Article

A new species of Andean poison frog, *Andinobates* (Anura: Dendrobatidae), from the northwestern Andes of Colombia

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Abstract

The poison frogs of the Colombian Andes, Pacific lowlands and Panama have been recently recognized as a new, monophyletic and well-supported genus: *Andinobates*. The species richness and distribution within *Andinobates* remain poorly understood due to the paucity of geographic, genetic and phenotypic data. Here we use a combination of molecular, bioacoustic and morphometric evidence to describe a new species of Andean poison frog: *Andinobates cassidyhornae* **sp. nov.** from the high elevation cloud forests of the Colombian Cordillera Occidental, in the northwestern Andes. The new species is associated to the *bombetes* group and characterized by a unique combination of ventral and dorsal color patterns. Data on 1119 bp from two mitochondrial markers allowed us to reject the null hypotheses that *A. cassidyhornae* **sp. nov.** is part of the phenotypically similar and geographically less distant species: *A. opisthomelas*, *A. virolinensis* or *A. bombetes*. The best available phylogenetic trees and the genetic distance to other *Andinobates* species further support this decision. Altogether, the advertisement call parameters unambiguously separated *A. cassidyhornae* **sp. nov.** calls from the calls of the three closest species. The new species adds to a poorly known and highly endangered genus of poison frogs that requires further studies and urgent conservation measures.

Key words: *Andinobates cassidyhornae* **sp. nov.**, dendrobatid frogs, poison frogs, phylogenetics, distribution, conservation, Colombia, Andes

Resumen

Las ranas venenosas diminutas de los Andes y Pacífico colombianos, y Panamá han sido recientemente reconocidas como un género nuevo, monofilético y bien soportado: las ranas venenosas andinas o *Andinobates*. La riqueza de especies y su distribución son poco conocidas, principalmente por la escasez de datos geográficos, genéticos y fenotípicos. Utilizamos una combinación de evidencia molecular, bioacústica y morfométrica para describir una nueva especie de rana venenosa andina, *Andinobates cassidyhornae* **sp. nov.**, de los bosques nublados en los Andes noroccidentales de Colombia. La nueva especie es parte del grupo *bombetes* y se caracteriza por una combinación única de coloraciones dorsal y ventral. El análisis de 1119 pb de dos marcadores mitocondriales nos permitió rechazar las hipótesis nulas de que *A. cassidyhornae* **sp. nov** era parte de las especies fenotípicamente similares y geográficamente próximas *A. opisthomelas*, *A. virolinensis* o *A. bombetes*. Los mejores árboles filogenéticos disponibles y las distancias genéticas a otras especies de *Andinobates* también soportan esa conclusión. En conjunto, los parámetros de las llamadas de anuncio separaron claramente las llamadas de *A. cassidyhornae* **sp. nov.** de las llamadas de las tres especies relacionadas. Esta nueva especie se suma a las otras de este género de ranas venenosas, poco conocido pero muy amenazado, que requiere mayor estudio y urgentes medidas de conservación.

Introduction

Neotropical poison frogs (Dendrobatidae) are one of the most charismatic and widely studied frog taxa. The number of new species descriptions has increased steadily since 1970, and dramatically since 1990 (Brown & Twomey *et al*. 2011). Most of the recently described species are minute poison frogs, a monophyletic group (Grant *et al*. 2006, Santos *et al*. 2009) consisting of two sister lineages with virtually non-overlapping distribution ranges: the species in the *ventrimaculata* and *minuta* groups, formerly placed within the genus *Ranitomeya* (Grant *et al*. 2006). A recent phylogenetic analysis, based on mitochondrial (cytochrome-b gene, 12S ribosomal RNA and 16S ribosomal RNA) and nuclear markers (rhodopsin exon 1, recombination activating gene 1, histone H3, 28S rDNA and seventh in absentia) led to the erection of a new genus, *Andinobates,* for the species in the former *minuta* group (Brown & Twomey *et al*. 2011). Whereas the species in the genus *Ranitomeya* (*sensu* Brown & Twomey *et al*. 2011) occur east of the Andes and throughout the Amazon basin, the *Andinobates* species occur in the Colombian Andes, the Colombian pacific lowlands, and northward into lowland Panama.

Species in the *A. bombetes* group are particularly threatened by intensive agricultural activity within the 1300–2300 m elevation range. Most of them are restricted to disjoint forest fragments surrounded by agriculture. The group currently includes seven species: *Andinobates abditus* (Myers & Daly 1976), *A. bombetes* (Myers & Daly 1980), *A. daleswansoni* (Rueda-Almonacid *et al*. 2006), *A. dorisswansonae* (Rueda-Almonacid *et al*. 2006), *A. opisthomelas* (Boulenger 1899), *A. tolimensis* (Bernal *et al*. 2007) and *A. virolinensis* (Ruiz-Carranza & Ramírez-Pinilla 1992). Many of them are currently diagnosed on the basis of coloration. The current trend to use molecular and bioacoustic analyses, in addition to morphological and meristic data, offers a pivotal insight into species limits and relationships, providing a clearer view of the degree of intra-and interspecific variation, which greatly facilitates taxonomic work.

During exploratory fieldwork at the recently created Mesenia-Paramillo Natural Reserve, in the Colombian Andes, Cordillera Occidental, we became aware of an *Andinobates* population that was tentatively assigned to *A. opisthomelas* on the basis of its dorsal coloration and geographic location. Further close examination of color combined with pattern distinguished it in our eyes as potentially new to science. Later molecular and bioacoustic analyses of frogs from four localities revealed a distinct lineage that should be considered a new species. Our aim here is to present the multi-trait evidence that supports the status of *Andinobates* **sp. nov.** *cassidyhornae* as a new species.

Material and methods

Specimens were collected in four localities in the Cordillera Occidental located in the northwestern Colombian Andes (Figure 1). The holotype and paratype specimens, from the Mesenia-Paramillo Natural Reserve, Antioquia, were sacrificed and preserved following standard procedures proposed by McDiarmid *et al*. (1994). The following measurements were taken according to Bernal *et al*. (2007), using a Mitutoyo Absolute CD-6"CSX digital caliper, to the nearest 0.01mm: SVL, snout-vent length; TL, tibia length measured between heel and outer surface of flexed knee; GBW, greatest body width taken just under the axillae; HW, head width between angles of jaws; IOD, interorbital distance; HL, head length, sagittal distance from tip snout to angle of jaw; TSCN, tip of snout to center of naris; NED, distance from center of nares to anterior edge of eye; IND, distance between centers of nares; EL, eye length; HDT, horizontal diameter of tympanum; MTD, distance from corner of mouth to lower edge of tympanic ring; HaL, hand length measured from the proximal edge of large medial palmar tubercle to tip of longest third finger; W3FD, width of third finger disc; W3F, width of third finger below disc; W3TD, width of third toe disc; W3T, width of third toe below disc; W4TD, Width of fourth toe disc; and W4T, width of fourth toe below disc. Body mass was measured with a DigiWeigh DW-100AS digital scale, to the nearest 0.01g.

Molecular and phylogenetic analyses. Tissue samples for molecular analyses were obtained from buccal swabs or toe-clippings of nine individuals of *A. cassidyhornae* **sp. nov.**, from four localities, and three individuals of *A. opisthomelas* from Amalfi (lat. 6.88, long. -75.09) and Guatapé (lat. 6.27, long. -75.19), Antioquia (Figure 1). Toe-clippings were preserved in 96% ethanol and swabs were stored dry. Genomic DNA was extracted using QIAGEN DNeasy Blood and Tissue kits. Samples and extractions were stored at -20° C. We amplified fragments of two mitochondrial loci: 16S rRNA (16S; 574bp) and the Cytochrome b gene (*Cytb*; 700bp) using primers 16Sar

and 16Sbr (Palumbi *et al.* 1991), and CytbDen3-L and CytbDen1-H (Santos & Cannatella 2011), respectively. PCR cycles for both loci consisted of an initial 2 min at 95° C and 30–35 cycles of 30 sec at 95° C, 1 min at 45° C, 1:30 min at 72° C and a final extension step of 6 min at 72° C. *Cytb* sequences were also obtained for frogs of several *Andinobates* species that had been sequenced for the 16S gene in a previous study (Brown & Twomey *et al.* 2011) using the protocol mentioned above. All sequences were deposited in GenBank under accession numbers JQ936619 – JQ936638, and JX879706 – JX879725.
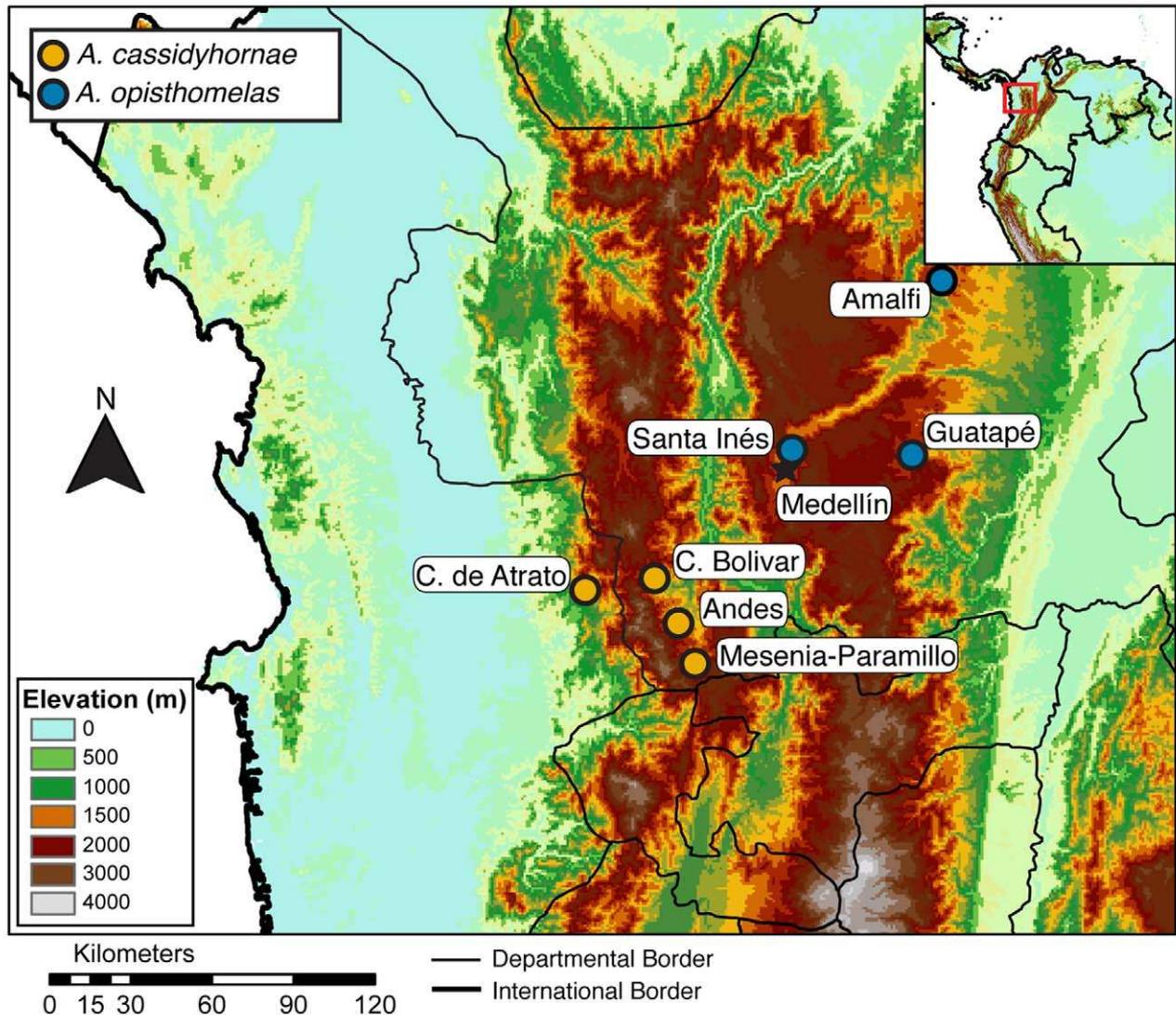
FIGURE 1. Sampled localities (in yellow, lat, long in degrees, elevation in m) of *A. cassidyhornae* **sp. nov.** in the northwestern Colombian Andes: Mesenia-Paramillo (5.52, -75.89, 2059 m), Andes (5.69, -75.92, 1367 m), Ciudad Bolivar (5.85, -76.04, 1260 m), and Carmen de Atrato (5.83, -76.26, 1248 m). As reference, we add three localities of *A. opisthomelas*: Santa Inés corresponds to the now extinct population from the type locality of *A. opisthomelas*, which was not sampled, Amalfi corresponds to the white venter form and Guatapé to the brown venter form.

The obtained homologous sequences, as well as sequences available in GenBank for *Ranitomeya fantastica*, used as outgroup, and most species of *Andinobates* (Table 1), were aligned using MUSCLE (Edgar 2004), implemented in Geneious (Drummond *et al.* 2010); the resulting alignments were visually corrected to resolve gap placements. The ends of alignments that contained information for less than 75% of the sequences were trimmed and excluded from subsequent analyses.

TABLE 1. Localities and GenBank accession numbers of the specimens used in molecular analyses.

Species	Locality	Study	GenBank Accession	
			16S	Cytb
A. bombetes	Colombia, Quindío, Barbas, Hacienda Lusitania	Santos *et al.* 2009	EU342669	JN 635880
A. bombetes	Colombia, Quindío, Barbas, Hacienda Lusitania	Santos & Cannatella, 2011	HQ290981	HQ290558
A. cassidyhornae **sp. nov.**	Colombia, Antioquia, Mesenia-Paramillo Natural Reserve	This study	JQ936636	JQ936622
A. cassidyhornae **sp. nov.**	Colombia, Antioquia, Mesenia-Paramillo Natural Reserve	This study	JQ936637	JQ936623
A. cassidyhornae **sp. nov.**	Colombia, Antioquia, Mesenia-Paramillo Natural Reserve	This study	JQ936638	JQ936624
A. cassidyhornae **sp. nov.**	Colombia, Antioquia, Andes	This study	JX879709	JX879719
A. cassidyhornae **sp. nov.**	Colombia, Antioquia, Ciudad Bolivar	This study	JX879710	JX879720
A. cassidyhornae **sp. nov.**	Colombia, Antioquia, Ciudad Bolivar	This study	JX879714	JX879724
A. cassidyhornae **sp. nov.**	Colombia, Antioquia, Ciudad Bolivar	This study	JX879715	JX879725
A. cassidyhornae **sp. nov.**	Colombia, Chocó, Carmen de Atrato	This study	JX879711	JX879721
A. cassidyhornae **sp. nov.**	Colombia, Chocó, Carmen de Atrato	This study	JX879712	JX879722
A. cassidyhornae **sp. nov.**	Colombia, Chocó, Carmen de Atrato	This study	JX879713	JX879723
A. claudiae	Panamá. Bocas del Toro	Roberts *et al.* 2006	DQ371315	DQ371334
A. dorisswansonae	Colombia, Tolima, Falan	Brown *et al.* 2011	JN635861	JQ936629
A. dorisswansonae	Colombia, Tolima, Falan	Brown *et al.* 2011	JN635863	JQ936630
A. fulguritus	Colombia, Chocó, Bahía Solano, Sierra Mecana	Grant *et al.* 2006	DQ502106	DQ502538
A. opisthomelas	Colombia, Antioquia, Guatapé	This study	JQ936633	JQ936619
A. opisthomelas	Colombia, Antioquia, Guatapé	This study	JQ936634	JQ936620
A. opisthomelas	Colombia, Antioquia, Guatapé	This study	JQ936635	JQ936621
A. opisthomelas	Colombia, Antioquia, Amalfi	This study	JX879706	JX879716
A. opisthomelas	Colombia, Antioquia, Amalfi	This study	JX879707	JX879717
A. opisthomelas	Colombia, Antioquia, Amalfi	This study	JX879708	JX879718
A. minutus	Panamá, Cocle, El Copé, Parque Nacional General de División Omar Torrijos Herrera	Grant *et al.* 2006	DQ502168	DQ502603
A. sp. Quibdó	Colombia, Chocó, Quibdó, La Troje	Santos *et al.* 2009	EU342670	JN635881
A. tolimensis	Colombia, Tolima, Falan	Brown *et al.* 2011	JN635857	JQ936632
A. tolimensis	Colombia, Tolima, Falan	Brown *et al.* 2011	JN635857	JQ936631
A. virolinensis	Colombia, Santander, Virolín, Costilla de Fara	Brown *et al.* 2011	JN635873	JQ936626
A. virolinensis	Colombia, Santander, Virolín, Costilla de Fara	Brown *et al.* 2011	JN635871	JQ936627
A. virolinensis	Colombia, Santander, Socorro	Brown *et al.* 2011	JN635875	JQ936625
A. virolinensis	Colombia Santander Socorro	Brown *et al.* 2011	JN635876	JQ936628
A. virolinensis	Colombia, Santander, Virolín, Costilla de Fara	Santos & Cannatella, 2011	HQ290994	HQ290571

In order to estimate the phylogenetic relationships between the putative new species and other *Andinobates*, we performed partitioned maximum likelihood (ML; Felsenstein 1981) and Bayesian (Rannala & Yang 1996)

analyses. The ML analysis was conducted in RaxML (Stamatakis 2006) under the GTR+Γ model, and with 1000 bootstrap replicates to assess nodal support. The Bayesian phylogeny was inferred using MrBayes (Ronquist & Huelsenbeck 2003) based on the best substitution models selected for each locus using the program PartitionFinder (Lanfear *et al.* 2012). Two replicates with four chains were run for 10,000,000 generations, sampling every 1000 and discarding the first 2500 trees as burnin. Chain convergence was examined using Tracer (Rambaut & Drummond 2007). For both analyses, the two alignments were concatenated and the dataset was partitioned using the best partition scheme according to PartitionFinder (Lanfear *et al.* 2012), which simultaneously selects the best partitioning strategy and evolutionary model for each partition. Kimura-two-parameter (K2P; Kimura 1980) genetic distances between *Cytb* sequences of individuals of the *bombetes* species group (Brown & Twomey *et al.* 2011) were estimated using MEGA 5 (Tamura *et al.* 2011).

To test whether the putative new species could be assigned to other species of *Andinobates* based on the best available genetic evidence, we ran Shimodaira-Hasegawa tests (SH tests; Shimodaira & Hasegawa 1999). In brief, they test whether a null phylogenetic hypothesis is significantly worse than an alternative one, based on resampling and maximum likelihood differences. We tested the topology of the best ML tree, inferred by RAxML (see previous paragraph), as the alternative hypothesis (H_a) against each of three null (H_0) hypotheses: *A. cassidyhornae* **sp. nov.** forming a single (polytomic) clade (the test clade) with (1) *A. opisthomelas*, (2) with *A. virolinensis* and (3) with *A. bombetes*. We chose these three species because they are part of the *bombetes* group, they occur at localities geographically close to the *A. cassidyhornae* **sp. nov.** type locality, and they all share roughly similar red dorsal coloration (patterned as longitudinal dorsolateral stripes in *A. bombetes*). SH tests were conducted using the R package Phangorn (R Development Core Team 2011, Schliep 2011), and likelihood was estimated under the GTR+Γ model with 100,000 bootstrap replicates. Test topologies were generated in Mesquite (Madisson & Madisson 2011), and consisted of a tree including the test clade, but otherwise identical to H_a.

Bioacoustic analyses. Males of *Andinobates* species utter advertisement calls of the 'buzz' type (Myers & Daly 1976, Brown & Twomey *et al.* 2011). Each trunk muscle contraction produces a single 'buzz' (i.e. call) consisting of a long series of pulses with amplitude but not frequency modulation (Erdtmann & Amézquita 2009). We recorded spontaneous advertisement calls of seven focal males (five from the type locality and two from Carmen de Atrato) with an Audio-Technica AT897 Shotgun microphone and a K-Tek KE-89CC boompole, connected to a Tascam DR-100 professional recorder. Air temperature was registered with a RH 101 Extech IR thermo-hygrometer. Digital recordings at 44 kHz were analyzed on Raven 1.4 (Cornell Laboratory of Ornithology, NY, USA). Three consecutive calls per male were measured in the following temporal and spectral parameters: number of pulses per call, call duration, intercall interval (silent interval between consecutive calls), rise time (percent of call duration until the point of highest amplitude), peak frequency, and the frequency bandwidth (interquartile range: the frequency range that encompasses the mid 50% of the energy in the selection).

To test whether the calls of the new species could be statistically distinguished from the calls of the phylogenetically and geographically closest species of the *bombetes* group (Brown & Twomey *et al.* 2011), we recorded males from four additional localities. Two of them represent the extremes of known variation in ventral coloration of *A. opishtomelas*, the external character that best allows to recognize the new species. Six males were recorded of the white-venter form (see Definition and Diagnosis) at Amalfi and six males of the brown-venter form at Guatapé (see Molecular and Phylogenetic Analyses), both in Departamento de Antioquia, Colombia. Seven males were additionally recorded from *A. bombetes* at Reserva Forestal Yotoco (around 4 km east to the type locality, Departamento del Valle del Cauca) and seven males from *A. virolinensis* at Virolín (the type locality, in Departamento de Santander).

Because anuran call traits can be affected by body temperature, we first regressed every call trait against environmental temperature. Where the regression was statistically significant, we saved the corresponding residuals and used them as new temperature-independent variables in our further analyses. To test whether the calls of *A. cassidyhornae* **sp. nov.** differ from the calls of the three closest species (*A. opisthomelas*, *A. bombetes*, and *A. virolinensis*), we ran a discriminant function analysis considering simultaneously all the call traits. To know the relative importance of each call trait in separating the species we compared the corresponding standardized discriminant coefficients and conducted species pairwise Tukey-Kramer HSD tests.

Results

Andinobates cassidyhornae sp. nov.

Dendrobates opisthomelas Silverstone 1975. Quebrada Arriba, bus stop 10 km by road from town of Andes, mountains near road (LACM 71962-70).

Andinobates opisthomelas Brown & Twomey *et al.* 2011. pp 33 Plate 3, Figure O. Guatapé Antioquia, Colombia, because of a mistake in the final manuscript. The correct locality according to one of the authors of that paper (D.M.-V.), who actually took the photograph, is Carmen de Atrato, Chocó, Colombia.

Holotype: An adult female (Figure 2) deposited in the amphibian collection of the Universidad de los Andes, Bogotá, Colombia, Andes-A1095 (Field number LUMA1001) is one of a series collected on March 30th 2012 at the Mesenia-Paramillo Nature Reserve, Municipality of Andes, Department of Antioquia, Colombia, by R. Medina, G. Suárez & L. Mazariegos.
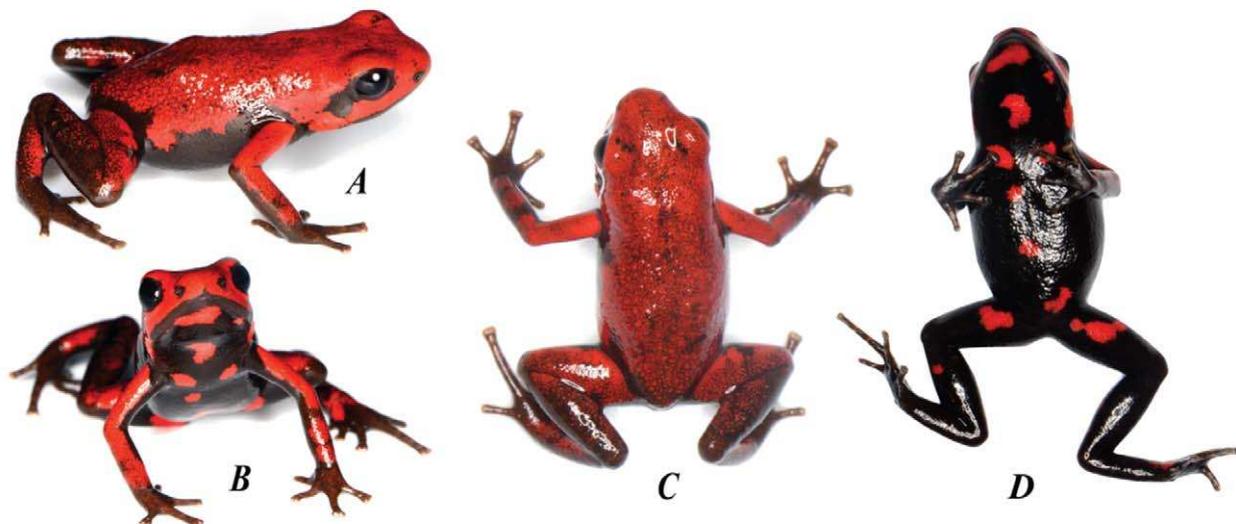
FIGURE 2. The holotype of *A. cassidyhornae* **sp. nov.** in A) lateral, B) partial ventral, C) dorsal, and D) ventral view (photos © 2012 L. Mazariegos).

Paratypes: Four adult females, and two adult males (Andes-A1088–1091, and 1093–1095) collected by R. Medina, G. Suárez & L. Mazariegos. Locality data is the same as the type locality (see below).

Type locality. Mesenia-Paramillo Nature Reserve, Vereda La Mesenia, about 12 km south of the municipality of Jardín but politically within the Municipality of Andes, (both in Departamento de Antioquia, Colombia), ca 5° 31´ N, 75° 53´ W at 2000 m elevation. Because of the heavy smuggling on dendrobatid frogs we refrain from providing more accurate coordinates.

Etymology. This specific epithet *cassidyhornae* is a patronym in honor of Cassidy Horn, for her passionate interest in poison frogs and her generous contributions to the conservation of cloud forests in Colombia.

Definition and diagnosis. A small-sized dendrobatid frog that we assign to the *Andinobates bombetes* species group (Brown & Twomey *et al*. 2011) based on the phylogenetic affinity (see *Molecular and Phylogenetic Analyses*) and on the following morphological characters: adult snout-vent length (SVL) <20.0 mm; adults with bright dorsal coloration; ventral coloration variable, usually with distinct bright markings; colored throat patch absent; head narrower than body; teeth absent; vocal slits present in males; first finger distinctly shorter than second (Figure 3); finger discs II and III weakly to moderately expanded; toe disc III and IV weakly expanded; toe V unexpanded; toe webbing absent; median lingual process absent (Brown & Twomey *et al.* 2011).

Andinobates cassidyhornae **sp. nov.** has an SVL of 19.03±0.31 mm (mean±SD, N = 12 frogs), a bright red dorsum with the color extending onto the upper front and hind limbs; lower forearms and hind limbs are dark brown. Ventral coloration is black with bright red irregularly sized and spaced ovoid or 'comma' shaped blotches or spots. It can be externally distinguished from other species in the *bombetes* group by the distinctive color

pattern: in *A. cassidyhornae* dorsum is bright red, and venter is black with well-defined bright red blotches or spots (Figure 4) vs. (1) in *A. opisthomelas* dorsum is red often with a posterior suffusion to brown, and venter is black with numerous white spots or reticulation (white-venter form) or venter is chocolate brown sometimes with red suffusion from the flanks (brown-venter form); (2) in *A. virolinensis* venter is whitish or bluish with black reticulation; (3) in *A. bombetes* the anterior half of dorsum exhibits bright red, yellow or rarely orange longitudinal and broad dorsolateral stripes; (4) in *A. tolimensis* the head is yellow, fading to brown towards the dorsum; (5) in *A. dorisswansonae* the dorsum is black or brown with red blotches, and the venter entirely black or black with few white or yellowish blotches; and (6) in *A. daleswansoni* the head is entirely red, and the body dull gold or brown.

Measurements of the holotype (mm). The holotype measurements correspond to an adult female with a SVL of 18.99 mm; TL of 8.10; HaL of 4.58; HL of 4.85; HW of 6.29; GBW of 7.54; IOD of 2.31; HDT of 1.23; ED of 2.15; TSCN of 1.40; NED of 1.52; IND of 2.36; MTD of 0.75; W3FD of 0.78; W3F of 0.46; W3TD of 0.83; W3T of 0.47; W4TD of 0.87; and W4T of 0.49. The corresponding measurements of all specimens collected are shown in Table 2.

TABLE 2. Measurements in millimeters for the type series of *Andinobates cassidyhornae,* **sp. nov.**

Measurements	Andes-A1093	Andes-A1088	Andes-A1089	Andes-A1090	Andes-A1094	Andes-A1091	Andes-A1095	Median	SD
SVL	19.36	19.34	19.17	18.92	18.45	18.99	18.99	18.99	0.31
TL	8.59	8.52	8.23	8.51	8.50	8.66	8.10	8.51	0.20
HaL	4.59	4.67	4.67	4.60	5.14	4.91	4.58	4.67	0.21
HL	6.06	5.59	5.17	4.75	5.09	5.00	4.85	5.09	0.46
HW	5.38	5.99	5.94	5.75	6.20	5.90	6.29	5.94	0.30
GBW	8.50	8.85	8.13	7.11	7.56	7.20	7.54	7.56	0.66
IOD	2.38	2.30	2.12	1.85	2.26	2.14	2.31	2.26	0.18
HTD	1.41	1.17	0.98	0.96	1.05	0.97	1.23	1.05	0.17
ED	2.49	2.31	2.12	2.45	2.47	2.21	2.15	2.31	0.16
TSCN	1.78	1.34	1.26	1.22	1.54	1.35	1.40	1.35	0.19
NED	1.71	1.68	1.54	1.57	1.78	1.68	1.52	1.68	0.10
IND	2.47	2.33	2.32	2.42	2.84	2.56	2.36	2.42	0.18
MTD	0.84	0.72	0.67	0.60	0.89	0.91	0.75	0.75	0.12
W3FD	0.94	0.99	0.85	0.95	1.12	1.10	0.78	0.95	0.12
W3F	0.49	0.44	0.48	0.53	0.65	0.52	0.46	0.49	0.07
W4TD	0.98	0.83	0.64	0.71	1.01	0.93	0.87	0.87	0.14
W4T	0.55	0.52	0.43	0.41	0.60	0.56	0.49	0.52	0.07
W3TD	0.69	0.91	0.66	0.79	0.83	0.78	0.83	0.79	0.09
W3T	0.50	0.54	0.54	0.52	0.68	0.57	0.47	0.54	0.07
BM in grams	0.64	0.62	0.58	0.59	0.53	0.58	0.60	0.59	0.03

Description of the holotype: The head is slightly wider than longer, and is narrower than the body. Snout subovoid in dorsal view and truncated in lateral view. *Canthus rostralis* subovoid, loreal region flat and vertical. Nares situated much closer to the end of the snout than to the eyes, ovoid in shape and directed posterolaterally. Eyes large and prominent with a diameter of 11.3% of SVL. The pupil is rounded and horizontally elliptical. Tympana and tympanic rings are in the posterolateral regions, ovoid and wider dorsoventrally, measuring 57.2 % of the diameter of the eyes. Supratympanic fold absent.

Rounded choanae, not visible in ventral view, as they are completely covered by the maxillary arch. Vomerine, maxillary and premaxillary teeth are absent. Tongue is elongated, almost two times longer than wide; the posterior margin of the tongue is not indented and its posterior third is not adhered to floor of mouth.
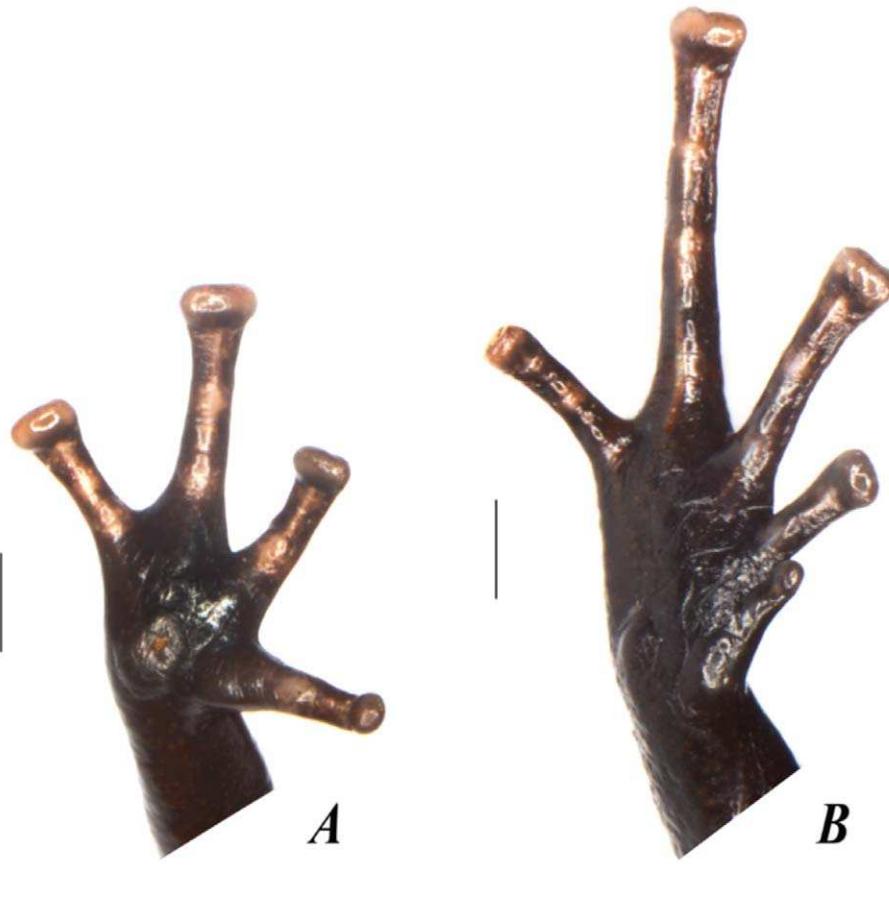
FIGURE 3. Palm (A) and sole (B) of the hand and foot of the type specimen of *A. cassidyhornae* **sp. nov.** (photo © 2012 L. Mazariegos). Scale = 1 mm.
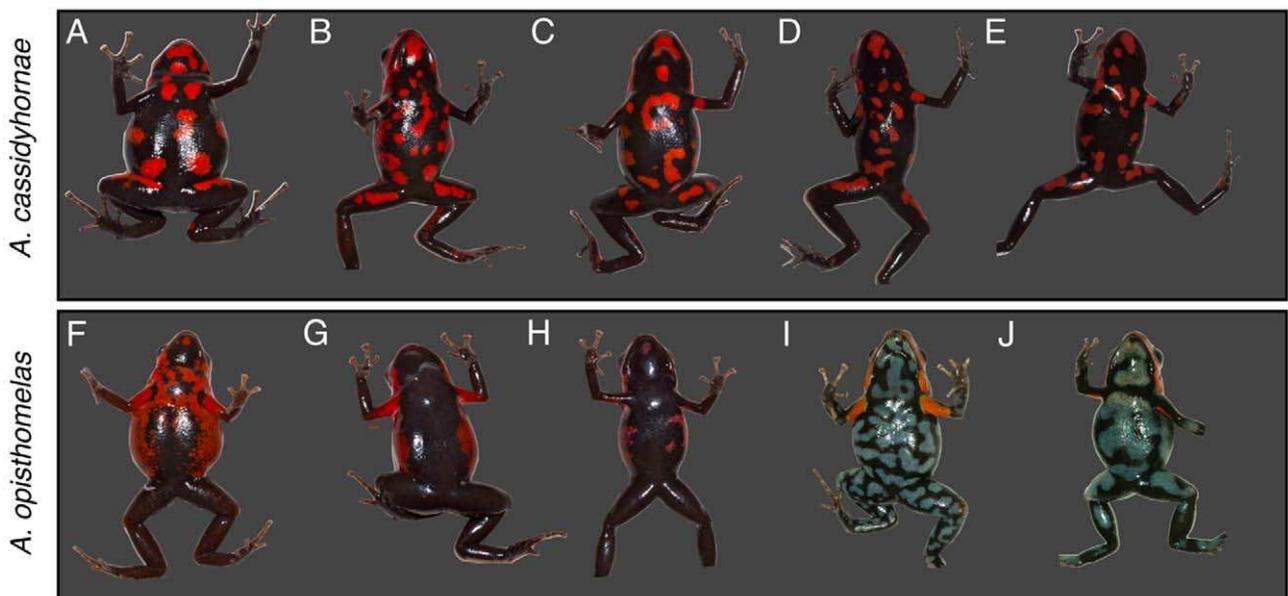
FIGURE 4. Distinctive ventral coloration of *A. cassidyhornae* **sp. nov.** from the type locality (A–B), Ciudad Bolívar (C), and Carmen de Atrato (D–E), compared to *A. opisthomelas*: the brown-venter form from Guatapé (F–H), and the white-venter form from Amalfi (I–J).

FIGURE 5. Male of *A. cassidyhornae* **sp. nov.** carrying a tadpole (photo © 2012 L. Mazariegos).

Hand relatively large (Figure 3), with a length equal to 24.1% of the snout-vent length. The relative length of the fingers in increasing order of size is: IV<II<I<III. The tip of the number IV digit reaches the middle of the second to last phalange of the number III digit and the distal extreme of the first digit reaches the base of the disc of the second digit. Finger discs moderately expanded on digits of the hand. Paired dorsal pads on dorsal surfaces of the discs are present. Outer metacarpal tubercle somewhat flat and rounded, inner metacarpal tubercles are elliptical and are located at the base of the first (I) fingers, basal subarticular tubercles are rounded and flat over fingers I and II. Two rounded and flattened subarticular tubercles appear on the number III and IV finger digits; the latter subarticular tubercle is not pronounced (Figure 3).

The relative length of the toes, in increasing size order is I<II<V<III<IV. The discs of toes are smaller than disks of fingers. Toes number II with the basal subarticular tubercle not protuberant. Toes III and V with two subarticular tubercles, and toes IV with three subarticular tubercles. Supernumerary plantar tubercles are absent. External metatarsal tubercle is smaller than the inner metatarsal tubercles (Figure 3).

Coloration of holotype in life: Iris very dark brown; almost indistinguishable from black pupil. Nares encircled by black; margins of upper and lower jaws are very dark brown; tympana are black. Dorsum primarily bright scarlet red, sharply defined along the margins with a few irregularly scattered black speckles and small irregular black markings; in other specimens, black marks may reflect attacks by predators and should therefore not be considered an element of the dorsal coloration. Flanks are red laterally and black ventrolaterally with no gradient merging of the two colors as is seen in *A. opisthomelas*. Venter ground color black with irregular sharply contrasting bright scarlet red irregularly shaped blotches or spots. Upper forelimbs bright scarlet red and tinged brown where the upper and lower colorations meet, with lower forearm dark brown below the elbow; wrists and hands brown with tips of toes and fingers beige. Thigh mostly red, irregularly speckled with brown; shanks mostly brown tinged with red irregularly (Figure 2).

Color in preservative (ethanol 70%): The dark brown and black colors turn dull black to dark olive in preservative. Discs and tubercules on hands and feet, pupil and cornea become grey or nearly white with time. The predominantly bright scarlet red dorsal coloration and ventral red blotches and spots turn metallic olive; pattern remains clearly distinguishable in preservative (ethanol 70%).

Natural history: The natural history of this species is poorly known. We found individuals in areas covered

with a thick layer of leaf litter and where abundant refuges were available. Males call regularly from the leaf litter or hidden amidst tree roots, throughout the day but prominently between 10h–14h and after periods of rain. Most calling males were observed accompanied by a female. We also encountered several courting pairs during a visit in March and April 2012. Males carry 1–3 tadpoles (Figure 5) on their dorsum. Males of other species of the *bombetes* group are known to release their tadpoles in bromeliad water tanks. Some tadpoles of the new species were found in water within the inflorescence husks of *Wettinia* palms. Whether this species displays biparental care is unknown.

Molecular and phylogenetic analyses. The final alignment consisted of 1119 bp (700bp unambiguously aligned from the Cytb gene and 419bp from the 16S gene). The chosen partitioning strategy was as follows: 16S unpartitioned under the GTR+ Γ model, and Cytb partitioned by codon position with K80+I, HKY+I and GTR+ Γ for the first, second, and third positions, respectively. Both ML and Bayesian phylogenies placed *A. cassidyhornae* **sp. nov.** as an independent, well-supported clade, separate from other species, within the *bombetes* species group. The two sequenced populations, the white- and the brown-venter forms, of *A. opisthomelas* formed a monophyletic albeit poorly supported group (Figure 6). The topology obtained is roughly consistent with the results of Brown & Twomey *et al*. (2011), but changes the relationships between *A. virolinensis, A. tolimensis* and *A. bombetes*, placing the first two as reciprocally monophyletic clades, and *A. bombetes* as the sister group of the two. However, the deeper phylogenetic relationships between the species in this group still require further study, since the obtained topology is highly polytomic and not well supported at this level. Within *A. cassidyhornae* **sp. nov.**, individuals from the Mesenia-Paramillo Natural Reserve, Antioquia, cluster together in a monophyletic group, whereas individuals from other localities do not show evidence of genetic structuring.

The SH tests rejected, at a very high level of statistical significance, two out of the three null topologies (H_0) that placed *A. cassidyhornae* **sp. nov.** within a clade with *A. opisthomelas* (P <0.00001), with *A. virolinensis* (P < 0.00001), and with *A. bombetes* (P = 0.05134). Additionally, pairwise genetic distances (K2P) between *A. cassidyhornae* **sp. nov.** and other species within the *bombetes* species group ranged between 0.0320–0.0677, well within the range of interspecific distances (Figure 7) observed for the group (0.0216–0.1053), and about tenfold the intraspecific distances observed (0–0.0091). Altogether, the reconstructed phylogenetic tree, the topology (SH) tests, and pairwise genetic distances offer strong support for *A. cassidyhornae* **sp. nov.** being a distinct species within the *bombetes* species group.

Bioacoustic analyses. All species' calls within the *Andinobates bombetes* species group consist of long and atonal series of pulses sounding like a 'buzz' or rattle, which is often longer than 1 sec. The advertisement call of *A. cassidyhornae* **sp. nov.** (Figure 8) follows roughly the same pattern. Calls consist of 234.3±20.3 (mean±SD) pulses, last 1.94±0.26 s, and are often uttered as series of calls separated by regular silent intervals of 10.1±2.1 s. The rise time is 50.0±13.2 % of the call duration. The peak call frequency averages 4.32±0.14 kHz and the frequency bandwidth 0.81±0.40 kHz.

Combining the calls of *A. cassidyhornae* **sp. nov.**, *A. opisthomelas* (two localities), *A. bombetes*, and *A. virolinensis*, we found that call duration (linear regression, R^2=0.31, F=13.9, P=0.0008, N=16 males), inter-call interval (R^2=0.17, F=6.5, P=0.0162), and the number of pulses per call (R^2=0.12, F=4.3, P=0.0472) decreased at higher temperatures. After removing the temperature effect by calculating regression residuals, the calls of *A. cassidyhornae* **sp. nov.** were clearly separated from the calls of the other species in a two-dimensional discriminant space (Figure 9, above, discriminant analysis, Wilks' Lambda approxim. F=13.9, P<0.0001). The first discriminant axis accounted for 87.7 % of variation and separated very well the calls of *A. cassidyhornae* **sp. nov.** from the call of any other species because the former were lower in peak frequency (F to enter=39.6, standardized discriminant coefficient=1.10, P<0.0001). The second discriminant axis accounted for 10.1% of variation and separated the calls of *A. cassidyhornae* **sp. nov.** from the call of *A. bombetes* because the former were longer in duration (F to enter=6.9, standardized discriminant coefficient=0.97, P=0.0013).

Regarding pairwise species comparisons by univariate (Figure 9, below) tests, the advertisement call of *A. cassidyhornae* **sp. nov.** was lower in peak frequency than the call of *A. opisthomelas* (Tukey-Kramer HSD test, - 0.97 kHz, P<0.0001), *A. virolinensis* (- 0.71, P<0.0001) and *A. bombetes* (- 0.53, P<0.0001). It was also longer in duration (+ 0.55 s, P=0.0058) and consisted of more pulses (+ 48 pulses, P=0.0356) compared to *A. bombetes*. Finally, its frequency bandwidth was wider compared to *A. virolinensis* (+ 0.40 kHz, P=0.0220) and *A. opisthomelas* (+ 0.35 kHz, P=0.0246).

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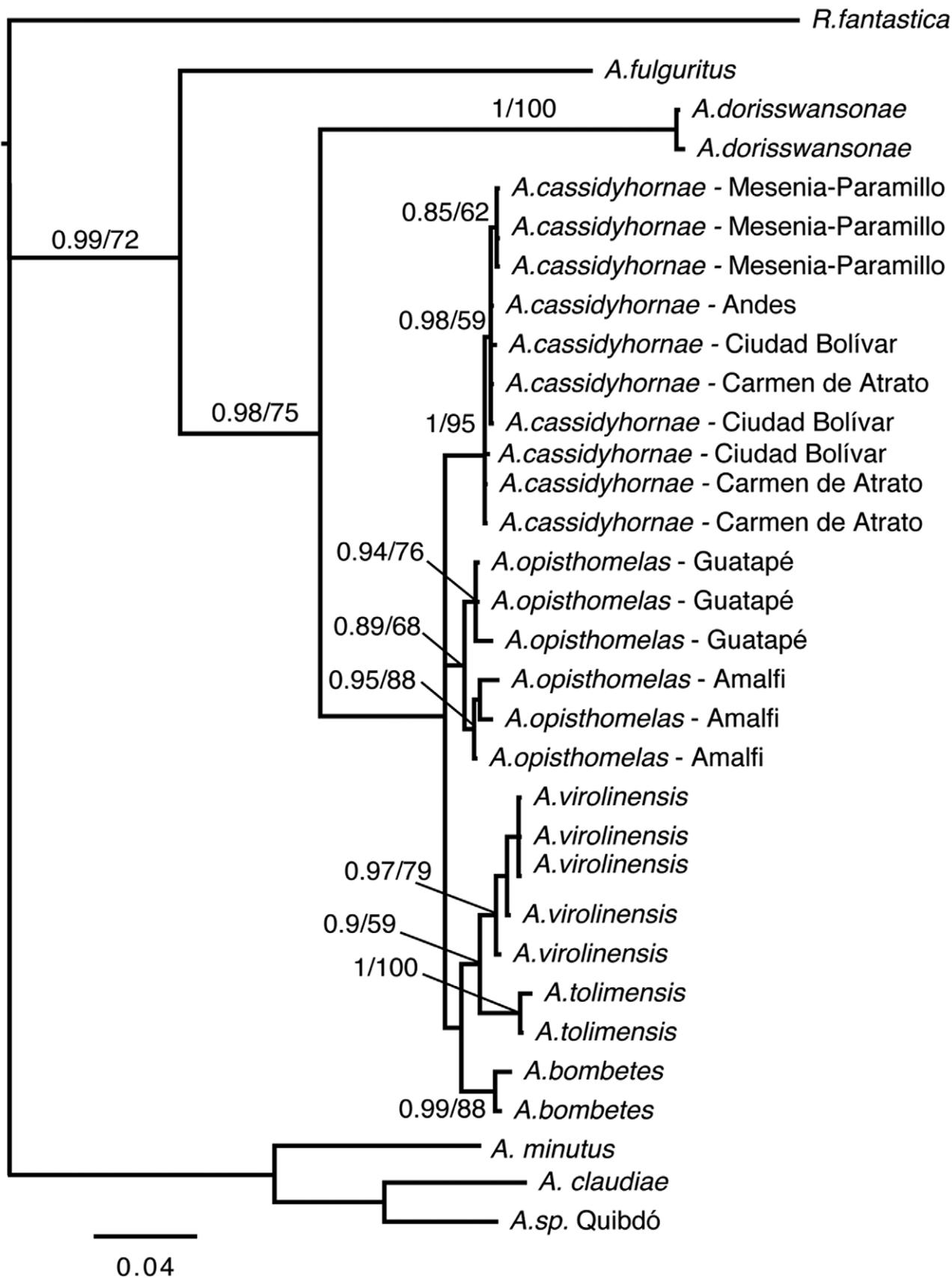
FIGURE 6. Bayesian phylogeny of 10 *Andinobates* species inferred using MrBayes. Numbers on internodes represent nodal support as follows: Bayesian posterior probability/RAxML bootstrap support. Support values for nodes with posterior probabilities under 0.8 and bootstrap supports below 70 are not shown. *Ranitomeya fantastica* was used as outgroup.
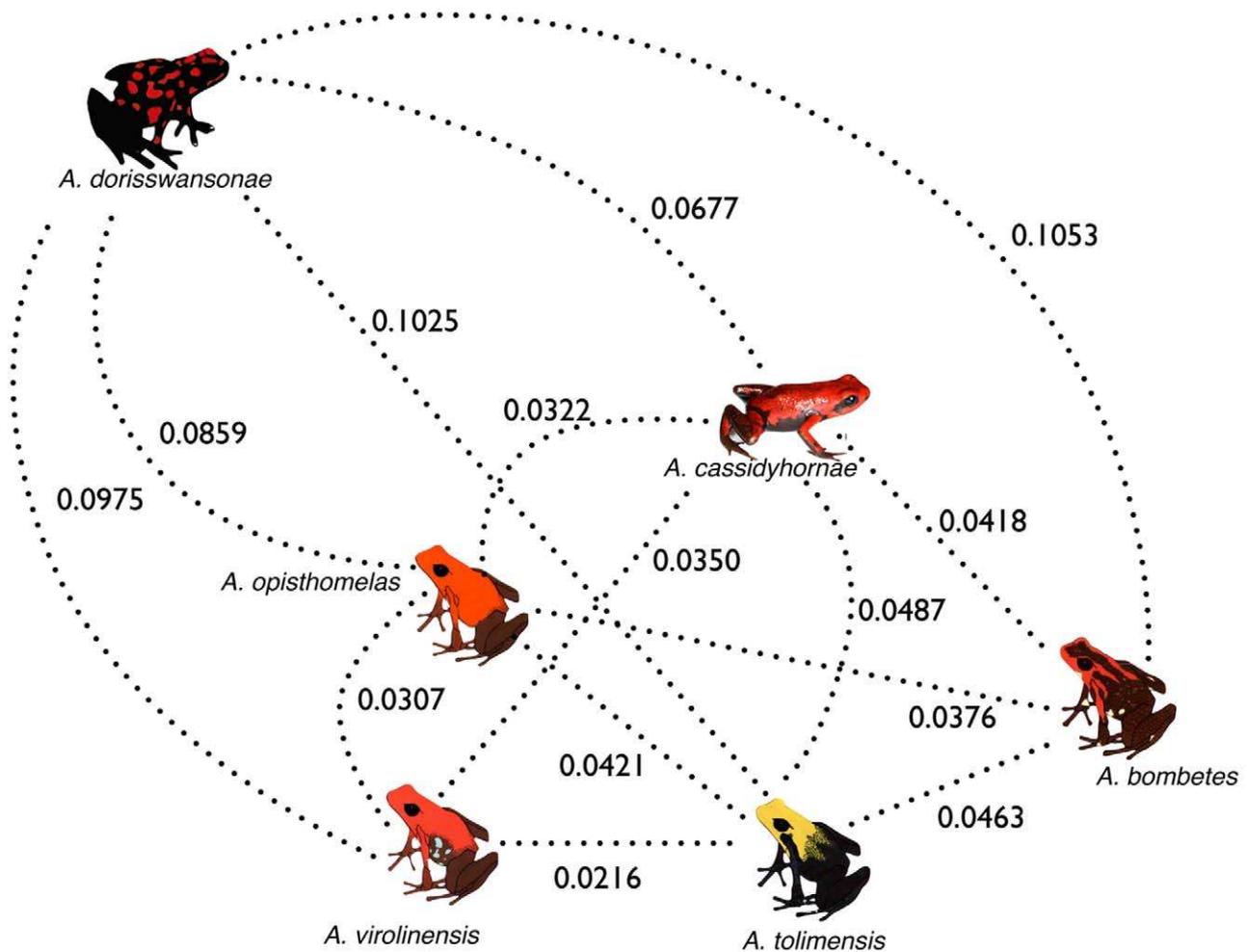
FIGURE 7. Mean interspecific K2P genetic distances of the amplified fragment of the *Cytb* gene between six geographically close species of the *bombetes* group, including *A. cassidyhornae* **sp. nov.** Line lengths are roughly proportional to pairwise genetic distances.

The strong among-species variation in call peak frequency could be partly attributed to concomitant variation in body size. Larger frogs usually produce calls at lower frequency values (e.g. Erdtmann & Amézquita 2009 for dendrobatid frogs) due to allometric constraints in larynx size. Indeed, increasing peak frequency is roughly related to decreasing body size in our study species: *A. cassidyhornae* **sp. nov.** is the largest species (mean±SD, 18.73±0.22 mm, N = 5 recorded males, this study), followed by *A. bombetes* (17.76±0.55, N = 28 males, Myers & Daly 1980), *A. opisthomelas* (16.80±1.24, N = 26 males, Silverstone 1975), and *A. virolinensis* (16.72±0.54, N = 127 males, Valderrama-Vernaza *et al.* 2009). We did not correct for body size effects on peak frequency, because we did not have all information on body size of recorded individuals. In any case, the difference in call frequency alone probably has important evolutionary implications. Across many frog species, ear sensitivity appears to match the peak frequency of the advertisement call (Capranica & Moffat 1983, see Amézquita *et al.* 2006, 2011 for examples on dendrobatid frogs). Thus, among-lineages differences in call frequency would imply a frequency mismatch between senders and receivers in the mate recognition signal, which could have promoted reproductive isolation between any pair of the *Andinobates* species we studied here.

Distribution, habitat and ecology. At the type locality, the Mesenia-Paramillo Natural Reserve, *Andinobates cassidyhornae* **sp. nov.** was found in two fragments of heavily disturbed cloud forests. The approximately 1.52 hectare site is located on a steep montane slope with a gradient of 65% (Figure 10). This mountain forest fragment is entirely surrounded by cattle grazing grasslands and agriculture crops. The forest fragment there has a dense, sometimes broken canopy with a complex stratification and emergent trees up to 20 m in height. The dominant canopy trees are Lauraceae (*Nectandra acutifolia, Nectandra laurel, Aniba coto, Aiouea dubia, Aniba perutiles* and *Ocotea sp.*), Oak (*Quercus humboldtii*) and Cedar (*Cedrela montana*). Shrubs and small trees in the families

Ericaceae, Gesneriaceae, Melastomataceae, Piperaceae and Rubiaceae dominate the understory. *Cyathea* sp. tree ferns are common and epiphytes are dense on most trees dominated by the families Araceae, Bromeliaceae, Dryopteridaceae and Orchidaceae. Bromeliads are predominantly of the genus *Guzmania*. The forest floor is covered with abundant leaf litter and decomposing wood. Remains of inflorescences of *Wettinia kalbreyerii* palms are common, and serve as terrestrial water reservoirs for extended periods of time. Near daily (primarily evening and early morning) cloud cover of fog, mist and precipitation provide a very cool and humid mesic environment. No additional water sources, i.e. streams or springs were found there. The average annual rainfall is 2500 mm. The average annual regional temperature is 15.0° C. There are two conspicuous wet seasons beginning in March and lasting until May, and another extending from October through December. The species is also known from other three localities visited by one of the co-autors (D. M.-V., Figure 1) that are much less known than the type localitity. They, however, look roughly similar in topography and frogs' microhabitat.
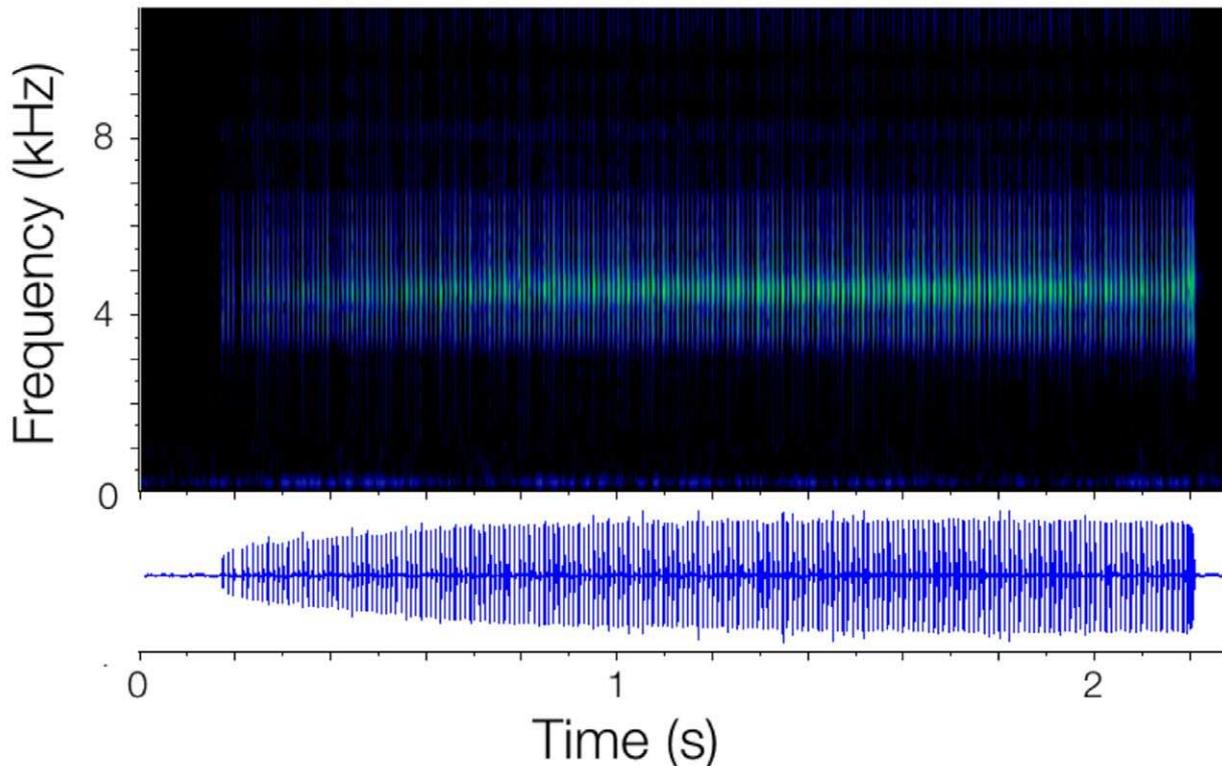
FIGURE 8. Sonogram (above) and oscillogram (below) of the advertisement call of *A. cassidyhornae* **sp. nov.** recorded by Ricardo Medina.

Conservation status. Almost all the known localities for species in the *A. bombetes* group are within the 1200–2100 m elevational belt. In Colombia, the forests within this range have been severely degraded by intensive agriculture, remarkably coffee plantations, which is a first order national product. *Andinobates cassidyhornae* **sp. nov.** was found at four localities within 1800–2059 m elevation (Figure 1). The minimum area of the elevational range encompassing these localities, equivalent to the area achieved by the sum of the occupied grid squares (Figure 2C in IUCN 2001), is between 200–300 km^2. However, to the best of or experience, the species distribution is sparsely patched including just few of the apparently suitable hills, probably occupying a minor fraction of the available habitat. In addition, most of the suitable forest below 2000 m elevation was cleared since many years ago at the type locality, which further limits the potential distribution of the new species.

Unfortunately, most localities included in this study are exposed to severe degradation by intensive agriculture. Contamination of watersheds by pesticides, herbicides, and soil degradation caused by agriculture and cattle grazing also degrade the environment here. At the type locality, there is an ongoing conservation project that involves the local community neighboring the Mesenia-Paramillo Nature Reserve and The Hummingbird Conservancy (THC) foundation. Based on their biogeographical, biological and hydrological importance, the project aims at increasing the percentage of protected conservation areas, thereby protecting from selective logging and massive deforestation, the old-growth cloud forests and sub-paramo ecosystems in the area.
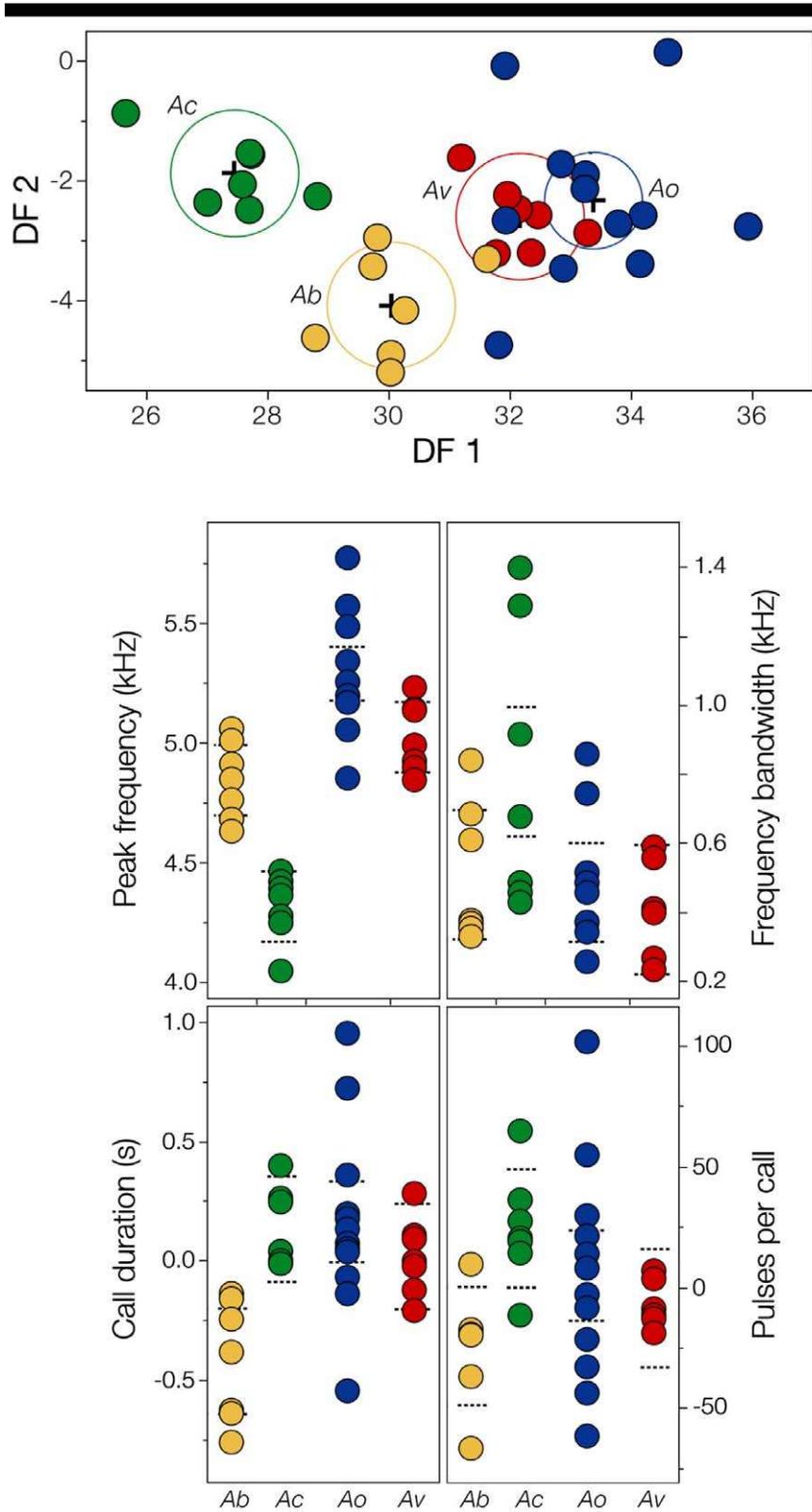
FIGURE 9. Above: Discriminant analysis of six spectral and temporal parameters of the advertisement calls of *A. cassidyhornae* **sp. nov.** (*Ac*, green dots), and the geographically closest species of the *bombetes* group: *A. bombetes* (*Ab*, yellow dots), *A. opisthomelas* (*Ao*, blue dots) and *A. virolinensis* (*Av*, red dots). Ellipses denote 95% confidence intervals of the multivariate mean. Non-overlapping ellipses thus indicate statistically significant differences at the 0.05 level. Below: Corresponding univariate analyses of the call parameters that most contributed in the discriminant analysis. Horizontal lines enclose 95% confidence intervals of the univariate mean.

FIGURE 10. View of the severely fragmented cloud forests at the type locality of *A. cassidyhornae* **sp. nov.** (photo © 2012 L. Mazariegos).

Summing up, the long-term and immediate survival of this species is threatened by its apparently small distribution combined with the heavy disturbance of primary forests where it lives in. The species is also highly vulnerable to massive smuggling in short time spans, as typically occurs when new forms of dendrobatid frogs become known by the illegal pet market. Based on the available information, we propose at least temporarily listing *A. cassidyhornae* **sp. nov.** as Critically Endangered (A1c, B2bi, ii, iv, IUCN 2001). Further phylogenetic, biogeographic, and taxonomic studies are urgently needed to develop appropriate conservation strategies for this group of frogs. The information obtained would greatly assist in the development and implementation of a species-specific management plan for *A. cassidyhornae* **sp. nov.**

Acknowledgments

References

Amézquita, A., Hödl, W., Lima, A.P., Castellanos, L., Erdtmann, L. & de Araújo, M.C. (2006) Masking interference and the evolution of the acoustic communication system in the Amazonian dendrobatid frog *Allobates femoralis*. *Evolution*, 60, 1874–1887.

Amézquita, A., Flechas, S.V., Lima, A.P., Gasser, H. & Hödl, W. (2011) Acoustic interference and recognition space within a complex assemblage of dendrobatid frogs. *Proceedings of the National Academy of Sciences*, USA, 108, 17058–17063. http://dx.doi.org/10.1073/pnas.1104773108

Bernal, M.H., Luna-Mora, V.F., Gallego, O. & Quevedo, A. (2007) A new species of poison frog (Amphibia: Dendrobatidae) from the Andean mountains of Tolima, Colombia. *Zootaxa*, 1638, 59–68.

Boulenger, G.A. (1899) Descriptions of new batrachians in the collection of the British Museum (Natural History). *Annals and*

Magazine of Natural History, 3, 273–277. http://dx.doi.org/10.1080/00222939908678122

Brown, J.L., Twomey, E., Amézquita, A., Barbosa De Souza, M., Caldwell, J.P., Lötters, S., Von May, R., Melo-Sampaio, P.R., Mejía-Vargas, D., Perez-Peña, P., Pepper, M., Poelman, E.H., Sanchez-Rodriguez, M. & Summers, K. (2011) A taxonomic revision of the Neotropical poison frog genus *Ranitomeya* (Amphibia: Dendrobatidae). *Zootaxa*, 3083, 1–120.

Capranica, R.R. & Moffat, J.M. (1983) Neurobehavioral correlates of sound communication in anurans. In: Ewert J.P. & Capranica R.R. (Eds.), *Advances in Vertebrate Neuroethology*, Ingle D, Plenum, New York, pp. 701–730. http://dx.doi.org/10.1007/978-1-4684-4412-4_36

Drummond, A., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Kearse, M., Moir, R., Stones-Havas, S., Sturrick, T., Thierer, T. & Wilson, A. (2010) Geneious v5.1. Available from http://www.geneious.com/ (accessed 26/08/2012).

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32, 1792–1797. http://dx.doi.org/10.1093/nar/gkh340

Erdtmann, L. & Amézquita, A. (2009) Differential evolution of advertisement call traits in dart-poison frogs (Anura: Dendrobatidae). *Ethology,* 115, 801–811. http://dx.doi.org/10.1111/j.1439-0310.2009.01673.x

Felsenstein, J. (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, 17, 368–376. http://dx.doi.org/10.1007/BF01734359

Grant T., Frost D., Caldwell J., Gagliardo R., Haddad C.F., Kok P.J., Means D.B., Noonan B.P., Schargel W.E. & Wheeler W.C. (2006) Phylogenetic systematics of dart-poison frogs and their relatives (Amphibia: Athesphatanura: Dendrobatidae). *Bulletin of the American Museum of Natural History*, 299, 1–262.
http://dx.doi.org/10.1206/0003-0090(2006)299[1:PSODFA]2.0.CO;2

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16, 111–120. http://dx.doi.org/10.1007/BF01731581

Lanfear, R., Calcott, B., Ho, S.Y.W. & Guidon, S. (2012) PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, 29, 1695–1701.
http://dx.doi.org/10.1093/molbev/mss020

Madisson, W. & Madisson, D. (2011) *Mesquite: A Modular System for Evolutionary Analysis*, ver. 2.75. http://mesquiteproject.org (accessed 26/08/2012).

McDiarmid, R.W. (1994) Preparing amphibians as scientific specimens. *In:* Heyer, W.R., Donnelly, M.A., McDiarmid, R.W., Hayek, L-A.C. & Foster, M.S. (Eds.), *Measuring and Monitoring Biological Diversity: Standard Methods for Amphibians*. Smithsonian Institution Press. Washington, pp. 289–297.

Myers, C.W. & Daly, J. (1976) A new species of poison frog (*Dendrobates)* from Andean Ecuador, including an analysis of its skin toxins. *Occasional Papers of the Museum of Natural History The University of Kansas Lawrence, Kansas,* 59, 1–12.

Myers, C.W. & Daly, J. (1980) Taxonomy and ecology of *Dendrobates bombetes,* a new Andean poison frog with new skin toxins. *American Museum Novitates,* 2694, 1–23.

Palumbi, S., Martin, A., Romano, S., McMillan, O., Stice, L. & Gabowski, G. (1991) *The Simple Fool's Guide to PCR.* Deppartment of Zoology, University of Hawaii, Hawaii, 45 pp.

R Development Core Team. (2011) *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing. Austria.

Rambaut, A. & Drummond, A. (2007) *Tracer v1.5*. Available from http://beast.bio.ed.ac.uk/Tracer (accessed 26/08/2012)

Rannala, B. & Yang, Z. (1996) Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. *Journal of Molecular Evolution*, 43, 304–311. http://dx.doi.org/10.1007/BF02338839

Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics,* 19, 1572–1574. http://dx.doi.org/10.1093/bioinformatics/btg180

Rueda-Almonacid, J.V., Rada, M., Sánchez, S., Velásquez-Alvarez, A. & Quevedo, A. (2006) Two new and exceptional poison dart frogs of the genus *Dendrobates* (Anura: Dendrobatidae) from the northeastern flank of the Cordillera Central of Colombia. *Zootaxa,* 1259, 39–54.

Ruiz-Carranza, P.M., & Ramírez-Pinilla, M.P. (1992) Una nueva especie de *Myniobates* (Anura: Dendrobatidae) de Colombia. *Lozania,* 61, 1–16.

Santos, J.C. & Cannatella, D.C. (2011) Phenotypic integration emerges from aposematism and scale in poison frogs. *Proceedings of the National Academy of Sciences*, 108, 6175–6180. http://dx.doi.org/10.1073/pnas.1010952108

Santos J.C., Coloma, L.A., Summers, K., Caldwell, J.P., Ree, R. & Cannatella, D.C. (2009) Amazonian amphibian diversity is primarily derived from late miocene Andean lineages. *PLoS Biology*, 7, 3. http://dx.doi.org/10.1371/journal.pbio.1000056

Schliep, K.P. (2011) Phangorn: phylogenetic analysis in R. *Bioinformatics*, 27, 592–593. http://dx.doi.org/10.1093/bioinformatics/btq706

Shimodaira, H. & Hasegawa, M. (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution*, 16, 1114–1116. http://dx.doi.org/10.1093/oxfordjournals.molbev.a026201

Silverstone P.A. (1975) A revision of the poison-arrow frogs of the genus *Dendrobates* Wagler. *Natural History Museum of Los Angeles County, Science Bulletin*, 21, 1–55.

Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, 22, 2688–2690. http://dx.doi.org/10.1093/bioinformatics/btl446

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739. http://dx.doi.org/10.1093/molbev/msr121

Valderrama-Vernaza M., Ramírez-Pinilla M.P. & Serrano-Cardozo V.H. (2009) Diet of the Andean frog *Ranitomeya virolinensis* (Athesphatanura: Dendrobatidae). *Journal of Herpetology*, 43, 114–123. http://dx.doi.org/10.1670/07-247R1.1