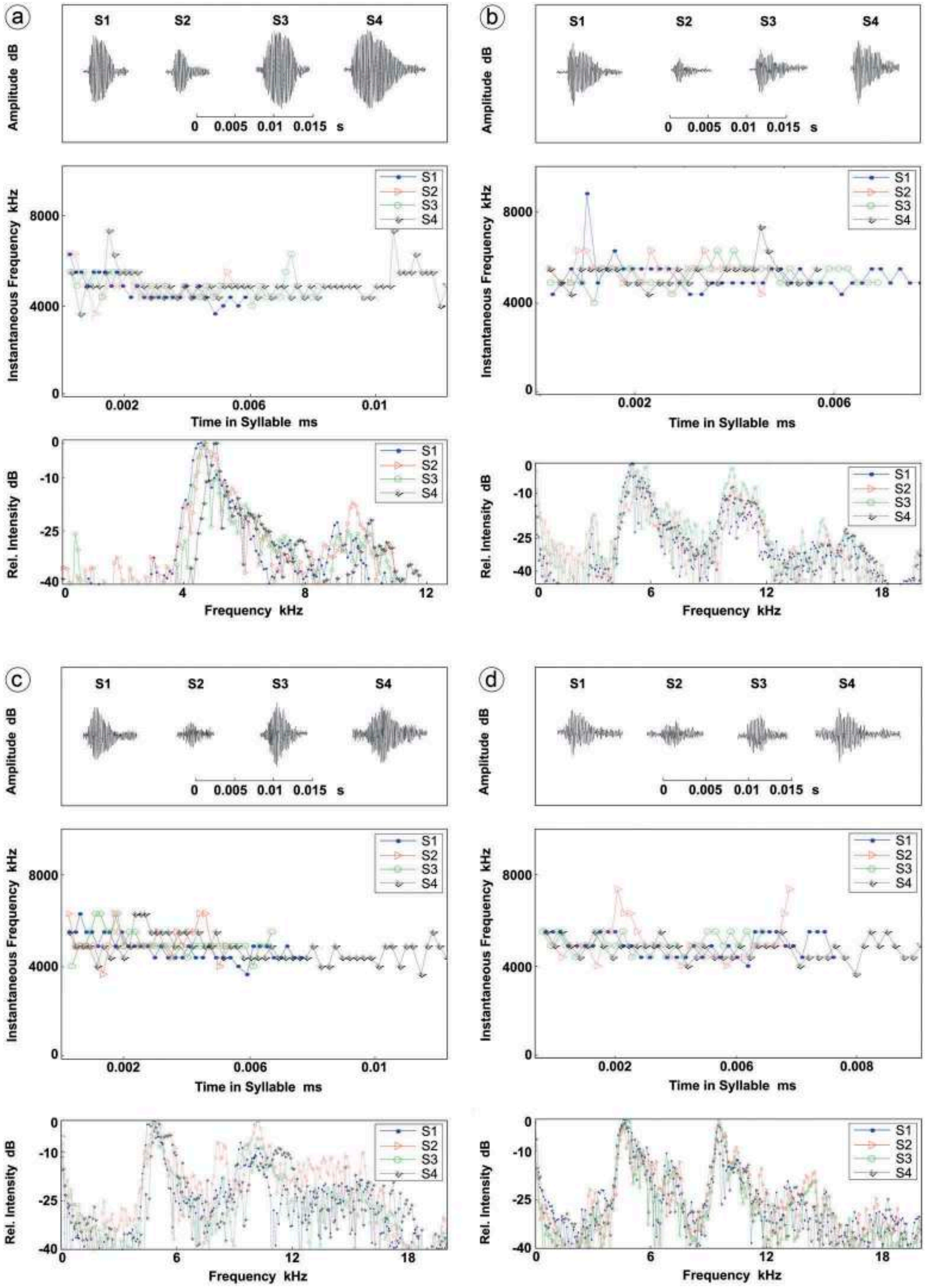

Figure 8. Analysis of selected calling song syllables of *Lerneca inalata beripocone* from four Pantanal study sites. Top: oscillograms; middle: frequency analyses based on zero-crossing; bottom: power spectra of selected syllables. S1–S4: Selected syllables of one echeme (first order assemblage of syllables). (a) SESC (flooded forest edge – FFE); (b) Pouso Alegre (flooded forest 1 – FF1); (c) Pouso Alegre (flooded forest 2 – FF2); (d) Pouso Alegre (flooded forest 3 – FF3).

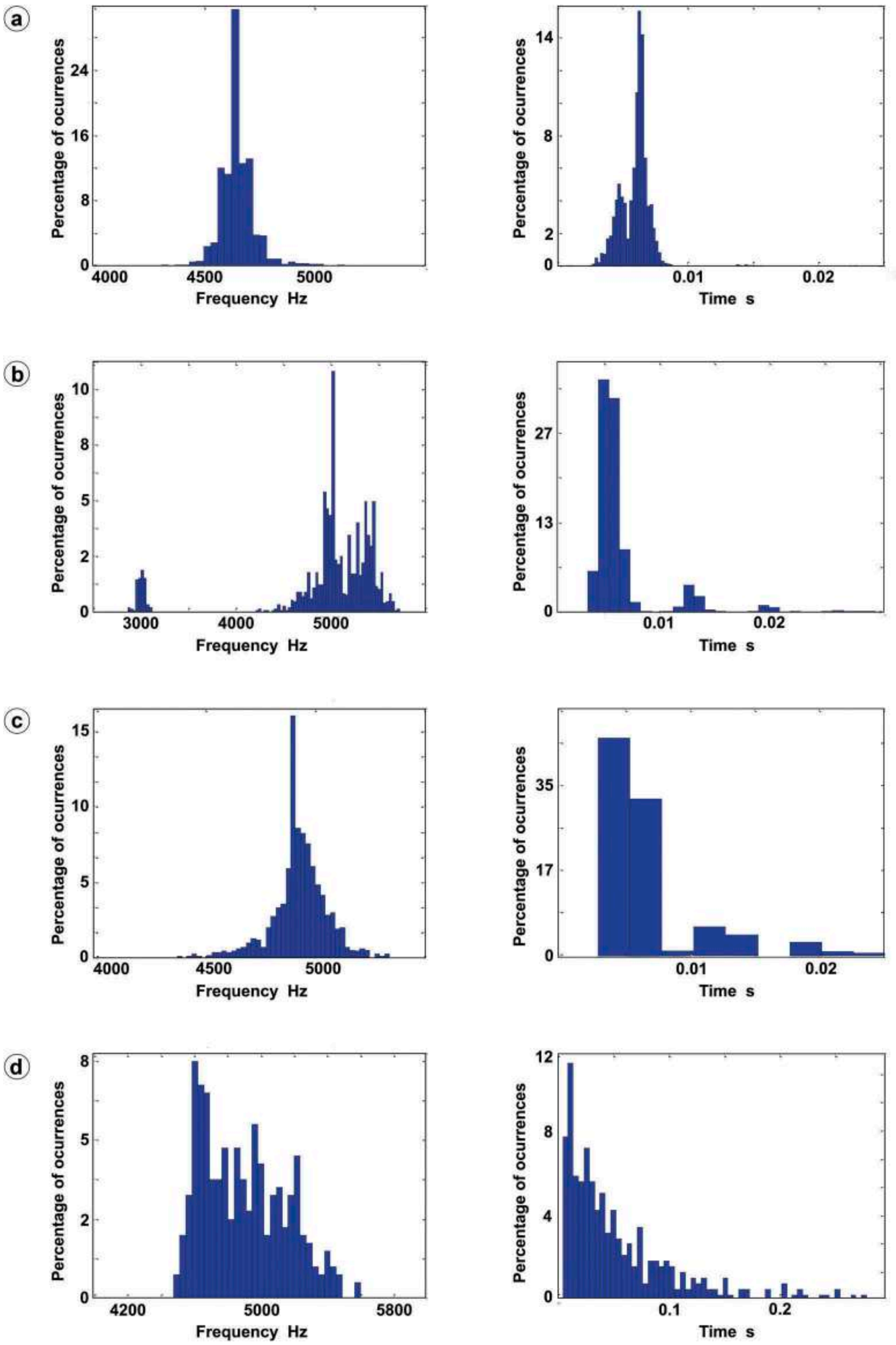

Figure 9. *Lerneca inalata beripocone*: Examples of dominant frequency (DF) (left) and syllable duration (right), based on individual calling song data shown in Figure 8. Study sites: (a) SESC, flooded forest edge (FFE); (b) Pouso Alegre, flooded forest 1 (FF1); (c) Pouso Alegre, flooded forest 2 (FF2); (d) Pouso Alegre, flooded forest 3 (FF3).

instantaneous frequency (kHz) compared to time in syllable (ms), and relative intensity (dB) related to syllable frequency (kHz) of *L. i. beripocone* individuals from different study sites demonstrate that frequency variations are morphology-based (tegmen-size variations).

Implications for such distinct intraspecific calling song properties are better understood when considering the hydrological cycle of the Pantanal which annually turns the savannah-like ecosystem into a wetland with a flood pulse of up to a 2 m rise in the northern Pantanal region, our study area. The monomodal inundation creates habitat fragmentations at a large scale leading to temporally isolated vegetation islands for 4–6 months, and resulting in a restricted gene flow between terrestrial animal populations (Junk et al. 2011). This stochastic isolation impact may contribute to evolutionary processes like rapid genetic changes, especially in insect populations, aided by their often rapid generation time and high rate of reproduction (overview Loxdale 2010). The result of such extreme environmental changes may be an important driver of intraspecific character plasticity initiating morphological and spectral diversification processes in *Lerneca*.

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