

Statistical differences and biological implications: a comparative analysis of the advertisement calls of two Andean stream treefrogs (Hylidae: *Hyloscirtus*) and the evolution of acoustic characters

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Abstract. We describe and compare the advertisement calls of *Hyloscirtus antioquia* and *H. larinopygion*, two sibling species of the Andean stream treefrogs of the *Hyloscirtus larinopygion* group. We recorded individual calls at seven localities in Colombia, including the type locality of *H. antioquia*. The advertisement calls of both species consist of a single, low-pitched, multi-pulsed note, with some overlaps in frequency and the duration of pulses (dominant frequency 1642.7–1756.5 Hz and 5–6 periodic pulses in *H. antioquia* and 1722.7–1894.9 Hz and 5–6 periodic pulses in *H. larinopygion*). We found significant statistical differences in spectral and temporal variables between both species; however, no acoustic trait can be considered diagnostic. Our results highlight an important topic in evolutionary biology that deserves thorough examination in Andean anurans; because not all statistically significant differences in auditory signals matter in male recognition and speciation. Furthermore, in the context of the most comprehensive phylogenetic hypothesis available for *Hyloscirtus*, the optimisation of acoustic characters suggests that call structure has a complex history that involved multiple transformations.

Key words. Amphibia, Anura, behaviour, bioacoustics, calls parameters, Colombia, *Hyloscirtus larinopygion* group, species diagnosis.

Introduction

Most anurans employ acoustic communication for territorial dominium and reproduction. Advertisement calls are acoustic signals usually emitted by male anurans to attract females and fend off other males (RYAN 1980, DUELLMAN & TRUEB 1994). Often they vary interspecifically and sometimes even intraspecifically, making them important sources of characters for species diagnosis (COCROFT & RYAN 1995, GERHARDT & HUBER 2002, PADIAL et al. 2008) and phylogenetic inference (DUELLMAN 2007, SCHNEIDER & SINSCH 2007). Vocalisations also play an important role in comparative behavioural studies with anurans as model system (BOSCH & BOYERO 2003, ROBILLARD et al. 2006, WELLS 2007, WOLLENBERG et al. 2007, BOTH & GRANT 2012).

Hyloscirtus antioquia RIVERA-CORREA & FAIVOVICH, 2013 is a colourful stream treefrog (Fig. 1a) allied to *H. la-*

rinopygion (DUELLMAN 1973) (Fig. 1b), with which it used to be confused previously (DUELLMAN & HILLIS 1990, ARDILA-ROBAYO et al. 1993, RIVERA-CORREA & FAIVOVICH 2013). *Hyloscirtus antioquia* and *H. larinopygion* are externally distinguishable by their colour patterns and the shape and keratin colour of the nuptial pads of adult males (RIVERA-CORREA & FAIVOVICH 2013). Both species are referred to the *H. larinopygion* group, and a recent phylogenetic analysis suggests they are sister species (BRUNETTI et al. 2015). *Hyloscirtus antioquia* is restricted to the northern parts of the Cordillera Central of Colombia, whereas *H. larinopygion* is widely distributed in the Cordilleras Central and Occidental of Colombia and northern Ecuador (LOMA et al. 2012, RIVERA-CORREA & FAIVOVICH 2013). Sympatry of *H. antioquia* and *H. larinopygion* is currently unknown, and the highlands of the Andes in the south of the Departamento de Antioquia (up to 3,300 m in Páramo

de Sonsón) seem to act as a geographic barrier between them.

The advertisement call of *H. antioquia* has not been described, and although that of *H. larinopygion* was described briefly by BERNAL et al. (2004), few quantitative data were provided. Thus, the objectives of this study are to 1) describe for the first time the advertisement call of *H. antioquia*, 2) provide a more detailed description of the advertisement call of *H. larinopygion*, and 3) compare the advertisement calls of these two closely related species and discuss patterns indicative of the evolution of advertisement call structures in species of the *H. larinopygion* species group.

Materials and methods

We recorded advertisement calls during eight field trips between February 2010 and December 2012 (Table 1, Fig. 1c). Recordings were made with a digital recorder (Sony PCM-D50) connected to a directional microphone (Sennheiser K6/ME66) at distances of 1.0–2.2 m from the calling male. In total, we analysed 234 calls obtained from six males of *H. antioquia* (106 calls) and six males of *H. larinopygion* (128 calls) recorded at several localities (Fig. 1c, Table 1). After each recording, we measured the male's body size (snout–vent length, SVL) to the nearest 0.1 mm with digital callipers (Mitutoyo) and air temperature with a digital thermometer (Casio Pathfinder Triple Sensor) (Table 1).

Specimens collected and voucher recordings are deposited in the Museo de Herpetología of the Universidad de Antioquia (MHUA) in Medellín, Colombia. Additional calls of *H. antioquia* were obtained from the audio file collection of the late P. M. RUIZ-CARRANZA deposited at the Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá, Colombia (see ICN Vouchers in Table 1). All recordings were analysed with Raven Pro 1.5 software for Mac OS X (Bioacoustics Research Program 2013) in Hann's sampling window, FFT window size of 256 points, sampling rate of 44.1 kHz, 16-bit precision, and overlap of 90%. Call parameter definitions follow MCLISTER et al. (1995), COCROFT & RYAN (1995), and GERHARDT & HUBER (2002). Following these authors, we consider in this study the dominant call frequency to be the frequency with the greatest amount of acoustic energy and a note as the sound unit produced by a single expiratory event of the frog.

We report numerical parameters as mean \pm SD and the respective range (Table 2). The following temporal parameters were measured in oscillograms: call duration (= note duration), number of pulses per call, and pulse duration. We used power spectra to calculate the dominant call frequency (= fundamental frequency) and frequency bandwidth (the difference between the 5 and 95% frequencies, hereafter referred to as low frequency and high frequency). We compared body sizes and each temporal and spectral acoustic traits between *H. antioquia* and *H. larinopygion* using the number of frogs recorded as sampling

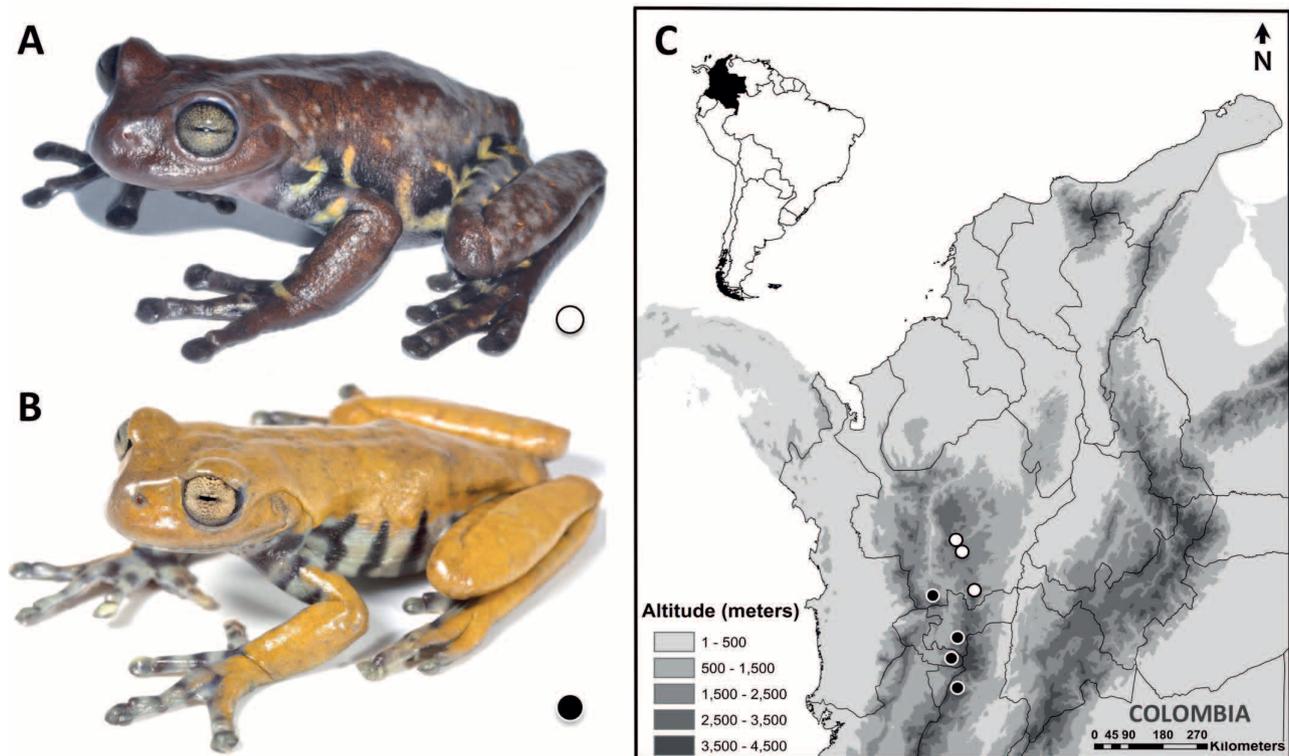


Figure 1. (A) *Hyloscirtus antioquia* in life (MHUA-A 8962, adult male, SVL 54.3 mm; photo: MRC); (B) *H. larinopygion* in life (QCAZ 41826, adult male, SVL 55.2 mm; photo: S. RON); (C) Map showing the localities of the recorded specimens. White dots – *Hyloscirtus antioquia*; black dots – *H. larinopygion*.

Table 1. Call recording localities of the specimens of *Hyloscirtus antioquia* and *H. larinopygion* analysed. Abbreviations used: Ind – Nº of individuals; T. Air – air temperature; NV – unvouchered specimen; Ant – Departamento de Antioquia; Cal – Departamento de Caldas; Ris – Departamento de Risaralda; Tol – Departamento de Tolima; * type locality.

| Species | Locality | Ind | Calls | T. Air (°C) | Acronym |
|------------------------|---|-----|-------|-------------|---------------------|
| <i>H. antioquia</i> | Serranía las Baldías*, Bello (Ant); 2,600 m | 4 | 76 | 15.6–16.2 | MHUA-A 7227–9 |
| | S. Sebastian-La Castellana, Envigado (Ant); 2,500 m | 1 | 14 | 15.7 | MHUA-A 5707 |
| | Páramo de Sonsón, Sonsón, (Ant); 2,800 m | 1 | 16 | 16.0 | ICN 18597 |
| <i>H. larinopygion</i> | Cuchilla Jardín-Támesis, Jardín, (Ant); 2,600 m | 1 | 50 | 16.0 | NV |
| | Villamaría, (Cal); 2,620 m | 1 | 18 | 16.2 | GGD022 |
| | Ucumarí, Pereira, (Ris); 2,500 m | 2 | 40 | 16.4 | MHUA-A 9423 MRC 575 |
| | Cajamarca, (Tol); 2,600 m | 2 | 20 | 15.8 | NV |

Table 2. Summary and results of statistical comparisons of call traits (mean ± standard deviation, range) between *Hyloscirtus antioquia* and *H. larinopygion*. N = Sample size.

| Call variables | <i>H. antioquia</i> | N | <i>H. larinopygion</i> | N | Resampling test (P) |
|-------------------------|----------------------------------|---------|-----------------------------------|---------|---------------------|
| Body size (mm) | 54.8±0.392 (53.8–56.6) | 6 | 56.5±0.286 (56.4–57.2) | 6 | 0.006 |
| Call duration (s) | 0.155±0.022 (0.122–0.181) | 106 | 0.197±0.016 (0.137–0.276) | 128 | 0.011 |
| No. of pulses/call | 5.515±0.069 (5.29–5.75) | 654/106 | 7.130±0.394 (6.0–8.0) | 816/128 | 0.001 |
| Pulse duration (s) | 0.016±0.0002 (0.0158–0.0176) | 492 | 0.018±0.0002 (0.0172–0.0188) | 612 | 0.002 |
| Low frequency (Hz) | 1497.6±22.387 (1464.2–1593.8) | 106 | 1659.4±34.915 (1550.4–1722.7) | 128 | 0.003 |
| Dominant frequency (Hz) | 1680.7±18.592 (1642.7–1756.5) | 106 | 1834.2±35.394 (1722.7–1894.90) | 128 | 0.003 |
| High frequency (Hz) | 1783.3±34.541 (1722.7–1919.4) | 106 | 2008.9±36.084 (1894.9–2067.2) | 128 | 0.004 |

unit in a resampling statistical test (BLANK 2011). In addition, we reduced the redundancy of measured acoustic traits by conducting a principal component analysis (PCA) with Varimax-rotation. We tested possible relationships between air temperature and acoustic trait values by conducting linear regressions on PCs. Finally, we used a PCA plot to visualize advertisement call divergence between species. Graphs of spectrograms, waveforms, and power spectra were obtained with Seewave 1.6 software, R 2.15.1 package Using Seewave settings, window name (Fourier transform window) = Hann; window length = 256 samples; and overlap = 90%.

Results

Hyloscirtus antioquia and *H. larinopygion* were found inside gallery forests alongside streams. Acoustic activity started at dusk and decreased toward early morning (18:00–03:00 h, approximately). Calling males of both species were observed vocalising from shrubs, low vegetation (< 2 m directly over slow-flowing streams), and common bamboo (*Chusquea* sp). The advertisement calls of both species consist of a low-pitched and pulsed single note that

resembles the sound produced by crickets through stridulation; this pulsed-note call is audible to the human ear as a melodious “brrrrrr”. The signal is degraded, and the pulses are not always distinguishable at a distance of ca 20 m. The amplitude modulation of each note increases in intensity in the first half of the call and decreases in the second half (Fig. 2). Pulses are rhombus-shaped (see oscillogram), with the third and fourth pulses containing the greatest energy. The fundamental frequency corresponds to the dominant frequency.

The advertisement call of *H. antioquia* consists of a single note of 5–6 periodic pulses (Figs 2a–c), with a gradual rise in frequency throughout the call. Call duration was 0.155 ± 0.022 s (0.122 – 0.181 s) with a call interval duration of 1.673 ± 0.357 s (1.055 – 2.687 s). The advertisement call of *H. larinopygion* consists of a single note of 6–8 periodic pulses (Figs 2d–f) with a gradual rise in frequency throughout the call. Call duration was 0.197 ± 0.016 s (0.137 – 0.276 s) with an interval duration of 1.079 ± 0.294 s (0.763 – 2.596 s). Advertisement calls were emitted by *H. antioquia* at a rate of 23–35 calls/minute (mean = 28 ± 4.12), while *H. larinopygion* emitted them at a rate of 20–48 calls/minute (mean = 34 ± 13.23). A summary of quantitative call traits of both species is provided in Table 2.

Male SVL was significantly greater (resampling test $P = 0.006$) in *H. larinopygion* than in *H. antioquia*, although the mean difference between species was only approximately 1.68 mm SVL. All temporal and spectral characteristics of calls were statistically significantly different (Table 2, Figs 3a–b). The variation in the measured call traits was successfully summarized by two principal components (Table 3); PC1 was mainly correlated with call frequency, whereas PC2 was mainly correlated with call duration and pulse number/call. Temperature and spectral call traits presented a marginal positive relationship ($\beta = 1.616$, $P = 0.056$). We did not remove the possible effect of temperature from our comparative analysis because (1) the predictive power of temperature on spectral trait variation was relatively low ($R^2 = 0.32$), (2) our results (Fig. 3c) did not change when we used residuals of the temperature-PC1 linear regression analysis instead of the original PC1 values (data not shown), and (3) air temperatures of recordings for the two species did not differ (resampling test $P = 0.422$).

Discussion

The spectral and temporal call traits were statistically significantly different between *H. antioquia* and *H. larino-*

Table 3. Results of a principal component analysis summarizing variation in the advertisement calls of *Hyloscirtus antioquia* and *H. larinopygion*. The highest loadings for each principal component are > 0.9 .

| Call variables | Principal component | |
|-------------------------|---------------------|--------|
| | PC1 | PC2 |
| Dominant frequency (Hz) | 0.982 | 0.130 |
| Low frequency (Hz) | 0.986 | 0.128 |
| High frequency (Hz) | 0.961 | 0.249 |
| Call duration (s) | 0.090 | 0.969 |
| Pulse duration (s) | 0.618 | 0.586 |
| No. of pulses /call | 0.208 | 0.963 |
| Eigenvalue | 4.044 | 1.555 |
| % of variance explained | 54.893 | 38.434 |

pygion (Table 2). However, these differences are too small to clearly diagnose the two species; similar results have been found in other Neotropical anurans (e.g., TSUJINISHIKIDO et al. 2012). Consequently, *H. antioquia* and *H. larinopygion* can only be diagnosed using morphological (RIVERA-CORREA & FAIVOVICH 2013) and molecular traits (RIVERA-CORREA unpublished data) at present. Call trait overlaps have been found previously between close-

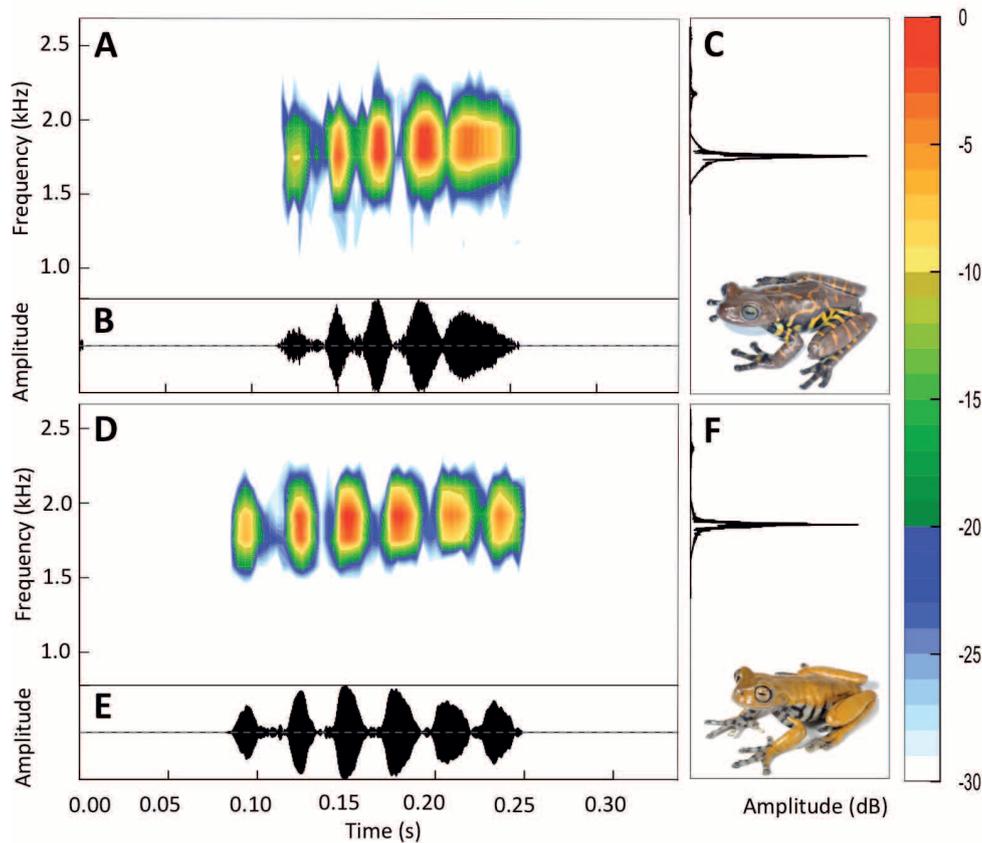


Figure 2. Advertisement calls of two recorded specimens of the *Hyloscirtus larinopygion* species group. *Hyloscirtus antioquia* (MHUA-A 7229): (A) spectrogram; (B) oscillogram; (C) power spectrum. *Hyloscirtus larinopygion* (MHUA-A 9423): (D) spectrogram; (E) oscillogram; (F) power spectrum.

ly related species of *Hyloscirtus* (COLOMA et al. 2012) and in other anuran species; they are associated with allopatric distribution patterns (DUELLMAN & PYLES 1983, COCROFT et al. 1990, KWET et al. 2005, PEREYRA et al. 2012, WEN et al. 2012) that render pre-mating isolation mechanisms to prevent hybridisation redundant. From this perspective, our results highlight an important topic in evolutionary biology that deserves more study in Andean anurans: not all statistically significant differences in auditory signals matter in mate recognition and speciation.

The higher call frequency in *H. larinopygion* than in *H. antioquia* is not attributable to differences in body size or ambient temperature. In anurans, an inverse relationship between body size and dominant call frequency is common (GERHARDT & HUBER 2002, VARGAS-SALINAS & AMÉZQUITA 2014); however, the males of *H. larinopygion* recorded in our study were larger (albeit slightly) than those of *H. antioquia*, yet the frequency of their calls was higher. Similarly, in some species it has been found that dominant call frequency is positively related with ambient temperature, but temperatures did not differ between our recordings of the two species; moreover, accumulated evidence suggests the relationship between spectral call traits and temperature to be weak or non-existent in many anuran species (GERHARDT 1978, RYAN 1987, GERHARDT & HUBERT 2002).

In addition to *H. antioquia* and *H. larinopygion* (BERNAL et al. 2004, this study), the advertisement calls of eight species of the *H. larinopygion* group have been described: *H. condor*, *H. criptico*, *H. lindae*, *H. pacha*, *H. pantostictus*, *H. psarolaimus*, *H. staufferorum*, and *H. tapichalaca* (KIZIRIAN et al. 2003, COLOMA et al. 2012, ALMENDÁRIZ et al. 2014). These calls all consist of a single note of short duration (133.0–542.0 ms; COLOMA et al. 2012, ALMENDÁRIZ et al. 2014, this study) and a broad band-pulse or multiple short pulses. Although ALMENDÁRIZ et al. (2014) described

the call of *H. condor* as pulsed, our re-analysis of their audio-recordings revealed that it is not pulsed (Fig. 4; see also their Figure 9).

Vocalisations can be informative for phylogenetic inferences (e.g., PÄCKERT et al. 2003, ROBILLARD et al. 2006, WOLLENBERG et al. 2007), although the reduced number of discrete characters in acoustic signals usually limits their usability. Nevertheless, the characterization of the advertisement call structure and studies on the acoustic behaviour can provide useful insights into the evolution of phenotypic characters in a group and contribute to clarifying the phylogenetic relationships between species (see GOICOECHEA et al. 2009). Based on current knowledge of advertisement call structures and the most comprehensive phylogenetic hypothesis available for *Hyloscirtus* (see ALMENDÁRIZ et al. 2014, BRUNETTI et al. 2015), the optimisation of acoustic characters indicates that call structure has a complex history that involved multiple transformations. Evidence indicates that the unpulsed call in *Hyloscirtus* is plesiomorphic, was replaced with multiple short pulses at least three times in the genus (Fig. 4), and includes at least two independent origins in the *H. larinopygion* species group: one in *H. psarolaimus* and another in the clade containing *H. antioquia*, *H. larinopygion*, *H. lindae*, and *H. pantostictus* (Fig. 4). Data on the advertisement calls of the remaining five species of the group (*H. caucanus*, *H. princecharlesi*, *H. ptychodactylus*, *H. sarampiona*, and *H. tigrinus*) and *H. charazani* in the *H. armatus* group are necessary to better understand the evolution of acoustic signals in these riparian species.

In contrast to *H. staufferorum*, whose advertisement calls can be heard from up to 500 m distance (COLOMA et al. 2012), *H. antioquia* and *H. larinopygion* calls are audible only at short distances (less than 50 m; M. RIVERA-CORREA and T. GRANT pers. obs.). Moreover, locating vocalizing individuals of these species, even at close proximity

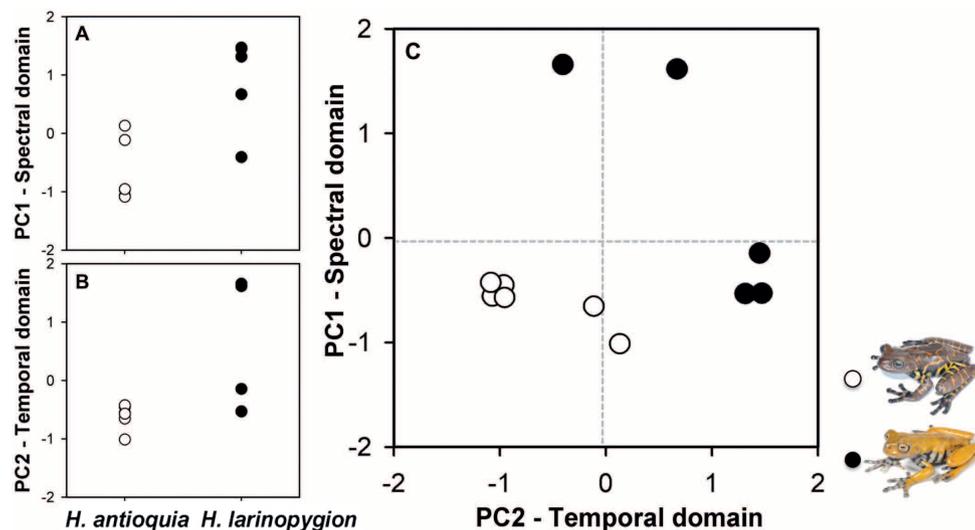


Figure 3. Differences in spectral (A), temporal (B), and both (C) advertisement call traits between *Hyloscirtus antioquia* (white dots) and *H. larinopygion* (black dots). The first principal component (PC1) represents call frequency whereas the second principal component (PC2) represents call duration (see also Table 2 for values). $N = 6$ for both species; some dots are superimposed.

(i.e., < 2 m), is extremely difficult. This could be due to the stream background noise masking the signal and/or vegetation scattering it (GERHARDT & HUBER 2002, WELLS 2007).

Finally, environmental noise and habitat structural complexity can be important selective forces that modulate signal evolution in species with acoustic communication (WILEY & RICHARDS 1982, PATRICELLI & BLICKLEY 2006, FENG et al. 2006, VARGAS-SALINAS & AMÉZQUITA 2013); however, the evolutionary pressures acting on the advertisement calls are unstudied in *Hyloscirtus*. The advertisement calls in this clade are a candidate model for future studies addressing the environmental factors that affect call structure, and we hope the information provided herein will help motivate further research on this topic.

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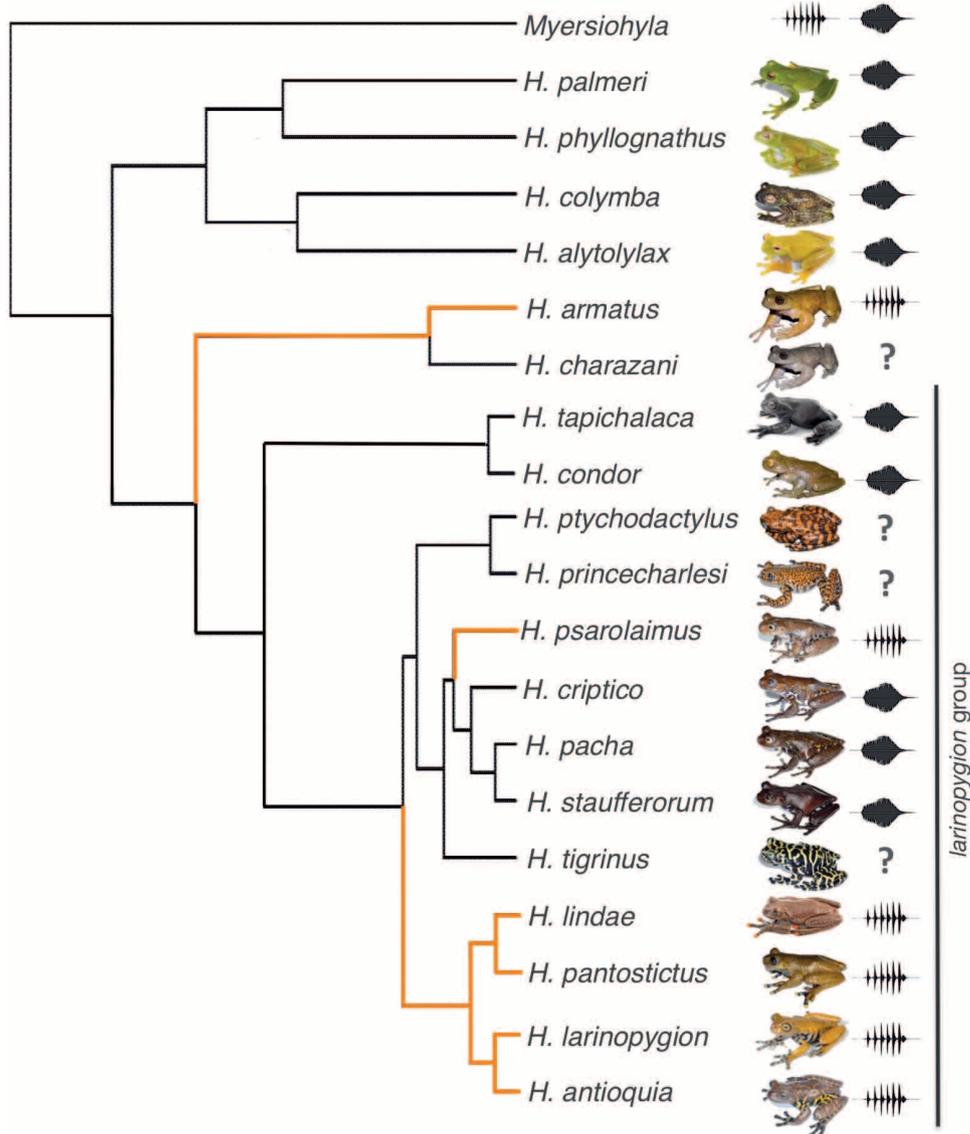


Figure 4. Phylogenetic relationships in the *Hyloscirtus larinopygion* species group, redrawn from ALMENDÁRIZ et al. (2014) and BRUNETTI et al. (2015) and showing the distribution of unpulsed (black branches) and pulsed (orange branches) advertisement call structures. The advertisement calls of some species of the group and the phylogenetic position of *H. caucanus* and *H. sarampiona* are currently unknown (see text).

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