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Cryptic diversity concealed in the Andean cloud forests: two new species of rainfrogs (*Pristimantis*) uncovered by molecular and bioacoustic data

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Two new species of frogs previously confused with *Pristimantis calcarulatus* are identified using molecular phylogenetics and described using an integrative taxonomic approach. The species are distributed in montane cloud forests of the northwestern Ecuadorian Andes. *Pristimantis pahuma* n. sp. and *Pristimantis cedros* n. sp. differ from their closest relatives by strongly supported reciprocal monophyly in mitochondrial genetic data (16S). Additionally, the genetic distance among *P. cedros* n. sp., *P. pahuma* n. sp. and *P. calcarulatus* (*sensu stricto*) is 7.1–9.5%. The advertisement call of *P. pahuma* n. sp. also differs from that of *P. calcarulatus*; the former emits single-note calls irregularly, whereas *P. calcarulatus* always calls in a series of 8–24 notes (calls for *P. cedros* n. sp. not recorded). Morphologically, the three species are almost undistinguishable; however, *P. cedros* n. sp. differs in life by having an iris with more numerous and smaller black reticulations, whereas the other two species have larger and fewer reticulations. Biogeographically, *P. cedros* n. sp. is separated from *P. pahuma* n. sp. and *P. calcarulatus* by the climatically dry and low elevation Río Guayllabamba Valley, which acts as a strong barrier to dispersal in these frogs. The results of this and other studies suggest that the true species richness of *Pristimantis* in the Andes is vastly underestimated. This underestimation may lead to declining protection for such cryptic species, many of which have smaller ranges than previously assumed. Species distributed across potential geographic barriers should be studied to detect the existence of cryptic species.

Keywords: amphibia; Andes; new species; *Pristimantis calcarulatus*; taxonomy

Se describen dos nuevas especies de ranas previamente confundidas con *Pristimantis calcarulatus*, mediante el uso de la taxonomía integrativa. Las dos nuevas especies habitan los bosques nublados de los Andes del noroccidente del Ecuador. La validez de los nuevos taxones, *Pristimantis pahuma* n. sp. y *Pristimantis cedros* n. sp. está sustentada por su monofilia recíproca en relación a sus congéneres más cercanos en el gen mitocondrial 16S. Adicionalmente, la distancia genética de *P. pahuma* n. sp., *P. cedros* n. sp., y *P. calcarulatus* (*sensu stricto*) es de 7.1–9.5%. *Pristimantis pahuma* n. sp. se caracteriza por emitir un canto con una única nota en intervalos irregulares, mientras que *P. calcarulatus* siempre emite series de cantos que contienen entre 8 y 24 cantos (el canto *P. cedros* n. sp. no ha sido grabado). Morfológicamente, las tres especies son prácticamente idénticas; sin embargo, *P. cedros* n. sp. tiene un iris con reticulaciones negras más numerosas y pequeñas que las que se observan en las otras dos especies. Biogeográficamente, *P. cedros* n. sp. se encuentra separado de *P. pahuma* n. sp. y *P. calcarulatus* por el valle del Río Guayllabamba, que presenta una elevación más baja y un clima más seco a los bosques aledaños, por lo que parece actuar como barrera a la dispersión de estos anfibios. Estos resultados, combinados con estudios previos, sugieren que la riqueza de especies de *Pristimantis* en los Andes se encuentra subestimada. Esto conlleva a errores en la evaluación del estado de conservación de las especies, ya que los rangos de cada especie son mucho más reducidos a lo que se asume. Se sugiere estudiar el efecto de potenciales barreras geográficas porque es muy probable que existan muchas más especies crípticas por descubrirse.

Palabras claves: Amphibia; Andes; nuevas especies; *Pristimantis calcarulatus*; taxonomía

<http://zoobank.org/urn:lsid:zoobank.org:pub:52E1ADFD-D585-499F-91C9-3D233B7CCA5F>

Introduction

Having nearly 1000 species, frogs of the clade Terrarana (Brachycephaloidea; *sensu* Padial et al. [1]) are among the most species-rich radiations of vertebrates in the world.[2,3] All members of this diverse group deposit their eggs in terrestrial habitats and the embryos develop directly into froglets, bypassing the tadpole stage.[4] The most diverse genus in this group is *Pristimantis* (in the family Craugastoridae;

sensu Pyron and Wiens [5]), which comprises nearly half of the known species of Terrarana (~475 species [3]). New species of *Pristimantis* are described regularly [6–9]; nonetheless, the number of species seems to remain severely underestimated.[1,2,10] In addition, intensive surveys and mitochondrial sequencing efforts suggest that there are many cryptic species of *Pristimantis*, with exceptional diversity occurring in the tropical Andes.[11–13]

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Two or more distinct species that resemble one another morphologically such that they are incorrectly classified as a single species are termed ‘cryptic species’ or ‘species complexes.’[14] Although cryptic species may seem to be identical in appearance, differences in their evolutionary history, reproductive behavior, or biogeography assure their recognition as separate species on distinct evolutionary trajectories.[16–18] The existence of cryptic species is problematic for taxonomists, ecologists and evolutionary biologists who, failing to distinguish the different taxa, underestimate species diversity, which thwarts conservation efforts.[10,15]

Herein, we identify and address the *Pristimantis calcarulatus* [19] complex, which is a clade of at least three cryptic species distributed in the montane cloud forests along the Pacific versant of the Andes of Colombia and Ecuador (Figure 1). We incorporate evidence from phylogenetics, bioacoustics, morphology and biogeography to describe two new species and clarify the status of *P. calcarulatus*. We also reassess the distributions and conservation status of these species, emphasizing the importance of studying cryptic diversity and its impacts on conservation.

Methods

Taxonomy

We follow the general lineage concept of species (i.e. unified species concept), which is a theoretical framework that defines a species as a separately evolving lineage within a temporal segment of a population.[16–18,20,21] Along with this species concept, we use an integrative taxonomic approach in assessing species boundaries.[22,23] Integrative taxonomy [22–24] is defined as the use of multiple data types (e.g. phylogenetics, bioacoustics, morphology, biogeography and ecology) to identify and name new species that meet multiple criteria. These multiple lines of evidence are used to test the hypothesis that a given population is an independently evolving lineage, and thus, a distinct species.

Generic and family names follow the taxonomy proposed by Hedges et al. [2], as modified by Pyron and Wiens.[5] We do not address group delimitation *sensu* Hedges et al. [2] because recent molecular studies [1,25] revealed that several subgeneric-level species groups are not monophyletic, including the group in which *P. calcarulatus* was placed (the *P. unistrigatus* group [2]).

Morphology

Traditionally, species have been diagnosed only on the basis of morphological differences because often these were the only available data. Morphology can provide

evidence that a population is a separately evolving lineage because phenotypic traits evolve through natural selection or by random drift. However, morphology alone often does not provide clear evidence of species boundaries. Many species can be extremely variable,[26–28] whereas other closely related species can have few or no morphological interspecific differences.[29–32]

Specimens were euthanized with 20% benzocaine, fixed for 24–48 h in 95% ethanol and stored in 70% ethanol. Diagnoses and descriptions mostly follow those of Lynch and Duellman [33], as modified by Duellman et al. [34]. We examined alcohol-preserved specimens from the amphibian collections at the Museo de Zoología of the Universidad Tecnológica Indoamérica (MZUTI) and the Biodiversity Institute at the University of Kansas (KU) (Appendix 1). All photographs were taken by CRH unless noted.

Continuous morphological traits were measured to the nearest 0.1 mm with Mitutoyo® digital calipers, following the protocols of Guayasamin and Bonaccorso [35]. We used the following measurements: (1) snout–vent length (SVL); (2) tibia length; (3) foot length; (4) head length; (5) head width; (6) interorbital distance; (7) upper eyelid width; (8) internarial distance; (10) eye diameter; (11) tympanum diameter; (12) radioulna length; (13) hand length; (14) Finger I length; (15) Finger II length = distance from outer margin of palmar tubercle to tip of Finger II; and (16) disk width of Finger III. Fingers are numbered preaxially to postaxially from I to IV to facilitate comparison with previous anuran literature. However, we stress that in an evolutionary perspective, anuran fingers should be numbered from II to V, consistent with the hypothesis that Digit I was lost in anurans.[36,37] Sexual maturity was determined by the presence of vocal slits in males and by the presence of eggs or convoluted oviducts in females. Shape of terminal phalanges was assessed via finger dissection and observation in a stereoscope Olympus SZ61.

Phylogenetics

To identify separately evolving lineages, we tested for strongly supported reciprocal monophyly in mitochondrial genetic data in phylogenetic analyses.[38] Reciprocal monophyly is supported when lineages have had enough time for complementary haplotype extinction to occur from the parent lineage.[21,24] Reciprocal monophyly is also evidence that reproductive isolation has occurred in sympatric species; however, hybridization, introgression and incomplete lineage sorting can obscure these relationships. In these situations, nuclear markers and coalescent-based species delimitation approaches are necessary.

We obtained genetic data for 23 specimens (Table 1) from the newly described taxa and *P. calcarulatus*. We

Table 1. Morphometrics (in mm) of adult males and females of *P. calcarulatus*, *P. cedros* n. sp. and *P. pahuma* n. sp. Range followed by mean and standard deviation.

Character	<i>P. calcarulatus</i> (<i>sensu stricto</i>)		<i>P. cedros</i> n. sp.		<i>P. pahuma</i> n. sp.	
	Male (<i>n</i> = 14)	Female (<i>n</i> = 1)	Male (<i>n</i> = 6)	Female (<i>n</i> = 7)	Male (<i>n</i> = 7)	Female (<i>n</i> = 2)
SVL	20.2–23.4 (21.6 ± 1.23)	23.2	21.5–23.0 (22.2 ± 0.60)	24.8–28.6 (26.7 ± 1.19)	20.2–22.8 (21.4 ± 0.88)	22.4–24.9
Tibia	10.9–12.5 (11.6 ± 0.49)	12.9	12.0–13.3 (12.7 ± 0.49)	13.7–15.2 (14.4 ± 0.51)	10.9–11.6 (11.3 ± 0.26)	12.7–12.9
Femur	9.4–11.1 (10.2 ± 0.48)	10.7	10.5–11.5 (10.9 ± 0.36)	12.4–13.5 (12.7 ± 0.37)	9.5–10.5 (9.9 ± 0.30)	10.8–11.6
Head length	7.9–9.2 (8.6 ± 0.33)	9.1	8.8–9.3 (9.0 ± 0.21)	10.6–11.4 (11.0 ± 0.33)	7.9–8.9 (8.5 ± 0.36)	9.0–10.0
Head width	7.2–8.7 (7.8 ± 0.42)	8.1	8.1–8.7 (8.4 ± 0.20)	9.6–10.5 (10.1 ± 0.29)	7.2–8.3 (7.8 ± 0.35)	8.3–9.1
Tympanum	0.86–1.17 (1.0 ± 0.11)	1.2	0.85–1.10 (1.0 ± 0.10)	1.08–1.49 (1.3 ± 0.16)	0.90–1.07 (1.0 ± 0.07)	1.2–1.5
Radiolula length	5.0–5.7 (5.3 ± 0.22)	5.7	5.0–5.4 (5.2 ± 0.15)	5.8–6.4 (6.2 ± 0.25)	4.9–5.5 (5.2 ± 0.23)	5.86.2
Specimens examined	MZUTI 500–507, 2050–53, 1883, 1899	MZUTI 1 508	MZUTI 1710–11, 1713, 1720, 1722–23	MZUTI 1714–18, 1724–25	MZUTI 486, 488–491, 493, 496	MZUTI 1 494, 499

collected samples from several localities, including the type locality of *P. calcarulatus*. We targeted a ~900 base pair (bp) segment of the mitochondrial ribosomal gene 16S. The methods for DNA extraction, primers used, PCR amplification and sequencing are described in Guayasamin et al.[39].

We supplemented these new data with published sequences of *Pristimantis* specimens from GenBank. Recent molecular studies have shown that some species groups need revision (see Taxonomy); therefore, the selection of genetic data can be challenging. Following the methods in Guayasamin et al.[27], we chose the most closely related specimens to *P. calcarulatus* from GenBank, regardless of prior species group designations. Our final sampling included eight species from the *Pristimantis unistrigatus*, *P. devillei* and *P. orcesi* species groups.[2] The distantly related *P. achatinus* was used as an out-group to root the phylogeny. GenBank accession numbers and their associated specimen data are included in Appendix 2.

The 16S rRNA sequence data was first aligned in Geneious [40] with MAFFT v7.[41] Then we manually aligned the MAFFT alignment to stem and loop secondary structures[42] because they can differ in their evolutionary model and substitution rate and should be partitioned separately.[43,44]

For maximum likelihood estimation, we used RAxML 7.2.0 [45] and the GTR + Γ model (Generalized Time-Reversible + Gamma) of nucleotide substitution, which accounts for variable base frequencies and rate heterogeneity across sites using four discrete rate categories to approximate a gamma-shaped distribution. Next, we applied the ‘-f a’ function to search simultaneously for the best-scoring likelihood tree and conduct a rapid bootstrap analysis (100 tree searches with 1000 bootstrap replicates).

Bayesian analyses were conducted with MrBayes 3.2.1 [46]. We used JMODELTEST 2.0 [47] and Akaike’s information criterion with the 16S stem and loop partitions to select the model of sequence evolution that best fits the data [48]. The model selected for the 16S stem partition was the SYM + I + G model (Symmetrical Model with equal base frequencies and a proportion of invariant sites and a gamma distribution for rates across sites; parameter estimates: $-\ln L = 1368.5$; $p = 69$). The best-fit model for the 16S loop partition was the GTR + G model (parameter estimates: $-\ln L = 3922.1$; $p = 71$). We analyzed 20 million generations (sampling every 1000) with four Markov chains and default heating values. We used a uniform Dirichlet prior for the rate matrix and did not incorporate prior information on the topology. The analysis was run twice to assess consistent convergence and stationarity, for which we examined the standard deviation of split frequencies and plotted the $-\ln L$ per generation. We

discarded the trees generated before stationarity as ‘burn-in,’ which was the first 25% of trees.

The final step was to test for reciprocal monophyly of each lineage by assessing whether each putative new species clustered with its conspecifics. Statistical support for this hypothesis is given through high bootstrap support (>75%) in maximum likelihood analyses and high posterior probability support (>0.95) in Bayesian analyses. We acknowledge that absolute thresholds of genetic distance (e.g. 2.5–3.0%) might be problematic because they do not address whether a lineage is independently evolving and statistical support cannot be acquired from this measure. Furthermore, distances may be underestimated as a result of introgression, incomplete lineage sorting, or low genetic variability of mitochondrial markers. These problems have been revealed in numerous frog species, especially in cryptic species complexes (e.g. *Hyloscirtus* [49]; *Pristimantis* [50]; *Mantella* [51]). Despite this possibility, pairwise genetic distances calculated from mitochondrial genetic markers (e.g. 12S, 16S, COI) usually delimit species accurately [51,52]; thus, we calculate and consider the distances along with other evidence.

Bioacoustics

Calls were recorded in the field with an Olympus LS-10 Linear PCM Field Recorder and a Sennheiser K6-ME66 shotgun microphone. The calls were recorded in WAV format with a sampling rate of 44.1 kHz/s with 16 bits/sample. We defined a call as the entire assemblage of acoustic signals emitted in sequence. Calls were considered as a ‘call series’ when a sequence of individual notes are separated by a consistent, short time interval with background noise between each note.[4] Distinguishable subunits of a call that are separated by temporally distinct segments are defined as notes. Notes were one of two types: (1) pulsed notes with one or more clear amplitude peaks; and (2) tonal notes with relatively constant amplitude throughout the call.

Call parameter definitions follow those of Hutter et al. [53] (Table 1); the following relevant parameters were selected: (1) note amplitude type (pulsed or tonal); (2) call arrangement type (singular or series); (3) series duration (ms); (4) series interval (s); (5) series rate (/s); (6) number of calls/series; (7) call duration (ms); (8) call rate (/s); (9) interval between calls (ms); (10) number of pulses; (11) pulse rate (/ms); (12) call envelope shape (time at peak amplitude divided by call duration); (13) dominant frequency (Hz); (14) fundamental frequency (Hz); (15) frequency modulation (Hz); and (16) first harmonic frequency (Hz). Measures are reported as the range followed by the mean $\pm$ two standard deviations from the mean.

Calls were analyzed with the R package SEEWAVE [54,55] in combination with a custom script (available upon request) to batch analyze hundreds of calls quickly. To measure durations and intervals, we considered sound below a relative amplitude threshold of 5% to be background noise. The analysis of calls was automated with the following routine: (1) audio file is normalized and filtered of background noise and other non-target sounds (e.g. insects, stream noise, rain) using a band-pass filter set to 2000–4000 Hz (removes sound that generates frequencies outside the species' frequency range); (2) target calls are located in the filtered audio file by recording the start time of each amplitude increase above a 5% threshold; (3) each call identified is vetted to ensure that it was a call of the target species and not other sounds; (4) each individual call is normalized to a relative scale to remove the effects of amplitude variation between calls; (5) using the call start times and the original recordings, each call is saved as an unfiltered file; (6) the separated calls are analyzed using various functions (for the call parameters above) available in SEEWAVE; and (7) significant outliers falling outside the 95% confidence intervals of the measurement data are inspected manually to ensure accurate measurement. To test the accuracy of this method, we used a prior data-set [53] based on manual measurement and found the results to be generally the same. Digital recordings are deposited at MZUTI and KU and are available upon request.

Results

Pristimantis calcarulatus [19]

Eleutherodactylus calcarulatus [19]

Pristimantis calcarulatus [55]

Diagnosis (Figures 2, 3)

The revised diagnosis of *P. calcarulatus* (*sensu stricto*) is based on specimens we attribute to this species after analysis of morphological, genetic and bioacoustic data (described below) from Tandapi (type locality), Reserva Otonga and Reserva Las Gralarias. Importantly, we also refer to the original description of *P. calcarulatus* [19] and type specimens, which only included specimens from the type locality. Further support for the identity of *P. calcarulatus* is that both new species do not occur at Tandapi and we found that these species are distributed allopatrically. Additionally, the previous morphological diagnosis is identical in most traits to the new species described below unless noted otherwise.

Adult *P. calcarulatus* are relatively small (details below) and can be distinguished from its congeners by the following combination of characters: (1) skin texture of dorsum finely shagreen, with few, low scattered tubercles; dorsal folds absent; venter smooth to areolate; (2)

tympanic membrane present with upper edge obscured by supratympanic fold; (3) snout rounded to subacuminate in dorsal view, rounded to protruding in profile, usually with papilla at tip; (4) upper eyelid with one conical tubercle and several low tubercles; cranial crests absent; (5) vomerine dentigerous process small, oblique in outline, positioned posterior to level of choanae, each process bearing 2–5 teeth; (6) males with a large subgular vocal sac, vocal slits present, nuptial pads absent; (7) Finger I conspicuously shorter than II, disks on fingers elliptical, laterally expanded, except for slightly expanded on Finger I; (8) fingers bearing narrow lateral fringes, palmar tubercle distally bifurcate; few supernumerary tubercles present, round, fleshy; (9) few, small ulnar tubercles present; (10) heel with elongate conical tubercle; low tarsal tubercles present; (11) toes bearing narrow fringes, webbing absent, Toe V longer than III; toe disks rounded and expanded laterally; (12) inner metatarsal tubercle elliptical, about 2–3 times the size of outer, rounded metatarsal tubercle, supernumerary plantar tubercles small, round, low, fleshy; (13) dorsum pale brown with dark brown marking; venter cream with brown flecks; posterior surfaces of thighs uniform brown; (14) SVL in adult females = 23.2–28.7 mm ($n = 9$; mean = 26.0 ± 1.5); SVL in adult males = 16.9–24.7 mm ($n = 33$; mean = 22.2 ± 1.6).

Coloration in ethanol

Dorsum cream to dark brown with darker brown markings, including an interorbital bar, chevrons, canthal and supratympanic stripes, labial bars, oblique limb bars and flanks with diagonal stripes. Posterior surfaces of thigh uniform brown; cloacal triangle dark brown edged with thin cream line; venter cream to pale gray peppered with brown (Figure 2).

Coloration in life

Dorsum yellowish, greenish, or reddish brown with darker brown or black pattern; flanks paler than dorsum. Vocal sac yellowish-cream to bright yellow. Venter pale yellow to greenish-brown with cream to pale gray flecks; posterior surfaces of thighs dull gray (Figure 3), Iris blue-gray with thin black reticulation and a reddish-brown horizontal stripe (Figure 4(A)).

Color variation

The following variation was noted in individuals from the type locality of *P. calcarulatus* (Tandapi): (1) uniformly colored dorsum (yellow or brown); (2) longitudinal stripes on the dorsum; (3) dorsal pattern that resembles that of *Dendropsophus leucophyllatus*; and (4) dorsum with a single, broad vertebral stripe ([19]; this

work). Additionally, the iris coloration is blue or golden, with a wide brownish-red stripe through the middle. Past studies have suggested a ratio of 80% blue to 20% golden for *P. calcarulatus* [26]; however, it is unclear how this is affected by the new taxonomic arrangements. Importantly, we note that iris coloration remains inadequate in distinguishing species in this complex. See Figures 3(A), 4(A), 5(A).

Phylogenetics

Within the *P. calcarulatus* complex, we identify three distinct reciprocally monophyletic mitochondrial lineages with strong bootstrap (>95%) and posterior probability (>0.95) support (Figure 6). We also find evidence for a fourth lineage (KU 177658), but lack sufficient samples to address reciprocal monophyly. Additionally, some population structure can be observed in the phylogenetic analyses for *P. calcarulatus*; individuals from Reserva Las Gralarias are monophyletic and have a genetic distance of 0.98–1.59% from the populations at Tandapi (type locality) and Reserva Otonga. Our phylogenetic results (among the nominal species) cannot be attributed to population structure or genetic isolation by distance because these species occur in a small geographic area ($\pm 7000 \text{ km}^2$); we sampled several localities, and at least two of the known species occur and were sampled within a kilometer at a single locality (Natural History).

Bioacoustics

We recorded 726 calls from seven individuals of *P. calcarulatus*. All calls were recorded at Reserva Las Gralarias April–June 2011. Calls were recorded at night with mist or light rain at 14–16°C. Males called on the upper surfaces of leaves 2–4 m above the ground and all recordings were made within 0.5–2.0 m of the calling male. The call of this species sounds like a rapid sequence of ‘tinks’ lasting several seconds.

P. calcarulatus emits pulsed notes (Figure 7(A)) that always are arranged in a series (Figure 8(A)) composed of 8–23 notes (14.82 ± 5.503) per series. The series duration is 1.417–5.230 s (3.696 ± 0.993). The interval between each note in the series is 0.165–0.210 s (0.186 ± 0.009), which contrasts with the larger and more variable interval of 2.2–97.2 s (33.810 ± 25.731) between each call series. Within a call series, the species emits notes at a rate of 1.9–5.9 notes/s (3.8 ± 1.0) and each call series is emitted at a rate of 0.87–1.557 series/min (1.181 ± 0.232).

An individual note within a series has 1–4 pulses/note (1.7 ± 1.0). The note typically has one strongly amplitude-modulated pulse and 1–3 weakly amplitude-modulated pulses (Figure 7(A)). The note has a shape of 0–0.243 (0.068 ± 0.05), with the peak amplitude

occurring in the first 25% of the note. The note duration is 25.22–57.32 ms (40.68 ± 6.413). Notes have little frequency modulation and the dominant frequency of a call is 3273–3359 Hz (3318 ± 143.5). The fundamental frequency is 2687–3150 Hz (2815 ± 87.041). See Table 2.

Distribution

Prior to this study, *P. calcarulatus* was known from the Pacific versant of the northwestern Andes in Ecuador and Colombia. In Ecuador, the species was considered restricted to an area of 7884 km², with a wide elevational distribution of 1460–2557 m [26] (Figure 1). The data presented herein indicate that *P. calcarulatus* (*sensu stricto*) is endemic to Ecuador and is confirmed to occur in the following localities: Reserva Orquideológica El Pahuma, Reserva Otonga, Reserva Las Gralarias (Canyon Trail, Río Chalguyacu, Kathy’s Creek, Lucy’s Creek, Nunbird Ridge and Puma Trail) and Tandapi (Appendix 1). All localities lie south of the Río Guayllabamba, which is a major barrier to dispersal in Andean frogs owing to the elevational and climatic characteristics of the river valley (discussed below). Therefore, we speculate that *P. calcarulatus* only occurs south of this river; all populations north of the Río Guayllabamba (including records from Colombia) currently identified as *P. calcarulatus* are likely a different species. The revised elevational distribution of this species is 1850–2072 m.

Natural history

P. calcarulatus inhabits undisturbed primary forest and mature secondary forest. The species does not call in pastures or other human-modified habitats. The species is abundant, and during periods of high rainfall, several males can be heard calling simultaneously with large distances (100–200 m) between them. They frequently call from the surfaces of leaves in bushes or trees at night at heights greater than 2 m above the ground. Females have been found on the surfaces of leaves lower than 2 m in height.

At Reserva Las Gralarias sympatric *Pristimantis* include: *P. achatinus*, *P. appendiculatus*, *P. eremitus*, *P. eugeniae*, *P. mutabilis*, *P. sobetes*, *P. verecundus* and *P. w-nigrum*. At Reserva Orquideológica El Pahuma sympatric *Pristimantis* include: *P. achatinus*, *P. appendiculatus*, *P. laticlavus*, *P. eremitus*, *P. eugeniae*, *P. sobetes*, *P. thymalopsoides* and *P. w-nigrum*. At Reserva Otonga congeneric species include: *P. achatinus*, *P. appendiculatus*, *P. eugeniae*, *P. hectus*, *P. parvillus*, *P. sobetes*, *P. verecundus* and *P. w-nigrum*.

Conservation status

P. calcarulatus [56] currently is categorized as Vulnerable B1ab (iii) under the following IUCN criteria: (1) the

extent of occurrence is less than 20,000 km²; (2) its distribution is severely fragmented; and (3) there is a continuing decline in the extent and quality of its primary forest habitat. Given that *P. calcarulatus* comprises at least three species, the distribution and available habitat of each species is reduced. We did not observe the three species in sympatry, which suggests that each species has a restricted distribution (Natural History, below). Additionally, *P. calcarulatus* is dependent on primary and secondary mature forest and was not found outside of this habitat type. *Pristimantis calcarulatus* is distributed in the protected areas of Reserva Las Gralarias, Reserva Orquideológica El Pahuma and Reserva Otonga. Finally, we noted that in the chytrid survey results of Guayasamin et al. [57] there was not a high prevalence of the devastating chytrid fungus in individuals sampled (3 individuals positive and 27 negative). Given the new distributional information, we recommend that this species be categorized as Endangered B1ab (iii), under the following IUCN criteria: extent of occurrence is less than 5000 km², its distribution is severely fragmented and there is a continuing decline in the extent and quality of its primary forest habitat.

***Pristimantis pahuma* n. sp.**

Zoobank registration number = urn:lsid:zoobank.org:act:F350B312-DED8-4215-8B2A-7868CC3B01C3

Common English name: El Pahuma Rainfrog.

Common Spanish name: Cutín de El Pahuma.

Holotype (Figure 9(A))

MZUTI 493, an adult male collected by Carl R. Hutter on 8 June 2012, at Reserva Las Gralarias (Tunnel Trail: 0.02961 S, 78.70462 W, 2235 m), Provincia de Pichincha, Ecuador.

Paratopotypes (Figure 9(B))

MZUTI 494, 499 (adult females), 486, 488–491, 493, 496 (adult males) collected by Carl R. Hutter 14 May–12 June, 2012 at Reserva Las Gralarias (Hercules Creek: 0.02694 S, 78.70475 W, 2186 m; Tunnel Trail: 0.02961 S, 78.70462 W, 2235 m), Provincia de Pichincha, Ecuador.

Referred material

QCAZ-A 42934, 42941, 42945, 42950, 42954, 42955, 42957 and 42975 collected by Carl R. Hutter and Juan F. Dueñas on 11 June 2009 at Reserva Orquideológica El Pahuma (Guarida del Oso: 0.018533 N, 78.637112 W, 2340 m; Yumbo trail: 0.008223 N, 78.641638 W, 2574 m), Provincia de Pichincha, Ecuador.

Generic placement

P. pahuma n. sp. is placed in the genus *Pristimantis* as diagnosed by Hedges et al.[2]. All *Pristimantis* share the absence of cranial crests, and presence of a dentigerous process on the vomer and T-shaped terminal phalanges.

Diagnosis

The diagnosis of *P. pahuma* n. sp. is the same as that of *P. calcarulatus* (*sensu stricto*), except for the size measurements of the frog. The SVL in adult females is 22.4–24.9 mm ($n = 2$) and in adult males is 20.2–22.8 mm ($n = 7$; mean = 21.4 ± 0.88).

Similar species

On the Pacific slopes of the Andes, *P. pahuma* n. sp. is most similar to species within the *P. calcarulatus* complex, including the second new species described and compared below. *P. pahuma* n. sp. and *P. calcarulatus* are morphologically undistinguishable. Furthermore, the two species occur in the same geographic area and can both be found within 1 km of one another (although likely not syntopically). However, *P. pahuma* n. sp. is easily distinguished from *P. calcarulatus* by bioacoustic and genetic traits. The new species differs from *P. calcarulatus* by emitting a single-noted call irregularly with inconsistent time intervals between calls that lasted several seconds or more. Conversely, *P. calcarulatus* emits its calls in a series, in which each note is separated by a consistent, and short interval (less than 1/5 s), with 8–24 notes per series (Figure 8; Bioacoustics, below). Each series was separated by a long period of silence typically lasting 30 s or more. Phylogenetically, *P. pahuma* n. sp. is reciprocally monophyletic with strong support, providing evidence that the lineage is evolving separately. As additional evidence, *P. pahuma* n. sp. and *P. calcarulatus* are separated by a substantial genetic distance of 7% or greater.

Outside the *P. calcarulatus* complex, *P. dissimulatus* is the most similar species of *Pristimantis* morphologically and genetically. Morphologically, *P. pahuma* n. sp. differs by having an ulnar tubercle (absent in *P. dissimulatus*) and a uniform brown coloration in the groin and on the concealed surfaces of the hind limbs (spots or lines on an orange background in *P. dissimulatus*). Phylogenetically, *P. dissimulatus* is sister to the *P. calcarulatus* complex of species and is separated by a genetic distance of 10% or greater. The only sympatric species that might be confused with *P. pahuma* n. sp. are *P. appendiculatus*, *P. eremitus*, *P. mutabilis*, *P. pteridophilus*, *P. sobetes* and *P. verecundus*. The new species is easily distinguished from the mentioned taxa by having a small papilla at tip

of snout and low tubercles on upper eyelid (elongated fleshy tubercle on tip of snout and conical tubercles on upper eyelid in *P. appendiculatus*), blue or golden iris (reddish iris in *P. eremitus*, *P. pteridophilus*, *P. sobetes* and *P. verecundus*), and by lacking flash coloration on the groin (reddish groin in females of *P. verecundus* and *P. mutabilis*). Additionally, *P. pahuma* n. sp. lacks dorsolateral folds, which are present in *P. appendiculatus*, *P. mutabilis*, *P. sobetes* and *P. verecundus*. Differences in body size are conspicuous among most species (Table 3).

Holotype description

Adult male (MZUTI 493; Figure 9(A)). Head longer than wide, narrower than body; upper eyelid bearing one conical tubercle and several low tubercles; head width 36.3% SVL; head length 40.1% SVL; snout moderately long (snout–eye distance 17.5% SVL), subacuminate in dorsal view and slightly protruding in profile; tongue longer than wide, with posterior margin notched; eye diameter greater than eye–nostril distance; nostrils slightly protuberant, directed anterolaterally; canthus rostralis weakly concave in profile; loreal region slightly concave; upper eyelid width 83% of interorbital distance; cranial crests absent; tympanic annulus distinct, except for upper border, which is obscured by supratympanic fold; tympanic membrane distinct; two postrectal tubercles situated posteroventrally to tympanic annulus; choanae elliptical, not concealed by palatal shelf of maxillary; dentigerous process of vomer posteromedial to choanae, low, oblique in outline, separated medially by distance less than width of dentigerous process, each bearing 2 or 3 teeth; skin on dorsum finely shagreen, flanks with scattered low tubercles; dorsolateral folds absent; skin of throat and venter smooth; discoidal and thoracic folds absent; cloacal sheath low; ulnar tubercles present, but low; outer palmar tubercle large, bifurcated distally; subarticular tubercles prominent, round; supernumerary palmar tubercles present, but few and low; fingers bearing narrow lateral fringes; Finger I shorter than Finger II (Finger I length 79.1% of Finger II length); disk of Finger I slightly expanded; all other disks conspicuously expanded, elliptical in shape; ventral pads defined by circumferential grooves.

Tibia length 54.7% SVL; foot length 47.2% SVL; heel tubercle large, conical; tarsal tubercles small; inner metatarsal tubercle oval, ~3× the size of the outer, rounded tubercle; subarticular tubercles round; plantar supernumerary tubercles indistinct; toes bearing narrow lateral fringes; webbing absent; all toe disks expanded, rounded to elliptical in shape; toes with ventral pads well defined by circumferential grooves; relative length of toes: I < II < III < V < IV; Toe V slightly longer than Toe III.

Holotype measurements (in mm)

SVL = 21.2, Tibia length = 11.6, Femur length = 9.9, Head length = 8.5, Head width = 7.7, Tympanum diameter = 1.03, Radioulna length = 5.0, Snout-to-eye distance = 3.7, Eye diameter = 2.9, Eye–nostril distance = 2.4, Interorbital distance = 2.3, Upper eyelid width = 1.9, Finger I length = 3.4, Finger II length = 4.3, Foot length = 10.0. Meristic variation of paratypes is shown in Table 1.

Coloration in preservative (Figure 9(A), (B))

In the holotype, dorsum light brown with dark brown markings, including an interorbital bar, chevrons, canthal and supratympanic stripes, labial bars, oblique limb bars and flanks with diagonal stripes; posterior surfaces brown, with minute cream flecks; cloacal triangle dark brown edged with thin cream line. Venter cream peppered with brown dots. The coloration of other preserved specimens varies in a similar fashion to *P. calcarulatus* described above.

Coloration in life (Figures 5(B), 10)

The dorsal coloration is individually variable and can change from yellow to reddish-brown in minutes (Figure 5(B)), as it does in *P. calcarulatus* and *Pristimantis cedros* n. sp. Vertebral and other dorsal stripes are sometimes present. Additionally, the iris coloration is blue or golden, with a wide brownish-red stripe through the middle.

Phylogenetics

The phylogenetic results place *P. pahuma* n. sp. in the genus *Pristimantis* (sensu [2]), and being most closely related to species in the *P. calcarulatus* complex (Figure 6). At the species level, *P. pahuma* n. sp. is reciprocally monophyletic, supporting our hypothesis that the new species is an independently evolving lineage. Substantial genetic distances also add support to our hypothesis because *P. pahuma* n. sp. has a genetic distance of 7.1–8.3% from *P. calcarulatus*.

Bioacoustics (Figures 7, 8 and 2)

We recorded 858 calls from 6 individuals of *P. pahuma* n. sp.; recordings were made within 0.5–2.0 m of the calling male. Calls were recorded at Reserva Orquideológica El Pahuma in June 2009 and at Reserva Las Gralarias April–June 2011. All these adult males were recorded at night with mist or light rain and a temperature of 14–16°C. Males called from the upper surfaces of leaves 2–4 m above the ground. The call of this species sounds like a ‘tink’ and is produced both irregularly and frequently.

P. pahuma n. sp. emits single-noted calls (Figure 7(B)), which do not occur in a series (Figure 8(B)). Notes have 1–3 pulses/note (1.6 ± 0.7), with one strongly amplitude-modulated pulse, and 1 or 2 weakly amplitude-modulated pulses. The note envelope shape is 0–0.276 (0.084 ± 0.058), with the peak amplitude occurring early in the note. Note duration is 33.9–65.0 ms (50.99 ± 6.89). Notes show little frequency modulation and the dominant frequency is 2929–3187 Hz (3091 ± 88). The fundamental frequency is 2577–2756 Hz (2655 ± 77).

Etymology

The specific epithet *pahuma* refers to Reserva Orquideológica El Pahuma (El Pahuma Orchid Reserve), where this new species was first discovered. This

species is named in honor of the reserve, the Lima family and the Ceiba Foundation for Tropical Conservation (Drs. Catherine Woodward and Joseph Meisel) for their outstanding commitment to conservation of primary montane cloud forest habitat of which this species requires. The epithet is also the Quechua word for ‘flattened peak’ and refers to the flat plateau at the highest elevation in Reserva Orquideológica El Pahuma, where the historic Yumbo Trail passes (see <http://www.ceiba.org/>).

Distribution (Figure 1)

On the basis of bioacoustic or genetic identification (Table 2), *P. pahuma* n. sp. occurs from the following Andean localities in the Provincia de Pichincha of north-western Reserva Orquideológica El Pahuma and Reserva

Table 2. Comparisons of call types recorded for the *P. calcarulatus* Complex.

Parameter	<i>P. calcarulatus</i> (<i>sensu stricto</i>)	<i>P. pahuma</i> n. sp.	<i>P. pahuma</i> n. sp. (El Pahuma)	<i>P. cf. calcarulatus</i> (KU 165179)
<i>n</i> – individuals (calls)	7 (726)	6 (858)	1 (158)	2 (48)
Note amplitude structure	Pulsed	Pulsed	Pulsed	Pulsed
<i>Series data</i>				
<i>n</i> – series	49	–	–	–
Notes/series	8–23 (14.82 ± 5.503)	–	–	–
Duration (s)	1.417–5.23 (3.696 ± 0.993)	–	–	–
Series interval (s)	2.244–97.2 (33.81 ± 25.731)	–	–	–
Note rate in series (/ms)	1.872–5.898 (3.746 ± 1.004)	–	–	–
Series rate (/s)	0.87–1.557 (1.181 ± 0.232)	–	–	–
<i>Individual note data</i>				
Note duration (ms)	25.22–57.32 (40.68 ± 6.413)	33.9–64.99 (50.99 ± 6.89)	36.55–63.47 (50.22 ± 7.186)	27.95–31.2 (29.54 ± 0.808)
Note rate (/s)	20.1–182.3 (75.7 ± 57.0)	16.76–35.45 (28.76 ± 6.76)	31.598	47.74–49.94 (48.84 ± 1.561)
Number of pulses (/note)	1–4 (1.714 ± 0.983)	1–3 (1.551 ± 0.683)	1–2 (1.487 ± 0.489)	2–3 (2.213 ± 0.414)
Pulse rate (/ms)	0.015–0.097 (0.041 ± 0.021)	0.015–0.061 (0.03 ± 0.013)	0.016–0.056 (0.031 ± 0.01)	0.059–0.105 (0.074 ± 0.013)
Call interval (s)	0.154–0.347 (0.214 ± 0.04)	1.52–7.391 (1.705 ± 0.766)	0.9–10.3 (1.8 ± 0.877)	0.885–1.666 (1.166 ± 0.166)
Note envelope	0–0.243 (0.068 ± 0.05)	0–0.276 (0.084 ± 0.058)	0–0.433 (0.108 ± 0.091)	0.027–0.103 (0.06 ± 0.023)
Dominant frequency (Hz)	3101–3618 (3318 ± 143)	2929–3187 (3091 ± 88)	3015–3101 (3084 ± 33.11)	3562–3562 (3562 ± 0)
Frequency modulation (Hz)	0–86 (52 ± 42)	0–86 (46 ± 43)	0–86.13 (68.03 ± 34.353)	0
Lower fundamental frequency (Hz)	2687–3150 (2815 ± 87)	2577–2756 (2655 ± 76)	2948–3095 (3026 ± 33.687)	3097–3097 (3097 ± 0)
Higher fundamental frequency (Hz)	3091–3594 (3283 ± 137)	2891–3228 (3074 ± 86)	2971–3158 (3075 ± 41.938)	3429–3509 (3472 ± 20.2)
First harmonic (Hz)	6311–7162 (6680 ± 214)	6092–6558 (6320 ± 85)	6155–6498 (6333 ± 78.629)	6330–6572 (6448 ± 56)

Notes: Calls were recorded from males calling at night and were subsequently collected as vouchers. ‘El Pahuma’ is a specimen of *P. pahuma* n. sp. from Reserva Orquideológica El Pahuma. *Pristimantis* cf. *calcarulatus* is a specimen recorded from lower elevations that might represent an additional divergent lineage. Note envelope is the ratio of the time of peak amplitude to note duration. Data are the range followed by the mean $\pm$ two standard deviations in parentheses.

Las Galarías (Appendix 1). The known elevational distribution of this species is 2186–2574 m.

Natural history (Figure 11)

P. pahuma n. sp. is found only in undisturbed, primary cloud forests. Female *P. pahuma* n. sp. are locally abundant and have only been found in habitat with calling males present. Females are typically found on the surfaces of leaves that are less than 2 m above ground. Males typically emit their single calls frequently from the surfaces of leaves in trees or bushes several meters above the ground at night (Figure 11).

The abundance of calling males creates a noticeable and resounding chorus that can be heard up to a kilometer away during seasonal periods of rainfall. (See Hutter and Guayasamin [30] for climatic data.) The chorus is loudest at dusk, when a large number of males begin calling and diminishes as the night continues. The

combined effect of the aggregated calling males within *P. pahuma* n. sp. populations differs markedly from that of *P. calcarulatus*. *P. calcarulatus* called more infrequently than *P. pahuma* n. sp.

At Reserva Las Galarías, sympatric frogs of the genus *Pristimantis* include: *P. achatinus*, *P. appendiculatus*, *P. eugeniae*, *P. hectus*, *P. pteridophilus*, *P. sobetes*, *P. vercundus* and *P. w-nigrum*. At Reserva Orquideológica El Pahuma, sympatric *Pristimantis* species include: *P. achatinus*, *P. appendiculatus*, *P. eremitus*, *P. eugeniae*, *P. floridus*, *P. pteridophilus*, *P. romanorum*, *P. sobetes*, *P. w-nigrum* and *P. yumbo*. *P. calcarulatus* was also recorded in these reserves, but was not found calling synoptically with *P. pahuma* n. sp. in the same habitat. Therefore, we have strong evidence that the two species are partitioned by elevation, with *P. calcarulatus* inhabiting lower (1850–2072 m) and *P. pahuma* n. sp. at higher (2186–2574 m) elevations. However, it is possible that their elevational distributions could vary at other localities.

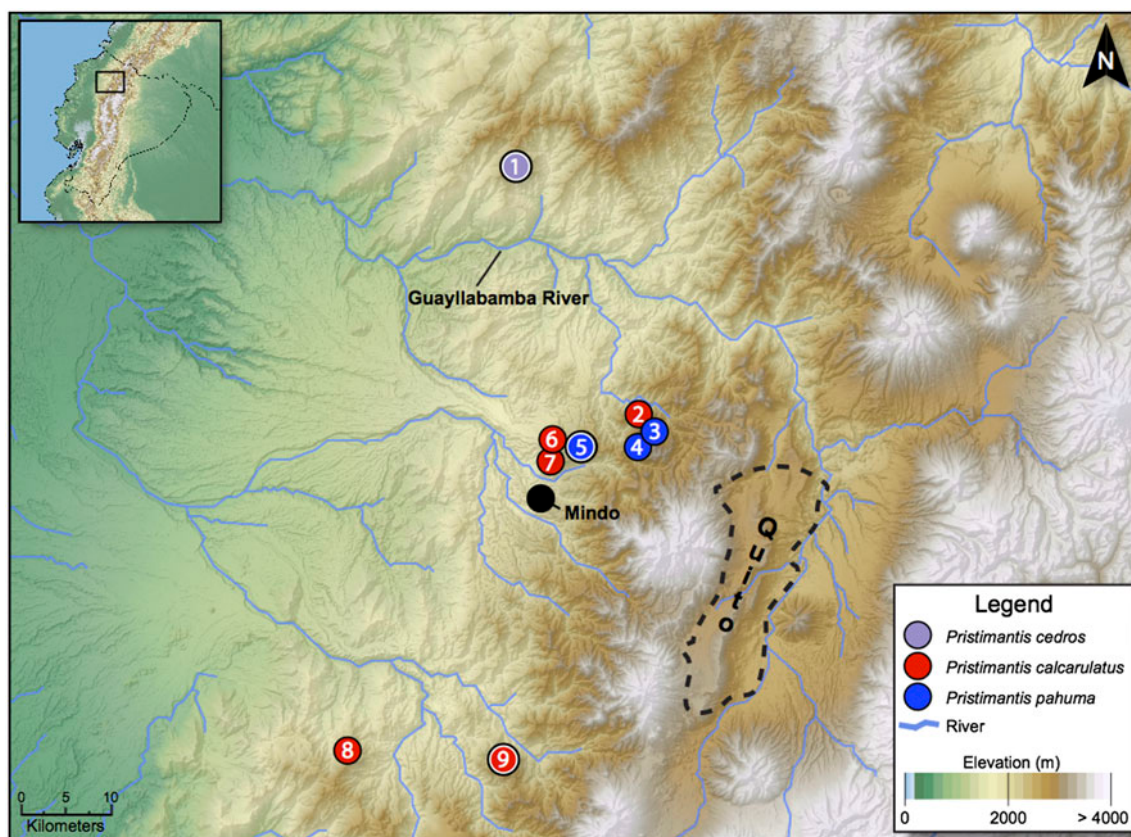


Figure 1. Distribution of *P. calcarulatus*, *P. cedros* n. sp. and *P. pahuma* n. sp. in the northwestern Andes of Ecuador.

Notes: The circles correspond to known localities and are colored for each species. The localities with a white and black outline are each species' type locality. The localities by number are as follows (see Appendix I for coordinates): (1) Reserva Los Cedros (*P. cedros* n. sp. type locality); (2) Reserva Orquideológica El Pahuma, research station; (3) Reserva Orquideológica El Pahuma, Guarida del Oso; (4) Reserva Orquideológica El Pahuma, Yumbo Trail; (5) Reserva Las Galarías, Tunnel Trail (*P. pahuma* n. sp. type locality) and Hercules Creek; (6) Reserva Las Galarías, Canyon Trail; (7) Reserva Las Galarías, Chaguayacu River; (8) Reserva Otonga; and (9) Tandapi (*P. calcarulatus* type locality).

Conservation status

Given the arguments presented above in the reassessment of *P. calcarulatus*, it seems likely that *P. pahuma* n. sp. also has a restricted distribution. The new species is known from only two localities (both protected areas) south of the Río Guayllabamba. Based on the information available for *P. pahuma* n. sp., we recommend that it be categorized as Endangered B1ab (iii) following IUCN (2001) criteria using the same justification for *P. calcarulatus*.

Pristimantis cedros n. sp.

Zoobank registration number = urn:lsid:zoobank.org:act:3A09AF93-EB72-4118-87EC-408BCAB5E7F2

Common English name: Los Cedros Rainfrog.

Common Spanish name: Cutín de Los Cedros.

Holotype (Figure 12(A))

MZUTI 1713, an adult male collected by Jaime Culebras, Fernando Rojas-Runjaic and Juan M. Guayasamin



Figure 2. Type series of *P. calcarulatus* in preservative. (A) Holotype KU 111218. (B) Paratypes KU 111221, KU 111230, KU 002240 and KU 002384.

on 14 March 2012 at Reserva Los Cedros (0.31943°N, 78.785°W, 1880 m), Provincia de Imbabura, Ecuador.

Paratopotypes (Figure 12(B))

MZUTI 1710 (adult male), 1714, 1715 (adult females), same data as holotype.

Paratypes (Figure 12(B))

All the following specimens (3 adult males; 5 adult females) were also collected at Reserva Los Cedros, Provincia de Imbabura, Ecuador, but at slightly different elevations than the holotype. MZUTI 1711 (adult male), 1716 (adult female) collected at 0.3184°N, 78.7837°W, 1790 m. MZUTI 1717–18 (adult females), 1720, 1722–23 (adult males) collected at 0.2193°N, 77.4746°W, 1621 m. MZUTI 1724–25 (adult females) collected at 0.2182°N, 77.4744°W, 1583 m. Collections were made on 13–15 March 2012.

Generic placement

P. cedros n. sp. is placed in the genus *Pristimantis* as diagnosed by Hedges et al.[2]. All *Pristimantis* lack cranial crests and possess dentigerous processes on the vomers and T-shaped terminal phalanges.

Diagnosis

The diagnosis of *P. cedros* n. sp. is the same as that of *P. calcarulatus* (*sensu stricto*), except for the following measurement details: (14) SVL in adult females 26.3–28.6 mm ($n = 7$; mean = 26.7 ± 1.19), in adult males 21.5–23.0 mm ($n = 6$; mean = 22.2 ± 0.60).

Similar species

On the Pacific slopes of the Andes, *P. cedros* n. sp. closely resembles species in the *P. calcarulatus* complex (*P. calcarulatus* and *P. pahuma* n. sp.). The new species differs subtly from *P. calcarulatus* and *P. pahuma* n. sp. by having smaller, more numerous black reticulations (Figure 5). However, this can be observed only in living individuals. Nevertheless, phylogenetic analyses and biogeography clearly distinguish *P. cedros* n. sp. from *P. calcarulatus* and *P. pahuma* n. sp. Phylogenetically, *P. cedros* n. sp. is reciprocally monophyletic with strong support (Figure 6) with respect to its sister taxa, thereby supporting our hypothesis that *P. cedros* n. sp. is a separately evolving lineage. Additionally, *P. cedros* n. sp. has a substantial genetic distance from *P. calcarulatus* (8.3–9.5%) and a distance of (7.3–8.4%) from *P. pahuma* n. sp. Biogeographically, the Río Guayllabamba Valley is a substantial barrier to dispersal. The populations of *P. cedros* n. sp. and *P. pahuma* n. sp. are separated by a short straight-line

distance of ~35 km; thus, isolation by distance neither explains reciprocal monophyly nor the elevated genetic differences. Last, although call recordings of *P. cedros* n. sp. were unavailable, field observations suggest that the call consists of a single note similar to that of *P. pahuma* n. sp. Although the call may not differ, *P. cedros* n. sp. is geographically isolated and likely speciated allopatrically from *P. pahuma* n. sp.; thus, the calls might not be expected to diverge from one another.

Outside the *P. calcarulatus* complex, the most similar species morphologically and genetically to the complex is *P. dissimulatus*. *P. cedros* n. sp. differs by having ulnar tubercle (absent in *P. dissimulatus*) and a uniform brown coloration in the groin and on the concealed surfaces of the hind limbs (spots or lines on an orange background in *P. dissimulatus*). Sympatric taxa that could be confused with *P. cedros* n. sp. are *P. appendiculatus*, *P. eremitus*, *P. mutabilis*, *P. pteridophilus*, *P. sobetes* and *P. verecundus*. Although the new species is distinguished by having a small papilla at tip of snout (elongated fleshy tubercle in *P. appendiculatus*), blue or golden iris (reddish iris in *P. eremitus*, *P. pteridophilus*, *P. sobetes* and *P. verecundus*) and by lacking flash coloration on the groin (reddish groin in females of *P. verecundus* and *P. mutabilis*). Additionally, *P. cedros* n. sp. lacks dorso-lateral folds, which are present in *P. appendiculatus*,

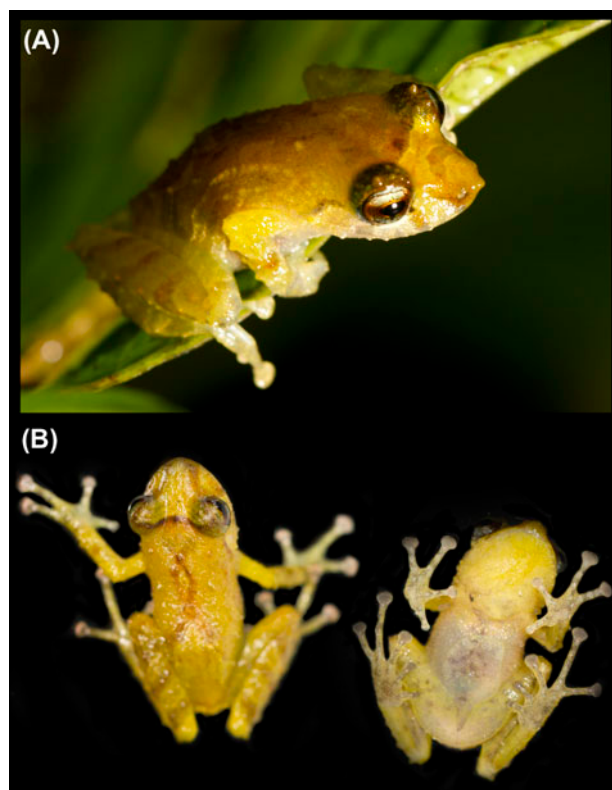


Figure 3. *P. calcarulatus* in life. (A) *in situ*, specimen not collected. (B) *ex-situ*, MZUTI 503.



Figure 4. Iris color variation in (A) *P. calcarulatus*, (B) *P. pahuma* n. sp. and (C) *P. cedros* n. sp. Note the smaller and more numerous reticulations in (C) *P. cedros* n. sp.

P. mutabilis, *P. sobetes* and *P. verecundus*. Differences in body size are conspicuous among most species (Table 3).

Holotype description

Adult male (MZUTI 1713; Figure 12(A)). Head longer than wide, narrower than body; upper eyelid bearing one conical tubercle and several low tubercles; head width

39.2% SVL; head length 41.9% SVL; snout moderately long (snout 17% SVL), rounded in dorsal and lateral views; tongue longer than wide, with posterior margin round, slightly notched; eye diameter larger than eye–nostril distance; nostrils slightly protuberant, directed anterolaterally; canthus rostralis weakly concave in profile; loreal region slightly concave; upper eyelid width 92% of interorbital distance; cranial crests absent;

Table 3. Adult male body size (SVL) in similar species of *Pristimantis*. Range followed by mean, standard deviation and sample size (when available).

Species	Adult male SVL	Literature source
<i>P. appendiculatus</i>	18.8–21.0	Lynch and Duellman [33]
<i>P. calcarulatus sensu stricto</i>	20.2–23.4 (21.6 ± 1.23; <i>n</i> = 14)	This study
<i>P. cedros</i> n. sp.	21.5–23.0 (22.2 ± 0.60; <i>n</i> = 6)	This study
<i>P. dissimulatus</i>	Unknown	–
<i>P. eremitus</i>	17.2–21.8	Lynch and Duellman [33]
<i>P. mutabilis</i>	17.2–17.4 (<i>n</i> = 2)	Guayasamin et al. [27]
<i>P. pahuma</i> n. sp.	20.2–22.8 (21.4 ± 0.88; <i>n</i> = 7)	This study
<i>P. pteridophilus</i>	17.6–25.1 (22.4 ± 0.3; <i>n</i> = 32)	Lynch and Duellman [33]
<i>P. sobetes</i>	20–23	Arteaga et al. [26]
<i>P. verecundus</i>	18.0–21.9 (20.2; <i>n</i> = 4)	Lynch and Burrowes [67]

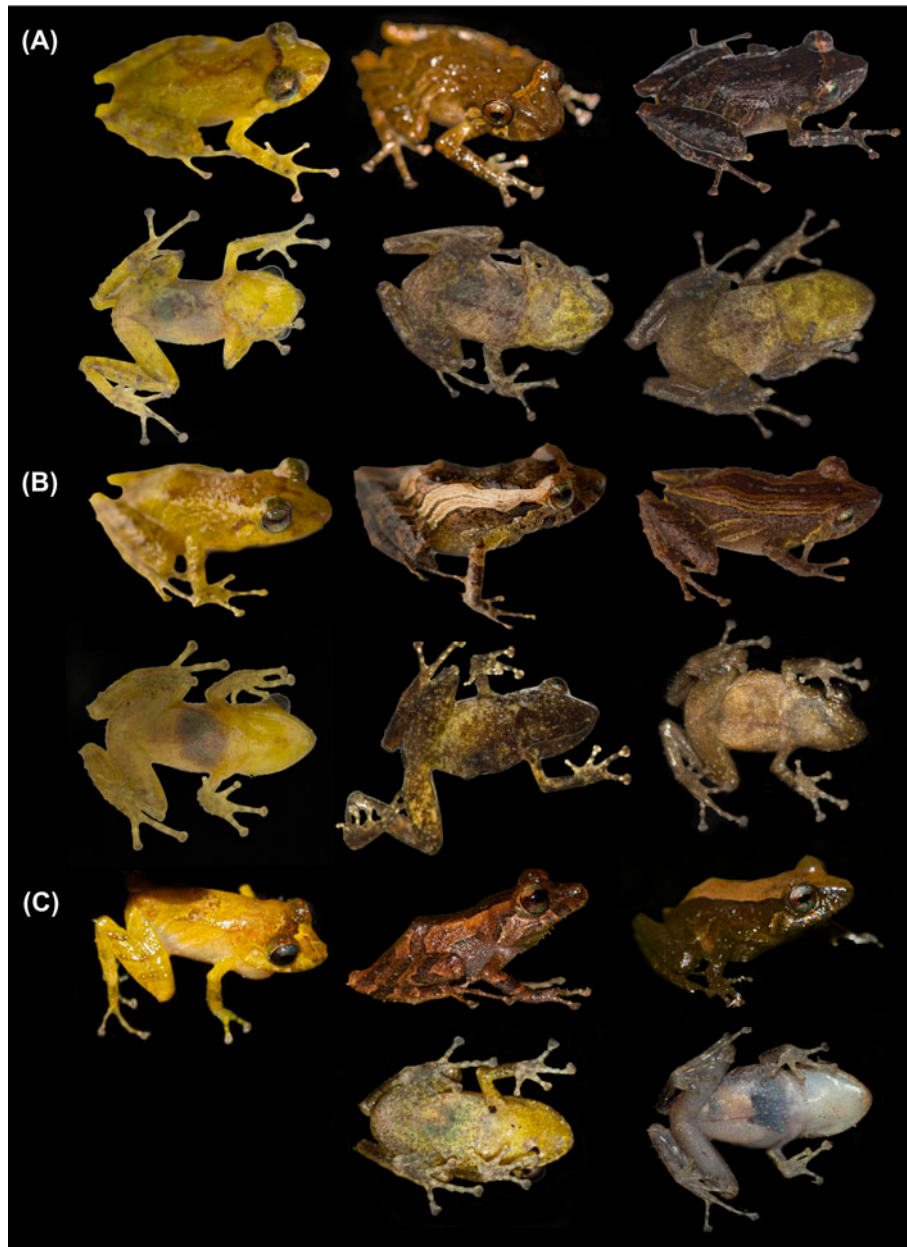


Figure 5. (A) Dorsal ventral color variation in (A) *P. calcarulatus*, (B) *P. pahuma* n. sp. and (C) *P. cedros* n. sp.

tympanic annulus distinct, except for upper border, which is obscured by supratympanic fold; tympanum of moderate size (tympanic diameter 29% of eye diameter); tympanic membrane distinct; two postictal tubercles situated posteroventral to tympanic annulus; choanae elliptical, not concealed by palatal shelf of maxilla; dentigerous processes of the vomers posteromedial to choanae, low, oblique in outline, separated medially by distance less than width of the dentigerous process, each bearing 3 teeth; skin on dorsum finely shagreen with scattered low tubercles; dorsolateral folds absent; skin of throat and venter smooth; discoidal and thoracic folds

absent; cloacal sheath low; ulnar tubercles present, but low; outer palmar tubercle large, distally bifurcate; subarticular tubercles prominent, round; supernumerary palmar tubercles present, but few and low; fingers bearing narrow lateral fringes; Finger I shorter than Finger II (Finger I length 82.6% of Finger II length); disk of Finger I slightly expanded; all other disks conspicuously expanded, elliptical in shape; ventral pads defined by circumferential grooves.

Tibia length 60.4% SVL; foot length 48.8% SVL; heel tubercle large, conical; tarsal tubercles small; inner metatarsal tubercle oval, $\sim 3\times$ the size of the outer, rounded

tubercle; subarticular tubercles round; plantar supernumerary tubercles indistinct; toes bearing narrow lateral fringes; webbing absent; all toe disks expanded, rounded to elliptical in shape; toes with ventral pads well defined by circumferential grooves; relative length of toes: $I < II < III < V < IV$; Toe V slightly longer than Toe III.

Holotype measurements (in mm)

SVL = 21.7, Tibia length = 13.1, Femur length = 11.0, Head length = 9.1, Head width = 8.5, Tympanum diameter = 0.85, Radioulna length = 5.0, Snout-to-eye distance = 3.7, Eye diameter = 2.9, Eye-to-nostril

distance = 2.6, Interorbital distance = 2.4, Upper eyelid width = 2.2, Finger I length = 3.8, Finger II length = 4.6, Foot length = 10.6. Meristic variation of paratypes is shown in Table 1.

Coloration in preservative (Figure 12)

In the holotype, dorsum pinkish-cream, with darker brown markings, including an interorbital bar, chevrons, canthal and supratympanic stripes, labial bars, oblique limb bars and flanks with diagonal stripes; posterior surfaces of thighs cream. Cloacal triangle brown to black edged with thin cream line. Venter uniform cream.

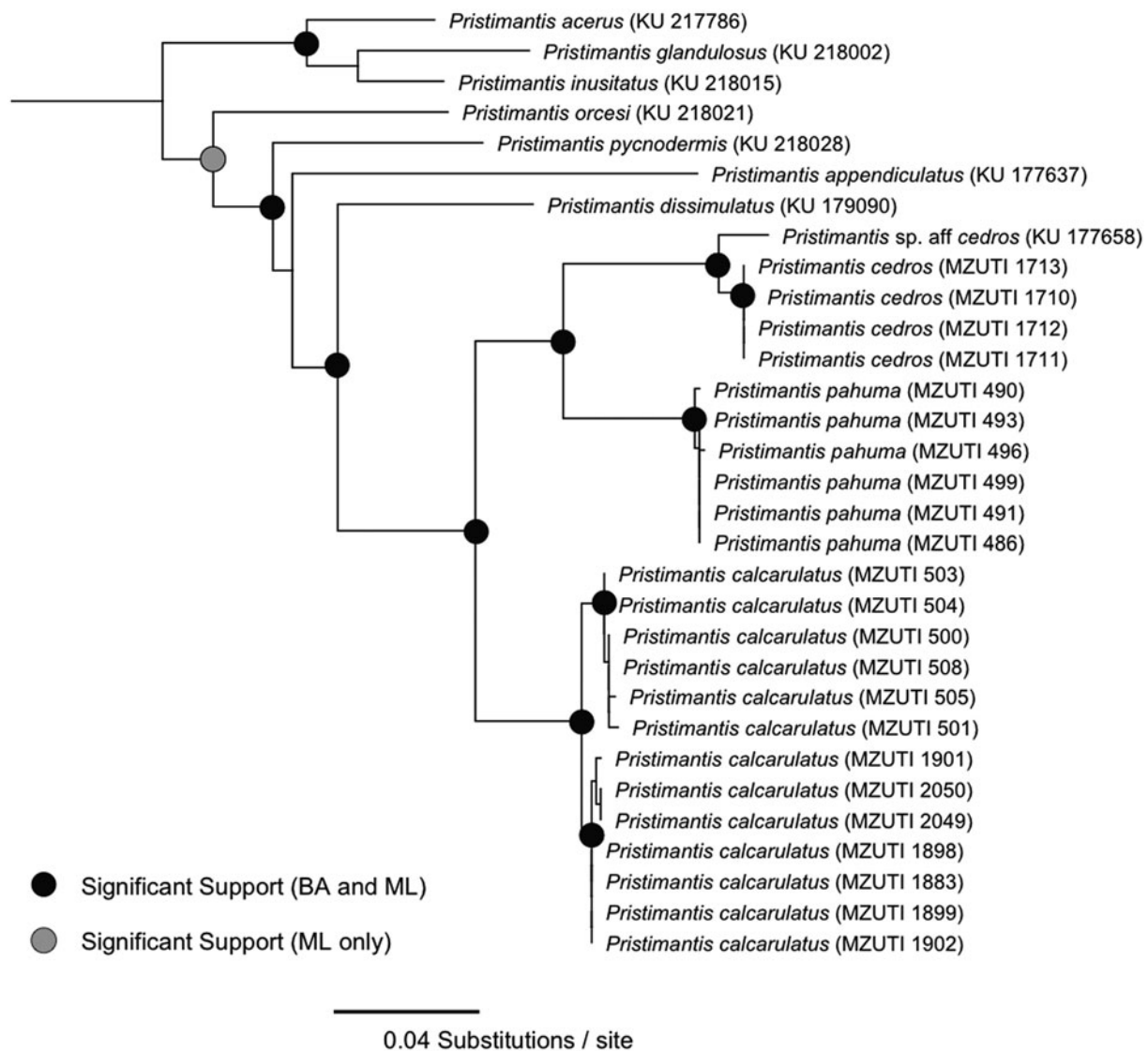


Figure 6. Phylogenetic maximum likelihood (ML) and Bayesian (BA) topology for species included in this study.

Notes: Significant support for relationships is shown through a circle at the node (ML: bootstrap > 75%; BA: posterior probability > 0.95). Nodes that were not significantly supported in either analyses lack a circle. Note the highly supported reciprocal monophyly of *P. calcarulatus*, *P. cedros* n. sp. and *P. pahuma* n. sp.

The variation in color pattern in other individuals resembles that described for *Pristimanti calcarulatus* (described above). Additionally, posterior surfaces of thighs vary from pale brown to dark brown, with minute cream flecks. Ventral surfaces vary from uniform cream, cream peppered with light brown, or brown with minute white dots.

Coloration in life (Figures 5(C) and 13)

Dorsum yellowish, greenish, or reddish brown with darker brown or black pattern; flanks paler than dorsum; vocal sac of males' yellowish-cream to bright yellow. Venter variable, including uniform cream or pale yellow to yellowish-brown with cream to pale gray flecks; posterior surfaces of thighs dull gray to brown, with minute white spots. Iris blue-gray to yellowish gray, with numerous thin and small black reticulations and a reddish-brown horizontal stripe. The color of individuals changes depending on light or temperature conditions, and they are paler during the night than the day.

Phylogenetics

The phylogenetic results support the morphological evidence by placing *Pristimantis cedros* n. sp. within the *P. calcarulatus* complex (Figure 6), with strong reciprocal

monophyly. Our phylogenetic analyses suggest that *P. pahuma* n. sp. and *P. cedros* n. sp. are evolutionary sister species (Figure 6), but additional genetic data and other cryptic species may modify this relationship. Genetic distances among the three species are also substantial: *P. cedros* n. sp. has a genetic distance of 8.3–9.5% from *P. calcarulatus*, and a genetic distance of 7.3–8.4% from *P. pahuma* n. sp.

Bioacoustics

While call recordings are not available, field observations suggest that this species has a similar call to *P. pahuma* n. sp. It is a single-noted 'tick' call that does not occur in a series, but could vary in other temporal or spectral parameters.

Etymology

The specific epithet *cedros* is the Spanish word for 'Cedar' and refers to Reserva Los Cedros, the type locality of the species. Reserva Los Cedros protects ~7000 hectares of premontane wet tropical forest and cloud forest in an area where illegal deforestation, hunting and settlements are constant threats to natural ecosystems. The reserve is the result of the outstanding work of Fundación Los Cedros, an organization directed by José DeCoux and

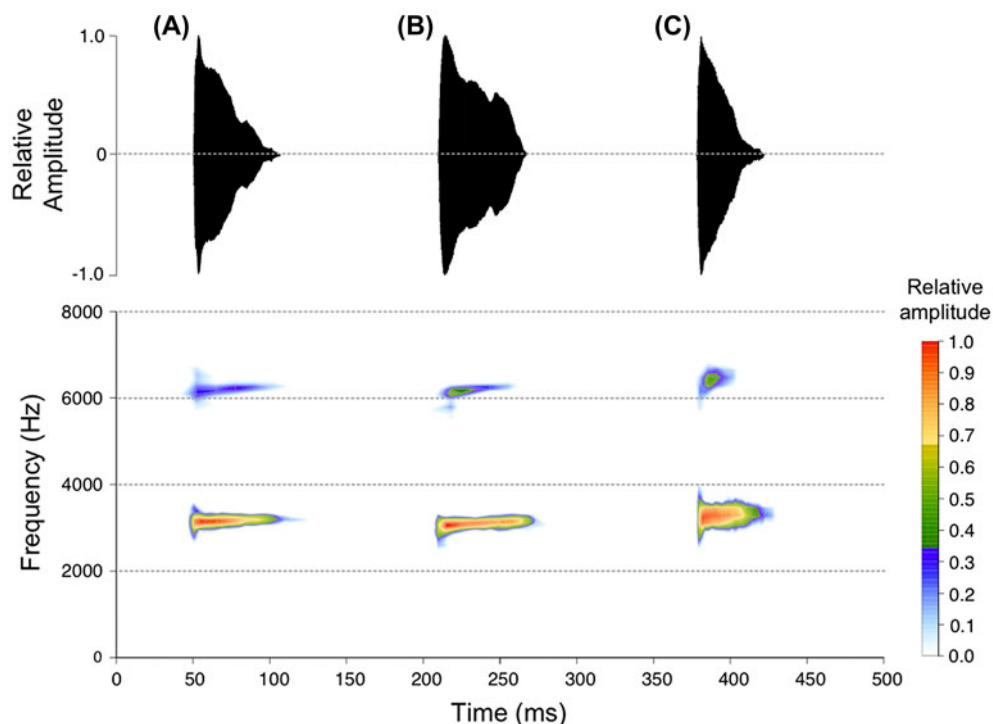


Figure 7. Advertisement call comparison in the *P. calcarulatus* complex. Species analyzed are: (A) *P. calcarulatus*, (B) *P. pahuma* n. sp. and (C) *P. aff. calcarulatus* (KU 165179).

composed of local community leaders, representatives from environmental groups and Los Cedros staff (see <http://reservaloscedros.org>).

Distribution

P. cedros n. sp. is known only from the cloud forests of Reserva Los Cedros, Provincia de Imbabura, Ecuador (Figure 1). The known elevational distribution of this species is 1583–1880 m.

Natural history

During the sampling period at Reserva Los Cedros (13–15 March 2012), *P. cedros* n. sp. was found only in undisturbed primary forest habitat. The species was locally abundant and several males were calling simultaneously. Males call from the surfaces of leaves of bushes

or trees at night. Males and females were found on the surfaces of leaves less than 2 m above the ground. Sympatric frogs of the genus *Pristimantis* include: *P. crenunguis*, *P. mindo*, *P. luteolateralis* and *P. parvillus*. *P. cedros* n. sp. was not documented to occur sympatrically with *P. calcarulatus* and *P. pahuma* n. sp.

Conservation status

Being consistent with the arguments presented for *P. calcarulatus* and *P. pahuma* n. sp., and following IUCN (2001) criteria, we recommend that *P. cedros* n. sp. is categorized as Endangered B1ab (iii).

Remarks

A genetic sequence obtained from GenBank and identified as *P. calcarulatus* (from Hedges et al.[2]) is of uncertain

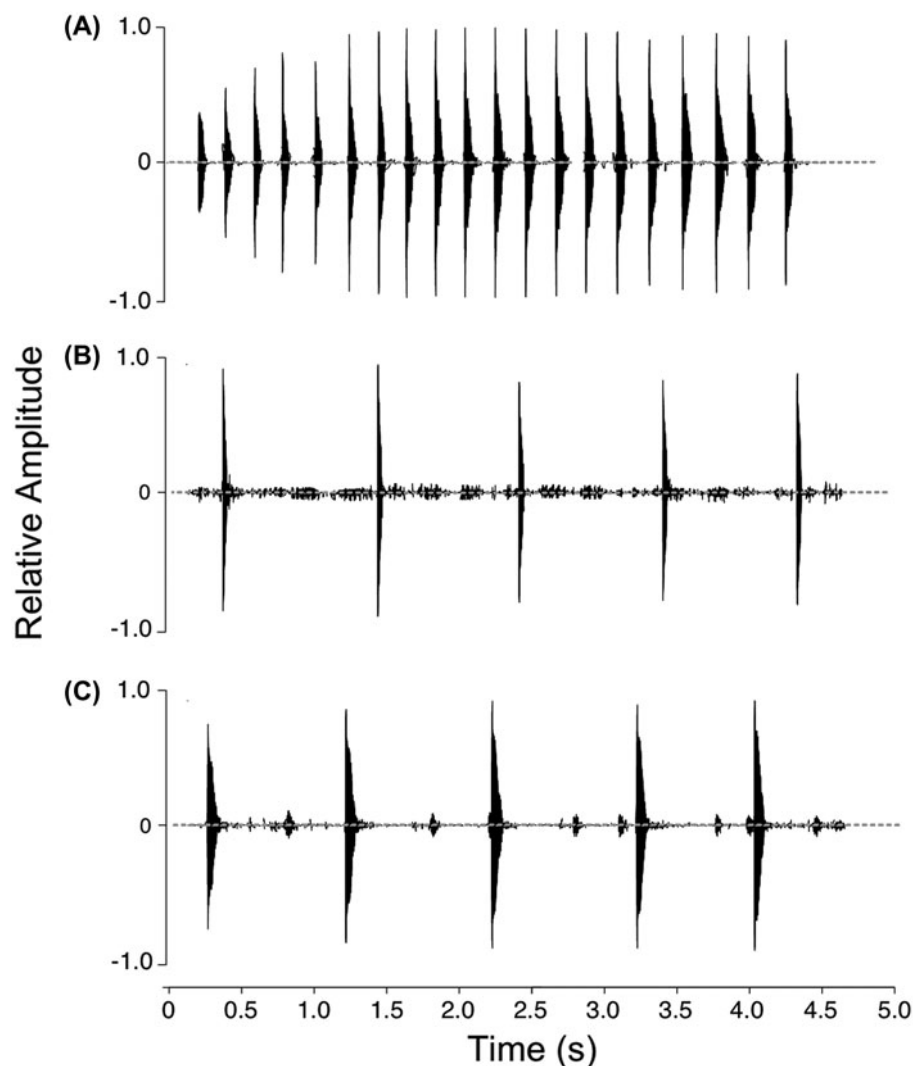


Figure 8. Comparison of a series of calls from (A) *P. calcarulatus* with the non-series calling of (B) *P. pahuma* n. sp. and (C) *P. aff. calcarulatus* (KU 165179).

identity. Morphologically, the specimen (KU 177658) is the same as the three identified species in the complex and genetically it is sister to *P. cedros* n. sp., with a genetic distance of 2.2–2.7%. Considering the lack of data and possible mitochondrial introgression, resolving the species status of this sample would require additional genetic and bioacoustic data. Therefore, at the moment we consider the specimen *Pristimantis* aff. *cedros*.

Discussion

We describe two new morphologically cryptic species from the *P. calcarulatus* complex, which are strongly supported by genetic and acoustic evidence; however, we could not diagnose the species with strong

morphological differences. We found that *P. cedros* n. sp. differs by having smaller and more numerous dark reticulations in the iris, which is a qualitative trait noted in other anurans (e.g. Hutter and Guayasamin [30]). We have argued that the lack of morphological differences among the members of the *P. calcarulatus* complex does not obviate their status as species because morphology may be conserved if genetic drift or natural selection is not substantial enough to influence morphological changes. Instead, we consider significant differences in evolutionary history (e.g. phylogenetic reciprocal monophyly) and pre-zygotic reproductive barriers (e.g. advertisement calls or geographic barriers) to be more relevant to species divergence and speciation.



Figure 9. Type series of *P. pahuma* n. sp. (A) Holotype, MZUTI 493. (B) Parapatotypes: MZUTI 494, 499 (adult females), 486, 488–491, 493, 496 (adult males). Photographs by Jaime Culebras.

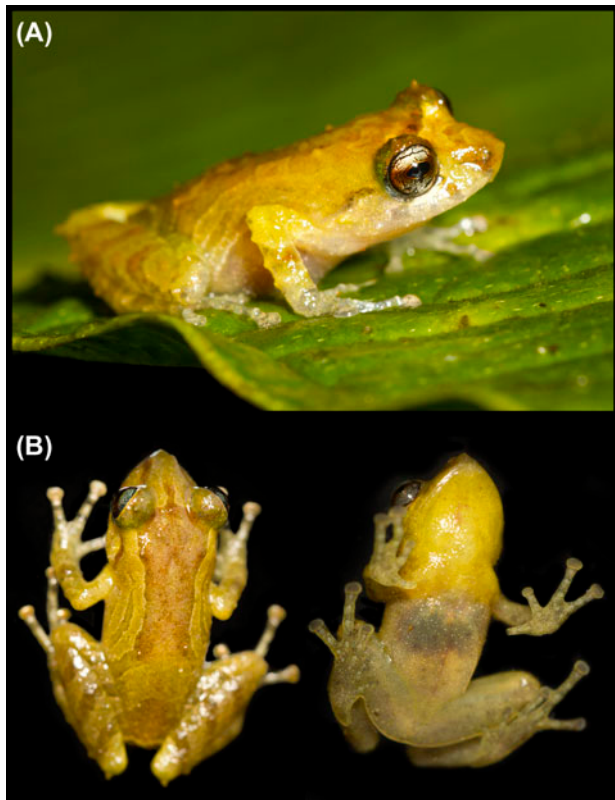


Figure 10. *P. pahuma* n. sp. in life. (A) *In-situ*, specimen not collected. (B) *Ex-situ*, MZUTI 488.

The divergence between *P. pahuma* n. sp. and *P. cedros* n. sp. is best explained by allopatric speciation occasioned by the formation of the Río Guayllabamba Valley. The valley separates the two species by a short airline distance of ~35 km; at its lowest point, the valley is ~600 m a.s.l., and has a significantly warmer, drier climate than the surrounding higher elevation Andean montane forests.[58,59] The Río Guayllabamba Valley shares these characteristics with other well-known biogeographic dispersal barriers in the Andes (e.g. Huancabamba Depression, Magdalena Valley), which are strong impediments to dispersal and gene flow in Andean taxa.[58,60–62] This is especially true for amphibians, which are dependent on humid conditions and have low vagility.[4,63] Only one previous study has provided evidence for substantial genetic divergence of a species distributed on both sides of the Río Guayllabamba Valley,[27] and we suggest that future studies should further assess its importance for promoting diversification and cryptic speciation in frogs and other taxa.

The widespread existence of cryptic species can have substantial implications for conservation management. Many recent studies have focused on the number of cryptic lineages within species with widespread distributions (e.g. *Dendropsophus minutus*,[64] *Rhinella margaritifera*,[10] *Pristimantis* [12]) and noted that each cryptic lineage fractures the total range size of each



Figure 11. Calling behavior of *P. pahuma* n. sp. (A) A calling male during a call; (B) after a call; and (C) and after calling behavior ceased due to disturbance.

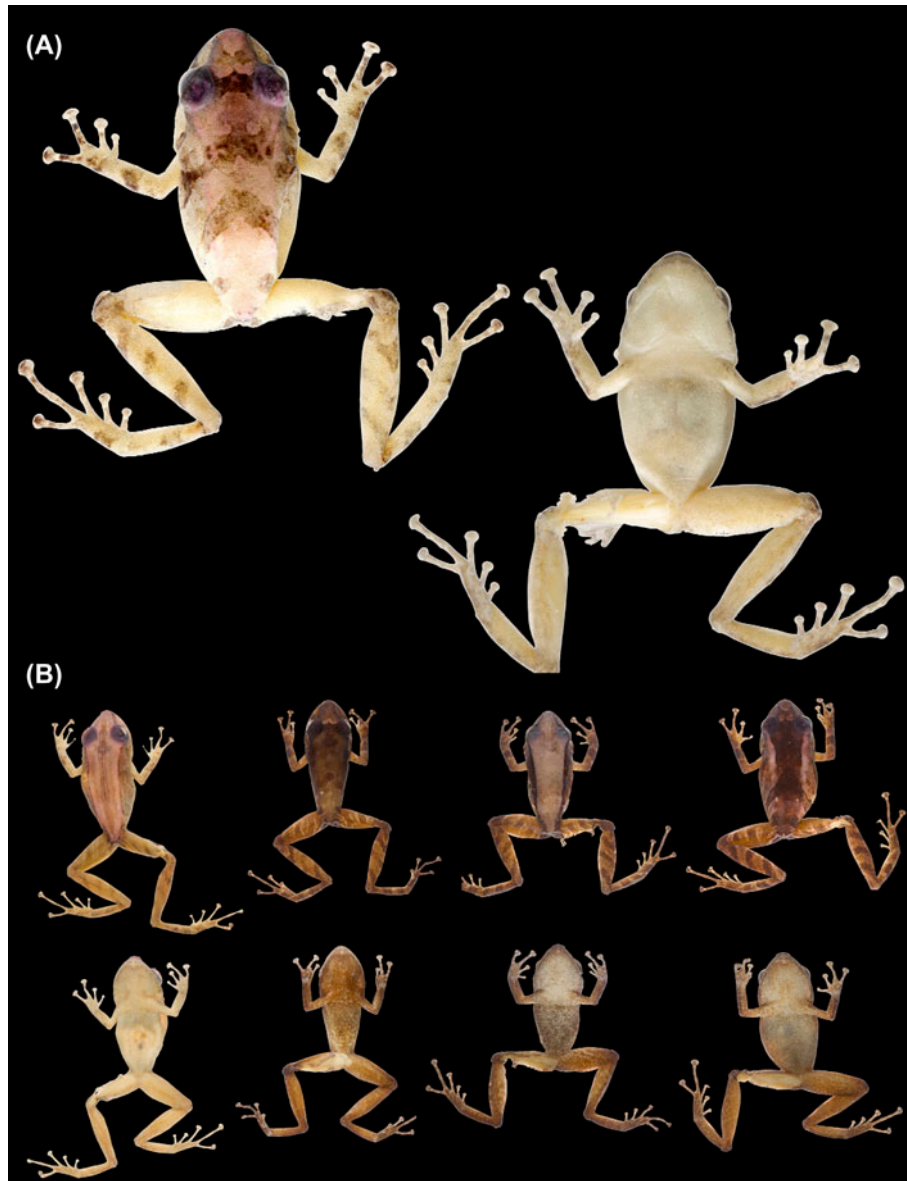


Figure 12. Type series of *Pristimantis cedros* n. sp. (A) Holotype, MZUTI 493. (B) Paratopotypes, MZUTI 486, 489, 491, 499. Photographs by Jaime Culebras.

species. However, most recognized Andean frogs are microendemic leading to small ranges; the presence of cryptic species further reduces these ranges, thereby making them more susceptible to extinction.[65,66] Because many cryptic lineages remain unrecognized, conservationists are unaware of their true population and range sizes.[10,15] This could result in an inappropriate conservation status and diminished protection for cryptic species with small ranges that might be decreasing in population size and losing habitat much faster than expected.

Our results also suggest opportunities for future research. First, we find evidence for additional cryptic lineages within the *P. calcarulatus* complex. Our phylogenetic results suggest the possibility of at least one additional lineage at Tandapi, which would require additional specimens and genetic data to assess species boundaries. Bioacoustic analyses for *P. calcarulatus* at nearby sites at lower elevations also suggest the possibility of an additional divergent lineage; genetic data are necessary to determine if it is a cryptic species (Figures 7, 8; Table 2). Additionally, *P. calcarulatus* has not been

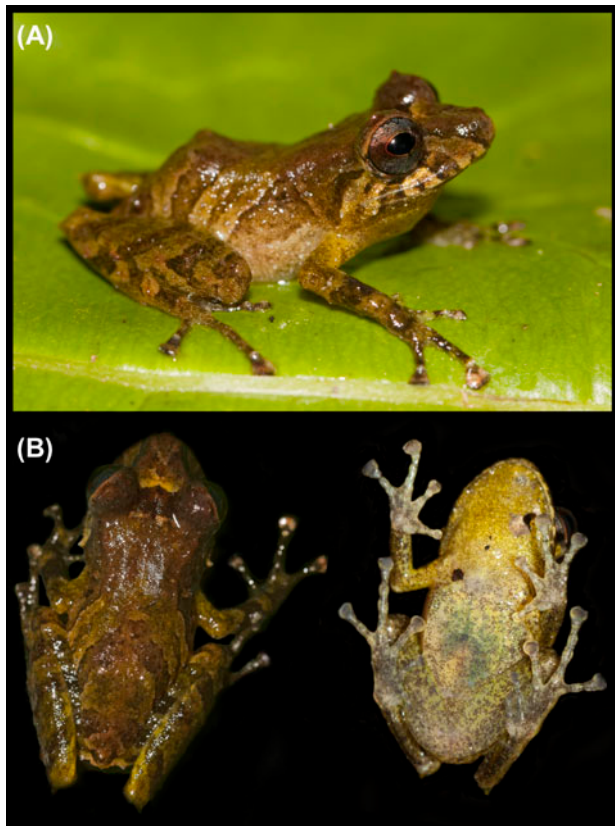


Figure 13. *Pristimantis cedros* n. sp. in life. (A) *In-situ*. (B) *Ex-situ* paratypes. Individuals not collected.

assessed in northern Ecuador and southern Colombia, which may contain additional cryptic lineages, or alternatively, represent populations of *P. cedros* n. sp. This study demonstrates the need for intensive study of cryptic diversity in Andean amphibians, as many more hidden species will likely be discovered.

Author contributions

CRH and JMG conceived and designed the study. CRH analyzed the data and wrote the first draft of the manuscript. JMG reviewed and improved the manuscript. CRH and JMG collected specimens, calls and sequences.

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of the new species were made by Jaime Culebras. This study was funded by the Universidad Tecnológica Indoamérica through the project 'Especiación y diversidad en gradientes elevacionales andinos.'

Associate Editor: Linda Trueb.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

- [1] Padial JM, Grant T, Frost D. Molecular systematics of terraranas (Anura: Brachycephaloidea) with an assessment of the effects of alignment and optimality criteria. *Zootaxa*. 2014;3825:1–132.
- [2] Hedges SB, Duellman WE, Heinicke P. New world direct-developing frogs (Anura: Terrarana): molecular phylogeny, classification, biogeography, and conservation. *Zootaxa*. 2008;1737:1–182.
- [3] Frost DR. Amphibian Species of the World: an Online Reference [Internet]. Version 6.0. New York: American Museum of Natural History; 2014 [cited 2014 Oct 9]. Available from: <http://research.amnh.org/herpetology/amphibia/index.html>.
- [4] Duellman WE, Trueb L. Biology of amphibians. London: Johns Hopkins University Press; 1994.
- [5] Pyron RA, Wiens JJ. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Mol. Phylogenet. Evol.* 2011;61:543–583.
- [6] Guayasamin JM, Arteaga A. A new species of the *Pristimantis orestes* group (Amphibia: Strabomantidae) from the high Andes of Ecuador, Reserva Mazar. *Zootaxa*. 2013;3616:345–356.
- [7] Rojas-Runjaic FJM, C. JA, Guayasamin JM. A new rainfrog of the *Pristimantis myersi* group (Amphibia: Craugastoridae) from Volcán Pichincha, Ecuador. *Zootaxa*. 2014;3780:36–50.
- [8] Urgilés VL, Sánchez-Nivicela JC, Nieves C, et al. Ranas terrestres en los ecosistemas surandinos de Ecuador I: Dos nuevas especies de *Pristimantis* (Anura: Craugastoridae) de la ladera oriental [Terrestrial frogs from the ecosystems of southern Ecuador I: Two new species of *Pristimantis* (Anura: Craugastoridae) of the eastern slope]. *Av. Cienc. Ing.* 2014;6:B51–B59.
- [9] Amphibia Web [Internet]. California: AmphibiaWeb; 2015 [cited 2015 Feb 12]. Available from: <http://amphibiaweb.org/>.
- [10] Fouquet A, Gilles A, Vences M, et al. Underestimation of species richness in neotropical frogs revealed by mtDNA analyses. *PLoS ONE*. 2007;2:e1109.
- [11] Duellman WE, Hedges SB. Three new species of *Pristimantis* (Lissamphibia, Anura) from montane forests of the Cordillera Yanachaga in Central Peru. *Phyllomedusa*. 2007;6:119–135.
- [12] Padial JM, De la riva I. Integrative taxonomy reveals cryptic Amazonian species of *Pristimantis* (Anura: Strabomantidae). *Zool. J. Linn. Soc.* 2009;155:97–122.
- [13] Kieswetter CM, Schneider CJ. Phylogeography in the northern Andes: complex history and cryptic diversity in a cloud forest frog, *Pristimantis w-nigrum* (Craugastoridae). *Mol. Phylogenet. Evol.* 2013;69:417–429.

- [14] Bickford D, Lohman DJ, Sodhi NS, et al. Cryptic species as a window on diversity and conservation. *Trends Ecol. Evol.* 2007;22:148–155.
- [15] Trontelj P, Fišer C. Perspectives: cryptic species diversity should not be trivialised. *Syst. Biodivers.* 2009;7:1–3.
- [16] Simpson GG. *Principles of animal taxonomy*. New York, NY: Columbia University Press; 1961.
- [17] Wiley EO. The evolutionary species concept reconsidered. *Syst. Zool.* 1978;27:17–26.
- [18] de Queiroz K. Ernst Mayr and the modern concept of species. *Proc. Natl. Acad. Sci. U.S.A.* 2005;102:6600–6607.
- [19] Lynch JD. New species of frogs (Leptodactylidae: *Eleutherodactylus*) from the Pacific versant of Ecuador. *Occas. Pap. Mus. Nat. Hist. Univ. Kansas.* 1976;55:1–33.
- [20] de Queiroz K. The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. In: Howard DJ, Berlocher SH, editors. *Endless forms: species and speciation*. Oxford: Oxford University Press; 1998. p. 57–75.
- [21] De Queiroz K. Species concepts and species delimitation. *Syst. Biol.* 2007;56:879–886.
- [22] Dayrat B. Towards integrative taxonomy. *Biol. J. Linn. Soc.* 2005;85:407–415.
- [23] Padial JM, Miralles A, De la Riva I, et al. Review: the integrative future of taxonomy. *Front Zool.* 2010;7:1–14.
- [24] Vences M, Guayasamin JM, Miralles A, et al. To name or not to name: criteria to promote economy of change in Linnaean classification schemes. *Zootaxa.* 2013;3636:201–244.
- [25] Pinto-Sánchez NR, Ibáñez R, Madriñán S, et al. The great American biotic interchange in frogs: multiple and early colonization of Central America by the South American genus *Pristimantis*. *Mol. Phylogenet. Evol.* 2012;62:954–972.
- [26] Arteaga A, Bustamante L, Guayasamin JM. The amphibians and reptiles of mindo: life in the cloudforest. Quito: Universidad Tecnológica Indoamérica; 2013.
- [27] Guayasamin JM, Krynak T, Krynak K, et al. Phenotypic plasticity raises questions for taxonomically important traits: a remarkable new Andean rainfrog (*Pristimantis*) with the ability to change skin texture. *Zool. J. Linn. Soc.* 2015;173:913–928.
- [28] Hutter CR, Lyons JA, Kell T, et al. The natural history, distribution, and conservation of the Lonely Rainfrog, *Pristimantis eremitus*. *Herpetologica.* 2015; in press.
- [29] Castroviejo-Fisher S, Vilà C, Ayarzagüena J, Blanc M, Ernst R. Species diversity of *Hyalinobatrachium* glassfrogs (Amphibia: Centrolenidae) from the Guiana Shield, with the description of two new species. *Zootaxa.* 2011;3132:1–55.
- [30] Hutter CR, Guayasamin JM. A new cryptic species of glassfrog (Centrolenidae: *Nymphargus*) from Reserva Las Gralarias, Ecuador. *Zootaxa.* 2012;3257:1–21.
- [31] Vences M, Gehara M, Koehler J, et al. Description of a new Malagasy treefrog (*Boophis*) occurring syntopically with its sister species, and a plea for studies on non-allopatric speciation in tropical amphibians. *Amphibia-Reptilia.* 2012;33:503–520.
- [32] Duellman WE, Barley AJ, Venegas PJ. Cryptic species diversity in marsupial frogs (Anura: Hemiphractidae: *Gastrotheca*) in the Andes of northern Peru. *Zootaxa.* 2014;3768:159–177.
- [33] Lynch JD, Duellman WE. Frogs of the genus *Eleutherodactylus* (Leptodactylidae) in western systematics, ecology, and biogeography. *Misc. Publ. Mus. Nat. Hist. Univ. Kansas.* 1997;23:1–236.
- [34] Duellman WE, Lehr E, Venegas PJ. Two new species of *Eleutherodactylus* (Anura: Leptodactylidae) from the Andes of northern Peru. *Zootaxa.* 2006;1285:51–64.
- [35] Guayasamin JM, Bonaccorso E. A new species of glass frog (Centrolenidae: *Cochranella*) from the lowlands of Northwestern Ecuador, with comments on the *Cochranella granulosa* GROUP. *Herpetologica.* 2004;60:485–494.
- [36] Fabrezi M, Alberch P. The carpal elements of anurans. *Herpetologica.* 1996;52:188–204.
- [37] Shubin N, Alberch P. A morphogenetic approach on the origin and basic organization of the tetrapod limb. In: Hecht M, Wallace B, Prance G, editors. *Evolutionary biology*. New York (NY): Plenum Press; 1986. pp. 319–387.
- [38] Avise JC. *Phylogeography: the history and formation of species*. Cambridge (MA): Harvard University Press; 2000.
- [39] Guayasamin JM, Castroviejo-Fisher S, Ayarzagüena J, et al. Phylogenetic relationships of glassfrogs (Centrolenidae) based on mitochondrial and nuclear genes. *Mol. Phylogenet. Evol.* 2008;48:574–595.
- [40] Biomatters. Geneious. Version 8.0.5. Auckland: New Zealand; [Cited 2015 Mar 1]. Available from: <http://www.geneious.com>
- [41] Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 2013;30:772–780.
- [42] Hutter CR, Guayasamin JM, Wiens JJ. Explaining Andean megadiversity: the evolutionary and ecological causes of glassfrog elevational richness patterns. *Ecol. Lett.* 2013;16:1135–1144.
- [43] Wiens JJ, Fetzner JW, Parkinson CL, et al. Hybrid frog phylogeny and sampling strategies for speciose clades. *Syst. Biol.* 2005;54:778–807.
- [44] Letsch HO, Kjer KM. Potential pitfalls of modelling ribosomal RNA data in phylogenetic tree reconstruction: evidence from case studies in the Metazoa. *BMC Evol. Biol.* 2011;11:146.
- [45] Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics.* 2006;22:2688–2690.
- [46] Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics.* 2001;17:754–755.
- [47] Posada D. jModelTest: phylogenetic model averaging. *Mol. Biol. Evol.* 2008;25:1253–1256.
- [48] Akaike H. A new look at the statistical model identification. *IEEE Trans. Autom. Control* 1974;19:716–723.
- [49] Coloma LA, Carvajal-Endara S, Dueñas JF, et al. Molecular phylogenetics of stream treefrogs of the *Hyloscirtus larinyopygion* group (Anura: Hylidae), and description of two new species from Ecuador. *Zootaxa.* 2012;3364:1–78.
- [50] Kok PJ, MacCulloch RD, Means DB, et al. Low genetic diversity in tepui summit vertebrates. *Curr. Biol.* 2012;22:R589–R590.
- [51] Perl RB, Nagy ZT, Sonet G, et al. DNA barcoding Madagascar's amphibian fauna. *Amphibia-Reptilia.* 2014;35:197–206. doi:10.15685381-00002942.
- [52] Vieites DR, Wollenberg KC, Andreone F, et al. Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proc. Natl. Acad. Sci. U.S.A.* 2009;106:8267–8272.
- [53] Hutter CR, Esobar-Lasso S, Rojas-Morales JA, et al. The territoriality, vocalizations and aggressive interactions of the red-spotted glassfrog, *Nymphargus grandisonae*, Cochran and Goin, 1970 (Anura: Centrolenidae). *J. Nat. Hist.* 2013;47:3011–3032.

- [54] Sueur J, Aubin T, Simonis C. Seewave: a free modular tool for sound analysis and synthesis. *Bioacoustics*. 2008;18:213–226.
- [55] R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available from: <http://www.R-project.org>
- [56] Castro F, Ron S, Coloma LA, et al. *Pristimantis calcarulatus*; 2004 [cited 2015 Feb 12]. The IUCN Red List of Threatened Species. Version 2014.2. Available from: www.iucnredlist.org
- [57] Guayasamin JM, Mendoza AM, Longo AV, et al. High prevalence of *Batrachochytrium dendrobatidis* in an Andean frog community (Reserva Las Galarías, Ecuador). *Amphib. Reptile Conserv*. 2014;8:33–44.
- [58] Herzog SK, Martinez R, Jørgensen PM, et al. Climate change and biodiversity in the tropical andes. Inter-American Institute for Global Change Research (IAI) and Scientific Committee on Problems of the Environment (SCOPE), Sao Jose dos Campos; Brazil, 2011. p. 348.
- [59] BirdLife International. Important Bird Areas factsheet: Valle de Guayllabamba; 2014 [cited 2014 Oct 11]. Available from: <http://www.birdlife.org>
- [60] Mothes PA, Hall ML, Janda RJ. The enormous Chillos Valley Lahar: an ash-flow-generated debris flow from Cotopaxi Volcano, Ecuador. *Bull. Volcanol*. 1998;59:233–244.
- [61] Marino J, Bennett M, Cossios D, et al. Bioclimatic constraints to Andean cat distribution: a modelling application for rare species. *Divers. Distrib*. 2011;17:311–322.
- [62] Anderson RP, Gomez-Laverde M, Peterson AT. Geographical distributions of spiny pocket mice in South America: insights from predictive models. *Global Ecol. Biogeogr*. 2002;11:131–141.
- [63] Duellman WE, Lehr E. Terrestrial breeding frogs (Strabomantidae) in Peru. Münster, Germany: Natur und Tier-Verlag, Naturwissenschaft; 2009.
- [64] Gehara M, Crawford AJ, Orrico VG, et al. High levels of diversity uncovered in a widespread nominal taxon: continental phylogeography of the neotropical tree frog *Dendropsophus minutus*. *PLoS ONE*. 2014;9:e103958.
- [65] Toledo LF, Becker CG, Haddad CF, et al. Rarity as an indicator of endangerment in neotropical frogs. *Biol. Conserv*. 2014;179:54–62.
- [66] Páez-Moscoso DJ, Guayasamin JM. Species limits in the Andean toad genus *Osornophryne* (Bufonidae). *Mol. Phylogenet. Evol*. 2012;65:805–822.
- [67] Lynch JD, Burrowes PA. The frogs of the genus *Eleutherodactylus* (family Leptodactylidae) at the La Planada Reserve in southwestern Colombia with descriptions of eight new species. *Occas. Pap. Mus. Nat. Hist. Univ. Kansas*. 1990;136:1–31.

Appendix 1. Specimens examined and corresponding locality data. All specimens are from Ecuador

Pristimantis calcarulatus (*sensu stricto*)

Provincia de Pichincha: Reserva Las Gralarias (0.01877°S, 78.73131°W; 2019 m), MZUTI 500–508. Nearby Tandapi (0.437°S, 78.811°W, 1939–2044 m), MZUTI 1883, 1887–88, 1898–99, 1901, Tandapi (type locality), KU 111218 (holotype), KU 111221, KU 111230, KU 165179, KU 002240 and KU 002384. *Cotopaxi Province*: Reserva Otonga (0.4194°S, 79.0033°W; 1980 m), MZUTI 2049. Reserva Otonga (0.4155°S, 79.0048°W; 2115 m), MZUTI 2050–53, 2201.

Pristimantis appendiculatus

Provincia de Pichincha: Mindo, Sachatamia Lodge (0.022°S, 78.759°W, 1734–1744 m), MZUTI 1377, 1379. *Provincia de Cotopaxi*: Tandapi (0.437°S, 78.811°W, 2059 m), MZUTI 1880–82; Reserva Integral Otonga (0.416°S, 79.005°W, 2115 m), MZUTI 2045, 2048.

Pristimantis eremitus

Provincia de Pichincha: Reserva Las Gralarias (0.00843°S, 78.73°W, 2063 m), MZUTI 426; Reserva Bellavista (0.0208°S, 78.685°W, 2028 m), MZUTI 2277–79.

Pristimantis sobetes

Provincia de Pichincha: Reserva Las Gralarias (0.008°S, 78.73°W, 1900–2100 m), MZUTI 432–36.

Pristimantis pteridophilus

Provincia de Pichincha: Reserva Las Gralarias (0.008°S, 78.73°W, 1900–2100 m), MZUTI 511–16.

Appendix 2. Genbank accession numbers for *Pristimantis* species from Ecuador used in the phylogenetic analyses **Bolded** accession numbers are new data added from this study

Museum No.	Species	Locality	GenBank accession
MZUTI 500	<i>P. calcarulatus</i>	Pichincha, Reserva Las Gralarias	KT210151
MZUTI 2050	<i>P. calcarulatus</i>	Cotopaxi, Reserva Otonga	KT210152
MZUTI 2049	<i>P. calcarulatus</i>	Cotopaxi, Reserva Otonga	KT210153
MZUTI 501	<i>P. calcarulatus</i>	Pichincha, Reserva Las Gralarias	KT210154
MZUTI 508	<i>P. calcarulatus</i>	Pichincha, Reserva Las Gralarias	KT210160
MZUTI 1902	<i>P. calcarulatus</i>	Pichincha, Tandapi	KT210161
MZUTI 1899	<i>P. calcarulatus</i>	Pichincha, Tandapi	KT210162
MZUTI 1883	<i>P. calcarulatus</i>	Pichincha, Tandapi	KT210163
MZUTI 1901	<i>P. calcarulatus</i>	Pichincha, Tandapi	KT210165
MZUTI 504	<i>P. calcarulatus</i>	Pichincha, Reserva Las Gralarias	KT210166
MZUTI 1898	<i>P. calcarulatus</i>	Pichincha, Tandapi	KT210167
MZUTI 503	<i>P. calcarulatus</i>	Pichincha, Reserva Las Gralarias	KT210172
MZUTI 505	<i>P. calcarulatus</i>	Pichincha, Reserva Las Gralarias	KT210173
MZUTI 1713	<i>P. cedros</i> n. sp.	Imbabura, Reserva Los Cedros	KT210155
MZUTI 1711	<i>P. cedros</i> n. sp.	Imbabura, Reserva Los Cedros	KT210156
MZUTI 1712	<i>P. cedros</i> n. sp.	Imbabura, Reserva Los Cedros	KT210157
MZUTI 1710	<i>P. cedros</i> n. sp.	Imbabura, Reserva Los Cedros	KT210170
MZUTI 493	<i>P. pahuma</i> n. sp.	Pichincha, Reserva Las Gralarias	KT210158
MZUTI 490	<i>P. pahuma</i> n. sp.	Pichincha, Reserva Las Gralarias	KT210159
MZUTI 496	<i>P. pahuma</i> n. sp.	Pichincha, Reserva Las Gralarias	KT210164
MZUTI 491	<i>P. pahuma</i> n. sp.	Pichincha, Reserva Las Gralarias	KT210168
MZUTI 499	<i>P. pahuma</i> n. sp.	Pichincha, Reserva Las Gralarias	KT210169
MZUTI 486	<i>P. pahuma</i> n. sp.	Pichincha, Reserva Las Gralarias	KT210171
KU 177658	<i>P. sp. aff. cedros</i>	Pichincha, Tandapi	EF493523
KU 179090	<i>P. dissimulatus</i>	Pichincha, Quebrada Zapadores	EF493522
KU 177637	<i>P. appendiculatus</i>	Pichincha, Quebrada Zapadores	EF493524
KU 218028	<i>P. pycnodermis</i>	Morona Santiago, Gualaceo-Limon Rd., 2.4 km E Azuay border	EF493680
KU 218021	<i>P. orcesi</i>	Pichincha, Bosque de Pasochoa	EF493679
KU 218015	<i>P. inusitatus</i>	Napo, 31 km N Jondachi	EF493677
KU 218002	<i>P. glandulosus</i>	Napo, 2.7 km W Cuyuja	EF493676
KU 217786	<i>P. acerus</i>	Napo, 6.8 km E Papallacta	EF493678