Zoologica Scripta

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Systematics of Andean gladiator frogs of the *Hypsiboas pulchellus* species group (Anura, Hylidae)

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Submitted: 18 March 2010
Accepted: 4 August 2010
doi:10.1111/j.1463-6409.2010.00448.x

Köhler, J., Koscinski, D., Padial, J. M., Chaparro, J. C., Handford, P., Lougheed, S. C. & De la Riva, I. (2010). Systematics of Andean gladiator frogs of the *Hypsiboas pulchellus* species group (Anura, Hylidae). — *Zoologica Scripta*, *39*, 572–590.

We revisit the taxonomic status of Andean species and populations of frogs of the *Hypsiboas pulchellus* group using multiple lines of evidence potentially indicative of evolutionary lineage divergence in anurans: differences in qualitative morphological or bioacoustic character states, no overlap in quantitative characters of advertisement calls, and monophyly of gene genealogies. We found qualitative and quantitative morphological characters to be extremely variable among species and populations of the group and thus of very limited use in assessing lineage divergence. In contrast, phylogenetic analyses based on 16S rRNA and cytochrome *b* sequences resolved highly supported clades that are in concordance with bioacoustic differences. The results support the specific distinctness of most nominal species recognized in the group, including the Bolivian *Hypsiboas balzani* and *Hypsiboas calliopleura*, two species that were considered to be synonymous, and revealed the presence of an undescribed species from southern Peru, which is here described as *Hypsiboas gladiator* sp. n. In contrast, *Hypsiboas andinus* and *Hypsiboas riojanus* were mutually paraphyletic, and showed no differences in morphology and acoustic characters. Consequently, we regard the former as a junior synonym of the latter. However, we discovered that populations of *H. riojanus* from central Bolivia exhibit some degree of genetic differentiation and advertisement call differences with respect to Argentine populations, but sampling of these Bolivian populations is too sparse to draw taxonomic conclusions. Our phylogenetic results support the hypothesis that ancestral lineages of the Andean *H. pulchellus* group experienced successive splitting events along a latitudinal gradient from north to south.

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Introduction

Andean frogs of the *Hypsiboas pulchellus* group occur in a broad latitudinal range of ca. 2000 km, that encompasses a high diversity of habitats, from the dry Chacoan forests of northern Argentina to the perhumid forests of the Amazonian eastern slopes of the Andes in southern Peru (Duellman *et al.* 1997; Köhler 2000; Faivovich *et al.* 2004). Together with other Neotropical species groups, they are

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referred to as 'Gladiator Frogs' because males possess well-developed prepollical spines that they use in territorial fighting (Faivovich *et al.* 2005).

Duellman *et al.* (1997) conducted the most comprehensive taxonomic review of Andean populations of the *H. pulchellus* group to date. Their study was based on morphology and bioacoustics, and resulted in preliminary delimitations of species groups of Andean hylids and the characterization, description and redescription of the six Andean species (*Hypsiboas alboniger*, *Hypsiboas andinus*, *Hypsiboas balzani*, *Hypsiboas marianitae*, *Hypsiboas melanopleura*, *Hypsiboas palaestes*) forming the group. A phylogenetic analysis based on mtDNA sequence variation (Faivovich *et al.* 2004) provided the first evidence for the monophyly of the Andean members of the *H. pulchellus* group and elucidated the status of several described taxa. The monophyly of the *H. pulchellus* group has been more recently supported by large-scale phylogenetic analyses of treefrogs (Faivovich *et al.* 2005; Wiens *et al.* 2005, 2010). Also, Faivovich *et al.* (2005) provided a clear molecular and morphological delimitation of the *H. pulchellus* species group.

Despite this recent increase in general knowledge, especially phylogenetic relationships, of Andean members of the *H. pulchellus* group, certain taxonomic problems remain. Indeed, one well-known problem in the study of the systematics of this group is the marked variation in morphological characters and colour pattern, with intra- and interpopulational variation often overlapping interspecific variation (Duellman *et al.* 1997), which has led to some taxonomic instability. For example, variation in the most conspicuous character states (e.g. colour, body shape) has been interpreted as intraspecific variation (Müller 1924), sometimes discrete enough to separate subspecies within a polytypic species (Barrio 1965), or as more or less fixed species-specific differences (e.g. Duellman *et al.* 1997). Also, the current application of the names *H. balzani* and *Hypsiboas callipleura* to frogs occurring along 800 km of humid forests of the Amazonian slopes of the Bolivian and Peruvian Andes is largely ambiguous (see Köhler 2000 for a discussion), and many recently discovered populations were misidentified or received preliminary identifications only (see De la Riva *et al.* 2000; Köhler 2000). Also, both Faivovich *et al.* (2004) and Koscinski *et al.* (2008) indicate that the distinctiveness of the polymorphic species *Hypsiboas andinus* and *Hypsiboas riojanus* is uncertain.

Here, we revisit the status of Andean species and populations of the *H. pulchellus* group by testing three hallmarks potentially indicative of species status in anurans (Padial *et al.* 2009): fixed differences in qualitative morphological or bioacoustic character states, as evidence of reduced or no gene flow; no overlap in quantitative characters of advertisement calls, indicative of prezygotic reproductive barriers; and monophyly of gene genealogies, as evidence of complete lineage sorting and lineage divergence. This approach is promising, since the combined use of molecular phylogenetics, bioacoustics and morphology has revealed cryptic species and resolved taxonomic problems in several amphibian groups (Vences & Wake 2007), and accelerated species discovery (e.g. Vieites *et al.* 2009). Our theoretical framework coincides with the cumulative approach described by Padial *et al.* (2010), where divergences in any of the organismal attributes that constitute taxonomic characters can provide evidence for the existence of a species. Under this approach character congruence is desired but not considered necessary. In practice, decisions are made based on the available information, which can lead to recognition of a species on the basis of a single set of characters if these characters are considered good indicators of lineage divergence. We show here that this approach is also helpful to solve several longstanding taxonomic problems surrounding Andean gladiator frogs of the *H. pulchellus* group. In addition, the use of molecular and bioacoustic evidence allowed us to discover and name a new species from the Amazonian slopes of the southern Peruvian Andes.

Materials and methods

Sampling

Fieldwork was conducted during different trips over several years during the rainy seasons. Voucher specimens were collected mainly by searching for calling males during day and night. Taxon sampling from own fieldwork includes the following currently recognized nominal species of Andean *Hypsiboas*: *H. andinus* (Müller 1924), *H. balzani* (Boulenger, 1898), *H. callipleura* (Boulenger, 1902), *H. marianitae* (Carrizo, 1992), *H. riojanus* (Koslowsky 1895) and *H. palaestes* (Duellman, De la Riva & Wild, 1997), plus samples from populations of unknown taxonomic status. A total of 73 samples from 42 localities were included in the analysis, covering most of the known ranges of these nominal species (Table S1). The number of samples and localities included for each taxon are as follows (number of samples/number of sampled localities): *H. andinus* (28/19), *H. balzani* (3/3), *H. callipleura* (12/4), *H. marianitae* (9/6), *H. palaestes* (6/2), *H. riojanus* (3/3), samples of unknown taxonomic status (12/5). We lack field and genetic data for two Andean species assigned to the group, *H. alboniger* (Nieden, 1923) and *H. melanopleura* (Boulenger, 1912), which were available only as museum vouchers (see Appendix). Sequences used were produced as described below or taken from GenBank.

Molecular genetics

For phylogenetic analyses we used two gene fragments, 16S rRNA and cytochrome *b* that have been broadly deployed to assess the monophyly and lineage divergence in anurans (Elmer *et al.* 2007; Fouquet *et al.* 2007a; Padial *et al.* 2008a, 2009; Vieites *et al.* 2009). We sequenced 61 specimens for the 16S and 33 for the cytochrome *b* regions, and used an additional eleven 16S and 26 cytochrome *b* sequences from GenBank (Table S1). Genomic DNA was extracted from ethanol-preserved tissues using standard phenol–chloroform protocols (Sambrook *et al.* 1989) or DNeasy Tissue Kit (QIAGEN, Venlo, The Netherlands). A fragment of ca. 560 bp of the 16S rRNA was amplified using the universal primers 16Sar-5′and 16Sbr-3′ (Hillis *et al.* 1996). In addition, a fragment of ca. 250 bp of cytochrome *b* was amplified using primers MVZ15-L from Goebel *et al.* (1999) and a modified version of H15149 (Lougheed *et al.* 1999), or additional primers designed for this study (cyt *b*-DK-L1 5′-GTAACAGYTCGTTTATTGATCTTC and cyt *b*-DK-H 5′-TGCAGCCCCTCAGAAATGATAT). PCR cycling was as follows: initial denaturation at 92 °C for 2 min; 35 cycles of denaturation at 92 °C for 15 s, annealing at 52 °C (16S) or 50 °C (cytochrome *b*) for 15 s, extension at 72 °C for 30 s; final extension at 72 °C for 2 min. PCR products were purified (Multiscreen; Millipore, Billerica, Massachusetts, USA), and sequencing was performed using BigDye Terminator chemistry (version 3.1) and analysed on ABI 3730xl sequencers (Applied Biosystems, Foster City, California, USA). Gene fragments were independently aligned through a web server with Mafft (Katoh *et al.* 2005) under the Q-INS-i strategy (Katoh & Toh 2008). Identical and thus redundant sequences were not all included in the phylogenetic analyses (see Table S1).

We assessed incongruence of phylogenetic signal between 16S and cytochrome *b* genes using the incongruence length difference test (IDL, Farris *et al.* 1994). We found no conflicting phylogenetic signal between the 16S and cytochrome *b* data ($P = 0.87$), and hence we combined datasets for both maximum parsimony (MP) and maximum likelihood (ML) analyses. For the cytochrome *b* gene we determined whether there was evidence of saturation at different codon positions by plotting uncorrected *p*-distances and modelled genetic distances by codon. We used three methods of phylogenetic inference (MP, ML and Bayesian), for the 16S and cytochrome *b* separately and for a combined dataset (see below).

Maximum parsimony phylogenetic analyses were performed in PAUP* version 4.0b10 (Swofford 2002). Strict and majority rule consensus trees were obtained using heuristic searches with tree bisection–reconnection and 5000 replicates. Clade support was calculated by nonparametric bootstrapping using heuristic searches of 1000 replicate datasets each with 10 random addition sequence replicates and no limit of trees imposed during tree search. Gaps were considered a fifth character state. Intraspecific and interspecific uncorrected *p*-distances for the 16S were also calculated in PAUP*.

Maximum likelihood analyses were performed in Garli 0.96 Beta (Zwickl 2006; available at http://www.nescent.org/informatics/download.php?software_id=4). We used default search parameters and implemented a GTR+I+G model (General Time Reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites calculated in MODELTEST 3.7; Posada & Crandall 1998) for the whole data matrix (data partitions are not yet available for this software). We performed a total of 100 runs to reduce the probability of inferring a suboptimal likelihood solution. Node support was assessed by 1000 bootstrap replicates.

Hypsiboas crepitans and *Hypsiboas faber*, two members of the sister clade of the *H. pulchellus* group (Faivovich *et al.* 2004, 2005), were used to root the 16S, cytochrome *b* and combined analyses for the MP and ML analyses.

For Bayesian phylogenetic analyses (Rannala & Yang 1996), we used MRBAYES version 3.2.1 (Ronquist & Huelsenbeck 2003). We used the program MODELTEST 3.7 (Posada & Crandall 1998) to select the model of sequence evolution for the 16S and for each of the codon positions of cytochrome *b* separately. We used separate models of evolution for each codon position of the cytochrome *b* because we found partial saturation in the third codon position. For the 16S data the selected model was GTR+I+G. For the first, second and third codon positions of cytochrome *b* the selected models were K80+I+G (Kimura two-parameters including invariable sites and rate variation among sites; Kimura 1980), JC (Jukes & Cantor 1969), and GTR+I+G, respectively. For the combined analysis, we partitioned the dataset by genes and codon positions. *Hypsiboas crepitans* was used to root the tree in all Bayesian analyses. For all analyses, the majority rule consensus tree was produced from two independent runs, each with eight Monte Carlo Markov chains with default heating parameters, run for 10 million generations (Metropolis-coupled MCMC). Trees were sampled every 1000 generations. Burn-in was evaluated by the examination of the standard deviation of split frequencies (<0.01) and by plotting the −ln L per generation using Tracer 1.2.1 (Rambaut & Drummond 2005). The first 1000 trees were discarded.

In phylogenetic analyses, bootstrap values ≥70% are considered to indicate strong support (Hillis & Bull 1993).

Bayesian posterior probabilities ≥0.95 are considered strongly supported, but it must be noted that relatively high posterior probabilities for short internodes may be overestimates of confidence (Alfaro *et al.* 2003; Erixon *et al.* 2003).

Bioacoustics

All recordings were taken in the field after identifying the calling male. Upon recording, the corresponding specimen was collected. Recording equipment included a Sony WM D6C tape recorder (Tokyo, Japan) and a Sennheiser Me-80 directional microphone (Hannover, Germany). The sounds were recorded on TDK SA60 cassettes, and digitized at a sampling rate of 44.1 kHz and 16 bit resolution with a Delta 66 digitizing board and Peak 3.2 for MacOS X (BIAS 2002) software (Fonoteca Zoológica, MNCN, Madrid, Spain), or Canary 1.2.4 (Cornell Bioacoustics Research Program, Ithaca, New York, USA). All calls were re-sampled at 22.5 kHz and 16 bit resolution and analysed using Adobe Audition 1.5 (San José, California, USA). Frequency information was obtained using a Hanning window function through Fast Fourier Transformations (FFT; width 1024). Audiospectrograms were generated at FFT width 256.

We analysed the following six quantitative characters (Table 1): number of notes per call, note duration (ms), call duration (ms), pulse rate (pulses per second within notes), and dominant frequency (Hz); and the following qualitative characters: presence/absence of frequency modulation in notes; presence/absence of amplitude modulation in notes; tonal vs. pulsatile character of notes. These acoustic characters are commonly used to diagnose and identify anuran species (e.g. Padial *et al.* 2008b). An additional source for information on calls was Duellman *et al.* (1997). Terminology and description of characters follow Köhler *et al.* (2005a).

Morphology

We follow Duellman *et al.* (1997) for terminology of qualitative and quantitative morphological character states used in the diagnoses and descriptions of *Hypsiboas*. Measurements were taken with digital calipers to the nearest 0.1 mm and are those provided in the species description below and Table S2. As a thorough morphological characterization based on large sample sizes of most Andean populations in the species group has already been provided by Duellman *et al.* (1997), we do not repeat complete descriptions here, but instead provide a synthesis of literature data and additional specimens examined subsequently. Specimens examined for this study are listed in the Appendix as well as museum abbreviations used throughout.

Results

Phylogeny

The resulting alignments contained 532, 254 and 786 characters for the 16S, cytochrome *b* and combined datasets, respectively. Ingroup 16S sequences collapsed into 37 unique haplotypes defined by 122 polymorphic sites (75 parsimony informative) and cytochrome *b* into 34 unique haplotypes defined by 98 polymorphic sites (72 parsimony informative). The 16S-cytochrome *b* data combined included 58 individuals, 41 haplotypes and 3 outgroup taxa. For both 16S and cytochrome *b*, many haplotypes were shared by individuals within the same localities. Only a few haplotypes were shared among localities. Within the *H. andinus* clade, 11 individuals from 11 localities in northwestern Argentina and southern Bolivia, and 11 individuals from five localities in central and northern Bolivia shared 16S haplotypes. Shared 16S haplotypes were also found in five *H. callipleura* from three localities in Bolivia, and six *H. marianitae* from four localities in Bolivia. Similar results were found for cytochrome *b* haplotype sharing: seven *H. andinus* individuals from seven localities in northwestern Argentina and southern Bolivia, 13 *H. andinus* from four localities in central and northern Bolivia, three *H. callipleura* from two localities in Bolivia, and six *H. marianitae* from four localities in Bolivia. No haplotypes were shared among species.

All phylogenetic analyses (not shown) resulted in similar tree topologies but the combined dataset of 16S and cytochrome *b* produced the best resolution of clades regardless of the type of analysis (MP, ML or Bayesian). Five distinct, strongly supported clades are evident (Fig. 1). Clade A contains individuals of *H. andinus* from Bolivia and northwestern Argentina as well as individuals of *H. riojanus* (as found by Koscinski *et al.* 2008). Clades B and C represent *H. marianitae* and *H. callipleura*, respectively. *Hypsiboas ericae*, although the most basal species in the *H. pulchellus* group in the analyses of Faivovich *et al.* (2004) and Wiens *et al.* (2010), is sister to the clades A–C, but this relationship received no significant support. Clade D represents individuals occurring in parapatry or sympatry with *H. callipleura* in Departamento La Paz, Bolivia, but it is clearly distinct from the *H. callipleura* clade and relates to *H. balzani* sensu stricto. Individuals belonging to clade E were collected from Peru and represent an undescribed species of Andean *Hypsiboas* plus *H. palaestes*. The first three clades, corresponding to *H. andinus/H. riojanus*, *H. callipleura* and *H. marianitae*, appear to be more closely related to each other than to the remaining two clades.

In analyses containing 16S or cytochrome *b* alone, *H. callipleura* and *H. marianitae* are nested with the *H. andinus/H. riojanus* clade including all Argentine samples, whereas *H. andinus* from mountain forest sites in central

Table 1 Qualitative and quantitative characters of advertisement calls of different populations of Andean species of the *Hypsiboas pulchellus* species group. Unless otherwise mentioned, data are based on own analyses.

Species	Locality (temperature)	Notes/call	Note duration (ms)	Call duration (ms)	Pulses/s	Pulsatile vs. tonal notes	Dominant frequency (Hz)	Frequency modulation in notes	Amplitude modulation in notes	Number of calls analysed (source)
Hypsiboas callipleura	Bolivia: Cochabamba: Chapare, 1250 m a.s.l. (16 °C)	1–5 (3.0 ± 0.9)	17–35 (24.6 ± 5.7)	29–244 (135 ± 47)	–	Tonal	1070–1380	No	Yes	23
H. callipleura	Bolivia: La Paz: Colonía Eduardo Avaroa, 1300 m a.s.l. (20 °C)	1–5 (3.1 ± 1.2)	15–29 (20.2 ± 4.6)	15–205 (115 ± 52)	–	Tonal	1140–1320	No	Yes	8
Hypsiboas balzani	Bolivia: La Paz: near Apollo, 1400 m a.s.l. (–)	1 (1.0 ± 0.0)	226–237	226–237	40–43	Pulsatile	1106–1132	No	Yes	2
Hypsiboas gladiator sp. n.	Peru: Puno: San Juan del Oro, 1200 m a.s.l. (20 °C)	1 (1.0 ± 0.0)	257–531 (374 ± 92)	257–531 (374 ± 92)	110–150	Pulsatile	770–910	No	Yes	9
H. cf. *gladiator*	Peru: Cusco: 4 km WSW Santa Isabel (21 °C)	1	170	170	App. 80	Pulsatile	1150	No	Yes	(Duellman *et al.* 1997)
Hypsiboas marianitae	Bolivia: Santa Cruz: El Fuerte de Samaipata, 1700 m a.s.l. (22 °C)	13–33 (21.1 ± 6.9)	8–15 (11.4 ± 2.1)	398–924 (590 ± 175)	–	Pulsatile	790–1100	No	Yes	18
H. marianitae	Bolivia: Cochabamba: La Siberia, 2800 m a.s.l. (12 °C)	11–13 (12.0 ± 1.4)	8–14 (10.8 ± 1.8)	248–594 (357 ± 137)	–	Pulsatile	840–926	No	Yes	5
Hypsiboas palaestes	Peru: Ayacucho: Tutumbaro, 1840 m a.s.l. (14 °C)	4–5	20–80 (34)	ca. 200	42–44	Pulsatile	4430–4450	No	Yes	(Duellman *et al.* 1997)
Hypsiboas riojanus	Argentina: La Rioja: near Sanogasta (11 °C)	1–4	No data	ca. 200	–	Tonal	2300	Yes	Yes	(Barrio 1965)
H. riojanus	Argentina: Tucumán: Río Los Sosa, 890 m a.s.l. (20 °C)	3–5 (4.4 ± 0.9)	21–133 (50 ± 35)	272–381 (312 ± 43)	–	Tonal	2200–2300	Yes	Yes	5
H. riojanus	Argentina: Jujuy: Lozano, 1550 m a.s.l. (20 °C)	2–4 (3.0 ± 0.4)	14–79 (47 ± 23)	101–336 (236 ± 76)	–	Tonal	2480–2800	Yes	Yes	8
H. riojanus	Argentina: Jujuy: Tilcara, 2500 m a.s.l. (8 °C)	2–7 (3.7 ± 1.6)	16–77 (42 ± 22)	159–592 (309 ± 136)	–	Tonal	1850–2210	Yes	Yes	11
H. riojanus	Argentina: Salta: Cuesta del Obispo, 1480 m a.s.l. (18 °C)	2–3 (2.8 ± 0.5)	17–61 (41 ± 16)	108–213 (174 ± 43)	–	Tonal	2000–2310	Yes	Yes	7
H. riojanus	Argentina: Salta: Cuesta del Obispo, 1670 m a.s.l. (12 °C)	2–5 (3.5 ± 1.1)	16–85 (41 ± 25)	151–416 (291 ± 112)	–	Tonal	1700–1920	Yes	Yes	9
H. riojanus	Bolivia: Santa Cruz: El Fuerte de Samaipata, 1700 m a.s.l. (20 °C)	2 (2.0 ± 0.0)	48–65 (57.0 ± 6.9)	176–184 (181 ± 4)	–	Tonal	2010–2400	Yes	Yes	5
H. riojanus	Bolivia: Santa Cruz: La Siberia, 2800 m a.s.l. (11 °C)	2 (2.0 ± 0.0)	67–153 (101 ± 34)	237–281 (259 ± 15)	–	Tonal	1800–2200	Yes	Yes	11

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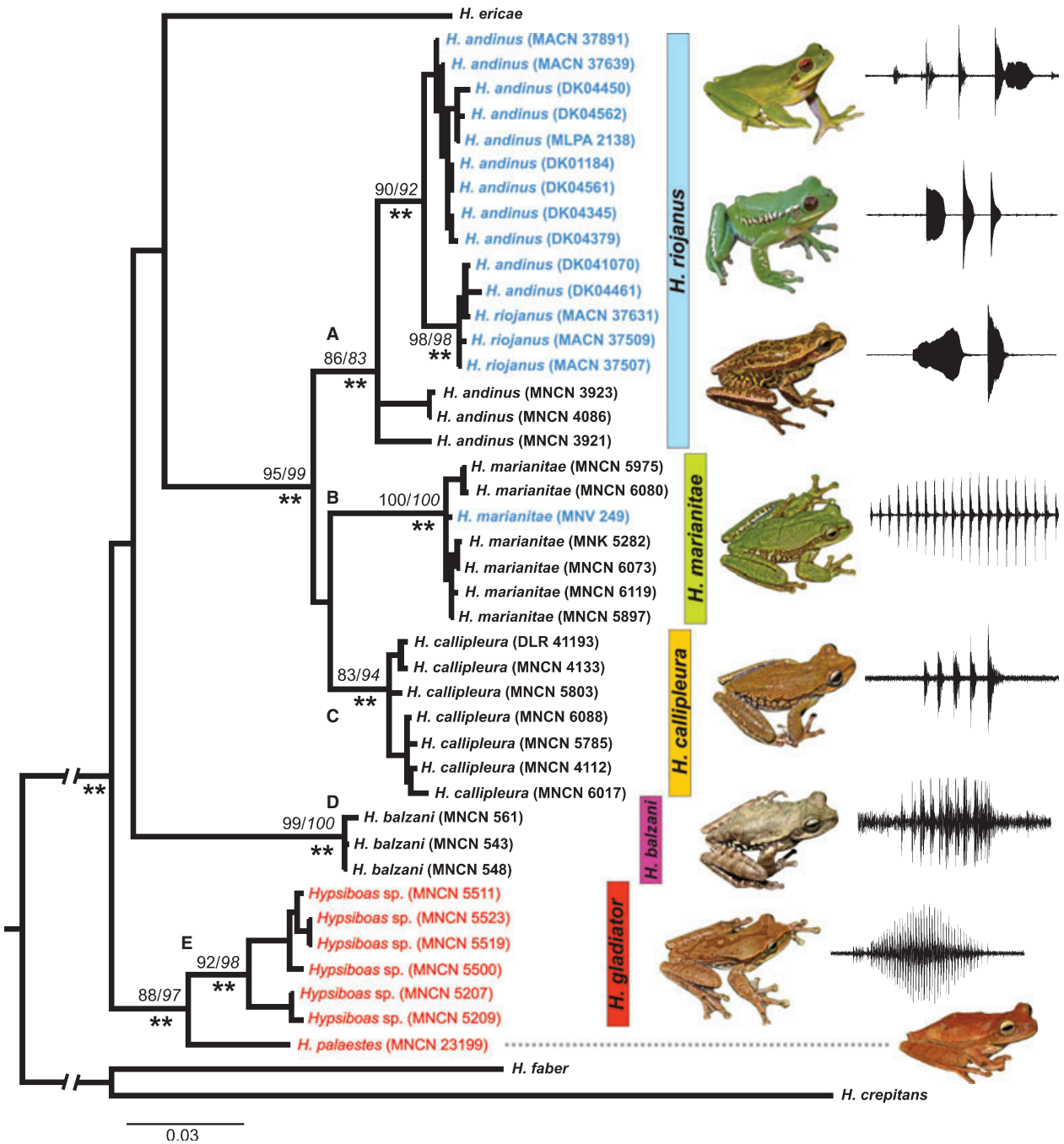

Fig. 1 Maximum likelihood tree based on 786 bp combined mitochondrial 16S and cytochrome *b* gene sequences of Andean populations of the *Hypsiboas pulchellus* group. *Hypsiboas faber* and *Hypsiboas crepitans* represent outgroup taxa. Values at nodes are bootstrap values in per cent from maximum likelihood (ML) and maximum parsimony (MP) (in italics), respectively; values below 80% not shown. Two asterisks denote a Bayesian posterior probability of 1.0. Coloured vertical bars correspond to the five species-level clades (A–E) referred to in the text (from top to bottom) and follow the taxonomy proposed herein. Photos depict representative specimens of respective clades to the left (not to scale), neighboured at the right by a respective 500 ms section of an oscillogram of the advertisement call of the taxon (not necessarily the figured specimen). For the clade of *Hypsiboas riojanus* several colour morphs and variation in advertisement call structure is depicted. Colours of sample names denote the country of origin as follows: black = Bolivia (except outgroup taxa and *Hypsiboas ericae*); blue = Argentina; orange = Peru. Numbers in parentheses refer to tissue IDs in respective collections. These numbers partly represent identical haplotypes shared among several specimens.

Bolivia are basal to these three clades. Also in the combined dataset, the Bolivian samples of *H. andinus* are basal to the clade with Argentine samples. However, support is rather low and relationships are not fully resolved (Fig. 1).

Given that the analyses of Faivovich *et al.* (2004, 2005) and Wiens *et al.* (2010) did not include samples of *Hypsiboas gladiator*, *H. palaestes* and *H. balzani* (their terminal for *H. balzani* actually corresponds to *H. callipleura* as identified herein; see Discussion), the general topology of our study is in agreement with those previous ones. Even the basal placement of *H. ericae* revealed by the mentioned former studies is apparent, although without support, when virtually considering the same reduced taxon sampling. However, our results indicate that an extended taxon sampling is required to resolve relationships within the entire *H. pulchellus* group including its low-elevation species.

Genetic divergence

The sequence divergence among studied species of Andean members of the *H. pulchellus* group was not exceptionally pronounced, given the uncorrected *p*-distances in the 16S gene fragment. Highest values were evident among individuals of the species pairs *H. marianitae–H. palaestes* (6.3–6.5%), *H. marianitae–H. gladiator* (5.2–5.4%), *H. marianitae–H. balzani* (5.0–5.2%), *H. palaestes–H. balzani* (5.0–5.4%), *H. palaestes–H. andinus/H. riojanus* (4.4–5.4%) and *H. palaestes–H. callipleura* (4.6–5.0%). Moderate values were found between *H. callipleura–H. gladiator* (4.0–4.4%), *H. andinus/H. riojanus–H. gladiator* (4.0–4.6%), *H. balzani–H. gladiator* (4.0–4.2%) and *H. andinus/H. riojanus–H. balzani* (3.2–4.0%). Comparatively low divergences were found between *H. balzani–H. callipleura* (3.0–3.6%), *H. marianitae–H. callipleura* (2.8–3.1%), *H. marianitae–H. andinus/H. riojanus* (2.5–3.2%), *H. palaestes–H. gladiator* (2.5–2.9%) and *H. callipleura–H. andinus/H. riojanus* (2.1–2.5%). The uncorrected *p*-distance between Bolivian and Argentine samples of *H. andinus/H. riojanus* varied from 1.5% to 2.3%. Within-clade divergences were all below 1.2%. The divergence of samples considered to represent *H. riojanus* from other Argentine samples of the *H. andinus/H. riojanus* clade was low (0.2–1.1%) and did not differ from values among samples within the whole clade.

Bioacoustics

In our analysis of vocalizations, we distinguished two groups of advertisement calls in Andean members of the *H. pulchellus* group. One group refers to calls of moderate duration with a strongly pulsatile nature, whereas the other group of calls is usually composed of short unpulsed harmonic notes. Pulsatile calls are emitted by frogs of the species *H. balzani*, *H. marianitae* and *H. gladiator* (described below), whereas short harmonic notes are emitted by *H. callipleura* and frogs in the *H. andinus/H. riojanus* clade. Comparison of call characteristics among all forms in this Andean species group revealed pronounced differences and allowed for unequivocal identification of most nominal species, except for differences of *H. andinus* and *H. riojanus* (see below). Differences among species are evident in temporal parameters, as well as in structure of calls (Fig. 2, Table 1). The greatest variability in temporal call parameters is exhibited by members of the geographically widespread *H. andinus/H. riojanus* clade. Differences within this clade are noticeable at the inter-populational level, but also among individuals within a single population (compare Duellman *et al.* 1997). However, the combination of two to seven moderately high-pitched harmonic notes with a characteristic shape in relative amplitude is shared by all *H. andinus/H. riojanus* populations studied. Despite this high variability, there appears to be some consistent differences between calls of Argentine and Bolivian populations considered here, with those from Bolivia emitting exclusively two-note calls, whereas the number of notes per call is highly variable among Argentine populations but typically calls are comprised of more than two notes.

Morphology

Our re-analysis of quantitative morphological characters in Andean species of the *H. pulchellus* group largely confirmed the results provided by Duellman *et al.* (1997). All measurements taken from specimens listed in the Appendix fall within the respective ranges provided by Duellman *et al.* (1997) and have broad overlap among nominal species. Therefore, we disregard quantitative measurements as being potentially useful to discriminate among Andean species of this group. In addition, we found extreme variability in colour pattern and dorsal skin texture among populations assigned to the *H. andinus/H. riojanus* clade. *Hypsiboas balzani* and *H. callipleura* were treated under the same name (*H. balzani*) by Duellman *et al.* (1997) and we were indeed unable to identify any reliable diagnostic morphological character distinguishing both clades. In both clades we noticed variability of dorsal colour (green, brown or beige), as well as different intensity and definition of dorsal markings. Similar intraspecific variability was evident in *H. marianitae*, with individuals exhibiting green, brown or beige dorsal colour, with different dorsal patterns ranging from uniformly coloured to strongly contrasting patterns with broad transverse bars. The undescribed species from Peru is also polymorphic in colour pattern. A summary of the qualitative characters of taxa (as defined by Duellman *et al.* 1997) in our morphological analysis is given in Table 2.

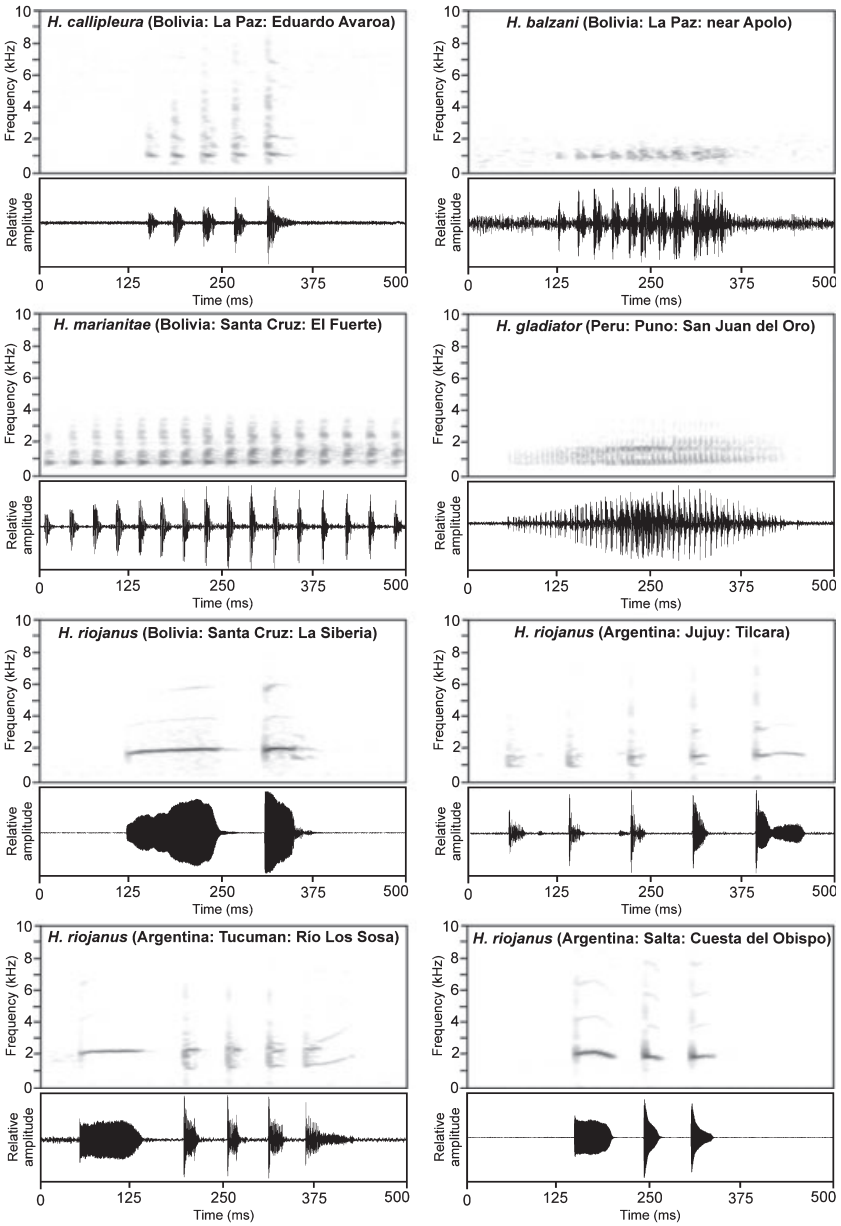

Fig. 2 Comparative spectrograms and oscillograms of advertisement calls of populations of Andean species of the *Hypsiboas pulchellus* group at the same time and frequency scale.

Discussion

The integrative approach and species diversity

For taxonomists, providing unequivocal diagnosis of interspecific differences and intraspecific polymorphism in anurans is often a difficult task when investigating morphology alone (Vences & Wake 2007). The diversity of evolutionary processes affecting character divergence, which may lead, for example, to striking cases of morphological crypsis or to situations where a single species may exhibit extraordinary levels of inter- or even intrapopulational polymorphism, call for the use of multiple lines of evidence to assess the status of species and to discover new ones (e.g. Djong *et al.* 2007; Brown & Twomey 2009; Vences *et al.* 2010). High levels of intraspecific and partly also intrapopulational polymorphism, in particular polychromatism, has been found for most Andean members of the *H. pulchellus* group for which there has been extensive sampling. The broad overlap in quantitative characters,

Table 2 Qualitative characters in external morphology of Andean members of the *Hypsiboas pulchellus* species group as used in the diagnostic scheme by Duellman *et al.* (1997). Data based on our examination of additional specimens (see Appendix).

Species	Snout shape in dorsal view	Snout shape in profile	Dorsal skin texture	Width of disc on finger III	Tarsal fold	Dorsal colour pattern	Flank colouration	Colour pattern of posterior surfaces of thighs
Hypsiboas alboniger	Rounded	Bluntly rounded	Coarsely granular	Equal to tympanum diameter	Elevated	Small irregular spots	Dark vertical bars	Dark vertical bars
Hypsiboas balzani	Rounded	Rounded to slightly truncate	Smooth to shagreen	Less than diameter of tympanum	Elevated	Irregular blotches and markings	Dark brown with cream blotches and flecks	Dark with cream flecks and spots
Hypsiboas callipleura	Subacuminate	Rounded	Smooth to shagreen	Less than diameter of tympanum	Elevated	Almost uniform, or with irregular blotches and (often transverse) markings	Dark brown with cream blotches and markings	Dark with cream flecks and spots
Hypsiboas gladiator sp. n.	Subacuminate	Rounded	Smooth	Equal to tympanum diameter	Elevated	Dark interorbital marking, irregular dark markings and blotches	Dark brown with cream blotches and markings	Dark with cream flecks and spots
Hypsiboas marianitae	Subacuminate	Rounded	Smooth	Less than diameter of tympanum	Low	Variable: uniform, or irregular dark markings, blotches and flecks	Dark brown with cream blotches and markings	Dark brown with cream blotches and markings
Hypsiboas melanopleura	Rounded	Bluntly rounded	Smooth	Less than diameter of tympanum	Low	Triangular interorbital bar, irregular dashes	Dark brown with small pale dots	Uniform
Hypsiboas palaestes	Rounded	Bluntly rounded	Smooth to shagreen	Equal to diameter of tympanum	Low	Irregular transverse marks or almost uniform	Uniformly dark, sometimes with small cream spots	Uniform
Hypsiboas riojanus (including its synonym *Hypsiboas andinus*)	Rounded	Bluntly rounded	Smooth to granular	Less than diameter of tympanum	Low	Extremely variable: small irregular spots, pale vertical bars, uniform	Extremely variable: small dark spots, pale blotches, uniform	Dark vertical bars

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and great intraspecific variability in qualitative and quantitative characters, impede our ability to unambiguously distinguish among *H. andinus/H. riojanus*, *H. balzani*, *H. callipleura*, *H. marianitae* and *H. gladiator* by morphology alone. The situation might be clearer for *H. alboniger*, *H. melanopleura* and *H. palaestes*, as all three species seem to exhibit a comparatively consistent colour pattern (Duellman *et al.* 1997; Lehr & von May 2004).

Advertisement calls within the group are well differentiated (Fig. 1) and strongly indicate prezygotic isolation. The species-level clades from our molecular phylogenetic analyses are supported by distinct advertisement calls. Thus, in addition to differences in morphology, analyses of vocalizations may help to assess divergence between species. A shift in temporal parameters (e.g. pulse rate, note repetition rate, note duration) is the most obvious difference of the calls investigated and is a common pattern among closely related frog species (see Padial *et al.* 2007, 2008b; Brown & Twomey 2009; Padial & De la Riva 2009). Here, the qualitative differences of tonal vs. pulsatile calls also proved to be a reliable character to distinguish among clades. However, our sample size does not allow for temperature or body size corrections. Generally, temperature (e.g. Blair 1958), body size (e.g. Márquez 1995) and hormonal conditions (e.g. Girgenrath & Marsh 2003), among others, are factors that can potentially affect quantitative parameters of frog calls. But the effect of temperature on frog calls strongly varies across species (Navas 1996). Unfortunately, there is no study assessing the effect of temperature, body size or other extrinsic or intrinsic factors on the call parameters of the frogs studied herein. Most of the calls analysed by us were recorded at temperatures ranging between 16 and 22°C, a difference potentially influencing some quantitative parameters in calls only to a slight extent. However, as far as is known, these temperature differences are not known to change qualitative parameters of calls substantially (e.g. Gerhardt & Huber 2002). Thus, we here use qualitative call parameters as main evidence for species-specific differentiation of sister clades (see Figs 1 and 2). Even when considering quantitative parameters, it can be demonstrated that the quantitative differences among calls of populations of *H. riojanus* recorded at 8 and 20 °C (both at Jujuy, Argentina) are rather low and by far less pronounced than geographical differences in calls of *H. riojanus* recorded at the same temperature (see Table 1). By demonstrating that temperature variation in quantitative parameters is lower than geographical call variation within one clade, we are convinced that our interpretation of call differences is not impaired by differences in recording temperatures.

Furthermore, our analysis suggests a case of independent evolution of pulsatile calls within the group. The phylogenetically most basal species emit pulsatile calls, and *H. marianitae* emits strongly pulsatile calls too, although it is nested within the clades representing *H. callipleura* and *H. riojanus*, both of which produce tonal notes.

Numbers of recognized amphibian species have recently increased steeply at the global scale (Köhler *et al.* 2005b; Vieites *et al.* 2009). The reasons for this are: (i) intensified exploration of poorly surveyed areas and (ii) use of a combination of character sets in taxonomic assessments (e.g. Padial & De la Riva 2009). This integrated approach, using different lines of evidence, allows for the identification of potential candidate species to be named (Vieites *et al.* 2009), as well as the classification of nominal species as stable or unstable in view of the probability of future changes according to the available lines of evidence and in case that more data accumulate (Padial *et al.* 2009).

Our integrative analysis of Andean members of the *H. pulchellus* species group provides evidence that among the nominal taxa recognized (Duellman *et al.* 1997), species diversity is actually higher than previously inferred. *Hypsiboas balzani* and *H. callipleura* could clearly be separated based on bioacoustics and molecular data, the new species *H. gladiator* could be identified from southern Peru (formerly partly considered to represent *H. balzani* by Duellman *et al.* 1997) and there is some indication that a further candidate species might occur along the Andean slopes of central Bolivia within what is here considered as *H. riojanus*. Furthermore, we corroborate a stable taxonomic status for *H. balzani*, *H. marianitae*, *H. callipleura*, *H. palaestes* and *H. gladiator* from southern Peru. In contrast, the taxonomic status of *H. riojanus* as recognized here should be considered unstable. A more dense sampling of populations across its supposed range would reveal a more detailed picture of actual genealogical relationships and patterns of differentiation, implied here by the divergence of the Bolivian samples included in our analyses. In addition, more species of this group are likely to be discovered, as field studies continue to sample poorly surveyed areas.

Molecular differentiation

DNA markers have increasingly been used to aid in delimitation of distinct lineages that may represent different species, especially for groups such as amphibians where other characters may not be as informative (Vences *et al.* 2005). In our study, between-species divergence (uncorrected *p*-distances) ranged between 2% and 6%, whereas within-species divergences were below 1.2%, except for the *H. riojanus* clade (1.5–2.3%), indicating the possible presence of an additional candidate species in Bolivia (see above). Our results on genetic divergences conform to

those of other studies of Neotropical frogs. Although divergence in mitochondrial markers both between and within Neotropical species varies widely, ranges reported in several studies are similar. Sequence divergence values of 2–3% or more in the 16S rRNA mitochondrial gene were interpreted to reflect distinct species of frogs by Chek *et al.* (2001) for the *Dendropsophus leucophyllatus* group, Symula *et al.* (2003) for Amazonian *Dendrobates* species, Camargo *et al.* (2006) for the *Leptodactylus fuscus* complex, Fouquet *et al.* (2007b) for *Scinax ruber* and *Rhinella margaritifera* species groups, and Funk *et al.* (2007) for the *Engystomops petersi* complex. High sequence divergence (>10%) between species has also been reported in certain groups (e.g. Chek *et al.* 2001; Symula *et al.* 2003; Fouquet *et al.* 2007b; Padial *et al.* 2009).

Systematics

Hypsiboas andinus, formerly a subspecies of *H. pulchellus*, was considered to represent a polymorphic and widespread species by Duellman *et al.* (1997). The elevation to species rank by Duellman *et al.* (1997) was based on some differences in colour (lack of the black bars on flanks and thighs of *H. riojanus*, implicitly considered a full species too) as well as on discontinuities in the distribution of the taxa. According to Langone & Lavilla (2002), the only recognizable difference between these species is the lack of dashed dorso-lateral lines in the population of *H. riojanus* from the type locality. *Hypsiboas riojanus* was originally described from La Rioja province, Argentina (Koslowsky 1895). Subsequently, in his revision of Andean members of the *H. pulchellus* group, Barrio (1965) considered this species to be a subspecies of *H. pulchellus*. Faivovich *et al.* (2004) formally elevated the taxon to species rank once again because they found that *H. riojanus* and *H. pulchellus*, a lowland species, were not sister taxa. The same analysis revealed a sister relationship of *H. riojanus* and *H. andinus*, and although the authors noted that morphological and acoustic characters of *H. andinus* and *H. riojanus* are very similar, they tentatively considered both taxa as valid. More recently, Koscinski *et al.* (2008), using a broader population sampling, found *H. riojanus* to be nested within *H. andinus* and suggested that the taxa might be conspecific.

Our molecular analyses, including three populations of *H. riojanus* (one sample from Sanogasta is only 20 km from the type locality) and 16 populations of *H. andinus*, support the conspecificity of both taxa. Furthermore, we found that the taxa possess no distinctive morphological characters. Indeed, we found that colour pattern, which has been the main characters used to distinguish the taxa (e.g. Barrio 1965; Cei 1980; Duellman *et al.* 1997), is highly variable. Finally, the broad overlap and variability of advertisement call characters among populations of the *H. andinus/H. riojanus* clade is inconsistent with the sympatric existence of two separate species. Consequently, we consider *H. andinus* and *H. riojanus* synonymous. As the name *H. riojanus* has priority, *Hyla andina* Müller 1924 (type locality: Caspinchango, Catamarca province, Argentina, by neotype designation; Langone & Lavilla 2002) becomes a junior synonym of *Hyla riojana* Koslowsky 1895.

In our phylogenetic analysis, Central Bolivian populations formerly referred to as *H. andinus* are sister to Argentine *H. riojanus*, although this relationship is not well supported. Genetic divergences between Bolivian montane forest populations and Argentine *H. riojanus* is moderate (1.5–2.3%), and advertisement calls seem to exhibit some quantitative differences, namely the number of notes per call tending to be higher in Argentine populations (Table 1). Thus, there is some indication that Bolivian mountain forest populations may represent an undescribed candidate species (see Vieites *et al.* 2009). However, it is also likely that populations from the highlands of southern and central Bolivia (e.g. terminal labelled as MNCN 4086 in Fig. 1) are conspecific with *H. riojanus* from Argentina. Both lineages may therefore occur in parapatry or even sympatry in Bolivian territory. As our sample size does not permit a sound taxonomic conclusion, we here tentatively consider Bolivian populations as a candidate species within *H. riojanus* pending further study, and following Padial *et al.* (2010), we identify it as *H. riojanus* (Ca1 Köhler *et al.* 2010).

According to morphological comparisons, Duellman *et al.* (1997) regarded *H. callipleura* as a synonym of *H. balzani*. The type locality of *H. balzani* – 'Prov. Yungas, 1600 m altitude' – lies within the surroundings of Coroico and Chulumani in the Departamento La Paz, Bolivia (Duellman *et al.* 1997). The type locality of *H. callipleura* was restricted to Charuplaya, 1350 m (Departamento Cochabamba, Bolivia) by lectotype designation (Duellman *et al.* 1997). By comparing advertisement call characters, Köhler (2000) provided evidence that specimens referred to as *H. balzani* from the Yungas de Cochabamba area probably represent a distinct species. Calls with the same characteristics as those described for the Cochabamba populations were later also recorded in the Departamento La Paz, close to the type locality of *H. balzani* (Köhler *et al.* 2006); given that the call of *H. balzani* was described as being rather different, these observations suggest that two related species currently classified under a single name occur in sympatry in the montane forests of northwestern Bolivia. Our molecular analyses now provide evidence for two distantly related clades that correspond to the two available species names. The molecular data are congruent

with the bioacoustic data, leaving no doubt about the existence of two species in the area investigated. However, as already discussed by Köhler (2000) the assignment of available names is hampered by the following facts: (i) there are no qualitative or quantitative morphological characters distinguishing *H. balzani* and *H. callipleura*; (ii) neither the different advertisement calls nor the molecular data are from the type specimens or topotypic material; and (iii) as both species apparently occur in sympatry at least in the Yungas de La Paz area (which also constitutes the vague type locality of *H. balzani*), we cannot exclude the possibility that both names are indeed synonymous and that there exists a second, as yet undescribed taxon. In conclusion, there is strong evidence from congruent bioacoustic and molecular analyses for the presence of two distinct species and there also are two available names, *H. balzani* and *H. callipleura*. Herein, we take the most conservative decision to confine the name *H. balzani* to the clade from the Yungas de La Paz with the pulsatile call and apply the name *H. callipleura* to the distantly allied clade with the more tonal call character. The main arguments for this action are the following: (i) the type locality of *H. balzani* is in the Yungas de La Paz area and it is reasonable to allocate the pulsatile call recorded by us in this area to *H. balzani* based on comparison of the type specimen (see Appendix) with our call vouchers; (ii) the type locality of *H. callipleura* is within the Departamento Cochabamba. In areas close to the type locality of *H. callipleura*, only melodic multiple note calls were heard and recorded, whereas pulsed calls are apparently missing. As a consequence of this new classification, most populations considered to represent *H. balzani* by Duellman *et al.* (1997) and those of Faivovich *et al.* (2004, 2005) and Wiens *et al.* (2010) now correspond to *H. callipleura*.

In addition, our analyses revealed the presence of an undescribed species of *Hypsiboas* occurring in the Andes of southern Peru. This new species (*H. gladiator*) is supported by differences in advertisement call, evidence of reciprocal monophyly, and genetic divergences (see description below).

Biogeography

Because basal nodes in our phylogenetic analysis are not strongly supported, the following biogeographic discussion should be considered only tentative. The most basal species in our phylogeny are those from southern Peru (*H. palaestes* and *H. gladiator*), which form the sister clade to the Bolivian-Argentine clade containing *H. balzani*, *H. callipleura*, *H. marianitae* and *H. riojanus*. Within the latter clade, *H. balzani* from northern Bolivia is basal to the species distributed in central and southern Bolivia and northern Argentina, although the position of *H. balzani* remains unresolved in our analyses. None of the southern ranging species occupies a basal position in the tree. This fact, together with evidence suggesting that the ancestor of Andean members of the *H. pulchellus* species group likely originated in the lowlands (Faivovich *et al.* 2004), suggest an Andean colonization in southern or central Peru with subsequent colonization and diversification towards the south along the Andean slopes. A latitudinal pattern of cladogenesis along the Andean hills from Peru and Bolivia has also been reported for other anurans (Padial *et al.* 2008a) and reptiles (Doan 2003). Doan (2003) predicted that because the Andean orogeny proceeded from south to north, we should expect a pattern of cladogenesis of Andean species following the rise of the Andes, with basal lineages occurring in the South and derived ones towards lower latitudes. Although she found some basal lineages with southern distributions, the pattern was not consistent enough to support her hypotheses. Contrarily to what is predicted by the South to North hypothesis, our results, as well as those of Padial *et al.* (2008a), indicate that at least certain basal lineages entered the Andes from northern South America and proceeded with diversification towards the South. Another interesting pattern is the latitudinal segregation of allo-parapatric sister species. *Hypsiboas marianitae* is a southern ranging species inhabiting dry forests of the inter-Andean valleys of Argentina and Bolivia, scarcely entering adjacent humid montane forests to the north, where it may meet the range of its sister *H. callipleura*, a species restricted to humid forests. Also, the populations of *H. riojanus* from the southernmost extent of the Yungas forest system and those populations from cloud forests and paramos of Bolivia form sister clades that might represent different, allo-parapatric species with some zone of contact at the upper limit of the Tucumanian forest in the South. The mostly parapatric species that share comparatively small areas of sympatry are the cryptic species pair *H. balzani* and *H. callipleura*, and the pair *H. marianitae* and *H. riojanus*. Nonetheless, despite the extreme level of morphological resemblance, these species pairs are not necessarily sister species (as in the case of *H. balzani* and *H. callipleura*) and they all differ from each other by considerable differences of their advertisement calls. *Hypsiboas palaestes* and *H. gladiator* from southern Peru, the most basal species, inhabit apparently similar humid montane forests of the Amazonian slopes in putative allopatry (nearest records between species span approximately 230 km). The described pattern among sister species – either occurring in allopatry or sharing comparatively small areas of sympatry only – is consistent with the idea that ancestral lineages experienced successive splitting events along a latitudinal gradient. However, whether the observed differ-

Fig. 3 Andean members of the *Hypsiboas pulchellus* group in life. —A–B. Male holotype of *Hypsiboas gladiator* sp. n. (MHNC 5391), from Valle de Marcapata, Departamento Cusco, Peru. —C. Amplectant pair of *H. gladiator* sp. n. (including female paratype MNCN 43703) from San Juan del Oro, Departamento Puno, Peru. —D. Male *Hypsiboas balzani* from near Apolo, Departamento La Paz, Bolivia. —E. Male *Hypsiboas callipleura* from near El Palmar, Departamento Cochabamba, Bolivia. —F. Male *H. callipleura* from Incachaca, Departamento Cochabamba, Bolivia. —G. Male *Hypsiboas marianitae* from Samaipata, Departamento Santa Cruz, Bolivia. —H. Amplectant pair of *H. marianitae* from Karahuasi, Departamento Santa Cruz, Bolivia. —I. Male *Hypsiboas alboniger* from Toro Toro, Departamento Potosí, Bolivia (photo by P.L. Ibisch). —J. Male *Hypsiboas palaestes* from Río Kimbiri, Cusco, Peru. —K. Male *Hypsiboas melanopleura* from Huancabamba, Departamento Piura, Peru (photo by E. Lehr). —L. Male *Hypsiboas riojanus* from P.N. Calilegua, Provincia Jujuy, Argentina. —M. Male *H. riojanus* from Molinos, Provincia Salta, Argentina. —N–O. Males of *H. riojanus* from Pomán, Provincia Catamarca, Argentina. —P–Q. Males of *H. riojanus* from Incachaca, Departamento Cochabamba, Bolivia. —R. Male of *H. riojanus* from Vallegrande, Departamento Santa Cruz, Bolivia.

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ences in call characters, habitat choice, or mtDNA sequences between sister parapatric species is the result of vicariant speciation with subsequent secondary contact (e.g. Ribas *et al.* 2007), or whether those differences evolved along a progressive process of dispersal to new areas (e.g. Elias *et al.* 2009), eventually enhanced by subsequent vicariant events, cannot be elucidated with the data at hand. A future analysis of ancestral areas reconstruction based on a fully resolved phylogeny with broader taxon sampling will shed light on the process responsible for this interesting pattern. Also, phylogeographic analysis combined with niche analyses may help to identify range expansion–contraction events, associated with wet and dry refugia along the Andean slopes.

Description of a new species

Genus *Hypsiboas* Wagler, 1830

Hypsiboas gladiator sp. n. (Fig. 3A–C)

Holotype. MHNC 5391 (field number 4639), adult male, from San Miguel, Valle de Marcapata, Province Quispicanchis, Departamento Cusco, Peru, 13°24′14.1"S, 70°53′57.7"W, 1155 m a.s.l., collected on 20 February 2006 by I. De la Riva, J. M. Padial, S. Castroviejo-Fisher and J. C. Chaparro.

Paratypes. MNCN 43710, adult female, from a point between San Miguel and Marcapata, Valle de Marcapata, Departamento Cusco, Peru, 13°28′26.2"S, 70°53′46.2"W, 1612 m a.s.l., collected on 21 February 2006; MNCN 44242, adult male, from Unión, Valle de Kosñipata, Departamento Cusco, Peru, 1800 m a.s.l., collected on 09 February 2007; MNCN 43706, adult male, from a point between Capiri and Mamabamba, Valle de Marcapata, Departamento Cusco, Peru, 13°28′24.0"S, 70°53′48.3"W, 1543 m a.s.l., collected on 20 February 2006 all by I. De la Riva, J. M. Padial, S. Castroviejo-Fisher and J. C. Chaparro. MNCN 43701–702, adult males, MNCN 43703, adult female, from a point between Santa Rosa and San Juan del Oro, Departamento Puno, Peru, 14°11′32.4"S, 69°04′46.2"W, 1097 m a.s.l., collected on 12 February 2006 by I. De la Riva, J. M. Padial, J. Bosch, S. Castroviejo-Fisher and J. C. Chaparro; MHNC 4192, 4303, 4326, 5049, adult males, MHNC 4296, subadult male, from San Pedro, Distrito Kosñipata, Provincia Paucartambo, Departamento Cusco, Peru, 13°03′47.5"S, 71°33′04.2"W, 1432 m a.s.l., collected on 13 November 1999, 26 June 2000, 01 July 2000, 15 April 2004, respectively, by J. C. Chaparro, J. A. Ochoa, J. A. Zegarra and O. D. Mujica; MHNC 5050, 5055, adult males, MHNC 5054, adult female, from Unión, Distrito Kosñipata, Provincia Paucartambo, Departamento Cusco, Peru, 13°04′17.2"S, 71°33′18.0"W, 1778 m a.s.l., collected on 10 May 2005 by J. C. Chaparro and J. A. Ochoa; MHNC 5075, adult male, 5074, adult female, from between Unión and San Pedro, Distrito Kosñipata, Provincia Paucartambo, Departamento Cusco, Peru, 13°04′03.5"S, 71°33′16.0"W, 1641 m a.s.l., collected on 15 April 2006 by J. C. Chaparro; MHNC 5661, adult male, from Santo Domingo de Carabaya, Distrito Coasa, Provincia Carabaya, Departamento Puno, Peru, 13°49′59.56"S, 69°38′31.39"W, 1669 m a.s.l., collected on 11 November 2006 by J. C. Chaparro; MHNC 5793, 5796, 5806, 5816, 5820, 5821, adult males, MHNC 5795, 5797, 5804, adult females, from Sirigua, Distrito Camanti, Provincia Quispicanchis, Departamento Cusco, Peru, 13°25′10.6"S, 70°54′23.9"W, 1220 m a.s.l., collected on 24–25 April 2007 by A. J. Delgado, A. Quiroz and R. Gutiérrez; MHNC 5956–5958, adult males, from Punto 1, near Santo Domingo de Carabaya, Distrito Coasa, Provincia Carabaya, Departamento Puno, Perú, 13°49′23.1"S, 69°38′30.3"W, 1975 m a.s.l., collected on 28 February 2007 by J. C. Chaparro, A. Quiroz and J. A. Zegarra.

Etymology. The specific epithet 'gladiator' is derived from Latin and refers to the fact that male–male combats involving the use of prepollical spines are a characteristic feature among species of the group, including this new one; hence, these species are called 'gladiator frogs'. The specific name is used as a noun in apposition.

Diagnosis. A moderate-sized species of the *H. pulchellus* species group characterized by: (i) snout subacuminate in dorsal view and rounded in profile; (ii) skin on dorsum smooth; (iii) width of disc on finger III equal to diameter of tympanum; (iv) tarsal fold low, weak; (v) dorsal colour pattern consisting of irregular blotches or reticulations; (vi) flanks brown with pale spots and flecks; (vii) posterior surfaces of thighs mottled brown with pale spots; (viii) advertisement call consisting of a single, long, pulsed note of 257–531 ms duration.

Comparisons. Apart from its genetic differentiation in the mitochondrial 16S and cytochrome *b* gene fragments, *H. gladiator* differs from other Andean species of the *H. pulchellus* species group in other ways. From *H. alboniger* it differs by having smooth dorsal skin (vs. coarsely granular) and a different colour pattern (large dark dorsal blotches lacking in *H. alboniger*); and from *H. callipleura* and *H. riojanus* mostly by having calls with pulsatile notes (vs. tonal). Morphologically, the new species is most similar to *H. balzani* and *H. marianitae*, but differs from both by advertisement call characteristics. The pulse rate in the call of *H. gladiator* is far higher compared to that of *H. balzani* (110–150 pulses/s vs. 40–43 pulses/s), and note duration is much longer compared to that in calls of *H. marianitae* (257–531 ms vs. 8–15 ms). Despite relatively low genetic divergence, *H. gladiator* differs from its sister species *H. palaestes* by the presence of white flecks and blotches on dark flanks (flecks and blotches lacking in

H. palaestes) and by the advertisement call. The call of *H. palaestes* (Duellman *et al.* 1997) consists of 4–5 pulsed notes, each of 20–80 ms duration (vs. single notes of 257–531 ms duration in *H. gladiator*), exhibiting a pulse rate of 42–44 pulses/s (vs. 110–150 pulses/s in *H. gladiator*), and a dominant frequency of 4430–4450 Hz (vs. 770–900 Hz in *H. gladiator*). Differentiation of *H. gladiator* and *H. melanopleura* is more difficult, as call recordings and phylogenetic analyses are lacking for *H. melanopleura*. However, none of the known specimens of *H. melanopleura* (n = 16; Duellman *et al.* 1997; Lehr & von May 2004) exhibit flanks with white blotches (only fine white spots are present in some individuals) and flecks on posterior surfaces of thighs, whereas in all known adult specimens of *H. gladiator* (n = 28) larger white blotches and flecks are present on the flanks and posterior surfaces of thighs. Individuals of *H. gladiator* usually exhibit a light dorsolateral line (27 out of 28 specimens) separating the dorsal colour pattern from that on flanks, which is absent in the known specimens of *H. melanopleura* (see Fig. 3). Furthermore, *H. melanopleura* has yellow dorsal colours at daytime (Lehr & von May 2004), which was not present in *H. gladiator*, and a less prominent tarsal fold. Although far from convincing when applying an integrative approach, we here consider it the most parsimonious choice to regard the constant differences in morphology observed among the voucher series of both species as an indication of inter-specific differentiation.

Description of the holotype. Body slender; head as wide as body, its width about equal its length; head width 33.9% of SVL, head length 34.3% of SVL; snout subacuminate in dorsal view, rounded in profile; interorbital distance 129% of width of eyelid; diameter of eye 121% of eye–nostril distance; tympanum separated from eye by distance about equal to diameter of tympanum; diameter of tympanum 44.7% of diameter of eye. Forearms hypertrophied, breadth of forearm 42.7% of its length; ulnar fold distinct, elevated; webbing formula for fingers: II1–1.5III1.5–1IV; width of disc on finger III equal to diameter of tympanum; prepollical spine blunt, projecting at right angle; nuptial excresences absent. Hind limb moderately slender; tibia length 54.8% of SVL; foot length 73.7% of SVL; inner tarsal fold distinct, straight; inner metatarsal tubercle ovoid, barely visible from above; webbing formula for toes: I0.5–0.75II0–1III0.25–1IV1–0 V. Skin on dorsum and flanks smooth, skin on venter granular. Dentigerous processes of vomers moderately long, transverse, narrowly separated medially, posteromedial to rounded choanae, each bearing six teeth.

Measurements (in mm): SVL 43.4; head width 14.6; head length 14.9; upper eyelid width 3.4; interorbital distance 4.4; tympanum diameter 2.1; eye diameter 6.3; eye–nostril distance 3.9; tibia length 23.8; foot length 18.8.

In preservative, dorsal surfaces dark cream with irregular brown markings and flecks; brown interorbital bar extending on upper eyelids and connected with brown marking posteriorly; upper surfaces of thighs dark cream with some rounded tan blotches; posterior surfaces of thighs brown with scattered white spots; a white supracloacal stripe; flanks dark brown with white flecks and spots, sharply contrasting with dorsum; tympanic region dark brown, loreal region brown; posterior regions of upper lip cream; ventral surfaces yellowish cream, throat with some brown mottling anteriorly; palmar and plantar surfaces greyish-brown.

In life, colouration differs mainly from that in preservative by having more vivid colours, an irregular green stripe in the loreal region, extending from eye to nostril, and a green fleck between eye and tympanum. The iris is silvery grey (Fig. 3A,B).

Variation. In general, *H. gladiator* is very variable with respect to colour pattern. Dorsal colour in preservative varies from brown to pale beige. Dark dorsal markings and blotches vary in size, outline and distinctness. Cream markings on flanks vary in size and shape, sometime being round spots only, sometimes being large irregular blotches, but are always present. In preservative, the throat can be white, cream or grey. Some male paratypes exhibit numerous transversal scratch markings on the dorsum, caused by male combats. In MNCN 43710, the snout appears more rounded in dorsal view compared to the holotype. Some specimens had predominantly green dorsal surfaces in life. Adult females are slightly larger than adult males (47.8–55.3 mm vs. 35.3–49.4 mm SVL). For measurements of type specimens, see Table S2.

Vocalization. The advertisement call of *H. gladiator* from San Juan del Oro consists of a single pulsed note with 257–531 ms duration, repeated at irregular intervals (Fig. 2). Each note contains approximately 37–62 (50 ± 9) pulses; pulse repetition rate within notes varies from 110 to 150 pulses/s. Apart from the pulsatile nature of notes, amplitude modulation is evident, with energy increasing and reaching its maximum at the middle of the note, then decreasing again. Frequency is distributed in a broad range from approximately 600–4000 Hz without distinct modulation, with maximum call energy at about 770–900 Hz. Air temperature during recording was 20 °C.

Distribution and ecology. The species is known only from the regions of Cusco and Puno, Peru, between 1097 and 1975 m a.s.l., from the localities of the type specimens (Fig. 4). The female KU 139212 figured by Duellman *et al.* (1997) as *H. balzani* from 4 km WSW from Santa Isabel, Region Cusco, Peru, is assignable to *H. gladiator* based on colour pattern and external morphology. *Hypsiboas gladiator* is fairly common along road ditches in primary and secondary rainforest of the Andean slopes,

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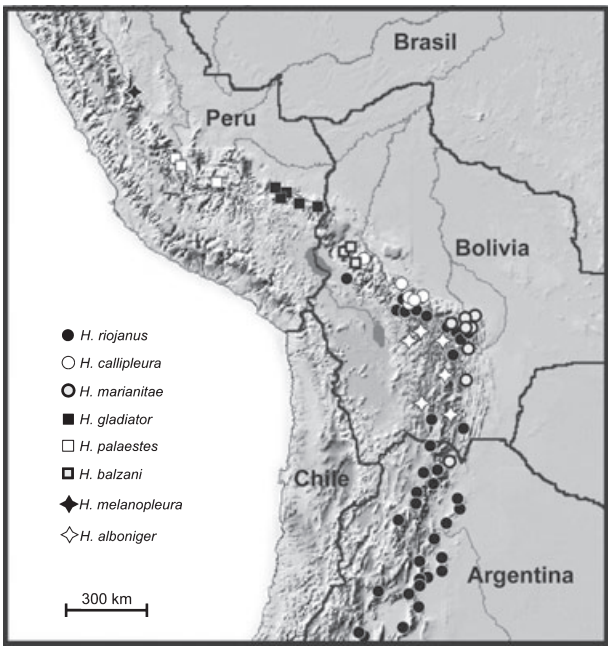

Fig. 4 Map of western-central South America indicating the known distributions of Andean species of the *Hypsiboas pulchellus* group. Placement of symbols is only approximate. Some species occur in sympatry at certain localities; the respective species' symbols are placed close to each other and overlapping. Localities of populations with uncertain specific identity are not shown.

where calling males are often heard. Tadpoles were abundant at the time of collecting. A dead adult was found between Capira and Mamabamba, Marcapata valley. Tests for infection by *Batrachochytrium dendrobatidis* based on qPCR were positive for adult individuals from San Juan del Oro area and Marcapata Valley (unpublished data: Jaime Bosch, pers. comm.).

Acknowledgements

We are grateful to E. O. Lavilla for providing literature, and to P. L. Ibisch and E. Lehr for providing photos of *Hypsiboas alboniger* and *Hypsiboas melanopleura*, respectively. O. Aguilar (MHNC), W. Böhme (ZFMK), B. T. Clarke (BM), G. Doria (MCSN) and W. E. Duellman (KU) provided access to specimens under their care, and J. A. Delgado provided material and data. For help during fieldwork, we are indebted to M. I. Bonansea, J. Bosch, S. Castroviejo-Fisher, S. Lötters, P. L. Tubaro and M. Vaira. Also, J. Bosch and S. Walker provided the data on chytrid infection of the new species. Three anonymous referees provided useful comments. We are grateful to the official authorities of Argentina, Bolivia and Peru for support and permissions. Collection and export permits for Peru are 008-2005-INRENA-IFFS-DCB and 002163-AG-DGFFS, respectively. Fieldwork of JK was supported by the German Academic Exchange Service (DAAD). JMP was funded by the EU Marie Curie Mobility and Training Programme (FP7, proposal 220714). This work was partially funded by projects CGL2005-03156 and CLG2008-04164 of the Spanish Ministry of Science and Innovation (IDlR, Principal Investigator), and NSERC grants to PH and SCL. DK was funded by NSERC, OGSST, Sigma Xi Grants in Aid of Research, Gaige Award from the ASIH, and various University of Western Ontario awards. DK wishes to thank M.-A. Lachance for providing expertise and laboratory space.

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Appendix

Museum abbreviations used

Museum abbreviations refer to: Natural History Museum, London, UK (BM); Canadian Museum of Nature, Ottawa, Canada (CMNAR); Colección Boliviana de Fauna, La Paz, Bolivia (CBF); Fundación Miguel Lillo, Tucumán, Argentina (FML); University of Kansas, Museum of Natural History, Lawrence, USA (KU); Museo Argentino de Ciencias Naturales, Buenos Aires, Argentina (MACN); Museo de Ciencias Naturales, Universidad de Salta, Salta, Argentina (MCN); Museo Civico di Storia Naturale, Genova, Italy (MCSN); Museo de Historia Natural, Universidad Nacional de San Antonio Abad del Cusco, Cusco, Peru (MHNC); Museo Nacional de Ciencias Naturales, Madrid, Spain (MNCN); Museo Noel Kempff Mercado, Amphibian Collection, Santa Cruz, Bolivia (MNK-A); Naturhistoriska Rijksmuseet, Section for Vertebrate Zoology, Stockholm, Sweden (NHRM); and Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany (ZFMK).

Additional specimens examined

Hypsiboas alboniger. BOLIVIA: Potosí: Toro Toro, ZFMK 54566; Puka Khara, NHRM 1873 (syntypes of *Hyla ocapia*); Sucre: Sucre, ZFMK 28601.

Hypsiboas balzani. BOLIVIA: La Paz: Prov. Yungas, 1600 m altitude [=Coroico and Chulumani], MCSN 28872 (holotype); between Río Ñeques and Apolo, CBF 2596; Arroyo Bilunto, close to Santa Cruz de Valle Ameno, CBF 2595; 5.2 km on road from Chaco to Chulumani, CBF 2585.

Hypsiboas callipleura. BOLIVIA: La Paz: between Caranavi and Palos Blancos, ZFMK 80582–583; Serranía Bellavista, Km 52, MNCN 42666, 42668, 43148; Serranía Bellavista, Km 40, MNCN 43301–302; Cochabamba: 120 km on road from Cochabamba to Chapare, 1050 m a.s.l., MNCN 43327–328; Chapare, between Paractito and El Palmar, MNCN 42662, ZFMK 66967, 72554, 72544, 72547–548, 72583–585, 72613; Chapare, road to San Onofre, MNCN 43110–111, 43113–117; near Tablasmontes, 2400 m a.s.l., MNCN 43298–300; Parajctí, ZFMK 80037; Incachaca, ZFMK 66942–945; Charuplaya, BM 1947.2.13.65 (lectotype), BM 1947.2.13.64 (paralectotype).

Hypsiboas cordobae. ARGENTINA: Córdoba: Pampa de Achala, ZFMK 57326, 59626.

Hypsiboas marianitae. BOLIVIA: Santa Cruz: El Fuerte, ZFMK 83310–313; La Hoyada, ZFMK 72634–635; Karahuasi, MNCN 43047, ZFMK 67103, 72651–652; Samaipata, ZFMK 60412–413, 60419, 66886–887; S of Cuevas, ZFMK 66881; 40 km W of Río Seco, ZFMK 67059–060.

Hypsiboas melanopleura. PERU: Pasco: Huancabamba, BM 1947.2.13.55 (paralectotype).

Hypsiboas palaestes. PERU: Cusco: La Convención, Río Kimbiri, Comunidad Machiguenga Pomoreni, 1100 m a.s.l., MHNC 6795, MNCN 44415, 44418–420; Ayacucho: SW Ayna, KU 163305 (holotype), KU 163306–314 (paratypes).

Hypsiboas riojanus. ARGENTINA: Salta: Santa Victoria, MCN 1259–60; San Andres, MCN 1203; Ruta de Cornisa, MCN 1201; Molinos, MCN 1209, 1223; S.F. de Escoipe, MACN 39035–36; Rosario de la Frontera, MCN 1223, 1253–58; Tucumán: Río San Ignacio, MCN 1206; Río el Nio, MCN 1208; Villa Nogués, MCN 1207; La Angostura, CMNAR 33140, MCN 1205; Río Los Sosa, FML 16112; Catamarca: Haulfín, MCN 1252; Las Estancias, MACN 39031; Pomán, MCN 1249–51; La Rioja: Sanogasta, MACN 39028 (tadpole); Jujuy: Tilcara, MACN 39037; P.N. Calilegua, MCN 1204; Lozano, MACN 39038; Villa Monte, MCN 1200; Maiz Gordo, MCN 1202. BOLIVIA: Cochabamba: near Corani, 3380 m a.s.l., ZFMK 60471–475; Incachaca, ZFMK 66947–952; Sehuencas, ZFMK 66828; Chapare 1800 m a.s.l., ZFMK 72580; Santa Cruz: Vallegrande, ZFMK 66901, 66903–904; La Siberia, ZFMK 66891; El Fuerte, ZFMK 60420–421.

Supporting Information

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

Table S1 Localities, voucher information and GenBank accession numbers for DNA sequences used in this study.

Table S2 Morphological measurements (in mm) of the type specimens of *Hypsiboas gladiator* sp. n. (M = adult male; F = adult female). Holotype voucher number in bold.

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