Vast cryptic diversity in direct-developing frogs *Pristimantis* (Anura: Strabomantidae): A new subgenus and the description of a new species from the eastern Andes of Ecuador (#124350)

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Vast cryptic diversity in direct-developing frogs *Pristimantis* (Anura: Strabomantidae): A new subgenus and the description of a new species from the eastern Andes of Ecuador

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Pristimantis , a genus of direct-developing frogs within the family Strabomantidae, comprises 617 recognized species, making it the most species-rich genus of vertebrates worldwide. This group include 264 described (43% of the world) species in Ecuador, being one of the countries in the region with the highest rate of species description, greatest diversity and endemism. In this study, we analyze the phylogenetic position and describe a new *Pristimantis* species from the Colonso Chalupas Biological Reserve and Llanganates National Park in northeastern Ecuador, using phylogenetic, genetic, morphological, and geographic evidence. Additionally, we propose a new subgenus within *Pristimantis* that includes the *Pristimantis prolatus* and *Pristimantis bicantus* species groups. Our results indicate that the new species and related species form a well-supported group with significant genetic divergence based on the 16S rRNA gene (Average uncorrected *p*-distance = 2.8% – 7.5%), within the *Pristimantis bicantus* species group. Morphologically, the new species is characterized by a black to dark-gray dorsum and marbled venter (less intense or brown in males), being endemic to the Guacamayos mountain range and the Llanganates region in the northeastern Andean foothills of Ecuador. We emphasize the importance of including topotypic specimens to analyze and compare specios groups to delimiting species, like *Pristimantis* ~~frogs~~.

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Abstract

Pristimantis, a genus of direct-developing frogs within the family Strabomantidae, comprises 617 recognized species, making it the most species-rich genus of vertebrates worldwide. This group include 264 described (43% of the world) species in Ecuador, being one of the countries in the region with the highest rate of species description, greatest diversity and endemism. In this study, we analyze the phylogenetic position and describe a new *Pristimantis* species from the Colonso Chalupas Biological Reserve and Llanganates National Park in northeastern Ecuador, using phylogenetic, genetic, morphological, and geographic evidence. Additionally, we propose a new subgenus within *Pristimantis* that includes the *Pristimantis prolatus* and *Pristimantis* *bicantus* species groups. Our results indicate that the new species and related species form a well-supported group with significant genetic divergence based on the 16S rRNA gene (Average uncorrected *p*-distance = 2.8% – 7.5%), within the *Pristimantis bicantus* species group. Morphologically, the new species is characterized by a black to dark-gray dorsum and marbled venter (less intense or brown in males), being endemic to the Guacamayos mountain range and the Llanganates region in the northeastern Andean foothills of Ecuador. We emphasize the importance of including topotypic specimens to analyze and compare specios groups to delimiting species, like *Pristimantis* frogs.

Key words: Integrative taxonomy; cryptic diversity; *Pristimantis paganus* sp. nov.*; Pristimantis prolatus* group; *Pristimantis bicantus* group, *Cryptomantis gen. nov*.

INTRODUCTION

Amphibians are one of the most diverse ~~components of the~~ vertebrate ~~fauna~~ in Ecuador, with a total of 700 species recorded *(Ron et al., 2024)*. The genus *Pristimantis* (Strabomantidae) is a group of terrestrial frogs with direct development, identified as the most diverse land-vertebrate genus in the Neotropics *(Waddell et al., 2018)*. *Pristimantis* ~~frogs~~ include 264 described (43% of the world) species in Ecuador *(Ron et al., 2024)*, being one of the countries in the region with the highest rate of species description, greatest diversity and endemism *(Hedges et al., 2008; Reyes-Puig & Mancero, 2022)*. However, it is suspected a vast richness underestimation *(Bickford et al., 2007)* due to the taxonomic complexity, incomplete phylogenies and the high phenotypic variation, with almost 60% of Ecuadorian species of this genus considered at risk of extinction *(Ortega-Andrade et al., 2021)*.

Over the past two decades, the description of new *Pristimantis* ~~species~~ has significantly increased *(Brito & Almendáriz, 2018; Brito & Pozo-Zamora, 2013; Reyes-Puig & Yánez-Muñoz, 2012; Yánez-Muñoz et al., 2010)*. Although taxonomic work based on morphology has notably advanced our understanding of this group *(Acevedo et al., 2014; Acosta-Galvis, 2015; Lehr & Coloma, 2008; Maciel et al., 2012; Reyes-Puig et al., 2015; Reyes-Puig & Yánez-Muñoz, 2012; Yánez-Muñoz, 2014)*, relying solely on morphological data can overlook cryptic diversity *(Lehr & Coloma, 2008; Ortega-Andrade et al., 2015; Páez & Ron, 2019)*. Recent studies have identified morphological synapomorphies in Brachicephaloidea, but no synapomorphies have been found for phenotypic or phylogenetic species groups within *Pristimantis*, except for the *P. conspicillatus* species group *(Heinicke et al., 2015; Ospina-Sarria & Grant, 2021; Ron et al., 2020)*. Conversely, some historical diagnostic traits, such as dorsolateral folds, coloration, dorsal tubercles, and cranial ridges *(Duellman & Lehr, 2009)*, have shown phenotypic plasticity and homoplasy *(Guayasamin et al., 2015)*.

Integrative taxonomy provide a more comprehensive understanding of species boundaries and relationships by cross-validating different types of data and complementary perspectives (phylogeography, comparative, morphology, population genetics, ecology, development, behaviour, etc.) *(Domínguez-Domínguez & Vázquez-Domínguez, 2009)*. From a taxonomic perspective, the description and delimitation of species is important since it provides information to estimate the conservation status of genealogical lineages *(Dayrat, 2005; de Queiroz, 2003; de Queiroz, 2007)*, especially of morphologically cryptic groups *(Ortega-Andrade et al., 2021)*.

A group of frogs related to *Pristimantis prolatus (Lynch & Duellman, 1980)* and *P. suetus (Lynch & Rueda-Almohadacid, 1980)*, found on the eastern slopes of the Andes in Ecuador, was reported by Ortega *et al. (~~Ortega et al.,~~ 2022)* to form a monophyletic group. Several species in this clade, including *Pristimantis bicantus (Guayasamin & Funk, 2009)*, *P. nelsongalloi (Valencia et al., 2019)* and *P. sacharuna (Reyes-Puig et al., 2015)*, were phylogenetically linked for the first time within a complex group that also includes at least three putative undescribed species. These species reveal hidden cryptic diversity along the montane forests of the eastern Andean slopes in Ecuador *(Ortega et al., 2022)*.

The Llanganates National Park and the Colonso Chalupas Biological Reserve are areas vastly unexplored in the northeastern Andes of Ecuador. Since 2016, research programs from Instituto Nacional de Biodiversidad (INABIO) and Universidad Regional Amazónica Ikiam, are dedicated to document the biodiversity from both conservation areas, where cryptic diversity in *Pristimantis* frogs has been recorded *(Guayasamin & Funk, 2009; Ortega et al., 2022)*. The conservation of these areas is important since they harbor a unique diversity of species belonging to sensitive ecosystems, including Páramo highlands and cloud forests. In contrast, some threats on biodiversity has been registered for those areas, related with illegal mining, habitat loss and forest exploitation *(Paz, 2022)*.

In this work, we describe a new subgenus ~~for~~ *Pristimantis* and a new *Pristimantis* ~~species~~ from Colonso Chalupas Biological Reserve and Llanganates National Park, northeastern Ecuador, based on phylogenetic, genetic, morphological and geographic lines of evidence.

MATERIAL AND METHODS

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix http://zoobank.org/. The LSID for this publication is: LSID urn:lsid:zoobank.org:pub:11445DCE-6CC1-4BA6-9ACC-1B070E79AA24. The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central SCIE and CLOCKSS.

Ethics statement

Specimens and tissue samples obtained following technical protocols proposed by *Heyer et al. (1994)*. Type specimens were deposited at the Instituto Nacional de Biodiversidad INABIO, Quito, Ecuador (DHMECN), under permits MAE-DNB-CM-2016-0045, 2017-0062 and 2019-0120 issued by the Ministry of Environment, Water and Ecological Transition of Ecuador.

Fieldwork

Collections were carried out in 2016 (0.93527° S, 77.92683° W, 2206 m a.s.l.), 2017, and 2021 (0.93829° S, 77.94833° W, 2157 m a.s.l.) at the Colonso Chalupas Biological Reserve, Napo Province, Ecuador, and at Cerro de Abitagua (1.36217° S, 78.10865° W, 2200 m a.s.l.) in Llanganates National Park, Pastaza Province, Ecuador, during the nights of December 12 to 24, 2021. Maps were created using the Digital Elevation Model (DEM) developed by the Ecuadorian Government through the Ministry of Agriculture's geospatial system, SIGTIERRAS, available at: http://geoportal.agricultura.gob.ec:8090/sinat_web_descarga/login.

Specimens were photographed alive, euthanized with 2% lidocaine by immersion (5-10 min), a sample of liver tissue was extracted, labeled with the respective field code and stored in 96% ethanol. A 10% solution of formalin was used to fix specimens left by 24h after capture and then preserved in 70% ethanol.

Taxon sampling

The protocol outlines steps for re-evaluating the taxonomy of a species complex *(Ortega-Andrade et al., 2015)*: 1) Selection of specimens for molecular analysis and review of the type series of taxa within the focus group, 2) conducting comparative molecular analyses (e.g., phylogenetics, genetic distances), and 3) performing comparative analyses of various lines of evidence to delimit species based on qualitative-quantitative morphological characters and biogeography. In general, specimens selected for morphological analyses and taxonomic descriptions were chosen after phylogenetic analyses (step two of the integrative protocol) and based on taxonomically assessed diagnostic characters for each species (step three of the protocol).

Molecular assay for tissue samples

Genomic DNA extraction was performed using the Wizard® Genomic DNA Purification kit (Promega, Madison, USA), with 5 - 10mg of liver or muscle tissue (see Supplementary Table ST1), following the manufacturer's protocol. The concentration and purity of genomic DNA were determined with the NanoDrop™ One/Onec Microvolume UV-Vis spectrophotometer (Thermo Scientific, Waltham, USA). We amplified DNA samples by Polymerase Chain Reaction (PCR) using three mitochondrial genes: 16S rRNA, 12S rRNA, and cytochrome oxidase subunit 1 (COI) and one nuclear gene recombination activating Gene 1 (RAG1). The final reaction volume was 15 µl and consisted of 2X DreamTaq Green (Invitrogen, Carlsbad, CA, USA), nuclease-free water, 50 ng/µL bovine serum albumin (BSA; Invitrogen, Carlsbad, CA, USA), 0.2µM forward primer, 0.2µM reverse primer and, 50 ng/µl of genomic DNA. PCR amplification was performed on 9 specimens according to the protocols of *Pinto-Sánchez et al. (2012)* and Sanger sequencing by Macrogen (Seoul, South Korea).

A second round of PCR amplification was performed on 20 specimens for sequencing using Oxford Nanopore Technology (ONT) at the Laboratory of Molecular Biology and Biochemistry, Universidad Regional Amazónica Ikiam (Supplementary Table ST1). For short amplicons, the following primers were used: 16sSar-L and 16sSbr-H for 16S rRNA; 12sL4E and 12sH10 for 12S rRNA; dgLCO-1490 and dgHCO-2198 for COI; and R182 and R270 for RAG1. Primers 12sL4E and 16sSbr were used to generate long fragments (approx. 2400 bp) of the 12S rRNA (868 bp) and 16S rRNA (1400 bp) genes (PCR conditions and primers are detailed in Supplementary Table ST2).

We performed two library preparations for ONT sequencing: the first was for short amplicons using the Ligation Sequencing Amplicons - PCR Barcoding protocol (SQK-LSK109 with EXP-PBC096, ONT, Oxford, UK), and the second was for long fragments, using the Ligation Sequencing Amplicon - Native Barcoding - 24 V14 protocol (SQK-NBD114.24, ONT, Oxford, UK). Both preparations followed the manufacturer's guidelines. The library preparations were purified with Agencourt 1.8x AMPure XP beads (Beckman Coulter, USA) and quantified using the Qubit 4 Fluorometer kit (Invitrogen, Carlsbad, USA).

We used two Flongle Flow Cells for sequencing: the R9 version (FLO-FLG001) for short fragments and the R10 version (FLO-FLG114) for long fragments. Prior to final library loading, the Flongle flow cells were conditioned with a mixture of 117 µl Flow Cell Flush (FCF) and 3 µl Flow Cell Tether (FCT) from the Flow Cell Priming Kit (EXP-FLP002). The sequencing mix had a final volume of 30 µl, consisting of 15 µl sequencing buffer (SB), 10 µl library beads (LIB), and 25 fmol of DNA library in a total volume of 5 µl.

Sequencing was performed on a MinION Mk1C device (ONT, Oxford, UK). The sequencer used MinKNOW v23.07.12 and Guppy v7.1.4 software to manage the processes during the run and perform basecalling and demultiplexing of the reads based on their barcodes. The sequencing run lasted 15 to 20 hours until all available pores were exhausted. The raw data were processed using the Porechop v0.2.4 (https://github.com/rrwick/Porechop) and NGSpeciesID v0.3.0 (https://github.com/ksahlin/NGSpeciesID) tools *(Egeter et al., 2022; Quilumbaquin et al., 2023)*. Nanopore sequences were edited and aligned in Geneious Prime v2024.0.1 *(Drummond et al., 2024)*.

Phylogenetic analysis

We generated a total of 99 consensus sequences (27 for 16S, 27 for 12S, 23 for COI, and 22 for RAG1) from 29 individuals (see Supplementary Table ST2). We performed an initial phylogenetic reconstruction to determine the position of the complex group studied within the ~~genus~~ *Pristimantis*. This analysis utilized a matrix that included 1155 taxa terminals and 2981 characters (see Supplementary Material SM1 for the matrix).

A second phylogenetic reconstruction, focusing on the complex group relationships, was developed using new sequences and genetic data deposited in GenBank. For the first time, we incorporated new sequences from *Pristimantis tungurahua (Reyes-Puig et al., 2010)*, *P. marcoreyesi (Reyes-Puig et al., 2014)*, *P. ganonotus (Duellman & Lynch, 1988), P. burtoniorum*

(Reyes-Puig et al., 2022), and additional sequences from *P. bicantus* and *P. prolatus*. DNA sequences were visually inspected and re-aligned to avoid non-homologous characters, following initial alignment with the MAFFT v7.017 (Multiple Sequence Alignment) algorithm *(Katoh et al., 2017)*, using default settings. To minimize unaligned characters in the concatenated matrix, sequence gaps were removed using Geneious Prime v2022.0.1 software *(Kearse et al., 2012)*. *Craugastor longirostris,* and *Niceforonia elassodiscus* were chosen as outgroups *(Duellman & Lehr, 2009)* in both phylogenetic analyses. The matrix, which included 64 taxa terminals and 3371 bp (Supplementary Material SM3), was concatenated and analyzed using Mesquite v3.61 *(Maddison & Maddison, 2019)*.

Phylogenetic trees were estimated to be using a maximum likelihood (ML) algorithm in IQTree v2.2.0 web-server *(Nguyen et al., 2015)*. Nucleotide substitution models were estimated with ModelFinder *(Kalyaanamoorthy et al., 2017)* implemented in the IQTree server, for a total of 8 partitions, one for each non-coding gene (12S and 16S) and one for each codon of the coding genes (COI and RAG1). Support for node values was estimated using the Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) *(Guindon et al., 2010)* with 1000 replicates and 1000 Ultrafast Bootstrap (UFBoot) *(Minh et al., 2013)*. The phylogeny was visualized and edited using FigTree v1.4.2 *(Rambaut, 2014)* and Inkscape v1.3.2. https://inkscape.org/es/. Uncorrected pairwise genetic distances (*p*-distances) between the new species and other species in the clade were estimated using 16S rRNA data from the aligned matrix in Geneious Prime v2024.0.7. https://www.geneious.com. Sequences that did not show homology were excluded from the analysis.

Definition of Candidate Species

We follow the concept defined by *Vieites et al. (2009)* to recognize Unconfirmed Candidate Species (CS) in this study. Specimens were characterized by a detectable genetic differentiation of more than 3%, or 2% according to several studies on *Pristimantis* ~~rainfrogs~~ *(Franco-Mena et al., 2023; Ortega-Andrade et al., 2015; Ortega et al., 2022)*. However, for some taxa, data could be deficient or incomplete for morphology, ecology, or distribution due to unavailability of voucher specimens or immature state of vouchers *(Vieites et al., 2009)*, that are commonly reported for this group *(Ortega et al., 2022; Páez & Ron, 2019; Ron et al., 2020)*.

Morphological analysis

The collected material was examined and compared with specimens deposited in the Herpetological Collection (DHMECN) from the Instituto Nacional de Biodiversidad, Quito (INABIO) and KU Herpetology Collection, University of Kansas, USA. The taxonomic terminology follows the proposal of *Duellman & Lehr (2009)*. The presence of the hyperdistal tubercle in the new species follows the codification by *Ron et al. (2020)*. Morphometric measurements were taken with an electronic caliper (accuracy ±0.01 mm). The following morphological measurements were taken *(Duellman & Lehr, 2009)*: (1) snout–vent length (SVL) = distance from snout tip to posterior margin of vent; (2) head width (HW) = greatest width of head measured at level of jaw articulation; (3) head length (HL) = from posterior margin of lower jaw to tip of snout; (4) horizontal eye diameter (ED) = distance between anterior and posterior borders of eye; (5) interorbital distance (IOD) = the breadth of the braincase between the orbits; (6) eye–nostril distance (EN) = distance from posterior margin of nostril to anterior margin of eye; (7) tympanic length (TD) = horizontal distance between external anterior and posterior margins of tympanic annulus; (8) tibia length (TL) = length of flexed leg from knee to heel; (9) upper eyelid width (EW) = perpendicular distance of the upper eyelid; (10) femur length (FL) = length of femur from vent to knee; (11) foot length (FoL) = distance from the proximal edge of the medial metatarsal tubercle to the tip of the fourth toe; (12) hand length (HaL) = distance from proximal edge of palmar tubercle to tip of Finger III; (13) disc width of Finger III (F3D) = measured across widest part of Finger disc III; (14) disc width of toe IV (T4D) = measured across widest part of toe disc IV. Fingers are numbered pre-axially to post-axially from I–IV. Comparative lengths of toes III and V were determined, both compared to toe IV; lengths of toes I and II were estimated compared to each other. Codification of subarticular tubercles follow the classification of *Ron et al. (2020)*. Sex, maturity of specimens and reproductive condition were delimited by identification of vocal slits, size and through direct observation of gonads by dorsolateral sectioning. Color in life was determined based on photographs taken in the field. Sexual dimorphism is analyzed with a Principal Component Analysis to detect the variables which explain the variation among sexes. Statistical analyses (PCA, U Mann-Whitney test) to compare morphometric measurements were performed in PAST software V4 *(Hammer et al., 2001)*.

RESULTS

Phylogenetic relationships

Our phylogeny identifies a high-supported monophyletic (bootstrap = 100) clade within ~~the genus~~ *Pristimantis* (Fig. 1), with two clades also defined with high support values (bootstrap = 99). The major clade is described herein as *Cryptomantis* subgenus nov. (See Systematic accounts, for taxonomy description). The first clade (A) is taxonomically assigned to the *Pristimantis prolatus* species group, which include four species (*P. ganonotus, P. burtoniorum, P. prolatus*, *P.* c.sp. 1), whereas the second clade (B), is composed of 10 species (*P.* c.sp. 2, *P. sacharuna, P.* c.sp. 3, *P.* c.sp. 4, *P*. c.sp. 5, *P. nelsongalloi*, *P. bicantus*, *P. tungurahua*, *P. marcoreyesi,* and *Pristimantis paganus* sp. nov.), being assigned to the *Pristimantis bicantus* species group (Fig. 1). The phylogenetic position for the holotype of *P. burtoniorum* and the topotypes of *P. tungurahua*, *P. marcoreyesi* and *P. prolatus* are reported for the first time.

We identified an undescribed species of *Pristimantis* (*Pr~~istimantis~~ paganus* sp. nov.; see Systematics account for description) grouped in a high-supported clade (bootstrap: 98%) together with its sister species, *Pristimantis marcoreyesi*, and *P.* c.sp. 2; all of them included in the *Pristimantis bicantus* species group.

The 16s rRNA uncorrected *p*-distance between *P. paganus* sp. nov. and related species within the *P. bicantus* species group range from 0.024–0.11 (0.06±0.018 Standard deviation [SD]); distances with *P. marcoreyesi* 0.024–0.031 (0.028±0.002 SD) with *P*. csp. 2 (0.03–0.062 (0.04±0.01)). The genetic distances compared with other species range from 0.024–0.17 (0.08±0.04); Supplementary Table ST4.

Systematic account

***Cryptomantis* subgenus nov.**

LSID urn:lsid:zoobank.org:act:AD83BF8F-72C0-4453-ABA8-705C2F258944

Type species. *Pristimantis prolatus (Lynch & Duellman, 1980).*

Definition. This clade is strongly supported by phylogenetic evidence (Fig. 1). Morphological synapomorphies are unknown**.** Members of this clade are characterized by: 1) head narrower than body, 2) tympanic membrane and tympanic annulus well defined, 3) cranial crests absent, 4) dentigerous processes of vomer present, 5) "S" condition of adductor muscle, 6) terminal discs of digits truncate, expanded, slightly expanded or lanceolate, with circummarginal grooves, 7) Finger I shorter than second, 8) Toe V longer than Finger III, 9) subarticular tubercles, present, prominent; hyperdistal subarticular tubercle, present 10) texture of dorsum variable, finely shagreen to tuberculate, usually with distinctive dorsolateral dermal folds and tubercles, 11) texture of venter areolate, 12) SVL range of adult individuals from 17 mm in males (*Pristimantis bicantus*) to 29.8 mm in females (*P. paganus* sp. nov.).

Diversity. Nine species (*P. ganonotus (Duellman & Lynch, 1988), P. burtoniorum (Reyes-Puig et al., 2022)*, *P. bicantus (Guayasamin & Funk, 2009), P. nelsongalloi (Valencia et al., 2019), P. prolatus (Lynch & Duellman, 1980)*, *P. sacharuna (Reyes-Puig et al., 2015)*, *P. tungurahua (Reyes-Puig et al., 2010)*, *P. marcoreyesi (Reyes-Puig et al., 2014)*, *P. paganus* sp. nov.) (Figs. 1–2).

Distribution. The distribution range of the subgenus *Cryptomantis* gen. nov. is restricted to the montane forests of the eastern foothills and adjacent to Amazonian Mountain ranges of Ecuador, ranging from the north, the upper basin of the Napo and Pastaza, including the headwaters of the Pastaza River, to the south, in the headwaters of the Nangaritza River.

Comments. Within the subgenus *Cryptomantis*, two well-supported clades are identified: the *Pristimantis (Cryptomantis) prolatus* species group and the *Pristimantis (Cryptomantis) bicantus* species group, including five Unconfirmed Candidate Species (Fig. 1).

***Pristimantis* (*Cryptomantis*) *prolatus* species group new taxon**

Definition: Small-sized frogs with relatively long limbs; SVL ranges from 13.7 mm in males of *P. prolatus* to 27.3 mm in females *P. burtoniorum*. Dorsolateral dermal folds present (except in *P. burtoniorum* and *P. ganonotus*); head width 32.5–39.6% of SVL. The tympanic membrane and annulus are distinctive. Dorsum is finely shagreen to tuberculate, venter areolate. The toes have no interdigital membranes, and ~~the~~ V toe is much longer than ~~the~~ III ~~toe~~. Lateral fringes are weakly defined or absent.

Diversity. ~~Three described species~~ (*P. ganonotus (Duellman & Lynch, 1988), Pristimantis burtoniorum (Reyes-Puig et al., 2022)*, *Pristimantis prolatus (Lynch & Duellman, 1980)*) and one Unconfirmed Candidate Species (Figs. 1–2).

Distribution. The distribution range of the group extends from the north, encompassing the montane forests of the eastern foothills of the Andes in the upper Napo basin, including the headwaters of the Pastaza River, to the south, reaching the headwaters of the Nangaritza River in Zamora Chinchipe.

Comments. The only available sequence of *Pristimantis prolatus* (KU 177433) *(Lynch & Duellman, 1980)*, collected near the type locality, has been shown to represent a lineage independent from other monophyletic groups within *Pristimantis (Hedges et al., 2008; Padial et al., 2014)*. The recent publication by *Ortega et al. (2022)*, provided more sequences from *P. prolatus* (*sensu lato*), excluding the sequence KU 177433 *(Hedges et al., 2008)*, shown that are related, with low support values, to *P. suetus (Lynch & Rueda-Almonacid, 1998)*, and a clade conformed by the *Pristimantis bicantus* species group. Our phylogeny contributes topotypic sequences of *Pristimantis prolatus* (*sensu stricto*), resulting in the identification of the Unconfirmed Candidate Species 1 and its relationship with *P. burtoniorum* in the group *(Reyes-Puig et al., 2022)*. Unlike *Ortega et al. (2022)*, we obtained a high support for the clade formed for the species corresponding to the *P. prolatus* and *P. bicantus* species groups. For *Pristimantis suetus* (MHUA 4404), a 502 bp fragment of 16S rRNA places it within a clade that also includes *P. platychilus (Lynch, 1996)* and *P. permixtus (Lynch et al., 1994)* which are from western and north-central Colombia, respectively (see Supplementary Figure SF1).

***Pristimantis* (*Cryptomantis*) *bicantus* species group new taxon**

Definition. Small-sized frogs with relatively long limbs; SVL ranges from 11.8 mm in males of *P. bicantus* to 29.8 mm in females of *P. paganus* sp. nov. Dorsolateral dermal folds are present (except in *P. bicantus*), but weakly defined in *P. nelsongalloi* and *P. paganus* sp. nov. Head width 37.7 – 42.4% of SVL. The tympanic membrane and annulus are distinctive. Dorsum finely shagreen with tubercles, venter areolate. The toes have no interdigital membranes, and ~~the~~

V ~~toe~~ is much longer than ~~the~~ III ~~toe~~. Lateral fringes are weakly defined or absent. Vocal slits and nuptial pads present or absent.

Diversity. Six described species (*Pristimantis bicantus (Guayasamin & Funk, 2009)*, *P. nelsongalloi (Valencia et al., 2019)*, *P. sacharuna (Reyes et al., 2015)*, *P. tungurahua (Reyes-Puig et al., 2010)*, *P. marcoreyesi (Reyes-Puig et al., 2014)*, *P. paganus* sp. nov) and four Unconfirmed Candidate Species (Fig. 1–2).

Distribution. The distribution range of the group is from the north, in the montane forests of the eastern foothills of the Andes in the upper Napo basin, including the headwaters of the Pastaza River, to the south, in the headwaters of the Nangaritza River in Zamora Chinchipe.

Comments. The recent publication by *Ortega et al. (2022)* provided the first sequences for *P. bicantus* (*sensu lato*), *P. nelsongalloi* (*sensu stricto*), *P. sacharuna* (*sensu stricto*), and a set of three unconfirmed candidate species. Their phylogenetic analysis showed that these specimens were strongly supported as part of a clade within the *Pristimantis bicantus* species group, which was left unnamed. Our phylogeny includes topotypic sequences of *P. bicantus* (*sensu stricto*) from Yanayacu Station, Napo, and positions them with a set of sister species (*P. tungurahua P. nelsongalloi and P. sacharuna*) and three unconfirmed candidate species (*P*. c.sp. 3 to *P*. c.sp. 5).

New species

***Pristimantis paganus* sp. nov.**

LSID urn:lsid:zoobank.org:act:D532641A-414E-4580-A506-FDAEFDA34B0E

Common name in Spanish: *Cutín pagano Amazónico*.

Suggested common English name: *Amazonian pagan rainfrog*

Holotype. DHMECN 16810, adult female, from the Colonso Chalupas Biological Reserve, camp 3, Alto Tena, San Juan de Muyuna, Napo province, Ecuador, (0.93158° S, 77.95659° W), 2495 m, collected on 24 November 2021, by H. Mauricio Ortega-Andrade, Keyko Loza, Mario H. Yánez-Muñoz, Miguel Urgilés, Jorge Brito, and Mauricio Herrera.

Paratypes. A total of 6 females, 13 males and 3 juveniles. Adult female collected at the same locality as holotype: DHMECN 16811, collected on 25 November 2021; Males collected at the same locality as holotype DHMECN 16812–4, collected on 25 November 2021 by Mario H. Yánez Muñoz, Miguel Urgilés, Jorge Brito, H. Mauricio Ortega-Andrade, Mauricio Herrera and Keyko Loza; Adult males: DHMECN 15592–3 (0.93824° S, 77.94553° W, 2200 m) collected by Jimmy Velasteguí, Grace Reyes, Michelle Guachamin and H. Mauricio Ortega-Andrade on 19 December 2016; DHMECN 15594 (0.93846° S, 77.94770° W, 2230 m) collected on 20 December 2016 by Jimmy Velasteguí, Grace Reyes, Michelle Guachamin, and H. Mauricio Ortega-Andrade; DHMECN 15595–15596, DHMECN 15598–15599 (0.93874° S, 77.94742° W, 2221 m) collected by Jimmy Velasteguí, Grace Reyes, Michelle Guachamin and H. Mauricio Ortega-Andrade on 18 June 2017; DHMEC 15600 and DHMECN 15603 (0.93685° S, 77.94993° W, 2216 m) collected by H. Mauricio Ortega-Andrade on 19 June 2017; DHMECN 15605 (0.93829° S, 77.94833° W, 2157 m) collected by Miguel Gómez, Grace Reyes, Salomón Ramírez and H. Mauricio Ortega-Andrade on 16 November 2018; Juveniles: DHMECN 15596, DHMECN 15599 (0.93844° S, 77.94641° W, 2223 m), DHMECN 15603 (0.93844° S, 77.94641° W, 2223 m) collected by H. Mauricio Ortega-Andrade on 18 June 2017; Females: DHMECN 15598 (0.93844° S, 77.94641° W, 2223 m) 18 June 2017, DHMECN 15600 (0.93685° S, 77.94993° W, 2216 m) 19 June 2017, DHMECN 15606 (0.93817° S, 77.94840° W, 2200 m) 16 November 2018, collected by H. Mauricio Ortega-Andrade, Grace Reyes, Miguel Gómez and Salomón Ramírez.

Referred specimens. DHMECN 17223 male collected on 01 December 2021, DHMECN 17221 on 28 November 2021, DHMECN 17222 on 29 November 2021 collected by Zane Libke, Eli Bieri, Kira Miller, Sara Dykman, Henry Sanchez, Vicente Sanchez, Jordi Salazar, Kira Miller, Sara Dykman (1.36217° S, 78.10865° W, 2221 m), Cerro de Abitagua, Llanganates National Park on 24 December 2021.

Diagnosis. *Pristimantis paganus* sp. nov. (Figs. 2–7) is a member of the subgenus *Cryptomantis* and *P. bicantus* species group, characterized by the following combination of characters: (1) dorsal skin finely granular with dorso-laterally aligned flattened warts, areolate venter, discoidal fold present and visible posteriorly, dorsolateral folds finely defined and discontinuous in females, defined continuous in males; scapular W-shaped fold defined in males; (2) tympanic membrane and tympanic annulus well defined, round, horizontal diameter of tympanum equal to 38% of eye diameter, antero-dorsal margin with a supratympanic fold and large subconical postrictal tubercles; (3) snout short, subacuminate in dorsal view, rounded in profile with slightly flared lips; (4) upper eyelid with at least three to four large rounded tubercles surrounded by several small rounded tubercles, two subconical tubercles behind each eye; no cranial crest; (5) dentigerous processes of vomers, oblique in outline each process with 3–8 teeth; (6) vocal slits indistinct; no nuptial pad and no vocal sac; (7) Finger I shorter than Finger II; broad, expanded disks dilated with circummarginal grooves; (8) Fingers with thin lateral cutaneous fringes; (9) subarticular tubercles, present, prominent; hyperdistal subarticular tubercle, present; ulnar tubercles absent; (10) heel with one subconical tubercle; outer edge with two flattened tubercles, tarsal fold absent; (11) inner metatarsal tubercle oval in females 3 times larger than rounded outer metatarsal tubercle; supernumerary tubercles absent; (12) toes with thin lateral fringes present, interdigital membrane absent, toe V longer than toe III; (13) females dorsally black with distinct pinkish (grayish cream in life) flank and throat markings. Males dorsally brown with dark brown W-shaped and inverted V-shaped markings on dorsum, with orange-brown blotches on canthal stripe and insertions of axillae and groin. Males with ventral coloration varying from light brown, dark brown or mottled gray and females with black mottling on white or cream venter; the iris is bicolor golden with fine black reticulations in the upper part and a horizontal wide copper medial band; (14) adult males, SVL= 17.20–21.5 mm (mean = 19.6, n = 13), females SVL= 24.8–29.8 mm (mean = 29.6 mm, n = 6), (Tables 1–2, Supplementary Table ST3).

Comparison with other species. The new species differs from its closer sister species (*P. marcoreyesi*, *P. tungurahua*, *P. nelsongalloi*, *P. sacharuna*, *P. bicantus*, *P. ganonotus, P. burtoniorum* and *P. prolatus*) by the presence of finely defined and discontinuous dorsolateral dermal folds in females, defined continuous with a W-shaped scapular fold in males (Weakly defined dorsolateral folds in *P. marcoreyesi*, W-shape very evident in males, not very evident in females in *P. tungurahua*, dorsolateral folds thin anteriorly, discontinuous posteriorly in *P. nelsongalloi*, W-shaped scapular fold followed by fine scapular fold in shaped of an inverted “V” in *P. sacharuna,* absent in *P. bicantus*, *P. burtoniorum, P. ganonotus* and H-shaped in *P. prolatus*). In addition, the distinctive coloration pattern in females with homogeneously black dorsum distinguishes it from its related species and other *Pristimantis* species from the eastern montane forests of Ecuador (Fig. 2).

Besides *Pristimantis paganus* is distinguished from *P. marcoreyesi, P. tungurahua*, *P. nelsongalloi* and *P. sacharuna* (characters of these species in parentheses) by lacking ulnar tubercles (small flattened ulnar tubercles in *P. marcoreyesi*, present low in *P. tungurahua*, small tubercles in *P. nelsongalloi*, subconical in *P. sacharuna*), rounded tubercles on upper eyelid (flattened to subconical in *P. marcoreyesi*, small and flattened in *P. nelsongalloi*, subconical in *P. sacharuna* and *P. tungurahua*), rounded external metatarsal tubercle (subconical in *P. marcoreyesi,* round subconical in *P. nelsongalloi and P. sacharuna*).

Likewise, the new species differs from *P. bicantus* by having truncated Fingers (discs on Fingers and toes not expanded or slightly expanded in *P. bicantus*), fine lateral fringes (absent in *P. bicantus*), males lack vocal slit (present in males from *P. bicantus*). Finally, it can be differentiated from *P. prolatus, P. ganonotus* and *P. burtoniorum* by having the snout short and subacuminate in dorsal view (acuminate in *P. prolatus and P. ganonotus*, snout large and subacuminate in *P. burtoniorum*), upper eyelid with rounded tubercles (one conical in *P. prolatus,* subconical in *P. burtoniorum* and absent in *P. ganonotus*). Other differences are mentioned in Supplementary Table ST3.

Description of the holotype. Adult female (Figs. 3–7), head longer than wide. Snout short, subacuminate in dorsal view, rounded in profile; lips flared, eye-nostril distance 12% of SVL, canthus rostralis straight, defined in dorsal and lateral view, loreal region concave, protruding nares directed laterally; interorbital area flat, no interorbital fold, interorbital distance wider than upper eyelid, ~90%; no cranial crest; upper eyelid with at least three to four large rounded tubercles, ~~tympanum present,~~ tympanic membrane differentiated from surrounding skin, tympanic ring evident surrounding ½ of tympanic membrane, upper margin covered by thick tympanic fold, tympanum visible, diameter of tympanum equals 38% of eye diameter, underside of tympanum with large subconical postrictal tubercles; choanae small, oval in outline, not covered by palatal floor of maxilla; dentigerous processes of vomers present oblique in outline with 4 to 8 teeth, tongue as broad as long, slightly heart-shaped 40% attached to floor of mouth.

~~Texture~~ of dorsum finely granular ~~and features~~ flattened warts arranged dorso-laterally, finely defined and discontinuous dorsolateral folds; belly strongly areolate; discoidal fold present and visible posteriorly, cloaca ~~has a~~ granular ~~texture~~, and the slender arms are free of tubercles on both the dorsal and ventral surfaces of the forearm. Ulnar tubercles absent; broad truncated disks on Fingers II to IV, with circummarginal indentations, subarticular tubercles rounded and flattened in lateral view with thin lateral cutaneous fringes; thenar tubercle oval with heart-shaped palmar tubercle, palmar surface without supernumerary tubercles with scattered micro-granulations on hands, hind limbs slender, length of tibia equals 56% of SVL, no tubercle on outer edge of tibia, with a small subconical tubercle on heel, inner tarsal fold absent, Fingers with thin cutaneous ridges, on Fingers I–V, without digital membranes; subarticular tubercles rounded and flattened in profile view; hyperdistal subarticular tubercle, present, elongated transversally; expanded disks on all toes, larger than those of the hand. Toe V longer than III, not extending beyond distal subarticular tubercle of toe IV.

Holotype coloration in preserved. Dorsal surface, forelimbs and hindlimbs homogeneously black with a thin creamy brown interorbital band. Inner edge of arm and forearm insertion, dorsal surfaces and bases of toes I and II grayish brown, flanks marbled with grayish cream markings, ventrally with grayish cream coloration on belly, throat, thigh and tibia surfaces, belly on cream background mottled with black markings, throat on cream background and brown spots with large black markings, dorsal surfaces of thighs with black bands separated by light brown interspaces. Grayish cream line on the upper lip interspersed with black interspaces. Lower lip with black background separated by three grayish cream bands (Figs. 3–4).

Holotype coloration in life. Dorsal surface forelimbs and hindlimbs homogeneously black with a thin interorbital band of light pinkish brown, irregular pattern extending on the front margin of the eyelid to the tip of the snout. Inner edge of arm insertion, forearm, lateral dorsal surfaces, thighs and belly pinkish brown with black blotches, ventrally pinkish cream coloration on surfaces of belly, throat, thighs and tibia, belly and throat on grayish cream background mottled with large black markings and light brown blotches, dorsal surfaces of thighs with black bands separated by pinkish brown interspaces. Pinkish-brown line with small pinkish blotches more pronounced on the upper lip interspersed, discontinuous by black interspaces. Lower lip on black background separated by cream bands (Fig. 5).

Measurements (in mm) of holotype. SVL = 27.23; HW = 10.24; HL = 11.21; ED = 3.92; IOD = 2.59; EN = 3.2; TD = 1.51; TL = 15.45; EW = 2.86; FL = 13.49; FoL = 13.54; HaL = 8.10; F3D = 1.18; T4D= 1.15

Variation. *Pristimantis paganus* shows sexual dimorphism in body size and coloration (Figs. 6–8). Males ~~of this species~~ are smaller in size than females (Fig. 8; See Table 2 for details in morphometric measurements of the type series). In ~~life, it has been observed that there is a notable variation in coloration between sexes.~~ Females have a light grayish brown color in the canthal stripe from the interorbital area of the head to the tip of the snout, this coloration is orangeness brown in males. In addition, an irregular blotch may or may not be present in the center of the canthal stripe, being black in females and dark brown in males (Fig. 7). The marbled irregular blotches on the ventral surfaces in females are black and in males ~~they~~ are grey, less intense in juveniles. Females have black dorsal coloration, while males have an orange-brown dorsum with dark brown W-shaped and inverted V-shaped marking on scapula. In ~~preserved~~, the dorsal coloration pattern varies from a homogeneously black, light gray to grayish brown dorsum with a cream interorbital bar. The ventral coloration differs in the intensity and abundance of the botches, females have black spots and in males this coloration varies from light brown, grayish brown to dark brown. In particular, the thighs and throat have a grayish cream coloration with large black spots in females, dark brown in males, and in both, throat dark gray or brown with pale flecks (Fig. 6).

Etymology. The specific epithet is from the Latin word "*paganus*", an adjective derived from "*pagus*", which refers to the inhabitants of the forest or village, far from civilization and towns, referring to the remote and unexplored sites where this species inhabits, in the montane cloud forests at the Colonso Chalupas Biological Reserve and Llanganates National Park, northeastern flanks of the Andes in Ecuador.

Distribution and natural history. *Pristimantis paganus* has been recorded from two localities separated by approximately 50 km, located in northeastern Ecuador in the provinces of Napo (Cordillera de Guacamayos = Guacamayos mountain range, Colonso Chalupas Biological Reserve) and Pastaza (Cerro de Abitagua, Llanganates National Park) (Fig. 9). These localities belong to the northern montane evergreen forest of the Eastern Cordillera of the Andes *(MAE, 2013)*. The type locality and other collection sites are characterized by primary forest with abundant epiphytic plants (bromeliads, ferns, and moss), typical of the Amazonian cloud forest (Fig. 10). The type series of the new species was collected at night between 22:00 and 01:00, on bromeliad leaves between 20–110 cm in height. The holotype, an adult gravid female with eggs ready to be laid, was recorded on the night of 21 November 2021.

Conservation status. During sampling in October 2016, July 2017 and November 2021, specimens ~~collected~~ of *Pristimantis paganus* were recorded infrequently (<25 individuals). However, this species is associated to primary forest in two protected areas Colonso Chalupas Biological Reserve and Llanganates National Park, on the northeastern flanks of the Amazonian Andes of Ecuador. Although viable populations (males, females and juveniles) of this species have been found within the National System of Protected Areas (SNAP), the region where it is distributed could be threatened by illegal mining, deforestation and climate change *(Paz, 2022)*. According to the IUCN Red List criteria *(IUCN, 2023)*, based on scarce number of localities and small distributional range with potential threats in the next decades, this species is proposed as "Near Threatened" (NT).

DISCUSSION

Cryptomantis, a new Subgenus for *Pristimantis*.

We described a new subgenus for *Pristimantis* and a new *Pristimantis* species from the Colonso Chalupas Biological Reserve and Llanganates National Park, northeastern Ecuador, based on phylogenetic, genetic, morphological and geographic lines of evidence. The clade described as subgenus *Cryptomantis*, include two intern clades described as the *Pristimantis prolatus* (three species) and the *P. bicantus* (six species) groups, shown strong phylogenetic support, confirming their monophyly (Fig. 1). However, no morphological synapomorphies have been identified to diagnose the subgenus *Cryptomantis* or its species groups within *Pristimantis (Hedges et al., 2008; Ospina-Sarria & Grant, 2021)*. *Padial et al. (2014)* reformulated the species series and species groups within *Pristimantis,* proposed by previous authors (i. e. *(Hedges et al., 2008; Lynch & Duellman, 1997; Lynch & Duellman, 1980; Pinto-Sánchez et al., 2012)*), to make them explicitly monophyletic. However, nominal species related to the *Cryptomantis* subgenus (*P. ganonotus, P. prolatus, P. tungurahua,*) were unassigned to any of the taxonomic groups in *Pristimantis (Padial et al., 2014)*. On the other hand, *P. bicantus* was described and assigned to the *Pristimantis myersi* species group *(Guayasamin & Funk, 2009; Padial et al., 2014)*, but excluded from this group and leave as unassigned by *(Ortega et al., 2022)*. *Franco-Mena et al. (2023)* recognized that *Pristimantis bicantus, P. nelsongalloi*, and *P. sambalan* form a well-supported clade nested within *P. caprifer*, suggesting that all of them should be assigned to the *Pristimantis* euphronides species group as defined by *Targino (2016)*. Our results discard a close phylogenetic relationship between *P. caprifer* and *P. euphronides + P. shrevei* clade*,* and all other members of the subgenus *Cryptomantis,* in agreement with previously phylogenetic studies *(Mendoza et al., 2015; Ortega-Andrade & Venegas, 2014; Padial et al., 2014; Waddell et al., 2018)* (Fig. SF1).

We observe two variant traits among species in the *Cryptomantis* clade. These include plump-bodied terrestrial frogs (mostly species of the *Pristimantis bicantus* species group), and slender-bodied bush frogs (distributed among the *P. prolatus* and *P. bicantus* species groups). The presence of dorsolateral dermal folds is a characteristic observed in both clades of *Cryptomantis*; however, it is not exclusive to this group and also occurs in other groups within

Pristimantis (Hedges et al., 2008; Páez & Ron, 2019). We advocate for more comprehensive future analyses to determine whether diagnostic characters, such as dorsolateral dermal folds or other dermal structures *(Guayasamin et al., 2015)*, are homologous or homoplastic among the *Pristimantis* clades *(Bejarano-Muñoz et al., 2022; Ospina-Sarria & Grant, 2021; Ron et al., 2020)*.

Currently, three subgenera are recognized within the genus *Pristimantis*: *Hypodictyon (Hedges et al., 2008; Heinicke et al., 2018), Huicundomantis (Ortega et al., 2022; Páez & Ron, 2019)* and *Trachyphrynus (Franco-Mena et al., 2023)* . The description of the new subgenus *Cryptomantis* does not cause taxonomic instability due to its strong monophyletic support; instead, it provides an additional step toward classifying and subdividing *Pristimantis* into well-supported cladistic groups.

Cryptic diversity and the need for further taxonomic work.

Species of the *Cryptomantis* subgenus are distributed in the eastern foothills of Ecuador. Speciation within this region is hypothesized to result from geographically restricted allopatry, with isolation occurring due to the uplift of the Cordillera Real Amazónica, the Abitagua and Guacamayos mountain ranges, and the occurrence of major Amazonian rivers such as the Napo and Pastaza (Fig. 9) *(Duarte, 2013)*. A striking pattern of cryptic diversity and high species replacement in small areas in the Pastaza basin reveals a complex biogeographical and evolutive history for terrestrial frogs in the region *(Reyes-Puig et al., 2022)*. The inclusion of topotypic sequences of *Pristimantis prolatus* (*sensu stricto*) and other *Pristimantis* species in our work, facilitated the understanding of species limits and the cryptic diversity of lineages, previously considered to have wide distributions in eastern Ecuador *(Bejarano-Muñoz et al., 2022; Ortega et al., 2022; Reyes-Puig et al., 2022)*. For example, in contrast to the phylogeny of *Ortega et al. (2022)*, we determined that the topotyptic populations of *Pristimantus prolatus* are sister of the *Pristimantis* Candidate Sp.1, and *Pristimantis bicantus* are sister of *Pristimantis* Candidate Sp. 5 (See comments for definition of species groups; Fig. 1). The genetic sequence of *Pristimantis prolatus* (KU 177433), as used in *Hedges et al. (2008)* and *Padial et al. (2014)*, places this population near the type locality (Río El Salado). It shows strong support for nesting within the topotypic series provided here from Río Reventador. Like other *Pristimantis* speciation events in the upper Napo River basin *(Bejarano-Muñoz et al., 2022)*, we identified cryptic diversity in

Pristimantis prolatus complex, with at least one Unconfirmed Candidate Species (Fig. 1), by determining broad phylogenetic divergences with isolated populations from Guacamayos mountain range to Sangay National Park, compared with topotypic populations. The inclusion of the *Pristimantis burtoniorum* holotype sequence provides confidence about the phylogenetic relationships in the *Pristimantis (Cryptomantis) prolatus* species group, determining it as a monophyletic, well-supported clade (Fig. 1). Our evidence is robust to assign *sensu stricto* populations of *Pristimantis bicantus* and their phylogenetic relationships (Fig. 1; See comments on species group definition) within a diverse clade that also includes *P. tungurahua, P. bicantus, P. sacharuna, P. nelsongalloi, P. marcoreyesi*, *P. paganus* and four candidate species (*P*. c.sp. 2, *P*. c.sp. 3, *P*. c.sp. 4, *P*. c.sp. 5) in the *Pristimantis (Cryptomantis) bicantus* species group (Fig. 1). The new species, *Pristimantis paganus,* is endemic to the Guacamayos mountain range and Llanganates in the northeastern Andean foothills of Ecuador, 2157 – 2500 m a.s.l.

We highlight the need to include topotypic specimens when working with speciose groups, like *Pristimantis*, integrating several lines of evidence to test cryptic diversity hypotheses, to avoid underestimating species limits, promoting taxonomic instability and infer erroneous phylogenetic relationships. The recognition of vast cryptic diversity in *Pristimantis* in a geographically restricted region promote efforts to taxonomic studies and research toward unexplored areas *(Ortega et al., 2022; Páez & Ron, 2019; Reyes-Puig et al., 2022; Reyes-Puig et al., 2014)*, like the Colonso Chalupas Biological Reserve and Llanganates National Park in Ecuador.

CONCLUSIONS

We described a new species, *Pristimantis paganus,* from the Colonso Chalupas Biological Reserve and Llanganates National Park, northeastern Ecuador. ~~Also, we propose~~ a new subgenus, *Cryptomatis*, within *Pristimantis* that includes the *Pristimantis prolatus* and *Pristimantis bicantus* species groups. Our results indicate at least five Candidate species within *Cryptomantis*. We emphasize the importance of including topotypic specimens to analyze and compare specios groups to delimiting species, like *Pristimantis* ~~frogs~~.

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Figure 1

Phylogenetic relationships of the genus *Pristimantis*

(A) Phylogenetic relationships of ~~the genus~~ *Pristimantis,* detailed in Supplementary Figure SF1. (B) Maximum likelihood tree of the subgenus *Cryptomantis*, whereas the position of *Pristimantis paganus* sp. nov. is shown in blue. SH-aLRT support (%) / ultrafast bootstrap support (%) values are shown for each branch. The voucher specimen number for each terminal is shown before the species name. Topotypes and localities of collection are shown associated to each clade, in colors. Unconfirmed candidate species are shown in red.

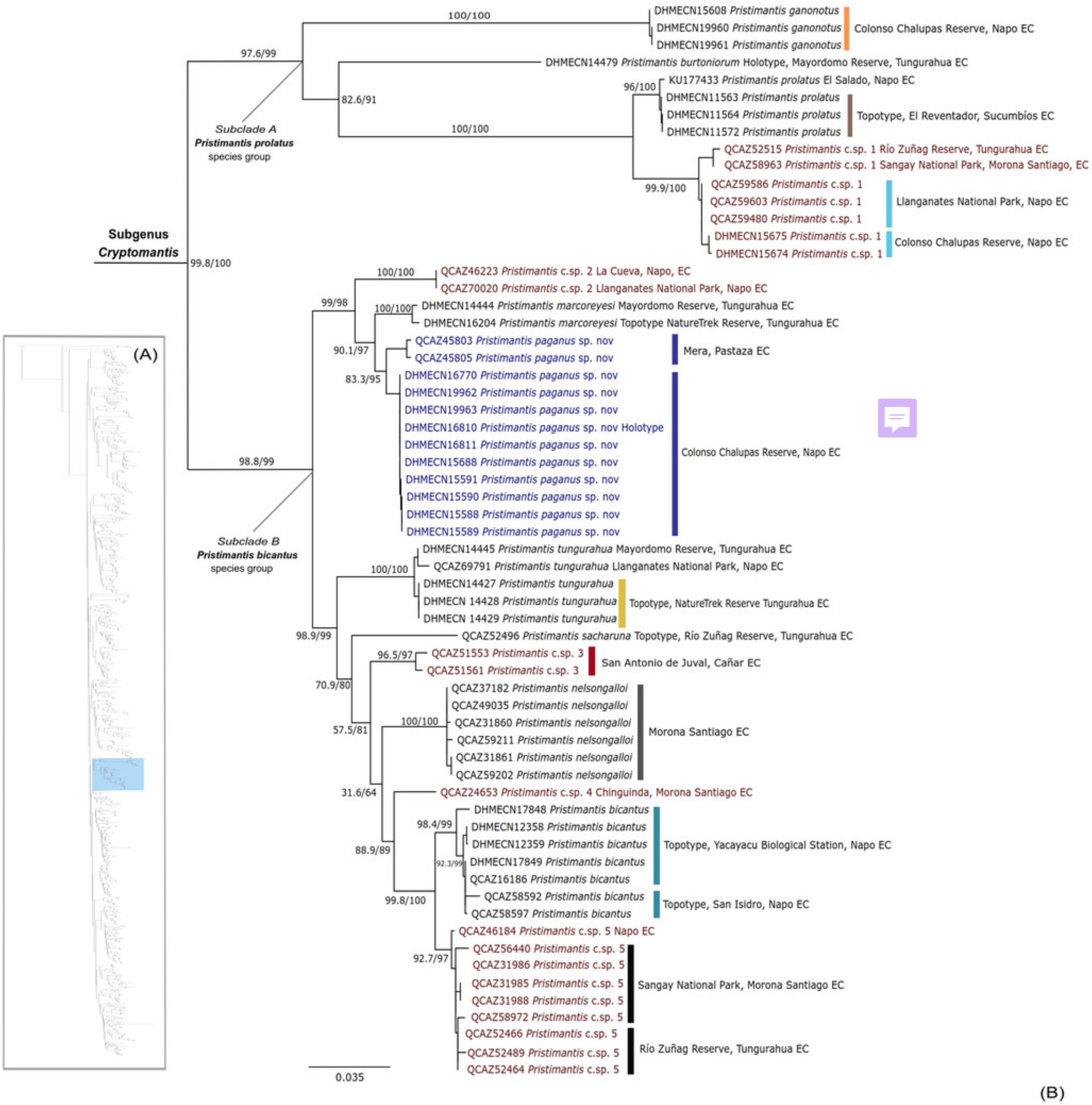
(A)
100/100
DHMECN15608 Pristimantis ganonotus
DHMECN19960 Pristimantis ganonotus
DHMECN19961 Pristimantis ganonotus
Colonso Chalupas Reserve, Napo EC
97.6/99
DHMECN14479 Pristimantis burtoniorum Holotype, Mayordomo Reserve, Tungurahua EC
96/100
KU177433 Pristimantis prolatus El Salado, Napo EC
82.6/91
DHMECN11563 Pristimantis prolatus
DHMECN11564 Pristimantis prolatus
DHMECN11572 Pristimantis prolatus
Topotype, El Reventador, Sucumbíos EC
100/100
Subclade A
Pristimantis prolatus
species group
QCAZ52515 Pristimantis c.sp. 1 Río Zuñag Reserve, Tungurahua EC
QCAZ58963 Pristimantis c.sp. 1 Sangay National Park, Morona Santiago, EC
99.9/100
QCAZ59586 Pristimantis c.sp. 1
QCAZ59603 Pristimantis c.sp. 1
QCAZ59480 Pristimantis c.sp. 1
Llanganates National Park, Napo EC
Subgenus
Cryptomantis
DHMECN15675 Pristimantis c.sp. 1
DHMECN15674 Pristimantis c.sp. 1
Colonso Chalupas Reserve, Napo EC
99.8/100
100/100
QCAZ46223 Pristimantis c.sp. 2 La Cueva, Napo, EC
QCAZ70020 Pristimantis c.sp. 2 Llanganates National Park, Napo EC
99/98
100/100
DHMECN14444 Pristimantis marcoreyesi Mayordomo Reserve, Tungurahua EC
DHMECN16204 Pristimantis marcoreyesi Topotype NatureTrek Reserve, Tungurahua EC
QCAZ45803 Pristimantis paganus sp. nov
90.1/97
QCAZ45805 Pristimantis paganus sp. nov
Mera, Pastaza EC
83.3/95
DHMECN16770 Pristimantis paganus sp. nov
DHMECN19962 Pristimantis paganus sp. nov
DHMECN19963 Pristimantis paganus sp. nov
DHMECN16810 Pristimantis paganus sp. nov Holotype
DHMECN16811 Pristimantis paganus sp. nov
DHMECN15688 Pristimantis paganus sp. nov
DHMECN15591 Pristimantis paganus sp. nov
DHMECN15590 Pristimantis paganus sp. nov
DHMECN15588 Pristimantis paganus sp. nov
DHMECN15589 Pristimantis paganus sp. nov
Colonso Chalupas Reserve, Napo EC
98.8/99
Subclade B
Pristimantis bicantus
species group
DHMECN14445 Pristimantis tungurahua Mayordomo Reserve, Tungurahua EC
QCAZ69791 Pristimantis tungurahua Llanganates National Park, Napo EC
100/100
DHMECN14427 Pristimantis tungurahua
DHMECN 14428 Pristimantis tungurahua
DHMECN 14429 Pristimantis tungurahua
Topotype, NatureTrek Reserve Tungurahua EC
98.9/99
QCAZ52496 Pristimantis sacharuna Topotype, Río Zuñag Reserve, Tungurahua EC
96.5/97
QCAZ51553 Pristimantis c.sp. 3
QCAZ51561 Pristimantis c.sp. 3
San Antonio de Juval, Cañar EC
70.9/80
QCAZ37182 Pristimantis nelsongalloi
QCAZ49035 Pristimantis nelsongalloi
100/100
QCAZ31860 Pristimantis nelsongalloi
57.5/81
QCAZ59211 Pristimantis nelsongalloi
QCAZ31861 Pristimantis nelsongalloi
QCAZ59202 Pristimantis nelsongalloi
Morona Santiago EC
31.6/64
QCAZ24653 Pristimantis c.sp. 4 Chinguinda, Morona Santiago EC
DHMECN17848 Pristimantis bicantus
98.4/99
DHMECN12358 Pristimantis bicantus
DHMECN12359 Pristimantis bicantus
88.9/89
92.3/99
DHMECN17849 Pristimantis bicantus
QCAZ16186 Pristimantis bicantus
Topotype, Yacayacu Biological Station, Napo EC
99.8/100
QCAZ58592 Pristimantis bicantus
QCAZ58597 Pristimantis bicantus
Topotype, San Isidro, Napo EC
QCAZ46184 Pristimantis c.sp. 5 Napo EC
QCAZ56440 Pristimantis c.sp. 5
92.7/97
QCAZ31986 Pristimantis c.sp. 5
QCAZ31985 Pristimantis c.sp. 5
QCAZ31988 Pristimantis c.sp. 5
QCAZ58972 Pristimantis c.sp. 5
Sangay National Park, Morona Santiago EC
QCAZ52466 Pristimantis c.sp. 5
QCAZ52489 Pristimantis c.sp. 5
QCAZ52464 Pristimantis c.sp. 5
Río Zuñag Reserve, Tungurahua EC
0.035
(B)

Figure 2

Representative species of the subgenus *Cryptomantis*.

Clade A-*Pristimantis prolatus* species group: (A) *Pristimantis ganonotus* DHMECN 16961, Colonso Chalupas Reserve, Napo; (B) *P. burtoniorum* DHMECN 14479, Paratype, Mayordomo Reserve, Tungurahua; (C) *P. prolatus* DHMECN 11564, Topotype, Río El Reventador, Sucumbíos; (D) *P*. c.sp. 1, DHMECN 15674, Colonso Chalupas Reserve, Napo. Clade B-*Pristimantis bicantus* species group: (E) *P*. c.sp. 2, QCAZ 70020, Llanganates Natinal Park, Napo; (F) *P. marcoreyesi*, DHMECN 13833, Tungurahua volcano, Tungurahua; (G) *P. paganus* sp. nov. DHMECN 15606, Colonso Chalupas Reserve, Napo; (H) *P. tungurahua*, DHMECN 14428, Vizcaya Reserve, Tungurahua; (I) *P. sacharuna*, QCAZ 52496, Zúñag Reserve, Tungurahua; (J) *P*. c.sp. 3, QCAZ 51553, San Antonio de Juval, Cañar; (K) *P. nelsongalloi*, DHMECN 5223, Zúñag Reserve, Tungurahua; (L) *P. bicantus*, DHMECN 12359, El Reventador, Sucumbíos; (M) *P*. c.sp. 5, QCAZ 52489, Sangay National Park, Morona Santiago. Photographs: Gustavo Pazmiño - Bioweb (E), Santiago Ron - Bioweb (I), Bioweb (J, M), Keyko D. Loza-Carvajal (A), Mario H. Yánez Muñoz (B, C, F-H, K, L), H. Mauricio Ortega-Andrade (D, G).

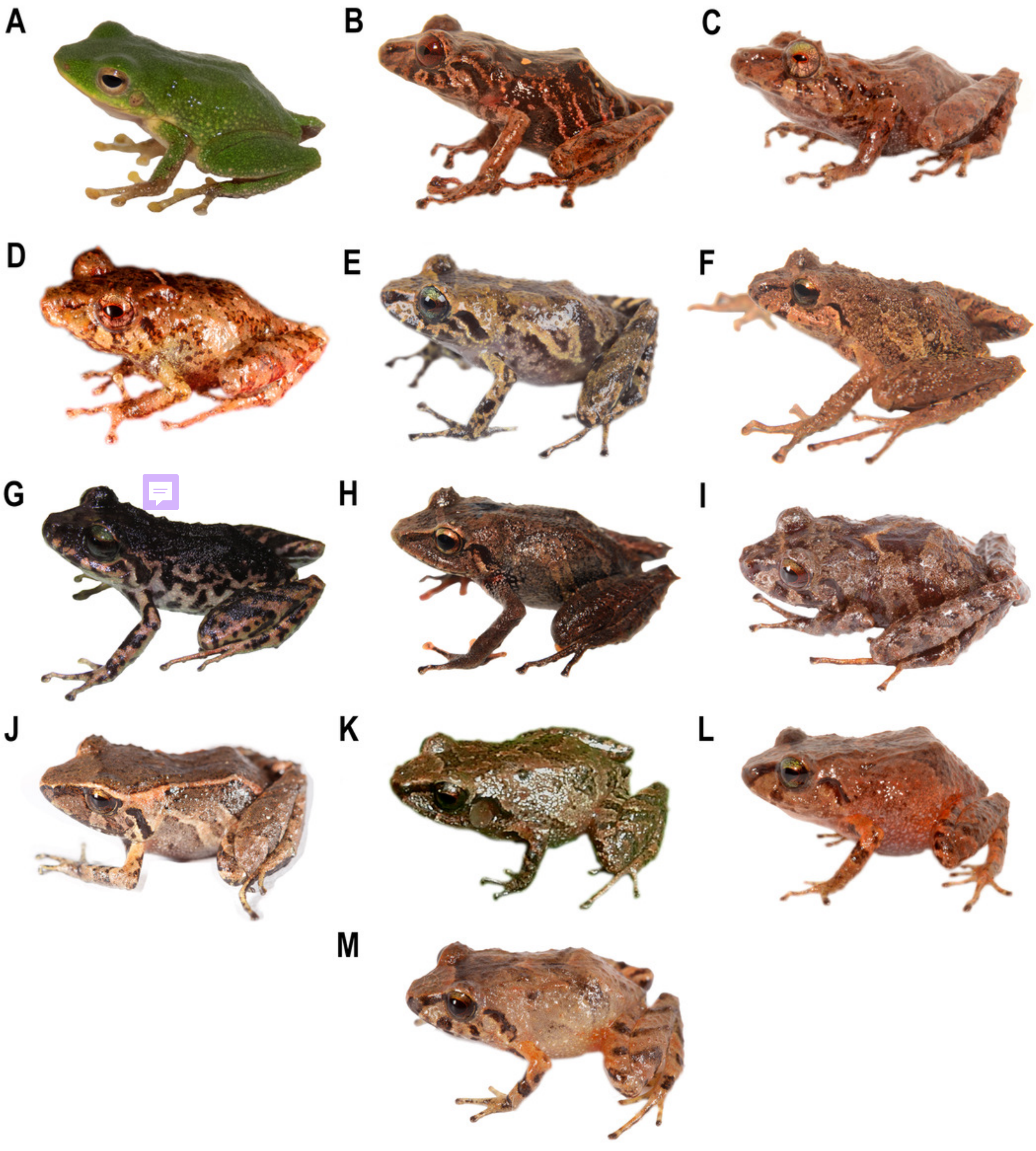
A
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Figure 3

Views of the holotype.

Dorsal, ventral and lateral views of the holotype (DHMECN 16810) of *Pristimantis paganus* sp. nov., an adult female in preserved (SVL = 27.2 mm). Photographs: Mario H. Yánez Muñoz.

Figure 4

Details of the Holotype.

Details of the hand, foot, dorsum and head are seen in profile of the holotype DHMECN 16810. Photographs: Mario H. Yánez Muñoz.


3 mm

Figure 5

Holotype in life of *Pristimantis paganus* sp. nov.

An adult female (DHMECN 16810, SVL = 27.2 mm). (A) dorsal view; (B) frontal view; (C) lateral view; and (D) ventral view. Photographs: Mario H. Yánez Muñoz.

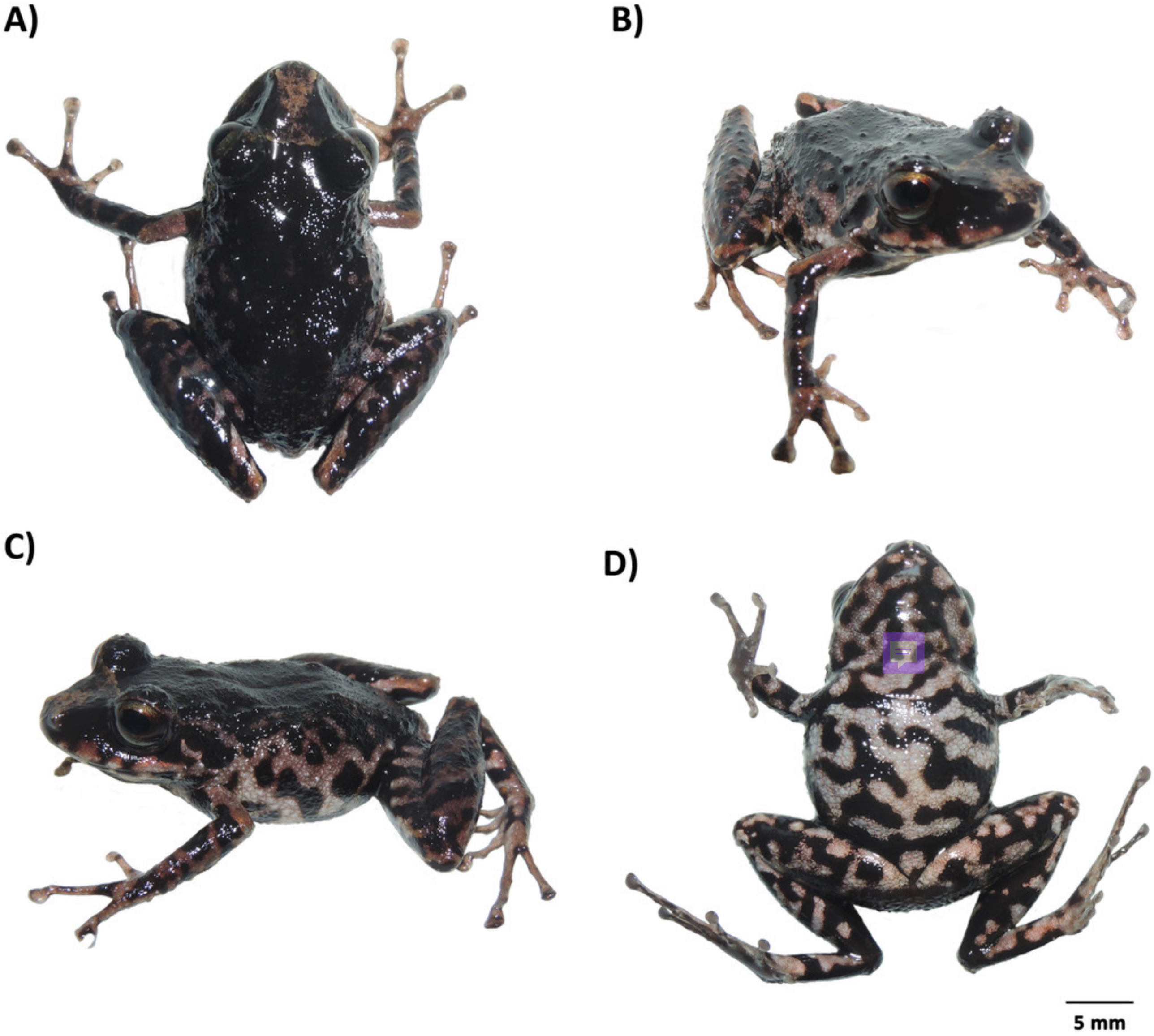

Figure 6

Variation of dorsal and ventral coloration in preserved specimens corresponding to the type series of *Pristimantis paganus* sp. nov.

From left to right: (A) DHMECN 16810, female, holotype, SVL = 27.2 mm; (B) DHMECN 15606, female, paratype, SVL = 29.8 mm; (C) DHMECN 16811, female, paratype, SVL = 24.8 mm; (D) DHMECN 15605, male, paratype, SVL = 21.4 mm; (E) DHMECN 16812, male, paratype, SVL = 20.2 mm; (F) DHMECN 16812, male, paratype, SVL = 20.2 mm; (G) DHMECN 16814, male, paratype, SVL = 19.1 mm. (H) DHMECN 16813, male, paratype, SVL = 17.2 mm. Photographs: Mario H. Yánez Muñoz (A, C, E-G), H. Mauricio Ortega-Andrade (B, D).

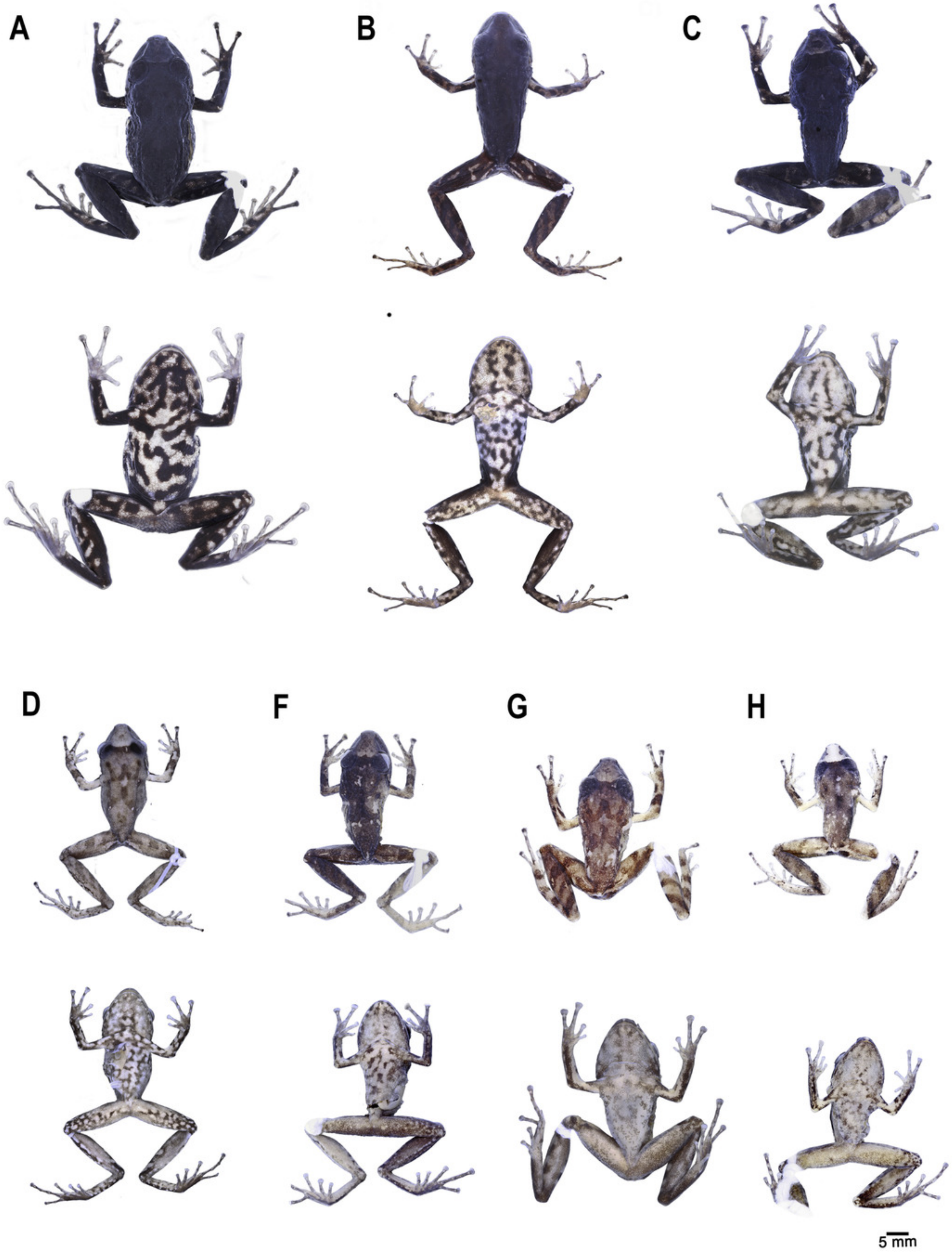
A
B
C
D
F
G
H
5 mm

Figure 7

Dorso-lateral and ventral coloration of *Pristimantis paganus* sp. nov. in life.

(A) DHMECN 15598, female, paratype, SVL = 29.2 mm; (B) DHMECN 15600, female, paratype, SVL = 29.8 mm; (C) DHMECN 15601, male, paratype, SVL = 20.1 mm; (D) DHMECN 16813, male, paratype, SVL = 17.2 mm. Photograph: H. Mauricio Ortega-Andrade (A-C), Mario H. Yánez Muñoz (D).

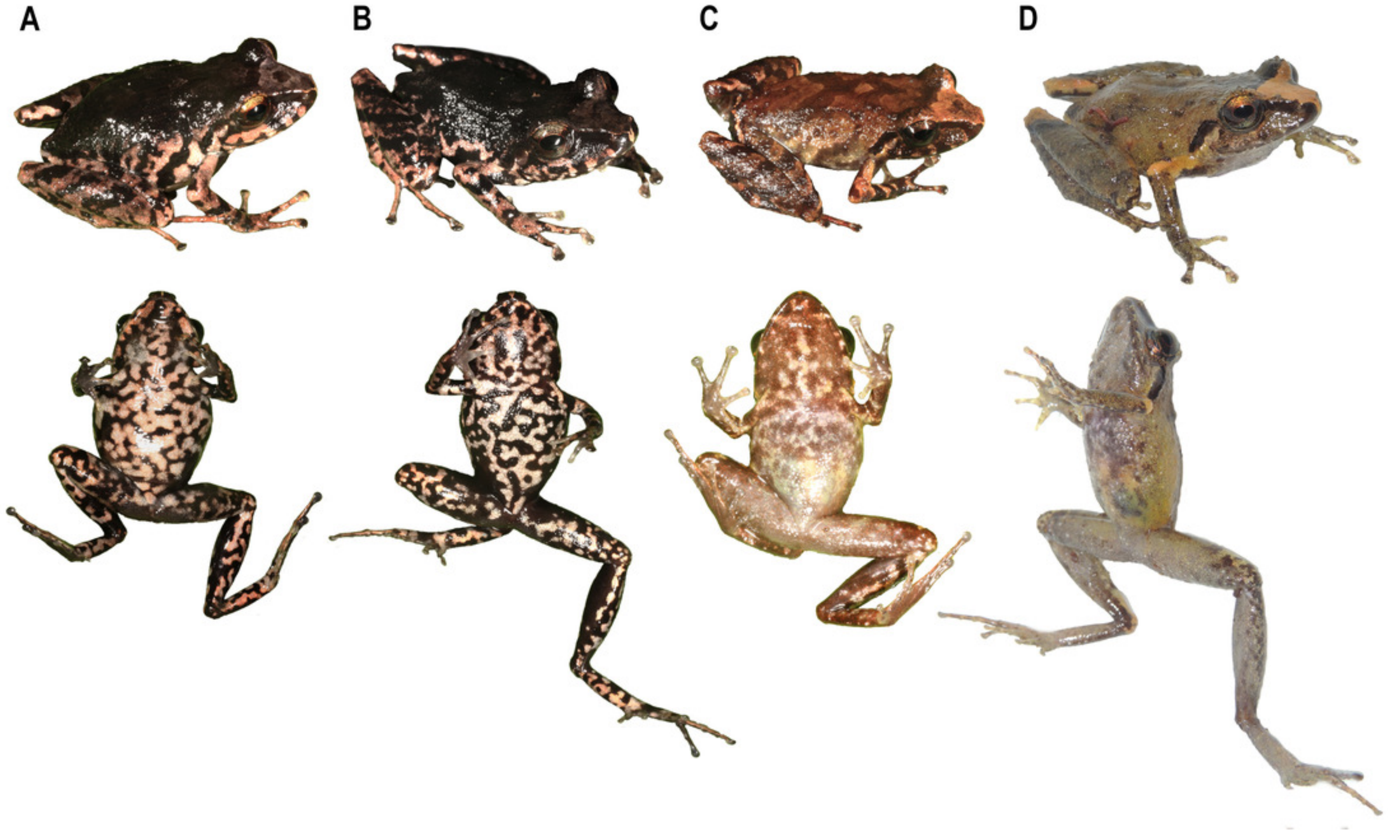

Figure 8

Morphometric comparisons between males and females of *Pristimantis paganus* sp. nov.

(A) Principal component analysis, with Snout vent length (SVL), Tibia length (TL) and Femur length (FL) as variables which most explain data variance (PC1 and PC2=98%); ellipsoids represent 95% of confidence limits; (B) Correlogram of morphometric measurements with Linear r statistics represented as colored ellipsoids; Boxplot comparisons of (C) Snout vent length (SVL) and (D) Femur length (FL) of males and females; asterisk represent statistical differences inferred by U Mann-Whitney test.

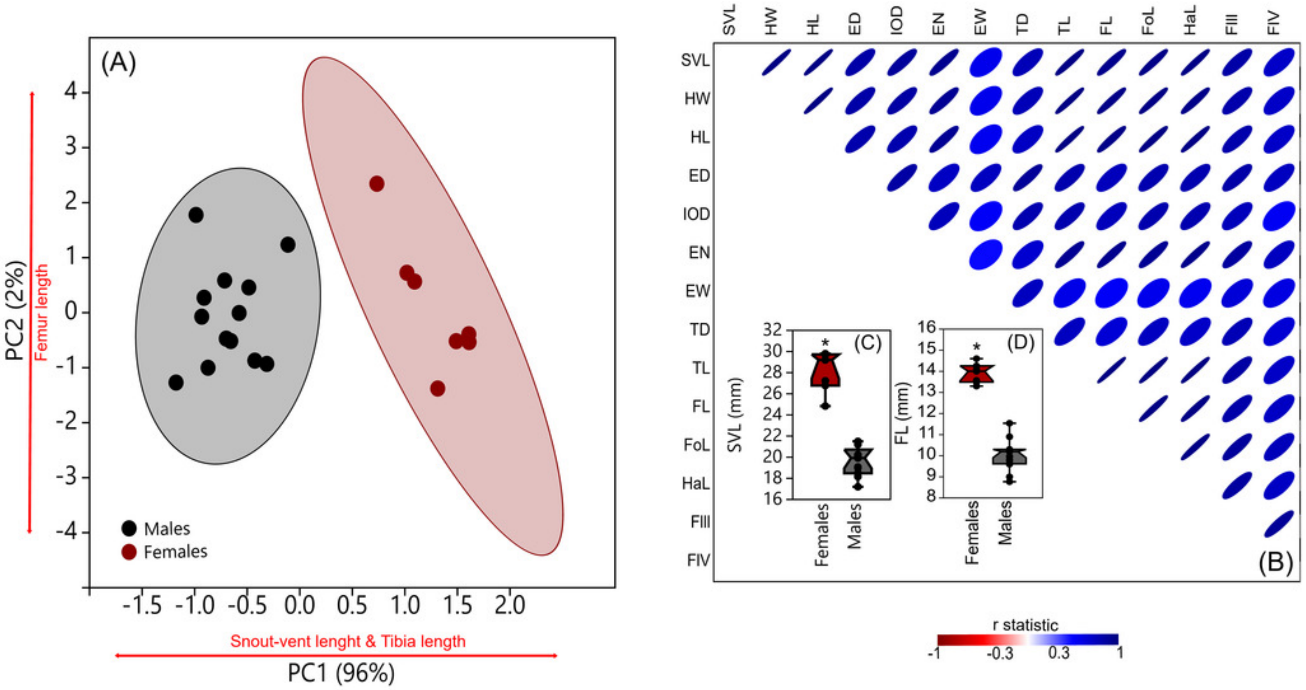

Figure 9

Distribution of the subgenus *Pristimantis* (*Cryptomantis*) ~~frogs~~ in eastern Ecuador (blue rectangle)

(A) Clade A, corresponding to localities of the *Pristimantis prolatus* species group; (B) Clade B, corresponding to localities of the *Pristimantis bicantus* species group; (C) Localities of species from the Clade B, *Pristimantis bicantus* species group in the Pastaza valley (Red rectangle in B). Major cities or towns in the eastern flanks of the Andes are marked as white circles with a dot. Colors refer to the clades in Figure 1.

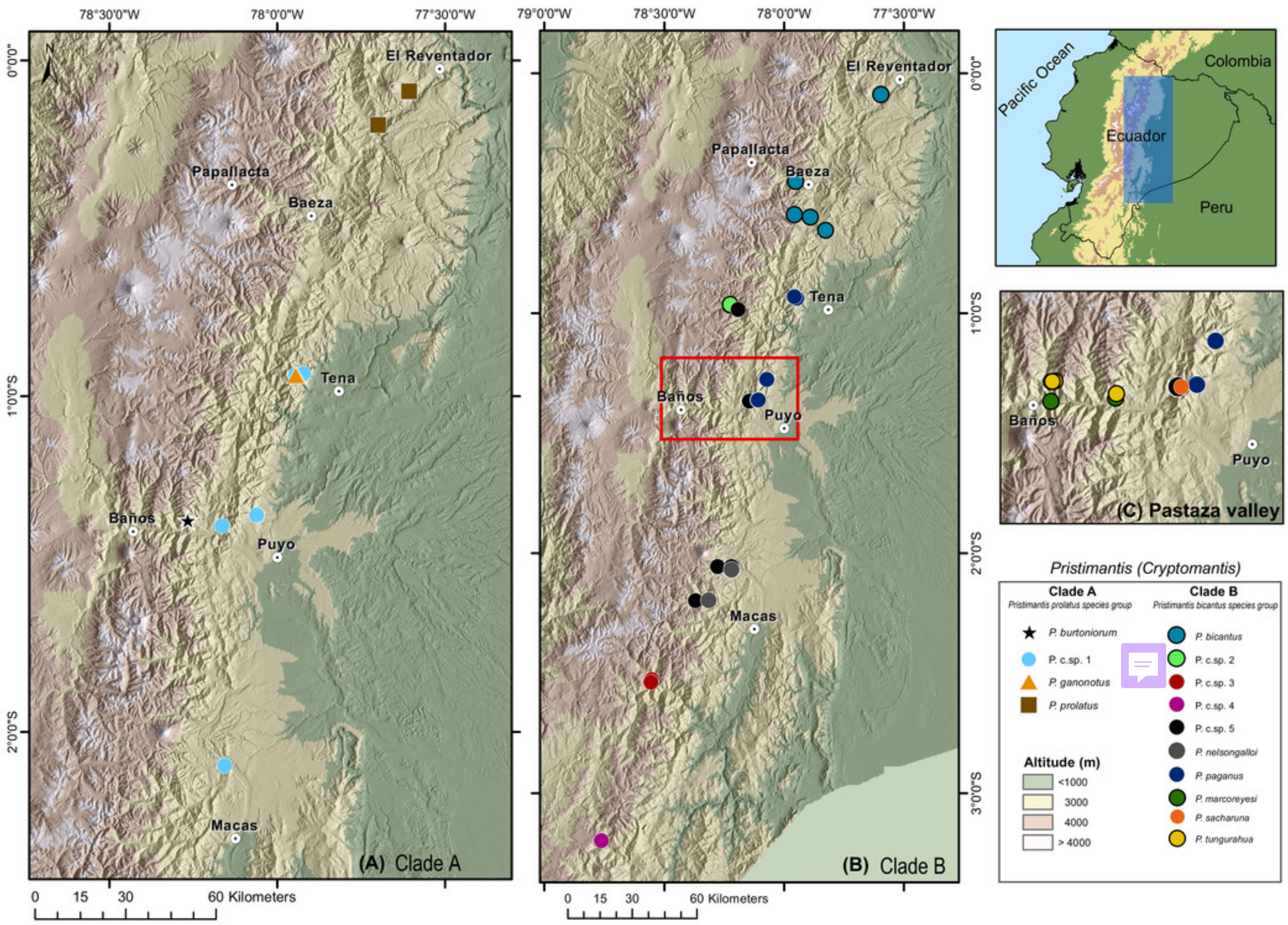

Figure 10

Habitat and specimens of *Pristimantis paganus* sp. nov.

(A) Cloud forest with epiphytic plants, type locality, Colonso Chalupas Biological Reserve; (B) Female, DHMECN19962; (C) Paratype, male, DHMECN 15602; (D) Paratype, adult female, DHMECN 17222. Photographs: Jorge Brito (A), Keyko D. Loza-Carvajal (B), H. Mauricio Ortega-Andrade (C), Zane Libke (D)

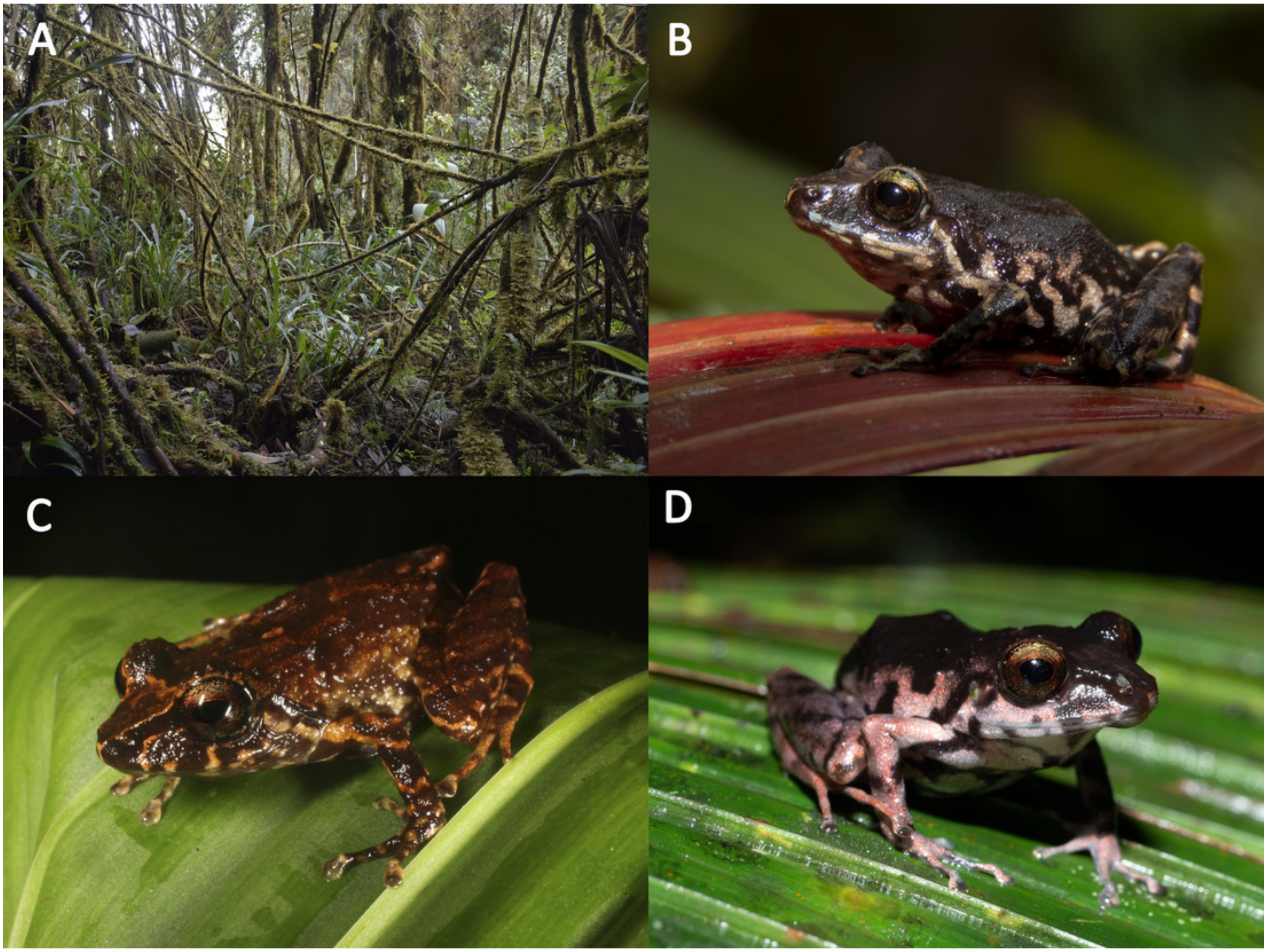

Table 1 (on next page)

Qualitative morphological diagnostic characters.

Comparison of *Pristimantis paganus* sp. nov. and its nominal sister species in the *Cryptomantis* subgenus.

Species	SVL (mm)		Snout in dorsal view	Snout in profile view	Disc shape	Upper eyelid tubercles	Lateral cutaneous ridges on hands	Ulnar tubercles	Tubercles in heels	Tubercles on external border of the tarsus	Altitudinal range (m.a.s.l.)
	Female	Male									
P. paganus sp. nov.	24.84–29.8	17.20–21.5	Subacuminate	Rounded	Truncated	Present, 3 to 4 rounded tubercles surrounded by several small ones.	Present	Absent	Subconical tubercle	Two flattened tubers	2157 – 2495
P. bicantus	17.0–21.7	12.0–15.8	Rounded	Rounded	Slightly expanded	Present, low tubers	Absent	Absent	Absent	Absent	2000 – 2300
P. burtoniorum	20.8–27.0	16.6–17.4	Subacuminate	Subacuminate	Expanded discs	Present, three to four large subconical tubers	Present	Small, conical	Small conical tuber	Present, round-shaped	2940 – 2970
P. ganonotus	-	14.0-15.2	Acuminate	Protruding	Expanded	Absent	Absent	Indistinct	Nonconical	Absent	2940 – 2970
P. marcoreyesi	22.8–30.8	14.3–22.2	Subacuminate	Angular	Slightly wider than the digits	Present, two or more flattened to subconical tubercles present	Present	Small, flattened	Presents, rounded	Present, weakly defined	2500 – 3100
P. nelsongalloi	12.0–17.0	18.5–21.7	Subacuminate	Rounded	Lanceolate discs	Present, four or five small supraoculars, flat and low	Absent	Present, small	One subconical and several small tubers	Absent	1627 – 1800
P. prolatus	20.8–24.1	13.7–18.4	Acuminate	Truncated	Expanded	Present, one conical	Absent	Absent	Present, conical	Present	1140 – 1933
P. sacharuna	18.5–19.5	-	Subacuminate	Slightly rounded	Slightly wider than the digits, expanded, rounded	Present, one subconical	Present	Present, subconical	Subconical tubercle	Two to 3 subconical tubers	2200

P. tungurahua	24.4–27.9	17.1–20.8	Subacuminate	Protuberant	Rounds, expanded	Present, one or two subconical tubercles	Absent	Present, low	Present, conical	Present, conical	2500 – 2750

Table 1. Qualitative morphological diagnostic characters used for comparison of *Pristimantis paganus* sp. nov. and its nominal sister species in the *Cryptomantis* subgenus.

Table 2(on next page)

Morphometric measurements (millimeters) of the type series of *Pristimantis paganus* sp. nov.

Range of measurements (maximum, minimum, mean and ± standard deviation).

TABLE 2. Morphometric measurements (millimeters) of the type series of *Pristimantis paganus* sp. nov. Range of measurements (maximum, minimum, mean and ± standard deviation).

Measures	Males n = 13	Females n = 7	Juveniles n = 3
Snout-vent length (SVL)	18.10-21.50 (19.59 ±1.33)	24.84-29.8 (28.18±1.93)	13.54-16.71 (15.46±1.37)
Head width (HW)	6.52-7.97 (7.6±0.56)	9.52-11.80 (10.66±0.76)	5.61-6.33 (5.96±0.29)
Head length (HL)	7.08-8.89 (8.3±0.56)	9.75-11.9 (10.97±0.6)	6.63-7.14 (6.80±0.23)
Horizontal eye diameter (ED)	2.89-4.00 (3.85±0.21)	3.73-4.9 (4.23±0.49)	2.35-3.24 (2.91±0.40)
Interorbital distance (IOD)	2.04-2.70 (2.6±0.14)	2.59-3.08 (3.32±0.55)	1.72-2.05 (1.96±0.17)
Eye-nostril distance (EN)	1.8-2.69 (2.2±0.56)	3.2-3.85 (2.53±0.40)	1.54-1.90 (1.77±0.16)
Eyelid width (EW)	1.76-3.00 (2.35±0.35)	1.92-3.20 (2.53±0.40)	1.26-1.47 (1.34±0.09)
Tympanic diameter (TD)	0.84-1.70 (1.70±0.2)	1.45-2.30 (1.70±0.26)	1.84-0.93 (0.89±0.04)
Tibia length (TL)	9.71-11.90 (11.5±0.56)	15.07-16.60 (15.72±0.59)	7.44-9.54 (8.69±0.90)
Femur length (FL)	8.77-1.54 (9.6±0.84)	13.30-14.60 (13.89±0.43)	7.13-8.64 (7.88±0.61)
Foot length (FoL)	8.23-10.77 (9.20±1.13)	13.38-14.7 (13.78±0.43)	6.76-7.87 (7.43±0.48)
Hand length (HaL)	4.78-6.76 (5.55±0.49)	7.27-8.40 (8.09±0.35)	3.83-4.99 (4.46±0.47)
Finger disc width III (F3D)	0.76-1.10 (0.95±0.21)	1.11-1.40 (1.20±0.10)	0.66-0.72 (0.68±0.02)
Finger disc width IV (T4D)	0.51-0.98 (0.8±0.14)	0.87-1.00 (1.04±1.10)	0.57-0.63 (0.60±0.02)