

## The phylogenetic relationships of the charismatic poster frogs, Phyllomedusinae (Anura, Hylidae)

Julián Faivovich<sup>a,\*</sup>, Célio F. B. Haddad<sup>b</sup>, Délio Baêta<sup>c</sup>, Karl-Heinz Jungfer<sup>d</sup>, Guilherme F. R. Álvares<sup>e</sup>, Reuber A. Brandão<sup>e</sup>, Christopher Sheil<sup>f</sup>, Lucas S. Barrientos<sup>g</sup>, César L. Barrio-Amorós<sup>h</sup>, Carlos A. G. Cruz<sup>c</sup> and Ward C. Wheeler<sup>i</sup>

<sup>a</sup>División Herpetología, Museo Argentino de Ciencias Naturales-CONICET, Angel Gallardo 470, C1405DJR, Buenos Aires, Argentina; Departamento de Zoología, I.B., Universidade Estadual Paulista, Rio Claro, CEP 13506-900, São Paulo, Brazil; and Herpetology, Division of Vertebrate Zoology, American Museum of Natural History, New York, USA; <sup>b</sup>Departamento de Zoologia, I.B., Universidade Estadual Paulista, Rio Claro, CEP 13506-900, São Paulo, Brazil; <sup>c</sup>Setor de Herpetologia, Departamento de Vertebrados, Museu Nacional, Universidade Federal do Rio de Janeiro, Quinta da Boa Vista, s/nº, São Cristóvão, CEP 20940-040, Rio de Janeiro, RJ, Brazil; <sup>d</sup>Panoramastr. 14, 74405 Gaildorf, Germany; <sup>e</sup>Laboratório de Fauna e Unidades de Conservação, Departamento de Engenharia Florestal, Universidade de Brasília, Brasília-DF, CEP 70910-900, Brazil; <sup>f</sup>Department of Biology, Dolan Center for Science & Technology, John Carroll University, 20700 North Park Boulevard, University Heights, OH 44118, USA; <sup>g</sup>Instituto de Ciencias Naturales, Universidad Nacional de Colombia, calle 53, 2º edificio Bogotá, Colombia; <sup>h</sup>Fundación Andígena, Apartado Postal 210, 5101-A Mérida, Venezuela; <sup>i</sup>Division of Invertebrate Zoology, American Museum of Natural History, Central Park West at 79th St, New York, NY 10024, USA

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### Abstract

The leaf or monkey frogs of the hylid subfamily Phyllomedusinae are a unique group of charismatic anurans. We present a molecular phylogenetic analysis that includes 45 of the 60 species of phyllomedusines using up to 12 genes and intervening tRNAs. The aims were to gain a better understanding of the phylogenetic position of *Phrynomedusa*, test the monophyly and explore the relationships among several putative lineages (*Hylomantis*, the *H. buckleyi* Group, *Phasmahyla*, the four species groups of *Phyllomedusa*, and the species of *Phyllomedusa* that remain unassigned to any group), and to examine the implications of our phylogeny for the evolution of several characters in phyllomedusines. The analyses resulted in a well-supported phylogenetic hypothesis that provides a historical framework for a discussion of the evolution of characters associated with reproductive biology, gliding behaviour, the physiology of waterproofing, and bioactive peptides. Implications include an earlier origin for eggless capsules than for leaf-folding behaviour during amplexus, two independent origins of gliding, and an earlier origin of reduction in evaporative water loss than uricotelism, which is a result that originally was predicted on the basis of physiology alone. Furthermore, our results support the prediction that bioactive peptides from different peptide families are to be expected in all species of Phyllomedusinae. *Hylomantis* (as recently redefined) is shown to be paraphyletic and the synonymy of *Agalychnis* is revised to remedy this problem by including both *Hylomantis* and *Pachymedusa*.

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Commonly known as leaf frogs, monkey frogs, and red-eyed tree frogs, phyllomedusines are possibly the most charismatic treefrogs with which the general public is familiar. In addition, several species of this subfamily have become popular in biochemical research over the past three decades due to the diversity of bioactive peptides that are stored in granular glands in the skin of

these frogs. However, the bioprospecting storm over these beautiful frogs had not until recently been accompanied by an equivalent increase in our knowledge of their phylogenetic relationships.

The first comprehensive hypothesis of relationships of Phyllomedusinae was that of Funkhouser (1957), who presented a precladistic narrative “phylogenetic arrangement” that included most species known at that time, and it was based mostly on perceived degrees of specialization of the foot (here reproduced as Fig. 1), a

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\*Corresponding author:

E-mail address: julian@macn.gov.ar

condensed version of which was also presented by Lutz (1968). A more recent hypothesis of the phylogenetic relationships of Phyllomedusinae (Faivovich et al., 2005) was presented in the broader context of relationships within Hylidae. This study was based on the analysis of partial or complete sequences of four mitochondrial and five nuclear genes for up to 5100 bp per species, and a dataset built from Burton's (2004) collection of observations on hyd foot musculature (the relevant section of their hypothesis is here reproduced as Fig. 2a). Furthermore, Faivovich et al. (2005) reviewed the available morphological evidence for the monophyly of Phyllomedusinae. Wiens et al. (2005) presented a separate phylogenetic analysis of hylids (the relevant section of their hypothesis is here reproduced as Fig. 2b) using fewer exemplars that included data from morphology for two phyllomedusines and several gene fragments (3367 bp from five gene fragments for four exemplars of Phyllomedusinae and 830 bp for ten other exemplars from the 12S ribosomal mitochondrial gene). Later, Wiens et al. (2006) combined most sequences generated by Faivovich et al. (2005) with those of Wiens et al. (2005) and presented a new phylogenetic hypothesis as supplementary data without substantial discussion (the relevant section of their hypothesis is reproduced as Fig. 2c). Moen and Wiens (2008) presented a reanalysis of the Phyllomedusinae available to Wiens et al. (2006) using Bayesian methods—the results of this latter study were presented as supplementary data and without discussion (here reproduced as Fig. 2d). More recently, Gomez-Mestre et al. (2008) presented a phylogenetic hypothesis for the genus *Agalychnis* on the basis of several mitochondrial and nuclear genes (here reproduced as Fig. 2e).

Taxon sampling of Phyllomedusinae included by Faivovich et al. (2005) comprised at least one exemplar of all but a single recognized genus within the subfamily and three of the four species groups of *Phyllomedusa*. Overall this sampling was adequate to test the monophyly of the subfamily and to gain a general perspective on the internal topology of the group. However, from the inception of that study, it was evident that the taxon sampling was insufficient to address certain phylogenetic problems within the subfamily. These problems ranged from the position of *Phrynomedusa* (no exemplars of this genus were available), to the monophyly of *Hylomantis* (only a single exemplar was available), to the individual monophyly of each of the species groups of *Phyllomedusa* (only one exemplar was available for each of the three of the four available species groups of *Phyllomedusa* then recognized). The results of Wiens et al. (2005, 2006) and Moen and Wiens (2008) shed some light on one of these issues because these analyses included an exemplar of *Phrynomedusa* (*P. marginata*) and one exemplar of the *Phyllomedusa perinesos* Group (*P. duellmani*), the only species group of *Phyllomedusa* for

which Faivovich et al. (2005) had no exemplars (Fig. 2d). However, for these two species Wiens et al. (2005, 2006) and Moen and Wiens (2008) were only able to include about 830 bp of the 12S mitochondrial gene, in comparison with the up to 8000 bp from several loci available for some species in their analyses. Considering the weakly supported results they obtained, the position of *Phrynomedusa* can still be considered to be poorly established.

The results of these phylogenetic analyses are difficult to compare because they resulted from different analytical methods and character weighting, particularly with regard to how nucleotide homologies and insertion/deletion events were considered during tree searches. Even though these analyses imply different relationships, they agree in several aspects.

1. Polyphyly of *Agalychnis*. The former *Agalychnis calcarifer* (and presumably also *A. craspedopus*) was inferred to be the sister taxon of all remaining Phyllomedusinae, and a new generic name (*Cruziophyla*) was introduced (Faivovich et al., 2005). However, the lack of any exemplar of *Phrynomedusa* in the analysis of Faivovich et al. (2005), with which both *C. calcarifer* and *C. craspedopus* share two character states of uncertain polarity (oral disc with complete marginal papillation, and bicoloured iris), left uncertainty in the analysis as to whether *Cruziophyla* is the sister taxon of all Phyllomedusinae, or if it is actually the sister taxon to *Phrynomedusa*. A solution to this question could not be hypothesized based on the taxonomic distribution of known morphological character states because Phyllomedusinae is the sister taxon of the large Australopapuan subfamily Pelodyadinae (Faivovich et al., 2005; Wiens et al., 2005, 2006). The internal relationships of Pelodyadinae remain to be studied and both character states that would support a sister-group relationship of *Cruziophyla* and *Phrynomedusa* do occur in some species of Pelodyadinae; therefore, the polarity of these character states could not be deduced at that time. In the results of Wiens et al. (2006), *Phrynomedusa* was obtained as the sister taxon of all Phyllomedusinae, whereas *Cruziophyla* was recovered as the sister group of *Phasmahyla*, with both nodes having less than 50% bootstrap support.

2. Polyphyly of *Phyllomedusa*. Duellman (1968, 1969), Cannatella (1980), and Jungfer and Weygoldt (1994) have suggested the polyphyly of *Phyllomedusa* and, corroborating this, the single available exemplar of the former *P. buckleyi* Group (*P. lemur*) was found to be only distantly related to all other exemplars of *Phyllomedusa* in these analyses (Fig. 2a–d). Furthermore, in the analysis of Faivovich et al. (2005), *P. lemur* formed a weakly supported clade with *Hylomantis granulosa*, the only available exemplar for this genus (Fig. 2a). For this reason, Faivovich et al. (2005) tentatively transferred the former *P. buckleyi* Group to *Hylomantis*, rather than continuing to recognize *Phyllomedusa* as a

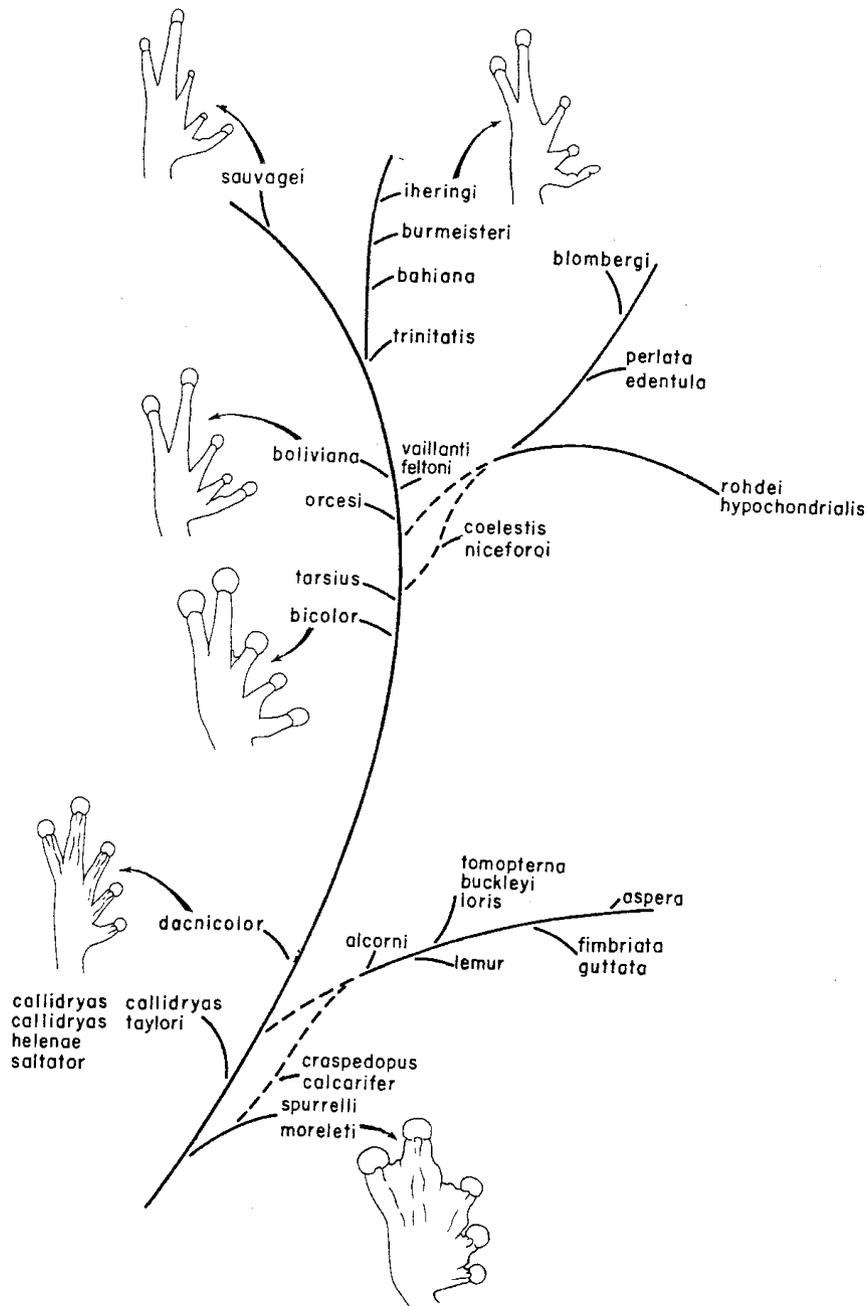


Fig. 1. “Proposed phylogenetic arrangement” by Funkhouser (1957, fig. 7) of species now included in Phyllomedusinae but then all included in *Phyllomedusa*; this illustration emphasizes some aspects of Funkhouser’s hypothesis on evolution of webbing in the feet of these frogs. *Phyllomedusa blumbergi*, *P. feltoni*, and *P. perlata* are junior synonyms of *P. vaillanti*. *Phyllomedusa edentula*, *P. niceforoi*, and *P. orcesi* are junior synonyms of *P. tarsius*. *Phyllomedusa helenae* is a junior synonym of *Agalychnis callidryas*. *Phyllomedusa alcorni* is a junior synonym of *Pachymedusa dacnicolor*. *Phyllomedusa loris* is a junior synonym of *Hylomantis buckleyi*.

paraphyletic group. Two important problems with this action are the low support for the monophyly of *Hylomantis* and the lack of evidence supporting the monophyly of the newly recognized *Hylomantis buckleyi* Group, as delimited by Cannatella (1982). In the hypotheses presented by Wiens et al. (2006; Fig. 2c) and Moen and Wiens (2008; Fig. 2d), *Hylomantis* (as

redefined by Faivovich et al., 2005) was not found to be monophyletic as relationships among *H. granulosa* and *H. lemur* were not resolved—these results were not discussed by either Wiens et al. (2006) or Moen and Wiens (2008).

Other pending issues in phyllomedusine systematics involve the internal relationships of the more species-

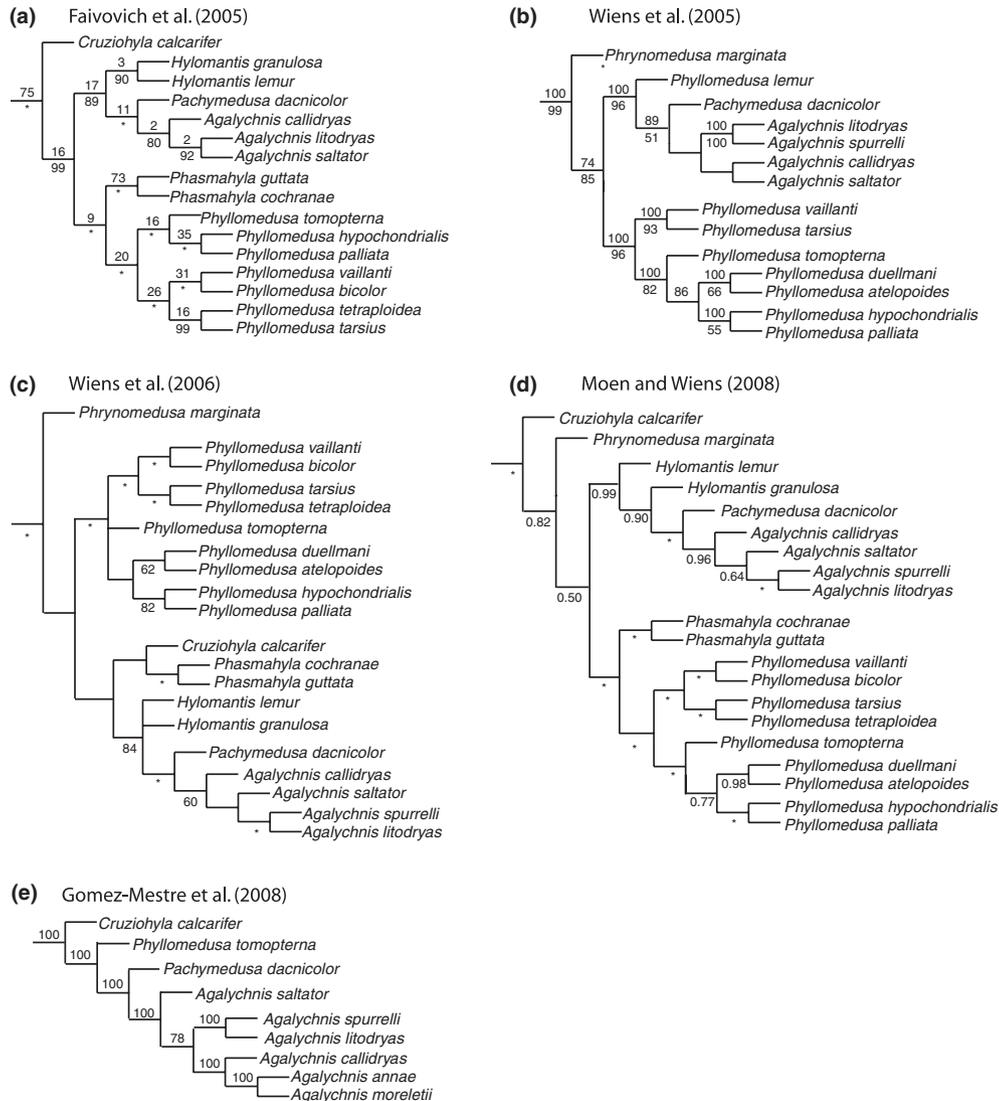


Fig. 2. Recent phylogenetic hypotheses of Phyllomedusinae. Note that *Agalychnis litodryas* has been considered a synonym of *A. spurrelli* by Ortega-Andrade (2008). (a) Section of the phylogenetic hypothesis of Faivovich et al. (2005) corresponding to Phyllomedusinae. Analysis based on partial or complete sequences of four mitochondrial genes (cytochrome *b*, 12S, the intervening tRNA<sup>Val</sup>, 16S) and five nuclear genes (portions of seven in absentia homolog 1, exon 1 rhodopsin, tyrosinase, RAG-1, 28S), as well as a dataset built from Burton's (2004) collection of observations on hylid foot musculature (information available for only five phyllomedusines). Analysis was performed using parsimony with direct optimization. Numbers above nodes are Bremer support values; numbers below are parsimony jackknife absolute frequencies (asterisks indicate 100% absolute frequency). Redrawn from Faivovich et al. (2005: figs 2 and 9). (b) A section of the phylogenetic hypothesis of Wiens et al. (2005) corresponding to Phyllomedusinae. Analysis based on four mitochondrial genes [12S, and a fragment including the complete upstream section of 16S, the intervening tRNA<sup>Leu</sup>, and NADH dehydrogenase subunit 1 (ND1)] and three nuclear genes [proopiomelanocortin A gene (POMC), exons 2 and 3 of cellular myelocytomatosis (c-myc)]. All genes were available for four exemplars of Phyllomedusinae; only 12S sequenced for the other ten species. The analysis also included a non-molecular dataset (scored for two phyllomedusines); they were performed with Bayesian Clade Posterior Probability and Parsimony, considering gaps as missing data. Numbers above nodes are Bayesian posterior probabilities; numbers below are bootstrap values for clades that were present in the parsimony analysis. Redrawn from Wiens et al. (2005: fig. 4). (c) A section of the phylogenetic hypothesis of Wiens et al. (2006) corresponding to Phyllomedusinae. This analysis incorporates most of the terminals and molecular data produced by Faivovich et al. (2005) to the database of Wiens et al. (2005). The analysis was performed with parsimony, considering gaps as missing data. Numbers below branches are bootstrap values (values < 50% not shown; asterisk indicates values ≥ 95%). Redrawn from Wiens et al. (2006, online supp. data: fig. 1a). (d) Phylogenetic hypothesis of Moen and Wiens (2008). This hypothesis include the same molecular data as that of Wiens et al. (2006), and was obtained using Bayesian Clade Posterior analysis. Numbers above nodes are Bayesian posterior probability values (Bpp). An asterisk denotes Bpp = 1.0. Redrawn from Moen and Wiens (2008, online supp. data: fig. S1). (e) Phylogenetic hypothesis of *Agalychnis* from Gomez-Mestre et al. (2008). Bayesian Clade Posterior Probability analysis based on mitochondrial genes (cytochrome *b*, and a fragment including the complete upstream section of 16S, the intervening tRNA<sup>Leu</sup>, and ND1) and nuclear genes (POMC, RAG-1, TNS3). Numbers above nodes are Bayesian posterior probability values. Redrawn from Gomez-Mestre et al. (2008: fig. 4a); outgroups not included.

rich genus, *Phyllomedusa*. Twenty-five of the 32 currently recognized species of *Phyllomedusa* have been placed among four species groups (*P. burmeisteri* Group (Pombal and Haddad, 1992), *P. hypochondrialis* Group (Caramaschi, 2007), *P. perinesos* Group (Cannatella, 1982), and *P. tarsiis* Group (Barrio-Amorós, 2006)), whereas seven species remain unassigned. Evidence of monophyly had been advanced in support of the *P. perinesos* and *P. tarsiis* Groups (Cannatella, 1982; Barrio-Amorós, 2006), whereas Faivovich et al. (2005) referred to some character states that could support the monophyly of the *P. hypochondrialis* Group, but suggested that more research was needed to assess the status of the latter group. Overall, the monophyly of none of these groups has been rigorously tested in a phylogenetic context.

Herein, we intend to expand upon the phylogenetic studies of Faivovich et al. (2005) and Wiens et al. (2006) to evaluate the evolutionary relationships within Phyllomedusinae. Our aims were to: (i) gain a better understanding of the position of *Phrynomedusa*, the only phyllomedusine genus that was not included in Faivovich et al. (2005); (ii) test the monophyly of the *Hylomantis buckleyi* Group, as well as the monophyly of *Hylomantis* as defined by Faivovich et al. (2005); (iii) test the monophyly of each of the proposed species groups within *Phyllomedusa*; (iv) explore the position of the *Phyllomedusa perinesos* Group; (v) explore the position of the species of *Phyllomedusa* that remain unassociated with any of the proposed species groups; (vi) explore relationships among species of the genus *Phasmahyla*, whose phylogenetic relationships remain poorly studied; and (vii) discuss the implications of the resulting hypothesis for our understanding of the evolution of several morphological, behavioural, and biochemical characters within the hylid subfamily, Phyllomedusinae. To address these goals, we present a phylogenetic analysis based on up to 12 genes plus intervening mitochondrial tRNAs for 45 of the 60 species within the subfamily.

## Materials and methods

### Taxon sampling

We included exemplars from all seven genera within Phyllomedusinae: *Agalychnis* (the five known species); *Cruziohyla* (one of the two known species; the unavailable species is *C. craspedopus*); *Hylomantis* (four of the eight species: the two species of the *H. aspera* Group, two of the six species of the *H. buckleyi* Group; the unavailable species are *H. buckleyi*, *H. medinae*, *H. psilopygion*, and *H. danieli*); *Pachymedusa* (a monotypic genus); *Phasmahyla* (five of the seven described species; the unavailable species are the recently described

*P. spectabilis* and *P. timbo* (Cruz et al., 2008a,b)); *Phrynomedusa* (one of the five species; the unavailable species are *P. appendiculata*, *P. bokermanni*, *P. fimbriata*, and *P. vanzolinii*); and *Phyllomedusa* (28 of the 32 species). The exemplars of *Phyllomedusa* include all seven species unassigned to any species group, the five species of the *P. burmeisteri* Group, three of the five species of the *P. tarsiis* Group (unavailable species are *P. coelestis* and *P. venusta*), the 11 species of the *P. hypochondrialis* Group, and two of the four species of the *P. perinesos* Group (unavailable species are *P. perinesos* and *P. ecuatoriana*). Overall, our sampling adds 27 species to the 16 species included by Faivovich et al. (2005) and 18 species to those included by Wiens et al. (2006) and Moen and Wiens (2008).

It is worth noting that Faivovich et al. (2005), when referring to the *Phyllomedusa tarsiis* Group, incorrectly assigned only four (*P. boliviana*, *P. camba*, *P. sauvagii*, and *P. tarsiis*) of the seven species that De la Riva (1999) included within the group, and they considered the other three species (*P. coelestis*, *P. trinitatis*, and *P. venusta*), previously assigned to the *P. tarsiis* Group (De la Riva, 1999) as unassigned to any species group. For the purposes of this paper, we follow De la Riva's (1999) allocation of these latter three species, as it was also supported in the recent redefinition of Barrio-Amorós (2006). We consider *P. camba*, *P. boliviana*, and *P. sauvagii* as unassigned to any species group, as they were excluded from the *P. tarsiis* Group by the latter author.

Outgroup sampling was drawn largely from the hylid subfamilies Pelodryadinae and Hylinae. This was based on the consensus view that Hylinae, Pelodryadinae, and Phyllomedusinae form a monophyletic group (e.g. Faivovich et al., 2005; Wiens et al., 2005, 2006; Frost et al., 2006). The single exception to this consensus is the recent study by Roelants et al. (2007), which recovered Phyllomedusinae and Pelodryadinae as sister taxa, but found Hylinae to be distantly related. Without discussion, Bossuyt and Roelants (2009) elevated the three hylid subfamilies to family rank to conform with Roelants et al.'s (2007) topology. Although this change in rank does not contradict prior findings, Roelants et al.'s (2007) result was based on a greatly reduced dataset that included far fewer hylid and, in particular, hylid (only eight exemplars, including one Phyllomedusinae and three Pelodryadinae) terminals than recent studies and excluded most of the DNA sequences published by Faivovich et al. (2005), Wiens et al. (2005, 2006) and Frost et al. (2006). We therefore followed the preponderance of the evidence in considering these three lineages to form a single clade, which we recognize as Hylidae.

Exemplars of Pelodryadinae on GenBank that had at least 1 kb of sequences from the 12S mt rRNA and 16S mt rRNA regions were included and, overall, 25 species within Pelodryadinae were found to be appropriate for this study and included. These included exemplars of 15

species groups of *Litoria*; three exemplars of the subgenus *Cyclorana*; and six species of the former *Nyctimystes* (see Frost et al., 2006), which now reside within *Litoria* and remain unassigned to any species group. Additionally, we included as other outgroup taxa eight hylids from within Hyliinae that represent exemplars of the four tribes recognized by Faivovich et al. (2005): Cophomantini (*Hypsiboas multifasciatus*, *Myersiohyala kanaima*); Dendropsophini (*Dendropsophus nanus*, *Pseudis minutus*, *Scinax staufferi*); Hylini (*Acris crepitans*); and Lophiohylini (*Phyllodytes luteolus*, *Trachycephalus venulosus*). Trees were rooted with *Myersiohyala kanaima*, an exemplar of the basal genus of the basal hyline tribe Cophomantini (Faivovich et al., 2005).

#### Character sampling

The present study is based on DNA sequence data representing up to 12 nuclear and mitochondrial genes plus three intervening tRNAs. Although we recognize the importance of morphological and other phenotypic evidence (e.g. Faivovich, 2002) and the conceptual superiority of total evidence analysis, much of the published information for Phyllomedusinae is based on observations of only a handful of species and must be complemented by extensive specimen-based research that was beyond the scope of this study. Throughout our discussion we refer to a few morphological observations made by others, and discuss the taxonomic distribution of bioactive peptides, certain physiological and behavioral characters, and characters associated with reproductive biology. These should serve as an aid to future, more comprehensive studies.

Whereas Wiens et al. (2006: Supp. Data) included up to 8000 bp for some of their terminals, only two of the phyllomedusines that they included (*Pachymedusa dactylos* and *Phyllomedusa tomopterna*) had actually been sequenced for this many base pairs. In most cases, the sequences available in their study were those from Faivovich et al. (2005) or a stretch of 830 bp of 12S sequenced by Wiens et al. (2005, 2006) that overlapped with the sequences generated by Faivovich et al. (2005). The present analysis is based on eight of the nine loci sequenced by Faivovich et al. (2005) plus one additional mitochondrial and two nuclear gene fragments [the ninth locus, a fragment of the 28S nuclear gene, shows almost no variation at this level (J.F., pers. obs.) so it is not included]. The analysis includes all relevant sequences produced by Faivovich et al. (2005) and Wiens et al. (2005). We also included GenBank sequences for non-overlapping fragments of the recombinase-activating gene 1 (RAG-1), tensin 3 (TNS3), and exon 2 cellular myelocytomatosis (c-myc) that were available for all species of *Agalychnis*, *C. calcarifer*, *H. lemur*, and *Phyllomedusa tomopterna* generated by Gomez-Mestre et al. (2008). The mitochondrial gene sequences produced for this project include

portions of cytochrome *b*, 12S, the intervening tRNA<sup>Val</sup>, 16S, and a fragment including the complete upstream section of 16S, the intervening tRNA<sup>Leu</sup>, NADH dehydrogenase subunit 1 (ND1), and tRNA<sup>Ile</sup> which was first incorporated by Wiens et al. (2005). The nuclear gene sequences produced include portions of seven in absentia homolog 1 (mistakenly called Seventh in Absentia by Faivovich et al., 2005), exon 1 rhodopsin, tyrosinase, RAG-1, proopiomelanocortin A gene (POMC) (first employed by Wiens et al., 2005), and exon 2 of chemokine receptor 4 (CXCR4) (first employed by Biju and Bossuyt, 2003). All the primers employed are the same as those employed by Faivovich et al. (2005), with the addition of 16S-frog and tMet-frog (fragment of 16S + tRNA<sup>Leu</sup> + ND1 + tRNA<sup>Ile</sup>; Wiens et al., 2005), CytbAR-H (used with MVZ15 to obtain a larger fragment of cytochrome *b* than the one employed by Faivovich et al., 2005; Goebel et al., 1999), POMC-1 and POMC-2 (Wiens et al., 2005), and CXCR4-C and CXCR4-G (Biju and Bossuyt, 2003).

#### DNA isolation and sequencing

Whole cellular DNA was extracted from ethanol-preserved tissues with the DNeasy (Qiagen, Valencia, CA, USA) isolation kit. Amplification was carried out in a 25- $\mu$ L reaction using puRe Taq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ, USA) or Fermentas Master Mix. For all amplifications, the PCR programme included an initial denaturing step of 30 s at 94 °C, followed by 35 (mitochondrial gene fragments) or 45 (nuclear gene fragments) cycles of amplification (94 °C for 30 s; 48–64 °C for 30 s; 72 °C for 60 s), with a final extension step at 72 °C for 6 min. PCR amplification products were desalted and concentrated using either an Ampure (Agencourt Biosciences, Beverly, MA, USA), or GE GFX PCR purification kit and labelled with fluorescent-dye labels terminators (ABI Prism Big Dye Terminators v. 1.1 cycle sequencing kits; Applied Biosystems, Foster City, CA, USA). The labelled PCR products were cleaned using cleanSEQ (Agencourt Biosciences, Beverly, MA, USA). The products were sequenced with an ABI 3730XL (Applied Biosystems), and all samples were sequenced in both directions to check for potential errors. Chromatograms obtained from the automated sequencer were read and contigs made using the sequence editing software Sequencher 3.0. (Gene Codes, Ann Arbor, MI, USA). Complete sequences were edited with BioEdit (Hall, 1999). See Appendix 3 for a list of specimens, locality data, and GenBank numbers.

#### Phylogenetic analysis

The rationale for using parsimony as an optimality criterion was advanced by Farris (1983) and recently

discussed by Goloboff (2003) and Goloboff and Pol (2005). The preference for the treatment of sequence data as dynamic homologies simultaneously with tree searches, as opposed to static homology hypotheses (multiple alignments) independent of tree searches, has been discussed and justified by Wheeler (1996, 2002) and De Laet (2005).

The phylogenetic analyses were performed with POY 4.1.1 (Varón et al., 2009a,b), using equal weights for all transformations (substitutions and insertion/deletion events). Sequences of 12S, 16S, ND1, and intervening tRNAs (valine, leucine, isoleucine) were preliminarily delimited in sections of putative homology (Wheeler et al., 2006), and equal-length sequences of nuclear protein-coding genes were considered as static alignments to accelerate the searches. Searches were performed using the command “Search”. This command implements a driven search building Wagner trees using random addition sequences (RAS), Tree Bisection and Reconnection (TBR) branch swapping followed by Ratchet (Nixon, 1999), and Tree Fusing (Goloboff, 1999). The command (Search) stores the shortest trees of each independent run and performs final tree fusing using the pooled trees as a source of topological diversity. Ten 12-h runs of Search were implemented in parallel at the American Museum of Natural History Cluster using 16 processors. The resulting trees were submitted to a final round of swapping using iterative pass optimization (Wheeler, 2003a). Bremer support indices (Bremer, 1988) were calculated with POY 4 by combining suboptimal trees generated with 100 RAS followed by TBR and keeping all the visited trees during the swapping as well as those generated from the TBR swapping of the optimal trees. Parsimony Jackknife (Farris et al., 1996) absolute frequencies were estimated from the implied alignment (Wheeler, 2003b) with T.N.T., Willi Hennig Society Edition (Goloboff et al., 2003, 2008), generating 50 RAS + TBR per replicate, for a total of 1000 replicates. Editing of trees was performed with Winclada (Nixon, 2002), and character optimizations and reconstructions with T.N.T.

## Results and discussion

The driven search resulted in four most parsimonious trees of length 22 959 steps that was hit 350 times during the series of RAS + TBR followed by different perturbations. Based on implied alignments, these trees have a consistency index (CI; Kluge and Farris, 1969) of 0.33 and a retention index (RI; Farris, 1989) of 0.67. Most of the 113 nodes present in the strict consensus are well supported, with 78 nodes having Parsimony Jackknife absolute frequencies  $\geq 95\%$  and 76 nodes with Bremer support  $\geq 10$ . TBR branch swapping using iterative pass optimization of these four topologies did not identify

novel topologies, but reduced tree cost to 22 914 steps. As there were no new topologies, Bremer support values were estimated using direct optimization without iterative pass, to speed up the search of suboptimals. The most parsimonious trees (Figs 3 and 4) conflict only in the internal relationships among exemplars of *P. aye-aye*, *P. itacolomi*, *P. megacephala*, and *P. oreades* (consensus of this sector shown in Fig. 5).

Our results recover a monophyletic Phyllomedusinae in which *Phrynomedusa* is sister to a group composed of all remaining genera, within which *Cruzirohyla* is the sister to all remaining genera (*Phasmahyla* + *Phyllomedusa*, and a clade containing a paraphyletic *Hylo mantis* with *Pachymedusa* + *Agalychnis*). These results are discussed in detail below.

## Outgroups

Relationships among the few hyline outgroups differ from those obtained by Faivovich et al. (2005) in that exemplars of the tribe Dendropsophini, *Pseudis*, *Scinax*, and *Dendropsophus*, are not monophyletic (Fig. 3). We consider this to be a result of differences in taxon sampling stemming from the radically different goals of the analyses performed in these studies, and we do not consider them to be significant. As recovered in several other analyses, Phyllomedusinae and Pelodyadinae are each monophyletic, and these two subfamilies were found to form a clade (Figs 3 and 4). The monophyly of Pelodyadinae is quite well supported (Jackknife resampling of 100% and Bremer support of 37). Given that we included only exemplars of 14 of the 37 species groups of Pelodyadinae, the relevance of the present analysis in exploring the relationships of this large Australopapuan group is minimal. Nonetheless, as in previous analyses (Faivovich et al., 2005; Wiens et al., 2005, 2006; Frost et al., 2006), exemplars of the former genera *Cyclorana* and *Nyctimystes* are nested within *Litoria* (Fig. 3), corroborating Frost et al.’s (2006) recent taxonomic changes to these taxa.

## The position of *Phrynomedusa*

The former *Agalychnis calcarifer* (and presumably also *A. craspedopus*) was found to be the sister taxon of all remaining Phyllomedusinae in the analysis of Faivovich et al. (2005), and a new genus (*Cruzirohyla*) was created for them. No exemplar of *Phrynomedusa* was available for Faivovich et al. (2005) and its position within Phyllomedusinae could not be established based on the taxonomic distribution of known morphological character states in that study. Although *Phrynomedusa* shares with *Cruzirohyla* two character states that are unique within Phyllomedusinae (oral disc with complete marginal papillation and bicoloured iris), the sister-group relationship of this subfamily with Pelodyadinae

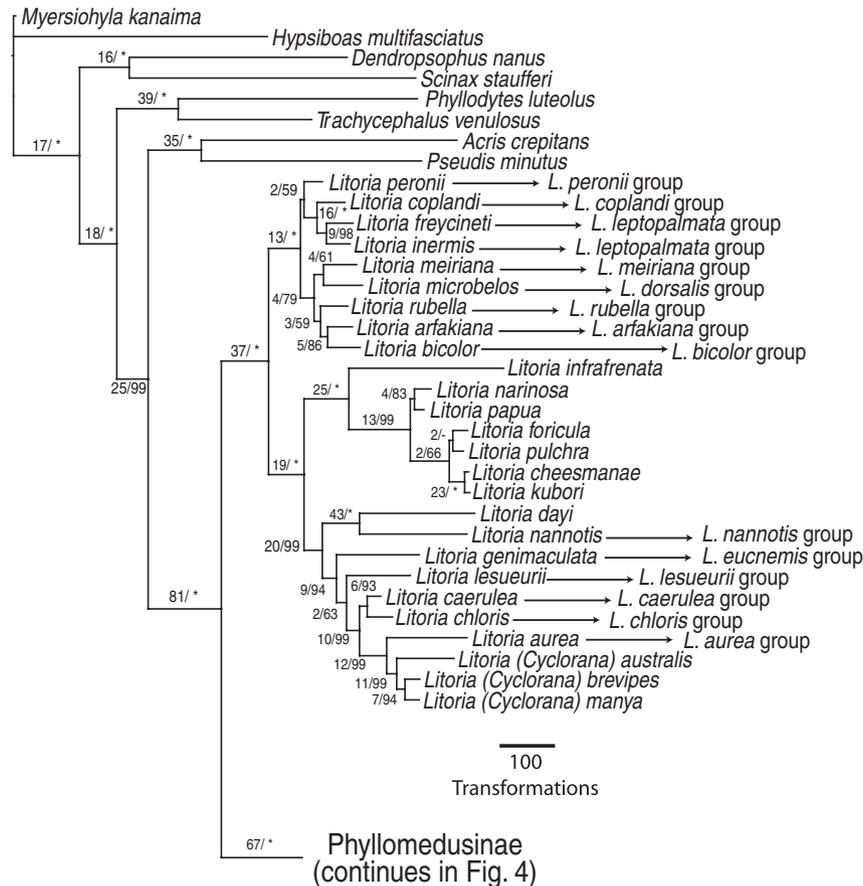


Fig. 3. Topology of the outgroups in one of the four most parsimonious trees (length 22 959 steps) resulting from the phylogenetic analysis of molecular data. This analysis includes the hyliid subfamily Phyllomedusinae plus several outgroups, including exemplars of the subfamilies Hyliinae and Pelodyrinae. This topology is constant in the four most parsimonious trees. Numbers adjacent to nodes are Bremer supports/parimony jackknife absolute frequencies. Asterisks indicate parsimony jackknife frequencies of 100%; dashes indicate frequencies  $\leq 50\%$ . Branch lengths are proportional to the number of unambiguous parsimony transformations; not all loci are available for all terminals.

complicated any interpretation regarding the origin and evolution of these character states. The presence of oral discs with complete marginal papillation and bicoloured irises in at least some species of Pelodyrinae (e.g. *Litoria dux*, *L. hunti*, and *L. citropa*), coupled with the almost complete ignorance about phylogenetic relationships within Pelodyrinae, imposes ambiguity in the polarity of these character states. This fact left uncertainty in the results of Faivovich et al. (2005) as to whether *Cruziophyla* actually is the sister group of all other phyllomedusines, or if it actually is the sister group of *Phrynomedusa*. In the results of Wiens et al. (2006), *Phrynomedusa* was found to be the sister taxon of all Phyllomedusinae, whereas *Cruziophyla* was recovered as the sister group of *Phasmahyla*. In the re-analysis of these data by Moen and Wiens (2008), using Bayesian methods and a much more restricted—albeit not identified—outgroup sampling, *Cruziophyla* was found to be the sister group of all Phyllomedusinae followed by

*Phrynomedusa*, the latter clade being poorly supported (Bayesian posterior probability of 0.82).

In the present analysis, which includes a substantially larger number of sequences of *Phrynomedusa* ( $\approx 5750$  bp from eight genes), the available specimens of *P. marginata* were found to form a clade with strong support and to be the sister taxon of all remaining phyllomedusines, followed by *Cruziophyla calcarifer* (Fig. 4). In the context of this topology, the optimization of the two character states unique for *Cruziophyla* and *Phrynomedusa* within Phyllomedusinae is dependent on their phylogenetic distribution within Pelodyrinae. Unfortunately, the sparse sampling available of this subfamily does not allow for a satisfactory optimization.

Two specimens of *Phrynomedusa marginata* were sequenced for this study. The specimens were from different localities in the State of São Paulo (Boraceia and São Luiz de Paraitinga), and 12S sequences from a

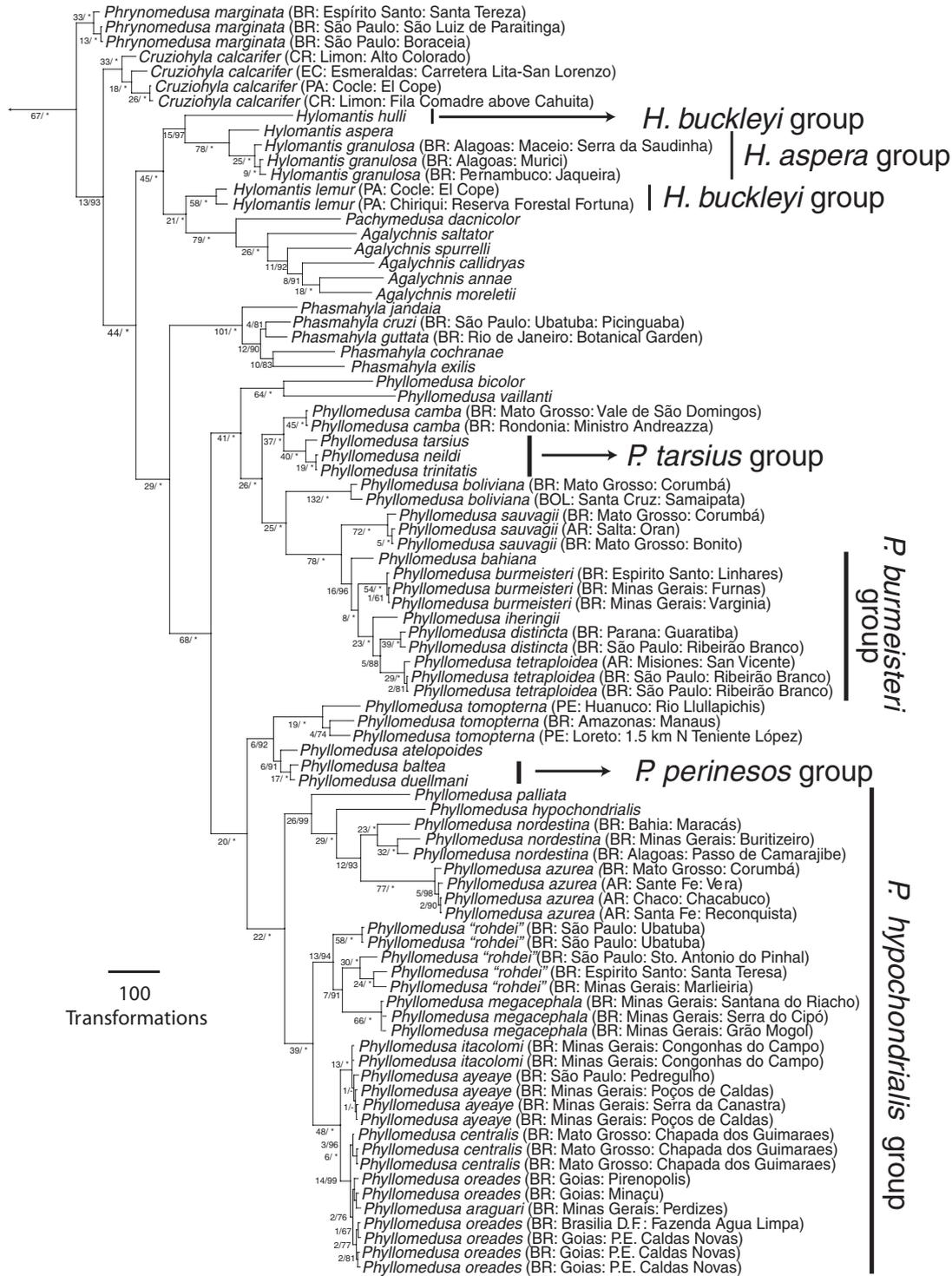


Fig. 4. Topology of Phyllomedusinae in one of the four most parsimonious trees (length 22 959 steps). Numbers close to nodes are Bremer support/Parsimony jackknife absolute frequencies. Nodes without values are those that collapse in the strict consensus (see Fig. 5). Asterisks indicate Parsimony jackknife frequencies of 100%; dashes indicate frequencies  $\leq 50\%$ . Branch lengths are proportional to the number of unambiguous parsimony transformations; not all loci are available for all terminals. Abbreviations: AR, Argentina; BOL, Bolivia; BR, Brazil; CR, Costa Rica; EC, Ecuador; PA, Panama; PE, Peru. See Appendix 2 for complete locality data.

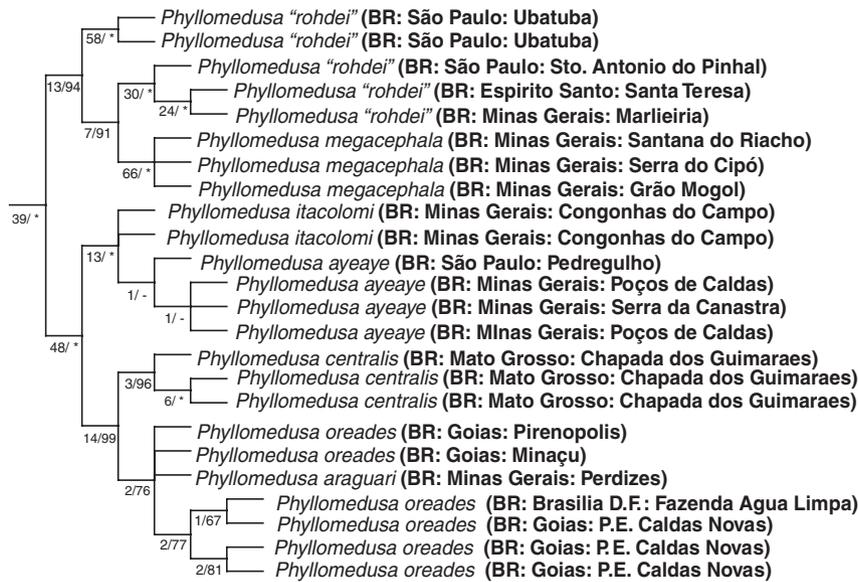


Fig. 5. Strict consensus of the conflicting sectors among the four most parsimonious trees (one of which is shown in Fig. 4); this region corresponds to the *Phyllomedusa hypochondrialis* Group. These results, in conjunction with the very reduced sequence divergence, corroborate the recent suggestion that *P. araguari* is a junior synonym of *P. oreades* (Brandão and Álvares, 2009), and indicates that *P. itacolomi* is a junior synonym of *P. ayeaye* as recently suggested by Baêta et al. (2009). Also note the paraphyly of the populations currently included under the name *P. 'rohdei'* with respect to *P. megacephala*. Numbers close to nodes are Bremer supports/parsimony jackknife absolute frequencies.

third specimen identified as *P. marginata* from the State of Espírito Santo (Santa Teresa, the type locality) produced by Wiens et al. (2005) were acquired from GenBank. We have not studied the voucher specimen for this sequence (USNM 217827), but the pairwise comparison of these 12S sequences indicates that they are somewhat divergent (uncorrected *P* distance = 3.0%). Interestingly, only *P. marginata* has been recorded from localities between the states of São Paulo and Espírito Santo, with the other four species of *Phrynomedusa* having very restricted distributions (Cruz, 1980, 1987, 1991). With the exception of *P. marginata*, species of *Phrynomedusa* are known from small samples, and some are known only from the type series. The observed divergence between samples from distant localities suggests that a careful taxonomic revision of the material assigned to *P. marginata* is necessary in order to understand the significance of these results. A better understanding of relationships within *Phrynomedusa* remains dependent on the collection of additional material of these infrequently encountered frogs.

In addition to the specimen of *Cruziohyla calcarifer* sequenced by Faivovich et al. (2005) from Panama (Cocle: El Cope: Parque Nacional “Omar Torrijos”) and the additional sequences of the same specimen produced for this project, two specimens were sequenced—one from Ecuador (Prov. Esmeraldas: Carretera Lita-San Lorenzo, km 49) and one from Costa Rica (Prov. Limón: Fila Comadre above Cahuita). Furthermore, there are sequences available from Gomez-Mestre

et al. (2008) from a specimen collected in Costa Rica (Prov. Limón: Alto Colorado, 3.5 km north-east of Guayacán, 710 m altitude). Our results found all of these exemplars to form a monophyletic group. The level of sequence divergence in cytochrome *b* (uncorrected *P* distance = 4.0%) among specimens from Ecuador and Costa Rica (Prov. Limón: Fila Comadre above Cahuita) and Panama (Cocle: El Cope: Parque Nacional “Omar Torrijos”) might be explained as geographical variation (there are roughly 1000 km between the closest localities) or could suggest that the taxonomy of the populations currently under the name *C. calcarifer* needs to be addressed. Furthermore, we were surprised to find important sequence divergence between two specimens from Provincia Limón Costa Rica (Fila Comadre above Cahuita, sequenced for this study; and Alto Colorado, 3.5 km north-east of Guayacán, 710 m altitude, sequence available on GenBank). Our specimen from Ecuador, which was collected close to the type locality of *C. calcarifer* (“the Rio Durango, 350 feet”, Provincia Esmeraldas, Ecuador; Boulenger, 1902), could well be considered from the area of the name-bearing population.

*Agalychnis*: absolute congruence with previous hypotheses

Phylogenetic relationships of *Agalychnis* found in this study (Fig. 4) are identical to topologies obtained by Gomez-Mestre et al. (2008; Fig. 2e), whose sequences

were included in our analysis. It is worth noting that a taxonomic revision of *A. litodryas* (included by Faivovich et al., 2005; Wiens et al., 2005, 2006; Moen and Wiens, 2008; Gomez-Mestre et al., 2008) recently considered this species to be a junior synonym of *A. spurrelli* (Ortega-Andrade, 2008).

Considering *Agalychnis*, the only phylogenetic hypothesis to date based on phenotypic characters is that of Duellman (2001). His analysis included 12 morphological characters scored for all species then included in *Agalychnis* (i.e. including the former *A. calcarifer* and *A. craspedopus*, which now are placed in *Cruziohyla*), plus *Pachymedusa dacnicolor*, and an outgroup vector built on the basis of character states of the former *Phyllomedusa buckleyi* Group. Duellman (2001) did not present his optimal trees, but judging from the 50% majority rule tree that he presents (Duellman, 2001; fig. 351a), it is evident that the strict consensus has no resolution. When the two species of *Cruziohyla* are eliminated from Duellman (2001, table 76) dataset, together with the characters that are informative only for these two species (Characters 5, 11, 12), four of the remaining characters (3, 4, 7, 8) are considered autapomorphies of *Pachymedusa*, and only four are considered phylogenetically informative for *Agalychnis*. However, there is a problem with the scoring for Character 7 (palpebral membrane): in the character matrix (Duellman, 2001, table 76) *P. dacnicolor* is scored as having a reticulated palpebral membrane (Char. 7.1), whereas all species then included in *Agalychnis* and the outgroup are scored as having the palpebral membrane not reticulated (Char. 7.0). Actually, the palpebral membrane is reticulated with gold in *A. annae*, *A. callidryas*, *A. moreletii*, *A. saltator*, and *A. spurrelli* (Duellman, 1970). It is reticulated with silvery grey in *C. craspedopus* (Duellman, 2005), and not reticulated in *Pachymedusa dacnicolor*, *Cruziohyla calcarifer* and species of the former *Phyllomedusa buckleyi* Group (Duellman, 1970; Cannatella, 1980). This revised taxonomic distribution plus those of the other four characters are fully congruent with our optimal topology for *Agalychnis*: characters 1.1 (Otic ramus of squamosal in narrow contact with crista parotica) and 6.1 (Cloacal sheath long and directed ventrally) are a synapomorphy of *A. annae* + *A. litodryas* + *A. moreletii* + *A. spurrelli*. Character 2.1 (Quadratojugal short not in bony contact with crista parotica) is a synapomorphy of *A. litodryas* + *A. spurrelli*. Characters 7.1 (Palpebral membrane reticulated) and 9.1 (Iris red or orange) are synapomorphies of *Agalychnis* (note that “Iris red or orange” actually are two character states, not one). In the context of our results, the reticulated palpebral membrane and the reddish hue of the iris are morphological synapomorphies that seem to corroborate the monophyly of *Agalychnis*, with a subsequent transformation into an

orange iris in *A. annae*. Even considering this, the evaluation of homologies among iris colours deserves further discussion, as some are consistently darker (e.g. *A. moreletii*) than others (e.g. *A. callidryas*), and the ancestral state proposed by Duellman (2001: 836, “not red or orange”) requires a careful redefinition.

*The paraphyly of Hylomantis sensu Faivovich et al. (2005)*

Faivovich et al. (2005) tentatively proposed the inclusion of the former *Phyllomedusa buckleyi* Group in the genus *Hylomantis*. This proposition was based on their optimal hypothesis (Faivovich et al., 2005; fig. 2) in which their exemplar of *Hylomantis* (*H. granulosa*) was found to be the sister taxon of the only available exemplar of the former *Phyllomedusa buckleyi* Group (*P. lemur*)—ultimately, the group remained within *Hylomantis* as the *H. buckleyi* Group. However, the only putative synapomorphy for the monophyly of the *H. buckleyi* Group that they could propose on the basis of the results of Cannatella (1980) was the bright orange flanks, although it was stated that the polarity of this transformation was not clear. Support for the redefined *Hylomantis* and the *H. buckleyi* Group was weak; therefore, the authors stated that the new arrangement was provisional until a denser sampling of the group became available.

In our optimal hypothesis (Fig. 4) the monophyletic *Hylomantis aspera* Group (comprising *H. aspera* and *H. granulosa*) is the sister taxon of *H. hulli*, one of the two exemplars from the *H. buckleyi* Group. This clade and *H. lemur* are successive sister taxa to the clade consisting of *Pachymedusa* + *Agalychnis*. These results indicate a lack of evidence for the monophyly of *Hylomantis* as redefined by Faivovich et al. (2005) and of the *H. buckleyi* Group as defined by Cannatella (1980). In addition to molecular data, the monophyly of the *H. aspera* Group is supported by recently published data on this species by Pimenta et al. (2007) who proposed that the liver covered by a white peritoneum is another morphological synapomorphy of the group. The tadpoles of the two species of the *H. aspera* Group (Nascimento and Skuk, 2007; Pimenta et al., 2007) share an oral disc that is relatively enlarged in comparison with that of most phyllomedusines without forming an anterodorsal oral disc modified as a funnel-shaped structure (as occurs in *Phasmahyla*). At this point it is unclear if this state can be considered an intermediate morphological step between those two oral disc configurations.

There are several ways to deal with the paraphyly of *Hylomantis*, as defined by Faivovich et al. (2005). The most conservative approach would be to ignore our results (Fig. 4) and preserve a paraphyletic *Hylomantis* until the remaining species become available in subse-

quent analyses. We do not consider this to be an intellectually viable option, although it would clearly be the least controversial approach, because it would ignore the existing phylogenetic knowledge of this group. This is particularly true given that the analysis presented herein represents the largest and most inclusive effort (considering taxon sampling) to test explicitly the hypotheses of relationships within Phyllomedusinae. If taxonomic changes cannot result from such a thorough and inclusive analysis, then discussions of taxonomic changes should move away from evidence-based methodologies to those of sociology and prior authority based on partial data (Frost et al., 2008).

We suggest that adequate evidence and support exists to follow the logical taxonomic consequences of our phylogenetic results, and we prefer to propose changes to resolve the paraphyly of *Hylomantis*. One solution would be to restrict *Hylomantis* to the *H. aspera* Group from Atlantic Forest remnants in north-east Brazil (*H. aspera* + *H. granulosa*), and to the fragments of the *H. buckleyi* Group associated with them (minimally *H. hulli*). This change also would require a revision of *H. lemur*, which on our tree (Fig. 4) is sister to *Pachymedusa* + *Agalychnis*—two alternatives exist for accommodating a required change to *H. lemur*: (i) describe a new genus to accommodate *H. lemur* and, if any, other components of the *H. buckleyi* Group found to be closely related with this species; or (ii) extend the definition of *Agalychnis* to include *Pachymedusa dacinicolor* and *H. lemur*. Both of these suggestions offer somewhat problematic solutions. With the first option, which requires the description of a new genus for *H. lemur*, there is a potential slippery slope that could lead to the description of additional monotypic genera for barely distinguishable species solely for purposes of preserving the relatively recent redefinition of *Agalychnis* and the monotypic *Pachymedusa*. A problem that is common to both solutions is how to proceed with the four species included in the *Hylomantis buckleyi* Group that were not available for this study (*H. buckleyi*, *H. danieli*, *H. medinae*, and *H. psilopygion*), and how to reconcile the anatomical similarities among the members of the *H. buckleyi* Group. For example, the close relationship between *H. buckleyi* and *H. hulli* seems reasonable, as these species are barely distinguishable. Cannatella (1980) noticed on the basis of overall similarity that *H. lemur* and *H. psilopygion* closely “resemble each other”, and that *H. buckleyi* is more similar to *H. medinae* whereas Cruz (1988, 1990) stated that *H. aspera* and *H. granulosa* closely resemble *H. buckleyi* and *H. psilopygion*, particularly on the basis of the “rugose” skin and the cream-coloured irises [Cruz (1990) also associated these with *Phasmahyla*]. No one has suggested a close relationship between *H. danieli* and any other species in this group using morphological similarity. Besides those suggestive—and contradic-

tory—associations, we are not aware of any putative synapomorphy that would allow us to associate the four unavailable species of the *H. buckleyi* Group with any of the identified clades. This could be dealt with by considering all these species to be *incertae sedis* until they become available and incorporated into a phylogenetic analysis. However, it should be taken into account that there is a possibility that these terminals would not group with any of the name-bearing clades, but rather may enlarge the grade leading to the taxa discussed, specifically *H. lemur*, (*Pachymedusa* + *Agalychnis*), or (*Agalychnis* + *Pachymedusa* + the paraphyletic *Hylomantis*). A radical solution to this potential instability would be to include both *Hylomantis* and *Pachymedusa* in the synonymy of *Agalychnis* and to retain species groups for those species within the two well-supported clades in this redefined *Agalychnis*—one for *Agalychnis* as currently defined (*A. saltator*, *A. spurrelli*, *A. callidryas*, *A. annae*, and *A. moreletii*) and another for those that reside in the *H. aspera* Group. Would this taxonomic change violate long-established notions of diversity in this group? The concept that most herpetologists have of *Agalychnis* (which has been demonstrated to be polyphyletic by Faivovich et al., 2005) is an artefact of a definition provided by Duellman (1968). The only phylogenetic analysis using morphology published for the genus (Duellman, 2001) employed only a handful of characters (12), and identified only a single synapomorphy for *Agalychnis* as redefined by Faivovich et al. (2005; see discussion above in the section “*Agalychnis*: absolute congruence with previous hypotheses”). It is worth noting that a second synapomorphy went unnoticed in the analysis of Duellman (2001) because of a typographical error (reticulated palpebral membrane) in his dataset; this character was identified earlier in the present paper.

Jungfer and Weygoldt (1994) explicitly rejected the possibility of including the former *Phyllomedusa buckleyi* Group in *Agalychnis*, because of “several morphological differences” between these groups, and because of the idea that observed similarities in reproductive biology between these two groups (eggs deposited on open leaves and absence of empty capsules) were putative plesiomorphies. Even though these polarities are corroborated herein, the molecular evidence supports the close relationship of *H. lemur* with *Pachymedusa* and *Agalychnis*, with a more distant relationship with *H. hulli*. For these reasons, the solution that seems most stable and that is consistent with our results is to support a taxonomic revision that includes both *Hylomantis* and *Pachymedusa dacinicolor* within *Agalychnis*. See Appendix 1 for our taxonomic conclusions. As the missing species of *Hylomantis* (*H. buckleyi*, *H. danieli*, *H. medinae*, and *H. psilopygion*) become available, it will be possible to study in more detail

the internal relationships of *Agalychnis*. From this point forward, we base our discussions on this new taxonomy.

### Relationships of *Phasmahyla*

The phylogenetic analysis of Faivovich et al. (2005; Fig. 2a) supported their two exemplars of *Phasmahyla* (*P. cochranae* and *P. cruzi* (as *P. guttata*)) as a well-supported monophyletic group. In addition to the molecular support for this clade, they suggested that a specialized larval oral disc (which is modified as a funnel-shaped structure in these species) and the lack of vocal sacs (Cruz, 1982, 1990) are putative synapomorphies for this genus. The optimal trees (Fig. 4) obtained here corroborated the monophyly of this genus with strong support. Although *Phasmahyla* is reported to lack a vocal sac and vocal slits (Cruz, 1990), males of *P. cochranae*, *P. exilis*, *P. guttata*, *P. jandaia*, and *P. timbo* do emit vocalizations that have been interpreted as advertisement calls (Bokermann, 1966; Bokermann and Sazima, 1978; Cruz, 1980; Cruz et al., 2008b), so the functional basis of this attribute warrants further research. Additionally, Faivovich et al. (2005) mentioned that although the laterodorsal glands (first noted in *Phasmahyla* by Cochran, 1955), could be considered a putative synapomorphy for this genus, further work would be needed to determine the homology of these glands with the parotoid glands, which are absent in this genus. Toledo and Haddad (2009) noticed that when walking, species of *Phasmahyla* sway slightly as if caught by the movement of the wind. The mimesis is increased by the leaf-like coloration of these species. The taxonomic distribution of this behaviour in other phyllomedusinae requires survey, as it could well be another synapomorphy of *Phasmahyla*.

Our results suggest that *Phasmahyla jandaia* is the sister taxon of the remaining species, with *P. cruzi* + *P. guttata* being the sister taxon of *P. cochranae* + *P. exilis*; however, these relationships are not particularly well supported (Fig. 4), and the relationships of the two unavailable species, *P. spectabilis* and *P. timbo*, are unknown. The sample of *P. guttata* included by Faivovich et al. (2005) from São Paulo (Ubatuba: Picinguaba) actually seems to be the newly described *P. cruzi*. The sample added here from Rio de

Janeiro could be considered a topotype of *P. guttata*, as the type locality is “the Carioca mountain at Tijuca, Rio de Janeiro” (Lutz and Lutz, 1939). Although these two similar species (Carvalho e Silva et al., 2009) were found to be monophyletic, the high sequence divergence between them for the cytochrome *b* fragment is striking (see Table 1).

### Phyllomedusa: relationships and species groups

The *Phyllomedusa burmeisteri*, *P. hypochondrialis*, *P. perinesos*, and *P. tarsius* Groups were recovered herein as monophyletic and are well supported (Fig. 4). Furthermore, *Phyllomedusa* was found to be composed of two major, well-supported clades. One of these includes the *P. burmeisteri* and *P. tarsius* Groups, plus *P. bicolor*, *P. camba*, *P. boliviana*, *P. sauvagii* and *P. vaillanti*. The other clade includes the *P. hypochondrialis* and *P. perinesos* Groups, plus *P. atelopoides* and *P. tomopterna*. None of these clades corresponds to previous attempts by Lutz (1950) to partition *Phyllomedusa* into smaller units that were alternatively considered as subgenera (Lutz, 1950) or genera (Lutz, 1966, 1968). In the subgenus *Phyllomedusa*, Lutz (1950) included *P. bicolor* and *P. loris* (a junior synonym of *P. buckleyi*); in the subgenus *Pithecopus* Cope, 1866, she included the *P. burmeisteri* and *P. hypochondrialis* Groups. The name *Pithecopus* (type species: *Phyllomedusa azurea* Cope, 1862) is available for the clade composed of the *P. hypochondrialis* Group, the *P. perinesos* Group, *P. atelopoides*, and *P. tomopterna*, but we do not see the need to resurrect it.

*The Phyllomedusa burmeisteri* group. Faivovich et al. (2005) could not advance any morphological synapomorphies for the *P. burmeisteri* Group, but followed Pombal and Haddad (1992) in retaining this group. Our results indicate a monophyletic *P. burmeisteri* Group comprising *P. burmeisteri*, *P. distincta*, *P. iheringii*, *P. tetraploidea*, and *P. bahiana* that is well supported by molecular evidence (Fig. 4).

The *Phyllomedusa burmeisteri* Group is unique within Phyllomedusinae for having a pair of species considered to hybridize throughout their overlapping ranges. The tetraploid (*P. tetraploidea*) and diploid (*P. distincta*) species produce triploid hybrids that apparently are

Table 1  
Percentage uncorrected pairwise distances between cytochrome *b* sequences of *Phasmahyla*. All localities are in Brazil

	1	2	3	4	5
1— <i>P. cochranae</i> CFBH 7307 (Minas Gerais: Poços de Caldas)	–				
2— <i>P. exilis</i> CFBHt 1448 (Espírito Santo: Cariacica)	11.2	–			
3— <i>P. cruzi</i> CFBH 5756 (São Paulo: Ubatuba)	11.2	12.3	–		
4— <i>P. guttata</i> MNRJ 41688 (Rio de Janeiro, Rio de Janeiro)	18	17.5	15.1	–	
5— <i>P. jandaia</i> MNRJ 39980 (Minas Gerais: Santana do Riacho)	11.5	13.6	11.5	18.2	–

sterile or have low fertility (Haddad et al., 1994). On the basis of indistinguishable vocalizations and their sympatric ranges, Haddad et al. (1994) suggested that *P. tetraploidea* originated through autopolyploidy from *P. distincta*.

*Phyllomedusa bahiana* was described originally as a subspecies of *P. burmeisteri* by Lutz (1950), and it was treated as such by Pombal and Haddad (1992). These authors recognized individuals with an intermediate pattern in the only diagnostic character among both subspecies of *P. burmeisteri* (pattern of the hidden areas of thigh: yellowish rounded blotches on a bluish background in *P. burmeisteri burmeisteri*; patternless in *P. b. bahiana*, smaller and fewer yellowish rounded blotches in the “intermediate”). This observation and the distribution of the frequencies of typical and intermediate individuals throughout their geographical range also prompted the hypothesis of extensive hybridization and existence of fertile hybrids. Silva-Filho and Juncá (2006) elevated *P. b. bahiana* to species status on the basis of differences in calls and larval morphology. They compared calls from a few typical specimens of each form and based their study of larval morphology on those of *P. bahiana* (described by them) and the description of larvae of *P. burmeisteri* (Cruz, 1982).

The scale of our sampling—species level—is too coarse to provide significant contribution towards discussions surrounding the origin and dynamics of these putative hybridization events. However, it is worth noting that the sister species relationship between *P. distincta* and *P. tetraploidea* is compatible with the hypothesis of an origin of the latter by autopolyploidy. Although our results support the notion that *P. bahiana* and *P. burmeisteri* are specifically distinct, we believe that the existence of specimens of intermediate thigh pattern throughout a large portion of the range of these two species—putative fertile hybrids according to Pombal and Haddad (1992)—has not been adequately explained and deserves further studies, possibly in an approach including a much denser sampling of critical specimens from throughout the distribution of these species.

*The Phyllomedusa hypochondrialis group.* Although quite characteristic morphologically and well supported in our results (Fig. 4), only ambiguous phenotypic evidence has been advanced for the monophyly of the *P. hypochondrialis* Group. Faivovich et al. (2005) restated some myological peculiarities originally described by Manzano and Lavilla (1995) and Manzano (1997) in *P. azurea* (using the name *P. hypochondrialis*) that might be considered synapomorphies of the group or one of its internal clades. One of these, the presence of the muscle *epicoracoideus*, was recorded by Faivovich et al. (2005) in *P. rohdei*. Subsequently, Caramaschi (2007) presented a taxonomic definition of the group

focused specifically on several species. In this study, Caramaschi (2007): (i) rediagnosed *P. megacephala* (Miranda-Ribeiro, 1926), which had been resurrected from the synonymy of *P. hypochondrialis* previously by Brandão (2002), but not fully diagnosed; (ii) resurrected and diagnosed *P. azurea* Cope, 1862; and (iii) described *P. nordestina*, a name that applies to the populations of north-eastern Brazil previously considered *P. hypochondrialis*. Furthermore, on the basis of the results of Faivovich et al. (2005), which demonstrated a sister relationship between *P. palliata* and *P. hypochondrialis* [the single exemplar of the group included by Faivovich et al. (2005)], Caramaschi (2007) included the former in the *P. hypochondrialis* Group. It is worth noting that Faivovich et al. (2005) did not consider their own results to be conclusive evidence to support the position of *P. palliata* within the *P. hypochondrialis* Group because they lacked adequate sampling of taxa within *Phyllomedusa* and did not assign species to particular groups. Caramaschi et al. (2007) described another new species, *P. itacolomi*. Subsequently, Giaretta et al. (2007) described *P. araguari*, raising the number of species included in the group to ten and making it the most species-rich group within *Phyllomedusa*. Brandão and Alvares (2009) recently suggested that *P. araguari* is a junior synonym of *P. oreades*.

With respect to non-molecular data and the characterization given by Caramaschi (2007), only one character state (the absence of vomerine teeth) could be considered a putative morphological synapomorphy of the *P. hypochondrialis* Group (Faivovich et al., 2005). However, this state also occurs in *P. atelopoides* (Sheil and Alamillo, 2005) and *Phasmahyla* (Cruz, 1990). Vomerine teeth are present in the *Phyllomedusa perinesos* Group (Cannatella, 1982) and in *P. tomopterna* (Funkhouser, 1957), and in the context of our optimal hypothesis, the absence of vomerine teeth is a putative synapomorphy of the *P. hypochondrialis* Group that is homoplastic with *P. atelopoides*. The lack of an omosternum, which was also mentioned by Caramaschi (2007), has been reported as well in *P. atelopoides*, *P. vaillanti* (Sheil and Alamillo, 2005), and *P. bicolor* (Funkhouser, 1957); there are no reports on its presence in the *P. perinesos* Group and its taxonomic distribution in phyllomedusines is not well known; postcranial osteology remains unknown in most species.

Our results suggest the existence of two well-supported clades within the *P. hypochondrialis* Group (Fig. 4). One of these is formed by the lowland species, *P. palliata* (western Amazon basin), *P. hypochondrialis* (Llanos, central and eastern Amazon basin, and areas of Amazonian influence in the Pantanal), *P. azurea* (Cerrado and Chaco), and *P. nordestina* (Caatinga). The other clade includes *P. rohdei* (a species from the Atlantic forest of Brazil) and the species from plateaus and mountain areas in central-eastern Brazil: *P. ayeaye*

(southern Minas Gerais, and neighbouring São Paulo; Araujo et al., 2007); *P. centralis* (known only for the type locality, Chapada dos Guimarães, in central Mato Grosso); *P. megacephala* (Serra do Cipó, Minas Gerais); and *P. oreades* (Goiás and Brasília). Most species of this latter clade are characterized by the presence of massive heads (superficial dissections show that this is the consequence of an unusual dorsal expansion of the parotoid glands, with the glandular acini sometimes extending even to the dorsal skin covering the snout; J.F., pers. obs.). Additionally, all but *P. rohdei* also have a reticulated colour pattern on their flanks. This pattern, however, seems to have originated independently in *P. megacephala* and the clade containing *P. ayeaye*, *P. centralis*, and *P. oreades* (*P. araguari* is considered a junior synonym of *P. oreades*; *P. itacolomi* is considered a junior synonym of *P. ayeaye*; see below).

The taxonomy of most species of the *P. hypochondrialis* Group is quite difficult. Within the lowland clade, our results are congruent with Caramaschi's (2007) recognition of *P. azurea* and *P. nordestina* as specifically distinct from *P. hypochondrialis*. However, future studies of this species group would benefit from a more extensive sampling of populations that currently are assigned to *P. hypochondrialis*, because this species exhibits considerable external morphological variation, rendering this species only weakly distinguishable from *P. azurea* and *P. nordestina* (Caramaschi, 2007). Although the exemplars from the three distant populations of *P. nordestina* are monophyletic in our analysis (Fig. 4), the level of sequence divergence among them is quite noticeable (see Table 2 for cytochrome *b* uncor-

rected *P*-distances; minimum *P*-distance = 10.4%), and the three samples (one of which is a topotype, Município de Maracás, Bahia; Caramaschi, 2007) span a linear range of more than 1300 km. We are quite surprised by this level of divergence and note that in separate analyses of each gene fragment available for the samples, we obtained the same topology as that shown in Fig. 3 (data not shown). Interestingly, the distance between the localities from which these samples were taken is similar to the distance (roughly 1150 km) spanned by the available samples of its sister taxon, *P. azurea*, in which sequence divergence in cytochrome *b* is much reduced (see Table 2; *P*-distance = 1.6%).

In the second major clade within the *P. hypochondrialis* Group, our results present a complex picture (Fig. 4) in which several exemplars that were assigned to *P. rohdei* form a clade with the three specimens of *P. megacephala*, such that *P. rohdei* is found to be paraphyletic. The topological results alone indicate that at least two species have been confused under the name *P. rohdei*. When also considering the high levels of sequence divergence in percentage uncorrected *P*-distances of cytochrome *b* (Table 3; minimum divergence among pairs = 8.1%), it seems that at least two different species should be recognized and that there is little justification to question the validity of *P. megacephala*. Furthermore, the uncorrected *P*-distances between the samples of São Paulo (Santo Antonio do Pinhal), Minas Gerais (Marlieiria), and Espírito Santo (Santa Teresa) suggest that they possibly represent more than one species. The type locality of *P. rohdei* is Rio de Janeiro (Mertens, 1926) and unfortunately none of our

Table 2

Percentage uncorrected pairwise distances between cytochrome *b* sequences of *Phyllomedusa azurea*, *P. hypochondrialis*, and *P. nordestina*

	1	2	3	4	5	6
1— <i>P. hypochondrialis</i> AMNH A-141109 (Guyana: Dubulay Ranch)	–					
2— <i>P. azurea</i> CFBH 2576 (Brazil: Mato Grosso: Corumbá)	14.3	–				
3— <i>P. azurea</i> MLP DB 2795 (Argentina: Chaco: Charata)	13.3	1.6	–			
4— <i>P. nordestina</i> CFBH 7330 (Brazil: Alagoas: Passo de Camarajibe)	16.4	15.1	14.6	–		
5— <i>P. nordestina</i> CHUNB 4443 (Brazil: Minas Gerais: Buritizeiro)	22.9	22.1	21.9	12	–	
6— <i>P. nordestina</i> CFBH 19538 (Brazil: Bahia: Maracás)	19.5	19.8	19.5	16.7	10.4	–

Table 3

Percentage uncorrected pairwise distances between cytochrome *b* sequences of *Phyllomedusa* “*rohdei*” and *P. megacephala*

	1	2	3	4	5	6	7	8
1— <i>P. “rohdei”</i> (São Paulo: Ubatuba: Itaguá)	–							
2— <i>P. “rohdei”</i> (São Paulo: Ubatuba: Itaguá)	0	–						
3— <i>P. “rohdei”</i> MNRJ 40691 (Espírito Santo: Santa Teresa: São Lourenço)	8.1	8.1	–					
4— <i>P. “rohdei”</i> CRR 18 (Minas Gerais: Marlieiria: Perdizes)	9.4	9.4	2.4	–				
5— <i>P. “rohdei”</i> CFBH 7196 (São Paulo: Santo Antonio do Pinhal)	9.1	9.1	3.7	5	–			
6— <i>P. megacephala</i> CFBH 10225 (Minas Gerais: Grão Mogol)	10.7	10.7	11.7	12	12.3	–		
7— <i>P. megacephala</i> MCNAM 6339 (Minas Gerais: Serra do Cipó: Cardeal Motta)	10.4	10.4	11.5	11.7	12	0.3	–	
8— <i>P. megacephala</i> MCNAM 6338 (Minas Gerais: Santana do Riacho)	10.2	10.2	11.2	11.5	11.7	0.6	0.3	–

exemplars was collected from this site. Do these results suggest the presence of cryptic species? Caramaschi (2007) studied specimens from throughout the range of *P. rohdei*, including material from the same localities that we sequenced, and suggested that although there are differences in coloration, there was no consistent pattern of variation that could support the idea of different species under the name *P. rohdei*. We propose that a thorough revision of all the material assigned to this species is now necessary, and until topotypes can be added to the present analysis, we refer to all these as *P. "rohdei"*.

Another component of this second major clade within the *P. hypochondrialis* Group is a clade composed of exclusively highland species that primarily inhabit plateaus or mountains of central eastern Brazil. The exemplars of *P. centralis* (topotypes) were found to be sister to a clade composed of all the exemplars of *P. oreades* from four localities, including the topotypes and one specimen from Perdizes, Minas Gerais (which is a topotype of *P. araguari*). This clade is sister to a clade composed of specimens of *P. ayeaye* from three localities (topotypes, plus specimens from Serra da Canastra, Minas Gerais, and Pedregulho, São Paulo) and the two specimens of *P. itacolomi* (which were collected very close to the type locality). Within this clade of montane species, we observed very low sequence divergence among the recognized species, despite the fact that many are from very distant localities. For example, the distance between the type locality of *P. centralis* (Chapada dos Guimarães, Mato Grosso; Bokermann, 1965) and the closest sample that we have of *P. oreades* (Pirenópolis, Goiás) is roughly 750 km. Our phylogenetic hypothesis, coupled with the extremely low levels of sequence divergence among these specimens (Table 4), corroborates the recent suggestion that *P. araguari* is a junior synonym of *P. oreades* (Brandão and

Álvares, 2009), whereas *P. itacolomi* should be considered a junior synonym of *P. ayeaye*, as is proposed by Baêta et al. (2009). Therefore, we treat these taxa as synonyms. Despite the low sequence divergence in cytochrome *b* between *P. centralis* and *P. oreades* (Table 4; maximum divergence among pairs,  $P = 0.977$ , minimum  $P = 0.991$ ), available evidence suggests that they are different species. Brandão et al. (2009) have recently shown that these species possess distinct vocalizations and differences in the larval oral disc. The distribution of these montane forms remains poorly known.

*The Phyllomedusa perinesos group.* Evidence of monophyly for this group of Andean cloud forest species (*P. baltea*, *P. duellmani*, *P. ecuatoriana*, *P. perinesos*) was first proposed by Cannatella (1982), who suggested that synapomorphies for this clade included the presence of purple coloration on the hands, feet, flanks, and concealed surfaces, as well as a purple venter with white granules. Our results recover the two exemplars of this group (*P. baltea* and *P. duellmani*) in a well-supported clade (Fig. 4), corroborating the monophyly of the group. No hypothesis of relationships of this group with other species of *Phyllomedusa* has been suggested; however, our results suggest that this clade is sister to *P. atelopoides* [a peculiar, terrestrial species (Duellman et al., 1988) with distinctive osteological characteristics (Sheil and Alamillo, 2005) from the western Amazon], which has remained unassigned to any species group. As the osteology of members of the *P. perinesos* Group are studied, it may be possible to discern if the unique characters states observed in *P. atelopoides* (Sheil and Alamillo, 2005), such as the non-exposed frontoparietal fontanelle and absence of palatines, are in fact autapomorphies of *P. atelopoides* or synapomorphies shared with members of the *P. perinesos* Group. In

Table 4

Percentage uncorrected pairwise distances between cytochrome *b* sequences of *Phyllomedusa araguari*, *P. ayeaye*, *P. centralis*, *P. itacolomi*, and *P. oreades*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1— <i>P. ayeaye</i> CFBHt 153 (Minas Gerais: Serra da Canastra)	–														
2— <i>P. ayeaye</i> CFBH 15672 (São Paulo: Pedregulho)	0.6	–													
3— <i>P. ayeaye</i> CHUNB 51414 (Minas Gerais: Poços de Caldas)	0.3	0.3	–												
4— <i>P. ayeaye</i> CHUNB 51413 (Minas Gerais: Poços de Caldas)	0.6	0.6	0.3	–											
5— <i>P. centralis</i> UFMT 6221 (Mato Grosso: Chapada dos Guimarães)	2.6	2.6	2.3	2.6	–										
6— <i>P. centralis</i> CHUNB 12570 (Mato Grosso: Chapada dos Guimarães)	2.6	2.6	2.3	2.6	0	–									
7— <i>P. centralis</i> CHUNB 12571 (Mato Grosso: Chapada dos Guimarães)	3.4	3.4	3.1	3.4	0.9	0.9	–								
8— <i>P. itacolomi</i> FSFL 858 (Minas Gerais: Congonhas do Campo)	0.6	0.6	0.3	0.6	2.6	2.6	3.4	–							
9— <i>P. itacolomi</i> FSFL 857 (Minas Gerais: Congonhas do Campo)	0.6	0.6	0.3	0.6	2.6	2.6	3.4	0	–						
10— <i>P. oreades</i> CHUNB 56869 (Goiás: P.E. Serra de Caldas)	2.3	2.3	2	2.3	0.9	0.9	1.7	2.3	2.3	–					
11— <i>P. oreades</i> CHUNB 56871 (Goiás: P.E. Serra de Caldas)	2.3	2.3	2	2.3	0.9	0.9	1.7	2.3	2.3	0	–				
12— <i>P. araguari</i> CHUNB 56879 (Minas Gerais: Perdizes)	2.3	2.3	2	2.3	1.5	1.5	2.3	2.3	2.3	0.6	0.6	–			
13— <i>P. oreades</i> CHUNB 51424 (Brasília D.F.: Fazenda Água Limpa)	2.3	2.3	2	2.3	0.9	0.9	1.7	2.3	2.3	0	0	0.6	–		
14— <i>P. oreades</i> CHUNB 49500 (Goiás: Pirenópolis)	2.9	2.9	2.6	2.9	1.5	1.5	2.3	2.9	2.9	1.2	1.2	1.7	1.2	–	
15— <i>P. oreades</i> CHUNB 56875 (Goiás: P.E. Serra de Caldas)	2.6	2.6	2.3	2.6	1.2	1.2	2	2.6	2.6	0.3	0.3	0.9	0.3	1.5	–

the context of our results, the terrestrial habits of *P. atelopoides* clearly are derived from arboreal ancestors.

*The Phyllomedusa tarsius group.* De la Riva (1999) included *P. boliviana*, *P. camba*, *P. coelestis*, *P. sauvagii*, *P. trinitatis*, and *P. venusta* in a poorly defined *P. tarsius* Group. Faivovich et al. (2005) stated that no synapomorphies were known for the group, but continued to recognize it following the suggestion of De la Riva (1999; but see comment in Materials and methods). Barrio-Amorós (2006) redefined the *P. tarsius* Group (comprising *P. coelestis*, *P. neildi*, *P. tarsius*, *P. trinitatis*, and *P. venusta*) and included a taxonomic characterization in which the single putative synapomorphy is the presence of an iris with fine black reticulations. *Phyllomedusa boliviana*, *P. camba*, and *P. sauvagii* were excluded from this species group because they lack black reticulations in the iris.

The exemplars of Barrio-Amorós's (2006) *P. tarsius* Group that were included in the present study (*P. neildi*, *P. tarsius*, and *P. trinitatis*) were recovered as a monophyletic group (Fig. 4) that excluded *P. boliviana* and *P. sauvagii* [a result also proposed by Barrio-Amorós (2006)]. Additionally, we obtained a well-supported sister-group relationship between *P. camba* and the *P. tarsius* Group. The striking sequence similarity among *P. neildi*, *P. tarsius*, and *P. trinitatis* is worth noting, suggesting the need of a fine-grained analysis of the *P. tarsius* Group and, perhaps, a taxonomic revision.

*Species of Phyllomedusa that remain unassigned to any species group.* We included six species of *Phyllomedusa* that historically have not been assigned to any species group (*P. atelopoides*, *P. bicolor*, *P. boliviana*, *P. camba*, *P. sauvagii*, and *P. vaillanti*). In our analysis, *P. atelopoides* was found to be sister to the *P. perinesos* Group. *Phyllomedusa bicolor* (the largest species of the genus) and *P. vaillanti* were recovered as monophyletic by Faivovich et al. (2005) and Wiens et al. (2006), a result that is corroborated here with much denser and more relevant sampling. These species were suggested on the basis of their “intermediate” level of specialization to be closely related by Funkhouser (1957), who placed them in a group that included *P. coelestis*, *P. tarsius* (both now in the *P. tarsius* Group), and *P. boliviana*.

*Phyllomedusa bicolor* and *P. vaillanti* share the presence of osteoderms that protrude beyond the skin as spines covered by a thin layer of epidermis, and that clearly are visible at low magnification. These spines and osteoderms first were noticed on these two species by Boulenger (1882), and were studied in detail by Ruibal and Shoemaker (1984). Funkhouser (1957) also reported osteoderms in *P. tarsius*, but like Ruibal and Shoemaker (1984), we could not confirm this occurrence in the material available to us.

*Phyllomedusa boliviana* and *P. camba* have been confused for many years (De la Riva, 1999), and Barrio-Amorós (2006: 64) stated that they seem to be “definitely very similar to each other”. Our analysis, however, does not recover them as sister species. Rather, *P. boliviana* is found to be the sister of a clade composed of *P. sauvagii* and the *P. burmeisteri* Group, whereas *Phyllomedusa camba* was found to be the sister of the *P. tarsius* Group.

Additionally, *P. sauvagii* has been considered “... one of the most highly specialized members of the evolutionary line” (Funkhouser, 1957: 18) on the basis of the reduced discs on the digits and the prominent parotoid glands. A similar argument has been presented by De la Riva (1999) and Barrio-Amorós (2006), adding that this species probably deserves to be placed in its own species group. Funkhouser (1957: 18) also stated that this species “... probably arose from a form similar to *P. boliviana*”, but did not elaborate on this comment. In our results, *P. sauvagii* is the sister species of the *P. burmeisteri* Group, and together these taxa form a clade that is the sister taxon of *P. boliviana*.

*Phyllomedusa tomopterna* is widely distributed in the Amazon basin, and a comprehensive study of this species that includes samples from throughout its distribution remains to be done. Our three exemplars (two from eastern Peru and the other from Manaus, Brazil) exhibit some sequence divergence (their cytochrome *b* sequences have a 6.4% uncorrected *P*-distance). This observation is consistent with the recent report of Fouquet et al. (2007), in which divergence in a short stretch of the 16S gene was observed between populations assigned to *P. tomopterna* of eastern Peru and the Guyanas. A denser sampling of sequences of this species and a taxonomic revision is necessary to understand better the meaning of this variation.

#### *Reproductive diversity: diversification of the terrestrial egg clutch*

Anuran reproduction resulting in terrestrial egg clutches of various levels of complexity (i.e. from eggs left in moss, moist soil, holes, open or folded leaves, to foam nests on top of water, natural burrows, holes, or trees), in which embryonic or early larval development is followed by passive dropping or active wriggling to a water body where free-living larval development occurs, has arisen independently several times (Noble, 1927; Salthe and Mecham, 1974; Duellman and Trueb, 1986; Wells, 2007). Phyllomedusinae is relatively diverse in terms of characters associated with reproduction, and although all phyllomedusines lay a terrestrial clutch, there is considerable variation in terms of where the clutch is laid (trunks, rock crevices, hanging roots, moss-covered lianas, open leaves, leaves carefully folded by the parents), the composition of the egg clutch (eggs laid

singly or accompanied by eggless capsules), and the site at which larval development occurs (ponds, streams, water trapped in the buttresses of trees, or depressions in fallen trees). Additionally, there are observations of adults that suggest differences among species in behaviour prior to oviposition. For example, in some species the female alone or the amplexing pair together will spend time in the water prior to oviposition so that the female can fill her urinary bladder with water, which subsequently is emptied during oviposition to hydrate the egg jelly capsules (Pyburn, 1970). Diversity also has been recorded at the level of transient embryonic structures associated with attachment to substrate, and in some species the embryo possesses a cement gland. Optimization of these characters on our optimal hypothesis (Fig. 4) provides some clues regarding the evolution of these characteristics and allows us to make predictions regarding their occurrence in species for which these aspects of reproductive biology remain to be observed or studied (Fig. 6). We have optimized and examined the evolution of five characters associated with reproduction and larval development: (i) bladder filling behaviour (absent or present); (ii) site of oviposition (on leaves, standing or fallen tree trunks, rocks, lianas or epiphyte roots); (iii) leaf folding behaviour (absent or present); (iv) eggless capsules (present or absent); and (v) site of larval development (in ponds, streams, water trapped in tree buttresses or fallen trunks).

Bladder-filling behaviour prior to oviposition has been observed in *Agalychnis callidryas* and *A. annae* (Savage, 2003), *A. dacnicolor* (Pyburn, 1970; Bagnara et al., 1986), and apparently *A. moreletii* (Pyburn, 1980) and *Cruziohyla calcarifer* (Roberts, 1994a, 1995). Hoogmoed and Cadle (1991) suggested that this behaviour also occurs in *C. craspedopus* (not included in our analysis). Females or amplexing pairs have been reported not to exhibit water-filling behaviour in *A. saltator* (Roberts, 1994b) and *Phrynomedusa marginata* (Weygoldt, 1991), and this behaviour has not been clearly observed in any published study of reproductive biology of *Phyllomedusa*, despite the fact that considerable attention has been paid to some members of this genus (see below). Gomez-Mestre et al. (2008) state that *A. spurrelli* does not exhibit this behaviour and cited Savage (2002) as evidence; however, we could not find any reference to support this statement in Savage's (2002) study, nor in Scott and Starrett (1974), the reference upon which much of Savage's (2002) account of the biology of this species is based. For this reason, we consider it still unknown whether *A. spurrelli* shows this behaviour. Pyburn and Glidewell (1971), and at least three of us (C.F.B.H., C.L.B.A., K.H.J.) have looked specifically for this behaviour while studying several species of *Phyllomedusa* (all species in the *P. burmeisteri* Group, *P. azurea*, *P. bicolor*, *P. hypo-*

*chondrialis*, *P. neildi*, *P. "rohdei"*, *P. tomopterna*, and *P. trinitatis*) and *Phasmahyla* (*Phasmahyla cochranae*), and it was not observed. Additionally, several careful observations on the courtship sequence of *P. bicolor* (Lescure et al., 1995), *P. boliviana* (Vaira, 2001) and *P. "rohdei"* (Wogel et al., 2005) did not indicate this behaviour. We consider it safe to assume that this behaviour does not occur in these species, and its distribution among these taxa across our phylogenetic hypothesis (Fig. 6) allows us to predict that it probably does not occur in any *Phasmahyla* or *Phyllomedusa*. The only possible exceptions are references by De la Riva (1999: 129) to several amplexant pairs of *Phyllomedusa camba* that were "... found on or near the ground close to the water, from where they climbed to the surrounding trees." De la Riva mentioned the possibility of bladder-filling behaviour, but the evidence that it actually occurs seems inconclusive as he did not report pairs in the water; although water could be absorbed directly from the ground as well, a more thorough study is necessary. The supposed absence of bladder-filling behaviour in *Phasmahyla* and *Phyllomedusa* and in the only studied species of *Phrynomedusa* (*P. marginata*) demonstrates at least two independent origins of this behaviour—one in *Cruziohyla* and the other in the clade of *A. dacnicolor* + the *A. callidryas* Group. Note, however, that its absence in *A. saltator* and unknown character states in *A. spurrelli* implies an ambiguous optimization regarding the origin of this state in *Agalychnis*. These results indicate that controversy remains concerning the evolution of bladder filling behavior, and it remains unclear on which nodes this characteristic was acquired and or lost. Likewise, it remains to be determined if water stored in the bladder by the female also plays a role in the hydration of the egg capsules in the groups in which bladder-filling behaviour prior to oviposition has not been observed. This is very likely as the bladder in terrestrial anurans functions as a water reservoir (for a review, see Jørgensen, 1997) and the eggs have highly hydrated capsules, besides the eggless capsules. It would be interesting to understand if the need to fill the bladder immediately before oviposition versus the use of water that presumably is already available in the bladder is related to differences in physiology of bladder water uptake or water balance in the different groups (see discussion below in the section "Evolution of water-proofing in Phyllomedusinae ..."). Additionally, it should also be considered whether this behaviour is facultative, depending on levels of environmental humidity, and therefore of hydration of the amplexing individuals.

Within Phyllomedusinae egg clutches have been reported to be laid on: (i) trunks, logs, stems, roots, or leaves (*Cruziohyla*: Marquis et al., 1986; Donnelly et al., 1987; Hoogmoed and Cadle, 1991; Caldwell, 1994; Roberts, 1994a, 1995; Block et al., 2003); (ii) rock



Fig. 6. A condensed version of the tree in Fig. 4, showing the taxonomic distribution and optimization of some characters associated with reproductive biology of Phyllomedusinae. Only a single specimen per species is included, and the taxonomy is updated on the basis of the proposals of the present paper. Note that for some characters the state of the condensed outgroup is unknown because due to the extremely scarce taxon sampling we do not consider our results regarding internal relationships of Pelodyadinae as a reliable hypothesis for inferring states at the level of its ingroup node. The characters that are scored for the outgroup correspond to states that have never been reported for Pelodyadinae. The dashed lines in the tree indicate areas of ambiguous optimizations: black, the origin of bladder-filling behaviour; light grey, the origin of post-hatching development in streams in some species of the *Phyllomedusa hypochondrialis* Group. Due to the unknown outgroup state, the optimization of the place of larval development at the base of the tree is ambiguous—it is not shown. A similar situation occurs with the optimization of the site of oviposition; because of the combination of unknown state in the outgroup and the various sites of oviposition in *Cruziophyla* and *Phrynomedusa*, it is not possible to establish at which node oviposition in leaves originated, and hence it is not shown. See Appendix 2 for the literature sources of observations for each species. Multistate characters are treated as non-additive. See text for further discussion.

crevices or standing or fallen trunks (*Phrynomedusa*: Lutz and Lutz, 1939; Cruz, 1982; Weygoldt, 1991; *Agalychnis aspera*: Skuk pers. com); (iii) moss-covered lianas, vines, open leaves, branches, and epiphyte roots (*Agalychnis annae*, *A. moreletii*, *A. saltator*: Roberts, 1994b; Gomez-Mestre et al., 2008); (iv) leaves (*A. callidryas*, *A. dacnicolor*, *A. hulli*, *A. lemur*, *A. spurrelli*; Duellman, 1970; Pyburn, 1970; Jungfer and Weygoldt,

1994; K.H.J., pers. obs); or (v) on a purse-like “nest” composed of one or more folded leaves, as occurs in all species of *Phasmahyla* and *Phyllomedusa* for which clutches have been described or observed (e.g. Bokermann and Sazima, 1978; Langone et al., 1985; Duellman et al., 1988; C.F.B.H., pers. obs.). In *Phyllomedusa*, the parents use their hind limbs to fold the leaf during oviposition. Note that these commonly used descriptors

of the site of oviposition do imply more than one character when they involve the active behaviour of the parents folding the leaf or leaves during mating; therefore, we consider the behaviour of leaf-folding to be different from the site of oviposition.

Although it seems evident that there is some level of intraspecific plasticity in the site of oviposition (particularly in *Cruziophyla* and *Phrynomedusa*), apparently there is little plasticity in *Phasmahyla* and *Phyllomedusa*, in which all evidence suggests that eggs are laid on leaves that are folded around the eggs. Clearly, however, at least two well-supported clades (*Phasmahyla* + *Phyllomedusa*; *A. lemur* + *A. dacnicolor* + *A. callidryas* Group) evolved from ancestors that exhibited oviposition on leaves; leaf-folding behaviour during amplexus evidently is a synapomorphy of *Phasmahyla* + *Phyllomedusa* (Fig. 6).

The issue of whether oviposition on leaves (which occurs in the latter clade and in *Agalychnis*, with the known exception of *A. granulosa*; polymorphic in *A. annae*, *A. moreletii*, and *A. saltator*) is a synapomorphy of these three genera is dependent on how the multiple places of oviposition in *Cruziophyla* are interpreted and coded. Oviposition on trunks, logs, stems, roots, or leaves could be seen as general use of vegetation and the oviposition on leaves among *Agalychnis*, *Phasmahyla*, and *Phyllomedusa* as a specialization, in which case this preference would optimize as a synapomorphy for this clade. It could also be interpreted as a character with multiple states that occur polymorphically in *Cruziophyla*, in which case the appropriate method of coding behavioural characters that relate to site of oviposition might be to combine the different character states that describe the individual site of oviposition into more inclusive categories (e.g. on rock crevices OR tree trunks OR hanging roots/branches OR leaves). With this coding, optimization of these data suggests that the ancestor of *Agalychnis* + *Phasmahyla* + *Phyllomedusa* could have deposited eggs on leaves. However, because of the polymorphic condition observed in *Cruziophyla* and *Phrynomedusa* it is not possible to establish if oviposition on leaves arose in that ancestor or earlier in the history of the group (non-additive optimization, and assuming that the character state in the ingroup node of Pelodyadinae is unknown). The latter coding scheme is shown in Fig. 6; the coding should be considered strictly tentative.

Egg clutches of several phyllomedusines are characteristic in that they contain viable eggs and empty jelly capsules. The presence of the empty capsules was first suggested by Agar (1910), and was then shown experimentally by Pyburn (1980), to play a key role in regulating levels of humidity in a clutch during pre-hatching development, thereby avoiding dehydration and reducing the concentration of diffusible, hazardous

metabolic wastes. A similar role for these capsules has been suggested for its presence in egg clutches of Brevicipitidae and Dendrobatidae, the only other groups of frogs in which eggless capsules are known to occur (Wager, 1965; Lötters et al., 2007; Müller et al., 2007). Additionally, in all known nests of *Phyllomedusa* and *Phasmahyla*, these eggless capsules act as a glue to hold the folded leaf or leaves around the eggs (Pyburn, 1980). During oviposition, the leaf is kept folded by the hind limbs of the parents; subsequent embryonic and early larval development occurs in the folded leaf, glued by the eggless capsules. No experimental study comparable with Pyburn's (1980) has been conducted to understand the physiological role that eggless capsules play, if any, in the exposed egg clutches of *Cruziophyla* and *Phrynomedusa*.

Eggless capsules have been reported in both species of *Cruziophyla* (Hoogmoed and Cadle, 1991; Caldwell, 1994; Roberts, 1994a, 1995), *Phrynomedusa marginata* (Weygoldt, 1991), *P. vanzolinii* (Lutz and Lutz, 1939; using the name *P. appendiculata*), and all species of *Phasmahyla* and *Phyllomedusa* for which clutches have been examined (e.g. Bokermann and Sazima, 1978; Langone et al., 1985; Duellman et al., 1988; C.F.B.H., pers. obs.). Empty capsules do not occur in the clutches of *Agalychnis annae* (Proy, 1993), *A. callidryas*, *A. dacnicolor* (Pyburn, 1970), *A. hulli* (K.H.J., pers. obs.), *A. lemur* (Jungfer and Weygoldt, 1994), *A. saltator* (Roberts, 1994b), and *A. spurrelli* (Scott and Starrett, 1974), and were not observed in a single clutch of eggs from *A. aspera* that were obtained from an amplexing pair held in a collecting bag (Pimenta et al., 2007). The optimization of eggless capsules in clutches suggests that this is a synapomorphy of Phyllomedusinae (no eggless capsules have been reported in pelodyadines with terrestrial clutches; Tyler, 1963; Menzies, 1993; Richards, 2002; Günther, 2006), with a subsequent loss in *Agalychnis* (Fig. 6).

Interestingly, eggless capsules were found to appear earlier during the evolutionary history of phyllomedusines than the behaviour of folding leaves to house the eggs, which is a behaviour seen in all species of *Phasmahyla* and *Phyllomedusa*. This suggests that the structure and biochemistry of the eggless capsules requires further attention, as it is unclear whether or not the eggless capsules afford functional advantages to those taxa that do not fold leaves around their eggs (e.g. allowing for increased adhesion of viable eggs to the leaf surface). The answer to this question would help to understand better the evolutionary origin of leaf-folding behaviour in *Phasmahyla* and *Phyllomedusa*, as this could be associated not only with a behavioural character state transformation, but also with transformations in the chemical nature of the eggless capsule.

Gomez-Mestre et al. (2008) described additional variation in egg clutch structure related to its overall

thickness as a result of the thickness of the gelatinous capsule and additional gelatinous material interspersed between individual eggs. They found that clutches of eggs from *Agalychnis saltator* and *A. spurrelli* are much thinner and less gelatinous than those of *A. annae*, *A. callidryas*, *A. dacnicolor*, *A. moreletii*, and *Cruziohyla calcarifer*. In the context of our results associated with the *A. callidryas* Group, the origin of the thinner clutches optimizes ambiguously (not shown in Fig. 6), as it does in the hypothesis of Gomez-Mestre et al. (2008). The taxonomic variation of this character needs to be assessed in exemplars of the other phyllomedusines for a broader understanding.

At some point early in larval development of phyllomedusines, the larvae wriggle out of their egg capsules and drop into the water where development proceeds to metamorphosis. The site of post-hatching larval development varies considerably from water trapped in fallen trees or tree buttresses (*Cruziohyla*, Hoogmoed and Cadle, 1991; Caldwell, 1994; Block et al., 2003), permanent or temporary slow-moving streams or backwaters of fast-moving streams (*Phrynomedusa*, *Phasmahyla* and polymorphically in some species of the *Phyllomedusa hypochondrialis* Group, *P. vaillanti*, and *A. lemur*; Cruz, 1982; Jungfer, 1988; K.H.J., pers. obs.) to development in still water that is permanent or temporary (most species of *Phyllomedusa* and *Agalychnis*, some occurrences in *Cruziohyla*; Duellman, 1970; Cruz, 1982; Hoogmoed and Cadle, 1991; K.H.J., pers. obs.). Optimization of character states associated with sites of post-hatching larval development (Fig. 6) indicates at least three independent origins of development in streams: once in the common ancestor of *Phasmahyla*, twice in the species of the *P. hypochondrialis* Group, and a possible origin of this behaviour in *Phrynomedusa*. Ultimately, the latter optimization will be dependent on the tree topology within Pelodryadinae, which cannot be determined from these results. The site of larval development in water trapped in fallen trees or tree buttresses that frequently occurs in the two species of *Cruziohyla* arose from development in still water trapped in puddles or small pools in the ground, where tadpoles of these species have also been found (Hoogmoed and Cadle, 1991; K.H.J., pers. obs.).

The reconstruction of two independent origins of post-hatching development in streams in a small clade within the *P. hypochondrialis* Group (*P. ayeaye*, *P. centralis*, *P. megacephala*, *P. oreades*, and *P. "rohdei"*) deserves comment. All species except *P. "rohdei"* occur in plateaus and mountain areas of East–Central Brazil, where they breed in temporary streams that are fed by rain water (Brandão and Álvares, 2009; Brandão et al., 2009). The species *Phyllomedusa ayeaye* and *P. oreades* also breed in pools (Lutz, 1966; Cardoso et al., 1989; Giaretta et al., 2007). Brandão et al. (2009) suggest that

breeding in pools occurs in altered environments but not in pristine areas. Regardless, variation in sites of post-hatching development in these two species determines an ambiguous optimization (Fig. 6) as to the exact node of origin in the clade containing these species and *P. centralis*. The two independent origins of development in streams implied by the optimization are coincident with the independent origins of these species—*P. megacephala* is nested in a clade including the Atlantic Forest species *P. "rohdei"*, whereas *P. ayeaye*, *P. centralis*, and *P. oreades* form a monophyletic group.

The cement gland is a transient embryonic and early larval structure that appears before hatching and which usually disappears at the onset of feeding. It produces a sticky mucous secretion that allows newly hatched larvae to hang motionless from the egg capsules, or from the surfaces of plants or rocks (Nokhbatolfoghahai and Downie, 2005). Information on embryonic and early larval development in phyllomedusines is poor, so available data concerning this character are scarce. The cement gland has been reported to be absent in *Phyllomedusa azurea* (Budgett, 1899) and *P. trinitatis* (Nokhbatolfoghahai and Downie, 2005), and we could not see this structure in embryos of *P. burmeisteri* and *Phasmahyla jandaia* (J.F., pers. obs.). The cement gland occurs in *Agalychnis callidryas* (Pyburn, 1963; Warkentin, 1999). Nokhbatolfoghahai and Downie (2005) related the absence of the cement gland in the studied species of *Phyllomedusa* with the fact that in this genus larvae are known to hatch at later developmental stages and start to feed early, and therefore there is "... no need of the CG [cement gland] to support the usual quiescent posthatching phase" (Nokhbatolfoghahai and Downie, 2005, p. 279). Plasticity in the time of hatching in response to risks such as predation or flooding in the *A. callidryas* Group and *Cruziohyla calcarifer* has been studied in detail (Gomez-Mestre et al., 2008). When variation exists in the time of hatching, a functional cement gland presumably would play a role in fixation to substrate in instances when hatchlings occurs at earlier stages. Clearly, more research is needed into the taxonomic distribution of the cement gland in Phyllo-medusinae. The minimal information currently available affords the opportunity to predict that the cement glands are absent in embryos of *Phasmahyla* and *Phyllomedusa*, and possibly present in the other phyllo-medusines.

#### Multiple origins of gliding

Gliding or directed aerial descent has been redefined as any controlled descent by an organism that converts gravitational energy to useful aerodynamic work (Dudley et al., 2007). The results of the present paper corroborate those of Faivovich et al. (2005) in suggesting a paraphyletic *Agalychnis* (now separated into

*Agalychnis* and *Cruziohyla*) and demonstrate that gliding arose independently in *Agalychnis* and *Cruziohyla*.

Gliding in phyllomedusines has been observed several times in *A. spurrelli* (Scott and Starrett, 1974) and *A. saltator* (Roberts, 1994b), and it is documented in the field only twice in *A. callidryas* (Pyburn, 1964). Duellman and Trueb (1986) include *A. moreletii* in a list of gliding species without further comment. Experimental data confirm that *A. callidryas* exhibits controlled descent behaviour, as it does in *Cruziohyla calcarifer* (McCay, 2001, 2003) and *C. craspedopus* (E. R. Wild, pers. comm.). Dudley et al. (2007) listed *A. dacnicolor* as a gliding species, followed by a string of citations (Pyburn, 1970; Scott and Starrett, 1974; Roberts, 1994b; Duellman, 2001; Faivovich et al., 2005; Pauly et al., 2005), none of which has any reference to gliding in *A. dacnicolor*; therefore, we consider that published evidence supporting this statement is lacking. There are no observations on occurrence of gliding in *A. annae*, a species for which there are no studies in the field. Morphologically, *A. annae* is similar to the gliding species in the development of webbing in the hands and feet (Duellman, 1970). On the basis of demonstrated gliding (albeit infrequently) in *A. callidryas* and *A. moreletii*, it is most parsimonious to predict its occurrence in *A. annae*.

A set of phenotypic character states have been associated with the ability to glide and arboreality: (i) enlarged hands and feet; (ii) full webbing on fingers and toes; and (iii) accessory skin flaps on margins of arms and legs (Emerson and Koehl, 1990). All characters occur in species of *Cruziohyla*, and in the context of our results, (i) and (ii) appeared independently in *Agalychnis*. Various species of *Agalychnis* that are known to glide show different levels of development of the webbing and size of hands and feet, with *A. saltator* having the less developed webbing (Duellman, 1970; Roberts, 1994b).

Gliding behaviour has been associated with synchronized descent to breeding sites in species that exhibit explosive reproduction and that live high in the forest canopy but that are dependent on standing water at ground level to reproduce (Roberts, 1994b; Wells, 2007). Our results offer some support to this hypothesis. *Agalychnis saltator* and *A. spurrelli* are explosive breeders (Scott and Starrett, 1974; Roberts, 1994b; Gomez-Mestre et al., 2008), whereas all other species that are known or predicted to glide (*A. annae*, *A. callidryas*, *A. moreletii*, *Cruziohyla calcarifer*, and *C. craspedopus*) are prolonged breeders (Pyburn, 1970; Caldwell, 1994; Gomez-Mestre et al., 2008). The fact that *A. saltator* and *A. spurrelli* are explosive breeders, gliders, and successive sister groups to a crown clade of prolonged breeders seems congruent with an origin associated with explosive breeding. Furthermore, it is noticeable that in

the prolonged breeders, *A. annae*, *A. callidryas*, and *A. moreletii*, records of gliding are so far only predicted by our hypothesis or rarely observed in nature (Pyburn, 1964). Note, however, that the optimization of the origin of explosive breeding is ambiguous, as it could be plesiomorphic for the *A. callidryas* Group or may have appeared independently in *A. saltator* and *A. spurrelli*. Gliding in *Cruziohyla* cannot be associated with explosive breeding, as available data indicate that these species are prolonged breeders.

Independent evolution of gliding behaviour has arisen relatively few times within Anura (see Dudley et al., 2007). Whereas it is remarkable that such an infrequent mode of locomotion has evolved independently at least twice in such a restricted clade, it is worth noting that, in the same way as with the diversity seen in terrestrial clutches, phyllomedusines also show a diversity of morphological characters associated with different forms of arboreality.

#### *Evolution of waterproofing in Phyllomedusinae: origins of the reduction of evaporative water loss and uricotelism*

Many comparative physiological studies have focused on members of Phyllomedusinae, particularly with species of *Phyllomedusa* and to a lesser extent with *Agalychnis*. Many of these studies were inspired by the simultaneous discovery in the early 1970s of the extraordinarily low rate of evaporative water loss and the presence of uricotelism in several species of *Phyllomedusa* (Shoemaker et al., 1972).

Evaporation of water through the skin (i.e. evaporative water loss, EWL) typically is very high in amphibians, and occurs at approximately the same rate in agar models of similar size and shape (Spotila and Berman, 1976; Wygoda, 1984; Shoemaker et al., 1992). Although it seems well established that, in general, arboreal frogs do exhibit lower EWL rates than non-arboreal frogs (Wygoda, 1984; Shoemaker et al., 1992; Young et al., 2005), EWL levels in studied species of *Phyllomedusa* [*P. azurea* (as *P. hypochondrialis*), *P. boliviana* (as *P. pailona*), *P. sauvagii*, and *P. tetraploidea* (as *P. iheringii*; Blaylock et al., 1976)] are even much lower. In fact, rates of EWL in *Phyllomedusa* are comparable with those of lizards (Shoemaker and McClanahan, 1975), and species of this genus sometimes are referred to as the “water-proof frogs” in physiological literature. Such highly efficient waterproofing in species of *Phyllomedusa* is achieved by means of a waxy skin secretion that is produced by skin lipid glands (Blaylock et al., 1976) and spread over the body by the action of their rear and hind limbs in a motion that is referred to as “wiping behaviour” (Blaylock et al., 1976). The waxy secretion is a heterogeneous mixture of wax esters and triglycerides (McClanahan et al., 1978) that block water evaporation up to 38–39 °C, after which evaporation

increases proportionally to the difference between body and air temperature (McClanahan et al., 1978; Shoemaker et al., 1987). Wiping behaviour has been described (but EWL unrecorded) in *P. iheringii* (Langone et al., 1985), *P. distincta*, *P. tarsius* (Castanho and De Luca, 2001), and *P. "rohdei"* from Espírito Santo, Brazil (D.B., pers. obs.). Evaporative water loss rates in *Agalychnis annae* and *A. dacnicolor* are much higher than in *Phyllomedusa* (Shoemaker and McClanahan, 1975; Bentley and Yorio, 1979), but still lower than in many other arboreal non-phyllomedusines (Wygoda, 1984). The source of the relatively low EWL rate in *Agalychnis* is not well understood as these species mostly lack lipid glands and waxy secretions (Shoemaker and McClanahan, 1975; Bentley and Yorio, 1979).

Shoemaker and McClanahan (1975) were reluctant to generalize that low EWL was characteristic of all species of *Phyllomedusa* because they could not examine species from the humid tropics. However, Castanho and De Luca (2001) reported wiping behaviour in *P. tarsius*, a species from the Amazon basin. As the waxy secretion and wiping behaviour are the main explanation for low EWL rates in this group, it seems reasonable to assume that this behaviour in Phyllomedusinae would indicate low EWL rates; a similar deduction could be made on the basis of the presence of lipid glands. These glands have been reported without any associated behavioural or EWL rate study in *P. trinitatis* (Thomas et al., 1990) and in *P. bicolor* (Lacombe et al., 2000), the former being distributed in humid forests in Trinidad and northern Venezuela, and the latter being a species from the Amazon basin. Considering the phylogenetic relationships of the species whose EWL has so far been studied or in which wiping behaviour has been reported, as implied by our optimal hypothesis, we can predict that the extremely low EWL also occurs in all species of *Phyllomedusa*. Concomitantly, we expect that as observations are carried out in other species of *Phyllomedusa*, the wiping behaviour will be found. In fact, we suggest that a critical test of this prediction may not even require actual observation of the wiping behaviour as a histological study demonstrating the presence or absence of lipid glands may suffice. EWL rates and presence or absence of wiping behaviour remain unknown in *Cruziohyla*, *Phrynomedusa*, and *Phasmahyla*. Higher EWL rates and absence of wiping behaviour in studied species of *Agalychnis* would allow the prediction of similar character states at least in the unstudied species of *Agalychnis*, *Cruziohyla*, and *Phrynomedusa*; however, there is some indication that this may not be the case. Observations indicate that there is a thick whitish precipitate that covers much of the dorsal skin on freshly fixed specimens of *A. granulosa*; (Marcelo G. de Lima, pers. comm.) we interpret this precipitate to be the waxy secretion.

*Phyllomedusa sauvagii* was reported to excrete its nitrogenous wastes mostly in the form of precipitated

uric acid (i.e. urate; Shoemaker et al., 1972), rather than as ammonia or urea (the typical excretion products of amphibians; Shoemaker et al., 1992). Subsequent to the study of urate precipitates, Shoemaker and McClanahan (1975) showed that *P. azurea*, *P. boliviana*, and *P. tetraploidea* also excrete urate, but at a lesser proportion in terms of total nitrogen waste produced by excretion (a minimum of 25%, compared with 80% in *P. sauvagii*). The excretion of urate consumes less water than does excretion of ammonia or urea, and therefore has been considered an adaptation to arid environments (Shoemaker et al., 1972; Duellman and Trueb, 1986; Campbell et al., 1987).

Concentrations of urate in excretions from *A. annae* and *A. dacnicolor* is negligible (Shoemaker and McClanahan, 1975), as in most other known amphibians. The limited knowledge on the taxonomic distribution of urate excretion in the context of our optimal hypothesis affords us the opportunity to predict its occurrence in species of *Phyllomedusa* that have not been studied. The distribution of urate excretion across the subfamily remains uncertain, but may be restricted to *Phyllomedusa* or *Phyllomedusa* + *Phasmahyla*. Until it is determined by studies of these species, we predict negligible concentrations of urate in the excretions of *Cruziohyla*, *Phrynomedusa*, and remaining species of *Agalychnis*, on the basis of its absence in *A. annae* and *A. dacnicolor*.

Blaylock et al. (1976) reasoned that the amount of water saved by uricotelism would be of little significance to amphibians with a freely evaporative skin because a negative water balance by means of EWL would far exceed the savings yielded by uricotelism; therefore, they suggested that the origin of uricotelism must have been simultaneous with (or preceded by) the origin of an impermeable skin. This prediction seems to be fully corroborated by our optimal hypothesis (Fig. 4), that would restrict the origin of uricotelism to the hypothetical ancestor of either *Phasmahyla* + *Phyllomedusa*, or perhaps only to *Phyllomedusa*. Depending upon which of these two alternatives receives greater support from future evidence (i.e. whether *Phasmahyla* is shown to be uricotelic at some level), the origin of a decreased EWL could be traced to at least one or two nodes deeper in the tree (e.g. the hypothetical ancestor of *Agalychnis* + *Phasmahyla* + *Phyllomedusa*).

The origin of reduced EWL could also be traced even earlier in the evolution of Phyllomedusinae. Buttemer and Thomas (2003) and Young et al. (2005) reported EWL in 19 species of pelodyadines, and most of them have at least low to moderate resistance to EWL that is well above levels of resistance typically observed among frogs that lack resistance to EWL; all the arboreal species studied by these authors [*Litoria gracilentata* (*L. gracilentata* Group), *L. xanthomera*, *L. chloris* (*L. chloris* Group), *L. splendida*, *L. gilleni*, *L. caerulea* (*L. caerulea* Group), *L. fallax*, *L. bicolor* (*L. bicolor* Group), *L. rothii*,

*L. peronii* (*L. peronii* Group), *L. rubella* (*L. rubella* Group)] have moderate to high resistance to EWL. Resistance to EWL in pelodyadines has been attributed to two factors: (i) proteinaceous and lipid skin secretions that have not been characterized completely (Amey and Grigg, 1995; Christian and Parry, 1997; Buttemer and Thomas, 2003), but that have not been associated with the waxy secretions of *Phyllomedusa*; and (ii) a very characteristic resting posture for these frogs, in which the ventral surfaces of the body are concealed and form a seal to the perching substrate, with the limbs folded underneath and the gular region flattened to the resting surface (see Young et al., 2005; for some variations). Studies on skin histology are notoriously scarce in pelodyadines, but an ultrastructural study of *Litoria caerulea* (Warburg et al., 2000) indicated the presence of lipid glands. The occurrence of levels of resistance to EWL in the sister group of Phyllomedusinae, with all studied arboreal species having moderate to high levels of resistance, *a priori* cannot be ruled out as homoplasy.

### Bioactive peptides

Phyllomedusines are well known for the large amount of bioactive peptides that have been isolated either from skin granular secretions or from structural deductions from skin cDNA libraries. Hundreds of papers have been published dealing with the isolation, structure, and pharmacology of these compounds. These peptides have various functions ranging from several potent antibiotics and antifungals (e.g. Mor et al., 1991; Thompson et al., 2007), some even with anti-HIV activity (VanCompernelle et al., 2005), to extremely potent  $\mu$ - and  $\delta$ -opioid receptor antagonists (such as dermorphins and deltorphins; Montecucchi et al., 1981; Wechselberger et al., 1998), and insulinotropic peptides (Marenah et al., 2004) (see Erspamer, 1994; Simmaco et al., 1998; Negri et al., 2000; and Pukala et al., 2006; for reviews of biological activity of several peptide families). The use of some of these peptides and/or their analogues or peptide mimics in human health is quite promising (Rotem and Mor, 2009). Some even have been shown to improve resistance to phytopathogens in transgenic potatoes (Osusky et al., 2005). Interestingly, pelodyadines (the sister group of phyllomedusines) have also been shown to be an extraordinary source of bioactive peptides, also with diverse functions (e.g. Erspamer et al., 1984; Doyle et al., 2002; Brinkworth et al., 2005).

Current knowledge of anuran bioactive peptides suggests that they are only partially amenable to phylogenetic interpretation. Most of the peptides isolated from phyllomedusines have been divided into several families on the basis of amino acid sequence and pharmacological characteristics (see Erspamer, 1994 for a review; Chen et al., 2004 for a further refinement; and Amiche et al., 2000; Leite et al., 2005; and Thompson

et al., 2007 for new families of antibiotic peptides), but there are various peptides that have not been associated with any of these families (e.g. Gebhard et al., 2004). The precursors of dermaseptins B, dermaseptins S, dermatoxins, dermorphins, deltorphins, hyposins, phyllokinins, phylloseptins, phylloxins, plasticins, and tryptophylins (11 of the peptide families shown to occur in Phyllomedusinae), caerins (a peptide family occurring in both Phyllomedusinae and Pelodyadinae), those of aureins, frenatins, and maculatin (peptide families occurring in Pelodyadinae), and those of several antimicrobial peptides isolated from ranoid frogs have been shown to belong to the same precursor gene family (preprodermaseptin), which is characterized by a conserved signal peptide (an acidic propiece) and peptide progenitor sequence (Amiche et al., 2000; Vanhoye et al., 2003; Zhou et al., 2005; Chen et al., 2006; Wang et al., 2008; Nicolas and El Amri, 2008). With few exceptions (e.g. caerulein and sauvagine), no particular peptide has been isolated from more than one species (see Pukala et al., 2006; Nicolas and El Amri, 2009). Currently, we consider that a species-level analysis of the evolution of bioactive peptides or its precursors in Phyllomedusinae should await much better knowledge of taxonomic distribution and genetic mechanisms.

However, when considered in the context of the phylogenetic hypothesis of Phyllomedusinae presented in this paper, the available information on taxonomic distribution of some bioactive peptides allows us to make some predictions about their occurrence, in a manner similar to Smith and Wheeler (2006) approach to venomous fishes. Additionally, this method may also allow us to tentatively consider their presence or absence as putative synapomorphies for particular clades. We recognize that our interpretations may be misled by the common custom of not publishing negative results. Note, however, that one of our interpretations (see below) is based on a published negative result.

Among phyllomedusines, the majority of the peptides so far described are from several species of *Phyllomedusa*, but more recently several have been described from *Agalychnis* (e.g. Wechselberger et al., 1998; Marenah et al., 2004; Conlon et al., 2007) and *Cruziohyala* (Abdel-Wahab et al., 2005). Peptides have been isolated or deduced from only 17 of 60 known phyllomedusines: five species of *Agalychnis* [*A. annae*, *A. callidryas*, *A. dacnicolor*, *A. lemur*, and *A. spurrelli* (using the name *A. litodryas*)]; 11 species of *Phyllomedusa* [*P. azurea* (in same cases using the name *P. hypochondrialis*), *P. bicolor*, *P. burmeisteri*, *P. distincta*, *P. hypochondrialis*, *P. nordestina* (using the name *P. hypochondrialis*), *P. orades*, *P. rohdei*, *P. sauvagii*, *P. tarsius*, and *P. trinitatis*], and *Cruziohyala calcarifer*. *Phasmahyla* and *Phrynomedusa* have not been prospected.

A first prediction suggested by the taxonomic distribution of all peptides so far isolated is that minimally all

species of Phyllomedusinae in the genera *Agalychnis* and *Phasmahyla* that remain un-prospected do, in fact, produce peptides of several of the peptide families already identified. This is evident in that peptides of the families of deltorphins, dermorphins, dermasseptins B and S, dermatoxins, phyllokinins, phylloseptins, plasticins, tryptophylins, and the peptide sauvagine have been isolated from species spread across the clade containing *Agalychnis*, *Phasmahyla*, and *Phyllomedusa*, and negative results regarding its occurrence have not been published. Phylloxins so far are known in *Phyllomedusa bicolor* and *P. sauvagii* (Pierre et al., 2000; Chen et al., 2005a,b). Our results suggest that minimally these peptides should be found in all other species of the clade that includes these species, *P. camba*, *P. boliviana*, and the *P. burmeisteri* and *P. tarsius* Groups, if not in a more inclusive clade. To date, only a single peptide has been isolated from *Cruziophyla*, and it has not been associated with any of the peptide families (Abdel-Wahab et al., 2005); therefore, it is difficult to predict if these peptide families occur further down the tree, in *Phrynomedusa*, as none of these peptide families has yet been reported in Pelodryadinae.

The presence of several of the peptide families listed above, upon better knowledge of their taxonomic distribution within Phyllomedusinae, could well be considered as synapomorphies of the clades where they are known to occur. With regard to biological activity, it seems worthy to note that although antibiotic peptides are known to occur in several other anurans (even if from other peptide families), the extremely potent and structurally unusual opioid peptides deltorphin and dermorphin and the seemingly extraordinary sauvagine (Erspamer, 1994) are so far known only in Phyllomedusinae—peptides of comparable biological activity have not been isolated from other anurans.

Peptides of the caerulein family (Phyllocaerulin) are absent at least in the *P. hypochondrialis* Group, as their content was < 1 µg/g in the exemplars studied and reported by Erspamer et al. (1985, 1986), *P. azurea* (as *P. hypochondrialis*), *P. palliata*, and *P. rohdei* (*P. atelopoides*, *P. tomopterna*, and the *P. perinesos* Group so far have not been prospected). Considering that its presence has been reported in three species of *Agalychnis* and several species of one of the two main clades of *Phyllomedusa* (*P. bicolor*, *P. sauvagii*, *P. burmeisteri*, *P. tarsius*, and *P. trinitatis*), our results predict its presence at least in the remaining species of this clade and *Agalychnis*, and in *Phasmahyla*. Furthermore, the presence of caerulein in several pelodryadines (Erspamer et al., 1984) could suggest its presence as well in *Cruziophyla* and *Phrynomedusa*, but this would depend on the optimal internal topology of Pelodryadinae.

The taxonomic distribution of other peptides, such as the bombesin-like peptides, represented by the phyllolitorins is more ambiguous. Phyllolitorins were isolated

from *Phyllomedusa sauvagii*, *P. burmeisteri*, *P. azurea*, and *P. “rohdei”*, and phyllolitorin-like activity was recorded in *P. trinitatis*; negative results were reported in *Agalychnis callidryas*, *A. dacnicolor*, and *Phyllomedusa bicolor* (Falconieri Erspamer and Severini, 1987; Mignogna et al., 1997). Although this could be the actual picture of the taxonomic distribution of this peptide family, implying some level of homoplasy in reference to our best hypothesis, Mignogna et al. (1997) stressed that litorins may not be detectable due to the oxidation of the C-terminal Met residue, which has been shown to provoke a total decay of activity in bombesin-like peptides. If we take into account that bombesin-like peptides have been isolated from some pelodryadines (where they are known as the litorin peptide family, see Erspamer et al., 1984), as in the case of caeruleins, the topology of pelodryadines would ultimately determine the limits of the group of phyllomedusines predicted to have litorins. Furthermore, if the presence of bombesin-like peptides in pelodryadines and phyllomedusines is shown to be explainable as a single historical event, it could well be considered as a synapomorphy of that clade.

#### *The monophyly of Pelodryadinae: a pending issue in the phylogeny of hylids*

Whereas the monophyly of Phyllomedusinae is highly corroborated on the basis of molecular data and several morphological character states of adults and larvae (Faivovich et al., 2005; Wiens et al., 2005), lack of detailed understanding about the internal topology of its sister group, the Australopapuan Pelodryadinae, imposes a degree of uncertainty for our understanding of relationships within Phyllomedusinae. To date, Pelodryadinae has been recovered as monophyletic in most molecular analyses (Faivovich et al., 2005; Frost et al., 2006; Wiens et al., 2005, 2006; this study), which have included (when most densely sampled) 24 of the 188 known species (Frost, 2009). In all these studies, the exemplars were chosen mostly on the basis of availability, and therefore were selected essentially at random with respect to the phylogenetic diversity of the subfamily. At most, these included exemplars of only 15 out of the 37 currently recognized species groups, the subgenus *Cyclorana*, and some (seven of 24) of the species of *Litoria* formerly included in the genus *Nyctimystes*. Morphological synapomorphies for Pelodryadinae are still dubious (see Faivovich et al., 2005: 53) and the only phylogenetic analysis that has employed morphological data to test the monophyly of this subfamily (Haas, 2003) failed to recover its monophyly. To this, we would add that some character states present in the two less inclusive clades of Phyllomedusinae (i.e. *Cruziophyla* and *Phrynomedusa*) occur in some pelodryadines: bicoloured iris occurs at least in *Litoria dux* and *L. hunti* (Richards and Oliver, 2006a; Richards et al., 2006); complete

marginal papillae occur in larvae of *L. booroolongensis*, *L. citropa*, *L. lessueuri*, *L. personata*, *L. rivicola*, *L. subglandulosa*, and *L. staccato* (Tyler et al., 1978; Anstis, 2002; Günther and Richards, 2005; Doughty and Anstis, 2007); well-developed hand and toe webbing (comparable with that of *Cruziophyla*) occurs in *L. dux*, *L. graminea*, *L. hunti*, *L. sauroni*, and several other species (Tyler, 1968; Richards and Oliver, 2006a; Richards et al., 2006). The fact that some species of Pelodyadinae that have not been included in any analysis lay terrestrial clutches of eggs [the *Litoria iris* and *L. prora* Groups, *L. longirostris*, and possibly *L. umarensis* (McDonald and Storch, 1993; Menzies, 1993; Günther, 2006)] does not allow us to infer at this point whether or not the behaviour of laying a terrestrial clutch within Phyllomedusinae is a synapomorphy of the group or of a more inclusive clade. These shared character states could be dismissed as homoplasies related perhaps to arboreal life or lotic environments in a different evidentiary context (such as a well-sampled phylogenetic hypothesis of Pelodyadinae in which all of the taxa with the relevant character states are far enough from the ingroup node so as to preclude any shared origin of these with Phyllomedusinae). However, in the current state of knowledge these seem to be appropriate reasons to question the monophyly of Pelodyadinae until it can be rigorously tested.

More generally, existing uncertainty of the phylogenetic relationships of Pelodyadinae also hinders our understanding of character evolution in all Hyalinae. For example, as with Phyllomedusinae, several pelodyadines lay large, yolk-rich, unpigmented eggs, including all known species formerly placed in *Nyctimystes* with known eggs, the *L. angiana*, *L. arfakiana*, *L. becki*, *L. dorsivena*, *L. iris*, *L. leucova*, *L. nannotis*, *L. napaea*, and *L. prora* species groups, *L. rivicola*, and *L. spartacus* (Tyler and Davies, 1978, 1979; Menzies, 1993; Günther and Richards, 2005; Richards and Oliver, 2006b). A similar situation is described in several cophomantaine hylids (the less inclusive tribe of Hyalinae), in the genera *Myersiophyla*, *Hyloscirtus*, *Aplastodiscus*, and *Hypsiboas* (the *H. benitezi* Group, Faivovich et al., 2006). Ignorance of the relationships of Pelodyadinae does not allow inference as to whether eggs with these characteristics are plesiomorphic for all hylids or not.

### Concluding remarks

This analysis included new sequence data from up to 10 mitochondrial and nuclear loci (including also GenBank sequences for a non-overlapping fragment of RAG-1, plus TNS3, and exon 2 from c-myc) as well as three intervening transfer RNAs (isoleucine, valine and leucine) for 45 of the 60 known species of Phyllomedusinae. Our analyses resulted in a well-supported phylogenetic hypothesis for phyllomedusines. The areas

of our hypothesis that need more attention in future studies include: testing the relationships of *Cruziophyla* and *Phrynomedusa* to the rest of Phyllomedusinae; evaluating internal relationships for *Agalychnis*, as redefined herein; and testing the position of those species of *Phasmahyla* for which character support is relatively low (Fig. 4). Our results underline interesting patterns of sequence diversity within *Phyllomedusa*, with cases of very low sequence divergence between recognized species (*P. centralis* and *P. oreades*), as well as cases with very high divergence between populations currently considered to be the same species (e.g. *P. nordestina* and *P. tomopterna*). The phylogenetic hypothesis proposed here (Fig. 4) provides a historical framework for a discussion of the evolution of characters associated with reproductive biology, gliding behaviour, the physiology of water loss control, and bioactive peptides. Regarding the last of these, phyllomedusines have been prominent stars in the history of bioactive peptide prospecting in amphibians, in terms both of diversity of biological activities and number of isolated peptides. Our results allow us, for the first time, to make several general predictions regarding their occurrence in still unprospected species. As more species are systematically prospected, peptides will offer additional sources of evidence to test phylogenetic hypotheses.

A non-molecular dataset is still the main pending issue for phylogenetic studies of Phyllomedusinae. The several character systems that have been studied with restricted taxonomic sampling [myology (Manzano and Lavilla, 1995; Manzano, 1997; Burton, 2004); chromosome morphology (e.g. Barrio, 1976; Schmid et al., 1995), spermatozoid ultrastructure (Costa et al., 2004), adult osteology (Sheil and Alamillo, 2005), larval external morphology (Cruz, 1982); larval internal oral morphology (Wassersug, 1980; Vera Candiotti, 2007), larval anatomy (Fabrezi and Lavilla, 1992; Haas, 2003; Sheil and Alamillo, 2005; Vera Candiotti, 2007), tongue morphology and feeding behaviour (Deban and Nishikawa, 1992; Gray and Nishikawa, 1995), vocalizations (e.g. Duellman, 1970), reproductive biology (e.g. Jungfer and Wegoldt, 1994)] should serve as a starting point to score informative phenotypic variation in phyllomedusines, which in turn will complement and test our phylogenetic knowledge for this unique and charismatic group of frogs in a total-evidence context.

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#### Note added in proof

While this paper was in press, a publication by Rosauer et al. (Rosauer, D., Laffan, S.W., Crisp, M.D.,

Donnellan, S.C., Cook, L.G., 2009. Phylogenetic endemism: a new approach for identifying geographical concentrations of evolutionary history. *Molecular Ecology* 18, 4061–4072) included as supplementary data a phylogenetic analysis including nearly 120 species of pelodyadines. The analysis is based on 1587 bp from the 12S and 16S mitochondrial genes, and includes five phyllomedusines (*Agalychnis dacnicolor*, *A. saltator*, *A. spurrelli*, *Phyllomedusa tomopterna* and *P. palliata*) as the only outgroups. The support for the internal relationships of Pelodyadinae in general is weak, and the results are not discussed in the publication. This contribution was published at the same time that we were correcting proofs, so its interesting implications could not be explored in the present paper.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

### Appendix S1. GenBank numbers

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

## Appendix 1

Taxonomic conclusions: a redefinition of *Agalychnis* Cope, 1864.

*Agalychnis* Cope, 1864.

Type species: *Agalychnis callidryas* Cope, 1862

*Hylomantis* Peters, 1873 “1872”. Type species: *Hylomantis aspera* Peters, 1873 “1872”, by monotypy. **New synonym.**

*Pachymedusa* Duellman, 1968. Type species: *Phyllomedusa dactylicolor* Cope, 1864 by original designation. **New synonym.**

**Diagnosis:** This genus is diagnosed solely on the basis of molecular data from the genes RAG-1, CXCR4, POMC, SIAH1, 12S, 16S, and ND1. No morphological synapomorphies are known.

**Characterization:** The genus is composed of a grade of similar looking, slender frogs that leads to a clade of the heavily built *Agalychnis dacnicolor* plus the *A. callidryas* Group. This genus includes phyllomedusines that lack a bicoloured iris (present in *Cruziophyla* and *Phrynomedusa*), parotid glands are absent or poorly developed, and egg-laying occurs on open leaves (i.e. not folded, as done by *Phasmahyla* and *Phyllomedusa*), tree trunks, or lianas. Extensive webbing on hands and feet is present in the *A. callidryas* Group but absent in all other species.

**Contents:** 14 species, eight divided in two species groups, and six unassigned to any group.

#### *Agalychnis aspera* Group

**Diagnosis:** This group is supported by molecular synapomorphies, lanceolate discs on digits, presence of a white peritoneum over the liver (Pimenta et al., 2007), and possibly the presence of a slip of the *M. depressor mandibulae* originating from the dorsal fascia at the level of the *M. dorsalis scapulae* [the condition is unknown in its sister taxon, *A. hulli*, and in *A. danieli*, but is known to be absent in all other species of *Agalychnis*, *Cruziophyla*, *Phasmahyla*, and *Phrynomedusa* (Duellman et al., 1988; Cruz, 1990)].

**Characterization:** See Cruz (1990).

**Comments:** The tadpoles of the two species of the *A. aspera* Group (Nascimento and Skuk, 2007; Pimenta et al., 2007) share an oral disc that is relatively large in comparison with that of most phyllomedusines, without forming an anterodorsal oral disc modified as a funnel-shaped structure, as seen in *Phasmahyla*. At this point it is unclear if it can be considered an intermediate morphological step between those two oral disc configurations.

**Contents:** *Agalychnis aspera* (Peters, 1882) new comb.; *Agalychnis granulosa* (Cruz, “1988” [1989]) new comb.

#### *Agalychnis callidryas* Group

**Diagnosis:** This group is supported mostly by molecular synapomorphies. The gold reticulated palpebral membrane and the red hue of the eyes are putative morphological synapomorphies (red hue with a subsequent transformation into orange iris in *A. annae*). Webbing on the hands and feet is more extensively developed than in the other species of the genus, but is still variable within the group (see Duellman, 1970)—this could be an additional putative synapomorphy.

**Characterization:** See Duellman (1970, 2001).

**Comments:** This group contains all species included in *Agalychnis* as redefined by Faivovich et al. (2005). It is not immediately clear whether the golden reticulated palpebral membrane should be considered homoplastic

within Phyllomedusinae with the silvery grey palpebral reticulation reported by Duellman (2005) for *Cruziophyla craspedopus*.

**Contents:** *Agalychnis annae* (Duellman, 1963); *Agalychnis callidryas* Cope, 1862; *Agalychnis moreletii* (Duméril, 1853); *Agalychnis saltator* Taylor, 1955; *Agalychnis spurrelli* (Boulenger, “1913” [1914]).

Species unassigned to any group

*Pachymedusa dacnicolor* and all species of the former *Hylomantis buckleyi* Group are included in *Agalychnis*, but remain unassigned to any species group. These are: *Agalychnis buckleyi* (Boulenger, 1882) new comb.; *Agalychnis dacnicolor* (Cope, 1964) new comb.; *Agalychnis danieli* (Ruiz-Carranza et al., 1988) new comb.; *Agalychnis hulli* (Duellman and Mendelson, 1993) new comb.; *Agalychnis lemur* (Boulenger, 1882) new comb.; *Agalychnis medinae* (Funkhouser, 1962) new comb.; *Agalychnis psilopygion* (Cannatella, 1980) new comb.

Inclusion of the former *Phyllomedusa danieli* in *Agalychnis* is as tentative as was its inclusion in the former *Phyllomedusa buckleyi* Group by Ruiz-Carranza et al. (1988) and in *Hylomantis* by Faivovich et al. (2005). This species has been known from a single specimen, and recent efforts by L. Barrientos to collect additional specimens in the type locality were unsuccessful.

## Appendix 2

Literature sources for the character states included in Fig. 6.

Sources for individual observations for each terminal taxon are: *Agalychnis aspera* and *A. granulosa* (Pimenta et al., 2007; Skuk and Nascimento, 2007); *A. lemur* (Jungfer and Weygoldt, 1994), *A. annae*, *A. dacnicolor*, *A. callidryas*, *A. moreletii*, *A. saltator*, *A. spurrelli* (Pyburn, 1963, 1964, 1970, 1980; Duellman, 1970; Bagnara et al., 1986; Roberts, 1994a,b; Gomez-Mestre et al., 2008); *Cruziophyla calcarifer* (Donnelly et al., 1987; Caldwell, 1994; Roberts, 1994b, 1995); *Phasmahyla jandaia* (Bokermann and Sazima, 1978); *P. cochraniae* (Bokermann, 1966; C.F.B.H., pers. obs.); *P. guttata* (Lutz and Lutz, 1939; Izecksohn and Carvalho e Silva, 2001; C.F.B.H., pers. obs.); *P. exilis* (Cruz, 1980); *Phyllomedusa azurea* (Budgett, 1899); *P. hypochondrialis* (Pyburn and Glidewell, 1971; Pyburn, 1980); *P. baltea* and *P. duellmani* (Cannatella, 1982); *P. bicolor*, *P. tomopterna*, *P. vaillanti* (Lescure, 1975; Duellman, 1978; Lescure et al., 1995); *P. camba* (De la Riva, 1999); *P. sauvagii* (Agar, 1910); *P. distincta* (Pombal and Haddad, 2005; C.F.B.H., pers. obs.); *P. burmeisteri* (Abrunhosa and Wogel, 2004); *P. boliviana* (Vaira, 2001); *P. tetraploidea* (Pombal and Haddad, 1992); *P. iheringii* (de Sá and Gehrau, 1983; Langone et al.,

1985); *P. trinitatis* (Kenny, 1968; C.L.B.A., pers. obs.); *P. tarsius* (Duellman, 1978); *P. neildi* (Barrio-Amorós, 2006); *P. atelopoides* (Duellman et al., 1988); *P. centralis* (Brandão et al., 2009); *P. oreades* (Brandão, 2001; Brandão and Álvares, 2009); *P. megacephala* (Eterovick and Sazima, 2004); *P. rohdei* (Lutz and Lutz, 1939; Wogel et al., 2005; C.F.B.H., pers. obs.).

### Appendix 3

Locality data (GenBank numbers for these specimens are provided as additional Supporting Information in Appendix S1)

Abbreviations are as follows: AM (Australian Museum, Sydney, Australia); AMNH (American Museum of Natural History, New York, USA); CFBH (Coleção Célio Fernando Baptista Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil); CFBHt Tissue collection, CFBH Collection (when tissue is voucher by itself, in the case of larvae); CHUNB (Coleção Herpetológica, Universidade Nacional de Brasília, Brasília, Brazil); CRR (Camila R. Rabelo Field Series); CVULA (Colección de Vertebrados, Universidad de los Andes, Mérida, Venezuela); DLS-N (David L. Scheltinga field series); FSFL (Felipe Sá Fortes Leite and Bruno Pacheco field series); JAC (Jonathan A. Campbell field series); JMR (Jeanne M. Robertson field series); KRL (Karen R. Lips field series); KU (The University of Kansas, Museum of Natural History, Lawrence, Kansas, USA); LSMZ (Louisiana State University, Museum of Zoology, Baton Rouge, Louisiana, USA); MACN (Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, Buenos Aires, Argentina); MCNAM (Museu de Ciências Naturais, Pontifícia Universidade Católica de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil); MJH (Martin J. Henzel field series); MLP DB (Colección Herpetología, Diego Baldo, Museo de La Plata, La Plata, Provincia de Buenos Aires, Argentina) MNRJ (Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil); QCAZ (Museo de Zoología de la Pontificia Universidad Católica del Ecuador, Quito, Ecuador); RDS (Rafael O. De Sá field series); ROM (Royal Ontario Museum, Toronto, Canada); SAMA (South Australian Museum, Adelaide, South Australia); SMNS (Staatliches Museum für Naturkunde, Stuttgart, Germany); TNHC (Texas Natural History Collection, Austin, Texas, USA); UFMT (Universidade Federal do Mato Grosso, Instituto de Biociências, Coleção Zoológica, Cuiabá, Mato Grosso, Brazil); UTA-A (University of Texas at Arlington, Texas, USA); ZU-FRJ (Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil).

Locality data

*Myersiohyala kanaima*: ROM39582: Guyana: Mount Ayanganna. *Hypsiboas multifasciatus*: AMNH A-141040: Guyana: Demerara: Ceiba Station, Madewini River, ca. 3 mi (linear) E Timehri Airport. *Scinax staufferi*: UTA A-50749: Guatemala: Zacapa: 2.9 km S Teculután, on road to Huit. *Dendropsophus nanus*: MACN 37785: Argentina: Entre Ríos: Depto. Islas del Ibicuy. *Acris crepitans*: LSMZ H-2164: USA: Alabama: De Kalb Co.: powerline access, 0.1 mi W of Lookout Mt. Boys Camp Rd. *Pseudis minutus*: MACN 37786: Argentina: Entre Ríos: Depto. Islas del Ibicuy: Ruta 12 vieja. *Phyllodytes luteolus*: N/A: Brazil: Espírito Santo: Setiba, Guarapari. *Trachycephalus venulosus*: AMNH A-141142: Guyana: Dubulay Ranch on the Berbice River, 200 ft. *Litoria peronii*: DLS n/n: Australia: no data. *L. arfakiana*: TNHC 51936: Papua New Guinea: Madang: ca. 10 km NW Simbai, Kaironk Village, 2000 m. *L. coplandi*: ABTC 12734: N/A. *L. chloris*: SAMA 25759: N/A. *L. rubella*: DLS-N72395: Australia: no data. *L. meiriana*: SAMA 17215: Australia: Western Australia: Black Rock, near Kununurra. *L. freycineti*: SAMA 12260: Australia: New South Wales: 16 km E Retreat. *L. inermis*: SAMA R53945: Australia: Western Australia: 24 km N Tunnel Creel Gorge. *L. infrafrenata*: N/A: Pet trade. *L. microbelos*: ABTC 12696: N/A. *L. pulchra*: SAMA 45335: Papua New Guinea: Magidobo, SHP. *L. foricula*: LM 424 (AA9697-99): N/A. *L. kubori*: AMNH A-82822: Papua New Guinea: Moroba: Wau. *L. cheesmanae*: AMNH A-82799: Papua New Guinea: Morobe: Garaina, ca. 2300 ft. *L. narinosa*: AMNH A-82845: Papua New Guinea: Tambul. *L. papua*: LM 425 (AA9400): N/A. *L. dayi*: SAMA R41010: Australia: Queensland: Pilgrim Sands. *L. nannotis*: SAMA R40266: Australia: Queensland: Paluma. *L. lesueurii*: SAMA R35012: Australia: New South Wales: Murrumbidgee River. *L. genimaculata*: SAMA R41068: Australia: Northern Territory: Mt. Lewis. *L. caerulea*: AMNH A-168409: Pet trade. *L. aurea*: AM 52744: New Caledonia: Province Nord: Valle Phaaye, Nomac River, 8 km E Poum. *L. (Cyclorana) brevipes*: DLS-N72023: N/A. *L. (Cyclorana) australis*: SAMA R16906: Australia: No data. *L. (Cyclorana) manya*: DLS-N72386: N/A. *Agalychnis annae*: Captive specimens, Henry Vila Zoo: Costa Rica: San Jose. *A. aspera*: MNRJ 35370: Brazil: Bahia: Itacarai: Parque Estadual Serra do Conduru, Setor Norte. *A. callidryas*: RDS795: Captive bred in the Baltimore National Aquarium. *A. dacnicolor*: JAC 22009: Mexico: Guerrero: Carretera Tierra Colorada-Ayutla, 187 m. *A. granulosa*: ZUFRJ 7926: Brazil: Pernambuco: Jaqueira. *A. granulosa*: FACN 075: Brazil: Alagoas: Maceio: Serra da Saudinha. *A. granulosa*: MNRJ 50123: Brazil: Alagoas: Murici: Fazenda Bananeira. *A. hulli*: To be deposited in SMNS: Ecuador: Prov. Napo: Selva Viva ca. 18 km ENE Ahuano. *A. lemur*: KRL 955: Panama: Cocle: El Cope: Parque

Nacional “Omar Torrijos”. *A. lemur*: MVZ210463: Panama: Chiriqui: Reserva Forestal Fortuna, 14 km N (by road) Los Planes, woods below Vivero Forestal, 1200 m. *A. moreletii*: N/A: Pettrade. *A. saltator*: JMR 609: Costa Rica: Prov. Heredia: La Selva Biological Station. *A. spurrelli*: QCAZ 13217: Ecuador: Unknown. *Cruziohyla calcarifer*: IGM15: Costa Rica: Limón, Alto Colorado, 3.5 km NE Guayacán, 710 m. *C. calcarifer*: KRL 800: Panama: Cocle: El Cope: Parque Nacional “Omar Torrijos”. *C. calcarifer*: To be deposited in SMNS: Costa Rica: Prov. Limón: Fila Comadre above Cahuita. *C. calcarifer*: To be deposited in SMNS: Ecuador: Prov. Esmeraldas: Carretera Lita-San Lorenzo, km 49. *Phasmahyla cochranæ*: CFBH 7307: Brazil: Minas Gerais: Poços de Caldas. *P. exilis*: CFBHt 1448: Brazil: Espírito Santo: Cariacica. *P. guttata*: MNRJ 41688: Brazil: Rio de Janeiro: Rio de Janeiro: Horto Botânico. *P. guttata*: CFBH 5756: Brazil: São Paulo: Ubatuba: Picinguaba. *P. jandaia*: MNRJ 39980: Brazil: Minas Gerais: Santana do Riacho: Serra do Cipó: Alto Palácio. *Phrynomedusa marginata*: CFBH 7613: Brazil: São Paulo: São Luiz do Paraitinga: Santa Virginia: PESM. *P. marginata*: MZUSP 137423: Brazil: São Paulo: Boracéia: Salesópolis. *P. marginata*: USNM 217827: Brazil: Espírito Santo: Santa Teresa, near edges of Reserva Biológica Nova Lombardia. *Phyllomedusa araguari*: CHUNB 56879: Brazil: Minas Gerais: Perdizes, 900 m. *P. atelopoides*: KU 215381: Peru: Madre de Dios: Cuzco Amazónico: 15 Km E Puerto Maldonado, 200 m. *P. ayeaye*: CFBHt 153: Brazil: Minas Gerais: Serra da Canastra. *P. ayeaye*: CFBH 15672: Brazil: São Paulo: Pedregulho: P. E. das Furnas do Bom Jesus. *P. ayeaye*: CHUNB 51421: Brazil: Minas Gerais: P.N. Serra da Canastra: São Roque de Minas, 1300 m. *P. ayeaye*: CHUNB 51414: Brazil: Minas Gerais: Poços de Caldas. *P. ayeaye*: CHUNB 51413: Brazil: Minas Gerais: Poços de Caldas, 1400 m. *P. azurea*: MZUSP 70801: Argentina: Santa Fe: Reconquista. *P. azurea*: CFBH 2576: Brazil: Mato Grosso: Corumbá. *P. azurea*: MLP DB 3449: Argentina: Santa Fe: Vera. *P. azurea*: MLP DB 2795: Argentina: Chaco: Chacabuco: Charata. *P. bahiana*: CFBH 2596: Brazil: Sergipe: Areia Branca: Serra de Itabahiana. *P. baltea*: To be deposited in SMNS: Peru: Depto. Pasco: Santa Cruz, near Oxapampa, 2050 m asl. *P. bicolor*: AMNH A-168459: Pet trade. *P. boliviana*: CFBH 2571: Brazil: Mato Grosso: Corumbá. *P. boliviana*: To be deposited in SMNS: Bolivia: Depto. Santa Cruz: Samaipata. *P. burmeisteri*: CFBHt152: Brazil: Espírito Santo: Linhares. *P. burmeisteri*: CFBH 17360: Brazil: Minas Gerais: Furnas. *P. burmeisteri*: FSFL 429: Brazil: Minas Gerais: Varginha. *P. camba*: UFMT 1909: Brazil: Mato Grosso: Vale de São Domingos. *P. camba*: CFBH 17278: Brazil: Rondonia: Ministro Andreazza. *P. centralis*: UFMT 6221: Brazil: Mato Grosso: Chapada dos Guimarães. *P. centralis*: CHUNB 12570: Brazil: Mato

Grosso: Chapada dos Guimarães, 750 m. *P. centralis*: CHUNB 12571: Brazil: Mato Grosso: Chapada dos Guimarães. *P. distincta*: CFBH 2658: Brazil: Paraná: Guaratuba. *P. distincta*: CFBH 2114: Brazil: São Paulo: Ribeirão Branco. *P. duellmani*: KU212206: Peru: San Martín: Rioja: Venceremos, 89 m NW Rioja, 1630 m. *P. hypochondrialis*: AMNH A-141109: Guyana: Dubu-lay Ranch on the Berbice River. *P. iheringii*: MNRJ 18782: Brazil: Rio Grande do Sul: Santa Maria. *P. itacolomi*: FSFL 858: Brazil: Minas Gerais: Congonhas do Campo. *P. itacolomi*: FSFL 857: Brazil: Minas Gerais: Congonhas do Campo. *P. megacephala*: MCNAM 6339: Brazil: Minas Gerais: PARNA Serra do Cipó: Cardeal Mota. *P. megacephala*: MCNAM 6338: Brazil: Minas Gerais: Santana do Riacho: Faz Calçada/Pico do Breu. *P. megacephala*: CFBH 10225: Brazil: Minas Gerais: Grão Mogol. *P. neildi*: CVULA 6503: Venezuela: Falcón: Municipio Petit: Vicinity of Murucusa. *P. nordestina*: CFBH 7330: Brazil: Alagoas: Passo de Camarajibe. *P. nordestina*: CHUNB 44443: Brazil: Minas Gerais: Buritizeiro. *P. nordestina*: CFBH 19538: Brazil: Bahia: Maracás: Fazenda Cana Brava. *P. oreades*: CHUNB 56869: Brazil: Goiás: PE Serra de Caldas, 1300 m. *P. oreades*: CHUNB 56871: Brazil: Goiás: PE Serra de Caldas, 1100 m. *P. oreades*: CHUNB 51424: Brazil: Brasília D.F.: Fazenda Agua Limpa, 1200 m. *P. oreades*: CHUNB 49500: Brazil: Goiás: Pirenópolis, 1300 m. *P. oreades*: CHUNB 56875: Brazil: Goiás: PE Serra de Caldas: Caldas Novas, 1100 m. *P. oreades*: CHUNB 49937: Brazil: Goiás: Minaçu, 950 m. *P. palliata*: To be deposited in SMNS: Bolivia: Beni: Rurrenabaque. *P. “rohdei”*: CFBHt 181: Brazil: São Paulo: Ubatuba: Itaguá. *P. “rohdei”*: CFBHt 93: Brazil: São Paulo: Ubatuba: Itaguá. *P. “rohdei”*: CFBH 7196: Brazil: São Paulo: Santo Antonio do Pinhal. *P. “rohdei”*: MNRJ 40691: Brazil: Espírito Santo: Santa Teresa: São Lourenço. *P. “rohdei”*: CRR-18: Brazil: Minas Gerais: Marliéria: Perdizes. *P. sauvagii*: CFBH 2573: Brazil: Mato Grosso: Corumbá. *P. sauvagii*: MACN 40002: Argentina: Salta: Oran: Pichanal: Ruta Prov. 5 y Rio San Francisco. *P. sauvagii*: CFBH 14250: Brazil: Mato Grosso: Bonito. *P. tarsius*: MJH 67: Brazil: Amazonas: Manaus: Reserva Duke. *P. tetraploidea*: CFBH 2464: Brazil: São Paulo: Ribairão Branco. *P. tetraploidea*: CFBH 1725: Brazil: São Paulo: Ribairão Branco. *P. tetraploidea*: MACN 37796: Argentina: Misiones: Guarani: San Vicente: Campo Anexo INTA “Cuartel Rio Victoria”. *P. tomopterna*: CFBH 2451: Brazil: Amazonas: Manaus. *P. tomopterna*: MJH 7076: Peru: Huanuco: Rio Llullapichis: Panguana. *P. tomopterna*: KU 221949: Perú: Loreto: 1.5 km N Teniente López, 310 m. *P. trinitatis*: CVULA 7086: Venezuela: Miranda: El Hatillo. *P. vail-lanti*: AMNH A- 166288: Guyana: Berbice River camp at ca. 18 mi (linear) SW Kwakwani (ca. 2 mi downriver from Kurundi River confluence), 200 ft.