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## Phylogenetic Relationships of Two Poorly Known Honduran Treefrogs (Hylidae: Hylinae: Hylini)

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### ABSTRACT

*Isthmohyla insolita* and *Exerodonta catracha* are Honduran hylids of uncertain phylogenetic relationships. In this paper we present a phylogenetic analysis of mitochondrial and nuclear genes to study their relationships. Our results show that these species are, respectively, the sister taxon of and nested among the few available species of *Plectrohyla*, possibly the least studied genus of Hylini in terms of phylogenetic relationships. To solve the polyphyly of *Exerodonta* and *Isthmohyla*, both species are provisionally transferred to *Plectrohyla*, pending a broader phylogenetic study including all species in the genus. Consequently, *Isthmohyla* is restricted to a group of hylids endemic to the Isthmian highlands of Costa Rica and Panama in lower Central America. The inclusion of *I. insolita* in *Plectrohyla* implies another instance of independent evolution of terrestrial egg clutches in Hylini.

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## INTRODUCTION

Faivovich et al. (2005) recognized the tribe Hylini, of the subfamily Hylinae, that included 16 genera, several of which resulted from the taxonomic rearrangement of most species of that tribe formerly included in *Hyla*. These taxonomic changes were based mostly on their phylogenetic results, but in the case of species not available for their study, the authors considered different sources of evidence to associate them with the genera and species groups recognized at that time. The study of Faivovich et al. (2005) was followed by a few publications that exposed a number of problems with the monophyly of the genera *Duellmanohyla*, *Ptychohyla*, and *Tripriion* (Wiens et al., 2005, 2006: suppl. information; Smith et al., 2007a; Duellman et al., 2016).

Faivovich et al. (2018) presented a phylogenetic analysis of Hylini that included a much denser taxonomic sampling for *Duellmanohyla*, *Isthmohyla*, and *Ptychohyla*. They also included several species of other genera (e.g., *Ecnomiohyla*, *Exerodonta*, *Megastomatohyla*, and *Sarcohyla*) that had sequences published in the context of more reduced phylogenetic analyses (Batista et al., 2014; Li et al., 2014; Köhler et al., 2016; Caviedes-Solis and Nieto-Montes de Oca, 2018) but that had not been included in the context of a broad phylogenetic study of Hylini.

The phylogenetic results of Faivovich et al. (2018) allowed them to provide a new taxonomic rearrangement, solving the nonmonophyly of *Charadrahyla*, *Duellmanohyla*, *Exerodonta*, *Isthmohyla*, and *Ptychohyla*, as defined at that time (definitions of Faivovich et al., 2005, for most genera, but also McCranie and Castañeda, 2006, for *Isthmohyla*). Furthermore, they discussed some of the doubts that still persist in the systematics of Hylini from a phylogenetic perspective, considering some poorly supported clades and several ambiguities stemming from clades whose monophyly requires further corroboration because some of its species had not been available for phylogenetic analyses and show complex character distributions. Two of these cases involve the monophyly of the genera *Exerodonta* and *Isthmohyla*.

*Exerodonta* was redefined by Faivovich et al. (2018), who showed that the genus as defined by Faivovich et al. (2005) was polyphyletic, given that *E. juanitae* was nested in *Charadrahyla*. To solve this problem, that species and the closely related *E. pinorum* were transferred to *Charadrahyla*. This rearrangement left the following species in *Exerodonta*: *E. abdivita*, *E. bivocata*, *E. catracha*, *E. chimalapa*, *E. melanomma*, *E. perkinsi*, *E. smaragdina*, *E. sumichrasti*, and *E. xera*. While several taxonomic problems persist in *Exerodonta*, Faivovich et al. (2018) noticed that several doubts involve species that are very similar with those that had been included in phylogenetic analyses, except for *E. catracha* and *E. melanomma*, whose inclusion is required for a proper test of the monophyly of *Exerodonta*.

*Isthmohyla* was also redefined by Faivovich et al. (2018), who showed that the genus was polyphyletic, as *I. melacaena* was nested in *Bromeliohyla*. To solve this problem, that species was transferred to *Bromeliohyla*. This rearrangement left in *Isthmohyla* 14 species, of which nine were included in their phylogenetic study (*I. debilis*, *I. graceae*, *I. infucata*, *I. lancasteri*, *I. picadoi*, *I. pseudopuma*, *I. rivularis*, *I. tica*, and *I. zeteki*). Faivovich et al. (2018) discussed the evidence for the association of the five species absent from their analysis with the different clades that they recognize in *Isthmohyla*, showing that there is evidence for the association of *I. angustilineata*, *I. calypsa*, *I. pictipes*, and *I. xanthosticta*, specifically with the *I. tica* group.

However, these authors stressed the fact that the inclusion of *I. insolita* in *Isthmohyla* was strictly tentative, as the available evidence was not decisive in favoring an association of *I. insolita* with this genus over an association with *Sarcohyala*.

*Exerodonta catracha* is a small treefrog (snout-vent length, [SVL] males = 22.6–28.7 mm, females = 28.6–32.1 mm) endemic to cloud forests of southwestern Honduras between 1700–2220 m along the continental divide of the Cordilleras Opalaca, Montecillos, and Montaña La Sierra (McCranie and Wilson, 2002; McCranie and Castañeda, 2007; Frost, 2024; J.R. McCranie, personal obs.). The species is associated with small ponds and slow-moving streams, where adults and juveniles are often found in low vegetation and bromeliads (Porrás and Wilson, 1987; McCranie and Wilson, 2002). *Isthmohyla insolita* is a small treefrog (SVL males = 30.0–36.0 mm, females = 34.3–38.0 mm) endemic to the cloud forests of the Cordillera Nombre de Dios, in northern Honduras between 1520–1690 m (McCranie and Wilson, 2002; McCranie and Castañeda, 2007; Castañeda and McCranie, 2011). Adults use leaves and mosses overhanging water for oviposition (Wilson et al., 1994a).

The recent availability of samples of *Exerodonta catracha* and *Isthmohyla insolita* provides the opportunity to test the monophyly of *Exerodonta* and *Isthmohyla*, explore the phylogenetic relationships of both species, and discuss its consequences for our knowledge of these and other genera of Hylini, particularly in terms of the evolution of reproductive biology in this tribe.

## MATERIALS AND METHODS

### TAXONOMIC SAMPLING

Voucher specimens of sequences produced for this study are housed at the Smithsonian National Museum of Natural History (USNM), the Museum of Zoology, Universidad de Costa Rica (UCR), and the University of Texas at Arlington (UTA). We produced sequences of one adult specimen (UCR 23564) and two tadpoles (UTA Tissue Collection 252948–49) of *Isthmohyla insolita* collected at the type locality (McCranie et al., 1993a), as well as of two adult specimens (USNM 570494–95) of *E. catracha* collected at El Rodeo, Intibucá, 2200 m, Honduras, 17 km NW from the type locality at Zacate Blanco, Intibucá, 2020 m, Honduras (Porrás and Wilson, 1987). These sequences were added to the dataset of Faivovich et al. (2018), expanded to include sequences of *Atlantihyla melissa* (produced by Townsend et al., 2020), some species of *Bromeliophyla*, *Charadrahyla*, *Duellmanohyla*, and *Quilticohyla* (produced by Caviedes-Solis et al., 2020, but not accessioned in GenBank), and *Tlalocohyla celeste* (produced by Varela-Soto et al., 2022). See appendix 1 for a list of voucher specimens and GenBank accessions (if available) other than those included by Faivovich et al. (2018: appendix S2).

### CHARACTER SAMPLING

Our analyses included the four mitochondrial genes 12–16S RNA, NADH ubiquinone oxidoreductase 1, their intervening tRNAs, cytochrome *b*, and 13 nuclear gene fragments from 12 genes: 28S RNA, beta-crystallin, *c-myc* gene exon 2, *c-myc* gene exon 3, proopiomelanocortin A

(POMC), prostaglandin E2 receptor EP4 subtype, protein tyrosine phosphatase non-receptor type 12, recombination activating-1 (RAG-1), rhodopsin exon 1, seven in absentia homolog 1 (SIAH1), sodium/calcium exchanger 1, tensin 3, and tyrosinase, as previously employed by Faivovich et al. (2005, 2018), Smith et al. (2007a, 2007b), and Wiens et al. (2005, 2010).

#### LAB PROTOCOLS

Whole cellular DNA was extracted from frozen and ethanol-preserved tissues (liver or muscle) using the DNeasy tissue kit (Qiagen Inc.). Primers used in PCR amplification are those used by Faivovich et al. (2018). PCR amplification was carried out in 25  $\mu$ L reactions using 0.2  $\mu$ L Taq (Thermo Fisher Scientific, Inc.). The PCR protocol consisted of an initial denaturation step of 3 minutes at 94° C, 35 (for mitochondrial genes) or 45 (for nuclear genes) cycles of 30 s at 94° C, 40 s at 48–62° C, and 30–60 s at 60°–72° C, and a final extension step of 10–15 minutes at 60°–72° C. The PCR amplified products were cleaned with 0.5  $\mu$ L of Exonuclease plus 1  $\mu$ L of Alkaline Phosphatase per 20  $\mu$ L of reaction and incubated for 30 min at 37° C. Sequencing was done on an automatic sequencer ABI 3730XL (Applied Biosystems) in both directions to check for potential errors and polymorphisms or by MacroGen Sequencing Team (MacroGen Inc., Seoul, Korea). The chromatograms obtained from the automated sequencer were trimmed for quality and the contigs for each gene fragment were assembled using Sequencher v5.3 (Gene Codes, Ann Arbor, MI).

#### PHYLOGENETIC ANALYSIS

We performed multiple alignments for each DNA fragment employing MAFFT version 7 (Kato and Standley, 2013) with G-INS-i (global homology considered), with default parameters for gap opening and extension. The alignments were visualized in BioEdit (Hall, 1999), and concatenated using SequenceMatrix (Vaidya et al., 2011).

Numerous authors have discussed the rationale for using the parsimony optimality criterion for inferring phylogenetic hypothesis (Farris, 1983; Goloboff, 2003; Goloboff and Pol, 2005; Kluge and Grant, 2006; Grant and Kluge, 2009), and we favored it because the cladogram that minimizes transformations to explain the observed variation is the simplest one, maximizes evidential congruence, and has the greatest explanatory power. The preferred relation used to treat indels (insertions and deletions) and all substitutions was 1:1:1 (transition: transversion: unit indels).

For the maximum parsimony phylogenetic analyses, we employed TNT v1.6 (Goloboff and Morales, 2023). Searches were done using the command “New Technology Search” under search level 15, which combines Ratchet, Tree Drifting, Sectorial Searches, and Tree Fusing (Goloboff, 1999; Nixon, 1999). For this driven search, we used the default options for these algorithms, hitting the most parsimonious trees 50 times. The resulting trees were submitted to a round of TBR branch swapping. Searches were done under the collapsing option “minimum length,” which collapses every node whose minimum length is 0. Parsimony jackknife

absolute frequencies (Farris et al., 1996) were calculated in TNT v1.6 using new technology requesting 5 hits with driven searches under search level 15, for a total of 1000 replicates. Gaps were treated as fifth state, but for comparative purposes with the maximum likelihood analysis (see below), we also performed the analyses with gaps treated as missing data.

For the graphic tree edition, we used the TNT script `forfai.run` written by Pablo A. Goloboff (available at <https://www.lillo.org.ar/phylogeny/tnt/scripts/forfai.run>) that takes as input the most parsimonious trees and the trees resulting from the resample pseudoreplicates, and outputs a strict consensus with branch lengths and support values in newick format. This file was read with FigTree v1.4.3 (Rambaut, 2014) for visualization and edition.

We performed a maximum likelihood analysis with IQ-TREE v1.6.12 (Nguyen et al., 2015). The optimal partition schemes and models for each partition were selected using ModelFinder (Kalyaanamoorthy et al., 2017). The maximum likelihood analysis was conducted with 1000 ultrafast bootstrap replicates (Minh et al., 2013; Hoang et al., 2018) using the option `-bnni` which reduces the risk of overestimating branch supports caused by severe model violations. The resulting tree (from the best scoring run with the highest log-likelihood value) was visualized and edited in FigTree 1.4.3 (Rambaut, 2014).

## RESULTS

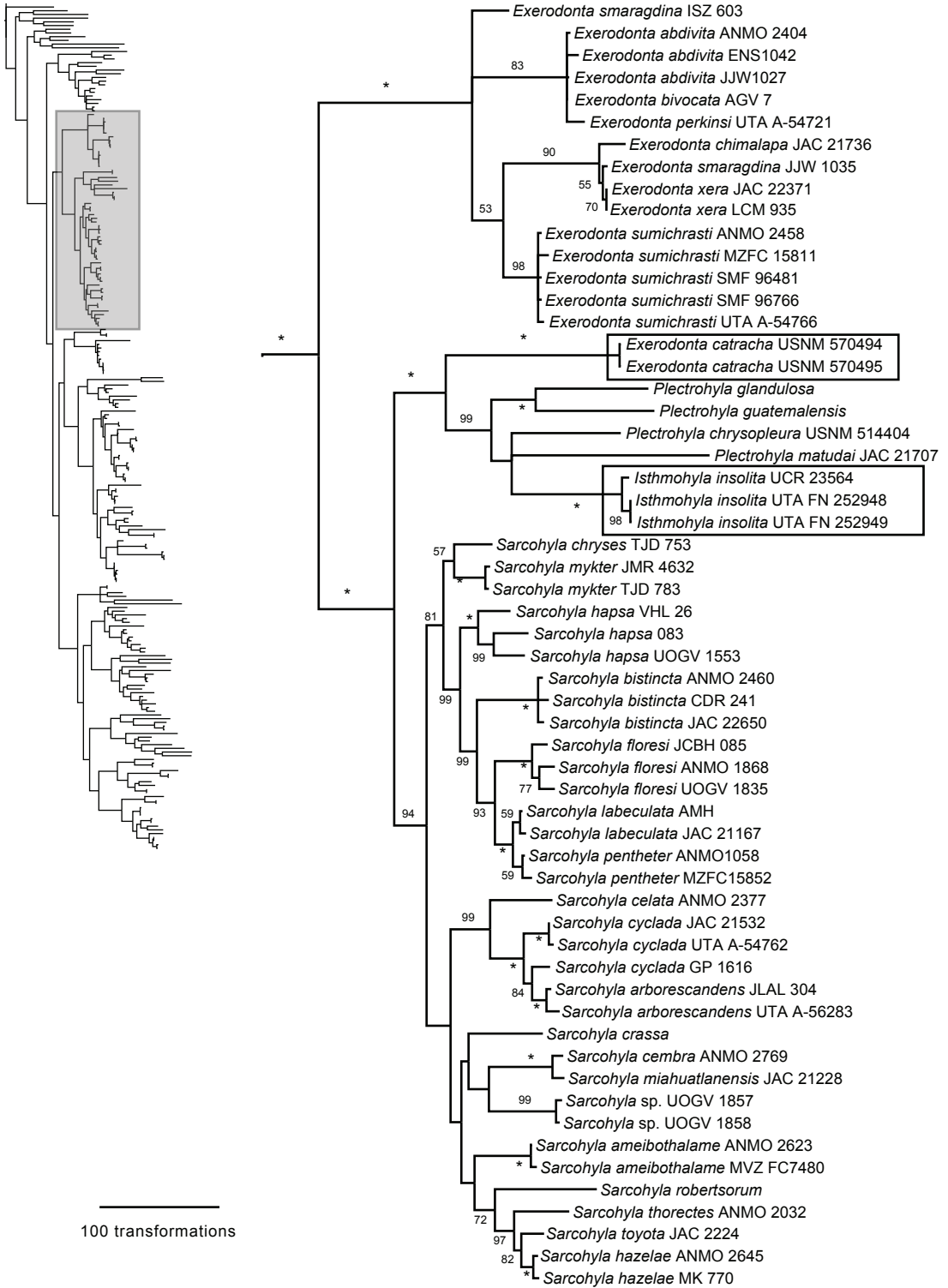
Our phylogenetic analysis using parsimony with gaps treated as fifth state resulted in 245 most parsimonious trees with 37,796 steps (fig. 1, supplementary data file 1 in the online supplement: <https://doi.org/10.5531/sd.sp.67>). Most conflict occurs at the level of internal relationships of some genera, such as *Charadrahyla*, *Exerodonta*, *Plectrohyla*, and *Tlalocohyla*, and among species represented by multiple specimens. These results are congruent with those of the parsimony analysis using gaps as missing data (217 most parsimonious trees with 36,182 steps; supplementary data file 2 in the online supplement), and, in terms of the well-supported clades, with ML results (supplementary data file 3 in the online supplement). Considering the goals of this project and the large size of the dataset, in figure 1 we show only that section of the parsimony results corresponding to the recovered position of *E. catracha* and *Isthmohyla insolita*.

Our results show that, as currently defined, *Isthmohyla* is polyphyletic with *I. insolita* recovered nested in *Plectrohyla*, in a poorly supported polytomy (<50% jackknife) with *P. chrysolepura* and *P. matudai* (fig. 1). Similarly, *Exerodonta* is polyphyletic, with *E. catracha* recovered as the well supported (99% jackknife support) sister taxon of the clade including *I. insolita* and the four species of *Plectrohyla* (fig. 1).

## DISCUSSION

### PHYLOGENETIC RELATIONSHIPS OF HYLINI

Our phylogenetic results are congruent in terms of the well-supported clades with those of Faivovich et al. (2018), as expanded with sequences (16S, Rag-1, and rhodopsin) of a number of additional species by Caviedes-Solis et al. (2020). They are also coincident with the latter





study in that *Duellmanohyla*, *Ptychohyla*, and *Quilticohyla* are not monophyletic (supplementary data file 2). *Duellmanohyla* is polyphyletic because *D. chamulae* and *D. schmidtorum* are recovered nested in *Ptychohyla*, and *D. ignicolor* in *Quilticohyla*. *Quilticohyla acrochorda* is recovered nested in *Ptychohyla*. The relationships of this clade are outside the scope of this paper, and they will be dealt with in a separate publication.

#### POLYPHYLY OF *EXERODONTA* AND *ISTHMOHYLA*

Porrás and Wilson (1987) commented on the similarity between their newly described *Hyla catracha* and the former *H. melanomma* in SVL, dorsum and thigh color pattern, finger and toe webbing, and osteological characters, therefore, including the new species in the former *H. pinorum* group as defined by Duellman (1970). This was followed by Campbell and Duellman (2000) and Duellman (2001) who considered these two species in the same species groups (first, by including them in the former *H. pinorum* group and subsequently in the former *H. miotypanum* group). Faivovich et al. (2005), based on the previous association and on their own results that recovered the former *H. melanomma* in the clade for which they resurrected the generic name *Exerodonta*, included the former *H. catracha* in this genus.

However, as noted by Faivovich et al. (2018), the sequences of *Exerodonta melanomma* included by Faivovich et al. (2005) actually correspond to *E. sumichrasti*, while those labeled as *E. melanomma* by Wiens et al. (2005) correspond to *E. abdivita*. Faivovich et al. (2018) also noticed that the tadpole of *E. catracha* described by McCranie et al. (1993b) differs from other larvae of *Exerodonta* in having a well-defined row of submarginal papillae on the anterior and posterior labia, a 2/3 labial tooth row formula, and a bluntly rounded tail tip. These character states are similar to those found in several species of *Plectrohyla* and *Sarcohyla* (see Duellman, 2001). Because of the confusing larval character distribution in *E. catracha* and the absence of this species and *E. melanomma* in the analyses, Faivovich et al. (2018) stressed the need for including sequences of both species for a proper test of the monophyly of *Exerodonta* as defined by them. Our results showing the polyphyly of *Exerodonta* as defined by Faivovich et al. (2018) and recovering *E. catracha*, *Isthmohyla insolita* and all available species of *Plectrohyla* in a well-supported clade (99% jackknife) support the inference that shared larval characters between *E. catracha*, *Plectrohyla*, and *Sarcohyla* actually indicate a closer relationship between these taxa.

The inclusion of the former *Hyla insolita* in *Isthmohyla* by Faivovich et al. (2005) was based on a number of shared characters that supported its association with *I. calypsa* and *I. lancasteri* in the former *H. lancasteri* group (McCranie et al., 1993a; Lips, 1996). These include the presence of granular dorsal skin, dark ventral pigmentation, unpigmented eggs, terrestrial oviposition on vegetation

←

FIG. 1. Strict consensus of the 245 most parsimonious trees of the analysis using gaps as fifth state (length 37,796 steps). Portion of the tree showing the results for the genera *Exerodonta*, *Plectrohyla*, and *Sarcohyla*. The left inset shows the section of the full tree of supplementary data file 1 that is shown here. The arrows point at the positions of *Exerodonta catracha* and *Isthmohyla insolita*, both recovered closely related with *Plectrohyla*. Values around nodes are jackknife absolute frequencies. Nodes lacking values have frequencies <50%. An asterisk (\*) indicates 100% frequency.



overhanging water bodies, and egg attendance by the adult (McCranie et al., 1993a; Wilson et al., 1994b; Lips, 1996; Castañeda and McCranie, 2011). Wilson et al. (1994b) also considered the possibility that the former *Hyla thorectes* (currently in *Sarcohyla*), a Mexican species having dark ventral pigmentation and terrestrial oviposition on vegetation overhanging water bodies (Duellman, 1970; Delia et al., 2013), might also be related to the former *H. lancasteri* group. The results of Faivovich et al. (2005) suggested that the former *H. hazelae* group as defined by Duellman (1970) and including the former *H. thorectes* and the nominal species, were actually members of the *P. bistincta* group, as corroborated by Caviedes-Solis and Nieto-Montes de Oca (2018), Faivovich et al. (2018), and the present results, rejecting a close relationship of these species with *I. insolita* or with the *I. tica* group (the clade that now includes the former *H. lancasteri* group; Faivovich et al., 2018).

Our results show that *Isthmohyla* is polyphyletic for having *I. insolita* nested in a well-supported clade (99% jackknife) including *Exerodonta catracha*, and the four available species of *Plectrohyla* (i.e., *P. glandulosa*, *P. guatemalensis*, *P. matudai*, and *P. chrysopleura*). This finding is remarkable in that it is an alternative relationship that had not been considered earlier; it also implies a third instance of convergence of a peculiar set of reproductive characters among Hylini and a seemingly perplexing scenario in the evolution of *Plectrohyla* in terms of character distribution.

#### PLECTROHYLA, A CINDERELLA OF HYLINI?

Despite the progress in our knowledge of phylogenetic relationships of Hylini in the last 18 years, *Plectrohyla* remains the major missing part of the picture. Even though it is one of the most speciose genera within the tribe with 19 currently recognized species (Frost, 2024), only four have been available for phylogenetic studies (see above; Faivovich et al., 2005; Wiens et al., 2005). This poor representation determines that our results, like previous analyses that included the same sampling of *Plectrohyla* (Faivovich et al., 2005, 2018; Duellman et al., 2016; Caviedes-Solis et al., 2020), are only a formal test of its monophyly. In the present case, there is the aggravating result that *Plectrohyla* is not monophyletic for having nested a species that has never been associated with this genus previously. It is precisely the limitation in taxonomic sampling of *Plectrohyla* that hinders a straightforward interpretation of our results.

Faivovich et al. (2005) considered the monophyly of *Plectrohyla* noncontroversial based on the list of phenotypic synapomorphies provided by Duellman and Campbell (1992) and Duellman (2001). These include bifurcate alary process of the premaxilla (actually, a posterior process of the medial margin of the alary process); sphenethmoid with anterior part ossified, incorporating the septum nasi and projecting forward to the leading margins of the nasals; frontoparietals abutting posteriorly, exposing only small part of the frontoparietal fontanelle; humerus having well-developed flanges; hypertrophied forearm; prepollex enlarged and ossified in both sexes; truncate prepollex; and absence of lateral labial folds in larvae. A critical reevaluation of these characters possibly will discard the truncate prepollex (as the morphology of the prepollex in the most recent common ancestor of *Plectrohyla* will depend on the internal topology, considering the known variation in prepollical structure within the genus: truncate, with one projecting spine or with a bifid spine; Duellman, 2001), and will require a clear definition of what larval labial folds

are, as a difference between the oral discs of *Plectrohyla* and *Sarcohyla* (differences are unclear in the descriptions and illustrations provided by Duellman, 1970, 2001).

However, it should be noticed that all synapomorphies of *Plectrohyla* were inferred as such in phylogenetic analyses that included as sole outgroups a terminal representing the known diversity of the former *Hyla bistincta* group (*Sarcohyla* today includes nine additional species) and rooted with a vector of hypothetical ancestral states (Duellman and Campbell, 1992; Duellman, 2001). For this reason, it is necessary to survey all these character states in the context of a reasonable representation of the diversity of Hylini, with emphasis on *Exerodonta* and *Sarcohyla*, to test whether they still optimize as synapomorphies of *Plectrohyla*. Evidently, this exercise will now have to consider the two species that our results indicate to be closely related with the available species of *Plectrohyla*.

Regardless of which of the synapomorphies inferred by Duellman and Campbell (1992) and Duellman (2001) end up optimizing for *Plectrohyla*, the scarce available information on the osteology of *catracha* and *insolita* suggest that most of them do not occur in these species (Porrás and Wilson, 1987; McCranie et al., 1993b). The species currently included in *Plectrohyla* are conspicuously different from *catracha* and *insolita* in characters such as body built (slender in *catracha*, moderate in *insolita*, and robust and heavily built in *Plectrohyla*), and prepollical morphology (elliptical in *catracha* and *insolita*; truncate, with one projecting spine, or a bifid spine in *Plectrohyla*). Furthermore, *catracha* (SVL males 24.9–27.4 mm, females 28.6–32.1 mm) is smaller than all species currently included in *Plectrohyla*, but body size of *insolita* overlaps (SVL males 30.0–36.0 mm, females 34.3–38.0 mm) with that of the three smaller species in the genus (*P. ixil*, *P. matudai*, *P. sagram*; combined SVL males 31.5–46 mm, females 39.6–51.9 mm; Duellman, 1970; Duellman and Campbell, 1992). Similarly to the smaller species of *Plectrohyla*, both *E. catracha* and *I. insolita* exhibit sexual dimorphism in body size, with females being somewhat larger than males (Duellman, 1970; Duellman and Campbell, 1992: table 7). The larval morphology of *catracha*, as mentioned earlier, and the scarce available information for *insolita* (Wilson et al., 1994a) are congruent with those of *Plectrohyla* + *Sarcohyla*.

With the strong indication of our results that *catracha* and *insolita* are more closely related with *Plectrohyla*, we include them provisionally in this genus, as *Plectrohyla catracha* (Porrás and Wilson, 1987), new combination, and *Plectrohyla insolita* (McCranie et al., 1993a), new combination, until a reasonable test of the monophyly of *Plectrohyla* could properly address the relationships of these two species with all other currently included in the genus.

The transference of *insolita* to *Plectrohyla* remedies the polyphyly of *Isthmohyla*, which is now monophyletic, and includes 13 species that are distributed exclusively in the Isthmian highlands of lower Central America in Costa Rica and Panama. The removal of *catracha* remedies the polyphyly of *Exerodonta*. However, as stressed by Faivovich et al. (2018) there are confusions with the taxonomy of some species of this genus. This is further reinforced by the recent addition by Caviedes-Solis et al. (2020) of a 16S sequence identified as *E. bivocata*, one of the species missing in the analysis of Faivovich et al. (2018) (the other being *E. catracha* and *E. melanomma*). That sequence is nearly identical to those identified as *E. abdivita* and *E. perkinsi* in our study (uncorrected p-distances 0.3%–0.5%).

THE EVOLUTION OF TERRESTRIAL EGG CLUTCHES  
AND EGG ATTENDANCE IN HYLIDS

The recognition that *insolita* is not associated with *Isthmohyla*, but rather with *Plectrohyla* has among its consequences that it represents an additional instance of independent evolution of terrestrial egg clutches in Hylidae, and the first report of this reproductive mode in *Plectrohyla*, a clade whose reproductive biology is scarcely known. Most available information comes from histological studies and observations on preserved specimens (Duellman and Campbell, 1992; Luna et al., 2019; Schulte et al., 2021), with field data available only for *P. avia* (Barrio-Amorós et al., 2016) and *P. guatemalensis* (McCranie et al., 1987; González-Mollinedo and Mármol-Katán, 2020), and recent data from transcriptomics of sexually dimorphic skin glands for *P. matudai* and *P. sagorum* (Schulte et al., 2021). The field observations indicated that *P. avia* and *P. guatemalensis* lay aquatic eggs scattered in streams, sometimes in roots, crevices, or cavities in rocks (McCranie et al., 1987; Barrio-Amorós et al., 2016; note that the latter authors were uncertain if the clutches belonged to *P. avia* or to *P. aff. sagorum*). Similar to all studied species of *Plectrohyla*, eggs in *P. insolita* are large and have an unpigmented animal pole (Duellman and Campbell, 1992; Wilson et al. 1994a; Castañeda and McCranie, 2011).

Besides *Plectrohyla insolita*, the other known instances of evolution of terrestrial egg clutches overhanging lotic or lentic water bodies in hylids are *Isthmohyla calypsa* (Hylini; Lips, 1996), *Tlalocohyla celeste* (Hylini; Varela-Soto et al., 2022), *Sarcohyla thorectes* (Hylini; Duellman, 1970; Delia et al., 2013), *Gabohyla pauloalvini* (Sphaenorhynchini; Bokermann, 1973; a second species, *Sphaenorhynchus carneus*, is reported to lay eggs in water and on leaves, see Araujo-Vieira et al., 2019), the *Dendropsophus decipiens* group (Dendropsophini; Lutz, 1947; Toledo et al., 2011), at least an internal clade of the *D. leucophyllatus* group (Dendropsophini; Orrico et al., 2021), an internal clade of the *D. parviceps* group (Dendropsophini; Duellman and Crump, 1974; Fouquet et al., 2015), and at least one species of the *D. ruschii* group (Dendropsophini; Weygoldt and Peixoto, 1987). As discussed by Orrico et al. (2021), the relationships of Dendropsophini with other tribes is unclear, and the reproductive modes of *Xenohyla* and some species of *Dendropsophus* are still unknown; therefore, it remains uncertain whether the occurrence of a terrestrial egg clutch in the *Dendropsophus decipiens* group, the *D. ruschii* group, *Gabohyla* and *Sphaenorhynchus* is homologous or represents independent origins. Among other hylids outside Hyliinae, terrestrial egg clutches evolved in the most recent common ancestor of Phyllomedusinae (Cruz 1990; Hoogmoed and Cadle, 1991), and apparently four times in *Litoria* (Pelodyadinae; *L. prora*, *L. havina*, *L. iris*, *L. gracilis*; Richards et al., 2023).

The egg clutches of some phytotelme breeding species are laid above the water level in the phytotelme tank; although comparable in being also terrestrial by definition, these clutches and those overhanging lotic or lentic water bodies as discussed above, have not been compared in terms of the physical constraints that they suffer. Given that a study of that sort is outside the scope of this paper, for the time being we also consider the known cases of terrestrial egg clutches in phytotelme breeding species. These are known so far among Hyliinae only in Hylini, in the two species of *Ecnomiohyla* with known reproductive biology *Ecnomiohyla rabborum* and *E. valancifer* (Mendelson et al., 2008; K.-H. Jungfer, personal commun.), *Isthmohyla zeteki*

(Dunn, 1937), and *Tripurion spinosus* (Jungfer, 1996), and, in Pelodyadinae, in the clade including *Litoria naispela*, *L. richardsi*, and *L. singadanae* (Richards et al., 2023). It should be taken into account that among the many reports of phytotelme breeding in hylids (see Blotto et al., 2021 for a summary in the family) in some species the actual place of oviposition is unknown, with some cases reported to be out of the water (e.g., at least one undescribed species of the *Ololygon perpusilla* group; Alves-Silva and Silva, 2009), and in other cases the eggs are in the phytotelme but after flooding of the tank, tadpoles complete their development in streams (*Bokermannohyla astarteae*; Malagoli et al., 2021).

The distant relationship of *Plectrohyla insolita* with *Isthmohyla*, also implies in the former another known instance of independent evolution of egg attendance in terrestrial clutches in Hylidae. Wilson et al. (1994a), citing a personal communication from K. Lips, noted similarities between the reproductive biology of *P. insolita* and *Isthmohyla calypsa*, as both species lay eggs on the upper surfaces of leaves and attend their egg clutches. Yet, the egg-guarding behavior described for these species seems to be substantially different. For instance, information on *I. calypsa* indicates that males stay close to their egg clutches at a distance of 1 to 2 meters from their offspring (K. Lips in Wilson et al., 1994a), whereas *P. insolita* has been reported to perform a repertoire of complex parental-care behaviors, such as commonly remaining with the eggs in close proximity during night- and daytime, homing to oviposition sites when disturbed, mechanical agitation of the eggs, engaging into physical contact with the eggs, and guarding multiple egg clutches on the same leaf at the same time (Wilson et al., 1994a; Castañeda and McCranie, 2011). Lips (2001) assessed over a hundred egg clutches of *I. calypsa* after a five-year sampling season without commenting on the presence of guarding adults, but rather on the fact that the species used alternative strategies to enhance offspring survival (e.g., variable egg and clutch size). The presence of adult males of the latter species guarding eggs was not noted in previous studies (Trueb, 1968; Lips, 1996); it should be established whether it actually performs egg attendance or instead shows site fidelity.

The male egg-guarding behavior of *Plectrohyla insolita* seems to be rather similar to that described for species of some genera of Centrolenidae (e.g. *Centrolene*, *Celsiella* and *Hyalinobatrachium*; Delia et al., 2017, 2020). Other hylid species known to perform parental care of terrestrial egg clutches are *Gabohyla pauloalvini*, where the care is given by the female (Carilo-Filho et al., 2021; Neto et al., 2022), and the phytotelme breeders *Ecnomihyla rabborum* and *E. valancifer*, where the care is provided by the male (Mendelson et al., 2008; K.-H. Jungfer, personal commun.). The basic aspects of the reproductive biology of *Plectrohyla* are still very poorly known and require additional research, both for increasing information of the natural history of this clade and for the understanding of the evolution of reproductive biology in hylids.

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## APPENDIX 1

## VOUCHER SPECIMENS AND GENBANK ACCESSIONS FOR THIS STUDY

Below are listed voucher specimens and GenBank accessions (if available) of sequences produced (in bold) or newly added for this study to the data-set of Faivovich et al. (2018; appendix S2); see that paper for the same information for all other terminals and genes included in that study and employed here. For the newly added sequences, the collection acronyms are those employed in the original publications (Townsend et al., 2020; Caviedes-Solis et al., 2020; Varela-Soto et al., 2022); those of sequences produced for this study correspond to United States National Museum (USNM), University of Texas at Arlington Tissue Collection (UTA FN), and Universidad de Costa Rica (UCR). Abbreviation: NA = not accessioned (see respective footnote for details).

	Voucher	12S-tVal-16S	16S-tLeu-ND1	Cyrb	POMC	RAG1	Rhod	SIAHI
<i>Atlantihyla melissa</i>	USNM 578679	MK176937/MK176946	—	—	MK177061	MW177730	MK177068	—
<i>Atlantihyla melissa</i>	USNM 578681	MW177722/MK177024	—	—	MW177743	MW177731	MW177726	—
<i>Bromeliophyla dendroscarta</i>	OFV 1079	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Charadrahyla altipotens</i>	ICS 680	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Charadrahyla esperancensis</i>	CE 2	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Exerodonta juanitae</i>	ICS647	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Charadrahyla sebbah</i>	VA 242	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Charadrahyla trux</i>	JCBH 1000	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Duellmanohyla chamulae</i>	ICS 591	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Duellmanohyla schmidtorum</i>	ICS 602	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Exerodonta abdivita</i>	ANMO 2404	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Exerodonta bivocata</i>	AGV 7	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Exerodonta smaragdina</i>	ISZ 603	NA <sup>1</sup>	—	—	—	NA <sup>2</sup>	NA <sup>3</sup>	—
<i>Plectrohyla catracha</i>	USNM 570494	<b>PP464052</b>	<b>PP490791</b>	<b>PP490808</b>	<b>PP490796</b>	<b>PP490806</b>	<b>PP490803</b>	<b>PP490799</b>
<i>Plectrohyla catracha</i>	USNM 570495	<b>PP464053</b>	<b>PP490792</b>	<b>PP490809</b>	<b>PP490797</b>	—	<b>PP490804</b>	<b>PP490800</b>
<i>Plectrohyla insolita</i>	UTA FN 252948	<b>PP464054</b>	<b>PP490788</b>	—	<b>PP490793</b>	—	<b>PP490801</b>	—
<i>Plectrohyla insolita</i>	UTA FN 252949	<b>PP464055</b>	<b>PP490789</b>	—	<b>PP490794</b>	—	<b>PP490802</b>	<b>PP490798</b>
<i>Plectrohyla insolita</i>	UCR 23564	<b>PP464056</b>	<b>PP490790</b>	<b>PP490807</b>	<b>PP490795</b>	<b>PP490805</b>	—	—

APPENDIX 1 *continued*

	Voucher	12S-tVal-16S	16S-tLeu-ND1	Cytb	POMC	RAG1	Rhod	SIAHI
<i>Tlalocohyla celeste</i>	YPM 13222	OM749744/OM749745	—	—	—	—	—	—
<i>Tlalocohyla celeste</i>	ECB-Anf-50-08-01	OM749742/OM749743	—	—	—	—	—	—

<sup>1</sup> Sequences produced by Caviades Solis et al. (2020) but not accessioned in Genbank. Available online ([https://static-content.springer.com/esm/art%3A10.1007%2Fs10531-020-01986-8/MediaObjects/10531\\_2020\\_1986\\_MOESM4\\_ESM.nex](https://static-content.springer.com/esm/art%3A10.1007%2Fs10531-020-01986-8/MediaObjects/10531_2020_1986_MOESM4_ESM.nex)).

<sup>2</sup> Sequences produced by Caviades Solis et al. (2020) but not accessioned in Genbank. Available online ([https://static-content.springer.com/esm/art%3A10.1007%2Fs10531-020-01986-8/MediaObjects/10531\\_2020\\_1986\\_MOESM7\\_ESM.nex](https://static-content.springer.com/esm/art%3A10.1007%2Fs10531-020-01986-8/MediaObjects/10531_2020_1986_MOESM7_ESM.nex)).

<sup>3</sup> Sequences produced by Caviades Solis et al. (2020) but not accessioned in Genbank. Available online ([https://static-content.springer.com/esm/art%3A10.1007%2Fs10531-020-01986-8/MediaObjects/10531\\_2020\\_1986\\_MOESM8\\_ESM.nex](https://static-content.springer.com/esm/art%3A10.1007%2Fs10531-020-01986-8/MediaObjects/10531_2020_1986_MOESM8_ESM.nex)).







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