**Variability in anuran advertisement call: A multi-level study with 15 species of monkey
tree frogs (Anura: Phyllomedusidae)**

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29 Abstract

Understanding the variability of acoustic signals is a first important step for the comprehension of the evolutionary processes that led to current diversity. Herein, we evaluate the variability of the advertisement call of the phyllomedusid species from the genera *Phyllomedusa* and *Pithecopus* at different levels: intra-individual, intra-population, inter-population, intra-species, and inter-specific. An analysis of coefficients of variation showed a continuum of variability between the acoustic parameters analyzed, from static to highly dynamic. Most of the variation was attributed to the inter-specific level while call parameters at the intra-individual level varied the least. However, each parameter behaved differently with call interval being the most variable across all levels. Most temporal acoustic parameters were affected by environmental temperature while pulse rate and dominant frequency were strongly influenced by body size. Only pulse rate was correlated to the geographic distance between populations, while all parameters presented a significant phylogenetic signal. Based on these results, we discuss the possible importance of different evolutionary forces and the usage of vocalizations for taxonomic purposes.

44 *Key words:* acoustic communication, evolution, *Pithecopus*, *Phyllomedusa*, phylogenetic
45 signal, trait diversification, variation

46 **Introduction**

47 Understanding variability is fundamental for the comprehension of evolution
48 (Hallgrimsson and Hall 2011). Darwin's observations on phenotypic variation were the basis
49 for the development of the concept of natural selection (Darwin 1859), and intra and inter-
50 specific variation were central for the modern evolutionary synthesis (Mayr 1966; Wright
51 1984). Acoustic signals are important for a large proportion of current fauna, and for most
52 groups it has a predominantly reproductive function (Bradbury and Vehrencamp 1998). There
53 is evidence that acoustic communication may not have increased the diversification rates of
54 terrestrial vertebrates at a macro evolutionary scale (Chen and Wiens 2020). However,
55 because these signals are involved in conspecific recognition, they may have a key role in
56 speciation of highly vocal groups, such as birds and frogs, and their importance as an
57 evolutionary force has been the focus of many studies (Wilkins et al. 2013).

58 Acoustic signals are the predominant form of communication for the vast majority of
59 anuran species. Although many species present more than one type of call, the advertisement
60 call emitted by males with the main function of attracting females can be considered the most
61 important acoustic signal for this clade (Wells 1977). Since the beginning of bioacoustic
62 studies on anurans, authors report that each species has a distinct advertisement call (Blair
63 1958, 1964; Duellman and Pyles 1983). Furthermore, it has been shown that females prefer
64 conspecific advertisement calls, even when considering sister taxa with relatively similar calls
65 (Gerhardt 1974; Backwell and Jennions 1993). Thus, this vocalization is considered an
66 important prezygotic reproductive barrier (Gerhardt and Huber 2002) and has been used as an
67 important taxonomic tool, helping overcome the lack of useful external morphological traits in
68 frogs (Padial and De la Riva 2009; Köhler et al. 2017).

69 The anuran advertisement call is considered stereotyped, especially in comparison to
70 other vertebrates such as birds and mammals. Still, there is considerable amount of intra-

71 specific variation at the individual level (Howard and Young 1998), at the population level
72 (Sullivan 1982), and among populations of the same species (Sullivan 1989). Although
73 several studies clearly demonstrate this variation in each level separately, few studies have
74 quantified how different acoustic parameters vary across multiple levels (within and between
75 individuals, populations and species) (Castellano et al. 2002; Bee et al. 2010). Different
76 acoustic parameters from the same call are semi-independent and might encode distinct
77 messages, thus evolving under differing selective pressures (Gerhardt and Huber 2002; Ryan
78 and Kime 2003). Therefore, a broad comprehension of variability at different levels enables
79 the proposition of various hypotheses about the evolutionary mechanisms that led to the
80 present acoustic diversity.

81 In general, variation in acoustic signals can be related to pleiotropic effects (Podos
82 2001), stochastic processes (Goicoechea et al. 2010), natural selection (Ryan et al. 1990; Röhr
83 et al. in press), and sexual selection (Gerhardt 2005). For anurans, pleiotropic effects of
84 morphology and physiology are exemplified by the well-known influence of body size and
85 temperature on acoustic parameters. The size of the vocal apparatus usually affects spectral
86 parameters, while temperature often influences temporal characteristics of the calls (Gerhardt
87 1994). Such relationships can affect the variability of different acoustic parameters at different
88 levels. For example, acoustic parameters that are more dependent on temperature should vary
89 more, especially intra-individually and in a shorter time scale. At the same time, parameters
90 that are highly dependent on morphology should be more stereotyped, with most of the
91 variation occurring at levels above individuals or ontogenetically. Therefore, it is important to
92 carefully consider the influence of temperature and body size in any bioacoustic study.

93 Stochastic processes also affect the evolution and variability of acoustic signals. For
94 anurans, these effects are tested by evaluating the geographic variation of acoustic parameters
95 (Pröhl et al. 2007) or the effect of phylogeny on inter-specific variation (Goicoechea et al.

2010). While some acoustic parameters vary mostly in response to stochastic processes, others are not correlated with phylogeny or biogeography, possibly because they are under strong selective forces (Erdtmann and Amézquita 2009). These different evolutionary pathways should also influence the amount of variation detected at different levels.

Selection may affect the variability of acoustic parameters in different ways (Wilkins et al. 2013). For example, acoustic parameters important for conspecific recognition are under selective pressure not to overlap with calls from sympatric species, possibly leading to more stereotypy and hence reducing the likelihood of hybridization (Lemmon 2009). Beyond this, in diverse acoustic communities, calls might also be more stereotyped in order to use silent windows and reduce masking interference (Chek et al. 2003; Bee 2008). At the same time, a higher stereotypy might be expected in habitats with dense vegetation coverage, because the acoustic propagation scenario is more stable compared to open areas, which are more susceptible to wind and temperature shifts (Wiley and Richards 1978; Ey and Fischer 2009).

Nevertheless, most studies on the variability of anuran advertisement call have focused on sexual selection (Gerhardt and Huber 2002). One key measurement of acoustic variation in frogs is the individual coefficient of variation. Based on this measure, extensive literature has shown that variability of different acoustic parameters follows a continuum from static to dynamic (Gerhardt 1991; Castellano et al. 2002; Bee et al. 2010). Static parameters should be under stabilizing or weakly directional sexual selection (Gerhardt 1991; Gerhardt and Huber 2002). Indeed, most studies confirm that females tend to prefer advertisement calls with values close to the species/population mean for such acoustic parameters (Castellano and Giacoma 1998; Rosso and Castellano 2006), and these parameters should be important for specific recognition and are more reliable taxonomic tools (Köhler et al. 2017). In contrast, acoustic parameters with high individual variability should be under directional selection due to female preferences for calls with more extreme values (Castellano and Giacoma 1998;

121 Bosch and Márquez 2005) which indicate male's quality or facilitate localization (Gerhardt
122 1994; Gerhardt and Huber 2002).

123 Herein, we evaluate the variability of different acoustic parameters of the
124 advertisement call of 15 tree frog species of the family Phyllomedusidae at different levels:
125 intra-individual, intra-population, inter-population, intra-specific, and inter-specific. We also
126 estimated what percentage of the total variation detected can be explained by each level.
127 Furthermore, we tested: 1) the effect of environmental temperature and body size on the
128 acoustic parameters, 2) if they present significant phylogenetic signal and 3) if they are
129 correlated to geographic distance between populations.

130

131 **Material and Methods**

132 *Study organisms and data collection*

133 The family Phyllomedusidae, commonly known as monkey tree frogs, currently
134 harbors 66 species distributed in the Neotropics (Duellman et al. 2016; Frost 2018). In Brazil,
135 the genera *Phyllomedusa* and *Pithecopus* are widely distributed, virtually found in all biomes.
136 The former is composed of 16 large species, while the latter comprises 11 smaller species. We
137 recorded the advertisement call of 15 species from both genera (Figure 1) across a large
138 spatial scale, encompassing 31 localities and 13 states throughout Brazil (Figure 2; Table S1
139 in Supplementary Material): *Phyllomedusa bahiana* Lutz, 1925, *P. burmeisteri* Boulenger,
140 1882, *P. camba* De la Riva, 1999, *P. distincta* Lutz, 1950, *P. iheringii* Boulenger, 1885, *P.*
141 *tetraploidea* Pombal and Haddad, 1992, and *P. vailantii* Boulenger, 1882; *Pithecopus ayeaye*
142 Lutz, 1966, *P. azureus* (Cope, 1862), *P. centralis* (Bokermann, 1965), *P. hypocondrialis*
143 (Daudin, 1800), *P. megacephalus* (Miranda-Ribeiro, 1926), *P. nordestinus* (Caramaschi,
144 2006), *P. oreades* (Brandão, 2002), and *P. rohdei* (Mertens, 1926). Although *P. tetraploidea*
145 and *P. distincta* can hybridize along a geographic contact zone (Haddad et al. 1994), our

sampling points comprised allopatric populations, therefore obviating the problem of hybrid specimens (see Figure 2 for the spatial distribution of samples).

We used a Marantz PMD 660 digital recorder with a sampling rate of 48 kHz and 16-bit resolution, connected to a Sennheiser ME66 directional microphone for most recordings. After recording most individuals, we measured the environmental temperature and snout-vent length (see Table S1 in Supplementary Material for data on each recording: species, locality, coordinates, temperature, body size, and total number of calls analyzed). Acoustic parameters were measured in Raven Pro 1.4 and spectrograms produced as follows: FFT window width = 256; Frame = 100; Overlap = 50%.

We analyzed a total of six acoustic parameters for all calls recorded, which are comparable at all levels, even between different species of these genera. We used the terminology for acoustic parameters after Köhler et al. (2017): peak of the dominant frequency (DF), call duration (CD), total number of pulses in the call (number of pulses - NP), average pulse duration considering all pulses from the call (pulse duration - PD), pulse rate (PR) as the ratio between NP and CD, and interval between calls (inter-call interval - CI) (see Figure 3 for details). Calls of *Phyllomedusa* and *Pithecopus* are structured in pulses that may be grouped into separate notes. Because calls of different species may or not present more than one note, making it difficult to establish homology between notes, the number of notes was not included in the analyses.

Acoustic variation analyses

To estimate the variability of acoustic parameters we calculated the coefficient of variation (CV), which is a standardized measure of dispersion calculated through the ratio of the standard deviation to the mean, for all acoustic parameters at five different levels: 1) intra-individual (different calls from a single individual), 2) intra-population (calls from different

170 individuals from a single population), 3) inter-population (calls from individuals from
171 different populations), 4) intra-species (calls from different individuals from the same species,
172 independent from which population), and 5) inter-specific (calls from individuals from
173 different species).

174 A stratified hierarchical subsampling method was implemented to deal with the
175 unbalanced sample sizes across species (see Table S2 in Supplementary Material). We
176 repeatedly drew from our data pool five different calls that were used to calculate the CV. We
177 ensured that calls were drawn from different entities forming the respective level of interest.
178 For instance, before drawing five calls at the intra-individual level, we drew a species, then a
179 population, then an individual, then five different calls. For the intra-population level, we first
180 drew a species, then a population, then five calls from different individuals and so on (see
181 Table 1 for a stepwise description of all steps used to obtain the calls used for each level). We
182 repeated each subsampling 1,000 times for each level to create five distributions of variation
183 for each acoustic parameter. For intra and inter-population levels, we only used data from
184 *Pithecopus nordestinus* due to the lack of sufficient population replicates for the other species.
185 Due to the low number of samples for *P. burmeisteri* and *P. vaillanti*, these species were only
186 included in the inter-specific variation level analysis.

187 Additionally, we performed a hierarchical ANOVA to calculate the amount of
188 variation that can be attributed to each level. For this analysis we used only four levels in
189 order to access the variability repartition: call, individual, population, and species. To test the
190 influence of body size (BS) and environmental temperature on acoustic parameters, we
191 performed linear mixed models using species as random effect. In this analysis, we used the
192 mean value from all calls from each individual, with the acoustic parameters as response
193 variables and BS and temperature as predictors. To test the correlation between acoustic
194 parameters and geographic distance between different populations of *P. nordestinus*, we

195 applied a Mantel test for each acoustic parameter. In the correlation matrix we included
196 geographical coordinates of each locality and the mean acoustic parameters for each
197 individual and population.

198 *Phylogenetic signal*

199 We tested for phylogenetic signal in all acoustic parameters analyzed using the K
200 statistic (Blomberg et al. 2003). K is a statistical metric that estimates the relative amount of
201 trait variance predicted under Brownian motion model (BM) compared to observed variance
202 in the data. It assumes that differences between species are proportional to divergence times,
203 while its significance can be tested by permuting traits across a phylogenetic tree. K values
204 vary continuously from 0, with K-values equal to 1 suggesting that traits are under BM; when
205 larger than one, related species present trait values more similar than expected by BM; and
206 when it is smaller than one, relatives are less similar than expected (Blomberg et al. 2003).
207 For this analysis we used the phylogeny from Pyron and Wiens 2011. All analyses were done
208 on the R 3.1.2. environment using the following packages: *lme4* (Bates et al. 2015); *ape*
209 (Paradis et al. 2019); *picante* (Kembel 2010); *phytools* (Revell 2012), and *tidyverse* (Wickham
210 et al. 2019).

211 *Sensitivity analysis*

212 To ensure that our results and the reported patterns were not an artefact of species with low
213 sampling, we repeated the coefficient of variation and hierarchical ANOVA analyses after
214 excluding data from species with less than 5 individuals and less than 30 different calls (Table
215 S2 in Supplementary Material): *P. burmeisteri*, *P. vaillantii*, *P. megacephalus*, and *P. azureus*.
216 Results from this sensitivity analysis remained substantively the same compared to the
217 analysis of the full dataset and, thus, we report the original result in the main text while results
218 from the sensitivity analysis are reported in the supplementary material (see Figures S2-3 in

219 Supplementary Material).

220 **Results**

221 We analyzed a total of 3,994 advertisement calls from 188 individuals of 15 different
222 species of phyllomedusids (Figure 1). Although we sampled calls from more than one locality
223 for various species, we only obtained enough samples from different populations for
224 *Pithecopus nordestinus*, for which we recorded various individuals from 14 populations from
225 different localities distributed throughout most of the species geographical distribution (Figure
226 2; Table S1 in Supplementary Material). The advertisement calls from all species recorded is
227 relatively simple, being composed of several similar pulses that can be grouped in different
228 notes (see Table 2 for the mean values of the acoustic parameters for the species recorded and
229 Figure S1 in Supplementary Material for spectrograms of one call from each species).

230 The multiple-level analyses of variability using CV showed that all acoustic
231 parameters presented the lowest variation intra-individually, but the intensity of this trend
232 varied between parameters. Dominant frequency, pulse duration, and pulse rate are the most
233 static parameters, especially intra-individually. Number of pulses and call duration present
234 intermediate values, while call interval is highly dynamic and shows less difference between
235 the intra-specific levels and the inter-specific level (Figure 4).

236 Call parameters also vary considerably below the interspecific level. In general, the
237 variability is smallest at the intra-individual level for all parameters and a tendency for the
238 intra-population level to show less variability than the inter-population, with the intra-specific
239 intermediate. Furthermore, the three most static parameters (dominant frequency, pulse
240 duration, and pulse rate) show the largest difference inter-specifically compared to other
241 levels (Figure 4).

242 The ANOVA confirmed the results from our multi-level CV evaluation, where most of

243 the advertisement call variability is explained by inter-specific differences. However, this
244 trend also varies a lot between parameters. There is a clear tendency for static acoustic
245 parameters to have a larger percentage of their variability credited to the inter-specific level,
246 with intermediate parameters having a little less of their variability explained by this level
247 (however more than 75%) and the dynamic parameter less than 50%. Below the inter-specific
248 level, pulse rate had a considerable part of its variation attributed to the population level
249 (Figure 5).

250 The linear mixed models showed that most of the acoustic parameters are significantly
251 affected by body size and/or environmental temperature. While temperature affected mostly
252 temporal acoustic parameters (CD, PN and PR), individuals body size affected the spectral
253 parameter (DF) and PR, but most of variation was attributed to species identity (Table 3).
254 Only pulse rate and call duration were significantly correlated to the geographical distance
255 between individuals of *P. nordestinus* (Table 4). Conversely, all acoustic parameters tested
256 presented a significant phylogenetic signal, with K values < 1 (Table 5).

257 Discussion

258 Acoustic parameters show a continuous variability from individuals to species, but
259 with varying degrees of differentiation according to each parameter. These results corroborate
260 most studies on acoustic variability of anuran advertisement calls (Castellano et al. 2002;
261 Reinhold 2009; Bee et al. 2010). We are unaware of others studies using, at the same time, so
262 many hierarchical levels in an evolutionary approach. Studying the variability of acoustic
263 parameters of anuran's advertisement calls in a multi-level approach has impact for both
264 taxonomy and evolutionary research, and even for studies which use automatic recording units
265 to monitor biodiversity. The parameters that presented the lowest CV were mainly those that
266 are considered important for frog specific recognition, especially dominant frequency and

267 pulse rate (Gerhardt 1994). Tests on female preference demonstrate that they generally prefer
268 calls with medium values for those parameters, exerting a stabilizing sexual selection over the
269 population (Castellano and Giacoma 1998). Furthermore, studies on reproductive character
270 displacement show that when populations occur in sympatry with sister taxa with similar
271 advertisement calls these preferences are stronger, confirming the importance of these
272 parameters for specific recognition and as reproductive barrier, diminishing hybridization
273 (Lemmon 2009). However, for some frog species females prefer calls with slightly lower
274 dominant frequencies, exerting weak directional sexual selection, possibly choosing for larger
275 males (Ryan 1980; Poole and Murphy 2007).

276 Considering the more variable parameters, call duration and number of pulses
277 presented intermediate values of CV, while call interval was highly dynamic. Although the
278 range of variation may vary according to species, this is a general tendency for parameter
279 variation in anurans (see review in Köhler et al. 2017). These acoustic parameters are
280 behaviorally controlled to some extent and are directly involved in the energetic expenditure
281 of call production (Gillooly and Ophir 2010; Ziegler et al. 2016). Accordingly, females
282 generally show directional preferences for these parameters (Sullivan 1992; Bosch and
283 Márquez 2005). These more extreme values from the population/species distribution could
284 indicate higher fitness males or just enhance probability of signal detection and facilitate
285 localization, and different sexual selection mechanisms might be involved (Ryan 1988;
286 Bruner et al. 2017).

287 As expected, the inter-specific level was the most variable and most of the
288 advertisement call variability for phyllomedusids is attributed to this level, while the lowest
289 CV was detected intra-individually, except for call interval, which has a high variability at all
290 levels (Figures 3 and 4). These results indicate that the advertisement call for these species
291 might be a reliable taxonomic tool (Köhler et al. 2017), yet, the influence of temperature and

body size should always be taken into consideration. Even though environmental temperature and body size significantly affected four from six acoustic parameters, species identity explained most of the variation of these parameters.

Variability in levels that compare calls from different individuals of the same species also changed as predicted. Parameters showed less variability at the intra-population level in relation to inter-population, with intermediate values for intra-specific, probably because it includes calls from individuals which may or not be from the same population. However, variation between populations was small when compared to studies on other anurans (Snyder and Jameson 1965; Hasegawa et al. 1999; Castellano et al. 2000; Kaefer et al. 2012). Inter-population variation may be associated to stochastic processes and be directly related to gene flow or selection (Smith et al. 2003; Bernal et al. 2005; Pröhl et al. 2007; Ohmer et al. 2009; Kaefer and Lima 2012), which may act directly on call characteristics or through pleiotropic effects (Ryan et al. 1990; Castellano et al. 1999). When selective pressures are weak, call variation is expected to be correlated with geographical distance between populations or associated to an important dispersal barrier (Pröhl et al. 2006; Maia et al. 2017). From the acoustic parameters evaluated for *P. nordestinus* only pulse rate and call duration were related to geographical distance between individuals, although there was no relationship at the population level. Conversely, pulse rate had a higher percentage of its variation explained by the population level when compared to the other static parameters. This low variability between different populations and its absent correlation with geographical distance may be related to conservative selective pressures acting upon all or most populations, to a large amount of gene flow, or because of recent population expansions. Thus, studies on geographic variation of anuran advertisement calls found contrasting results on the relationship between call variation and geographic distances (Snyder and Jameson 1965; Hasegawa et al. 1999; Castellano et al. 2000; Smith et al. 2003; Bernal et al. 2005; Pröhl et al. 2007; Ohmer et al.

317 2009; Kaefer and Lima 2012; Kaefer et al. 2012).

318 Conversely, when considering the variation between calls from the different species
319 studied, all acoustic parameters presented a significant phylogenetic signal. Nevertheless,
320 parameters had K-values lower than one, suggesting that traits varied more than expected by
321 Brownian motion. Because behavioral traits are generally more labile (Blomberg et al. 2003),
322 and call parameters can be under different selective pressures, it's possible to have directional
323 selection even with a phylogenetic signal in advertisement call of phyllomedusids. These
324 results corroborate most studies on phylogenetic signals for anurans (Wollenberg et al. 2007;
325 Erdtmann and Amézquita 2009; Goicoechea et al. 2010; Gingras et al. 2013). Although the
326 anuran advertisement call traditionally is seen as a rapidly evolving trait and under strong
327 selective forces, especially sexual selection (Gerhardt 1994), studies have found surprisingly
328 strong phylogenetic signals for most acoustic parameters tested, calling attention to the
329 importance of stochastic processes, such as genetic drift, in the evolution of these
330 vocalizations (Cocroft and Ryan 1995; Wollenberg et al. 2007; Erdtmann and Amézquita
331 2009; Goicoechea et al. 2010).

332 Our results confirm that the advertisement call of the species studied from the family
333 *Phyllomedusidae* may be used as a reliable taxonomic tool, because all acoustic parameters
334 analyzed, except call interval, presented a much higher inter-specific variation in comparison
335 to all intra-specific levels. However, considering the variation detected for the different intra-
336 specific levels, it is important to have adequate replicates from different localities.
337 Furthermore, it is recommended to consider temperature and body size, because most of the
338 acoustic parameters were influenced by one or another variable. CV at different levels shows
339 that, between the analyzed acoustic parameters, dominant frequency and pulse rate are the
340 most reliable parameters for taxonomy. The variation is a central component of evolution.
341 Thus, studies that take into account how and how much traits vary across multiple levels can

342 help us to understand which factors (e.g. genetic drift, sexual selection, environment, etc.) can
343 shape diversification of species.

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360 **Data availability**

361 The raw data and the code used in the analyses are available online at
362 https://github.com/paternogbc/ms_variability_anuran_call

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556 **Figure 1.** Phylogenetic tree based on Pyron and Wiens (2011) including all species of phyllomedusid recorded
 557 and a representation of their advertisement call (oscillogram). Species are represented as: *Pithecopus*
 558 *megacephalus* (Miranda-Ribeiro, 1926) (a), *Pithecopus ayeaye* Lutz, 1966 (b), *Pithecopus oreades* (Brandão,
 559 2002) (c), *Pithecopus centralis* (Bokermann, 1965) (d), *Pithecopus rohdei* (Mertens, 1926) (e), *Pithecopus*
 560 *nordestinus* (Caramaschi, 2006) (f), *Pithecopus azureus* (Cope, 1862) (g), *Pithecopus hypocondrialis* (Daudin,
 561 1800) (h), *Phyllomedusa vaillantii* Boulenger, 1882 (i), *Phyllomedusa camba* De la Riva, 1999 (j),
 562 *Phyllomedusa iheringii* Boulenger, 1885 (k), *Phyllomedusa distincta* Lutz, 1950 (l), *Phyllomedusa tetraploidea*
 563 Pombal and Haddad, 1992 (m), *Phyllomedusa burmeisteri* Boulenger, 1882 (n), *Phyllomedusa bahiana* Lutz,
 564 1925 (o).

565 **Figure 2.** Sampling points of all populations recorded and phylogenetic relationship among 15 species of
566 monkey tree frogs (family Phyllomedusidae) used in this study, in Brazil.

567 **Figure 3.** Acoustic parameters analyzed for *Phyllomedusa* and *Pithecopus*. Temporal parameters evaluated are
 568 shown in the oscillogram (A) and spectral parameters in the spectrogram (B).

569 **Figure 4.** Variability of six acoustic parameters from the advertisement call of 15 species of monkey tree frogs
 570 (family Phyllomedusidae) at different levels. Coefficient of variation was calculated based on a stratified
 571 hierarchical subsampling method in which we repeatedly drew from our data pool five different calls. This
 572 procedure was repeated 1,000 times for each parameter at each level. *Levels in which the coefficient of
 573 variation was calculated only with samples from *Pithecopus nordestinus*.

574 **Figure 5.** Results of the hierarchical ANOVA showing what percentage of the variability of the advertisement
575 call of 15 species of monkey tree frogs (family Phyllomedusidae) is attributed to each level for all acoustic
576 parameters: dominant frequency; number of pulses; pulse duration; pulse rate; call duration; interval between
577 calls.

578 **Table 1.** Detailed step by step procedure used to draw calls from our data pool in the stratified hierarchical
579 subsampling method used to calculate the coefficient of variation of the advertisement call of phyllomedusid
580 species at different levels for each acoustic parameter.

Level	Step 1	Step 2	Step 3	Step 4	Step 5
Intra-individual	Draw one specie	Draw one individual	Draw five different calls from this individual	Calculate CV	Repeat 1,000 x
Intra-population	Draw one population of <i>P. nordestinus</i>	Draw five different individuals from this population	Draw one call from each individual	Calculate CV	Repeat 1,000 x
Inter-population	Draw five different populations of <i>P. nordestinus</i>	Draw one individual from each population	Draw one call from each individual	Calculate CV	Repeat 1,000 x
Intra-specific	Draw one species	Draw five different individuals	Draw one call from each individual	Calculate CV	Repeat 1,000 x
Inter-specific	Draw five different species	Draw one individual from each specie	Draw one call from each individual	Calculate CV	Repeat 1,000 x

581 **Table 2.** Advertisement call description for all species of monkey tree frogs (family Phyllomedusidae) recorded,
582 with number of individuals and calls per species. Mean, standard deviation, maximum and minimum values for
583 each acoustic parameter, air temperature (Temp) and body size (BS). Acoustic parameters are abbreviated as:
584 call duration (CD), dominant frequency (DF), call interval (CI), number of pulses (NP), pulse rate (PR) and pulse
585 duration (PD).

Species	N ind.	N of calls	CD (s)	DF (Hz)	CI (s)	NP	PR (pulse/s)	PD (s)	Temp (°C)	BS (mm)
<i>Pithecopus nordestinus</i>	100	2227	0.04 ± 0.02	2079.1 ± 194	9.09 ± 26.60	3.81 ± 0.91	109.91 ± 32.99	0.005 ± 0.001	20.39 ± 2.23	34.36 ± 2.43
(Caramaschi, 2006) <i>Pithecopus azureus</i> (Cope,	4	45	(0.01-0.09) 0.03 ± 0.00	(1500-2625) 2291.2 ± 59	(0.02-290.30) 21.60 ± 20.66	(2-8) 4 ± 0	(40-230.77) 135.71 ± 7.61	(0.0005-0.01) 0.005 ± 0.001	(16-25.4) 22.5 ± 1.71	(29.57-40.31) 34.09 ± 1.56
1862) <i>Pithecopus ayeaye</i> Lutz, 1966	5	96	(0.03-0.03) 0.09 ± 0.04	(2153-2412) 1471.4 ± 168	(1.43-88.38) 7.3 ± 12.85	(4-4) 5.63 ± 1.14	(111.11-150) 71.52 ± 40.51	(0.004-0.006) 0.008 ± 0.002	(20-23.9) 17.6	(31.79-35.27) 40.49 ± 1.43
<i>Pithecopus megacephalus</i>	2	20	(0.03-0.16) 0.32 ± 0.09	(1206-1723) 1809.4 ± 140	(0.25-60.04) 42.74 ± 34.67	(3-10) 13.4 ± 3.59	(30.30-208.33) 39.78 ± 7.99	(0.003-0.015) 0.005 ± 0.001	23	(37.97-41.43) 38.49 ± 0.11
(Miranda-Ribeiro, 1926) <i>Pithecopus centralis</i>	9	215	(0.25-0.57) 0.04 ± 0.01	(1688-2063) 1271.5 ± 175	(7.03-137.45) 7.55 ± 12.43	(8-22) 4.42 ± 0.84	(23.38-54.14) 115.82 ± 28.47	(0.004-0.006) 0.004 ± 0.001	NA	(38.56-38.41) NA
(Bokermann, 1965) <i>Pithecopus oreades</i> (Brandão,	7	200	(0.02-0.1) 0.03 ± 0.01	(938-1550) 1679.6 ± 153	(0.2-79.32) 15.36 ± 42.19	(3-7) 3.93 ± 0.64	(45.98-166.67) 1140.52 ± 23.25	(0.002-0.008) 0.004 ± 0.001	NA	NA
2002) <i>Pithecopus hypocondrialis</i>	11	354	(0.02-0.07) 0.03 ± 0.01	(1125-2067) 1970.3 ± 98	(0.19-352.91) 8.16 ± 19.46	(3-7) 4.07 ± 0.75	(79.37-285.71) 141.15 ± 14.44	(0.002-0.007) 0.005 ± 0.001	22.66 ± 0.53	35.85 ± 1.62
(Daudin, 1800) <i>Pithecopus rohdei</i> (Mertens,	7	47	(0.02-0.05) 0.15 ± 0.07	(1688-2250) 2014.6 ± 189	(0.20-195.22) 37.13 ± 43.13	(3-7) 5.23 ± 2.40	(103.45-200) 33.16 ± 4.56	(0.003-0.008) 0.018 ± 0.003	(22.2-23.5) 23.51 ± 0.23	(32.67-37.6) 44.15 ± 2.03
1926) <i>Phyllomedusa bahiana</i> Lutz,	10	115	(0.08-0.42) 0.31 ± 0.17	(1688-2250) 1141.3 ± 148	(2.77-183.16) 18.41 ± 14.26	(3-11) 14.50 ± 4.17	(25.16-46.39) 43.09 ± 9.25	(0.011-0.024) 0.014 ± 0.004	(23.2-23.8) 21.43 ± 2.9	(41.63-46.36) 75.32 ± 2.99
1925 <i>Phyllomedusa burmeisteri</i>	1	5	(0.13-0.99) 0.12 ± 0.01	(938-1313) 1102.5 ± 154	(0.99-74.14) 8.40 ± 4.86	(7-26) 6.8 ± 0.45	(20.62-63.49) 51.45 ± 0.78	(0.006-0.021) 0.004 ± 0.000	(15.3-24.2) 25.7	(69.31-78.48) 63
Boulenger, 1882 <i>Phyllomedusa camba</i> De la	5	260	(0.1-0.13) 0.04 ± 0.01	(1034-1378) 669 ± 105	(3.72-15.22) 5.81 ± 15.20	(6-7) 4.71 ± 1.12	(50.42-52.17) 112.59 ± 8.56	(0.004-0.004) 0.005 ± 0.001	22.9 ± 1.09	83.03 ± 2.25
Riva, 1999 <i>Phyllomedusa distincta</i> Lutz,	9	113	(0.02-0.07) 0.23 ± 0.05	(5623-1125) 1299.2 ± 99	(0.09-121.42) 22.98 ± 12.38	(3-9) 6.83 ± 1.06	(88.88-142.86) 27.88 ± 3.64	(0.003-0.006) 0.017 ± 0.001	(22.1-24.1) 18.57 ± 1.14	(79.49-85.34) 50.51 ± 2.52
1950 <i>Phyllomedusa iheringii</i>	7	164	(0.13-0.35) 0.32 ± 0.07	(1125-1875) 1240.5 ± 132	(9.2-82.56) 17.35 ± 12.20	(5-10) 9.31 ± 1.80	(15.24-35.71) 27.74 ± 3.04	(0.013-0.024) 0.017 ± 0.003	(16-19.8) 15.59 ± 0.74	(47.46-53.98) 54.26 ± 2.91
Boulenger, 1885 <i>Phyllomedusa tetraploidea</i>	7	121	(0.19-0.49) 0.03 ± 0.09	(938-1500) 1182.3 ± 108	(1.01-109.48) 18.04 ± 13.75	(6-14) 9.28 ± 2	(20.75-36.36) 29.39 ± 3.81	(0.012-0.025) 0.015 ± 0.001	(14.9-16.7) 18.97 ± 1.56	(49.69-58.13) 58.91 ± 4.39
Pombal and Haddad, 1992 <i>Phyllomedusa vaillantii</i>	4	12	(0.19-0.65) 0.04 ± 0.01	(938-1313) 1609.4 ± 149	(2.24-77.32) 64.12 ± 65.69	(5-15) 7.92 ± 1.31	(20.634-38.095) 178.12 ± 15.68	(0.010-0.022) 0.004 ± 0.001	(16-20.6) 23.05 ± 1.37	(54.52-65.48) 49.76 ± 2.1
Boulenger, 1882			(0.03-0.053)	(1313-1875)	(11.99-180.15)	(5-10)	(155.56-212.12)	(0.002-0.005)	(22-25)	(47.08-51.9)

Table 3. Influence of body size and environmental temperature on the acoustic parameters of the advertisement call of 15 species of monkey tree frogs (family Phyllomedusidae). Linear mixed models included the mean value of the acoustic parameter for each individual and the species was included as a random effect. Acoustic parameters are abbreviated as: call duration (CD), dominant frequency (DF), call interval (CI), pulse duration (PD), number of pulses (NP) and pulse rate (PR). In bold *P*-values ≤ 0.05 .

	Body size					Temperature				
	Estimate	Chisq	<i>P</i> -value	<i>r</i> ² conditional	<i>r</i> ² marginal	Estimate	Chisq	<i>P</i> -value	<i>r</i> ² conditional	<i>r</i> ² marginal
DF	-23.885	64.313	< 0.001	0.878	0.643	9.474	2.638	0.104	0.908	0.002
CD	0.001	1.285	0.257	0.860	0.024	-0.008	21.518	< 0.001	0.871	0.028
CI	-0.074	0.074	0.790	0.300	0.002	0.412	0.305	0.581	0.277	0.002
PD	< 0.001	0.151	0.697	0.915	0.002	< 0.00001	0.035	0.851	0.917	< 0.001
NP	0.049	1.808	0.179	0.827	0.038	-0.164	9.186	0.002	0.848	0.014
PR	-1.296	5.041	0.025	0.855	0.095	2.869	11.075	0.001	0.836	0.018

591 **Table 4.** Results of Mantel test evaluating which acoustic parameters of the advertisement call of *Pithecopus*
592 *nordestinus* (Caramaschi, 2006) are correlated to the geographic distance between populations. In bold *P*-values
593 ≤ 0.05 .

Source	Individuals		Populations	
	<i>r</i>	<i>P</i> - value	<i>r</i>	<i>P</i> - value
Call Duration	0.064	0.053	0.096	0.175
Dominant				
Frequency	0.035	0.190	0.068	0.307
Number of Pulses	0.018	0.316	0.130	0.116
	-			
Pulse Duration	0.017	0.601	0.064	0.282
Pulse Rate	0.109	0.004	0.137	0.135
	-			
Call Interval	0.004	0.459	0.058	0.248

594 **Table 5.** Phylogenetic signal for acoustic parameters and body size of monkey tree frogs *Phyllomedusa* and
595 *Pithecopus* calculated through K statistic (Blomberg et al., 2003). PIC.mean is the mean observed PIC
596 (phylogenetic independent contrasts) variance and PIC.rdn.mean is the mean random PIC variance.

Source	K statistic	PIC.mea	PIC.rdn.mean	P-
		n		value
Call Duration	0.74	0.001	0.004	0.010
Dominant	0.64	16689.36	56643.05	0.005
Frequency				
Call Interval	0.69	23.48	72.39	0.013
Number of Pulses	0.54	0.96	2.68	0.017
Pulse Rate	0.79	198.23	714.60	0.006
Pulse Duration	0.57	3.29E-06	9.18E-06	0.031
Body Size	1.16	11.44	40.84	0.003