

# Neotropical frogs and mating songs: The evolution of advertisement calls in glassfrogs

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## Abstract

Anurans emit advertisement calls with the purpose of attracting mates and repelling conspecific competitors. The evolution of call traits is expected to be associated with the evolution of anatomical and behavioural traits due to the physics of call emission and transmission. The evolution of vocalizations might imply trade-offs with other energetically costly behaviours, such as parental care. Here, we investigated the association between body size, calling site, parental care and call properties (call duration, number of notes, peak frequency, frequency bandwidth and call structure) of the advertisement calls of glassfrogs (Centrolenidae)—a family of Neotropical, leaf-dwelling anurans—using phylogenetic comparative methods. We also explored the tempo and mode of evolution of these traits and compared them with those of three morphological traits associated with body size, locomotion and feeding. We generated and compiled acoustic data for 72 glassfrog species (46% of total species richness), including representatives of all genera. We found that almost all acoustic traits have significant, but generally modest, phylogenetic signal. Peak frequency of calls is significantly associated with body size, whereas call structure is significantly associated with calling site and paternal care. Thus, the evolution of body size, calling site and paternal care could constrain call evolution. The estimated disparity of acoustic traits was larger than that of morphological traits and the peak in disparity of acoustic traits generally occurred later in the evolution of glassfrogs, indicating a historically recent outset of the acoustic divergence in this clade.

## KEYWORDS

acoustic communication, amphibians, comparative method, Neotropics, phenotypic disparity

## 1 | INTRODUCTION

Animal communication is an important behavioural feature that allows individuals to exchange information about their location, territory, condition, sex, size and the presence of potential predators (Barnard, 2004; Wells, 2007). Acoustic communication is mediated through sound signals that have temporal, spectral and structural components, which are determined by multiple intrinsic and extrinsic factors. For example, closely related species are predicted to have

similar vocalizations due to the shared ancestry of the behaviour and anatomy of sound-producing organs (Cocroft & Ryan, 1995; Panhuis, Butlin, Zuk, & Tregenza, 2001). Also, some spectral properties of acoustic signals depend on the mass of the vibrating structure of the vocal apparatus, which is often correlated with body size and, therefore, may represent an index signal (since there is a correlation between properties of the signal and a given trait of interest to the receiver evaluated based on said signal—Maynard Smith & Harper, 1995, 2003). For example, the dominant frequency of vocalizations

is usually negatively correlated with the body size of the emitter in anurans (Gingras, Boeckle, Herbst, & Fitch, 2013; Goutte et al., 2016, 2018; Zweifel, 1968), birds (Gonzalez-Voyer, den Tex, Castelló, & Leonard, 2013; Podos, 2001; Ryan & Brenowitz, 1985; Seddon, 2005; Wallschläger, 1980) and mammals (Barclay & Brigham, 1991; Fitch, 1997; Pfefferle & Fischer, 2006).

Environmental conditions of the habitat where the species is found can also influence acoustic signals. Some species vocalize from sites that enhance or reduce the attenuation or the masking of sounds by environmental background noise (Arak & Eiriksson, 1992; Chaverri & Gillam, 2013; Erdtmann & Lima, 2013; Hödl, 1977; Morton, 1975; Muñoz & Penna, 2016; Ryan & Kime, 2003). Hence, variation in acoustic signals among species can be the result of a combination of environmental conditions during signal emission and the morphology of the signaller or receiver (Endler, 1992).

Acoustic mating signals provide information about an individual's reproductive disposition and as such are directly involved in mate choice (Gerhardt, 2010). Calling behaviour is energetically costly (Crump, 1996; Prestwich, 1994; Townsend, 1986); therefore, energy allocated to other activities related to reproduction, such as brooding, may constrain investment in call emission and potentially decrease mate attraction (Townsend, 1986). Thus, a trade-off could be expected between investment into emission of mating signals and other costly behaviours such as parental care. Since the amount of energy necessary to produce acoustic mating signals often relates to temporal traits such as call length or note emission rate (Gerhardt & Huber, 2002), the predicted trade-off should be reflected in these call components. Mating signals are also involved in mate attraction and mate choice, and are likely under sexual selection, which could lead to rapid divergence (Gonzalez-Voyer & Kolm, 2011; Wilkins, Seddon, & Safran, 2013). In contrast, nonsexually selected traits such as morphological traits involved in locomotion or feeding are subjected mainly to natural selection and more likely to be experiencing stabilizing selection (Gonzalez-Voyer & Kolm, 2011; Ridley, 2004). Hence, it is expected that acoustic traits will show a different tempo and mode of evolution than ecology-related traits.

Most anuran males emit advertisement calls, which serve to attract mates and repel conspecific males (Wells, 1977). Such calls are genetically determined and highly stereotyped, playing an important role in species recognition and reproductive isolation (Duellman & Trueb, 1986; Wells, 2007; but see Köhler et al., 2017, pp. 39–42). Differentiation of these signals among populations may result in speciation in relatively short evolutionary timeframes (Vences & Wake, 2007). Thus, understanding call evolution could be important to gain better understanding of anuran speciation.

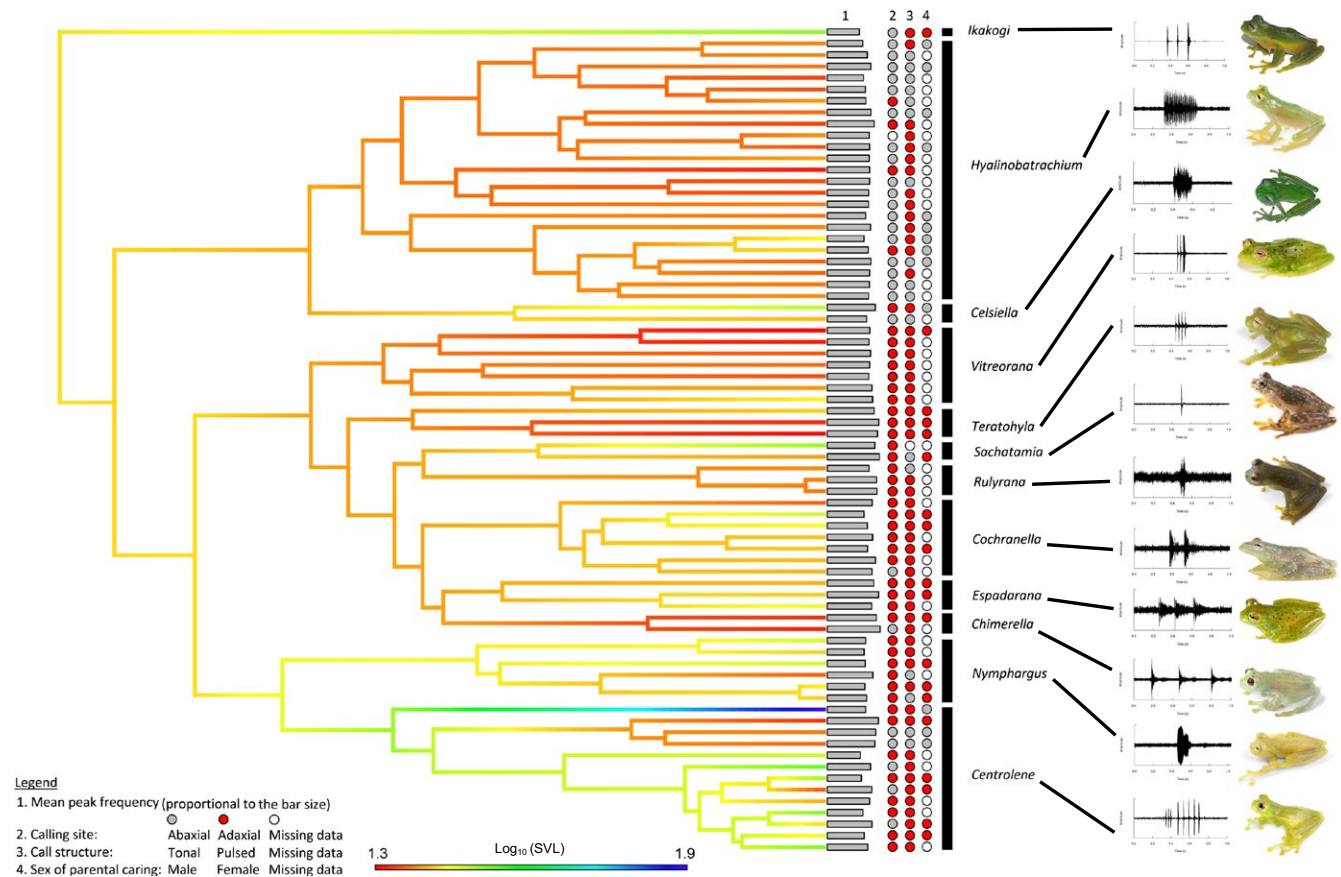
The few studies that have investigated the evolution of anuran advertisement calls in species-rich clades at deep evolutionary timescales have revealed complex associations between temporal call traits and shared ancestry among species (Erdtmann & Amézquita, 2009), spectral call traits and body size (Erdtmann & Amézquita, 2009; Goutte et al., 2016, 2018) and acoustic specificities of noisy torrent habitats (Goutte et al., 2016, 2018). In at

least one Neotropical clade (Superfamily Dendrobatoidea), the evolution of call traits is also apparently related to the occurrence of aposematic dorsal colour patterns, with calls of brightly coloured species diversifying at faster rates than calls of cryptically coloured species (Santos et al., 2014).

These studies relied on phylogenetic comparative methods (PCM), a number of analytical approaches that consider a set of species in a historical framework in order to analyse the evolutionary mechanisms involved in their diversification (Garamszegi, 2014; Pagel, 1999). These methods require previous knowledge of the genealogical relationships among species and a representative sample of the phenotypic traits of interest for the target taxon. These limitations have resulted in only a handful of empirical studies investigating the evolution of acoustic signals in large anuran clades, which jointly encompassed three of the 56 currently recognized anuran families (Frost, 2018), despite the important roles that mating calls play in anuran speciation.

Glassfrogs (Centrolenidae) are anurans characterized by having transparent ventral skin, which inhabit streamside vegetation alongside lotic environments (Castroviejo-Fisher, Guayasamin, Gonzalez-Voyer, & Vilà, 2014; Guayasamin, Castroviejo-Fisher, Trueb, Rada, & Vila, 2009). They constitute a clade of 155 species, distributed in humid forests from Mexico to Bolivia, along the Andes and across the Amazon, with vicariant species distributed in the island of Tobago and in the Brazilian Atlantic Forest (Castroviejo-Fisher et al., 2014; Frost, 2018). Glassfrogs most likely started to diversify roughly 25 million years ago in the Miocene in South America and the phylogenetic relationships among species are relatively well-explored and stable (Figure 1), with the most complete phylogeny to date including more than 73% of the described species diversity (Castroviejo-Fisher et al., 2014; Delia, Bravo-Valencia, & Warkentin, 2017). Males of most glassfrog species vocalize while perched on vegetation overhanging streams, except some *Centrolene*, *Rulyrana* and *Sachatamia* species (roughly 10 spp.) that call from rocks near water (Guayasamin et al., 2009). Male glassfrogs may assume a calling position on the upper or lower surface of a leaf, and calling position is generally fixed within species (Cisneros-Heredia & McDiarmid, 2007; Guayasamin et al., 2009). In some anuran species, mating calls of males are highly adapted to the structure of the calling site (Lardner & bin Lakim, 2002; Muñoz & Penna, 2016). Hence, leaf-dwelling could influence call evolution in glassfrogs. During reproduction, eggs are deposited by females near the male's calling site and eggs are attended by one or both parents for a period of time that can vary from a few hours to the hatching of tadpoles, depending on the species (Delia et al., 2017).

To date, the calls of 48% of glassfrog species have been described. In combination with a well-resolved phylogeny, this makes centrolenids an ideal model to study the relative effects that historical and ecological factors might have had on the evolution of their acoustic signals. Using PCM, we herein evaluate the influence of historical (phylogeny), intrinsic (body size, exclusively male parental care) and extrinsic (calling site)



**FIGURE 1** Maximum clade credibility tree of Centrolenidae modified from Delia et al. (2017). Only the species included in this study ( $n = 72$ ) are represented as terminals. Internal branch colours represent ML reconstructions of body size ( $\text{Log}_{10}$  of snout-vent length in mm), whereas terminal branch colours represent the average body size of each species. Mean peak frequency, calling site (abaxial = underside of leaves and adaxial = upper side of leaves), call structure and sex of caring parent are shown at tips. Graphs and photographs represent call waveforms ( $x$ -axis = 1 s) and dorsolateral views of representative species in Centrolenidae. Photographs: Santiago R. Ron (Anfibios del Ecuador; <http://www.bioweb.bio/faunaweb/amphibiaweb/>), Marco Rada, and Juan Guayasamin

factors to explain the evolution of advertisement call traits. We devise five predictions on how call traits and intrinsic and extrinsic explanatory variables potentially relate to each other. First, we predict that peak frequency of calls will be higher in smaller-sized species, given the association between the size of the vibrating apparatus and body size. Second, we predict that similar calling positions led to the evolution of similar advertisement calls because of similar selective pressures on signal transmission. Third, we predict that species with males providing parental care should emit shorter and simpler (i.e., of one note or tonal) calls than species with maternal care, as a result of the energetic trade-off between calling and brooding. Fourth, we expect acoustic divergence in glassfrogs was not constant through the group's evolution, and we predict that major shifts in the disparity of acoustic traits may be coincident with dispersal events into new ecological regions and/or major geological events that transformed the landscape. Fifth, we predict that acoustic traits will show a different tempo and mode of evolution than ecology-related traits (i.e., body size, head width, length of limbs).

## 2 | MATERIALS AND METHODS

### 2.1 | Taxon sampling and acoustic data

We analysed a total of 830 advertisement calls from 67 species, plus a putative undescribed species (*Hyalinobatrachium* aff. *bergeri*), which were compiled from audio recordings gathered from personal and museum collections (origin of recordings, authorship, and other relevant information are detailed in Dataset S1). This covered 43% of the species richness in Centrolenidae, including representatives of all genera. We analysed calls of as many individuals as possible for each species (mean = 3 individuals;  $SD = 2.5$ ; min.-max. = 1-12 individuals) to obtain a good estimate of the central tendency statistics for call properties of each species. We analysed five calls per individual and used the average value for each call property measured among all individuals of each species in all analyses.

We measured temporal and spectral call traits from oscillograms and power spectra, respectively. We estimated power spectra using a Fast Fourier Transform (FFT) analysis with a Blackman window of 5 ms, 80% of overlap in time grid and a DFT size of

1,024 samples in frequency grid. We carried out all bioacoustic analyses using RavenPro v. 1.4 (Bioacoustics Research Program, 2011).

Acoustic trait definition and terminology followed Köhler et al. (2017). We measured four acoustic traits: call duration (CD), measured as the time elapsed between the beginning and the end of the call as viewed in the oscillogram; number of notes (NN), measured as the number of tonal notes or pulse groups that are temporally separated from other notes or pulse groups by large silent intervals; peak frequency (PF), measured as the frequency emitted with the highest energy; and bandwidth (BW), which is the difference between the upper and lower frequency bounds of the notes, as measured 20 dB below the peak frequency. The -20 dB threshold was adopted in order to avoid considering frequencies originated from background noise in the analyses. Additionally, we categorized the call structure (CS) as pulsed (when at least one note presented sequential 100% amplitude modulation between pulses) or tonal (when no sequential amplitude modulation was present within any note that formed the advertisement call).

Other call variables commonly used in bioacoustics (e.g., number of harmonics in a note, duration of individual notes in a call and duration of the silent intervals between notes) were not considered because of the impossibility of verifying the homology of different call elements (individual notes or intervals) among all species. The display of note harmonics on audiospectrograms or power spectra is also strongly affected by low signal-to-noise ratio. Hence, these elements were more or less detectable depending on the intensity of background environmental noise captured in each sound recording.

We compiled data on the same call traits (except for BW) from the literature for 10 glassfrog species for which we could not access original recordings (Dataset S2). Hence, call traits were obtained for a total 78 glassfrog taxa. Nevertheless, only 72 of those taxa are represented in the phylogenetic tree considered in the PCM analyses (see below).

In one species, *Centrolene lynchi*, three different calls were found among recordings, associated with three different localities in Ecuador: (a) "3.5 km NE Mindo" (voucher: KU 164710; sound record: Fonozoo 194-8176); (b) "Reserva Las Galarias" (voucher: unknown; sound records: LS00018, 00020, 00029, 00031, and ASL013; source: Ana Salgado); (c) "Tandayapa, 1.0 km SW of on Mindo road" (voucher: RWM 12178; sound record: 201486\_44k\_C\_lynchi; source: Macaulay Library). We interpreted this marked variation associated with geographic locality as potentially indicating the existence of three different species. Accordingly, we only analysed calls corresponding to specimens included in the molecular phylogeny, which were obtained from Reserva Las Galarias (Castroviejo-Fisher et al., 2014).

## 2.2 | Morphological, behavioural and ecological data

We obtained morphometric data for 67 glassfrog species from the literature and for 11 species from fellow researchers (Dataset S3).

We selected three morphometric traits of biological relevance in anurans, which were shared among all references, to compare their evolution with acoustic traits: (a) snout-vent length (SVL), the most common descriptor of body size in anuran bioacoustics studies (Köhler et al., 2017), (b) head width (HW), which is related to feeding habits, as it limits maximum prey size (Emerson, 1985; Toft, 1980, 1981), and (c) tibia length (TL), a hindlimb measurement, which is related to jumping performance (Emerson, 1978, 1991; Zug, 1978). We considered the average values of SVL and the average values of the ratios HW/SVL and TL/SVL among males of each species for all analyses. Ratios were used instead of the raw values of HW and TL in order to rule out collinearity with SVL.

We compiled the predominant calling site of each species from the literature and complemented it with field observations provided by fellow researchers (Dataset S3). We categorized calling site in a binary way as either the underside of leaves or the upper side of leaves or rocks, because there was no reason to assume that call traits would be differently affected by different substrates considering the frog's calling position above them (as opposed to a calling frog positioned on the underside of a leaf forming a dome, which could potentially reverberate or differentially absorb certain spectral bands of a sound signal). Parental care (i.e., prolonged attendance) data were obtained from Delia et al. (2017) (Dataset S3), based on field observations of 39 species, distributed in 11 genera. Thirty-three of these species were represented in the acoustic dataset described above.

## 2.3 | Phylogeny

To establish the evolutionary relationships among glassfrog species, we used the most recent and complete molecular ultrametric phylogeny of Centrolenidae (Delia et al., 2017). This phylogeny includes 111 of the 155 currently named species, plus 10 putative undescribed species, and includes representatives of all currently recognized genera. This accounts for 72% of glassfrog species diversity. The phylogeny was based on DNA sequences of up to three mitochondrial and seven nuclear genes (totalling 6,645 bp) and was inferred from a Bayesian analysis with the dataset partitioned by gene using a GTR + I +  $\Gamma$  nucleotide substitution model, and dated with the relaxed-clock method (Delia et al., 2017). The topology used in all analyses corresponds to the maximum clade credibility tree of 10,001 trees sampled from the posterior distribution, with mean branch lengths (Dryad; <https://doi.org/10.5061/dryad.16vc0>). The topology is completely resolved, and only 13% of nodes have a posterior probability <0.90. For the purpose of the PCM, we trimmed the original tree by removing the species for which we had no phenotypic data (Figure 1), using the "drop.tip" function as implemented in the package APE (Paradis, Claude, & Strimmer, 2004) in R (R Core Team, 2017).

## 2.4 | Phylogenetic comparative analysis

We used PCM to estimate phylogenetic signal of acoustic traits, to measure correlated evolution between acoustic traits and other

variables (i.e., morphology, calling site and parental care) and to infer the tempo of phenotypic evolution of acoustic and morphometric variables.

We coded categorical variables (e.g., call structure, calling site) as numeric discrete variables. Continuous traits were log- or square-root-transformed (Table 1) in order to fulfil requirements of the statistical methods (i.e., normality of the residuals) (Freckleton, 2009) and the assumptions of the evolutionary model (Felsenstein, 1985; Freckleton, 2009). For all phylogenetic linear models, we visually assessed distribution of residuals and tested the correlations between the absolute values of the phylogeny-corrected residuals and their predicted values, in order to assess homogeneity of variance (Freckleton, 2009).

We estimated the value of the evolutionary parameter  $\lambda$ , a measure of the phylogenetic signal, for each acoustic trait separately. This parameter indicates the extent to which closely related species tend to resemble each other in relation to a given trait (Pagel, 1999). A value of  $\lambda = 1$  indicates high phylogenetic signal in a trait (i.e., phylogenetically closer species are more similar to each other), whereas a value of  $\lambda = 0$  indicates no phylogenetic signal (i.e., trait similarity among species is independent of shared ancestry; Freckleton, Harvey, & Pagel, 2002). The value of  $\lambda$  was estimated through a maximum-likelihood approach assuming a constant-rate Brownian motion model (BM) of evolution (Symonds & Blomberg, 2014), which considers that traits undergo a random process of successive and independent small changes (Felsenstein, 1985). Thus, the magnitude of evolutionary change of a phenotypic trait along a branch will be proportional to the branch length (Symonds & Blomberg, 2014). The  $\lambda$  parameter was estimated using the "phylosig" function as implemented in the package PHYTOOLS (Revell, 2012) in R (R Core Team, 2017).

We estimated the phylogenetic signal of binary traits using the D parameter (Fritz & Purvis, 2010). A value of  $D = 0$  indicates high phylogenetic signal in a trait (i.e., phylogenetically closer species are more similar to each other), whereas a value of  $D = 1$  indicates no phylogenetic signal (i.e., trait similarity among species is independent of shared ancestry; Fritz & Purvis, 2010). The D parameter was estimated using the "phylo.d" function as implemented in the package CAPER (Orme et al., 2013) in R.

In order to address each question of correlated evolution between continuous acoustic traits and other phenotypic or environmental variables, we used phylogenetic generalized least squares models (PGLS). PGLS incorporates phylogenetic relationships among species into the error structure of the linear model, considering a model of phenotypic evolution that best fits the observed data (Grafen, 1989; Martins & Hansen, 1997). We used PGLS models considering the  $\lambda$  transformation because they provided a better fit to our data. When  $\lambda$  is not statistically different from 1, a Brownian model of phenotypic evolution is assumed, whereas when  $\lambda = 0$  the residuals are independent (i.e., no covariance due to phylogenetic relatedness). The value of  $\lambda$  was estimated by maximum likelihood. Analyses were carried out using the "pgls" function as implemented in the package CAPER (Orme et al., 2013) in R.

We assessed associations between binary acoustic traits and phenotypic variables using phylogenetic logistic regressions (Ives & Garland, 2009). The alpha ( $\alpha$ ) parameter estimates the level of phylogenetic correlation in the regression (Ives & Garland, 2009). Analyses using phylogenetic logistic regressions were carried out using the "phyloglm" function as implemented in the package PHYLLOM (Ho & Ane, 2014) in R.

We studied the tempo of evolution of acoustic (CD, NN, PF, BW) and morphometric (SVL, HW/SVL, TL/SVL) traits by estimating their phenotypic disparity index (DI) (Harmon, Weir, Brock, Glor, & Challenger, 2007). The DI is an estimate of the temporal dynamic of phenotypic evolution that describes how phenotypic disparity is partitioned along the phylogeny. DI values  $>0$  suggest that most of the phenotypic disparity is distributed within clades; negative DI values suggest that disparity is distributed among clades, implying an early divergence of the trait; DI values near 0 indicate accumulation of phenotypic disparity among and within clades that is consistent with the Brownian motion model. We used disparity-through-time (DTT) plots to visually represent the average relative disparity of each subclade, which is estimated by dividing the average disparity of all subclades whose ancestral lineages were present at that time by the average disparity of the clade as a whole, and repeating this procedure at each node moving up from the root to the tip of the phylogeny (Harmon, Schulte, Larson, & Losos, 2003).

**TABLE 1** Maximum-likelihood estimates of lambda ( $\lambda$ ) and disparity index (DI), with the corresponding  $p$  values, for acoustic and morphometric traits for Centrolenidae

Trait	N	$\lambda$	$p$	DI	$p$
Log <sub>10</sub> (CD)	72	0.45	0.11	0.26	0.97
Sqrt (NN)	72	0.21	0.04*	0.16	0.96
Sqrt (PF)	72	0.72	$1.56 \times 10^{-5*}$	0.03	0.79
Sqrt (BW)	64	0.73	$1.02 \times 10^{-7*}$	0.27	0.97
Log <sub>10</sub> (SVL)	72	0.94	$1.06 \times 10^{-5*}$	$-9.9 \times 10^{-4}$	0.76
Log <sub>10</sub> (HW/SVL)	72	0.77	$4.08 \times 10^{-6*}$	0.004	0.75
Log <sub>10</sub> (TL/SVL)	72	0.28	0.004*	0.23	0.81

BW: bandwidth frequency; CD: call duration; HW: head width; NN: number of notes; PF: peak frequency; SVL: snout-vent length; TL: tibia length.

\*Significant results.

We estimated the DI and produced DTT for only the first 4/5 of the phylogeny to avoid artefacts due to incomplete species sampling and overestimation of disparity between closely related species (Gonzalez-Voyer & Kolm, 2011; Harmon et al., 2003). The DI and the DTT plots were obtained using the “*dt*” function as implemented in package GEIGER (Harmon et al., 2007) in R. The values of the timescale generated by default by the DTT plot were replaced with the adjusted first 4/5's of the glassfrog radiation chronogram estimated by Castroviejo-Fisher et al. (2014).

### 3 | RESULTS

The final dataset contained 72 glassfrog species. Forty-three species (60%) vocalized from the upper surface of leaves, whereas 28 species (39%) vocalized from the lower surface of leaves. Only one species (*Centrolene geckoideum*) vocalized from rocks. Seventeen species had tonal advertisement calls, whereas 54 species had pulsed calls. In one species for which call parameters were obtained from the literature, *Sachatamia ilex*, data on call structure was not available; hence, this terminal was coded as missing data for this trait. The mode for number of notes was 1 (range: 1–13). Mean call duration estimated among glassfrogs was 0.274 s (range: 0.006–1.443 s), mean peak frequency was 4,880.1 Hz (range: 2,713.2–7,407.4 Hz), and mean frequency bandwidth was 1,226.3 Hz (range: 810.4–2,476.1 Hz). The average SVL among glassfrogs was 23.8 mm (range: 18.4–75.2 mm), the mean HW/SVL ratio was 0.36 (range: 0.32–0.41), and the mean TL/SVL was 0.56 (range: 0.50–0.67). Thirteen species were described as having the male taking part in parental care, whereas in 20 species parental care is conducted exclusively by the female (Dataset S3).

#### 3.1 | Phylogenetic signal

Estimates of  $\lambda$  indicated that two acoustic traits (peak frequency and frequency bandwidth) had an elevated phylogenetic signal, significantly different from 0 ( $\lambda = 0.72$ ,  $p = 1.56 \times 10^{-5}$  and  $\lambda = 0.73$ ,  $p = 1.02 \times 10^{-7}$ , respectively). The value of  $\lambda$  for number of notes was significantly different from 0, although the maximum-likelihood  $\lambda$  value was lower than for the other components ( $\lambda = 0.21$ ,  $p = 0.04$ ), while the  $\lambda$  value for call duration was not significantly different from 0 ( $\lambda = 0.45$ ,  $p = 0.11$ ) (Table 1). The values of  $D$  for call structure and calling site indicated that both have significant phylogenetic signal ( $D = 0.43$ ,  $p_{\text{Random}} = 0.01$  and  $D = 0.04$ ,  $p_{\text{Random}} = 0$ , respectively)

**TABLE 2** Estimates of the parameter  $D$  for binary variables assessed for Centrolenidae. Call structure was coded as tonal (0) or pulsed (1). Calling site was coded as “upper side of leaves or rocks” (0) or “underside of leaves” (1)

Trait	N	D	$p_{\text{Random}}$	$p_{\text{Brownian}}$
Call structure	71	0.43	0.01	0.16
Calling site	72	0.04	0	0.48

(Table 2), although modest for call structure. Hence, closely related glassfrog species tend to call while perched in similar places (on leaves or under the surface of leaves) and to emit calls with similar structure (e.g., tonal or pulsed), with more similar peak frequencies and bandwidths.

Two of the evaluated morphometric traits, SVL and HW/SVL, had strong phylogenetic signal ( $\lambda = 0.94$ ,  $p = 1.06 \times 10^{-5}$  and  $\lambda = 0.77$ ,  $p = 4.08 \times 10^{-6}$ , respectively), whereas TL/SVL had a weak but significant phylogenetic signal ( $\lambda = 0.28$ ,  $p = 0.004$ ) (Table 1). This indicates that glassfrog species sharing a more recent common ancestor are generally more similar in body size (Figure 1) and head width than species with a more distant common ancestor.

#### 3.2 | Environmental, behavioural and morphological correlates of call traits

The PGLS analyses revealed a significant correlation between SVL and peak frequency (Table 3), which suggests that peak frequency could represent an index signal (Gonzalez-Voyer et al., 2013; Maynard Smith & Harper, 1995, 2003) in glassfrogs providing honest information about body size.

Calling site only influenced among-species differences in call structure. The results indicate that glassfrogs that call from the underside of leaves tended to emit tonal calls, whereas glassfrogs that call from the upperside of leaves tended to emit pulsed calls (Tables 4 and 5). Finally, calls of species with male brooding were generally characterized by tonal calls (Table 4). We found no additional associations between acoustic variables and male investment in parental care (Table 6), indicating that the structure of advertisement calls represents a trade-off with investment in parental care.

#### 3.3 | Tempo and mode of evolution of call and morphological traits

The disparity-through-time analyses suggested that the evolution of call duration, number of notes and frequency bandwidth differs from that of two morphometric traits (i.e., SVL and HW/SVL) but not from that of TL/SVL in terms of the tempo and mode of evolution. Similarly, peak frequency mirrors the temporal pattern of evolution of SVL and HW/SVL. Acoustic traits had disparity indices that were nearly an order of magnitude higher than those of morphometric traits (0.03–0.27 vs.  $-9.9 \times 10^{-4}$ –0.004, respectively; Table 1), except for TL/SVL, which had a DI = 0.23, close to the maximum DI for acoustic traits. Nonetheless, the DI was not significantly different from zero for any of the traits studied. Peak frequency had the lowest value of DI among acoustic traits. Considering the disparity-through-time (DTT) analysis, disparity of all phenotypic traits peaked (with different magnitudes) during the first third of the centrolenid radiation (around 15 MYA) and then decreased (Figure 2). In contrast, all acoustic traits except peak frequency increased their relative DI towards the present, around 10–8 MYA—approximately half-way along the radiation of glassfrogs—deviating from DI values predicted under a Brownian

**TABLE 3** Phylogenetic generalized least squares model of the relationship between peak frequency and snout-vent length (SVL) for Centrolenidae

	N	$\lambda$	R <sup>2</sup>	$\beta \pm SE$	t value	p value
Sqrt (Peak frequency)	72	0.76	0.155			
Intercept				117.476 ± 14.359	8.818	8.47 × 10 <sup>-12</sup>
Predictor: Log <sub>10</sub> (SVL)				- 36.255 ± 10.116	- 3.584	6.21 × 10 <sup>-4*</sup>

\*Significant correlations.

**TABLE 4** Phylogenetic logistic regressions of associations between call structure and calling site (underside of leaves vs. upper side of leaves or rocks), and between call structure and male attendance during brooding of egg clutch for Centrolenidae

	N	$\alpha$	b ± SE	z value	p value
Call structure	70	26.57			
Intercept			1.787 ± 0.552	3.237	0.001
Predictor: Calling site			-1.458 ± 0.617	-2.363	0.018*
Call structure	29	80.59			
Intercept			1.951 ± 0.695	2.805	0.005
Predictor: Male brooding			- 1.784 ± 0.921	- 1.934	0.053*

\*Significant correlations.

motion model (BM) (Figure 2a-d). A similar pattern is also observed in TL/SVL. This indicates that (a) for most acoustic traits assessed and TL/SVL, a greater proportion of the disparity was concentrated within subclades and (b) that acoustic divergence did not follow a purely Brownian model and, instead, results suggest most divergence is recent, compared to the temporal patterns observed for two morphometric traits (i.e., SVL and HW/SVL)—which present a model of evolution better approximated by a Brownian model.

## 4 | DISCUSSION

### 4.1 | Phylogenetic signal in acoustic traits

The idea that behavioural characters, such as anuran vocalizations, carry phylogenetic information has a relatively old origin in evolutionary biology (Blair, 1958) and the similarity of acoustic traits among close relatives had been previously suggested for glassfrogs (McDiarmid, *in* Cocroft & Ryan, 1995; Wen, Vasquez, & Castroviejo-Fisher, 2012), but this is the first time it has been quantitatively evaluated. Although behavioural traits are generally deemed more labile than morphological, ecological and physiological traits in vertebrates (Blomberg, Garland, & Ives, 2003; but see Wenzel, 1992; Gonzalez-Voyer et al., 2013), our results indicate that all acoustic traits but one have significant phylogenetic signal; a pattern reported in studies of other anuran groups (Cocroft & Ryan, 1995; Wollenberg, Glaw, Meyer, & Vences, 2007; Erdtmann & Amézquita, 2009; Goicoechea, De La Riva, & Padial, 2010; Gingras, Mohandesan, Boko, & Fitch, 2013; but see Cannatella et al., 1998; Tobias, Evans, & Kelley, 2011).

The strength of the phylogenetic signal varied among acoustic traits, with number of notes having the lowest phylogenetic signal (Table 1). On the other hand, call duration is not phylogenetically constrained. Previous studies have found individual, intraspecific and interspecific variation of temporal traits such as call duration, call rate and pulse rate to be correlated with environmental temperature (Bellis, 1957; Blair, 1958; Gillooly & Ophir, 2010; Goutte et al., 2018; Köhler et al., 2017; Zweifel, 1959). Unfortunately, analyses of correlations between acoustic variables and environmental temperature were precluded by (a) the lack of associated temperature data of most recordings and (b) the different sample sizes of recordings available for each species, some of which were represented by recordings spanning several dates and geographic locations, rendering an average temperature estimation uninformative. Further studies, ruling out the temperature, could indicate if modifying call duration is an avenue for divergence of calls in glassfrogs. In sum, our analyses indicate that each acoustic trait had a particular pattern of evolution.

Phylogenetic signal was also weaker among acoustic traits when compared to that of morphological traits (Table 1), which may indicate that the evolution of traits under sexual selection is less constrained than other characters (Gonzalez-Voyer & Kolm, 2011), most likely because sexual selection does not impose stabilizing selection but rather directional or disruptive selection (Civetta & Singh, 1998; Masta & Maddison, 2002). Little variation in ecological traits and more variation in sexual traits are predicted for an evolutionary process led by divergent sexual selection (Safran, Scordato, Symes, Rodríguez, & Mendelson, 2013). Disruptive sexual selection could be an important driver for speciation in some animal clades because traits involved in mate choice facilitate reproductive isolation (Boake, 2005; Coyne

& Orr, 2004; Higashi, Takimoto, & Yamamura, 1999; Kraaijeveld, Kraaijeveld-Smit, & Maan, 2011; Masta & Maddison, 2002; Payne & Krakauer, 1997; Seehausen, 2000; Seehausen et al., 2008; Streebman & Danley, 2003). The lower phylogenetic signal of the acoustic traits, compared to most morphological traits measured, suggests that sexual selection could be an important driver of the diversification of glassfrogs.

#### 4.2 | Correlated evolution of peak frequency and body size

A general principle in bioacoustics is that the mass of the structure producing a sound is inversely correlated with the peak frequency of the sound (Wilkins et al., 2013). Our results show a negative correlation between SVL and PF in glassfrogs when considering the phylogeny. Similar relationships were also found in other anuran clades (Erdtmann & Amézquita, 2009; Gingras, Boeckle, et al., 2013; Goutte et al., 2018; Röhr, Paterno, Camurugi, Juncá, & Garda, 2016), birds (Gonzalez-Voyer et al., 2013; Podos, 2001; Ryan & Brenowitz, 1985; Seddon, 2005; Wallschläger, 1980) and mammals (Barclay & Brigham, 1991; Fitch, 1997; Pfeufferle & Fischer, 2006). However, the variation explained by our PGLS analysis is relatively small

( $R^2 = 0.155$ ) if compared to other anuran clades, where variance explained spanned a range of 38%–66% (Erdtmann & Amézquita, 2009; Gingras, Boeckle, et al., 2013; Röhr et al., 2016), which could indicate variation in the degree of constraint imposed by body size on the evolution of peak frequency across taxa.

The limited body size variation in glassfrogs is striking when compared to other anuran groups in which the evolution of call parameters has been investigated (Erdtmann & Amézquita, 2009; Gingras, Boeckle, et al., 2013; Gingras, Mohandesan, et al., 2013; Goutte et al., 2016; Röhr et al., 2016). Male glassfrogs are small (male SVL interquartile range: 20.82–24.86 mm), with a single outlier—the giant *Centrolene geckoideum* (male SVL range: 70.20–80.70 mm; Guayasamin, Varela-Jaramillo, & Frenkel, 2018). The restricted variance in body size may be explained by a developmental constraint (Gould, 1980; Smith et al., 1985; Stearns, 1986) and testing this hypothesis would require detailed developmental studies. Nevertheless, mapping SVL evolution on the phylogeny reveals several independent evolutionary transitions between relatively large and small species (Figure 1), which would not be expected if a common developmental constraint limited the distribution of body size among species of glassfrogs. Furthermore, several species emit calls at frequencies higher than those expected by the PGLS model

**TABLE 5** Phylogenetic generalized least squares models of associations between advertisement call traits and calling site (underside of leaves vs. upper side of leaves or rocks) for Centrolenidae

	N	$\lambda$	$R^2$	$\beta \pm SE$	t value	p value
Log <sub>10</sub> (Call duration)	71	0.33	0.007			
Intercept				−0.842 ± 0.145	−5.826	1.71 × 10 <sup>−7</sup>
Predictor: Calling site				0.092 ± 0.130	0.710	0.480
Sqrt (Number of notes)	71	0.23	0.007			
Intercept				1.330 ± 0.170	7.847	4.15 × 10 <sup>−11</sup>
Predictor: Calling site				0.116 ± 0.173	0.668	0.507
Sqrt (Peak frequency)	71	0.746	0.016			
Intercept				66.300 ± 3.351	19.788	<2 × 10 <sup>−16</sup>
Predictor: Calling site				1.992 ± 1.889	1.055	0.295
Sqrt (Bandwidth frequency)	63	0.703	0.002			
Intercept				34.107 ± 1.738	19.621	<2 × 10 <sup>−16</sup>
Predictor: Calling site				−0.358 ± 1.110	−0.322	0.748

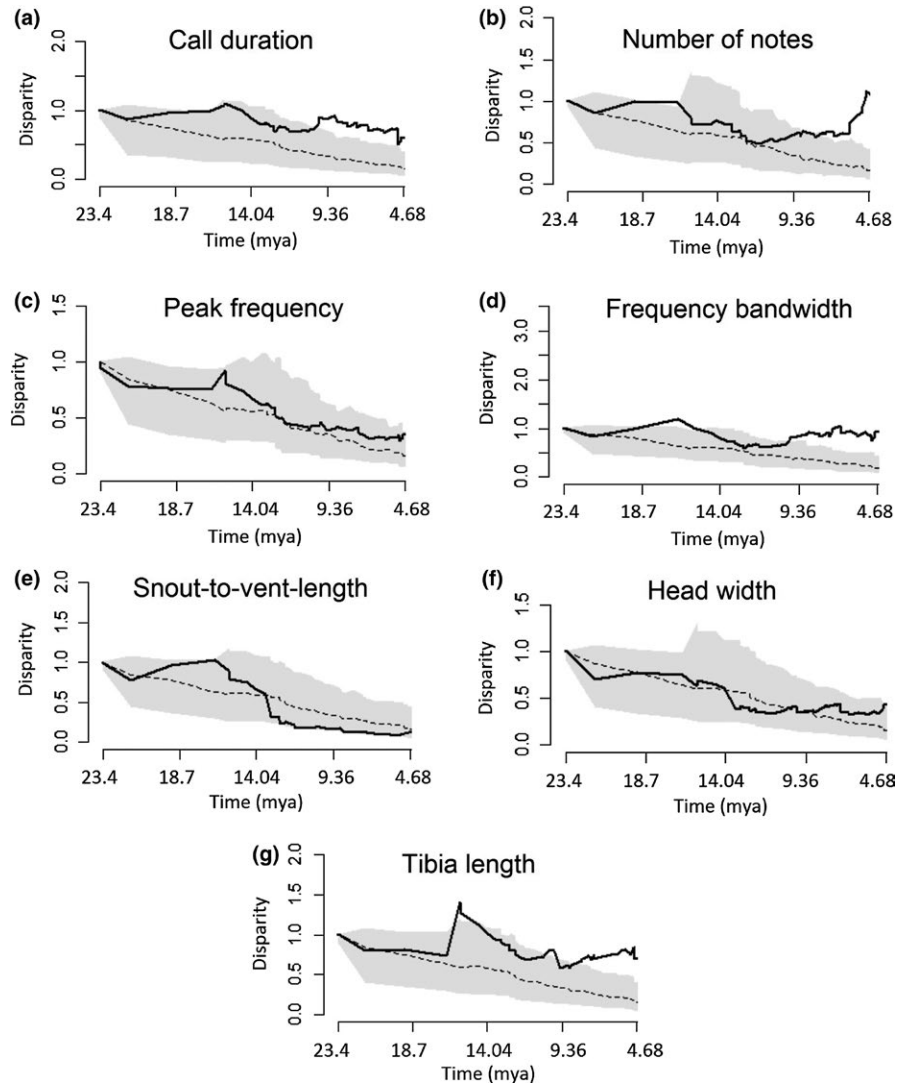
\*Significant correlations.

**TABLE 6** Phylogenetic generalized least squares models of the relationship between advertisement call traits with male attendance during brooding of egg clutch for Centrolenidae

	N	$\lambda$	$R^2$	$\beta \pm SE$	t value	p value
Log <sub>10</sub> (Call duration)	29	0	0.002			
Intercept				−0.649 ± 0.116	−5.595	6.20 × 10 <sup>−6</sup>
Predictor: Male brooding				−0.050 ± 0.198	−0.254	0.801
Sqrt (Number of notes)	29	0	0.084			
Intercept				1.583 ± 0.137	11.591	5.46 × 10 <sup>−12</sup>
Predictor: Male brooding				−0.367 ± 0.232	−1.578	0.126

\*Significant correlations.





**FIGURE 2** Disparity-through-time (DTT) plots for acoustic and morphometric traits of Centrolenidae. Vertical axis represents average subclade disparity (an estimate of relative disparity among species within a subclade compared with total clade disparity). (a) Call duration; (b) number of notes; (c) peak frequency; (d) frequency bandwidth; (e) snout-to-vent length (SVL); (f) head width/SVL; and (g) tibia length/SVL. The timescale was estimated from the chronogram of Castroviejo-Fisher et al. (2014). The solid line represents the relative disparity through the time estimated from the dataset, the dashed line represents a null (Brownian) model, and the grey zone represents the 95% confidence intervals estimated from the simulations under Brownian motion

(Figure S1), and a historical constraint may not be the only factor at play behind the observed pattern. Glassfrog body size could be alternatively limited by their arboreal, leaf-dwelling habit, with leaf surfaces acting as calling perches, oviposition sites and background camouflage (Kubicki, 2007; Señaris & Ayarzagüena, 2005).

The predominance of small glassfrog species only explains a flatter slope in the correlation between SVL and PF, and not the low variance explained. A factor that could explain PF variation beyond size is background noise. Some studies have suggested that anurans inhabiting lotic environments tend to emit calls with higher frequencies as a result of acoustic adaptation to reduce interference with the noise produced by waterfalls and rapids (Boonman & Kurniati, 2011; Feng et al., 2006; Goutte et al., 2016, 2018; Narins et al., 2004; Röhr et al., 2016; Vargas-Salinas & Amézquita, 2014). Thus, the high-pitched calls of some relatively large species could be an adaptation to outcompete the masking interference of torrent noise. As background noise data were missing for all calls, future studies could benefit from collecting data on the intensity and bandwidth of waterfall and rapids noise (Goutte et al., 2016, 2018; Narins et al., 2004; Schwartz & Bee, 2013), as well as information about height

of calling perches (Kubicki, 2007; Señaris & Ayarzagüena, 2005) and standardized measurements of call amplitude along the background noise gradient (Schwartz & Bee, 2013).

### 4.3 | Advertisement call and calling site

Several anuran species vocalize from positions that maintain the integrity of information contained in acoustic signals along the transmission path (Hödl, 1977; Schwartz, Huncce, Lentine, & Powers, 2016) or enhance its intensity (Bailey & Roberts, 1981; Lardner & bin Lakim, 2002; Muñoz & Penna, 2016). Our results indicated that the acoustic structure of calls (pulsed or tonal notes) was the only trait associated with the type of calling site.

Males that signal from the lower surface of leaves may emit tonal calls to minimize the energetic budget, since pulsed calls potentially require more contractions of muscles associated with sound production and clinging upside-down may be energetically demanding, since frogs splay out their limbs (and occasionally reposition them when these slip) in order to reduce the peeling angle and consequently to increase attachment (Endlein et al., 2013). However, the

ability to adhere to lower leaf surfaces or to stand on leaves while not bending them may be limited by body mass (Barnes, Oines, & Smith, 2006) which, in anurans, is strongly correlated with SVL (Santini, Benítez-López, Ficetola, & Huijbregts, 2018). Nonetheless, we found no significant differences in SVL between species that use different calling sites (PHYLANOVA:  $F_{1,71} = 1.43$ ;  $p = 0.63$ ).

Calling site choice could also be driven by predation risk (Richards & Bull, 1990; Travis, Keen, & Juillianna, 1985). Bats are sound-oriented predators, discriminating edible anuran prey from their calls (Tuttle & Ryan, 1981). In *Hyalinobatrachium fleischmanni*, males tend to call from the lower side of leaves when frog-eating bats (*Trachops cirrhosus*) occur nearby (Delia, Cisneros-Heredia, Whitney, & Murrieta-Galindo, 2010). Future work would greatly benefit from measuring the size and the resistance of leaves used as calling perches, as well as from analyses of potential vibrational responses of these leaves, for example, by means of laser Doppler vibrometry, and from experimental approaches investigating the role of bats as glassfrog predators.

#### 4.4 | Evolution of parental care and advertisement calls

Glassfrog species with paternal care normally invest more time brooding than species with exclusive maternal care (Delia et al., 2017). Parental care by males may impose trade-offs with acoustic signalling during reproduction, since both activities are energetically demanding (Ryan & Kime, 2003; Townsend, 1986). In agreement with that expectation, we found that species in which males are involved in parental care generally produce tonal calls, which demand a smaller number of muscle contractions than calls containing multiple pulses (Wells, 2007).

Although call or note emission rates could be better descriptors of energy spent in calling by male glassfrogs than call structure or call duration, they were not considered in this study due to limitations of the sound recordings, which were generally too short to allow their estimation. We recommend that, in the future, researchers should aim for longer, at least ten-minute-long recordings, as well as noting ambient temperature to rule out potential confounding effect of temperature on temporal traits (Goutte et al., 2018).

#### 4.5 | Tempo and mode of phenotypic evolution

The study of the evolution of phenotypic characters in evolutionary radiations has focused on morphology, with few studies assessing acoustic traits (Gonzalez-Voyer et al., 2013; Simões et al., 2016). Our results (Table 1, Figure 2) showed two clear patterns of phenotypic evolution. The first is dominated by morphological characters (body and head size), although it also includes peak frequency. It is characterized by a relatively constant rate of evolution, with disparity comparable with that under a BM model. This suggests that the evolution of these traits could be explained by gradual fluctuations in selection regimes (O'Meara, Ané, Sanderson, & Wainwright, 2006; Revell, Harmon, & Collar, 2008), rendering high values of

phylogenetic signal (Revell et al., 2008), as effectively observed in the corresponding estimates of  $\lambda$ . The second pattern includes most acoustic traits (call duration, number of notes and frequency bandwidth) and TL/SVL. Their phenotypic disparity is more partitioned within subclades, indicating that the bulk of their variation occurred around 10–8 MYA, when observed disparity values surpassed the null models' 95% confidence intervals (Figure 2), possibly indicating a marked change in the selection regime.

Streelman and Danley (2003) proposed that differentiation in communication traits occurs at the last stage of vertebrate evolutionary radiations, generally following differentiation in habitat and morphology. The predominance of morphological and acoustic traits in the first and second pattern (with constant rate of evolution and with peak of disparity in recent evolutionary time, respectively) could be due to different types of selection acting upon each group of traits. Traits directly involved in mate choice are often under sexual selection, having disruptive or directional selection (Civetta & Singh, 1998; Masta & Maddison, 2002), whereas traits that are not involved in mate choice are often under natural selection, which can be stabilizing, disruptive or directional (Coyne & Orr, 2004; Gonzalez-Voyer & Kolm, 2011; Ridley, 2004). Otherwise, distinct patterns of disparity found among exclusively sexual and other traits may reflect differences in ecological, physiological or genetic constraints.

Following the idea of "ecological opportunity" (Stroud & Losos, 2016), the strong and temporarily coincidental significant increase in disparity observed in call duration, number of notes, bandwidth frequency and TL/SVL could have been triggered by past environmental changes or dispersals into new areas. When considering the historical biogeography of Centrolenidae (Castroviejo-Fisher et al., 2014), the time estimates for the onset of increasing disparity coincide with (a) major changes within biogeographic areas already inhabited by ancestral glassfrogs, such as the main orogeny of the Andes and the formation of the modern Amazon basin (Hoorn & Wesselingh, 2010) and (b) the dispersal into new biogeographic areas such the large radiation of Cochranellini and the colonization of the MRCA of *Teratohyla* of the Amazonian lowlands and of *Celsiella* of the highlands. Variation of vegetation structure along elevational gradients and biogeographic regions is a strong candidate variable selecting aspects of locomotion and vocalizations (Morton, 1975; reviews in Morinay, Cardoso, Doutrelant, & Covas, 2013; Wilkins et al., 2013). However, data on streamside forest structure is lacking for the vast majority of sites (if not all) where glassfrog species were recorded.

## 5 | CONCLUSIONS

To date, this study is the most comprehensive investigation on anuran call evolution at a macroevolutionary scale considering the available coverage of species and acoustic data sampling. Our results allow us to propose a general scenario which provides a theoretical background for tests of associations between shared ancestry, morphology, behaviour and the evolution of acoustic signals that are key to reproductive isolation and speciation in anurans. Among our most striking

findings is the one that the negative body size-peak frequency relationship, strong in many anuran lineages, is very weak in glassfrogs. We tentatively hypothesize that this pattern is due to morphological constraints that cause the small interspecific variation in size among species, which could be mediated by predation risk and other ecological pressures selecting for small size. Regarding call structure, we postulate that the energetic costs associated with clinging upside-down on leaves and with parental care exerted by males limit their ability to produce complex, amplitude-modulated calls. Although recognizing that reconstruction of disparity through time applies exclusively to the direct ancestors of extant species and that disparity in the past might have been higher than estimated in our analyses, the tempo of acoustic evolution in glassfrogs is not discordant with the hypothesis that acoustic differentiation in this group was associated with the occupation of new habitats, filling in vacant acoustic spaces.

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## SUPPORTING INFORMATION

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