

## A molecular perspective on the phylogeny of the *Hyla pulchella* species group (Anura, Hylidae)

Julián Faivovich,<sup>a,b,c,\*</sup> Paulo C.A. García,<sup>d,e</sup> Fernando Ananias,<sup>f,g</sup> Laura Lanari,<sup>c</sup>  
Néstor G. Basso,<sup>h</sup> and Ward C. Wheeler<sup>i</sup>

<sup>a</sup> Division of Vertebrate Zoology (Herpetology), American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA

<sup>b</sup> Department of Ecology, Evolution, and Environmental Biology, Columbia University, USA

<sup>c</sup> División Herpetología, Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, Ángel Gallardo 470, 1405 Buenos Aires, Argentina

<sup>d</sup> Curso de Pós-Graduação em Zoologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), C.P. 199, 13506-900, Rio Claro, São Paulo, Brazil

<sup>e</sup> Universidade de Mogi das Cruzes, Área de Ciências da Saúde, Curso de Ciências Biológicas, Av. Dr. Cândido Xavier de Almeida e Souza, 200, 08780-911, Mogi das Cruzes, SP, Brazil

<sup>f</sup> Universidade Braz Cubas, Curso de Ciências Biológicas, Rua Francisco Rodrigues Filho, 1233—CEP 08773-380, Mogi das Cruzes, SP, Brazil

<sup>g</sup> Programa de Pós-Graduação em Biologia Celular e Estrutural, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil

<sup>h</sup> Centro Nacional Patagónico (CENPAT)—CONICET, Boulevard Brown s/n., 9120 Puerto Madryn, Chubut, Argentina

<sup>i</sup> Division of Invertebrate Zoology, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA

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### Abstract

A molecular phylogenetic analysis of the *Hyla pulchella* species group was performed to test its monophyly, explore the interrelationships of its species, and evaluate the validity of the taxa that were considered subspecies of *H. pulchella*. Approximately 2.8 kb from the mitochondrial genes 12s, tRNA valine, 16s, and Cytochrome *b* were sequenced. The analysis included 50 terminals representing 10 of the 14–15 species currently recognized in the *H. pulchella* group, including samples from several localities for some taxa, several outgroups, as well as two species previously suspected to be related with the group (*Hyla guentheri* and *Hyla bischoffi*). The results show that the *H. pulchella* and *Hyla circumdata* groups are distantly related, and, therefore, should be recognized as separate groups. As currently defined, the *H. pulchella* group is paraphyletic with respect to the *Hyla polytaenia* group; therefore, we recognize the *Hyla polytaenia* clade in the *H. pulchella* group. Two subspecies of *H. pulchella* recognized by some authors are considered full species including *Hyla pulchella riojana* because it is only distantly related to *H. pulchella*, and *Hyla pulchella cordobae* because molecular and non-molecular evidence suggests that it is specifically distinct. With the inclusion of the *H. polytaenia* clade, *H. guentheri*, and *H. bischoffi*, and the recognition of the two former subspecies of *H. pulchella* as distinct species, the *H. pulchella* group now comprises 25 described species. All representatives of the *H. pulchella* group with an Andean distribution are monophyletic and nested within a clade from the Atlantic forest from south-southeastern Brazil/northeastern Argentina, and Cerrado gallery forest from central Brazil.

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### 1. Introduction

The large, clearly non-monophyletic genus *Hyla* (Campbell and Smith, 1992; Duellman, 2001; Duellman and Campbell, 1992; Faivovich, 2002), currently includes 335 species (Frost, 2002), most of which are placed into approximately 40 species groups. Slightly

\* Corresponding author.

E-mail addresses: [julian@amnh.org](mailto:julian@amnh.org) (J. Faivovich), [pcgarcia@hotmail.com](mailto:pcgarcia@hotmail.com) (P.C.A. García), [feanancias@brazcubas.br](mailto:feanancias@brazcubas.br) (F. Ananias), [nbasso@cenpat.edu.ar](mailto:nbasso@cenpat.edu.ar) (N.G. Basso), [wheeler@amnh.org](mailto:wheeler@amnh.org) (W.C. Wheeler).

more than half of these are confined to South America. For its local abundance and number of species, one of the most conspicuous species groups of *Hyla* in Southern South America, is the *Hyla pulchella* species group.

This group was recognized by Bokermann (1963), without comment, while describing *Hyla cymbalum*. (Bokermann used the name *Hyla raddiana* Fitzinger, 1826, instead of *H. pulchella* Duméril & Bibron, 1841; see Garcia et al., 2003 for a detailed nomenclatural history.) The group was studied in detail by Barrio (1965) who focused on what he considered to be a single polytypic species, *H. pulchella* with 5 subspecies: *Hyla pulchella andina* L. Müller, 1924; *Hyla p. cordobae* Barrio, 1965; *Hyla p. riojana* Koslowsky, 1895; *Hyla pulchella prasina* Burmeister, 1856; and the nominal subspecies. Later, Lutz (1968) added *Hyla pulchella joaquini* B. Lutz, 1968.

Lutz (1973) proposed the formal recognition of the “cycle” of *H. pulchella*, elevating *H. p. prasina* to species status, and including in this group *H. cymbalum*, *Hyla marginata* Boulenger, 1887, and *Hyla semiguttata* A. Lutz, 1925. Faivovich (1996) included *Hyla caingua* Carrizo, 1991 in the group, without further comment; this action was corroborated by Cruz and Caramaschi (1998). Langone (1997) considered *H. p. joaquini* and *H. semiguttata* as synonyms of *H. marginata*. Furthermore, Langone suggested that *Hyla guentheri* Boulenger 1886, a species that Lutz (1973) included in the *H. polytaenia* group, could be related to the *H. pulchella* group, although he stressed the difficulties of this association due to the lack of a clear definition of the *H. pulchella* and *H. polytaenia* species groups. Boulenger (1887) stated in its original description that *Hyla bischoffi* was allied to *H. pulchella*. Lutz (1973) stated that *H. bischoffi* and *H. pulchella* were similar, but she associated the former species with the *H. polytaenia* species group.

Duellman et al. (1997) considered *Hyla pulchella andina* to be a distinct species and redefined the *H. pulchella* group to include: *Hyla albonigra* Nieden, 1923; *H. andina*; *Hyla balzani* Boulenger, 1898 (a senior synonym of *H. callipleura* Boulenger, 1903, fide Duellman et al., 1997); *H. cymbalum*; *H. marginata*; *Hyla marianitae* Carrizo, 1992; *H. melanopleura* Boulenger, 1912; *H. palaestes* Duellman et al., 1997; *H. prasina*; *H. p. pulchella*; *H. p. cordobae*; *H. p. joaquini*; *H. p. riojana*, and *H. semiguttata*. Duellman et al. (1997) also proposed the inclusion of the *Hyla circumdata* group in the *H. pulchella* group and considered the possibility that *Hyla armata* Boulenger, 1902, may be a highly derived species related to the *H. pulchella* group. However, their cladogram showed *H. armata* as sister species of the *Hyla larinopygion* group, and Kizirian et al. (2003) stated that this group cannot be diagnosed exclusive of *H. armata*. Cruz and Caramaschi (1998, 2000), Garcia et al. (2001, 2003), and Cabral Eterovick and Brandão (2001) did not follow the proposed merging of the *H. pulchella* and the *H. circumdata* groups.

The contents of the *Hyla pulchella* group were further modified by Caramaschi and Cruz (2000), who included *Hyla ericae* Caramaschi and Cruz, 2000. Garcia et al. (2001) redescribed *H. marginata* and Garcia et al. (2003) redescribed *H. p. joaquini*, which they considered to be a distinct species. Köhler (2000) found some differences among populations of *H. balzani* in Bolivia, for which he tentatively used the name *H. cf. callipleura*.

Duellman et al. (1997) proposed that the *Hyla pulchella* group is related to other Neotropical *Hyla* species in which males have a greatly enlarged prepollex with a projecting prepollical spine (the so called “gladiator frogs,” Kluge, 1979). The species groups explicitly or implicitly proposed to have close relationships with this putative clade are the *H. albomarginata*, *H. albopunctata*, *H. boans*, *H. circumdata*, *H. claresignata*, *H. geographica*, *H. granosa*, *H. pulchella*, *Hyla punctata*, *H. pseudopseudis*, and *H. polytaenia* groups (Bokermann, 1972; Cabral Eterovick and Brandão, 2001; Cruz and Caramaschi, 1998; Duellman, 1970, 2001; Duellman et al., 1997; Hoogmoed, 1979). In a recent species description, Kizirian et al. (2003) discussed possible relationships between *Hyla tapichalaca*, the *H. larinopygion* group, and the Andean members of the *H. pulchella* group.

The goals of the present study are: (i) to test the monophyly of the *H. pulchella* species group, with special attention to the *H. circumdata* and *H. polytaenia* groups, (ii) to infer interrelationships of its species, and (iii) to evaluate the status of the subspecies (Barrio, 1965) of *H. pulchella*.

## 2. Material and methods

### 2.1. Taxon sampling

Ten of the 14 (or 15, if *Hyla callipleura* is different from *H. balzani*, as suggested by Köhler, 2001) currently recognized species of the *H. pulchella* group were included in this study. Among species not represented here, *H. cymbalum*, *H. melanopleura*, and *H. palaestes*, are known only from the type material; tissues of *H. albonigra* were not available. We also included sequences from four localities of *H. andina* (including a topotype) and two localities of *H. marianitae* (including a topotype). In addition, we included samples from two of the subspecies of *H. pulchella* recognized by Barrio (1965) (*H. p. cordobae* and *H. p. riojana*), as well as samples from five localities for *H. p. pulchella*, three localities for *H. p. riojana*, and two localities for *H. p. cordobae*. Lastly, we also included sequences of a new species from northeastern Argentina, *H. sp. 1*, which is related to *H. semiguttata*.

We included *Hyla bischoffi* and *Hyla guentheri* because both have been indirectly associated with the *H.*

*pulchella* group. Two representatives of the *H. polytaenia* species group, *Hyla leptolineata* and *H. sp. 2*, an unnamed species of the group, were included because both Lutz (1973) and Langone (1997) expressed doubts on the limits between this group and the *H. pulchella* group. Given that Duellman et al. (1997) suggested that the *H. pulchella* group should also contain the species included in the *H. circumdata* group, we included three representatives of this group (Table 1) to test this possibility. Considering that Kizirian et al. (2003) expressed doubts regarding the group assignment of *H. tapichalaca*, it was included in the analysis. *H. armata* was included because Duellman et al. (1997) discussed a possible relationship to the *H. pulchella* group.

Considering the proposed relationship of the *Hyla pulchella* group to gladiator frogs, we included one representative of every species group that had ever been associated with this putative clade, with the exception of the *H. claresignata*, *H. miliaria*, and *H. pseudopseudis*

groups, for which no representatives were available. Because the phylogenetic relationships within Hylidae, and particularly within Hylinae are poorly known, we included sequences of 10 representatives of other species groups of *Hyla* and other genera to increase sampling of hylid diversity. Trees were rooted with *Phyllomedusa vaillanti* following Duellman (2001) and Haas (2003) who suggested that *Phyllomedusa* could be a suitable outgroup for the clade containing Hylinae and Pseudinae. See Table 1 for a list of the included species and the taxa of which they are exemplars. A list of the voucher specimens of all the tissues and locality data is provided in Appendix A.

## 2.2. DNA extraction and sequencing

Whole cellular DNA was extracted from frozen and ethanol preserved tissues (usually liver or muscle) using either phenol–chloroform extraction methods or the DNeasy QIAGEN isolation kit. The mitochondrial 12s, tValine, and 16s genes were amplified using the overlapping primer pairs MVZ59–MVZ50, L13–TITI–TUSI, L2A–H10, and AR–BR (see Table 2 and Goebel et al., 1999). The concatenation of the amplified fragments results in ca. 2420 bp that span most of the 12s, all tRNA valine, and 1500 out of the roughly 1800 bp of the 16s gene. The mitochondrial cytochrome *b* gene was amplified using the primer pairs MVZ15–Cytb2, which results in a 385 bp product. Amplification was carried out in a 25- $\mu$ l volume reaction, with puRe Taq Ready-To-Go PCR beads (Amersham–Biosciences, Piscataway, New Jersey). For all the amplifications, the PCR program included an initial denaturing step of 60 s at 94 °C, followed by 35 cycles of amplification (94 °C for 60 s, 50 °C for 60 s, 72 °C for 60 s), with a final extension step at 72 °C for 6 min. In a few cases, slight adjustments of  $\pm 2$  °C were made to the annealing temperature.

PCR-amplified products were cleaned either with a QIAquick PCR purification kit or with ARRAY-IT (TeleChem International, Sunnyvale, California), and labeled with fluorescent-dye labels terminators (ABI Prism Big Dye Terminators v. 3.0 cycle sequencing kits). The labeled PCR products were isopropanol-precipitated following the manufacturer's protocol. The sequencing products were run on ABI 3700 or ABI Prism 377 automatic sequencer. Most samples were sequenced in both directions. Fragments were built using chromatograms obtained from the automated sequencer and Sequencher 3.0. Complete sequences were edited with BioEdit (Hall, 1999).

Despite repeated attempts, we were unable to sequence the fragment L13–TITI for a specimen of *H. pulchella* (MLP-A 2148), L13–TITI for the single available specimen of *H. guentheri* (CFBH 3386), and the first 500 bp of the fragment MVZ59–MVZ50 for *H.*

Table 1

A list of the species included in this analysis and the species groups or genera to which they are presently referred

Species	Current placement
<i>Duellmanohyla ruftoculis</i>	<i>Duellmanohyla</i>
<i>Hyla albomarginata</i>	<i>Hyla albomarginata</i> group
<i>Hyla albopunctata</i>	<i>Hyla albopunctata</i> group
<i>Hyla andina</i>	<i>Hyla pulchella</i> group
<i>Hyla armata</i>	<i>Hyla armata</i> group
<i>Hyla astartea</i>	<i>Hyla circumdata</i> group
<i>Hyla balzani</i>	<i>Hyla pulchella</i> group
<i>Hyla bischoffi</i>	Unassigned
<i>Hyla caingua</i>	<i>Hyla pulchella</i> group
<i>Hyla cinerea</i>	<i>Hyla cinerea</i> group
<i>Hyla circumdata</i>	<i>Hyla circumdata</i> group
<i>Hyla ericae</i>	<i>Hyla pulchella</i> group
<i>Hyla faber</i>	<i>Hyla boans</i> group
<i>Hyla fasciata</i>	<i>Hyla geographica</i> group
<i>Hyla granosa</i>	<i>Hyla granosa</i> group
<i>Hyla guentheri</i>	Unassigned
<i>Hyla hylax</i>	<i>Hyla circumdata</i> group
<i>Hyla joaquina</i>	<i>Hyla pulchella</i> group
<i>Hyla leptolineata</i>	<i>Hyla polytaenia</i> group
<i>Hyla marginata</i>	<i>Hyla pulchella</i> group
<i>Hyla marianitae</i>	<i>Hyla pulchella</i> group
<i>Hyla minuta</i>	<i>Hyla minuta</i> group
<i>Hyla nana</i>	<i>Hyla microcephala</i> group
<i>Hyla prasina</i>	<i>Hyla pulchella</i> group
<i>Hyla pulchella</i>	<i>Hyla pulchella</i> group
<i>Hyla punctata</i>	<i>Hyla punctata</i> group
<i>Hyla semiguttata</i>	<i>Hyla pulchella</i> group
<i>Hyla sp. 1</i>	<i>Hyla pulchella</i> group
<i>Hyla sp. 2</i>	<i>Hyla polytaenia</i> group
<i>Hyla tapichalaca</i>	Unassigned
<i>Osteocephalus leprieurii</i>	<i>Osteocephalus</i>
<i>Phrynohyas venulosa</i>	<i>Phrynohyas</i>
<i>Phyllomedusa vaillanti</i>	<i>Phyllomedusa</i>
<i>Pseudis paradoxa</i>	<i>Pseudis</i>
<i>Scinax ruber</i>	<i>Scinax</i>
<i>Smilisca baudinii</i>	<i>Smilisca</i>

The subspecies of *Hyla pulchella* are not included.

Table 2

A list of the primers employed in this study

Primer name	Primer sequence	Author
MVZ59	5'-ATAGCACTGAAAAYGCTDAGATG-3'	Graybeal (1997)
12SA-L	5'-AAACTGGGATTAGATACCCCCTAT-3'	Palumbi et al. (1991)
12SF-H	5'-CTTGGCTCGTAGTTCCTGGCG-3'	Goebel et al. (1999)
MVZ50	5'-TYTCGGTGTAAGYGARAKGCTT-3'	Graybeal (1997)
12sL13	5'-TTAGAAGAGGCAAGTCGTAACATGGTA-3'	Feller and Hedges (1998)
TitusI	5'-GGTGGCTGCTTTTAGGCC-3'	Titus and Larson (1996)
16L2A	5'-CCAAACGAGCCTAGTGATAGCTGGTT-3'	Hedges (1994)
16SH10	5'-TGATTACGCTACCTTTGCACGGT-3'	Hedges (1994)
16Sar	5'-CGCCTGTTTATCAAAAACAT-3'	Palumbi et al. (1991)
16Sbr	5'-CCGGTCTGAACTCAGATCACGT-3'	Palumbi et al. (1991)
Wilkinson2	5'-GACCTGGATTACTCCGGTCTGA-3'	Wilkinson et al. (1996)
MVZ15	5'-GAACTAATGGCCACACWWTACGNAA-3'	Moritz et al. (1992)
Cytb2	5'-AAACTGCAGCCCCTCAGAAATGATATTTGTCCTCA-3'	Kocher et al. (1989)

The primer Wilkinson 2 was used on occasions instead of 16Sbr; 12SA-L and 12SF-H were used as internal primers.

*fasciata*; therefore, those fragments were treated as missing data in the phylogenetic analyses.

For five terminals (*Hyla caingua*, *H. p. cordobae*, *Hyla faber*, *H. joaquina*, and *H. semiguttata*; see Appendix A), we sequenced two specimens from the same locality. In the case of *H. caingua*, where the sequences of both specimens were identical, only one was included; in the other cases, in which the differences ranged between 1 and 10 nucleotides, consensus sequences were created to reduce the number of terminals analyzed by POY.

### 2.3. Phylogenetic analysis

Phylogenetic analyses employed Direct Optimization (Wheeler, 1996, 1998, 2002) using POY (Wheeler et al., 2002). Sequence alignment and tree searching have traditionally been treated as two independent steps in phylogenetic analyses: sequences are first aligned, and that fixed or static alignment is then treated as a standard character matrix that is the basis for tree searching in the test of character congruence. However, there may be other, equally defensible multiple sequence alignments that would require fewer hypothesized transformations to explain the observed sequence variation; an explanation that requires fewer transformations is more parsimonious and is therefore, objectively preferred over explanations that require a greater number of transformations. Given that it is impossible to assess the number of transformations required by a given alignment without a topology, and that the optimal alignment will vary from cladogram to cladogram, Direct Optimization seeks the cladogram-alignment combination that minimizes the total number of hypothesized transformation events required to explain the observations. Within this framework, insertion/deletion events (indels; gaps) are historical evidence that is equally important to substitutions when hypothesizing common ancestry. To maximize explanatory power and descriptive efficiency,

searches were conducted under equal weights for transitions, transversions, and indels (Frost et al., 2001); differential costs for gap opening and gap extension were not explored. Heuristic algorithms (see below for description and references) applied to both tree searching and alignments were employed throughout the analysis; as with any heuristic solution, the optimal solution from these analyses represents the upper bound. This study is guided by the idea that a simultaneous analysis of all the available evidence maximizes explanatory power (Kluge, 1989; Nixon and Carpenter, 1996); therefore, we analyzed all four loci simultaneously.

The analysis was performed using a sub-cluster of 15 processors of the American Museum of Natural History parallel cluster. Our search strategy included building Wagner trees using 60 random addition sequences followed by (1) a round of tree-bisection and reconnection (TBR) branch swapping, and (2) 20 parsimony ratchet replicates (Nixon, 1999a), re-weighting 15% of the characters (fixing the weight multiplier to 2), keeping one tree per replicate, with the resulting trees subjected to a final round of TBR branch swapping. This search strategy was done under the command "iterativepass." Under this command, the optimization heuristics commonly employed in Direct Optimization are reduced and, therefore tree length is evaluated more rigorously (Wheeler, 2003b). Of course, this implies a much higher cost than regular Direct Optimization in computation times, so there is a tradeoff between exhaustiveness of tree calculation and the tree space that is explored.

Bremer support (Bremer, 1988) was calculated in POY under the same optimization routine (iterativepass). Parsimony Jackknife percentages (Farris et al., 1996) were calculated from 500 replicates. Both measures of clade support were calculated using standard Direct Optimization, because iterativepass optimization is computationally too costly to perform the minimum number of replicates required by the method. In turn,

this implies that the Parsimony Jackknife values could be overestimated. Extra length imposed by alternative hypotheses were examined through constrained searches that included 10 random addition sequences followed by a round of TBR and 20 ratchet replicates using the same settings as the unconstrained search, all under iterativepass. Since these constrained searches are not as rigorous as the unconstrained one, its results are approximate.

For the analysis, the whole mitochondrial ribosomal cluster was cut into five fragments coincident with primer pairs (Giribet, 2001); although this constrains homology assessment, the universe of alternative ancestral sequences that has to be explored is a more tractable problem than using a single fragment of approximately 2400 bp. The six sequence files are available from the first author. WinClada (Nixon, 1999b) was used for tree edition.

### 3. Results

The Optimization Alignment-based heuristic search strategies under equal weights for transitions, transversions, and gaps, resulted in two most parsimonious trees of 7442 steps, found in 1 of 60 replicates of random addition sequences followed by 20 ratchet replicates each. The implied alignment (Wheeler, 2003a) has 3209 bp, of which 1240 are informative characters.

Overall, most of the 45 nodes of the strict consensus are well supported, with 28 nodes having a Bremer support  $\geq 10$  and 16 nodes having a Bremer support  $\geq 20$ ; additionally 33 nodes have a Jackknife value  $\geq 75\%$  and 27 nodes have a Jackknife value  $\geq 90\%$ .

The strict consensus of the two topologies (Fig. 1), is completely resolved, with the exception of internal conflict among representatives of *H. p. pulchella* and *H. andina*.

The strict consensus of the resulting topologies contains a well-supported clade composed of all the representatives of groups that historically have been associated with gladiator frogs: the *Hyla albomarginata*, *H. albopunctata*, *H. boans*, *H. circumdata*, *H. geographica*, *H. granosa*, *H. polytaenia*, *H. pulchella*, and *H. punctata* species groups. Among outgroup taxa, *H. armata* + *H. tapichalaca*, are sister to the entire gladiator-frog clade, and the representatives of the 30-chromosome *Hyla* groups, *H. nana* and *H. minuta*, form the next most closely related clade. This clade is, in turn, most closely related to a clade composed of *Sphaenorhynchus lacteus* plus two sister groups, one composed of *Osteocephalus lepreurii* and *Phrynohyas venulosa*, and the other composed of *Duellmanohyla rufioculis*, *H. cinerea*, and *Smilisca baudinii*. *Scinax ruber* and *Pseudis paradoxa* are the two most basal terminals.

Within the gladiator frog clade, the three representatives of the *H. circumdata* group form a monophyletic group at the base of the clade, followed by four successive monophyletic groups: (*H. granosa* + *H. punctata*), (*H. albopunctata* + *H. fasciata*), (*H. albomarginata* + *H. faber*), and *H. pulchella* group (including *H. bischoffi* and *H. guentheri*) which is paraphyletic with respect to the *H. polytaenia* group.

*Hyla ericae* is sister taxon to a clade composed of the two representatives of the *H. polytaenia* group (*H. sp. 2*, and *H. leptolineata*), *H. semiguttata*, *H. joaquinii*, and *H. sp. 1*, and another clade containing all the remaining representatives of the *H. pulchella* group. Within the latter, is a monophyletic group composed of the Andean representatives of the *H. pulchella* group, including *H. p. riojana*, *H. andina*, *H. marianitae*, and *H. balzani* and a clade composed of some species from the Brazilian Atlantic forest and its remnants (*H. caingua*, *H. marginata*, *H. bischoffi*, *H. guentheri*, *H. prasina*), the lowlands of southern Brazil, Uruguay, and Argentina (*H. pulchella*), and the highlands of central Argentina (*H. p. cordobae*).

All the populations of *Hyla pulchella pulchella* form a monophyletic group, as do the populations of *H. p. riojana*, *H. p. cordobae*, *H. andina*, and *H. marianitae*.

### 4. Discussion

#### 4.1. Outgroup topology

The topology of the outgroup taxa shows some interesting and some unexpected results. We mostly attribute them to taxon sampling, as this analysis was not designed to address the relationships among these taxa. Some of the results, though, are noteworthy. The sister group relation between the two representatives of the thirty chromosome *Hyla* species groups is consistent with the long-held idea that the group is monophyletic (Bogart, 1973; Duellman, 1970; Duellman and Crump, 1974; Duellman and Trueb, 1983). The *Osteocephalus lepreurii* + *Phrynohyas venulosa* clade is congruent with a monophyletic group composed of frogs with double, bilateral vocal sacs (Trueb, 1970). The clade containing *Duellmanohyla rufioculis*, *Smilisca baudinii*, and *H. cinerea* is consistent with the proposal of Duellman (2001) that most of the North American and Middle American hylines are monophyletic. The basal position of *Scinax ruber* is an unexpected result probably related to taxon sampling.

Because we included only a small subset of all hylid frogs, we do not consider the present analysis to be a particularly strong test of the monophyly of gladiator frogs. However, it does corroborate hypotheses based on morphological (Duellman, 1970, 2001; Kluge, 1979) and behavioral (Kluge, 1979) evidence. Duellman et al. (1997) found support for a clade composed of the *H.*

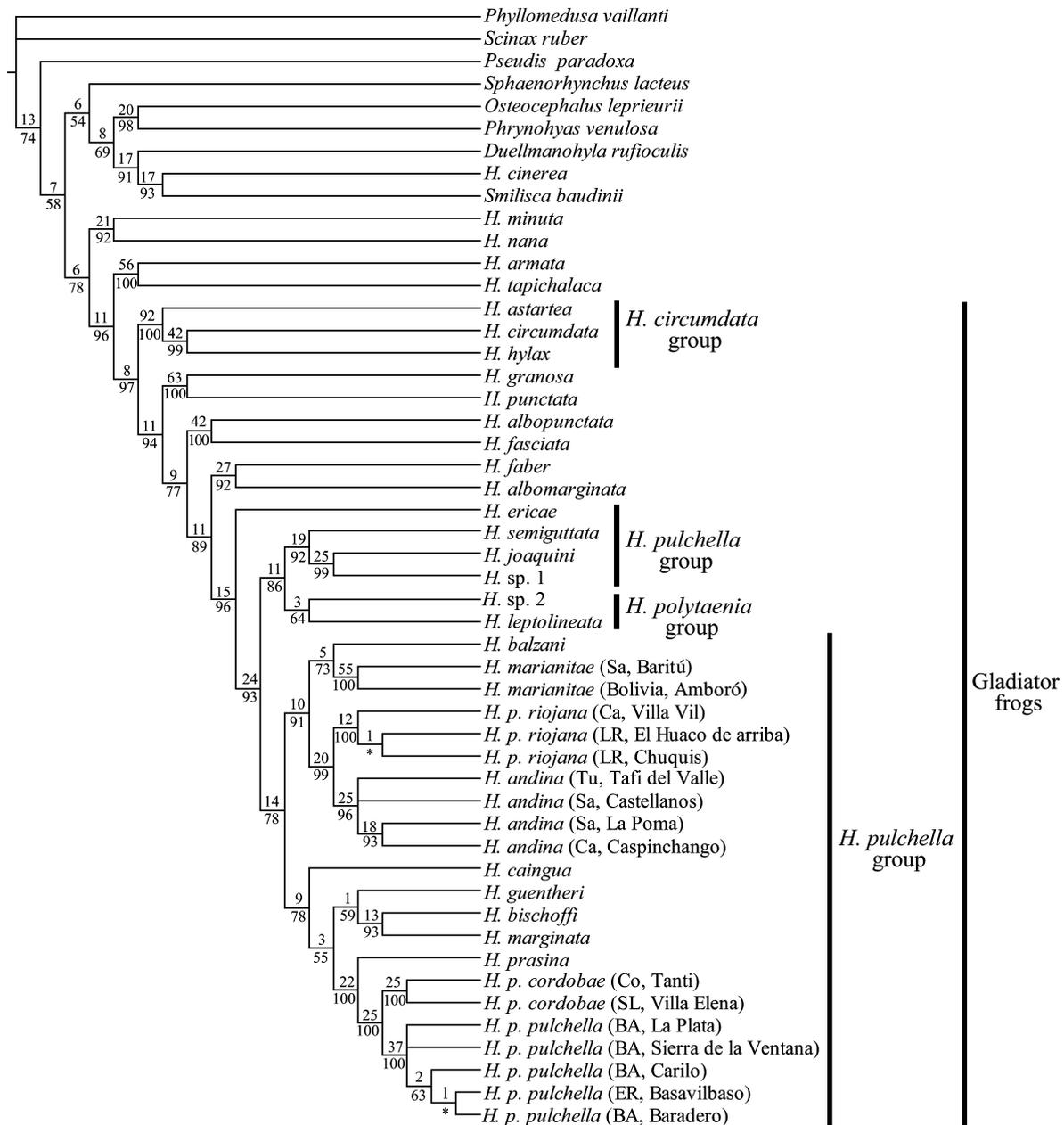


Fig. 1. Strict consensus of the two most parsimonious trees found in the analysis of the *Hyla pulchella* species group and outgroups. Numbers above nodes are Bremer supports; numbers below nodes are Parsimony Jackknife values. Nodes with an asterisk have Parsimony Jackknife values <50%. All the localities shown are from Argentina unless otherwise stated. Abbreviations are as follows: BA, Buenos Aires; Ca, Catamarca; Co, Córdoba; ER, Entre Ríos; LR, La Rioja; Sa, Salta; SL, San Luis; Tu, Tucumán.

*albomarginata*, *H. albopunctata*, *H. boans*, *H. circumdata*, *H. geographica*, and *H. pulchella* groups in the presence of an enlarged prepollical spine lacking a quadrangular base. The inclusion of *H. punctata* and the *H. polytaenia* group in this clade supported by molecular data is coincident with both showing the same morphology of the prepollical spine as the other included species (Garcia and Faivovich, pers. obs). It is unclear why Duellman et al. (1997) did not include the *H. polytaenia* group in this putative clade. *Hyla punctata* was excluded, presumably, because it is incorrectly re-

ported to lack a prepollical spine (Duellman et al., 1997, p. 33).

Kizirian et al. (2003) considered that *H. tapichalaca* has character states diagnostic of both the *H. larinopygion* and *H. pulchella* groups, and so were unsure about its relationships. The present analysis suggests a closer relation to *H. armata*, a species allied with the *H. larinopygion* group (Duellman et al., 1997; Kizirian et al., 2003). The only morphological character state that *H. tapichalaca* shares with the *H. pulchella* group is the presence of an enlarged, curved, and

pointed prepollex (Kizirian et al., 2003), which is also shared with all other groups of gladiator frogs (Duellman et al., 1997).

#### 4.2. A test of Duellman et al.'s (1997) hypotheses

Duellman et al. (1997) proposed the merging of the *H. circumdata* and *H. pulchella* species groups under the latter name. This suggestion was ignored by Cruz and Caramaschi (1998, 2000) and Cabral Eterovick and Brandão (2001), and contested by Garcia et al. (2001, 2003). This study consistently recovers the representatives of the *H. circumdata* group as a monophyletic group distantly related to the *H. pulchella* group, therefore we continue to recognize both species groups.

Duellman et al. (1997) presented a cladistic analysis, based on 14 morphological characters, of most Andean groups of *Hyla*. Their most parsimonious tree depicts *H. armata* as sister species of the *H. larinopygion* group, and this clade as sister-taxon of the *H. bogotensis* group. However, Duellman et al. (1997, p. 36) also suggested that “*Hyla armata* may have been derived from a lowland lineage or be a highly derived species related to the *H. pulchella* group.” The present study clearly rejects the hypothesis of a close relationship between *H. armata* and the *H. pulchella* group.

#### 4.3. Paraphyly of the *Hyla pulchella* group

Based on our results, the *Hyla pulchella* group is paraphyletic, because the two representatives of the *H. polytaenia* group, *H. sp. 2*, and *H. leptolineata*, form a monophyletic group nested within it. To correct this, we propose the inclusion of all the species of the *H. polytaenia* group in the *H. pulchella* group and to continue to recognize a putatively monophyletic “*H. polytaenia* clade” within the group. Cruz and Caramaschi (1998) presented a formal diagnosis of the *H. polytaenia* group, mostly based on color pattern. Putative synapomorphies of this clade are the absence of any pattern on the thighs and the mostly striped dorsal pattern. Currently, it comprises seven species (Caramaschi and Cruz, 2000): *H. buriti* Caramaschi and Cruz, 1999; *H. cipoensis* B. Lutz, 1968; *Hyla goiana* B. Lutz, 1968; *H. leptolineata* Braun and Braun, 1977; *Hyla phaeopleura* Caramaschi and Cruz, 2000; *H. polytaenia* Cope, 1870; and *H. stenocephala* Caramaschi and Cruz, 1999, however, the taxonomy of the clade is complex, and there are still several species to be described.

#### 4.4. The (end of the) subspecies of *Hyla pulchella*

Barrio (1965) stated that *H. p. cordobae* was intermediate between the Andean forms (at that time, only

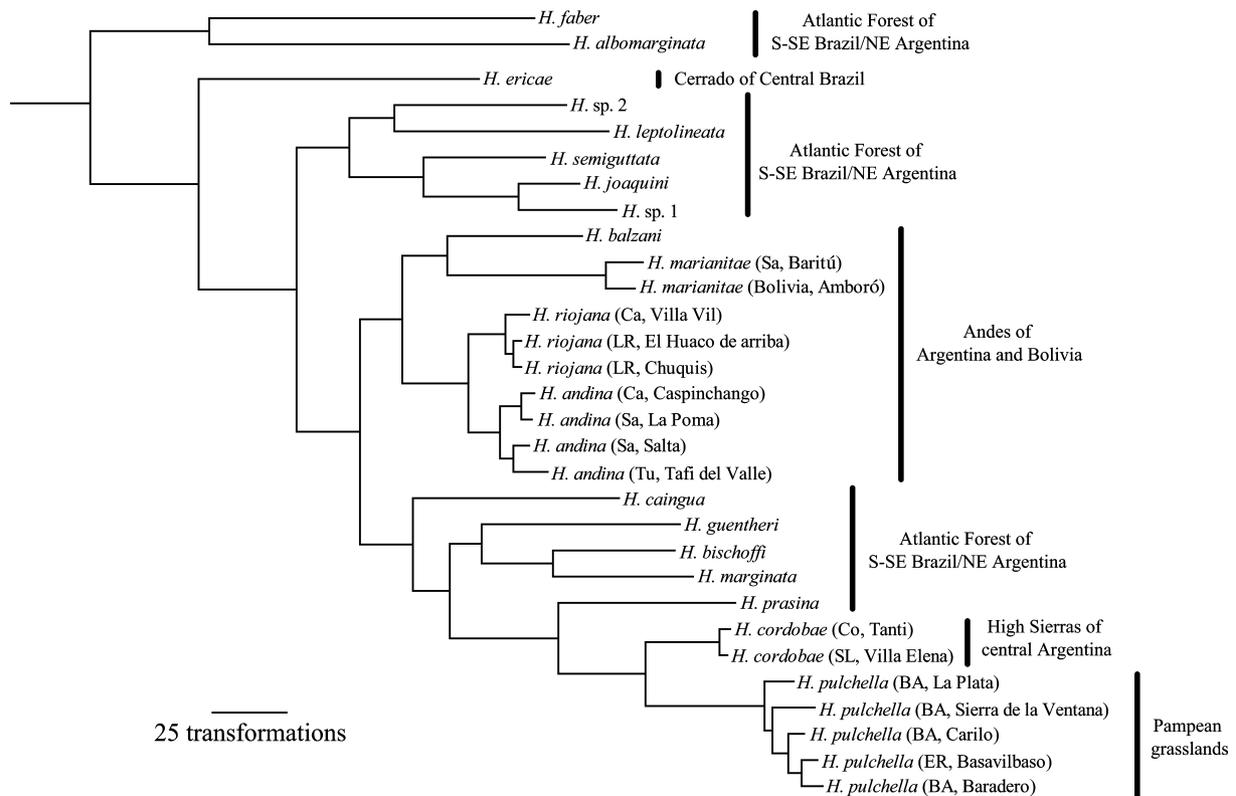


Fig. 2. Partial view of one of the two most parsimonious trees found in the analysis, showing the geographic distribution of the species of the *Hyla pulchella* group included in the analysis. Abbreviations as in Fig. 1.

*H. p. riojana* and *H. p. andina*) and the eastern forms (at that time, only *H. p. pulchella* and *H. p. prasina*). However, in this study (Figs. 1 and 2) the *H. p. cordobae* samples form a well-supported monophyletic group that is sister of the clade containing all *H. p. pulchella*. This result by itself does not reject the possibility that *H. p. cordobae* and *H. p. pulchella* are conspecific. However, if we take into account external evidence such as the morphological differences between the two forms and the different advertisement call structure (Barrio, 1965; Basso and Basso, 1987; Di Tada et al., 1996), then the results are highly suggestive of different species. Therefore, we consider *H. cordobae* to be a distinct species.

Barrio (1965) originally considered *H. p. riojana* to be most closely related to (then) *H. p. andina*. While our study corroborates this proximity, they show that there is no basis for considering *H. riojana* as a subspecies of *H. pulchella*. We therefore, consider *H. pulchella* to be distinct from *H. riojana*, being the important question whether *H. andina* is specifically distinct from this form.

*Hyla riojana* and *H. andina* are morphologically very similar, as are their vocalizations (see Barrio, 1965). They differ externally only in that the former lacks the dashed white or cream dorsolateral stripes that begin behind the eyes and that are characteristic, at least of the Argentinean populations, of *H. andina*, including the type locality (see Langone and Lavilla, 2002). The two species are apparently parapatric (the southern limit of the distribution of *H. andina* and the northern limit of the range of *H. riojana* are not well known). *Hyla andina* is a variable species especially regarding color pattern, coloration, vocalization (De La Riva et al., 2000; Duellman et al., 1997; Köhler, 2000; Reynolds and Foster, 1992), and larval morphology (Lavilla, 1984). In our analysis, the three populations of *H. riojana* form a monophyletic group, as do the four populations of *H. andina*. Both clades in turn form a monophyletic group. This result does not imply that *H. andina* and *H. riojana* are necessarily different species. Furthermore, the four specimens of *H. andina* of this study are by no means a thorough sampling of the species in terms of its broad variation and distribution (from Catamarca, Argentina, to northern Bolivian Andes). Because our results do not contradict the existence of these two species, we tentatively recognize them as valid taxa. However, a denser sampling is required to adequately test the possibility that *H. riojana* is not nested within *H. andina* (making the latter a junior synonym).

*Hyla pulchella* is a fairly variable species that ranges from southern Buenos Aires, in Argentina, through Uruguay and southern Brazil, north to Santa Catarina. In the east, it reaches the province of Chaco, Argentina. Barrio (1965) mentioned that specimens from the population of Sierra de la Ventana, a mountainous outcrop in the southern part of the province of Buenos Aires, were slightly larger and show differences in the dorsal pattern from other populations. The results

presented here are ambiguous as to whether the population of Sierra de la Ventana is a different species. It should also be taken into account that for the analyzed specimen the DNA fragment delimited by the primers L13-TitI could not be sequenced. For these reasons, we consider this as an open problem. Additional data and increased sampling of populations are required for further testing.

#### 4.5. The taxa of Central, southeastern, and southern Brazil

The species of central, southeastern, and southern Brazil form a paraphyletic assemblage that contains a clade of the Andean representatives of the *Hyla pulchella* group, and another clade with species of the pampean grasslands and the high Sierras of central Argentina (see Fig. 2).

This study supports the tentative association of *H. bischoffi* and *H. guentheri* with the *H. pulchella* group proposed by Boulenger (1887) and Langone (1997).

Garcia et al. (2001) tentatively suggested that within the *H. pulchella* group, *H. joaquini* (as *H. p. joaquini*), *H. ericae*, *H. marginata*, *H. semiguttata*, and other undescribed species (including *Hyla* sp. 1, of this analysis plus some undescribed species close to *H. semiguttata*), could together form a clade diagnosed by (1) the lack of dark blotches or bands on the sides and hidden areas of thighs, (2) vocalizations with long and multi-pulsed notes, and (3) reproduction at streams. Our results indicate that while *H. joaquini*, *H. semiguttata*, and *H. sp. 1* are a monophyletic group, they are not particularly closely related to either *H. ericae* or *H. marginata*. The close relationship between *Hyla joaquini*, *H. semiguttata*, and *H. sp. 1* is not surprising, since these species are externally similar (Garcia et al., 2003); however, at this point no morphological synapomorphies are known for this clade.

*Hyla bischoffi* appears as the sister species of *H. marginata* and together they form a clade with *H. guentheri*. This latter species and *H. bischoffi* were suggested to be closely related to the *H. polytaenia* group by Lutz (1973). Cruz and Caramaschi (1998) excluded *H. bischoffi* and *H. guentheri* from the *H. polytaenia* group because these two species have transversal bars on hidden surfaces of the thigh. The results of the present analysis corroborate the position of Cruz and Caramaschi (1998).

It is noteworthy that although *H. ericae*, a species from central Brazil, occurs in an area that belongs to the morphoclimatic domain of the Cerrado (Ab'Saber, 1977), it inhabits gallery forests (P.C.A. Garcia, pers. obs.), which have denser vegetation than the surrounding open formations (Eiten, 1992). This preference for streamside forest is generally similar to that of most other species of the group that inhabit the Atlantic forest domain of southern and southeastern Brazil, and its remnants in northeastern Argentina (P.C.A. Garcia, pers. obs.).

#### 4.6. Biogeography

Three results from the phylogenetic analysis have noteworthy biogeographic significance (Fig. 2): (1) the monophyly of the Andean members of the *H. pulchella* group; (2) the Andean clade nested within an Atlantic forest/Cerrado clade; and (3) the *H. cordobae* + *H. pulchella* clade nested within an Atlantic forest clade.

An interesting result emerging from the analysis is the monophyly of all the included Andean species of the *H. pulchella* group, which suggest a single event of colonization of the Andes and subsequent speciation.

The Andean clade nested within the Atlantic forest/Cerrado clade (Fig. 2) constitutes an instance of a biogeographic pattern that we are not aware of in other vertebrate groups. Because several species in the *H. pulchella* group occur in the Atlantic forest, as do the close outgroup taxa *H. albomarginata* and *H. faber* (although these are representatives of two groups that have some species outside southern-southeastern/central Brazil), there are few alternative hypotheses where this pattern does not hold. Constraining the monophyly of the Andean clade + *H. pulchella* results in a topology 85 steps longer than the best trees; constraining the monophyly of the Andean clade + (*H. cordobae* + *H. pulchella*) is 42 steps longer.

Despite its occurrence in the high Sierras of central Argentina, *H. cordobae* does not belong to the Andean clade. This is surprising given its acoustic and morphological similarity to *H. riojana* and, to a lesser extent, *H. andina* (Barrio, 1965). Our results also contradict Ceï (1980) who suggested that the elevational communities of the high Sierras of Cordoba and San Luis (1000–2000 m.s.l.) should be related to the sub-Andean frog fauna and suggest instead a mixed origin of this fauna, with at least one element (*H. cordobae*) more related to species in the pampean grasslands and litoral-mesopotamic region.

The *H. cordobae* + *H. pulchella* clade is nested within an Atlantic forest clade, and therefore represents a secondary invasion of non-forested regions. This is not an uncommon pattern. For instance, a similar pattern has been observed in the genus *Scinax*, where *Scinax berthae* is nested within the *Scinax catharinae* group (a group mostly confined to the Atlantic forest), but its distribution reaches Buenos Aires province (Faivovich, 2002).

#### 4.7. Missing taxa

Considering the composition of the *H. pulchella* group before this phylogenetic analysis, there are four species that could not be included. Three of these, *H. albonigra*, *H. melanopleura*, and *H. palaestes*, also have an Andean distribution, and we think that they might be associated with the Andean clade. The fourth species, *H. cymbalum*, is known only from the type locality in

southeastern Brazil (Campo Grande, Santo André, São Paulo). According to Bokermann (1963), it is closely related to *H. pulchella*, from which it differs in having a different advertisement call; Caramaschi (in Frost, 1985, 2002) suggested that probably they are conspecific.

Taking into account the inclusion in the *H. pulchella* group of the seven described species previously assigned to the *H. polytaenia* group, then there are other five species that were not included in this analysis. This makes a total of nine known, out of the now 25 or 26 included in the group (this figure includes *H. guentheri*, *H. bischoffi*, and the recognition of the two former subspecies of *H. pulchella* as distinct species) that are absent. What is the impact of the absence of nearly 35% of the known species presumed to be in this clade? The only way of knowing it is to include these taxa. In the meantime, we consider it the main limitation of this study.

Despite recent efforts studying the taxonomy of several species of the *H. pulchella* group (see Section 1), it is evident that our knowledge is still quite rudimentary. This study provides a first phylogenetic framework, which will be necessarily enriched as more characters (particularly morphology) and the remaining taxa of the group are added.

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## Appendix A

A list of all specimens, collection numbers, localities, and GenBank Accessions of the sequences included in this analysis. Vouchers followed by an asterisk (\*) are topotypes. In a few cases, the tissues have separate numbers of official tissue collections. These are given as footnotes. Collection abbreviations are as follows: AM-CC: Ambrose Monell Cryo Collection, AMNH. AMNH-A: American Museum of Natural History, New

York, USA. CFBH: Collection of Célio F.B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil. DLR: Ignacio De La Riva field series (to be deposited in the Museo Nacional de Ciencias Naturales, Madrid, Spain). IWK: Field tags used by Maureen A. Donnelly; to be accessed to the Herpetological Collection of the Florida International University. MACN: Museo Argentino de Ciencias Naturales “Bernardino Rivadavia,” Buenos Aires, Argentina. MCN: Museo de Ciencias Naturales de la Universidad Nacional de Salta, Salta, Argentina. MNCN ADN: Collection of DNA samples of the Museo Nacional de Ciencias Naturales, Madrid, Spain. MNK: Museo “Noel Kempff Mercado,” Santa Cruz, Bolivia. MVZ: Museum of Vertebrate Zoology, University of California, Berkeley, California, USA. MVZFC: MVZ tissue Collection. MLP-A: Museo de La Plata, La Plata, Argentina. MLP-DB: Collection Diego Baldo, at MLP. MZUSP: Museu de Zoologia, Universidade de São Paulo, São Paulo, Brazil. QCAZ: Museo de Zoología de la Pontificia Universidad Católica del Ecuador. USNM: National Museum of Natural History, Smithsonian Institution, Washington DC, USA. ZUEC: Museu de Historia Natural, Universidade de Campinas, Campinas, São Paulo, Brazil.

Collection number	Species	mtRibosomal cluster	Cytochrome <i>b</i>	Locality
MVZ 207194 <sup>a</sup>	<i>Duellmanohyla rufiocularis</i>	AY549315	AY549368	Costa Rica: Guanacaste: Volcan Cacao
USNM 284519	<i>Hyla albomarginata</i>	AY549316	AY549369	Brazil: Pernambuco: Near Caraurucu, on way to Serra dos Cavalos
ZUEC 12053	<i>Hyla albopunctata</i>	AY549317	AY549370	Brazil: São Paulo: Campinas
MACN 37639	<i>Hyla andina</i>	AY549318	AY549371	Argentina: Salta: Dto. La Poma: Río Calchaquí, km 1234 Ruta Nac. 40.
MACN 37891	<i>Hyla andina</i> *	AY563626	AY563628	Argentina: Catamarca: Dto. Santa Maria: Caspinchango
MLP A 2138	<i>Hyla andina</i>	AY549319	AY549372	Argentina: Tucumán: Tafi del Valle
MCN 800	<i>Hyla andina</i>	AY549320	AY549373	Argentina: Salta: Dto Vaqueros: 5 km from Castellanos in the road to Lesser
AMNH-A 165163 <sup>b</sup>	<i>Hyla armata</i>	AY549321	AY549374	Bolivia: Santa Cruz: Caballero: Canton San Juan: Amboro National Park
USNM 303032	<i>Hyla astartea</i>	AY549322	AY549375	Brazil: São Paulo: Near Salesopolis, Estação Biologica de Boraceia
DLR 4119 <sup>c</sup>	<i>Hyla balzani</i>	AY549323	AY549376	Bolivia: Depto. La Paz: Prov. Noryungas: Serrania Bellavista
CFBH 3356	<i>Hyla bischoffi</i>	AY549324	AY549377	Brazil: Santa Catarina: Rancho Queimado
MLP-DB 1083	<i>Hyla caingua</i>	AY549325	AY549378	Argentina: Misiones: Posadas
MLP-DB 1084	<i>Hyla caingua</i>	AY549326	AY549379	Argentina: Misiones: Posadas
MVZ 145385 <sup>d</sup>	<i>Hyla cinerea</i>	AY549327	AY549380	United States: Texas: Travis Co. Austin, Municipal Golf Course
CFBH 3621	<i>Hyla circumdata</i>	AY549328	AY549381	Brazil: Santa Catarina: São Bento do Sul
MLP A 2139	<i>Hyla cordobae</i>	AY549329	AY549382	Argentina: Córdoba: Tanti
MACN 37692	<i>Hyla cordobae</i>	AY549330	AY549383	Argentina: San Luis: Dto. Chacabuco: Villa Elena: Arroyo “La calera”
MACN 37693	<i>Hyla cordobae</i>	AY549331	AY549384	Argentina: San Luis: Dto. Chacabuco: Villa Elena: Arroyo “La calera”
CFBH 3599	<i>Hyla ericae</i> *	AY549332	AY549385	Brazil: Goias: Alto Paraiso de Goias
MACN 36999	<i>Hyla faber</i>	AY549333	AY549386	Argentina: Misiones: Depto. Guarany: San Vicente
MACN 37000	<i>Hyla faber</i>	AY549334	AY549387	Argentina: Misiones: Depto. Guarany: San Vicente

## Appendix A (continued)

Collection number	Species	mtRibosomal cluster	Cytochrome <i>b</i>	Locality
AMNH-A 164081	<i>Hyla fasciata</i>	AY549335	AY549388	Guyana: Iwokrama: Cowfly camp.
AMNH-A 164105	<i>Hyla granosa</i>	AY549336	AY549389	Guyana: Iwokrama: Muri Scrub camp
CFBH 3386	<i>Hyla guentheri</i>	AY549337	AY549390	Brazil: Rio Grande do Sul: Terra de Areia
		AY549421		
USNM 303036	<i>Hyla hylax</i> *	AY549338	AY549391	Brazil: São Paulo: Near Salesopolis, Estação Biológica de Boraceia
CFBH 3625	<i>Hyla joaquina</i>	AY549339	AY549392	Brazil: Santa Catarina: Urubici
CFBH 3280	<i>Hyla joaquina</i>	AY549340	AY549393	Brazil: Santa Catarina: Urubici
CFBH 3848	<i>Hyla leptolineata</i>	AY549341	AY549394	Brazil: Santa Catarina: Município de São Domingos
CFBH 3098	<i>Hyla marginata</i>	AY549342	AY549395	Brazil: Rio Grande do Sul: São francisco de Paula
MNK 5282 <sup>c</sup>	<i>Hyla marianitae</i>	AY549343	AY549396	Bolivia: Santa Cruz: Caballero: Canton San Juan: Amboró National Park
MV0249	<i>Hyla marianitae</i> *	AY549344	AY549397	Argentina: Salta: Baritú
MACN 33799	<i>Hyla minuta</i>	AY549345	AY549398	Argentina: Misiones: Depto. Guarany: San Vicente
MACN 37785	<i>Hyla nana</i>	AY549346	AY549399	Argentina: Entre Rios: Dto. Islas del Ibicuy
CFBH 3388	<i>Hyla prasina</i>	AY549347	AY549400	Brazil: Santa Catarina: Rio Vermelho
MLP A 2140	<i>Hyla pulchella</i>	AY549348	AY549401	Argentina: Buenos Aires: La Plata
MLP A 2148	<i>Hyla pulchella</i>	AY549349	AY549402	Argentina: Buenos Aires: Sierra de la Ventana
		AY549422		
MACN 37790	<i>Hyla pulchella</i>	AY549350	AY549403	Argentina: Buenos Aires: Escobar: El Cazador
MACN 37664	<i>Hyla pulchella</i>	AY549351	AY549404	Argentina: Entre Rios: Dto. Uruguay: Basavilbaso
MACN 37788	<i>Hyla pulchella</i>	AY549352	AY549405	Argentina: Buenos Aires: Carilo
MACN 37792	<i>Hyla punctata</i>	AY549353	AY549406	Argentina: Chaco: Resistencia: Camino a Isla del Cerrito
MACN 37631	<i>Hyla riojana</i>	AY549354	AY549407	Argentina: Catamarca: Depto. Belen: Río El Bolsón, próx. Villa Vil
MACN 37509	<i>Hyla riojana</i>	AY549355	AY549408	Argentina: La Rioja: Sanogasta: El Huaco de Arriba
MACN 37507	<i>Hyla riojana</i>	AY549356	AY549409	Argentina: La Rioja: Castro Barros: Chuquis
CFBH 3579	<i>Hyla semiguttata</i>	AY549357	AY549410	Brazil: Parana: Piraquara
CFBH 3705	<i>Hyla semiguttata</i>	AY549358	AY549411	Brazil: Parana: Piraquara
MACN 37793	<i>Hyla sp. 1</i>	AY549359	AY549412	Argentina: Misiones: Depto. Guarany: San Vicente
MZUSP 111556	<i>Hyla sp. 2</i>	AY549360	AY549413	Brazil: Minas Gerais: Município Itamontes
QCAZ 16704	<i>Hyla tapichalaca</i> *	AY563625	AY563627	Ecuador: Zamora-Chinchipec: Reserva Tapichalaca: Road bet. Yangana and Valladolid
AMNH-A 131254 <sup>f</sup>	<i>Osteocephalus leprieurii</i>	AY549361	AY549414	Venezuela: Amazonas: Neblina Base Camp on Rio Mawarinuma (= Rio Baria)
AMNH-A 141142 <sup>g</sup>	<i>Phrynohyas venulosa</i>	AY549362	AY549415	Guyana: Dubulay Ranch on the Berbice River, 200 ft.
AMNH-A 166288 <sup>h</sup>	<i>Phyllomedusa vaillanti</i>	AY549363	AY549416	Guyana: Berbice River camp at ca.18 mi (linear) SW Kwakwan
MACN 38584	<i>Pseudis paradoxa</i>	AY549364	AY549417	Argentina: Formosa: Laguna Yema
IWK 109	<i>Scinax ruber</i>	AY549365	AY549418	Guyana: Iwokrama: Muri Scrub camp
MVZ 133014 <sup>i</sup>	<i>Smilisca baudinii</i>	AY549366	AY549419	Mexico: Sonora: 10.6 mi W (by road) Alamos
USNM 268930	<i>Sphaenorhynchus lacteus</i>	AY549367	AY549420	Peru: Madre de Dios: Tambopata Reserve

<sup>a</sup> MVZFC 14249.<sup>b</sup> AM-CC 107397.<sup>c</sup> MNCN ADN 593.<sup>d</sup> MVZFC11676.<sup>e</sup> AM-CC 107481.<sup>f</sup> AM-CC 106117.<sup>g</sup> AM-CC 101455.<sup>h</sup> AM-CC 107020.<sup>i</sup> MVZFC 12876.

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