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Molecular phylogenetics of the *Pristimantis lacrimosus* species group (Anura: Strabomantidae)

Proyecto de investigación

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A mi madre y hermana, Laila Samuelsson y Emelie Samuelsson

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RESUMEN

El género *Pristimantis* es uno de los vertebrados más diversos a lo largo del Neotrópico. Sin embargo, su filogenia aun no es concluyente y se les ha posicionado en distintos grupos de acuerdo con sus caracteres genéticos y morfológicos. En este estudio, se realizó un análisis genético del grupo de especies *Pristimantis lacrimosus*. Este grupo está compuesto por 25 especies que habitan en el neotrópico y se caracterizan por ser bromelícolas. Estudios recientes apuntan que este es un grupo no monofilético; sin embargo, muchas de las especies que tentativamente pertencen al grupo, no han sido incluídas en los análisis de su filogenia. En este estudio se incluyó por primera vez a *Pristimantis lacrimosus* y cinco especies adicionales, de las estribaciones occidentales andinas y tierras bajas del Amazonas de Ecuador y Perú. Obtuvimos secuencias mitocondriales (16S y COI) e inferimos un nuevo a árbol, bajo un modelo bayesiano. Los resultados sugieren que existen dos clados no relacionados entre si. El Clado A se posiciona como un nuevo grupo (*Pristimantis boulengeri*) e incluye a una nueva especie aun no descrita que habita en las estribaciones andinas del Ecuador. Por otro lado, el Clado B corresponde al grupo de especies *Pristimantis lacrimosus*, el cual redefinimos.

Palabras clave: Filogenia, Taxonomia, *Pristimantis lacrimosus*, marcadores moleculares, citocromo oxidasa I, 16S.

ABSTRACT

The genus Pristimantis is one of the most diverse vertebrates along the Neotropics. However, their phylogeny is not yet conclusive and they have been placed in different groups according to their genetic and morphological characteristics. In this study, a genetic analysis of the species group *Pristimantis lacrimosus* was carried out. This group is composed of 25 species that inhabit the Neotropics and are characterized by being bromeliads. Recent studies indicate that this is a non-monophyletic group; however, many of the species that tentatively belong to the group have not been included in the analysis of their phylogeny. This study included for the first time *Pristimantis lacrimosus* and five additional species from the western Andean foothills and Amazon lowlands of Ecuador and Peru. We obtained mitochondrial sequences (16S and COI) and inferred a new tree, under a Bayesian model. The results suggest that there are two non- related clades. Clade A is positioned as a new group (*Pristimantis boulengeri*) and includes a new species not yet described that inhabits the Andean foothills of Ecuador. On the other hand, Clade B corresponds to the group of species *Pristimantis lacrimosus*, which we redefine. In this work we redefine the group *P. lacrimosus* including its type species.

Keywords: Phylogeny, Taxonomy, *Pristimantis lacrimosus*, molecular markers, cytochrome oxidase I, 16S.

TABLE OF CONTENTS

Resumen **6**
Abstract **7**
Introduction **11**
Objectives **13**
Methods **14**
Field work……..... **14**
Laboratory procedures……..... **14**
Phylogenetic analysis……. **15**
Results..... **17**
Discussion **19**
Referencias bibliográficas..... **20**

LIST OF TABLES

Appendix 1. GenBank accession number of each gen used in our phylogenetic analysis **.....24**

Appendix 2. Best fit model for each partition name obtained with partition finder 2.1.1. **.....37**

Appendix 3. Morphological characteristics of species that belongs to the *Pristimantis lacrimosus* species group**.....38**

Appendix 4. Uncorrected genetic distance from P. lacrimosus species group. Lower left matrix presents the genetic distance for 16S. Upper right matrix presents the genetic distance of COI (Citocrome c oxidase)**……...39**

LIST OF FIGURES

Figure 1. Map of the Chamana Reserve at the province of Tungurahua, Ecuador **.....41**

Figure 2. Map of Rio el Topo (Reserva Zuñac) at the province of Tungurahua, Ecuador**……42**

Figure 3. phylogenetic relationships of the genus *Pristimantis* based on a concatenated matrix of the mitochondrial gene 16S and COI**.** Species in the *P. lacrimosus* (purple) and *P. boulengeri* (pale green) groups, and new species (yellow) are highlighted**……...43**

INTRODUCTION

Pristimantis is the most diverse amphibian genus, with 533 described species, and a Neotropical distribution (Frost, 2019). Although no morphological trait has been identified as a synapomorphy for the genus, its monophyly is well-supported by several molecular studies (Heinicke *et al*., 2007; Pyron & Wiens, 2011; Canedo & Haddad, 2012; Pinto-Sánchez *et al*., 2012; Padial *et al*., 2014). Moreover, the loss of the D-stem of the tRNAcys gene has been proposed as molecular synapomorphy for *Pristimantis* (Crawford *et al*., 2010).

Because of the taxonomic complexity, and gargantuan diversity; of *Pristimantis,* the genus has been divided into eleven species groups, where only seven have been tested for monophyly (Rivera- Correa, *et al*., 2017; Padial, *et al*., 2014). Also, most *Pristimantis,* 312 species have not been assigned to any group (Padial *et al*., 2014).

In this study, I focus on *Pristimantis lacrimosus* group, which was first recognized as the *lacrimosus* assembly by Lynch and Duellman (1980), and that now contains the following 29 species: *P. acuminatus* (Shreve, 1935), *P. apiculatus* (Lynch and Burrowe, 1990), *P. aureolineatus* (Guayasamin, Ron, Cisneros-Heredia, Lamar and McCracken, 2006), *P. boulengeri* (Lynch, 1981), *P. brevifrons* (Lynch, 1981), *P. bromeliaceus* (Lynch, 1979), *P. dorsopictus* (Rivero and Serna, 1988 "1987"), *P. ecuadorensis* (Guayasamin, Hutter, Tapia, Culebras, Peñafiel, Pyron, Morochz, Funk, and Arteaga-Navarro, 2017), *P. enigmaticus* (Ortega-Andrade, Rojas-Soto, Valencia, Espinosa de los Monteros, Morrone, Ron, and Cannatella, 2015), *P. eremitus* (Lynch, 1980), *P. deyi* (Lehr, Gregory and Catenazzi, 2013), *P. lacrimosus* (Jiménez de la Espada, 1875), *P. latericius* (Batallas and Brito, 2014), *P. limoncochensis* (Ortega-Andrade, Rojas-Soto, Valencia, Espinosa de los Monteros, Morrone, Ron, and Cannatella, 2015), *P. mendax* (Duellman, 1978), *P. mindo* (Arteaga, Yánez-Munoz and Guayasamin, 2013), *P.*

olivaceus (Köhler, et al., 1998), *P. omeviridis* (Ortega-Andrade, Rojas-Soto, Valencia, Espinosa de los Monteros, Morrone, Ron, and Cannatella, 2015), *P. padiali* (Moravec, Lehr, Pérez-Peña, López, Gagliardi-Urrutia and Arista-Tuanama, 2010), *P. pardalinus* (Lehr, Lundberg, Aguilar and von May, 2006), *P. petersi* (Lynch and Duellman, 1980), *P. phoxocephalus* (Lynch, 1979), *P. royi* (Morales, 2007), *P. pseudoacuminatus* (Shreve, 1935), *P. schultei* (Duellman, 1990), *P. tantanti* (Lehr, Torres-Gastello and Suárez-Segovia, 2007), *P. tayrona* (Lynch and Ruiz-Carranza, 1985), *P. urani* (Rivera-Correa and Daza-R, 2016) *P. waoranii* (McCracken, Forstner and Dixon, 2007), and *P. zimmermanae* (Heyer and Hardy, 1991).

Most species in the lacrimosus Group have a shared morphology that has been attributed to their habitat preferences (i.e., bromeliads; Lynch & Duellman, 1980; Lynch 1980). These ecomorphological traits include, most conspicuously, a flat and pointy head. Padial et al. (2014) redefined the *P. lacrimosus* Group and proposed the following diagnostic traits: acuminate snout, smooth dorsal skin, round and ovate finger and toe discs. Although their final redefinition includes 25 species (see below), only a fraction (8 species) were included in their phylogeny. Subsequently, Rivera-Correa & Daza (2016), with an improved taxon sampling, obtained a topology where the *P. lacrimosus* Group (sensu Padial et al. 2014) is formed by two non-sister clades; thus, the lacrimosus Group is not monophyletic (Rivera-Correa & Daza, 2016).

Herein, we infer the evolutionary relationships of the the *lacrimosus* group, with an expanded taxon sampling that includes, for the first time, the type species of the group (i.e., *Pristimantis lacrimosus*). This addition allows us to unambiguosly define the group. It is also relevant to highlight that *Pristimantis lacrimosus* is the type species of the genus *Cyclocephalus* (Jímenez de la Espada, 1875); thus, in the hypothetical scenario of a splinting of *Pristimantis*, the genus *Cyclocephalus* could be assigned and defined easily.

OBJECTIVES

2.1 GENERAL OBJECTIVE

Evaluate the phylogeny and species diversity of the species group, with and increased taxon sampling.

2.2 SPECIFIC OBJECTIVES

- Analyze the monophyly of the *Pristimantis lacrimosus* species group.
- Redefine the species content of the *Pristimantis lacrimosus* group.
- Asses the undescribed diversity of the *Pristimantis lacrimosus* group

METHODS

Field work

Taxon sampling was increased through field surveys conducted at Reserva Bosque Chamana (1°26'24.2"S 78°22'04.1"W) (Fig 1) and Río Topo (1°24'38.23"S 78°11'33.65"W) (Fig 2) located in the province of Tungurahua, Ecuador.

The specimen collection (anurans) consisted in the capture of each individual and placed in a plastic bag adding leaf litter. Location, elevation and coordenates were taken with GPS for each specimen. All samples were obtained during the night from 7:00 to 11:59 p.m. Specimens were sacrificed with 2% lidocaine and preserved in 70% alcohol. Tissue were stored in 95% alcohol for further genetic analysis. Sex was determined by the presence of vocal slits, a trait present only in adult males; adult females were identified by the presence of convoluted oviducts. All collected specimes were stored at the Museo de Zoologia USFQ (ZSFQ), Universidad San Francisco de Quito and Instituto Nacional de Biodiversidad (INABIO), Quito, Ecuador.

Laboratory procedures

For DNA extraction we followed the Guanidinium Thiocyanate protocol described by Chomczynski (1993) for the isolation of biological samples. To analyze the quality and concentration of DNA, each extraction was quantified in the Nanodrop 2000. Then, we amplified the mitochondrial region fragment of cytochrome c oxidase subunit 1 (COI) gene, and a partial sequence of rRNA gene 16S.

The DNA amplification for cytochrome c oxidase (COI) followed the protocol of Pinto-Sanchez *et al*. (2012), using the forward primer dgLCO 1490 (GGTCAACAAATCATAAAGAYATYGG) and reverse primer dgHCO 2190 (TAAACTTCAGGGTGACCAAARAAYCA) (Meyer & Paulay, 2005). Each PCR reaction contained a final concentration of 2 mM MgCl2, 0,5 mM dNTP, 0,10 U / µL *Taq*

DNA polymerase (Invitrogen) and 1 µM of each primer, in a total volume of 25 µL. DNA amplification for this gene included an initial denaturation for 5 min at 94°C followed by 32 cycles of 94°C for 30 s, annealing at 52°C and extension at 72°C for 60 s. Afterwards a final extension of 72°C for 7 min was performed (Pinto- Sanchez *et al*., 2012).

The amplification for 16S gene followed the protocol of Guayasamin *et al*. (2017) with the primers 16SC (GTRGGCCTAAAAGCAGCCAC) and 16Sbr-H (CCGGTCTGAACTCAGATCACGT) designed by Darst & Canatella (2004) and Palumbi *et al* (1991). Each PCR reaction contained a final concentration of 1,5 mM MgCl2, 0,5 mM dNTP, 0,25 U / µL *Taq* DNA polymerase (Invitrogen) and 0,2 µM of each primer, in a total volume of 25 µL. DNA amplification for this gene included an initial denaturation for 4 minutes at 94 ° C; followed by 1 minute at 94 ° C, 30 seconds at 57 ° C, 2 minutes at 72 ° C for 30 cycles, and a final extension of 8 minutes at 72 ° C.

PCR amplicon results were confirmed through electrophoresis in a 2% agarose gel. All samples were cleaned with Exosap and sent to Macrogen Inc for sequencing. Chromatographs were manually edited and aligned in Geneious 11.1.5 (Kearse, *et al*. 2012).

Phylogenetic analysis

As outgroups, we included all *Pristimantis* with COI and 16S sequences available at GenBank (Appendix 1). Our dataset included 227 terminals, with some species having more than one sequence (Appendix 1). Sequences were aligned using MAFFT version 7 under the structural alignment Q-INS-I (Avaliable at: https://mafft.cbrc.jp/alignment/server/index.html). Additional manual verification of the alignment was performed in Mesquite project version 3.6 (Maddison and Maddison, 2018). To obtain the best partition scheme, we used Partition Finder under the Bayesian information criterion (BIC) (Lanfear *et al*., 2012). We implemented the best model for

phylogenetic analysis in IQ-Tree v1.6.11 (Ronquist *et al*., 2012). Phylogenetic inferece was analized under a Maximum likelihood (ML) criterium. Robustness and reliability were performed under 10000 bootstrap approximation (Hoang *et al*., 2017).

RESULTS

Evolutionary relationships

The concatenated results of both genes contained 1556 aligned sites: 896 characters for 16S and 660 characters for COI. The best fit model scheme for each gene is shown in Appendix 2. The inferred bayesian tree is shown in Figure 3. The *lacrimosus* group *sensu lato* is formed by two non- sister monophyletic clades. Therefore, a redefintion of *lacrimosus* group is necessary.

The *Pristimantis lacrimosus* group

Species content: Given the phylogeny shown in figure 3, the redefined species content of the *lacrimosus* groups is the following: *Pristimantis acuminatus, P. aureolineatus, P. bromeliaceus, P. ecuadorensis, P. enigmaticus, P. galdi, P. lacrimosus, P. limoncochensis, P. cf. mendax, P. mindo, P. moro, P. omeviridis, P. ornatissimus, P. petersi, P. schultei, P. subsigillatus, P. tantanti, P. waoranii* and *P. zeuctotylus.*

Morphological diagnosis

Considering the redefined species composition of the *P. lacrimosus*, we define it as following: dorsal skin shagreened or smooth; absence of dorsolateral folds (present in *omeviridis* and *zeuctotylus*); skin of venter areolate or coarsely aerolate*;* tympanic annulus and membrane evident (absent in *acuminatus* and not visible in *limoncochensis*); snout truncate, acuminate or subacuminate; upper eyelids lack tubercles; cranial crests absent (present in P. *galdi*); vomerine teeth present; vocal slits and nuptial pads present (absent in *enigmaticus, omeviridis, acuminatus, limoncochenis* and *tantanti*); first finger shorter than second; toes with lateral fringes; Toe V longer than Toe III. For more information see Appendix 3.

Species content of the *Pristimantis boulengeri* group

Species content: Our analysis supports the validity of the *Pristimantis boulengeri* group, first proposed by González-Duran *et al.* (2017). The species included in this group are: *Pristimantis angustilineatus, P. boulengeri, P. brevifrons, P. dorsopictus* and *P. urani.*

Morphological diagnosis: The *P. boulengeri* species group diagnosis is based on the following traits: snout with small papilla; absence of dorsolateral folds; ventral skin coarsely areolate; males with nuptial pads; presence of vocal slits and extended external vocal sac; ulnar tubercles present (absent in *P. brevifrons*); double distal subarticular tubercle on Fingers III and IV (polymorphic in *P. brevifrons*); heel tubercle present (absent in *P. angustilineatus*); first finger shorter than second; digital disc round and expanded except in Toe V and Finger I in *P. brevifrons*; Toe III shorter than Toe IV; and absence of cranial crests.

DISCUSSION

In amphibians there are many cases in which morphology has been used as equivalent to synapomorphies without carrying out any formal study. This is also the case for the *lacrimosus* group *sensu lato*, which, as we have shown, is not monophyletic. This opens up the possibility of evaluating the morphological traits for convergences. We highlight that, in frogs, convergent evolution is common (see Guayasamin *et al.* 2008; Santos *et al*. 2009; Vences *et al.* 2003). The *lacrimosus* group *sensu lato* included species that are bromeliad specialist; thus, the shared morphology of these species (i.e., (flattened and pointed head, flattened body) might be a result of common selective preasures and not shared ancestry.

This study also reveals several candidate new species. *Pristimantis* is thought to present low vagility, small home ranges, and narrow elevational ranges (Duellman, 2005), traits that favor diversification (Polato et al. 2018). Also, speciation processes may be acelerated because of local competition and microhabitat preferences (Ortega- Andrade *et al*., 2015; Peterson *et al*., 2011).

Another source of new species is field work concentrated in the canopy. There is a conspicuous sampling bias, concentrated on strate below 3 meters; thus, a large number of new species can be found in the canopy (Guayasamin *et al*., 2006; Almendariz & Cisneros-Heredia, 2005; Ortega- Andrade *et al*., 2015). Sampling effort in bromeliads, combined with molecular techniques, surely will facilitate species discovery and description, specially in the Andean foothills (McCracken *et al*., 2007; Guayasamin *et al.,* 2006).

REFERENCIAS BIBLIOGRÁFICAS

Almendáriz, A., & Cisneros- Heredia, D. F. (2005). Nuevos datos sobre la distribución e historia natural de Gastrotheca longipes (Boulenger, 1882), una rana marsupial amazónica poco conocida (Amphibia: Anura: Hylidae). Revista Politécnica. 2005; 26(1):20–27.

Arteaga-Navarro, A. F., M. R. Bustamante, and J. M. Guayasamin. (2013). The Amphibians and Reptiles of Mindo. Life in the Cloudforest. Serie de Publicaciones Científicas, Universidad Tecnológica Indoamérica. Publicación no. 1. The Amphibians and Reptiles of Mindo. Life in the Cloudforest. Serie de Publicaciones Científicas, Universidad Tecnológica Indoamérica. Publicación no. 1 : 1–257.

Batallas-R., D., Brito-M., J. (2014): Nueva especie de rana del género Pristimantis del grupo lacrimosus (Amphibia: Craugastoridae) del Parque Nacional Sangay, Ecuador. Pap. Avulsos Zool. 54: 51-62.

Canedo, C. & Haddad, C.F.B. (2012) Phylogenetic relationships within anuran clade Terrarana, with emphasis on the placement of Brazilian Atlantic rainforest frogs genus Ischnocnema (Anura: Brachycephalidae). Molecular Phylogenetics and Evolution, 65, 610–620.

Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 15, 532-534

Crawford, A.J., Ryan, M.J. & Jaramillo, C.A. (2010). A new species of Pristimantis (Anura: Strabomantidae) from the Pacific coast of the Darien Province, Panama, with a molecular analysis of its phylogenetic position. Herpetologica, 66, 192–206. http://dx.doi.org/10.1655/09-018r1.1

Darst, C. R., & Cannatella, D. C. (2004). Novel relationships among hyloid frogs inferred from 12S and 16S mitochondrial DNA sequences. *Molecular phylogenetics and evolution*, *31*(2), 462-475.

Duellman, W.E. (1990): A new species of Eleutherodactylus from the Andes of northern Peru (Anura: Leptodactylidae). J. Herpet. 24: 348-350.

Duellman, W.E. (1978) The biology of an equatorial herpetofauna in Amazonian Ecuador. Miscellaneous Publications of the University of Kansas, Museum of Natural History, Lawrence, 65, 1–352

Duellman, W.E. (2005). Cusco Amazónico: The lives of amphibians and reptiles in an Amazonian rainforest. Ithaca, New York: Cornell University

Frankel, C., Yánez-Muñoz, M. H., Guayasamin, J. M., Páez-Rosales, N., Varela-Jaramillo, A. y Ron, S. R. (2018). Pristimantis pseudoacuminatus En: Ron, S. R., Merino-Viteri, A. Ortiz, D. A. (Eds). Anfibios del Ecuador. Version 2019.0. Museo de Zoología, Pontificia Universidad Católica del Ecuador. https://bioweb.bio/faunaweb/amphibiaweb/FichaEspecie/Pristimantis%20pseudoacuminatus. (accesed 24 June 2019).

Frost, D.R. (2019): Amphibian Species of the World: an Online Reference. Version 6.0. <http://research.amnh. org/herpetology/amphibia/index.php> (accessed 20 June 2019).

González-Duran, G. A., Targino, M., Rada, M. A. R. C. O., & Grant, T. (2017). Phylogenetic relationships and morphology of the Pristimantis leptolophus species group (Amphibia: Anura: Brachycephaloidea), with the recognition of a new species group in Pristimantis Jiménez de la Espada, 1870. Zootaxa, 4243(1), 42-74.

Guayasamin, J. M., Castroviejo-Fisher, S., Ayarzagüena, J., Trueb, L., & Vilà, C. (2008). Phylogenetic relationships of glassfrogs (Centrolenidae) based on mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution*, *48*(2), 574-595.

Guayasamin, J. M., Hutter, C. R., Tapia, E. E., Culebras, J., Peñafiel, N., Pyron, R. A., & Arteaga, A. (2017). Diversification of the rainfrog Pristimantis ornatissimus in the lowlands and Andean foothills of Ecuador. *PloS one*, *12*(3), e0172615.

Guayasamin, J. M., Ron, S. R., Cisneros-Heredia, D. F., Lamar, W., & McCracken, S. F. (2006). A new species of frog of the Eleutherodactylus lacrimosus assemblage (Leptodactylidae) from the western Amazon Basin, with comments on the utility of canopy surveys in lowland rainforest. Herpetologica, 62(2), 191-202.

Hedges, S. B., Duellman, W. E., & Heinicke, M. P. (2008). New World direct-developing frogs (Anura: Terrarana): molecular phylogeny, classification, biogeography, and conservation. Zootaxa, 1737(1), 1-182.

Heinicke, M. P., Barrio-Amoros, C. L., & Hedges, S. B. (2015). Molecular and morphological data support recognition of a new genus of New World direct-developing frog (Anura: Terrarana) from an under-sampled region of South America. *Zootaxa*, *3986*(2), 151-172.

Heyer, W. R., and L. M. Hardy. (1991). A new species of frog of the Eleutherodactylus lacrimosus assembly from Amazonia, South America (Amphibia: Anura: Leptodactylidae). Proceedings of the Biological Society of Washington 104: 436–447.

Hoang, D. T., Chernomor, O., Von Haeseler, A., Minh, B. Q., & Vinh, L. S. (2017). UFBoot2: improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution*, *35*(2), 518-522.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., ... & Thierer, T. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647-1649.

Köhler, J., V. R. Morales, S. Lötters, S. Reichle, and J. Aparicio. (1998). A new green species of frog, genus Eleutherodactylus, from Bolivia and Peru (Amphibia, Anura, Leptodactylidae). Studies on Neotropical Fauna and Environment. Amsterdam 33: 93–99.

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., Calcott, B. (2016). PartitionFinder 2: new methods for selecting partitioned models of evolution for

molecular and morphological phylogenetic analyses. Molecular biology and evolution. DOI: dx.doi.org/10.1093/molbev/msw260

Lehr, E., C. Gregory, and A. Catenazzi. (2013). A new species of Pristimantis (Amphibia: Anura: Strabomantidae) from the Río Abiseo National Park, Peru . Zootaxa 3731: 201–211.

Lynch, J. D., and M. S. Hoogmoed. (1977). Two species of Eleutherodactylus (Amphibia: Leptodactylidae) from northeastern South America. Proceedings of the Biological Society of Washington 90: 424–439.

Lynch, J.D. (1979): Leptodactylid frogs of the genus Eleutherodactylus from the Andes of southern Ecuador. Miscellaneous Publication. Misc. Publ. Mus. Nat. Hist. Univ. Kansas 66: 1-62.

Lynch, J. D., & Duellman, W. E. (1980). The Eleutherodactylus of the Amazonian slopes of the ecuadorian Andes (Anura: Leptodactylidae) (No. 59 UNI).

Lynch, J. D., & Duellman, W. E. (1997). Frogs of the genus Eleutherodactylus (Leptodactylidae) in western Ecuador: systematic, ecology, and biogeography. Natural History Museum, University of Kansas.

Maddison, W. P. and D.R. Maddison. (2018). Mesquite: a modular system for evolutionary analysis. Version 3.51 http://www.mesquiteproject.org

McCracken, S.F., Forstner, M.R., Dixon J.R. (2007). A new species of the Eleutherodactylus lacrimosus assemblage (Anura, Brachycephalidae) from the lowland rainforest canopy of Yasuni National Park, Amazonian Ecuador. Phyllomedusa 6: 23-36

Meyer, C., Paulay, G. (2005). DNA barcoding: error rates based on comprehensivesampling. PLoS Biol. 3, 2229

Meza-Joya, F. L., & Torres, M. (2016). Spatial diversity patterns of Pristimantis frogs in the Tropical Andes. Ecology and evolution, 6(7), 1901-1913.

Ortega-Andrade, H. M., Rojas-Soto, O. R., Valencia, J. H., de los Monteros, A. E., Morrone, J. J., Ron, S. R., & Cannatella, D. C. (2015). Insights from integrative systematics reveal cryptic diversity in Pristimantis frogs (Anura: Craugastoridae) from the Upper Amazon Basin. *PloS one*, *10*(11), e0143392.

Padial, J., Grant, T. & Frost, D. (2014) Molecular systematics of terraranas (Anura: Brachycephaloidea) with an assessment of the effects of alignment and optimality criteria. Zootaxa, 3825 (1), 1-132. https://doi.org/10.11646/zootaxa.3825.1.1

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., & Grabowski, G. (1991). The Simple Fool's Guide to PCR. Version 2.0. Honolulu: University of Hawaii.

Peterson, A.T., Soberon J, Pearson R.G., Anderson R.P., Martínez-Meyer E, Nakamura, M., et al. (2011). Ecological niches and geographic distributions. United States of America: Monographs in Population Biology. Princeton University Press.

Pinto-Sánchez, N. R., Ibáñez, R., Madriñán, S., Sanjur, O. I., Bermingham, E., & Crawford, A. J. (2012). The great American biotic interchange in frogs: multiple and early colonization of Central America by the South American genus Pristimantis (Anura: Craugastoridae). *Molecular Phylogenetics and Evolution*, *62*(3), 954-972.

Polato, N. R., Gill, B. A., Shah, A. A., Gray, M. M., Casner, K. L., Barthelet, A., ... & Kondratieff, B. C. (2018). Narrow thermal tolerance and low dispersal drive higher speciation in tropical mountains. Proceedings of the National Academy of Sciences, 115(49), 12471-12476.

Pyron, R.A. Wiens, J.J. (2011): A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol. Phylogenet. Evol. 61: 543-583.

Reyes- Puig, J.P. (2018). Fundacion Ecominga. News about our efforts to conserve Ecuadorian forests. <https://ecomingafoundation.wordpress.com/category/reserves/chamana-reserve/> (Accesed 24 June 2019).

Rivera-Correa, M., & Daza, J. M. (2016). Molecular phylogenetics of the Pristimantis lacrimosus species group (Anura: Craugastoridae) with the description of a new species from Colombia. *Acta Herpetologica*, *11*(1), 31-45.

Rivera-Correa, M., Jimenez-Rivillas, C., & Daza, J. M. (2017). Phylogenetic analysis of the Neotropical Pristimantis leptolophus species group (Anura: Craugastoridae): molecular approach and description of a new polymorphic species. *Zootaxa*, *4242*(2), 313-343.

Ronquist, F., M. Teslenko, P. van der Mark, D.L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M.A. Suchard, and J.P. Huelsenbeck. (2012). MRBAYES 3.2: Efficient Bayesian phylogenetic inference and model selection across a large model space. Syst. Biol. 61:539-542.

Santos, J. C., Coloma, L. A., Summers, K., Caldwell, J. P., Ree, R., & Cannatella, D. C. (2009). Amazonian amphibian diversity is primarily derived from late Miocene Andean lineages. PLoS biology, 7(3), e1000056.

Savage, J. M. (1965). A new bromeliad frog of the genus Eleutherodactylus from Costa Rica. Bulletin of the Southern California Academy of Sciences 64: 106–110.

Shepack, A., von May, R., Ttito, A., & Catenazzi, A. (2016). A new species of Pristimantis (Amphibia, Anura, Craugastoridae) from the foothills of the Andes in Manu National Park, southeastern Peru. ZooKeys, (594), 143.

Valencia, J.H., Yánez-Muñoz, M. H., Betancourt-Yépez, R., Terán-Valdez, A., & Guayasamin, J.M. (2010). Una llamativa nueva especie de Pristimantis (Anura: Terrana: Strabomantidae) de las estribaciones noroccidentales de los Andes de Ecuador. Avances. 2 (3): 41- 45

Vences, M., Vieites, D. R., Glaw, F., Brinkmann, H., Kosuch, J., Veith, M., & Meyer, A. (2003). Multiple overseas dispersal in amphibians. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1532), 2435-2442.

Yánez-Muñoz, M. H., & Bejarano-Muñoz, E. P. (2013). Lista actualizada de ranas terrestres Pristimantis (Anura: Craugastoridae) en las estribaciones occidentales del Distrito Metropolitano de Quito, Andes de Ecuador. Boletín Técnico, 11, 125-150.

APPENDIX

Appendix 1: GenBank accession number of each gen used in our phylogenetic analysis.

Species	Gen Bank accesion no		Museum voucher	Country	Province/ Department: Locality	Lat	Lon
	16S	COI					
Bufo melanostictus	FJ882791	-	VUB 0052	Sri Lanka	-	-	-
Craugastor longirostris	EF493395	-	KU177803	Ecuador	Pichincha: Santo Domingo	-	-
Craugastor longirostris	JN991417	JN991346	AJC 1336	Colombia	Antioquia: Maceo	6.54N	-74.64W
Craugastor podiciferus	EF493360	-	MVZFC13463	Costa Rica	Heredia: Chompipe, vicinty of volcan Barba	-	-
Eleutherodactylus caribe	EF493385	-	USNM 314179	Haiti	Dept. delaGrand: Dame- Marie	-	-
Eleutherodactylus marnockii	DQ283101	-	USNM 331345	USA	Texas: Travis Co, Austin	-	-
Oreobates cruralis	EU186666	-	KU215462	Peru	Madre de Dios: Puerto Maldonado	-	-
Oreobates saxatilis	EU186708	-	KU212327	Peru	San Martin: Tarapoto	-	-
Phyllomedusa hypochondrialis	AY948748	-	VUB 0990	-	-	-	-
Pristimantis acerus	EF493678	-	KU217786	Ecuador	Napo: Papallacta	-	-
Pristimantis achatinus	EF493660	JN991349	KU217809	Ecuador	Manabi: Rio Cuaque, pedernales	-0.06	-80.05
Pristimantis actites	EF493696	-	KU217830	Ecuador	Cotopaxi: Pilalo	-0.95	-78.99
Pristimantis acuminatus	DQ195448	-	MC11555	Colombia	Bogota	-	-
Pristimantis acuminatus	EU130579	-	QCAZ 19664	Ecuador	Napo: Parque Nacional Yasuni	-	-
Pristimantis acuminatus	-	-	MZUTI 3796	Ecuador	Pastaza: Oglan	-1,325960	-77.686887
Pristimantis acuminatus	-	-	MZUTI 3702	Ecuador	Zamora- Chinchipe: Maycu	-4,20719	-78,63987

Pristimantis acuminatus	MH516156	-	QCAZ 17510	Ecuador	Orellana	0.6766667S	76.3952778 W
Pristimantis acuminatus	-	-	CORBIDI 4769	Perú	Loreto: Andoas	-2,351	-75,816
Pristimantis acuminatus	-	-	CORBIDI 7469	Perú	Loreto: Datem, Sargento Puño	-3,219	-77,585
Pristimantis acuminatus	-	-	CORBIDI 7579	Perú	Loreto: Sector 2	-3,187	-77,433
Pristimantis acuminatus	-	-	QCAZ 53263	Ecuador	Morona Santiago: Tukupi	-2,794	-77,483
Pristimantis aff. ardyae	-	-	ZSFQ 582	Ecuador	-	-	-
Pristimantis aff. ardyae	-	-	SCA 0454	Ecuador	Tungurahua: Baños/ Reserva Chamana	-	-
Pristimantis s aff. ardyae	-	-	SCA 0455	Ecuador	Tungurahua: Baños/ Reserva Chamana	-	-
Pristimantis aff. ardyae	-	-	SCA 0702	Ecuador	Tungurahua: Baños/ Reserva Chamana	-	-
Pristimantis aff. ardyae	-	-	SCA 0703	Ecuador	Tungurahua: Baños/ Reserva Chamana	-	-
Pristimantis aff. ardyae	-	-	SCA 0706	Ecuador	Tungurahua: Baños/ Reserva Chamana	-	-
Pristimantis aff. subsigillatus	-	-	ZSFQ 1023	Ecuador	-	-	-
Pristimantis affinis	JN991424	-	nrps 0031	Colombia	Cundinamarca: Parque Nacional Natural Chinagaza	4.628 N	73.725 W
Pristimantis altae	-	JN991361	AJC 0398	Costa Rica	Alajuela: Monumento Natural Histórico LaPaz	10.20 N	84.53 W
P. altamazonicus	EF493670	-	KU215460	Peru	Madre de Dios: Puerto maldonado	-	-
P. altamazonicus	-	-	MF20218	Ecuador	Orellana: Tiputini	-0,637096	-76,150119

P. altamazonicus	-	-	MF20289	Ecuador	Orellana: Tiputini	-0,637096	-76,150119
P. altamazonicus	-	-	MF20510	Ecuador	Orellana: Tiputini	-0,635504	-76,163995
P. angustilineatus	JN371034	-	UVC:15828	Colombia	Valle del Cauca: El Cairo	4.8103 N	76.1722 W
P. angustilineatus	JN104677	-	UVC:15888	Colombia	Valle del Cauca: El Cairo	4.8103 N	76.1722 W
P. angustilineatus	JN371035	-	UVC:15941	Colombia	Valle del Cauca: El Cairo	4.8103 N	76.1722 W
P. aniptopalmatus	EF493390	-	KU291627	Peru	Pasco: Oxapampa	-	-
P. appendiculatus	EF493524	-	KU177637	Ecuador	Pichincha: Quebrada Zapadores	-	-
P. ardalonychus	EU186664	-	KU212301	Peru	San Martin: Rio Cerranyacu	-	-
P. aureolineatus	-	-	MZUTI 3072	Ecuador	Orellana: Estacion Biodiversidad Tiputini	-0,632938	-76,14532
P. aureolineatus	-	-	MZUTI 3795	Ecuador	Pastaza: Oglán	-1,318608	-77,699208
P. aureolineatus			SFM1152	Ecuador	Orellana: Tiputini	-0,67703	-76,38036
P. aureolineatus			SFM1158	Ecuador	Orellana: Tiputini	-0,67703	-76,38036
Pristimantis bipunctatus	EF493702	-	KU291638	Peru	Pasco: Oxapampa		
Pristimantis bogotensis	JN991432	JN991362	nrps 0033	Colombia	Cundinamarca: Parque Nacional Natural Chinagaza	4.628 N	73.725 W
Pristimantis boulengeri	KU724435	KU724444	MHUAA 8951	Colombia	Pereira: Parque Regional Natural Ucumarí	-	-
Pristimantis boulengeri	KU724436	KU724445	MHUAA 8952	Colombia	Pereira: Parque Regional Natural Ucumarí	-	-
Pristimantis boulengeri	DQ195480	DQ195452	MAV257	Colombia	Bogota	-	-
Pristimantis brevifrons	JN370973	JN371055	UVC:15904	Colombia	Cali: Valle del Cauca	3º 25' 43.9"	76º 39' 33.4"

Pristimantis brevifrons	JN991433	-	nrps 0059	Colombia	Valle del Cauca : El Cairo	4.74 N	76.295 W
Pristimantis brevifrons	JN370957	JN371041	UVC:15856	Colombia	Valle del Cauca: El Cairo	4º 44' 33.9"	76º 17' 45.0"
Pristimantis bromeliaceus	EF493351	-	KU291702	Peru	Pasco: Oxapampa	-	-
Pristimantis buccinator	EU712631	-	MHNC 4921	Peru	Cusco: Cocha Camungo	-	-
Pristimantis buckleyi	EF493350	-	KU217836	Ecuador	Carchi: El Angel	-	-
P. cajamarcensis	EF493663	-	KU217845	Ecuador	Loja: Yangana	-	-
Pristimantis calcarulatus	EF493523	-	KU177658	Ecuador	Pichincha: Tandapi	-0.15	-78.84
Pristimantis caprifer	EF493391	-	KU177680	Ecuador	Pichincha: La palma	-0.32	-78.84
P. caryophyllaceus	EU186686	JN991363	MVZ203810	Costa Rica	Cartago: Tapanti Bridge over Rio Grande de Orosi	-	-
P. caryophyllaceus	JN991434	JN991363	AJC0486	Costa Rica	San Jose: Los Juncos	9.98 N	83.84 W
Pristimantis celator	EF493685	-	KU177684	Ecuador	Carchi: Maldonado	-	-
Pristimantis cerasinus	JN991437	JN991366	AJC 0527	Costa Rica	Limón: CRARC, Guayacán, Siquirres	10.04 N	83.55 W
Pristimantis ceuthospilus	EF493520	-	KU212216	Peru	Cajamarca: Chota, 12 Km W llama	-	-
Pristimantis cf. mendax	EU186659	-	MTD45080	Peru	Pasco: Aquimarca	-	-
Pristimantis cf. mendax	EU186659	-	MTD45080	Peru	Pasco: Aquimarca	-	-
Pristimantis cf. mendax	KY006097	-	IWU12	Peru	Pasco: Yanachaga-Chemillén National Park	-	-
Pristimantis cf. mendax	-	KY962786	MUSM31157	Peru	Junin, Concepcion: Mariscal Castilla	11.651278 S	75.087472 W
Pristimantis chalceus	EF493675	-	KU177638	Ecuador	Carchi: Maldonado	-	-
Pristimantis chiastonotus	EU201061	-	162AF	Suriname	Brokopondo: Parque Natural Brownsberg	-	-
Pristimantis chloronotus	AY326007	-	WED 52959	Ecuador	Napo: Santa Barbara	-	-

Pristimantis citriogaster	EF493700	-	KU212278	Peru	San Martin: Cataratas Ahnashiyacu, 14 km NE Tarapoto	-6.56	-77.12
Pristimantis colomai	EF493354	-	QCAZ 17101	Ecuador	Esmeraldas: Alto Tambo	0.9	-78.55
Pristimantis condor	EF493701	-	KU217857	Ecuador	Morona- Santiago: 4.6 km N Gualaquiza	-3.43	-78.56
P. conspicillatus	EF493529	-	QCAZ 28448	Ecuador	Sucumbios: Monte Tour	-0.15	-76.27
Pristimantis cremnobates	EF493528	-	KU177252	Ecuador	Napo: Rio Salado	-0.73	-77.01
P. croceoinguinis	EF493665	-	KU217862	Ecuador	Morona- Santiago: 53.8 km E Bella Union via Santiago	-3.43	-78.56
Pristimantis crucifer	EU186718	-	KU:177733	Ecuador	Pichincha: Tandapi	-0.15	-78.84
Pristimantis cruentus	EF493697	JN991369	AMNH A12444	Panama	Ratibor: Finca Ojo de Agua	-	-
Pristimantis cryophilius	EF493672	-	KU217863	Ecuador	Azuay: 4 km W Laguna Torcadorn	-2.96	-79.11
Pristimantis curtipes	EF493513	-	KU217871	Ecuador	Pichincha: Bosque de Pasochoa	-0.43	-78.48
Pristimantis danae	EU192272	-	IDLR 4825	Peru	Cusco: Union, Valle de Kosnipata	-	-
Pristimantis devillei	EF493688	-	KU217991	Ecuador	Napo: Papallacta	-0.37	-78.14
Pristimantis diadematus	EU186668	-	KU221999	Peru	Loreto: Teniente Lopez	-4.23	-74.22
Pristimantis dissimulatus	EF493522	-	KU179090	Ecuador	Pichincha: Quebrada Zapadores	-0.245	-78.726
Pristimantis dorsopictus	KU724437	KU724446	MHUAA 7455	Colombia	Medellin: Corregimiento de Santa Elena	-	-
Pristimantis dorsopictus	KU724440	-	MHUAA 8960	Colombia	Antioquia: San Felix	-	-
Pristimantis duellmani	AY326003	-	WED 53050	Ecuador	Carchi: 5 km W La Gruel	-	-

P. ecuadoriensis	KX785344	-	CJ 5351	Ecuador	Cotopaxi: San Francisco de las Pampas	-0.42415	-78.9572
P. ecuadoriensis	KX785343	-	CJ 5350	Ecuador	Cotopaxi: San Francisco de las Pampas	-0.42415	-78.9572
Pristimantis enigmaticus	-	-	QCAZ 38771	Ecuador	Pastaza: Alrededores de Villano, campamento petrolero de AGIP, Villano B-II Unidad 3	-1. 456	-77.445
Pristimantis enigmaticus	-	-	QCAZ 40935	Ecuador	Pastaza: Finca km 6 vía San Ramón-El Triunfo. Cooperativa La Mariscal Sucre.	-1.37	-77.86
Pristimantis eremitus	-	-	MZUTI 2278	Ecuador	Pichincha: Antpitta trail. Bellavista	-0.02077	-78.68517
Pristimantis eremitus	-	-	MZUTI 2279	Ecuador	Pichincha: Antpitta trail. Bellavista	-0.02077	-78.68517
Pristimantis eriphus	EU186671	-	QCAZ 32705	Ecuador	Napo: Yanayacu	-	-
Pristimantis euphronides	EF493527	-	BWMC6918	Grenada	Grand Etang National Park	-	-
Pristimantis fenestratus	EF493703	-	MHNSM9298	-	-	-	-
Pristimantis gaigae	JN991448	-	CH 6471	Panama	Bocas del Toro: Distrito de Changuinola, Corregimiento Valle de Risco, Quebrada Pinolillo	9.134 N	82.497 W
Pristimantis galdi	EU186670	-	QCAZ 32368	Ecuador	Zamora Chinchipe: El Pangui	-4.17	-78.69
Pristimantis gentryi	EF493511	-	KU218109	Ecuador	Cotopaxi: 27.6 km E Pilabo	-0.933	-78.85

Pristimantis glandulosus	EF493676	-	KU218002	Ecuador	Napo: 2.7 km W Cuyuja	-0.399	-78.044
Pristimantis imitatrix	EF493667	-	KU215476	Peru	Madre de dios: Puerto Maldonado	-12.04	-70.42
Pristimantis inguinalis	EU186676	-	ROM40164	Guyana	Sierra Pacaraima: Monte Ayanganna	5.38	-59.93
Pristimantis inusitatus	EF493677	-	KU218015	Ecuador	Napo: 31 km N Jondachi	-0.72	-77.8
Pristimantis jaguensis	KP082865	-	MHUAA7239	Colombia	Antioquia: Sector Arenosas, Embalse de San Lorenzo	-	-
Pristimantis jaguensis	KP082869	-	MHUAA7248	Colombia	Antioquia: Sector Arenosas, Embalse de San Lorenzo	-	-
Pristimantis koehleri	FJ438799	-	MNCN 42983	Bolivia	Santa Cruz: La Chonta	-	-
Pristimantis labiosus	EF493694	-	QCAZ 19771	Ecuador	Pichincha: 4 km NW La Florida. Finca Gloria	-0.1	-78.96
Pristimantis lacrimosus	MH516181	-	QCAZ 15982	Ecuador	Sucumbios: Añangu	-0.167	-77.65
Pristimantis lacrimosus			MF19455	Ecuador	Orellana: Tiputini	-0.635722	-76.147000
Pristimantis lanthanites	EF493695	-	KU222001	Peru	Loreto: San Jacinto	-4.67	-73.96
Pristimantis latidiscus	EF493698	-	KU218016	Ecuador	Pichincha: 5 km W La Florida	-0.1	-78.96
Pristimantis leoni	EF493684	-	KU218227	Ecuador	Carchi: 51.3 km W Tulcan	0.82	-77.73
Pristimantis librarius	JN991451	JN991379	QCAZ 25852	Ecuador	Napo: Pano	1.10 S	77.92 W
P. limoncochensis	-	-	QCAZ 19180	Ecuador	Sucumbíos: Saladero de Dantas, Reserva de Producción Faunística Cuyabeno	-0.002	-76.177
P. limoncochensis	-	-	QCAZ 52987	Ecuador	Sucumbíos: Limoncocha	-0.407	-76.621

Pristimantis lirellus	EF493521	-	KU212226	Peru	San Martin: Rioja, Rio Cerranayaca	-7.24	-76.83
Pristimantis llojsintuta	EU712642	-	FMK 72610	Bolivia	Cochabamba: Vieja Carretera de Chapare		
Pristimantis luteolateralis	EF493517	-	KU177807	Ecuador	Pichincha: Tandapi	-0.416	-78.799
Pristimantis lymani	EF493392	-	KU218019	Ecuador	Loja: 3.9 km E Loja	-3.99	-79.2
Pristimantis malkini	EU186663	-	QCAZ 28296	Ecuador	Sucumbios: Monte Tour	-0.15	-76.31
Pristimantis marmoratus	EU201063	-	110bm	French Guiana	Regina: Kaw		
Pristimantis martiae	-	JN991380	QCAZ 17998	Ecuador	Napo	-1.07	-77.62
Pristimantis melanogaster	EF493664	-	WED56846	Peru	Chachapoyas: Slobe Abra Barro Negro, 28 km SSW Leimebambe	-2.2	-73.46
Pristimantis mindo	KF801584	-	MZUTI 1382	Ecuador	Pichincha: Mindo/ Reserva Los Cedros	-0.02470	-78.75
Pristimantis mindo	KU999211	-	MZUTI 2284	Ecuador	Pichincha: Mindo/Yellow House	-0.04462	-78.75407
Pristimantis miyatai	JN991452	JN991382	RC 610	Colombia	Santander: Florida Blanca, El Diviso	4.63	-73.73
Pristimantis moro	JN991453	JN991383	AJC 1753	Panama	Chilibre: Cerro Azul	9.17	-79.42
Pristimantis museosus	JN991455	JN991385	AJC 1210	Panama	Chame: Altos del Maria, ~7.5 km NE of El Valle de Anton	8.64	-80.07
Pristimantis nervicus	JN991456	JN991386	nrps 0048	Colombia	Cundinamarca: PN Natural Chingaza	4.63	-73.73
Pristimantis nyctophylax	EF493526	-	KU177812	Ecuador	Pichincha: Tandapi	-0.416	-78.799
Pristimantis ockendeni	EF493519	JN991387	KU222023	Peru	Loreto: 1.5 km N Teniente Lopez	-4.26	-74.22
Pristimantis ockendeni	JN991457	JN991387	QCAZ 25428	Ecuador	Orellana	-	-

Pristimantis ocreatus	EF493682	-	KU208508	Ecuador	Carchi: 26.6 km W Tulcan	-	-
Pristimantis omeviridis	-	-	QCAZ 17510	Ecuador	Orellana: Estación Científica Yasuní PUCE	-0.677	-76.395
Pristimantis omeviridis	-	-	QCAZ 55392	Ecuador	Orellana: Tambococha	-0.978	-75.426
Pristimantis orcesi	EF493679	-	KU218021	Ecuador	Pichincha: Bosque Pasochoa	-0.43	-78.48
Pristimantis orestes	EF493388	-	KU218257	Ecuador	Azuay: 7 km E Sigsig	-3.017	-78.767
Prismantis ornatissimus	KX785341		MZUTI 4806	Ecuador	Cotopaxi: La mana	-0.83142	-79.213368
Prismantis ornatissimus	KX785342		MZUTI 4807	Ecuador	Cotopaxi: La mana	-0.83142	-79.213368
Pristimantis paisa	JN991389	JN991459	AJC 1344	Colombia	Antioquia: Maceo	6.55	-74.78
Pristimantis pardalis	-	JN991390	CH 6284	Panama	Darien, Chepigana: Rio congo Arriba, Tent camp, Reserva Natural Privada Chucanti	8.8	-78.46
Pristimantis parvillus	EF493352	-	KU177821	Ecuador	Pichincha	-0.21	-78.87
Pristimantis peruvianus	EF493707	JN991392	MHNSM9267	Peru	-	-	-
Pristimantis petersi	-	-	MZUTI 893	Ecuador	Napo: El Chaco	-0,25582	-77,85938
Pristimantis petersi	-	-	MZUTI 894	Ecuador	Napo: El Chaco	-0,25582	-77,85938
Pristimantis petersi	-	-	MZUTI 4021	Ecuador	Tungurahua: La Candelaria	-	-
Pristimantis petersi	-	-	MZUTI 4022	Ecuador	Tungurahua: La Candelaria	-	-
Pristimantis petersi	-	-	ZSFQ 0576	Ecuador	Pastaza: Mera	-1,35721	-78,05620
Pristimantis petrobardus	EF493367	-	KU212293	Peru	Cajamarca: Chota, ca 2 km W Huambos	-6.56	-78.65
P. pluvicanorus	AY843586	-	AMNH-A 165195	Bolivia	Santa Cruz: Caballero, Canton San Juan, Amboro National Park	-	-

P. phoxocephalus	EF493349	-	KU218025	Ecuador	Chimborazo: 70 km W Riobamba via Pallatanga	-1.983	-78.967
P. phoxocephalus	-	-	MZUTI 3936	Ecuador	Azuay: Cuenca	-	-
Pristimantis pirrensis	JN991462	JN991393	AJC 0594	Panama	Darien: Cana, Pirre high camp	7.77	-77.73
Pristimantis platydactylus	EU192255		MNCN43248	Peru	Quispicanchis: between San Miguel and Marcapata	-	-
Pristimantis platydactylus		JN991394	MBH 5746	Colombia	Santander: Serrania de Bellavista	15.84 S	67.56 W
Pristimantis prolatus	EU186701	-	KU177433	Ecuador	Napo: Rio Salado, 1 km upstream from Rio Coca	-0.73	-77.01
Pristimantis ptochus	-	JN991395	nrps 0058	Colombia	Valle del Cauca: El Cairo, Vereda El Brillante, Corregimiento El Boqueron, Cerro El Ingles	4.74	-76.3
Pristimantis pulvinatus	EU186723	-	KU181015	Venezuela	Bolivar: km 127, El Dorado-Santa Elena de Uairen Rd	6.36	-63.58
Pristimantis pycnodermis	EF493680	-	KU218028	Ecuador	Morona Santiago: Gualaceo-Limon Rd., 2.4 km E Azuay border	-2.56	-78.11
Pristimantis pyrrhomerus	EF493683	-	KU218030	Ecuador	Bolivar: Bosque Protector Cashca Totoras	-1.57	-79.11
P. quaquaversus	JN991463	JN991396	QCAZ 25676	Ecuador	Pastaza	-0.31	-78.88
P. quaquaversus			QCAZ 16150	Ecuador	Sucumbios	-	-
P. quinquagesimus	EF493690	-	KU179374	Ecuador	Pichincha: Quebrada Zapadores	-0.43	-78.48

P. rhabdocnemus	EU186706	-	KU291651	Peru	Pasco: 2.9 km N, 5.5 km E Oxapampa	-10.45	-75.15
P. rhabdolaemus	EF493706	-	KU173492	Peru	Cuzco: Buenos Aires	-13.52	-71.97
Pristimantis rhodoplichus	EF493674	-	KU219788	Peru	Piura: Le Tambo	-5.17	-80.64
Pristimantis ridens	EF493355	JN991398	AMNH A124551	Panama	Cocle: El Valle	8.604	-80.126
Pristimantis ridens	JN991464	JN991398	ENS 10722	Honduras	Olancho: Sierra de Agalta	14.96 N	86.14 W
Pristimantis riveti	EF493348	-	KU218035	Ecuador	Azuay: 8.1 km W Morona-Santiago border on Gualaceo-Limon Rd.	-2.67	-77.9
Pristimantis rozei	EF493691	-	-	Trinidad and Tobago	Tobago: Road to King's Bay Reservoir"	10.5	-61.23
Pristimantis sagittulus	EF493705	-	KU291635	Peru	Pasco: 0.9 km N, 2.1 km E Oxapampa	-10.45	-75.15
Pristimantis samaipatae	FJ438803	-	MNCN 42989	Bolivia	Santa Cruz: Km 6 Angostura-Samaipata road	-	-
Pristimantis savagei	JN991467	JN991401	nrps 0085	Colombia	Villavicencio: Restrepo	4.25	-73.58
Pristimantis schultei	EF493681	-	KU212220	Peru	Chachapoyas: 5 km N Levan	-6.22	-77.85
Pristimantis schrevei	EF493692	-	-	Saint Vincent and the Grenadines	St. Vincent: 1.6 mi. NE Vermont	n/a	n/a
P. simonbolivari	EF493671	-	KU218254	Peru	Madre de Dios: Bosque Protector, Cashca Totoras	-1.7	-78.883
Pristimantis simonsii	EU186665	-	KU212350	Peru	Cajamarca: slope Abra Quilsh	-6.45	-78.84
Pristimantis skydmainos	EF493393	-	MHNSM10071	Peru	-	-9.09	-75.16

Pristimantis spinosus	EF493673	-	KU218052	Ecuador	Morona- Santiago: 10.6 km W Plan de Milogio	-2.56	-78.11
Pristimantis stictogaster	EF493704	-	KU291659	Peru	Pasco: 2.9 km N, 5.5 km E Oxapampa	-10.45	-75.15
Pristimantis subsigillatus	KF801580	-	MECN 10117	Ecuador	Manabi: Reserva Ayampe	-	-
Pristimantis subsigillatus	EF493525	-	KU218147	Ecuador	Azuay: Luz Maria	-2.701	-79.466
Pristimantis suetus	JN991469	-	MHUA 4404	Colombia	Antioquia: Guatape	6.23	-75.16
Pristimantis supernatis	AY326005	-	KU202432	Ecuador	Napo: 3.5 km E Santa Barbara	-	-
Pristimantis surdus	EF493687	-	KU177847	Ecuador	Imbabura: La Delicia	-	-
Pristimantis taeniatus	JN991470	JN991403	AJC 1839	Panama	Darien: Cana, Sendero Mina	8.05	-77.58
Pristimantis taeniatus	JN991473	JN991407	CJD 069	Colombia	Santander: Mesa de los Santos	6.766 N	73.083 W
Pristimantis tantanti	-	-	CORBIDI 12987	Peru	Ucayali: Rio Abujao	-8.309	-76.665
Pristimantis tantanti	-	-	MHNC 12845	Peru	Ucayali: Yahuish 10C, cuenca del rio Abujao	-8.349	-73.68
P. terraebolivaris	EU186650	-	-	Trinidad and Tobago	Tobago: Road to King's Bay Reservoir"	10.5	-61.23
P. thymalopsoides	EF493514	-	KU177861	Ecuador	Cotopaxi: Pilalo	-0.91	-78.97
Pristimantis thymelensis	AY326009	-	WED 53004	Ecuador	Carchi: 12 km W Tufino	-	-
Pristimantis toftae	EF493353	-	KU215493	Peru	Madre de Dios: Puerto Maldonado	-11.77	-70.81
Pristimantis truebae	EF493512	-	KU218013	Ecuador	Cotopaxi: 24.6 km E Pilalo	-0.571	-77.869
Pristimantis unistrigatus	EF493387	-	KU218057	Ecuador	Imbabura: 35 km E Pasquela	0.35	-78.5
Pristimantis urani	KU724443	KU724450	MHUAA 7472	Colombia	Antioquia: Urrao, Corregimiento La	6.440725	-76.206295

					Encarnación, Vereda El Maravillo		
Pristimantis urani	KU724442	-	MHUAA 7467	Colombia	Antioquia: Urrao, Corregimiento La Encarnación, Vereda El Maravillo	6.440725	-76.206295
Pristimantis urani	KU724441	KU724449	MHUAA 7471	Colombia	Antioquia: Urrao, Corregimiento La Encarnación, Vereda El Maravillo	6.440725	-76.206295
Pristimantis urichi	EF493699	-	USNM336098	Trinidad and Tobago	Trinidad: Arima Vallley	10.63	-61.28
Pristimantis verecundus	EF493686	-	QCAZ 12410	Ecuador	Cotopaxi: Otonga	-0.81	-78.93
Pristimantis versicolor	EF493389	-	KU218096	Ecuador	Zamora- Chinchipe: 1.7 km E Loja border	-3.983	-79.117
Pristimantis vertebralis	EF493689	-	KU177972	Ecuador	Imbabura: La Delicia	0.37	-78.38
Pristimantis viejas	JN991476	JN991409	EMM 250	Colombia	Antioquia: San Rafael, San Lorenzo	6.29	-75.03
Pristimantis viejas	JN991409		AJC 1352	Colombia	Tolima: Falan	5.12	-74.95
Pristimantis w-nigrum	AY326004	-	WED 53045	Ecuador	Carchi: 5 km W La Gruel	0.58	-78.06
Pristimantis walkeri	EF493518	-	KU218116	Ecuador	Pichincha: 5 km W La Florida	0.257	-79.054
Pristimantis waoranii			SFM0944	Ecuador	Orellana: Tiputini	-0,64087	-7614588
Pristimantis waoranii			SFM1243	Ecuador	Orellana: Tiputini	-0.682550	-76.40804
Pristimantis wiensi	EF493668	-	KU219796	Peru	Piura: 12.7 km E Canchaque	-4.92	-80.67
Pristimantis zeuctotylus	EU186678	-	ROM43978	Guyana	Sierra Pacaraima: Mount Wokomung	5.08	-59.83
Pristimantis zophus	JN991479	JN991413	nrps 0060	Colombia	Antioquia: Caldas, Reserva Ecologica Alto de San Miguel	6.03	-75.1

Strabomantis anomalus	EF493534	-	KU177627	Ecuador	Pichincha: Santo Domingo	-	-
Strabomantis biporcatus	EU186691	-	CVULA7073	Venezuela	Sucre: PN de Paria	-	-
Yunganastes ashkapara	FJ438796	-	ZFMK 70318	Bolivia	Cochabamba: Carrasco National Park	-	-
Yunganastes bisignatus	EU192235	-	CBF5719	Bolivia	La Paz: Toriri	-	-
Yunganastes fraudator	FJ539065	-	MNCN 43107	Bolivia	Cochabamba: Villa Tunari	-	-
Yunganastes mercedesae	FJ539066	-	ZFMK 72572	Bolivia	Cochabamba: Old Chapare Road	-	-
Pristimantis sp1.	-	-	ZSFQ 584	Ecuador	Napo: Quijos	-	-
Pristimantis sp1.	-	-	ZSFQ 585	Ecuador	Napo: Quijos	-	-
Pristimantis sp1.	-	-	ZSFQ 580	Ecuador	Napo: Quijos	-	-
Pristimantis sp2.	-	-	ZSFQ 583	Ecuador	-	-	-
Pristimantis sp.	-	-	MZUTI 761	Ecuador	Napo: El Chaco	-0,24569	-77,90772

Appendix 2: Best fit model for each partition name obtained with partition finder 2.1.1.

Best model	*Partition name*	*Number of sites*
GTR+I+G	16S	896
HKY+I+G	COI_1stops	220
GTR+G	COI_2stops	220
SYM+I+G	COI_3stops	220

Appendix 3: morphological characteristics of species that belongs to the *Pristimantis lacrimosus* species group. Source numbers: (1) Ortega-Andrade *et* al., 2015; (2) Guayasamin *et* al., 2006; (3) Lynch, 1979; (4) Lehr *et* al., 2013; (5) Guayasamin *et* al., 2017; (6) Lynch y Duellman, 1980; (7) Duellman, 1978; (8) Arteaga- Navarro *et* al., 2013; (9) Savage, 1965; (10) Köhler *et* al., 1998; (11) Lynch, 1997; (12) Moravec *et* al., 2010; (13) Duellman, 1990; (14) Frankel *et* al., 2018; (15) Valencia *et* al., 2010; (16) McCracken *et* al., 2007; (17) Lynch and Hoogmoed, 1977; (18) Heyer and Hardy, 1991.

Species name	Dorsal skin	snout shape	dorsolateral folds	ventral skin	Rostral papilla	Source
P. acuminatus	shagreened	acuminate	absent	areolate	Present	1
P. aureolineatus	shagreened	acuminate	absent	areolate	Present	2
P. bromeliaceus	smooth	acuminate	absent	Coarsely areolate	Present	3
P. deyi	shagreened	acuminate	weakely visible	areolate	Present	4
P. ecuadorensis	shagreened	acuminate	absent	areolate	Present	5
P. enigmaticus	shagreened	acuminate	absent	areolate	Present	1
P. galdi	smooth	acuminate	absent	areolate	Present	6
P. lacrimosus	smooth	acuminate	absent	areolate	Present	6
P. limoncochensis	smooth	acuminate	absent	areolate	Present	1
P. mendax	shagreened	acuminate	absent	Coarsely areolate	Present	7
P. mindo	smooth	acuminate	absent	Weakely areolate	absent	8
P. moro	smooth	subacuminate	absent	Coarsely areolate	Present	9
P. olivaceus	shagreened	subacuminate	absent	Coarsely areolate	Present	10
P. omeviridis	smooth	acuminate	present	areolate	Present	1
P. ornatissimus	shagreened	acuminate	absent	smooth	Present	11
P. padiali	shagreened	acuminate	absent	areolate	Present	12
P. petersi	smooth	acuminate	absent	Coarsely areolate	Present	6
P. pseudoacuminatus	smooth	acuminate	absent	areolate	Present	1

P. schultei	shagreened	acuminate	absent	areolate	Present	13
P. subsigillatus	smooth	subacuminate	absent	areolate	Present	14
P. tantanti	shagreened	acuminate	absent	areolate	Present	1
P. tribulosus	shagreened	acuminate	absent	areolate	Present	15
P. waoranii	shagreened	subacuminate	absent	areolate	absent	16
P. zeuctotylus	shagreened	subacuminate	present	areolate	absent	17
P. zimmermanae	smooth	acuminate	absent	Coarsely areolate	Present	18

Appendix 4: uncorrected genetic distance from *P. lacrimosus* species group. Lower left matrix presents the genetic distance for 16S. Upper right matrix presents the genetic distance of COI (Citocrome c oxidase).

	P. acuminatus	*P. aureolineatus*	*P. bromeliaceus*	*P. ecuadorensis*	*P. enigmaticus*	*P. galdi*	*P. lacrimosus*	*P. lacrimosus*	*P. limoncochensis*	*P. mendax*
P. acuminatus	-	-	-	-	0.07306813	-	0.22357865	-	0.04413817	0.21042538
P. aureolineatus	0.16666586		-	-	0.22047666	-	0.18996960	-	0.22188450	0.21597297
P. bromeliaceus	0.14500604	0.13169570		-	-	-	-	-	-	-
P. ecuadorensis	0.13691516	0.13169447	0.11273394		-	-	-	-	-	-
P. enigmaticus	0.04235940	0.13851343	0.12685779	0.11433458		-	0.23418899	-	0.08509787	0.20026778
P. galdi	0.15263118	0.13465924	0.11686937	0.09668741	0.13555419		-	-	-	-
P. lacrimosus	0.16071600	0.12991533	0.12655596	0.12792880	0.13571578	0.14304276		-	0.21732523	0.17426779
P. lacrimosus	0.11900401	0.13116683	0.12442339	0.11225115	0.12336025	0.10784198	0.13019333		-	-
P. limoncochensis	0.03119181	0.14201896	0.13170070	0.12617349	0.04342088	0.14162626	0.14448896	0.12551612		0.20688554
P. mendax	0.11838393	0.10898201	0.00566840	0.09407241	0.12168439	0.10947917	0.11199526	0.12544586	0.13073041	
P. mindo	0.15788234	0.12401914	0.11168628	0.08845434	0.13687082	0.09258132	0.12957678	0.11443694	0.15118956	0.09670441
P. moro	0.09061119	0.09969258	0.08650429	0.06830622	0.09499558	0.07706324	0.08838836	0.12205559	0.09903807	0.08719286
P. omeviridis	0.01800683	0.13923187	0.12577506	0.11481085	0.03415339	0.13713231	0.14089430	0.11820118	0.03439730	0.12404298
P. ornatissimus	0.14363901	0.14884321	0.11639499	0.05613140	0.13696079	0.10958995	0.14262878	0.12118457	0.13442456	0.10138042
P. petersi	0.14286811	0.13043864	0.12237473	0.09885549	0.11977062	0.11108225	0.13381770	0.11468149	0.12775267	0.10957260

P. schultei	0.13458630	0.14191382	0.10740770	0.09272491	0.13460075	0.11145460	0.13732104	0.11158590	0.14020111	0.09719030
P. subsigillatus	0.17103475	0.14238846	0.14513494	0.12615456	0.15038493	0.12373086	0.15992561	0.12980092	0.15976897	0.12535269
P. tantanti	0.07958178	0.16090798	0.14596225	0.14543439	0.08183892	0.14788696	0.15100290	0.13350446	0.08004880	0.15601880
P. waoranii	0.16484859	0.11080043	0.13190104	0.11772927	0.14938233	0.11424250	0.13007221	0.13361524	0.16083666	0.11664328
P. zeuctotylus	0.17580422	0.10778651	0.14360204	0.11828705	0.15110535	0.13533930	0.12907270	0.13806109	0.15420484	0.12615000
	P. mindo	*P. moro*	*P. omevirids*	*P. ornatissimus*	*P. petersi*	*P. schultei*	*P. subsigillatus*	*P. tantanti*	*P. waoranii*	*P. zeuctotylus*
P. acuminatus	-	0.20092410	0.01087634	-	0.19171381	-	-	0.10132698	0.24793633	-
P. aureolineatus	-	0.20288968	0.21752828	-	0.19907033	-	-	0.25290037	0.17629179	-
P. bromeliaceus	-	-	-	-	-	-	-	-	-	-
P. ecuadorensis	-	-	-	-	-	-	-	-	-	-
P. enigmaticus	-	0.18304694	0.07442032	-	0.19263537	-	-	0.11057278	0.25237300	-
P. galdi	-	-	-	-	-	-	-	-	-	-
P. lacrimosus	-	0.19387814	0.22648788	-	0.20058852	-	-	0.24277119	0.20060790	-
P. lacrimosus	-	-	-	-	-	-	-	-	-	-
P. limoncochensis	-	0.19281201	0.04808264	-	0.20339908	-	-	0.11888128	0.24924012	-
P. mendax	-	0.19401927	0.21146805	-	0.18719394	-	-	0.21318077	0.20969865	-
P. mindo		-	-	-	-	-	-	-	-	-
P. moro	0.07475440		0.19934069	-	0.19174597	-	-	0.20692992	0.22406314	-
P. omeviridis	0.13930008	0.09018246		-	0.19246734	-	-	0.10427358	0.24473298	-
P. ornatissimus	0.10007176	0.07898686	0.13409497		-	-	-	-	-	-
P. petersi	0.10198628	0.08655455	0.12658239	0.10469321		-	-	0.21274515	0.21884790	-
P. schultei	0.10533375	0.07042748	0.13357793	0.09108173	0.08774179		-	-	-	-
P. subsigillatus	0.10009214	0.08696929	0.15049525	0.12534390	0.12766634	0.12560446		-	-	-
P. tantanti	0.15760012	0.10914088	0.08045283	0.13906407	0.13126174	0.14798275	0.15797708		0.25276337	-
P. waoranii	0.11592182	0.10074019	0.15544884	0.13395393	0.12448763	0.11428126	0.14264876	0.15761792		-
P. zeuctotylus	0.11452036	0.10089438	0.15306576	0.12181070	0.12195969	0.11656332	0.14019451	0.15827347	0.11321065	0

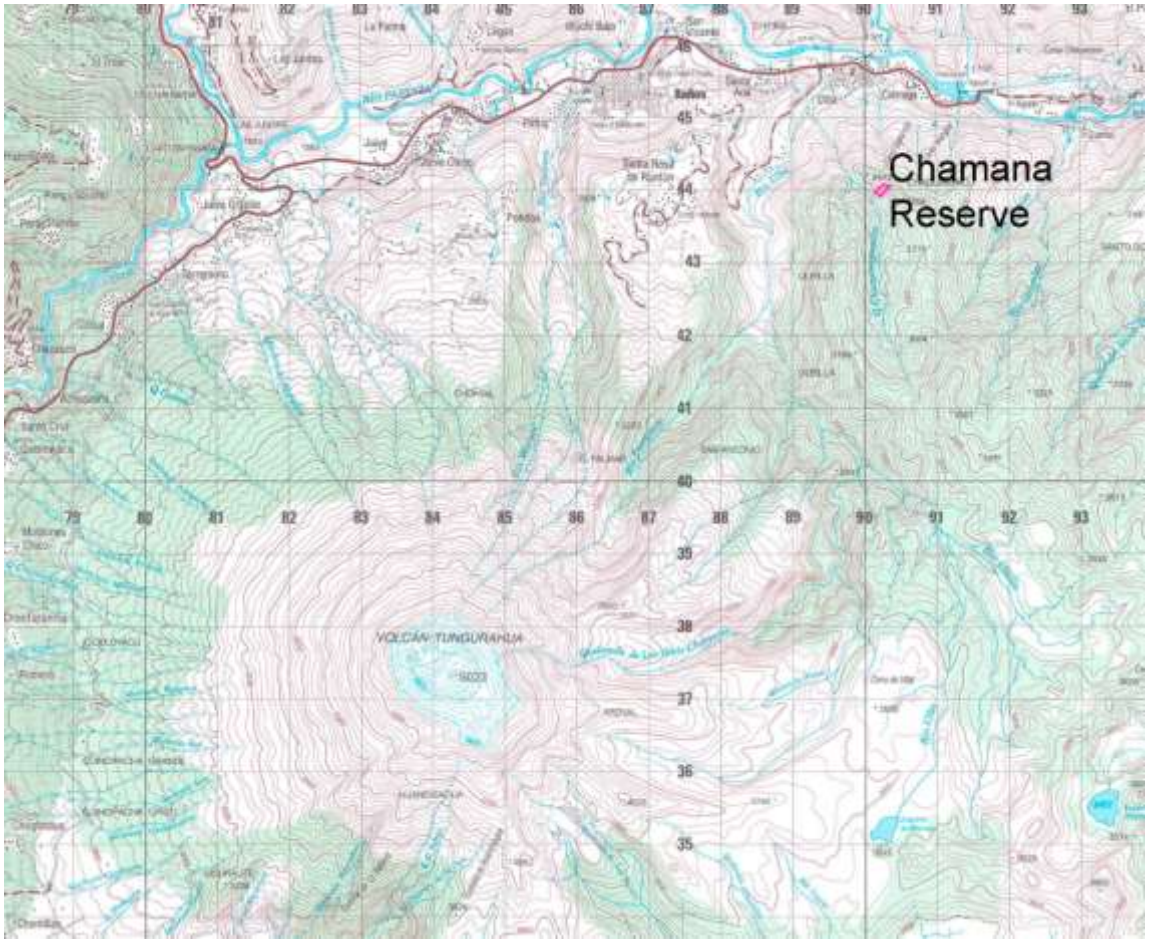

Figure 1: Map of the Chamana Reserve at the province of Tungurahua, Ecuador (Reyes- Puig, 2018). A candidate new species was discovered at this locality.

Figure 2: Map of Rio el Topo (Reserva Zuñac) at the province of Tungurahua, Ecuador. A candidate new species was discovered at this locality.

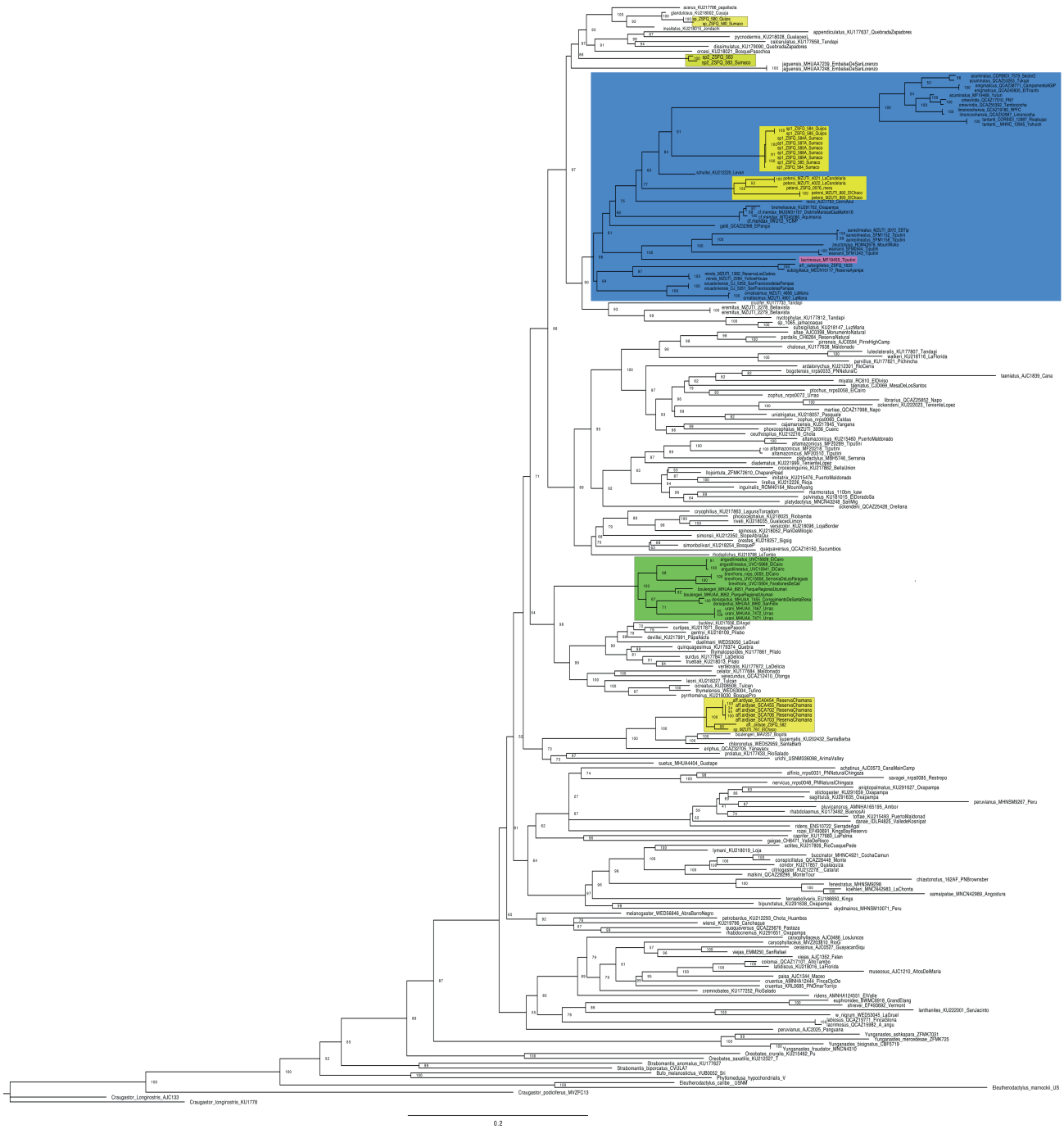

Figure 3: phylogenetic relationships of the genus *Pristimantis* based on a concatenated matrix of the mitochondrial gene 16S and COI. Species in the *P. lacrimosus* (blue) and *P. boulengeri* (green) groups, and new species (yellow) are highlighted.