

# Evolution of advertisement signals in North American hyliid frogs: vocalizations as end-products of calling behavior

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## Abstract

We studied the advertisement signals in two clades of North American hyliid frogs in order to characterize the relationships between signal acoustic structure and underlying behavior. A mismatch was found between the acoustic structure and the mechanism of sound production. Two separate sets of phylogenetic characters were coded following acoustic versus mechanistic criteria, and exploratory treatments were made to compare their respective phylogenetic content in comparison with the molecular phylogeny (Faivovich et al., 2005). We discuss the consequences of the acoustic/mechanistic mismatch in terms of significance of acoustic characters for phylogenetic and comparative studies; and the evolution of vocalizations in North American treefrogs. Considering only the acoustic structure of frog vocalizations can lead to misleading results in terms of both phylogenetic signal and evolution of vocalizations. In contrast, interpreting the acoustic signals with regard to the mechanism of sound production results in consistent phylogenetic information. The mechanistic coding also provides strong homologies for use in comparative studies of frog vocalizations, and to derive and test evolutionary hypotheses.

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Many animals depend on acoustic communication for reproductive success and survival. Both field and laboratory studies have shown the importance of signals of communication for sexual and natural selection and, more broadly, for the evolution and diversification of taxa (Gerhardt and Huber, 2002, and references within).

With the development of comprehensive methods of phylogenetic reconstruction and the use of phylogeny for testing evolutionary hypotheses, there is a recent tendency to extrapolate populational results on acoustic communication using a comparative approach. This is usually done by optimizing signal characters and receiver preferences on the phylogeny, and by the subsequent comparison of their patterns of transformation (Basolo, 1990, 1995; Ryan and Rand, 1993).

Behavioral characters can be defined as any other characters, either morphological or molecular, following the classical rules of observation, definition and analysis,

with the same problems, requirements and pitfalls (Wenzel, 1992; Greene, 1994; Grandcolas et al., 2001; Brooks and McLennan, 2002; Stuart et al., 2002; Desutter-Grandcolas and Robillard, 2003; Desutter-Grandcolas et al., 2003). Application of the principle of homology to behavior requires a careful examination of behavioral features and not only to define broad functional classes (Wenzel, 1992). However, acoustic signals are not strictly behavioral characters, but rather constitute end-products of the calling behavior. As emphasized by Stuart and Currie (2001, 2002), it is necessary to relate end-products with the behavior and structure involved in their production to make sure that homologous traits are compared.

The relationship between signal and calling behavior/mechanism is straightforward in certain groups that use stereotyped mechanisms of sound production. In crickets, for example, signals correspond to repetitions of syllables, which are always produced by stridulation during a single closure of the forewings (e.g., Bennet-Clark, 1989). The cricket syllable is thus a significant

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homologous unit among species in terms of both signal and behavior. All higher-order temporal patterns correspond to groups of syllables (chirps) or, in turn, groups of chirps: all temporal patterns can thus be compared among taxa on the syllable basis.

The signal/mechanism relationship is much less direct in vertebrates where signals are emitted using much more complex and flexible systems (e.g., Bradbury and Vehrencamp, 1998). However, the basic mechanism of sound production is similar in most frogs, and consists in a modified breathing mechanism (Martin and Gans, 1972; Schneider, 1988), with a few exceptions in the family Pipidae (Rabb, 1960; Yager, 1992). In most families of frogs, the calling apparatus involves the same muscles, cartilages and membranes, and most variation is restricted to size and shape of common structures (Trewavas, 1933; Schmid, 1976).

From a mechanistic and behavioral point of view, each act of sound emission corresponds to a cycle of exhalation–inhalation. The notion of “note”, which refers to the total amount of sound energy generated during a single airflow cycle (McLister et al., 1995), is an appropriate acoustic unit to use when comparing the sound production in frogs.

From a strictly acoustic point of view, the notions of “pulse” and “call” are convenient units for comparison of acoustic signals in frogs, as “syllables” and “chirps” are used to describe cricket songs. The main difference between frogs and crickets is that the mechanistic (note) and acoustic (pulse, call) units may not always match in the same way among frogs, even in closely related species. As discussed by McLister et al. (1995), McLister (2001) and Gridi-Papp (2003), the temporal structure of an anuran call can correspond either to a single note or to a repeated train of notes. Furthermore, as shown by Martin (1971), the notes (= pulses in Martin’s terminology) can be unmodulated or modulated, in amplitude, frequency, or both. In other words, a consideration of the mechanisms of sound production rather than acoustic characteristics alone is required to specify homologies in frog vocalization that can be used in phylogenetic analyses.

In the present study we focus on two closely related species groups of North American hylids (*Hyla squirella* (*Hyla cinerea*–*Hyla gratiosa*)) and (*Hyla avivoca* (*Hyla versicolor*–*Hyla chrysoscelis*)). Our first goal is to describe and compare for each species the advertisement signals in terms of mechanistic and acoustic units, in order to characterize the relationships between signal and emitting behavior. Secondly, we design two separate sets of phylogenetic characters coded following the acoustic versus mechanistic criteria. Phylogenetic treatments and the optimization of these characters on the phylogeny allow us to examine their patterns of transformations and to address the following question: What are the consequences of the alternative codings (acoustic

versus mechanistic) on the phylogenetic significance of behavioral characters, and on the signal evolution?

## Materials and methods

### *Taxonomic model and phylogeny*

The recent phylogenetic study of Hylidae by Faivovich et al. (2005) resulted in a revision of the taxonomic delimitation of the genus *Hyla*. Strong molecular evidence based on the analysis of approximately 5100 base pairs from four mitochondrial (12S, tRNA valine, 16S and cytochrome *b*) and five nuclear genes (rhodopsin, tyrosinase, RAG-1, seventh in absentia, and 28S) supports a clade including all North American and Eurasian species of *Hyla* (Fig. 1). According to this study, *Hyla* now includes four species groups and a few non-assigned species, whose relationships will be reconsidered in future analyses.

In the present study we focused on two monophyletic species groups from Faivovich et al.’s (2005) study: (1) the *Hyla cinerea* group, includes three North American species, *H. cinerea* (Schneider, 1799), *H. gratiosa* LeConte, “1856” [1857], and *H. squirella* Bosc, 1800; and (2) the *Hyla versicolor* group, three North American species, *H. avivoca* Viosca, 1928, *H. chrysoscelis* Cope, 1880, and *H. versicolor* LeConte, 1825. Although *H. versicolor* includes at least four independently evolved tetraploid lineages, male calls have differentiated very little and there is genetic evidence for extensive interbreeding among these lineages (Ptacek et al., 1994; Holloway et al., 2006); individuals of both genders are also morphologically cryptic. Although *H. chrysoscelis* was not included in Faivovich et al.’s (2005) study, its status as the sister species of *H. versicolor* is well supported by numerous studies (Ptacek et al., 1994; Smith et al., 2005; Wiens et al., 2005; Holloway et al., 2006). We used the species *Pseudacris crucifer* (Wied-Neuwied, 1838) as an outgroup for the phylogenetic treatments. According to the phylogeny of Hylidae (Faivovich et al., 2005), an ideal outgroup for our study would consist in several members of the *H. arborea* species group, which is the sister group of the clade including all the other species of *Hyla*; however, no data are available about the mechanism of sound production of these species.

### *Acoustic data*

We used recordings of the advertisement calls made by HC Gerhardt and colleagues (now located at the MacCauley Laboratory at Cornell University). These recordings allowed analyses of the temporal patterns of the calls of each species of interest. The acoustic analysis was performed using standard software, such as Raven

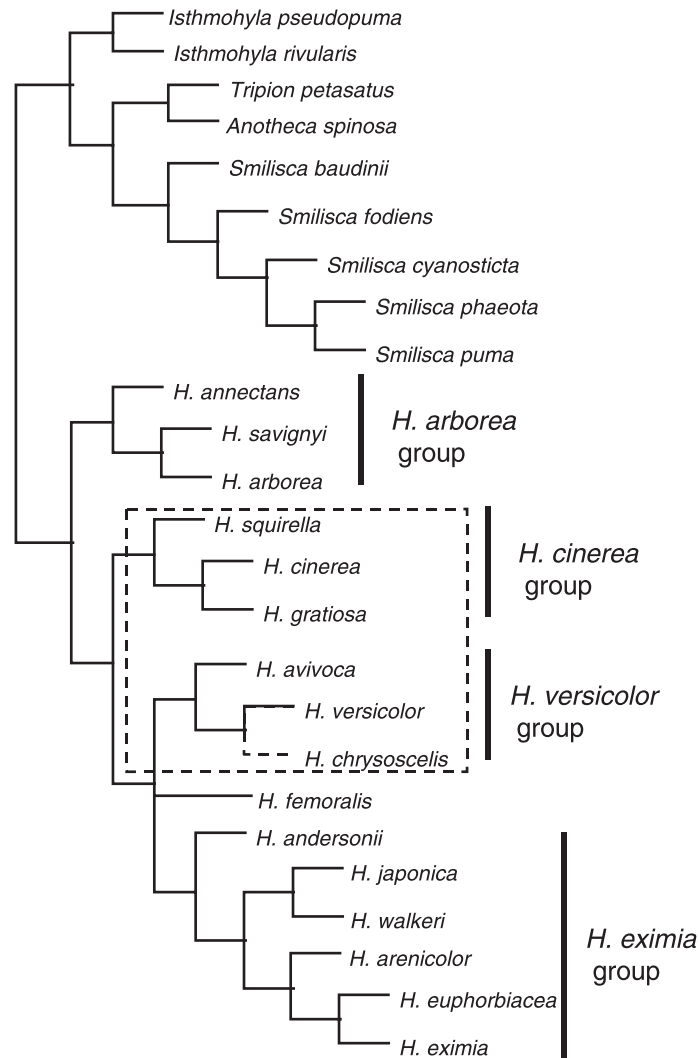


Fig. 1. Phylogeny of *Hyla* based on Faivovich et al. (2005) showing the clades under study (dotted square). The species *Hyla chrysoscelis* is tentatively placed as the sister group of *Hyla versicolor* (see text).

version 1.2.1 (Cornell Laboratory of Ornithology Bioacoustics Research Program, New York) and Cool Edit 2000 (Syntrillium Software Corporation, Phoenix, AZ). We analyzed 20 calls of 10 individuals per species, and made 20 replicates of each measurement per recording. The measured parameters are listed in Table 1. After correction for temperature, mean values and standard deviation per individual and grand means and standard deviations for each species were calculated for use in character state delimitation (Appendix 1).

#### Data on mechanism of sound production

The mechanism of sound production in anurans, including some North American treefrogs, has been examined in studies addressing questions about physiology (McLister et al., 1995; McLister, 2001; Gridi-Papp 2003) and the energetic cost of calling behaviors

(Prestwich et al., 1989). The authors of these studies concur that an advertisement call corresponds to a single exhalation movement (= 1 note) in *H. cinerea* (Prestwich et al., 1989; McLister et al., 1995; Gridi-Papp 2003), *H. gratiosa* (Prestwich et al., 1989), *H. squirella* (Prestwich et al., 1989), and in the outgroup *P. crucifer* (Prestwich et al., 1989); an advertisement call corresponds to a variable number of exhalation movements (= train of notes) in *H. chrysoscelis* (McLister et al., 1995; Girgenrath and Marsh, 1997; McLister, 2001), *H. versicolor* (McLister et al., 1995; Girgenrath and Marsh, 1997; McLister, 2001; Gridi-Papp 2003), and *H. avivoca* (J. McLister, pers. comm.; H.C.G., pers. obs.).

#### Coding of signal characters

The aim of this study is to compare character coding of acoustic signals that considers primarily either

Table 1

Alternative character codings. The characters numbers (A1–8; M1–10) refer to the columns in the character matrix (Table 2)

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Acoustic coding	
A1—Advertisement call: pulsed (0) (see characters A5–A7), unpulsed (1) (see character A8)	
A2—Call duration: 150–300 ms (0), 500–1100 ms (1), 3000–4000 ms (2)	
A3—Call repetition rate: 4–15 (0), 19–27 (1), 31–85 (2) (in call per minute)	
A4—Call duty cycle (ratio of call duration and intercall duration): 12–17 (0), 20–40 (1)	
A5—Pulse number per call: 11–20 (0), 24–40 (1)	
A6—Pulse duration: 5–8 ms (0), 13–16 ms (1), 27–33 ms (2), 69–77 ms (3)	
A7—Pulse rate: 4–40 (0), 85–100 (1) (in pulse per second)	
A8—Unpulsed call with pseudo-pulses at the beginning (1), no pseudo-pulses (0)	
Mechanistic coding	
M1—Call consisting of a single note (0), multinote (1) (see characters M2–M4)	
M2—Number of note per multinote call: 11–20 (0), 30–40 (1)	
M3—Duration of multinote call: 500–1100 ms (0); 3000–3500 ms (1)	
M4—Multi-note call duty cycle: 14–17% (0); 20–35% (1)	
M5—Note duration: 10–80 ms (0), 150–300 ms (1)	
M6—Note duty cycle: 6–40% (0), 50–67% (1)	
M7—Note emission rate: 20–330 (0), 1200–1300 (1), 2200–2600 (2) (in note per minute)	
M8—Note pulsed (1) (see characters M9–M10), not (0). The pulsation is characterized by a rapid amplitude modulation	
M9—Note pulsation homogeneous along the note (0), limited to the beginning (1) (see character M10)	
M10—Initial note pulsation followed by a weak amplitude modulation (1), not (0)	

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acoustic criteria alone, or mechanistic/behavioral criteria. For the “acoustic” coding, we use acoustic units such as pulses, calls and silences to make up homologies. For the “mechanistic” coding, we use notes as units, i.e., the amount of sound produced during a single expiration.

In both cases we applied Remane’s (1952) criteria for homology (Atz, 1970; Munding, 1979; Wenzel, 1992; Price and Lanyon, 2002; Desutter-Grandcolas and Robillard, 2003) in the same way as for morphological characters. These criteria are: (1) similarity of relative position, (2) special quality, and (3) continuity through intermediate forms (Remane, 1952). In comparisons of behavioral displays and end-products, “position” can be interpreted as the temporal position of a sound or movement in a sequence of behaviors (Tinbergen, 1959). Thus, by this criterion, displays that are used in the same behavioral context, or components with the same relative position within a display, can be hypothesized to be homologous in different species. According to the criterion of “special quality”, sounds or behaviors that are highly stereotyped and that share complex details are more likely to be homologous than ones that are variable and relatively simple (Slikas, 1998). The presence of “intermediate forms” in patterns of vocal evolution is also strongly indicative of homology and can help in identifying signal components that are relatively derived or ancestral.

Quantitative characters were coded using gap coding (Archie, 1985; Stevens, 1991), with the criterion of non-overlap of 95% confidence intervals to define gaps, and minimizing the number of uninformative states. All characters were equally weighted and treated as non-additive.

### *Phylogenetic analyses*

We made separate treatments for each data set (acoustic versus mechanistic), in order to compare their respective phylogenetic content. The aim of this study was clearly not to reconstruct hylid phylogeny using signal characters only, but rather to estimate the phylogenetic consistency of each coding strategy and to compare the results with the strong molecular phylogeny (Faivovich et al., 2005). No simultaneous analysis was done using both character sets because they were based on alternate coding schemes that used partly overlapping information. We also did not perform simultaneous analyses combining molecular data and behavioral characters; however, because the behaviors are heritable trait and the characters are defined using explicit homology criteria, there is no theoretical reason to exclude these data from the phylogenetic reconstruction (Grandcolas et al., 2001), and they will be considered in further phylogenetic studies of hylids. Characters were polarized through outgroup comparison (e.g., Nixon and Carpenter, 1993) using the taxon *Pseudacris crucifer*. Phylogenetic analyses were performed using NONA version 2.0 (Goloboff, 1999) run with Winclada version 1.00.08. (Nixon, 2002), using parsimony with a branch-and-bound algorithm. Searches were performed using the “mult\*max\*N” commands with 1000 replicates and the “hold10000” and “hold/100” options, and exact solutions were obtained with the “mswap+” command. Both the consistency index (CI: Kluge and Farris, 1969) and the retention index (RI: Farris, 1989) were computed.

Character optimizations on the phylogenetic tree were done with both Fast and Slow procedures of optimization

using Winclada. All alternative scenarios for each character were considered because they represent as many hypotheses of character change (Brooks and McLennan, 1991). The outgroup was not taken into account for *a posteriori* character optimizations (Grandcolas et al., 2004) because the outgroup used here for character polarization is not representative of the sister group of North American *Hyla*, but is only one example of the diversity of frog advertisement signals.

## Results

### Signal temporal structure and definition of characters (Fig. 2)

A mismatch occurs when character definitions are based on the acoustic structure of the advertisement signal as opposed to the mechanism of sound production. The *H. cinerea* group is heterogeneous in term of

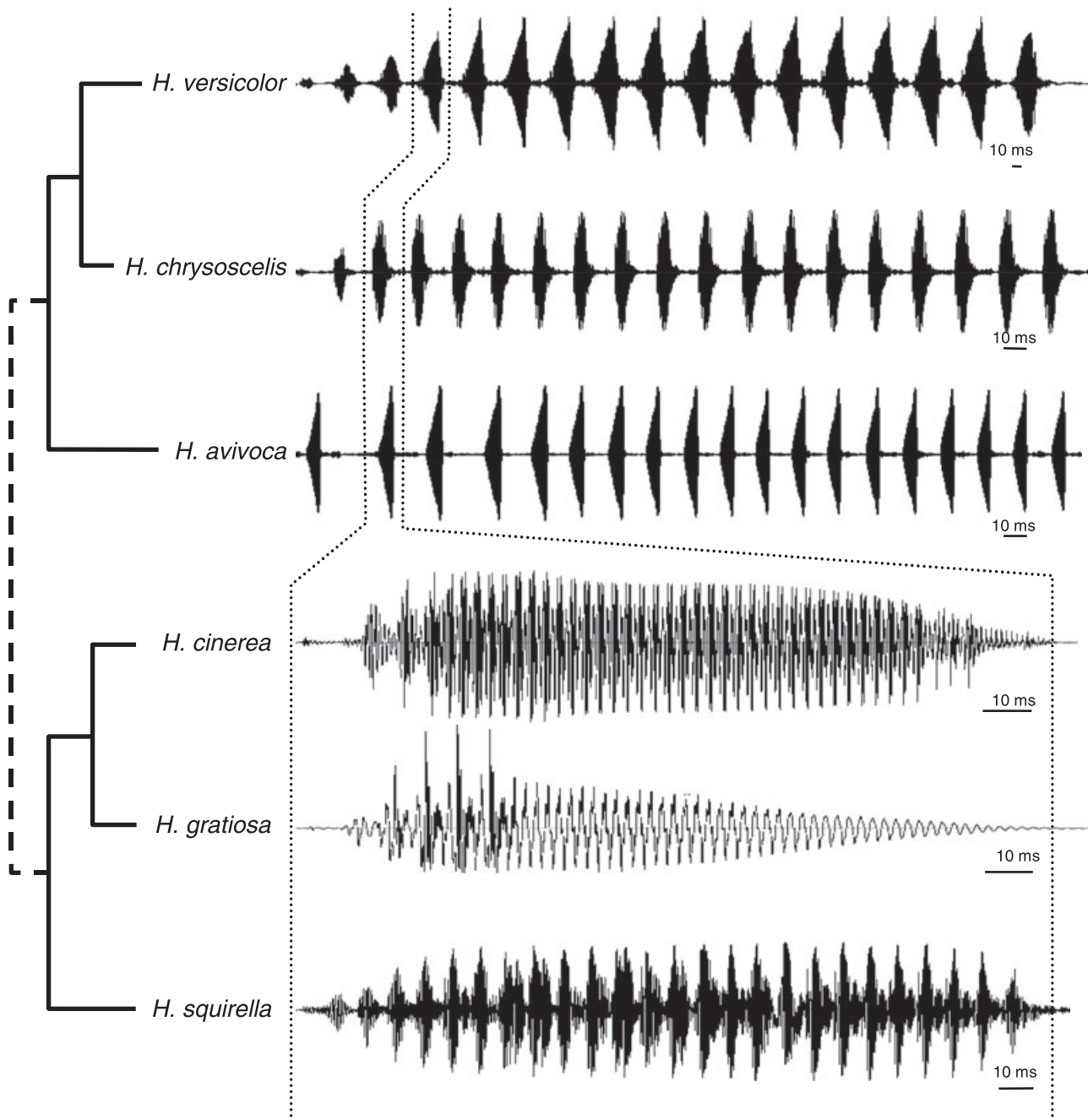


Fig. 2. Advertisement calls of six species of North American *Hyla* mapped on to the phylogenetic hypothesis. Dotted lines delimit one note, i.e., a mechanistic unit of sound emission.

acoustic structure: the call of *H. squirella* is pulsed, whereas the calls of *H. cinerea* and *H. gratioiosa* are unpulsed, despite the presence of two to three pseudo-pulses at the beginning of the call, and followed by an imperfect amplitude modulation in *H. cinerea* (Gerhardt et al., 1980). However, from a mechanistic point of view, all the species within the *H. cinerea* group emit single-note calls, which means that the pulsed call of *H. squirella* is homologous to the unpulsed calls of *H. cinerea* and *H. gratioiosa*. Detailed observation of the oscillograms reveals an imperfect pulsation in these two species and forming pseudo-pulses at the beginning of the note (Gerhardt, 1974, 1981); on the basis of the mechanistic criterion, these pseudo-pulses could be homologous to the pulses in the call of *H. squirella*. Similarly, the amplitude modulation following the pseudo-pulses in *H. cinerea* but not in *H. gratioiosa* (Gerhardt et al., 1980), could be homologous to the pulses in *H. squirella*.

By contrast the advertisement calls of the three species in the *H. versicolor* group show the same acoustic/mechanism pattern. They have multinote calls, composed of a variable number of pulses, each pulse being emitted as a single note, i.e., as a behavioral calling unit.

These observations and the resulting acoustic and mechanistic codings led to the definition of eight acoustic characters (A1–8) and 10 mechanistic characters (M1–10) (Tables 1 and 2). These sets of characters were used in exploratory cladistic treatments and were optimized on the molecular phylogeny to determine their patterns of transformation.

### Phylogenetic results

#### Acoustic characters (Fig. 3A)

One tree was obtained (14 steps, CI 85, RI 75). A basal multifurcation separates *P. crucifer*, *H. gratioiosa* and all the other species, with the following relationships [*H. cinerea* (*H. squirella* (*H. chrysoiscelis* (*H. avivoca*–*H. versicolor*))]. Compared with the molecular phylogeny (Faivovich et al., 2005; Fig. 1), only the

*H. versicolor* species group is recovered, with different internal relationships on the basis of call duration (character A2: 1) and call rate (character A3: 0), although its internal relationships differ from the previous hypothesis (Fig. 1), with *H. avivoca* and *H. versicolor* monophyletic on the basis of the number of pulses per call (character A5: 2). The *H. cinerea* group is not recovered, the species *H. gratioiosa* being at the unresolved base of the tree, *H. cinerea* being the sister group of all the other species on the basis of the call duty cycle (character A4: 1), and *H. squirella* being the sister group of the *H. versicolor* clade on the basis of call pulsation (character A1: 0).

#### Mechanistic characters (Fig. 3B)

One tree was obtained (11 steps, CI 100, RI 100). A basal trifurcation separates *P. crucifer*, the *H. cinerea* group with an internal trifurcation, and the *H. versicolor* species group, with the following relationship: [*H. avivoca* (*Hyla chrysoiscelis*–*H. versicolor*)]. Compared with the molecular phylogeny, both species groups and the internal topology of the *H. versicolor* group are recovered; only the internal relationships within the *H. cinerea* group are not recovered. The monophyly of the *H. cinerea* group is supported by the pulsation of the note (character M8); that of the *H. versicolor* group by the call/note relationship (multinote calls: character M1: 1) and the note duration (character M5: 0). The clade [*Hyla chrysoiscelis*–*H. versicolor*] is supported by the note duty cycle (character M6: 1).

#### Character optimizations on the molecular phylogeny

##### Acoustic characters (A1–A8; Fig. 4)

According to the optimization of the character A1 (one scenario; one step), the call is ancestrally “pulsed”, and becomes “unpulsed” in [*H. gratioiosa*–*H. cinerea*].

The call duration (character A2; five scenarios; two steps) gives ambiguous results of optimization, the three character states being possibly ancestral.

The call rate is either low or high ancestrally (character A3; two scenarios; three steps). If low ancestrally (ancestral state “0”), it increases independently in *H. versicolor* and at the base of the *H. cinerea* group, with a reversal toward a lower value in *H. gratioiosa*; if high ancestrally (ancestral state “2”), there is a slight decrease in *H. gratioiosa* and a more important one at the basis of the *H. versicolor* group, with a subsequent reversal in *H. versicolor*.

The call duty cycle (character A4; one scenario; two steps) is ancestrally comprised between 20% and 40% and shows two parallel decreases in *H. gratioiosa* and *H. chrysoiscelis*.

The pulse number per call (character A5; three scenarios; two steps) is either low or high ancestrally: when low ancestrally, the scenario shows two parallel

Table 2

Data matrix of characters from the acoustic coding (A1–A8) and the mechanistic coding (M1–M10) of advertisement vocalizations of six North American *Hyla* species and one outgroup. (–), inapplicable characters

	AAAAAAAAMMMMMMMMM
	1
	123456781234567890
<i>Pseudacris crucifer</i>	1020---00---1000--
<i>Hyla avivoca</i>	0201030-10110000--
<i>Hyla chrysoiscelis</i>	0100110-11000120--
<i>Hyla cinerea</i>	1021---10---100111
<i>Hyla gratioiosa</i>	1010---10---100110
<i>Hyla squirella</i>	0021101-0---10010-
<i>Hyla versicolor</i>	0111020-10010110--

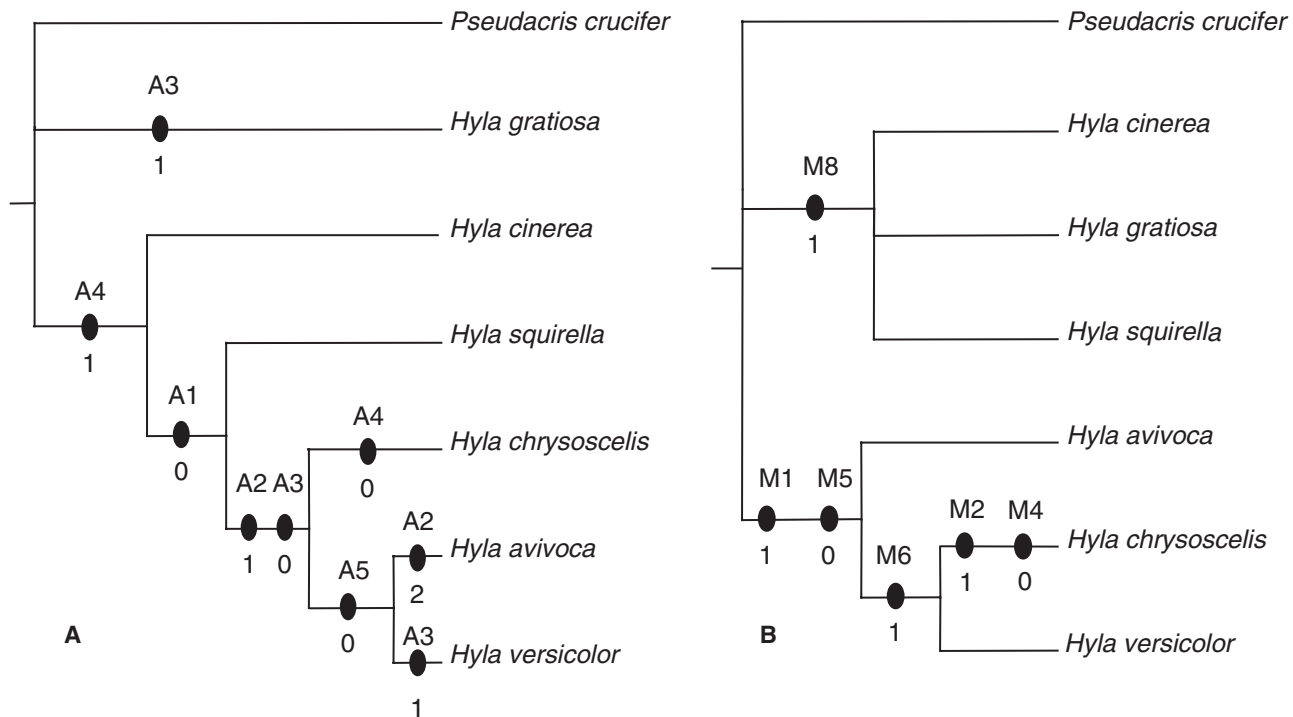


Fig. 3. Phylogeny six species of North American *Hyla* and one outgroup based on: (A) characters defined using the acoustic coding (one most parsimonious tree, 14 steps, CI 85, RI 75); (B) characters defined using the mechanistic coding (one most parsimonious tree, 11 steps, CI 100, RI 100). Unambiguous apomorphies supporting the branches are indicated; the character states (below) and numbers (above) refer to the character descriptions and data matrix (Tables 1 and 2).

increases in *H. chrysoscelis* and *H. squirella*; when high ancestrally the pulse number shows either a decrease at the basis of the *H. versicolor* group followed by a reversal toward high values in *H. chrysoscelis*, or two parallel decreases in *H. versicolor* and *H. avivoca*.

As the pulse duration (character A6) varies greatly among species, each taxon shows a different value with no possible association between species (uninformative character; four scenarios; three steps).

The pulse rate is either low or high ancestrally (character A7; three scenarios; one step), with, respectively, either an increase in *H. squirella* (or in the *H. cinerea* group), or a decrease in the *H. versicolor* group (uninformative character).

The character A8 is uninformative, as the pseudo-pulses at the beginning of the call concern the two species characterized by unpulsed calls.

#### Mechanistic characters (M1–M10; Fig. 4)

Mechanistically, the call is ancestrally either multinote or single-note (character M1; two scenarios; one step), with, respectively, a shift toward a single-note call in the *H. cinerea* group, or toward a multinote call in the *H. versicolor* group.

The number of notes per multinote call (character M2; one scenario; one step) is low ancestrally and shows

one autapomorphic increase in *H. chrysoscelis* (uninformative character).

The duration of multinote calls (character M3; two scenarios; one step) is either low or high ancestrally, with, respectively, an increase in *H. avivoca*, or a decrease in [*H. chrysoscelis*–*H. versicolor*] (uninformative character).

The duty cycle of multinote calls (character M4; one scenario; one step) is high ancestrally, and shows an autapomorphic decrease in *H. chrysoscelis* (uninformative character).

The note duration (character M5; two scenarios; one step) is either low or high ancestrally, with, respectively, an increase in the *H. cinerea* group, or a decrease in the *H. versicolor* group.

The note duty cycle (character M6; one scenario; one step) is low ancestrally, with an increase at the basis of the clade [*H. versicolor*–*H. chrysoscelis*].

The note emission rate (character M7; three scenarios; two steps) is low ancestrally and shows either two autapomorphic increases in *H. versicolor* and *H. chrysoscelis*, one large increase at the basis of the clade [*H. versicolor*–*H. chrysoscelis*] followed by a slight decrease in *H. versicolor*, or two consecutive increases, first in [*H. versicolor*–*H. chrysoscelis*], then a larger one in *H. chrysoscelis* (uninformative character).

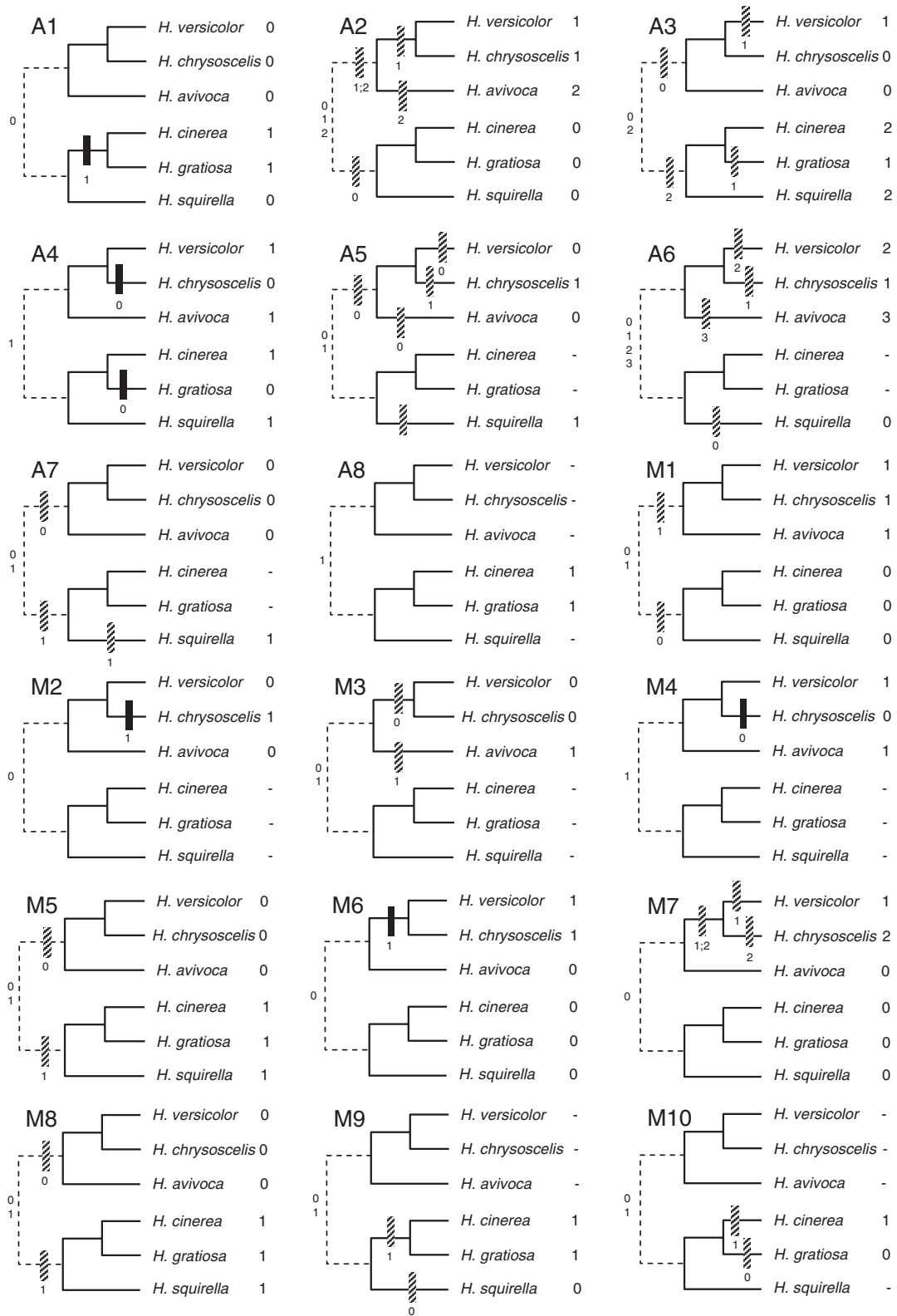


Fig. 4. Character optimizations on the molecular phylogeny (Fig. 1). Putative ancestral states are figured at the basis of the tree and all possible transformations are given (obtained character state shown below the transformation). Symbols: dotted line, non-sister relationship between the species groups; black rectangle, unambiguous transformation; dotted rectangle, ambiguous transformation.



The note pulsation (character M8; two scenarios; one step) is ambiguous ancestrally; it is either unpulsed with a pulsation occurring at the basis of the *H. cinerea* group, or pulsed, with a loss of the pulsation at the basis of the *H. versicolor* group.

The note pulsation (character M9; two scenarios; one step) is ancestrally either limited to the beginning and extends to the whole note in *H. squirella*, or homogeneous along the note and becomes restricted to the beginning in the clade [*H. gratiosa*–*H. cinerea*] (uninformative character).

The amplitude modulation following the initial pulsation in *H. gratiosa* and *H. cinerea* (character M10; two scenarios; one step) is either absent or present ancestrally, with, respectively, an autapomorphic occurrence in *H. cinerea* or loss *H. gratiosa* (uninformative character).

## Discussion

The present study shows that there is a mismatch between the acoustic signal structure and the mechanism of production of advertisement vocalizations in hylid frogs. These discrepancies have important implications for our understanding of frog signals in a comparative context, which may ultimately affect homology statements, definition of phylogenetic characters and transformation series, and, in turn, conclusions about the evolution and diversification of acoustic signals. We now discuss the consequences of the acoustic/mechanistic mismatch in terms of significance of acoustic characters for phylogenetic and comparative studies; and the evolution of vocalizations in North American treefrogs.

### *Comparative study and phylogenetic significance of vocalizations*

The mismatch between the acoustic structure and emission mechanism of the advertisement signals in treefrogs corroborates the hypothesis that end-products may be unpredictable and misleading compared with the underlying behaviors that create them (Stuart and Currie, 2001). From this observation arises the question of which level of observation best fits the study of end-products of behavior. As argued by Freudenstein et al. (2003), it is crucial to look at different levels in parallel, especially because not all character information is directly encoded in genomes (Mahner and Bunge, 1997; Eisthen and Nishikawa, 2002 in Wray and Abouheif, 1998). This principle is clearly applicable to behavior, which is, at some level, independent from morphology (Wenzel, 1992), and cannot be completely explained in terms of genes and/or fine neural mechanisms. However, the direct relationship existing between

end-products and underlying behaviors is far more restrictive. In the case of advertisement signals in treefrogs, the question is whether or not we should compromise straightforward information brought by the sound production behavior to consider acoustic categories, which are obviously artificial. We thus assert that end-products per se do not constitute a strong basis for deriving hypotheses of homology (Stuart and Hunter, 1998; Stuart and Currie, 2001, 2002). Comparative studies of the acoustic structure of vocalizations can still be performed using acoustic properties of vocalizations provided that appropriate characters are identified using behavioral/mechanistic references. Thus our position is similar to the widely acknowledged fact that homology of morphological characters must be defined in a correct anatomical framework and not merely based on broad functional similarity.

To further the comparison of these two levels of observations, we made separate phylogenetic analyses based on the characters defined by each alternate coding strategy. In both cases, the resulting topologies may either be weakly supported or completely unresolved, mainly because of the low number of characters and taxa. Nevertheless, each treatment gives only one unambiguous topology, poorly but surely supported by changes in vocalizations. These results, like other previously published studies, empirically show that behavior can introduce as informative variation in phylogenetic analyses as any other type of data (De Queiroz and Wimberger, 1993; Wenzel, 1992; Stuart and Hunter, 1998; *contra* Cannatella et al., 1998).

The tree resulting from the acoustic coding (Fig. 3A) does not recover the topology obtained by the molecular phylogeny (Faivovich et al., 2005; Fig. 1). Although the *H. versicolor* group is monophyletic, its internal relationships differ from our hypothesis, with *H. chrysoseelis* and *H. versicolor* not being sister taxa. The *H. cinerea* group is not recovered, and *H. squirella* is the sister group of the *H. versicolor* clade, with *H. cinerea* being the sister group of *H. squirella* and the *H. versicolor* clade. Based on acoustic criteria, the calls of *H. squirella* and of the species within the *H. versicolor* group are incorrectly considered pulsed the same way. This clearly illustrates how ill-conceived hypotheses of homology and characters can result in a misleading phylogenetic signal, if not no signal at all.

The tree resulting from the mechanistic coding (Fig. 3B) conversely recovers most of the relationships from the molecular study. Both species groups are indeed monophyletic, and the internal topology of *H. versicolor* group is similar to our hypothesis (Fig. 1). The only relationship that is not recovered by this analysis is the internal topology of the *H. cinerea* clade, which is unresolved, probably due to the low number of characters. Nevertheless, the corroboration of the molecular tree by the characters derived from the

mechanistic coding reinforces the idea of a strong, consistent phylogenetic content of the advertisement signals. Our results also emphasize the importance of defining behavioral characters using clear and explicit criteria of homology, and to relate end-products such as acoustic signals to the underlying behaviors, at least when conducting comparative studies.

Until now, few phylogenetic studies of anurans have included characters based on vocalizations (Cocroft and Ryan, 1995; Cannatella et al., 1998; Heyer, 1998), and only some of these considered the mechanisms of sound emission related to the acoustic signals. As mentioned by Cannatella et al. (1998), the advertisement calls of the *Physalaemus pustulosus* species group and the outgroups they considered are very similar in structure, which suggests that the calls may all be produced the same way. Not considering the mechanism would thus have no negative repercussions in this study.

In their paper on the evolution of vocalizations in *Pseudacris* and *Bufo*, Cocroft and Ryan (1995) put on the same level of comparison the calls of *Pseudacris* species (including the mono-note call of *P. crucifer*) and that of *H. chrysoscelis* (multinote call) taken as an outgroup, which is misleading according to the present study. However, they were careful to restrict comparisons of character evolution to the species sharing a common mechanism of call production in order to compare only homologous traits, based on what was known about the calling mechanism. For *Pseudacris*, they excluded the outgroup *H. chrysoscelis* from the comparative study while all *Pseudacris* species were observed to share a call mechanism consisting of a unidirectional flow of air. In the case of *Bufo*, they cite the work of Martin (1971), who clearly showed that the mechanism of call production is comparable among species.

Finally, in a study of *Leptodactylus* species, Heyer (1998) seems to have considered both “acoustic” and “mechanistic” units because he used the terms calls, pulses and notes for defining phylogenetic characters. However, he did not specify if he actually made any observation on the mechanism of sound emission, which is unlikely given that he mixed the concept of notes and call, apparently without considering potential problems of homology. Assuming that Heyer’s (1998) use of the term “note” refers to the same mechanistic unit as presented here, most of the characters he made up for describing temporal patterns of advertisement signals should be revised for sake of homology.

#### *Evolution of vocalizations based on acoustic and mechanistic codings*

The first step when studying the evolution of certain traits of taxa is to understand the organization, structure and/or variation of the traits in question. This under-

standing leads to comprehensive hypotheses of homology, thereafter used for definition of characters, which can then be used in phylogenetic analyses. The resulting patterns of transformations finally inform us about the evolution of the traits under study. Considering this succession of steps, comparative studies heavily depend on the criteria used to “understand” the traits of interest. This is particularly problematical when several alternative criteria can be justified, as the use of non-matching criteria can lead to drastically different hypotheses of homology, character definition, and in turn, patterns of transformation.

In some cases the competing criteria for trait comparison are matching. In crickets for instance, the basic acoustic units of the songs, the syllables, are always produced by the same mechanism of stridulation, and there is a one-to-one correspondence between the unitary act of calling (the to-and-fro movement of the forewings) and the basic unit of emitted sound (Robillard et al., submitted). As we demonstrate here in North American *Hyla*, there can be a mismatch between the acoustic and mechanistic criteria in trait comparisons that can result in misleading inferences about the evolution of the signals.

The most convincing example of the negative influence of this mismatch appears when we compare the evolution of the basic structure of the signal according to each criterion. Considering the signal structure with the acoustic criterion only (character A1; Fig. 4), one would conclude that the call is ancestrally pulsed, and that it becomes unpulsed once in [*H. cinerea*–*H. gratiosa*]. However, the mechanistic criterion shows that the call is either multi- or mono-note ancestrally (character M1; Fig. 4). The pulses that make up the calls are thus clearly of different natures in *H. squirella* and the *H. versicolor* group (Fig. 2); in *H. squirella*, they are subunits of one single note, while each pulse is a separate note in the *H. versicolor* group. Consequently, they have to be considered as different characters, which means that they have evolved convergently (Desutter-Grandcolas et al., 2005).

Most signal parameters are similarly affected by the mismatch between the acoustic and mechanistic criteria, mostly because the acoustic coding compares quantitative parameters that belong to single-note calls and multinote calls. For example, according to the acoustic coding, the call duty cycle (character A4; Fig. 4) shows two parallel decreases in *H. gratiosa* and *H. chrysoscelis*. According to the mechanistic coding, the call in fact corresponds to one note in *H. gratiosa* and to a group of notes in *H. chrysoscelis*. The decreases in call duty cycle are thus not parallel but convergent, as different characters are concerned. In such a case, it would be better to consider the note duty cycle instead of the call duty cycle (character M6, Fig. 4) for sake of homology; the optimization of the note duty cycle shows one

increase at the basis of the clade [*H. versicolor*–*H. chrysoscelis*] only. A similar call duty cycle thus appears twice, not because of the repetition of the same evolutionary change, but because of two independent changes of two different parameters: a slight decrease in the note duty cycle in *H. gratioiosa* not considered significant during the discretization of the character M6, and a decrease of the duty cycle of multinote calls (character M4). Such convergent evolutionary patterns for the temporal structures of advertisement calls were also found in eneopterinae crickets (Robillard, 2004).

Finally, unexpected hypotheses of homology came out from the mechanistic coding. In particular, as the pulses in *H. squirella* are note subunits, they correspond to a deep amplitude modulation that can be compared with the initial pseudo-pulses occurring in the notes/calls of *H. gratioiosa* and *H. cinerea*. The phylogenetic pattern (character M9; Fig. 4) corroborates this hypothesis of homology. Similarly, the weak amplitude modulation following the pseudo-pulses in *H. cinerea* may be homologous to the pulses of *H. squirella*.

## Conclusions

In the present study we showed that considering only the acoustic structure of frog vocalizations can lead to misleading results in terms of both phylogenetic signal and interpretation of the modalities of evolution of acoustic signals. In contrast, interpreting the acoustic signals with regard to the mechanism of sound production results in consistent phylogenetic information, which can be combined with other kinds of data. The mechanistic coding also provides strong homologies for use in comparative studies of frog vocalizations, and to derive and test evolutionary hypotheses. This leads to the question of how to compare biologically significant traits when they are clearly of a different nature according to the most trusted homology criteria. This issue will be of importance in future studies aiming at transposing and testing populational results at the phylogenetic scale.

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## Appendix 1

Vocalization measurements summary (mean  $\pm$  standard deviation) for one outgroup species and six *Hyla* species, adjusted for a common temperature of 20 °C

	A2	A3	A4	A5	A6	A7
<i>P. crucifer</i>	193.1 $\pm$ 43.2	45.1 $\pm$ 13.6	12.8 $\pm$ 4.1	–	–	–
<i>H. avivoca</i>	3472.8 $\pm$ 181.5	5.2 $\pm$ 1.1	31.4 $\pm$ 2.3	17.8 $\pm$ 1.7	73.1 $\pm$ 3.2	5.2 $\pm$ 0.4
<i>H. chrysoscelis</i>	937.4 $\pm$ 108.5	12.1 $\pm$ 2.1	16 $\pm$ 1.6	35 $\pm$ 3.9	14.7 $\pm$ 1	40.1 $\pm$ 2.2
<i>H. cinerea</i>	216.2 $\pm$ 16	73.6 $\pm$ 7.6	25.8 $\pm$ 1.9	–	–	–
<i>H. gratiosa</i>	190.3 $\pm$ 8.1	23.9 $\pm$ 3.1	8.9 $\pm$ 1.2	–	–	–
<i>H. squirella</i>	279 $\pm$ 16.8	70.7 $\pm$ 9.6	33.1 $\pm$ 2.3	25.6 $\pm$ 1.2	7 $\pm$ 0.5	92.2 $\pm$ 5.6
<i>H. versicolor</i>	614.7 $\pm$ 76.4	21.1 $\pm$ 2.1	25.7 $\pm$ 5	13.1 $\pm$ 1.4	29.6 $\pm$ 2.5	20.9 $\pm$ 0.5

	M2	M3	M4	M5	M6	M7
<i>P. crucifer</i>	–	–	–	193.1 $\pm$ 43.2	12.8 $\pm$ 4.1	45.1 $\pm$ 13.6
<i>H. avivoca</i>	17.8 $\pm$ 1.7	3472.8 $\pm$ 181.5	31.4 $\pm$ 2.3	73.1 $\pm$ 3.2	37.6 $\pm$ 2.2	309.3 $\pm$ 23.9
<i>H. chrysoscelis</i>	35 $\pm$ 3.9	937.4 $\pm$ 108.5	16 $\pm$ 1.6	14.7 $\pm$ 1	57.4 $\pm$ 3.4	2407.8 $\pm$ 132.4
<i>H. cinerea</i>	–	–	–	216.2 $\pm$ 16	25.8 $\pm$ 1.9	73.6 $\pm$ 7.6
<i>H. gratiosa</i>	–	–	–	190.3 $\pm$ 8.1	8.9 $\pm$ 1.2	23.9 $\pm$ 3.1
<i>H. squirella</i>	–	–	–	279 $\pm$ 16.8	33.1 $\pm$ 2.3	70.7 $\pm$ 9.6
<i>H. versicolor</i>	13.1 $\pm$ 1.4	614.7 $\pm$ 76.4	25.7 $\pm$ 5	29.6 $\pm$ 2.5	63.7 $\pm$ 2.6	1251.7 $\pm$ 29.3