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Altitudinal distribution and advertisement call of *Colostethus latinasus* (Amphibia: Dendrobatidae), endemic species from eastern Panama and type species of *Colostethus* , with a molecular assessment of similar sympatric species

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Abstract

We conducted a molecular assessment of *Colostethus*-like frogs along an elevational gradient in the Serranía de Pirre, above Santa Cruz de Cana, eastern Panama, aiming to establish their species identity and to determine the altitudinal distribution of *C. latinasus.* Our findings confirm the view of *C. latinasus* as an endemic species restricted to the highlands of this mountain range, *i.e.*, 1350–1475 m.a.s.l., considered to be type locality of this species. We described the advertisement call of *C. latinasus* that consists of a series of 4–18 single, short and relatively loud "peep"-like notes given in rapid succession, and its spectral and temporal features were compared with calls of congeneric species. For the first time, DNA sequences from *C. latinasus* were obtained, since previously reported sequences were based on misidentified specimens. This is particularly important because *C. latinasus* is the type species of *Colostethus*, a genus considered paraphyletic according to recent phylogenetic analyses based on molecular data.

Key words: Neotropical, Anura, systematics, taxonomy, DNA barcoding, *Silverstoneia*

Resumen

Realizamos una evaluación molecular de ranas parecidas a *Colostethus*, a lo largo de un gradiente de elevación en la Serranía de Pirre, arriba de Santa Cruz de Cana, en el este de Panamá, con el objetivo de establecer la identidad de las especies y determinar la distribución altitudinal de *C. latinasus*. Nuestros hallazgos confirman la opinión de *C. latinasus* como una especie endémica restringida a las tierras altas de esta serranía, *i.e.*, 1350–1475 m.a.s.l., considerada la localidad tipo de esta especie. Describimos el llamado de anuncio de *C. latinasus* que consta de una serie de 4–18 notas sencillas, cortas y relativamente ruidosas, semejantes a un "piip", dadas en una sucesión rápida; además, comparamos sus características espectrales y temporales con los llamados de especies congéneres. Por primera vez, se obtuvieron secuencias de ADN de *C. latinasus*, ya que las secuencias previamente reportadas provienen de especímenes identificados erróneamente. Esto es particularmente importante debido a que *C. latinasus* es la especie tipo de *Colostethus*, un género considerado parafilético de acuerdo con análisis filogenéticos recientes, basados en datos moleculares.

Introduction

Colostethus latinasus was originally described by Cope (1863) as a *Phyllobates* from the Truandó region of northern Chocó, Colombia. Since its description, the identity of this species has been elusive, being frequently confused with other Central and South American dendrobatoid species (Savage 1968; Grant 2004), a confusion

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aggravated by the loss of the type specimen (Barbour & Noble 1920; Savage 1968). Aiming to stabilize the nomenclature of this species, Savage (1968) designated as the neotype a female specimen (National Museum of Natural History specimen USNM 50198) collected from Cana (= Santa Cruz de Cana), Darién Province, Republic of Panama. Despite doubts about the precise collecting locality of the neotype, Grant (2004) examined museum specimens from the Serranía de Pirre that agreed in all aspects with the neotype and concluded that it was probably collected from the highlands above Cana.

In the past two decades, the systematics of the anuran family Dendrobatidae has been comprehensively revised (*e.g.*, Grant *et al.* 2006). Even though several new species of this family have been described (*e.g.*, Grant & Myers 2013), many distinctive frogs remain undescribed (*e.g.*, Grant *et al.* 2006). Therefore, it is important for future systematic and taxonomic studies to clearly establish the identity of *C. latinasus*, the type species of the genus *Colostethus* Cope, 1866 (Savage 1968, Grant 2004, Grant *et al.* 2006, Frost 2016). Moreover, *Colostethus* is currently considered to be paraphyletic with respect to the genus *Ameerega* (Santos *et al.* 2009, Pyron & Wiens 2011), a condition still waiting to be resolved (Frost 2016).

The geographic distribution of *C. latinasus* is restricted to the mountain range of Serranía de Pirre. Other specimens previously assigned to this species (*e.g.*, Savage 1968) from nearby Cerro Malí, a peak in the Altos de Puna region of the Serranía del Darién (Myers & Lynch 1997), are considered to represent a similar yet undescribed species (Grant 2004). Given the well-established role of advertisement calls in species recognition and reproductive isolation (Wells 2007), and the relevance of call characters in anuran phylogenetic and systematic studies (Padial *et al.* 2008, Goicoechea *et al.* 2010), we describe here the advertisement calls emitted by males of *C. latinasus* from the Serranía de Pirre, above Cana, currently considered to be the type locality of this species (Grant 2004). In addition, we conducted a molecular assessment of individuals of similar sympatric species along an elevational gradient in this region, aiming to establish their identity and to determine the altitudinal distribution of *C. latinasus*.

Materials and methods

Survey area and specimen collection. To complement our observations on the altitudinal distribution of *C. latinasus* and establish the identity of similar sympatric species present in this geographic area, we collected tissue samples from individuals of *Colostethus*-like frogs and tadpoles, along a 449–1606 m.a.s.l. elevational gradient, from the surroundings (i.e., < 5 km) of Cana field station (7.755800ºN 77.684583ºW) to the ridge of the adjoining Serranía de Pirre (7.774500ºN 77.733200ºW). We followed the American Society of Ichthyologists and Herpetologists guidelines for use of live amphibians in field and laboratory research, and our protocol was approved by the Smithsonian Tropical Research Institute's IACUC. Field-caught specimens were euthanized with Benzocaine. Their tissues were stored in a NaCl-saturated buffer containing 0.25 M EDTA and 20% dimethyl sulfoxide (DMSO) (Seutin *et al.* 1991). All specimens were vouchered in museum collections. We used the following collection abbreviations: AJC = Andrew J. Crawford field series, CH = Círculo Herpetológico de Panamá, and MVUP = Museo de Vertebrados de la Universidad de Panamá. Snout-vent length (SVL) of voucher specimens were measured using a Mitutoyo Absolute digital caliper (model CD-6"CX) to the nearest 0.01 mm.

DNA barcoding and analysis. DNA extraction, PCR and sequencing protocols followed those of Crawford *et al.* (2010) for obtaining mitochondrial DNA sequence data from the COI 'Barcode of Life' fragment (Hebert *et al.* 2003) and the 16S ribosomal gene (Kessing *et al.* 2004). We obtained 657 aligned base pairs (bp) for the COI gene fragment, and 556 bp from the 16S gene after excluding sites with gaps.

Candidate evolutionary models were evaluated using *jModeltest* version 0.1.1 applied to each gene separately based on the corrected Akaike Information Criterion (AICc) to identify the model that simultaneously minimized bias and variance in parameter estimates (Anderson 2008, Posada 2008). As a model of intermediate complexity, the 2-rate HKY+G model, was recommended for the COI gene, and therefore was not partitioned further. The 6-rate GTR+G model was recommended for the 16S data subset. A 2-gene partitioned likelihood phylogenetic analysis (Felsenstein 1981) was conducted with *GARLI* version 2.0 using 20 replicate searches and default settings (Zwickl 2006). Parameters and rates were independent between the two data partitions. A bootstrap likelihood analysis was conducted in *GARLI*, with two replicate searches conducted per each of 300 bootstrapped datasets. To accelerate search times, parameter values for the best-fit unpartitioned evolutionary model (according to *jModeltest*) were set to their ML values.

Statistical support for clades was also evaluated by Bayesian Markov chain Monte Carlo (MCMC) phylogenetic inference (Yang & Rannala 1997) with *MrBayes* version 3.1.2 (Ronquist & Huelsenbeck 2003) on the CIPRES portal (Miller *et al.* 2010), assuming default parameters except the prior distribution on the rate heterogeneity shape parameter, alpha, was given a maximum value of 5.0, and three Metropolis-coupled chains were run with the temperature parameter lowered to 0.05 to increase swap rate among chains, based on trial runs. Two independent MCMC analyses were run for 5 million generations each with posterior probabilities estimated from samples taken every 1,000 generations following a burn-in of 3 million generations, at which point average standard deviation of split frequencies between runs had dropped below 0.015 and the chains had converged to similar likelihood scores.

Acoustic recordings and analysis. We used a SONY IC Recorder ICD-P320 with its built-in microphone to record the calls of the male preserved as voucher specimen CH 6400 (MVUP 2342, see below). The compressed format DVF files from this recorder were converted to 44.1 kHz and 16-bit WAV files, using the Digital Voice Editor 2 software. The calls of additional, unvouchered males were recorded using a Roland's Edirol 24-bit wave/ mp3 recorder R-09 with a Realistic directional microphone (Cat. No. 33-1062). These recordings were made at a sampling frequency of 44.1 kHz and a wav-16-bit mode. The air temperature was registered during recordings.

All recordings were analyzed with the sound analysis and synthesis laboratory software Avisoft-SASLab Pro version 5.1.23. We used the waveform display with a resolution of 0.2–0.3 milliseconds (ms) to measure the temporal variables of the call, and used the spectrogram display with a Hamming window, 1024 points FFT size, 56 Hz bandwidth and 43 Hz resolution for measuring frequency variables, and used the power spectrum to determine frequency peaks. For the spectral analysis of notes and creating graphics, only the best quality, uncompressed audio recordings were used. With the spectrogram display we determined the maximum and minimum frequencies of each note to calculate its bandwidth. Data extracted from calls of different males were pooled for descriptive statistical analyses. The potential effects of temperature on the temporal features of these calls could not be adequately assessed due to the small sample size. However, differences in air temperature of these call recordings were relatively small, i.e., maximum $\Delta T = 4°C$. Moreover, any intraspecific temporal variation in calls that could be attributed to temperature appeared to be minimal, especially for interspecific comparisons.

Results

Altitudinal distribution. We collected 46 individuals, comprising 5 to 24 specimens of four species (Table 1). Out of these 46 individuals, only 5 of these could be grouped as *C. latinasus* and were found at a 1350–1475 m.a.s.l. elevation range (Fig. 1). The other individuals could be grouped as *C.* aff. *panamansis* (7 individuals with a 494–1246 m.a.s.l. range), *C.* aff. *pratti* (24 individuals with a 449–1246 m.a.s.l. range), and *Silverstoneia* aff. *nubicola* (10 individuals with a 462–1597 m.a.s.l. range). These three species had a more extensive distribution along the elevational gradient compared to *C. latinasus*. *Colostethus* aff. *panamansis* and *C.* aff. *pratti* showed a similar altitudinal distribution pattern, while *Silverstoneia* aff. *nubicola* had the most extensive vertical distribution along the elevational gradient, overlapping the high-altitude distribution of *C. latinasus.* At high-altitude, individuals of *C. latinasus* can be distinguished from these three species by their relatively larger size (SVL of adult males = 29.40–32.39 mm, n = 3; adult female SVL = 29.83–30.16 mm, n = 2) and more terrestrial habits. In addition, adult individuals of *C. latinasus* have a marbled throat and chest, being more darkly marbled in males (Figs. 2, 3).

Vocalization. Males of *C. latinasus* were often observed in shaded and very humid areas with abundant leaf litter and fallen branches and logs, away from streams, calling on the forest floor or on fallen vegetation during 1130–1245 h, above Cana, near the ridge of Serranía de Pirre (7.76551ºN 77.72418ºW to 7.76893ºN 77.72758ºW, datum WGS84, 1350–1475 m.a.s.l.), Darién Province, Republic of Panama, on August 8, 2007.

A male (29.40 mm in SVL, preserved as a voucher specimen CH 6400 in the Círculo Herpetológico de Panamá collection (later deposited in the Museo de Vertebrados de la Universidad de Panamá as MVUP 2342, Fig. 3) and other calling males were recorded at an air temperature of approximately 23°C. The digital audio file of advertisement calls of male MVUP 2342 can be heard online at http://biogeodb.stri.si.edu/bioinformatics/dfm/metas/view/48934. Additional males were recorded at an air temperature of 19°C.

TABLE 1. Samples of dendrobatid (*Colostethus*-like) frogs and tadpoles used in molecular phylogenetic analyses. All samples were collected in and around the Cana field station, Darién National Park, Darién Province, Republic of Panama. AJC = Andrew J. Crawford field series, CH = Círculo Herpetológico de Panamá, and MVUP = Museo de Vertebrados de la Universidad de Panamá. Asterisk (*) indicates a tadpole specimen.

Genus	Species	Field series number	Collection specimen number	Elevation (meters)	GenBank numbers	
					COI	16S
Colostethus	*latinasus*	CH 6381	CH 6381	1475 m	KF806998	KF807037
Colostethus	*latinasus*	CH 6389	CH 6389	1400 m	KF806996	KF807035
Colostethus	*latinasus*	CH 6400	MVUP 2342	1350 m	KF806999	KF807038
Colostethus	*latinasus*	CH 6402	CH 6402	1475 m	KF806997	KF807036
Colostethus	*latinasus*	CH 6405	CH 6405	1475 m	KF806995	KF807034
Colostethus	aff. *panamansis*	AJC 1816	CH 9628	550 m	KC129188	KC129295
Colostethus	aff. *panamansis*	AJC 1856*	CH 9654	1246 m	KC129215	KC129323
Colostethus	aff. *panamansis*	AJC 1857*	CH 9655	1246 m	KC129214	KC129322
Colostethus	aff. *panamansis*	AJC 1859	CH 9634	1246 m	KC129213	KC129321
Colostethus	aff. *panamansis*	CH 5546 [a]	CH 5546	1246 m	KC129211	KC129319
Colostethus	aff. *panamansis*	CH 6356	CH 6356	494 m	KC129212	KC129320
Colostethus	aff. *panamansis*	CH 6448	CH 6448	1246 m	KC129189	KC129296
Colostethus	aff. *pratti*	AJC 1697	CH 10087	525 m	KF807020	KF807059
Colostethus	aff. *pratti*	AJC 1698	CH 10088	525 m	KF807023	KF807062
Colostethus	aff. *pratti*	AJC 1699	CH 10089	525 m	KF807021	KF807060
Colostethus	aff. *pratti*	AJC 1700	CH 10090	525 m	KF807000	KF807039
Colostethus	aff. *pratti*	AJC 1801	CH 10091	525 m	KF807002	KF807041
Colostethus	aff. *pratti*	AJC 1802	CH 10092	525 m	KF807004	KF807043
Colostethus	aff. *pratti*	AJC 1803	CH 10093	525 m	KF807005	KF807044
Colostethus	aff. *pratti*	AJC 1804	CH 10094	525 m	KF807006	KF807045
Colostethus	aff. *pratti*	AJC 1805	CH 10095	525 m	KF807007	KF807046
Colostethus	aff. *pratti*	AJC 1806	CH 10096	525 m	KF807008	KF807047
Colostethus	aff. *pratti*	AJC 1807	CH 10097	525 m	KF807009	KF807048
Colostethus	aff. *pratti*	AJC 1808	CH 10098	525 m	KF807010	KF807049
Colostethus	aff. *pratti*	AJC 1810*	CH 10106	526 m	KF807011	KF807050
Colostethus	aff. *pratti*	AJC 1811*	CH 10107	525 m	KF807012	KF807051

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TABLE 1. (Continued)

Genus	Species	Field series number	Collection specimen number	Elevation (meters)	GenBank numbers	
					COI	16S
Colostethus	aff. *pratti*	AJC 1812	CH 10099	550 m	KF807013	KF807052
Colostethus	aff. *pratti*	AJC 1815	CH 10100	550 m	KF807022	KF807061
Colostethus	aff. *pratti*	AJC 1877	CH 10101	1246 m	KF807015	KF807054
Colostethus	aff. *pratti*	CH 5524 [b]	CH 5524	550 m	KF807003	KF807042
Colostethus	aff. *pratti*	CH 5598 [c]	CH 5598	535 m	KF807017	KF807056
Colostethus	aff. *pratti*	CH 5601 [c]	CH 5601	525 m	KF807019	KF807058
Colostethus	aff. *pratti*	CH 5602 [d]	CH 5602	550 m	KF807018	KF807057
Colostethus	aff. *pratti*	CH 6330	CH 6330	525 m	KF807016	KF807055
Colostethus	aff. *pratti*	CH 6363	CH 6363	449 m	KF807001	KF807040
Colostethus	aff. *pratti*	CH 6446	CH 6446	1246 m	KF807014	KF807053
Silverstoneia	aff. *nubicola*	AJC 1870	CH 10102	1246 m	KF807030	KF807069
Silverstoneia	aff. *nubicola*	AJC 1880	CH 10103	1246 m	KF807031	KF807070
Silverstoneia	aff. *nubicola*	AJC 1881	CH 10104	1246 m	KF807026	KF807065
Silverstoneia	aff. *nubicola*	AJC 1889	CH 10105	1246 m	KF807027	KF807066
Silverstoneia	aff. *nubicola*	CH 5558	CH 5558	1130 m	KF807024	KF807063
Silverstoneia	aff. *nubicola*	CH 5599 [e]	CH 5599	525 m	KF807028	KF807067
Silverstoneia	aff. *nubicola*	CH 5600	CH 5600	525 m	KF807029	KF807068
Silverstoneia	aff. *nubicola*	CH 6369	CH 6369	462 m	KF807033	KF807072
Silverstoneia	aff. *nubicola*	CH 6370	CH 6370	462 m	KF807032	KF807071
Silverstoneia	aff. *nubicola*	CH 6404	CH 6404	1597 m	KF807025	KF807064

[a] COI was sequenced for the same individual by Grant *et al.* (2006), referred to as *C. panamensis*. The mitochondrial genome region 12S-16S was also sequenced for this same individual by Santos *et al.* (2009) and referred to as *C. panamensis* or *C. inguinalis*, and referred to as *C. panamansis* in Pyron & Wiens (2011). This individual was collected in the headwaters of Río Cana, Serranía de Pirre.

[b] COI was sequenced for the same individual by Grant *et al.* (2006), referred to as *C.* "*pratti*-like". This individual is from Cana, not from "Jungurudó", Darién, as reported previously, *i.e.*, sample 1144 in Grant *et al.* (2006). This specimen was referred to as *C. pratti* by Pyron & Wiens (2011).

[c] Referred to as *C.* "*pratti*-like" by Grant *et al.* (2006). 12S-16S was sequenced for the same individual and referred to as *C. latinasus* by Santos *et al.* (2009); subsequently, included in Pyron & Wiens (2011).

[d] Referred as *C.* "*pratti*-like" by Grant *et al.* (2006).

[e] 12S-16S was sequenced for the same individual by Santos *et al.* (2009), referred as *S. nubicola* or *C. nubicola*. This individual was collected in Cana on the trail to Boca de Cupe.

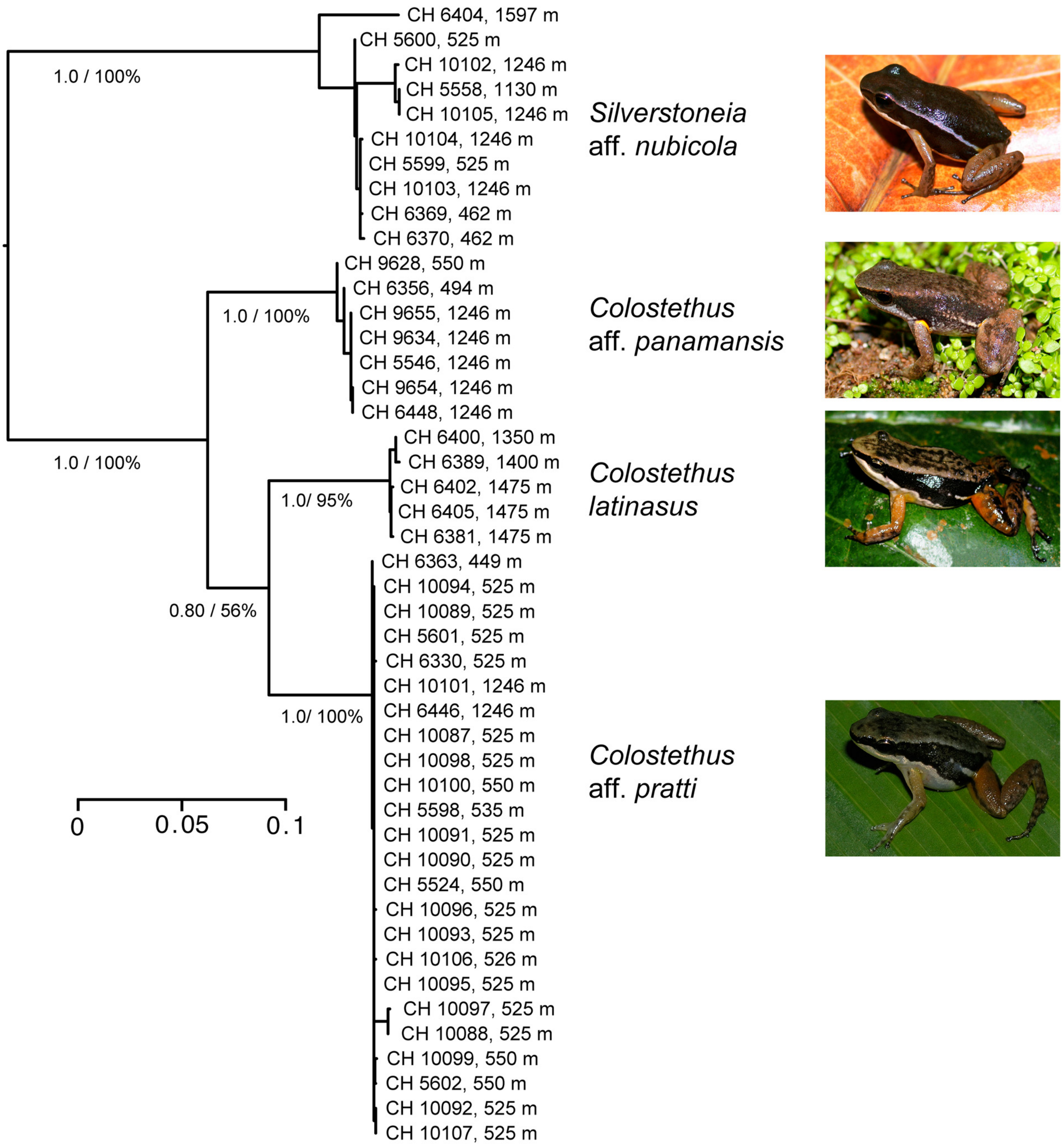

FIGURE 1. Likelihood-based phylogeny inferred from a partitioned analysis of partial DNA sequences of the COI and 16S mitochondrial genes. Scale bar indicates patristic distances estimated from partitioned likelihood analysis. Clade support values indicate Bayesian marginal posterior probabilities followed by likelihood bootstrap support values as percentage. Unscaled photographs of *Silverstoneia* aff. *nubicola* (CH 5558, SVL = 20.48 mm), *Colostethus* aff. *panamansis* (CH 5546, SVL = 25.40 mm), *C. latinasus* (CH 6389, SVL = 30.16 mm) and *C.* aff. *pratti* (CH 1087, SVL = 18.48 mm).

The advertisement call of *C. latinasus* consisted of a series of single and short, relatively loud “peep”-like notes given in rapid succession (Fig. 4). Males called at a rate of 4.0 ± 1.4 calls/min (mean ± standard deviation, n = 5 males), apparently synchronizing their calls to those of neighboring males but often partially overlapping them. The advertisement calls had 4–18 notes (10 ± 5 notes, mode = 7 notes, n = 15 calls of 8 males), 0.3–1.6 second (s) duration (975 ± 468 ms) and a note repetition rate of 11.3 ± 0.9 notes/s (n = 11 calls of 4 males). The dominant frequency of calls had one major peak at 2658 ± 79 Hz (n = 14 calls of 7 males). The notes had a duration of 41 ± 6 ms (n = 79 notes of 8 calls from 2 males), and inter-note intervals of 57 ± 9 ms (n = 71 inter-note intervals of 8 calls from 2 males). These notes were slightly upwardly frequency modulated. The lower frequency of the notes was 2427 ± 26 Hz, their higher frequency is 2878 ± 69 Hz, their bandwidth was 451 ± 66 Hz and the dominant frequency of notes peaked at 2673 ± 40 Hz (n = 56 notes of 5 calls from 1 male). The first note of calls often had a slightly lower frequency peak, less frequency modulation and lasted a few ms longer than the other notes.

FIGURE 2. Color in life of *Colostethus latinasus* individuals. Unscaled photographs. A–C) adult female (CH 6381), SVL = 29.83 mm; D–E) adult female (CH 6389), SVL = 30.16 mm; F) adult male (CH 6402), SVL = 31.72 mm.

Discussion

As commented in Grant (2004), the presence of *C. latinasus* at Cana (500–550 m.a.s.l.) is unlikely. We have not seen individuals nor heard any calls of this species at this site, despite of approximately 18 days of surveys collectively accumulated during years 2002–2010. However, we have routinely heard males calling from the slopes high above Cana, along a trail to the top of the Serranía de Pirre ridge, *i.e.*, approx. 1350–1500 m.a.s.l. Our observations support the view that *C. latinasus* is a species restricted to the highlands. Therefore, specimens that were previously assigned to *C. latinasus* from lower elevations around Cana (*e.g.*, Santos *et al.* 2009, Pyron & Wiens 2011), as well as their associated tissues and DNA sequences (see Table 1), do not belong to this species. In the present study, DNA sequences of *C. latinasus* are published for the first time.

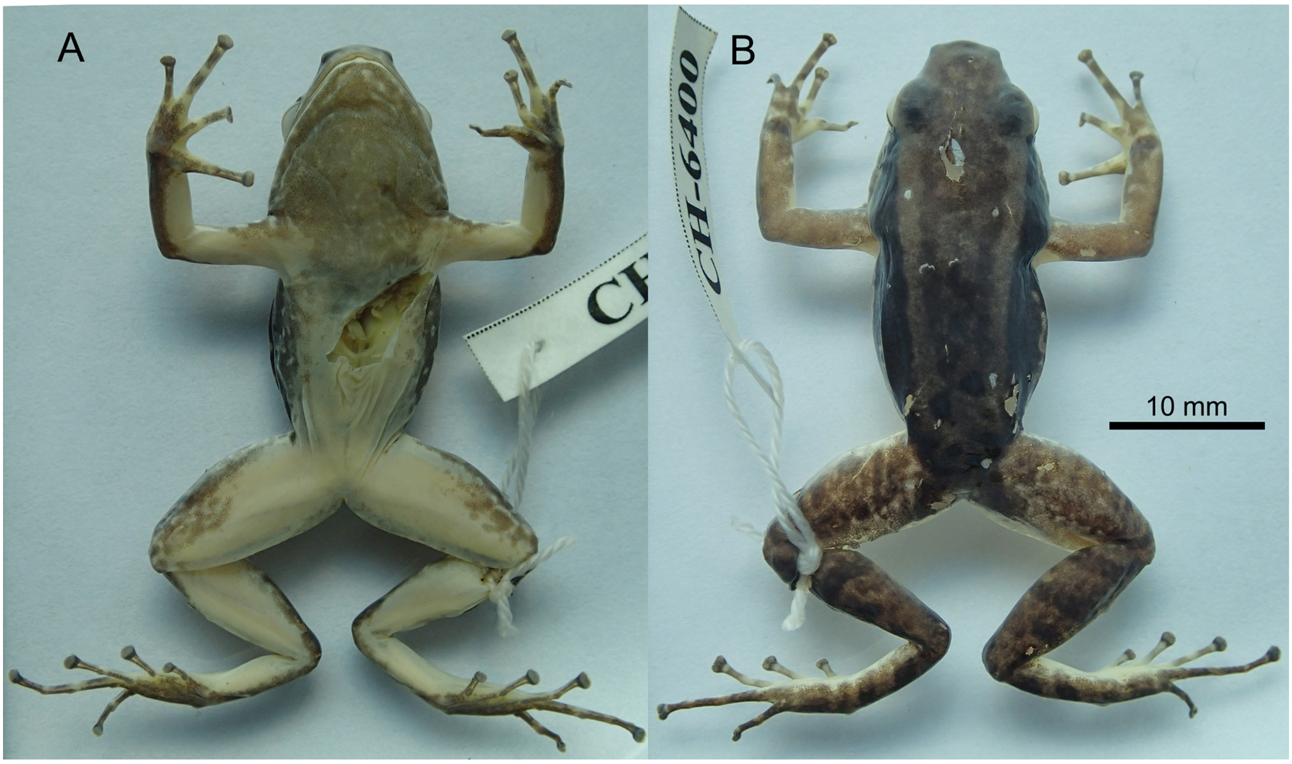

FIGURE 3. Voucher specimen of *Colostethus latinasus* (CH 6400, MVUP 2342), adult male. A) ventral view; B) dorsal view.

As with other dendrobatoid species (Ibáñez & Smith 1995), the description of the advertisement call of *C. latinasus* may be helpful for species identification in studies of the diversity and systematics of anurans from Darién and adjacent Colombia. We analyzed two advertisement calls recorded by William E. Duellman from a male specimen (University of Kansas Natural History Museum KU 76830) on Cerro Malí, at a temperature of 20°C, and deposited as recording No. 7227 in the Fonoteca Zoológica, Museo Nacional de Ciencias Naturales de Madrid (www.fonozoo.com). We assumed no errors in the available recording, and this specimen was previously considered to represent *C. latinasus* (Savage 1968). Nonetheless, these calls differ from those of *C. latinasus*, described here, mainly in having a longer duration (mean 8.4 *vs*. a maximum of 1.6 s in *C. latinasus*), slower note repetition rate (mean 6.6 *vs*. 11.3 notes/s in *C. latinasus*) and notes lacking evident frequency modulation (present in *C. latinasus*). Therefore, morphological data (Grant 2004) and call characteristics reported here support specimens from Cerro Malí as being another species.

The advertisement call of *C. latinasus* is also distinct from other species of the genus *Colostethus*. Detailed descriptions of the vocalizations, based on spectral and temporal characteristics, from *C. panamansis* (as *C. inguinalis*) have been published (Wells 1980). No voucher specimens are associated with any of these calls, and the reported air temperature was 25°C (Wells 1980). The advertisement call from a population of *C. panamansis* at Cerro Campana, in central Panama, was described as a whistled trill composed of 2–5 frequency modulated notes, each note with an approximate duration of 60 ms (Wells 1980). It differs from *C. latinasus* by having fewer notes (mode of 3 *vs.* mode of 7 notes in *C. latinasus*) with a longer duration (60 *vs.* 41 ± 6 ms in *C. latinasus*) and a higher frequency (3200–4547 *vs.* 2427 ± 26 to 2878 ± 69 Hz in *C. latinasus*) (Wells 1980). In addition, males of *C. panamansis* are known to emit advertisement calls at a higher rate (46–58 *vs.* 4.0 ± 1.4 calls/min in *C. latinasus*) (Wells 1980).

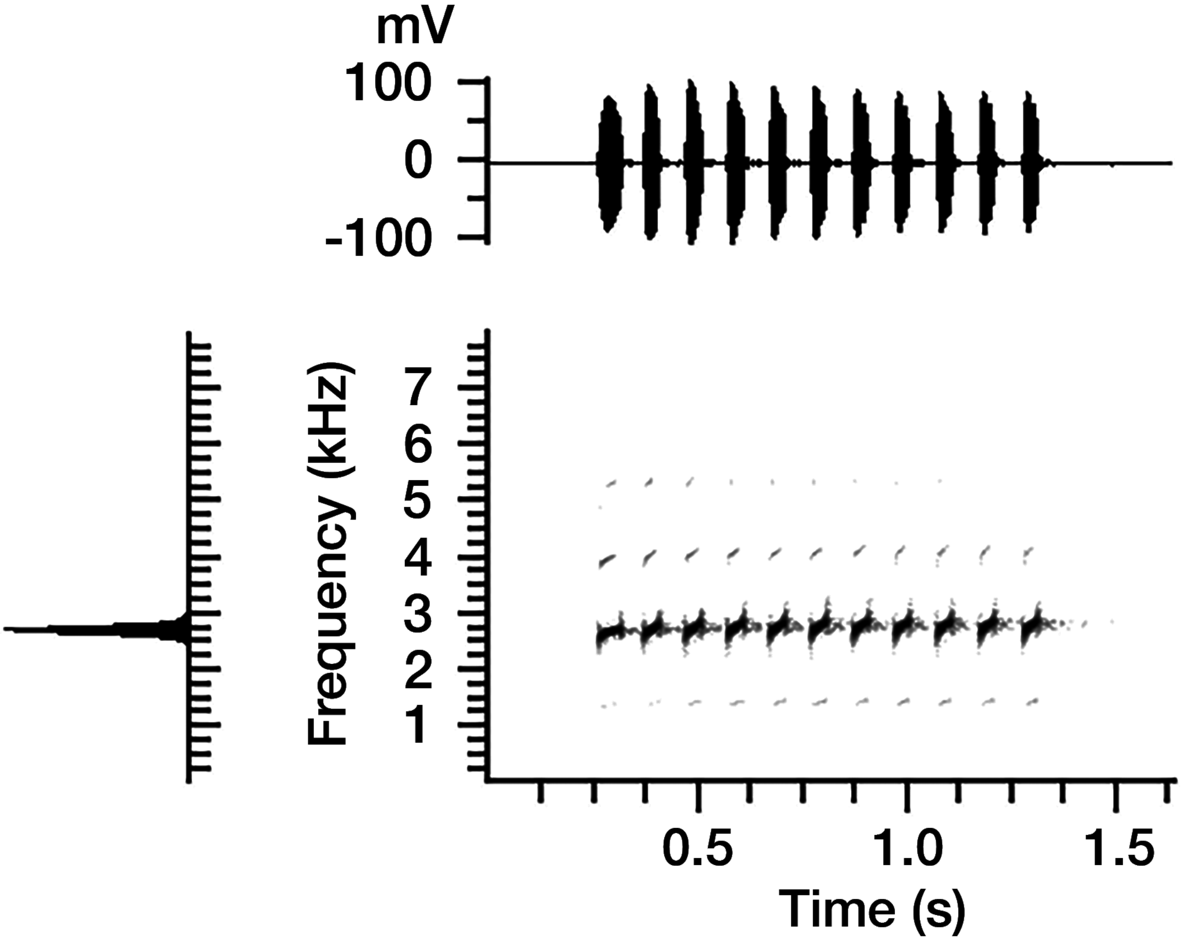

FIGURE 4. Waveform (upper), power spectrum (lower left) and spectrogram (lower right) of the advertisement call produced by a male *Colostethus latinasus*, recorded at 19°C air temperature. This is one call in the series of five calls that can be heard online at http://biogeodb.stri.si.edu/bioinformatics/dfm/metas/view/48935.

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