Rediscovery of Laura's glassfrog *Nymphargus laurae* (Anura: Centrolenidae) with new data on its morphology, colouration, phylogenetic position and conservation in Ecuador

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Abstract

We report the rediscovery of Laura's Glassfrog, *Nymphargus laurae* Cisneros-Heredia and McDiarmid, 2007, based on two female specimens collected at the Colonso-Chalupas Biological Reserve, province of Napo, Ecuador. The species was described and known from a single male specimen collected in 1955 at Loreto, north-eastern Andean foothills of Ecuador. Limited information was available about the colouration, systematics, ecology, and biogeography of *N. laurae*. We provide new data on the external morphology, colouration, and distribution and comment on its conservation status and extinction risk. We discuss the phylogenetic relationships of *N. laurae*, which forms a clade together with *N. siren* and *N. humboldti*. The importance of research in unexplored areas must be a national priority to document the biodiversity associated, especially in protected areas.

Key words: Phylogeny, taxonomy, glassfrogs, Colonso-Chalupas Biological Reserve, systematics.

Introduction

Nymphargus Cisneros-Heredia & McDiarmid, 2007 currently includes 42 described species of glassfrogs (family Centrolenidae), and 21 of them occur in Ecuador (Guayasamin et al., 2020; Frost, 2021). Despite increasing efforts to better understand the diversity, natural history, ecology and distribution of glassfrogs in the tropical Andes, several species of *Nymphargus* remain known only from their type localities or few collected specimens [e.g., *Nymphargus buenaventura* Cisneros-Heredia & Yanez-Muñoz, 2007 (Cisneros-Heredia & Yánez-Muñoz, 2007; Yánez-Muñoz et al., 2014), *N. laurae* Cisneros-Heredia & McDiarmid 2007 (Cisneros-Heredia & McDiarmid, 2007), *N. lindae* Guayasamin in Guayasamin et al. 2020 (Guayasamin et al., 2020), *N. manduriacu* Guayasamin, Cisneros-Heredia, Vieira, Kohn, Gavilanes, Lynch, Hamilton, and Maynard, 2019 (Guayasamin et al., 2019)].

Laura's Glassfrog *Nymphargus laurae* was described based on a male specimen collected in 1955 at Loreto, on the north-eastern foothills of the Andes of Ecuador (Cisneros-Heredia & McDiarmid, 2007). More than 60 years have passed since the collection of the holotype and single known specimen of *N. laurae*, and no additional individuals or information has become available for the species (Guayasamin et al., 2020). Since its description, numerous herpetologists have searched for *N. laurae* along the eastern slopes of the Andes of Ecuador without success. Due to its apparent rarity, restricted distribution, and extensive habitat change and loss at the type-locality, *N. laurae* was classified as Endangered at the national level (Ortega-Andrade et al., 2021) and Critically Endangered at the global level (Cisneros-Heredia 2008).

Between 2016–2018, we collected two individuals of *Nymphargus laurae* at the Colonso-Chalupas Biological Reserve. In this context, we provide new information about the morphological and chromatic variation, natural history, conservation status and extinction risk and reveal for the first time the phylogenetic position of *N. laurae* in Centrolenidae.

Materials & Methods

Study area and field surveys

The Colonso-Chalupas Biological Reserve is a national protected area located on the foothills of the north-eastern Andes of Ecuador, province of Napo. This biological reserve protects 932.46 km^2, extending between 560–4432 m elevation, and being home to a variety of ecosystems, from tropical evergreen forests to paramos (van der Hoek et al., 2018). It is part of an ecological corridor with two neighbouring protected areas: Antisana Ecological Reserve, to the north, and Llanganates National Park, to the south (Ramis et al., 2018; van der Hoek et al., 2018). Montane evergreen cloud forests are characterised by a great variety of trees of the families Melastomataceae, Solanaceae, Myrsinaceae, Aquifoliaceae, Araliaceae, Rubiaceae. Those trees reach up to 15–25 m in height, showing gnarled trunks and branches and dense and compact crowns, covered by epiphytes, including orchids, bromeliads, aroids, and ferns (MAE, 2012,

2013). This ecosystem is commonly covered by mist, either constantly or during the early morning and late afternoon (Ramis et al., 2018). The average annual rainfall is 4620 mm, and the average annual temperature is 28.7 °C. The rainy season extends between March and July, with 448 mm of monthly average rainfall and 23.5 °C of monthly average temperature. The dry season is between August and January, with 353 mm of average monthly precipitation and 23.9 °C of average monthly temperature (INAMHI, 2015). Nocturnal surveys for collection of amphibians and reptiles were conducted at the Colonso-Chalupas Biological Reserve from 19h00-02h00, at a stream nearby to Ikiam's Scientific Station, on 17 October 2016 (0.9348°S 77.9270°W, 1506 m) and at the Narpa stream, on 09 June 2018 (0.9353°S 77.9268°W, 1440 m) (Fig. 1). Environmental Ministry of Ecuador provided full approval for this research (MAE-DNB- CM- 2017- 0062).

Morphological characteristics

Terminology, characters and measurements follow formats and definitions described by Cisneros-Heredia & McDiarmid, 2007, Watters et al. (2016) and Guayasamin et al. (2020). Examined frogs were photographed in life, anaesthetised with lidocaine 2%; specimens and tissues were fixed in 96% ethanol and preserved separately in 75% ethanol. Sex and maturity were determined by directly examining gonads through dissections and noting secondary sexual characters (i.e., vocal slits and nuptial pads). Colour patterns are based on photographs and annotations of living specimens taken in the field. Adjective "enamelled" is used to describe the shiny white colouration produced by the accumulation of iridophores (Lynch & Duellman, 1973; Cisneros-Heredia & McDiarmid, 2007).

The following measurements were taken with a digital calliper (0.02 mm accuracy, rounded to nearest 0.1 mm) under a stereomicroscope: snout-vent length (SVL); head width (HW); head length (HL), snout length (SL); interorbital distance (IOD), horizontal eye diameter (ED), internarial distance (IND), eye-nostril distance (EN), horizontal tympanum diameter (TD); upper eyelid width (UEW), forearm length (FLL), hand length (HAL), finger IV disk width (Fin4DW), thigh length (THL), tibia length (TL), foot length (FL).

Morphological data used for comparisons were obtained from the direct examination of specimens deposited in the following collections: The Natural History Museum, Department of Zoology, London (BMNH); División de Herpetología, Instituto Nacional de Biodiversidad, Quito (DHMECN); Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá (ICN); University of Kansas Natural History Museum, Lawrence, KS (KU); Museum of Comparative Zoology, Harvard University, Cambridge, MA (MCZ); Museo de Zoología, Pontificia Universidad Católica del Ecuador, Quito (QCAZ); National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM); Museo de Zoología, Universidad San Francisco de Quito, Quito (ZSFQ).

DNA extraction, amplification, sequencing, and phylogenetic analyses

Genomic DNA was extracted from hepatic tissue preserved in 96% ethanol from specimen DHMECN 15383, using the "Isolation of Genomic DNA" protocol, Wizard Genomic DNA Purification Kit (Promega, 2019). Three mitochondrial gene fragments of 12S ribosomal rRNA gene, 16S ribosomal rRNA gene, Cytochrome C Oxidase Subunit 1 (COI), and the Recombination Activating Protein 1 gene (RAG1) were amplified using the Polymerase Chain Reaction (Saiki et al., 1988). Primers for 12s gene (12sH10-FWR 5'-CACYTTCCRGTRCRYTTACCRTGTTACGACTT-3' / 12sL4E-REV 5'-TACACATGCAAGTYTCCGC-3'), 16s gene (16Sar-L-FWR 5'-CGCCTGTTTATCAAAAACAT-3' / 16Sbr-H-REV 5'-CCGGTCTGAACTCAGATCACG T-3'), COI (dgLCO-1490-FWR 5'-GGTCAACAAATCATAAAGAYATYGG-3' / dgHCO-2198-REV 5'-TAAACTTCAGGGTGACCAAARAAYCA-3') and RAG1 (RAG1-R182 5'GCCATAACTGCTGGAGCATYAT3' / RAG1-R270 5'AGYAGATGTTGCCTGGGTCTTC3'), were used. Each PCR reaction was composed of a 25 µl reaction mix containing: 12.5 µl GoTaq Green Master Mix, 4.5 µl H_20, 1.5 µl on 10 µM of Forward and Reverse primers, and 5 µl of purified DNA. We perform amplification on an Applied Biosystems GeneAmp PCR System 9700 thermal cycler. The amplification program was set with an initial denaturation of 95°C (5 min) followed by 35 cycles of 95°C (30 sec), 57°C (30 sec), 72°C (15 sec), with a final extension temperature of 72°C (5 min) and 4°C for an unlimited period. Amplified DNA products were visualised by electrophoresis on a 2.5% agarose gel and post-staining with Tris/Borate/EDTA buffer (TBE) under blue light. PCR-amplified sequences were purified using Illustra™ ExoProStar™ Enzymatic PCR and Sequencing Clean-Up Kit. Sequencing was performed in both DNA strain directions and undertaken by Macrogen, Seoul, South Korea (http://www.macrogen.com). Chromatographs resulting from sequencing were revised and edited using Geneious Prime v.2020.0.5 software (Kearse et al., 2012). New sequences were deposited in GenBank with the following accession numbers: 12S MZ820691; 16S MZ831508; COI MZ828399; RAG1 MZ835991).

To infer the phylogenetic position of *N. laurae*, we included sequences for 119 species (Table S1) selected from Guayasamin et al. (2020), obtained from the NCBI GenBank database (National Center for Biotechnology Information NCBI, 2020). We included two species for Allophrynidae as outgroups (Table S1) and rooted the phylogeny with *Allophryne ruthveni*. Alignments were reviewed and edited manually to remove regions with a high proportion of missing data at the edges and hypervariable regions with Geneious Prime v.2020.0.5 software (Kearse et al., 2012). We used Mesquite v3.61 software (Maddison & Maddison, 2019) to store the sequences and to create a concatenated matrix of all genes (12s, 16s, COI and RAG-1), with partitions for codons of the coding genes (COI and RAG-1). Because our combined data set comprised two ribosomal genes (12s and 16s), one protein-coding mitochondrial gene (COI) and one nuclear gene (RAG-1), we used PartitionFinder2 v2.1.1 (Lanfear et al., 2012), in the CIPRES Science Gateway V.3.3 (Miller, Pfeiffer & Schwartz, 2010), to select the GTR + I + G

as the optimal model for nucleotide substitution per partition.

Phylogenetic analyses were conducted using Maximum Likelihood (ML) and Bayesian Methods (BA) on the aligned sequences in the CIPRES Science Gateway V.3.3. ML analyses were performed using Garli v2.0 [Genetic Algorithm for Rapid Likelihood Inference; (Zwickl, 2006)]. We perform a total of 10 runs to reduce the probability of inferring a suboptimal probability solution. Node support was evaluated using 1000 bootstrap replicates. Bayesian phylogenetic analyses were performed in MrBayes v3.2.2 (Ronquist & Huelsenbeck, 2003), using five runs of the Monte Carlo Markov Chain (MCMC) algorithm for 20 million generations each, with four heated chains (0.2 heating parameter). Trees were sampled every 20000 generations, with burning of 25% of the total trees. To evaluate the effective sampling size of the five independent, uncorrelated runs, we used the statistical number of effectively independent draws from the posterior (ESS> 200) visualised with Tracer v1.6. (Rambaut et al., 2013). Phylogenetic trees were edited using FigTree v1.4.2. (Rambaut, 2014).

Conservation Status

We assessed the conservation status of *N. laurae* based on an environmental risk surface model (0=no threats, 1=maximum threat value) produced for Ecuadorian amphibians by (Ortega-Andrade et al., 2021), a satellite image (2019, Google Earth) from the type locality of the species, and a map of the National System of Protected Areas (http://ide.ambiente.gob.ec/mapainteractivo/). We classify the extinction risk of *N. laurae* based on the categories and criteria presented by (IUCN, 2012, 2019).

Results

Surveys at the Colonso-Chalupas Biological Reserve resulted in the collection of two specimens of *Nymphargus laurae*. A subadult female (DHMECN 15383) was found at a stream near the Ikiam´s Scientific Station on 17 October 2016 by H. Mauricio Ortega-Andrade. An adult male (DHMECN 15384) was collected at Narpa stream, on 09 June 2018 by Miguel Gómez Laporta and H. Mauricio Ortega-Andrade. Both specimens were found active at night on leaves of riverine vegetation up to 4.5 meters, next to small creeks, between 22h00–23h00. *Nymphargus laurae* was in syntopy with *Nymphargus cochranae*, *Pristimantis quaquaversus*, *P. malli*, *P. incomptus*, *P. ventrimarmoratus*, *P.* sp. aff. *petersi*, and *P.* sp. aff. *conspicillatus*. The female was evaluated as subadult by having unconvoluted oviducts and immature ovarian eggs.

Both specimens are similar to the holotype in their anatomy and colouration, showing all diagnostic characters described for *N. laurae*: (1) vomerine teeth absent; (2) snout truncated in dorsal and lateral views; (3) tympanic annulus evident; (4) dorsal skin shagreen with slightly elevated warts corresponding to ocelli and scattered spicules, (5) ventral skin granular, without cloacal ornamentation except for a pair of large flat tubercles; (6) parietal peritoneum white,

198 covering 2/3 of the abdomen; all other peritonea clear; (7) liver lobed, hepatic peritoneum clear;
199 (8) humeral spine absent; (9) webbing basal between fingers; (10) webbing on feet similar to
200 holotype (see below); (11) no dermal folds or tubercles on hands, arms, feet or tarsi; (12)
201 unpigmented nuptial pad Type-I, concealed prepollex; (13) Finger II longer than Finger I; (14)
202 eye diameter larger than the width of disc on Finger III; (15) colouration in life, green with ocelli
203 (yellow spots surrounded by black), and in preservative, lavender with ocelli (cream-coloured
204 centre surrounded by dark lavender).
205
206 The male specimen (DHEMCN 15384) has SVL = 22.1 mm, slightly larger than the male
207 holotype (USNM 288453, 19.9 mm SVL), and both are smaller than the subadult female
208 (DHMECN 15383, 22.3 mm SVL). Differences in measurements and proportions between males
209 are probably due to interspecific variation (Table 1). Male has a combination of large and small
210 spicules (visible under magnification) on the head, dorsum, and flanks, but spicules on the lower
211 part of dorsum and eyelids are smaller. The female has smaller spicules compared to the male in
212 the parts of the body. A spicule is present in the centre of each ocellus, being more prominent
213 and pointed when compared to other body spicules. The female has the tympanic annulus is
214 proportionally covered by the supratympanic fold than in in males. Hand webbing in the new
215 specimens (III 2 2/3 – 2 1/3; Fig. 2A) is very similar to the holotype (III 2 2/3 – 2 ½ IV), and feet
216 webbing shows slight variation: I 2 - 2 ¾ II 1 ½ - 2 ¾ III 1 ½ - 2 ¾ IV 2 ¾ - 1 ½ V in the female
217 and I 2 ¾ - 2 ¾ II 1 ½ - 2 ¾ III 1 ½ - 2 ¾ IV 2 ½ - 1 ¾ V in the male (Fig. 2B) (I2-–2+II11/2–
218 2+III1+–21/2IV21/2–11/2V in the male holotype). The male has two papillae on discs of Toe I
219 and II (Fig. 2C). The female lacks papillae on toes. The holotype of *N. laurae* has two papillae
220 on each toe disc, except for Toe V. Absence of papillae in the female and in some toes in the new
221 male (Fig. 2B) suggest that papillae show intraspecific variation or is of external origin.
222
223 In preservation, the new specimens show similar colourations to the holotype. However, the
224 female shows a lavender dorsum, while the new male and the holotype have cream dorsum with
225 lavender tones. The female has 19 ocelli on the body and eight on the head (Fig. 3A), and the
226 male has six ocelli on the body and three on the head (Fig. 3B) (14 on the body and five on the
227 head of the holotype). Upper eyelids are dark lavender. Fingers and toes lack melanophores. All
228 ventral surfaces are cream. The parietal peritoneum and sclera are white, covering 2/3 of the
229 abdomen; pericardium, digestive peritonea, hepatic peritoneum, and urogenital peritonea are
230 clear.
231
232 The colouration in life of *N. laurae* remains known only from the brief description provided by
233 Gustavo Orcés-Villagómez, Ecuadorian zoologists who donated the specimen to James A.
234 Peters, USNM curator, and reported in the original description of the species: “green with yellow
235 spots surrounded by black” (Cisneros-Heredia and McDiarmid 2007). The new specimens allow
236 for a complete description: Head green, darker than the body, lip greenish cream; dorsal surfaces
237 of body, arms and legs green; ocelli on head and body having yellow spots surrounded by black;

ocelli absent on arms and legs; upper flanks coloured as dorsum but lower flanks cream, with a sharp division between both; hands, finger, feet and toes yellowish-green, with yellow discs; nuptial pad cream (Fig. 4). Throat greenish-white, all other ventral surfaces cream white. Yellow circumpupillary ring and whitish iris with thin dark reticulations and dark flecks concentrated towards the middle (Fig. 4). Nictitating membrane yellowish, without reticulations. Green bones.

We reconstructed the evolutionary tree (Fig. 5) of *N. laurae* with a dataset including 120 taxa and 2823 base pairs. ML and BA analyses are both congruent and recovered the phylogenetic position of *N. laurae* as sister species of *N. siren* and together as sister to *N. humboldti*. The clade *N. laurae* + *N. siren* + *N. humboldti* has high posterior probability (0.9 node value in Fig. 5), while the ML bootstrap value has relative low support (<0.7). This clade is closely related to *Nymphargus megacheirus* and *N. anomalus* (Fig. 5). Phylogenetic relationships among major groups to genus level are supported with high values, in *Hyalinobatrachium, Centrolene, Cochranella, Espadarana, Rulyrana, Sachatamia, Teratohyla,* and *Vitreorana* (Fig. S1).

Nymphargus laurae is known from two localities in the province of Napo, on the north-eastern flanks of the Andes of Ecuador, at elevations between 700–1500 m (Fig. 1). The type locality, Loreto, was originally covered by Lowland Evergreen forests, and it is located on the lower slopes of the Sumaco volcano, on the upper Napo valley. Satellite images (Fig. 1) show that less than 10% of the natural forests remains at the type locality. The new locality, Colonso-Chalupas, is still covered by Evergreen montane forest (Fig. 1). The environmental risk surface (ERS) results in threat values from 0 (Colonso Chalupas) to 0.37 (Loreto), due to habitat loss and fragmentation for cattle raising and agriculture, deforestation, roads, oil pipelines, and stochastic events related with explosions of the Sumaco Volcano.

Discussion

The records of *Nymphargus laurae* presented in this paper correspond to the first report of the species after 66 years from its original collection. The Colonso-Chalupas Biological Reserve is the second known locality of *N. laurae*, extending its geographic range in ca. 77 km SW from the type locality, at Loreto, province of Orellana, Ecuador (Cisneros-Heredia & McDiarmid, 2007). These records also extend the altitudinal range of the species from ca. 700 m (see comments on the elevation of Loreto by Urgilés et al. 2017) up to 1500 m. *Nymphargus laurae* maybe more widespread than currently known, but possibly it is endemic to north-eastern Ecuador.

Dorsal colouration pattern showing ocelli with yellow centre surrounded by black on a green dorsum is shared by three ocellated glassfrogs: *N. cochranae*, *N. laurae* and *N. lindae*. These species share a common biogeographic pattern across the eastern Andean slopes in Ecuador, with *N. cochranae* being widespread across the eastern Andean slopes of the Andes of Ecuador and southern Colombia and sympatric with *N. laurae* (Cisneros Heredia & McDiarmid, 2005; 2006; 2007, this paper). All three species are very similar in their morphology and colouration, and when a single known specimen was available for *N. laurae*, the differentiation between *N. laurae*

and *N. lindae* was weak and there was the possibility that *N. cochranae* and *N. laurae* may be synonyms (Guayasamin et al. 2020). Now we can provide strong evidence for the distinctiveness of *N. laurae*, which is not closely related to *N. cochranae* nor *N. lindae*, based on morphological, chromatic, and molecular data. Externally, *N. laurae* differs from *N. cochranae* by having much larger ocelli (ocelli in *N. cochranae* are small, and in some specimens they are so small that without close inspection, they appear to be just dark spots); ocelli with yellow centre (orange centre in *N. cochranae*), Finger II longer than Finger I (Figer I > Finger II in *N. cochranae*); distal subarticular tubercle of fourth finger bifurcate; indistinct outer metatarsal tubercle; supernumerary tubercles present; no ocelli on forearms and shanks (present in some *N. cochranae*); no vomerine teeth (present in some *N. cochranae*); and smaller body size (23.8–31.6 mm SVL in males of *N. cochranae* vs. 19.9–22.3 mm SVL in males of *N. laurae*). *Nymphargus laurae* and *N. lindae* are very similar, but *N. lindae* is diagnosable due to the present of vomerine teeth (absent in *N. laurae*), low ulnar and tarsal folds present (absent in *N. laurae*), and slightly larger body size (19.9–22.3 mm SVL in males of *N. laurae* vs. 23.0–26.5 mm SVL in males of *N. lindae*). The condition of the papillae at the tip of toes was used as a diagnostic character in the original description of *N. lindae*, but it is not a useful taxonomic character due to its variation in *N. laurae*.

Phylogenetic analyses place *N. laurae* in a clade with *N. siren* and *N. humboldti* (Fig. 5). These results are interesting due to the colouration differences among *N. laurae, N. humboldti* and *N. siren* and their close distribution in nearby areas at the Sumaco volcano and the Guacamayos mountain range. *Nymphargus siren* and *N. humboldti* are almost identical, the only phenotypic diagnostic character being the smaller body size of *N. siren*. However, our phylogenetic information shows that, despite their similarities, they are not sister to each other. *Nymphargus siren* is distributed on the eastern Andean slopes from southern Colombia to northern Ecuador, at elevations between 1410–2000 m; *N. humboldti,* is known from two localities on the eastern Andean slopes of central Ecuador, at elevations between 1770–2400 m (Guayasamin et al., 2020); and *N. laurae* is restricted to lowland and foothill forests along the Upper Napo River basin (Cisneros-Heredia and McDiarmid 2007, this paper). Our results suggest the dispersal of this clade occurred in the northern Andes, along montane forest in the upper Napo River basin, Guacamayos mountain range and Sumaco volcano (Fig. 1A). Although these species have similar elevations and distributional ranges, the role of morphological, behavioural, bioacoustics and physiological features (i.e., climatic tolerances) is still intriguing, regarding their evolution and biogeographical diversification in eastern Andes of Ecuador.

Based on data provided herein, we propose the following extinction risk assessment for *N. laurae*: (1) *N. laurae* has suffered population reductions, based on the continuous decline in habitat quality at its type locality and surroundings, where no recent record for the species have been obtained despite surveys. Habitat quality at Colonso-Chalupas is better by being part of a protected area. However, since only three specimens are known for the species, we refrain from

using criterion A until more data are available to at least inferred the population status of the species; (2) the species is known from just two localities with different conservation conditions, thus each one should be evaluated as a different threat-defined location; (3) an EOO cannot be estimated with two localities but the estimated AOO is 8 km^2, which is within the threshold for Critically Endangered (< 10 km^2). However, we consider that it is possible that the geographic range of *N. laurae* is larger, closer to the threshold for Endangered (10–500 km^2) under criterion B2; (4) the type locality and any potential locality outside of Colonso Chalupas are under ongoing habitat decline due to forest loss and water pollution. This information suggests the extinction risk of N. laurae is relatively high and we propose that it should be classified under the IUCN category of Endangered (EN) based on criteria B2ab(iii,iv). Although N. laurae now is expected to have a wider distribution, urgent conservation actions are encouraged for this species and other range-restricted amphibians the eastern Andes slopes of Ecuador.

Conclusions

We provide new information about *Nymphargus laurae*, a species previously known from a single specimen collected decades ago. Our new specimens collected at the Colonso Chalupas Biological Reserve increase the geographic range of the species along the north-eastern slopes of the Ecuadorian Andes. New insights into the morphology, colouration, and phylogeny of *N. laurae* demonstrate its distinctiveness among other ocellated glassfrogs, with which it is not closely related because it is part of a clade with *N. siren* and *N. humboldti*. Although now known from a second locality, the geographic range of *N. laurae* is still limited and habitat loss and fragmentation are threatening the long-term survival of populations outside of protected areas, thus we suggest that the species' extinction risk should be categorised as Endangered at the global and national level and conservation actions are urgently encouraged. The importance of research in unexplored areas must be a national priority to document the biodiversity associated, especially for range-restricted species and in little-explored protected areas.

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Figure legends

Figure 1. (A) Map of Ecuador showing the distribution ranges of *Nymphargus laurae*: type locality (yellow star) and new locality (specimens INABIO15383-84; yellow dot), and phylogenetic sister species of *N. laurae*: *N. siren* (red dots) and *N. humboldti* (green dots). Interlined rectangle in A delimit the area shown in B. (B) Environmental risk surface (Ortega-Andrade et al., 2021) and protected areas in the distribution range of *N. laurae*. Numbers correspond to the following protected areas: (1) Cayambe-Coca National Park, (2) Sumaco-Napo-Galeras National Park, (3) Antisana Ecological Reserve, (4) Colonso-Chalupas Biological Reserve, (5) Llanganates National Park. Note high risk modelled for the type locality, which is excluded from the National System of Protected Areas of Ecuador. (C) Satellite image (2019, Google Earth) of the Upper Rio Napo valley, type locality near the town of Loreto (ca. 0.666670° S, 77.316700°W, ca. 500 m elevation), slopes of the Sumaco Volcano, on the Cordillera Oriental, eastern slopes of the Andes, Provincia de Orellana, República del Ecuador.

Figure 2. Hand (A), foot (B) and papillae (C) of *Nymphargus laurae* (INABIO15384). Tags and background color have been digitally removed.

Figure 3. Views of the body (dorsum and venter), of (A) INABIO15383 and (B) INABIO15384 in specimens of *Nymphargus laurae*. Tags and background colour have been digitally removed.

Figure 4. *Nymphargus laurae* (INABIO15383), (A) dorsal view, (B) side view, (C) front view and (D) ventral view.

Figure 5. Optimal maximum likelihood tree (log likelihood= -28155.635) of clade Nymphargus

(orange rectangle) inferred from a partitioned analysis of the 12s, 16s, COI and RAG-1 genes. Circles indicate significant support values for clades recovered by Bayesian (BA) and Likelihood (ML) analyses.

Supplemental information

Supplemental Table S1. Taxon and genetic markers used in this study.
Sequences generated in previous studies were downloaded from GenBank. Newly generated sequences are in bold blue.

Supplemental Figure S1. Optimal maximum likelihood tree (log likelihood=-28155.635), showing the phylogenetic relationships among 119 species of Centrolenidae and one outgroup taxa. Values above nodes are posterior probabilities resulting from Bayesian phylogenetic analyses (values < 0.9 not shown, = 1). Numbers bellow nodes correspond to non-parametric bootstraps (values < 0.70 not shown, = 1).

Figure 1

Figure 1

(A) Map of Ecuador showing the distribution ranges of *Nymphargus laurae*: type locality (yellow star) and new locality (specimens INABIO15383-84; yellow dot), and phylogenetic sister species of *N. laurae*: *N. siren* (red dots) and *N. humboldti* (green dots). Interlined rectangle in A delimit the area shown in B. (B) Environmental risk surface (Ortega-Andrade et al., 2021) and protected areas in the distribution range of *N. laurae*. Numbers correspond to the following protected areas: (1) Cayambe-Coca National Park, (2) Sumaco-Napo-Galeras National Park, (3) Antisana Ecological Reserve, (4) Colonso-Chalupas Biological Reserve, (5) Llanganates National Park. Note high risk modelled for the type locality, which is excluded from the National System of Protected Areas of Ecuador. (C) Satellite image (2019, Google Earth) of the Upper Rio Napo valley, type locality near the town of Loreto (ca. 0.666670° S, 77.316700°W, ca. 500 m elevation), slopes of the Sumaco Volcano, on the Cordillera Oriental, eastern slopes of the Andes, Provincia de Orellana, República del Ecuador.

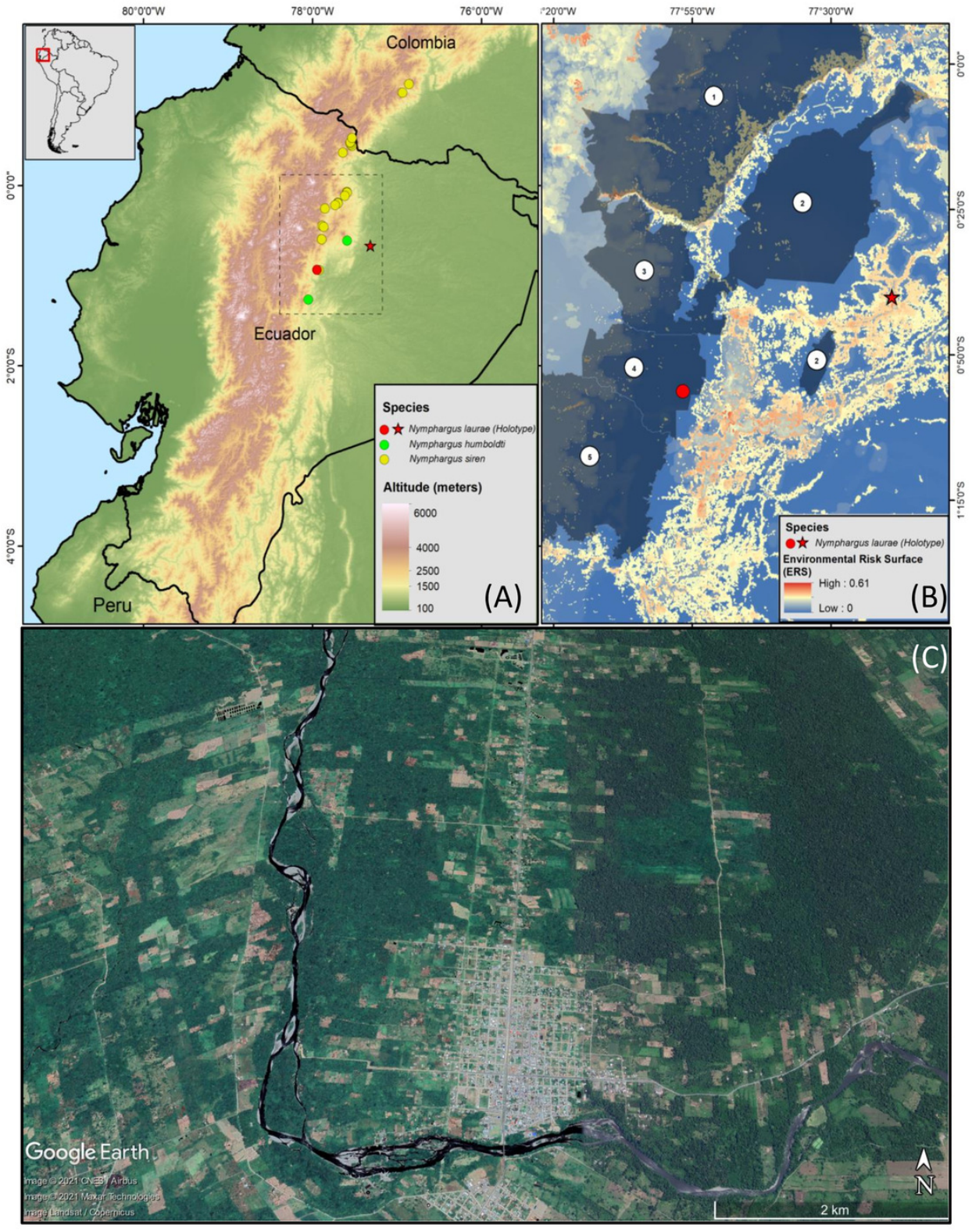
80°0'0"W
78°0'0"W
76°0'0"W
0°0'0"
2°0'0"S
4°0'0"S
Colombia
Ecuador
Peru
Species
Nymphargus laurae (Holotype)
Nymphargus humboldti
Nymphargus siren
Altitude (meters)
6000
4000
2500
1500
100
(A)
77°20'0"W
77°55'0"W
77°30'0"W
0°0'0"
0°25'0"S
0°50'0"S
1°15'0"S
1
2
3
4
5
Species
Nymphargus laurae (Holotype)
Environmental Risk Surface (ERS)
High : 0.61
Low : 0
(B)
(C)
Google Earth
image © 2021 CNES / Airbus
Image © 2021 Maxar Technologies
Image Landsat / Copernicus
2 km
N

Figure 2

Figure 2

Hand (A), foot (B) and papillae (C) of *Nymphargus laurae* (INABIO15384). Tags and background color have been digitally removed.

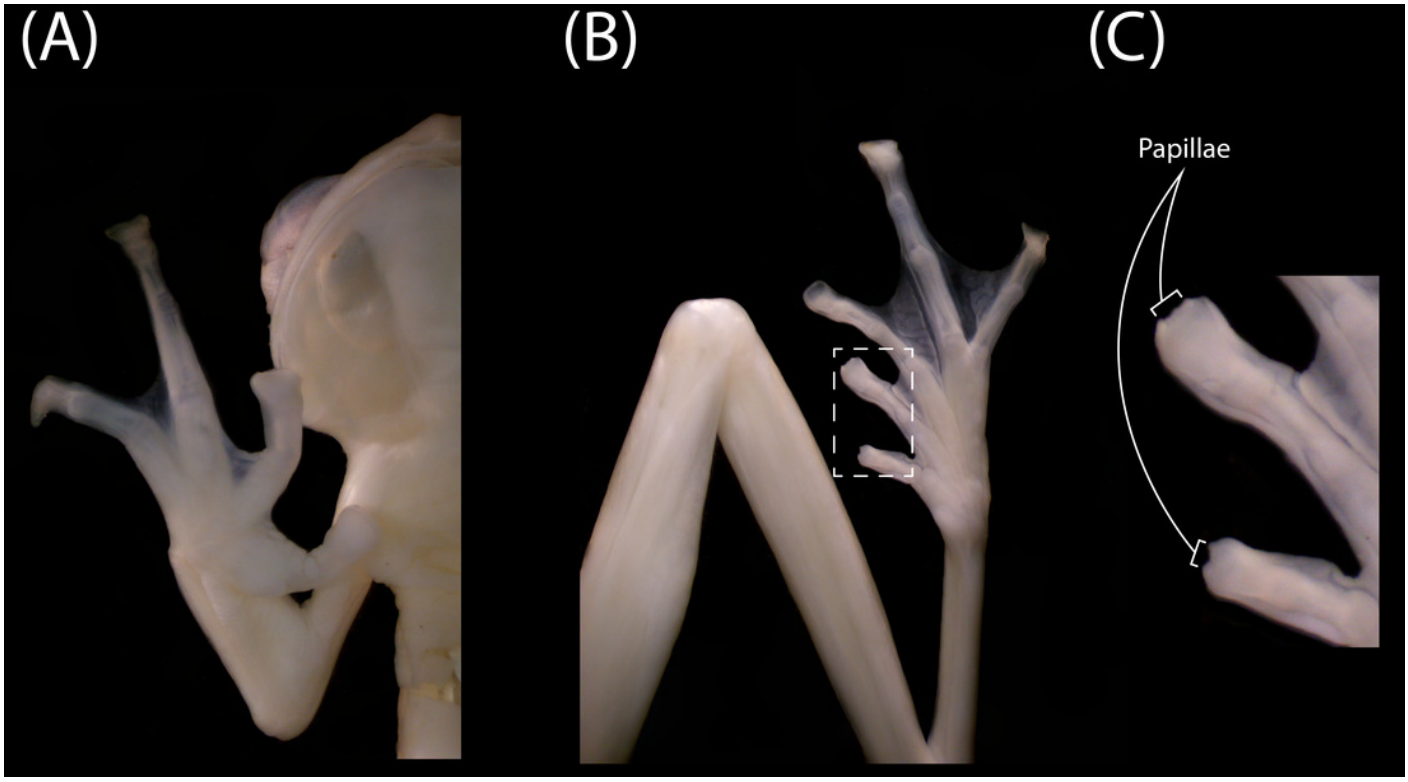

Figure 3

Figure 3

Views of the body (dorsum and venter), of (A) INABIO15383 and (B) INABIO15384 in specimens of *Nymphargus laurae*. Tags and background colour have been digitally removed.

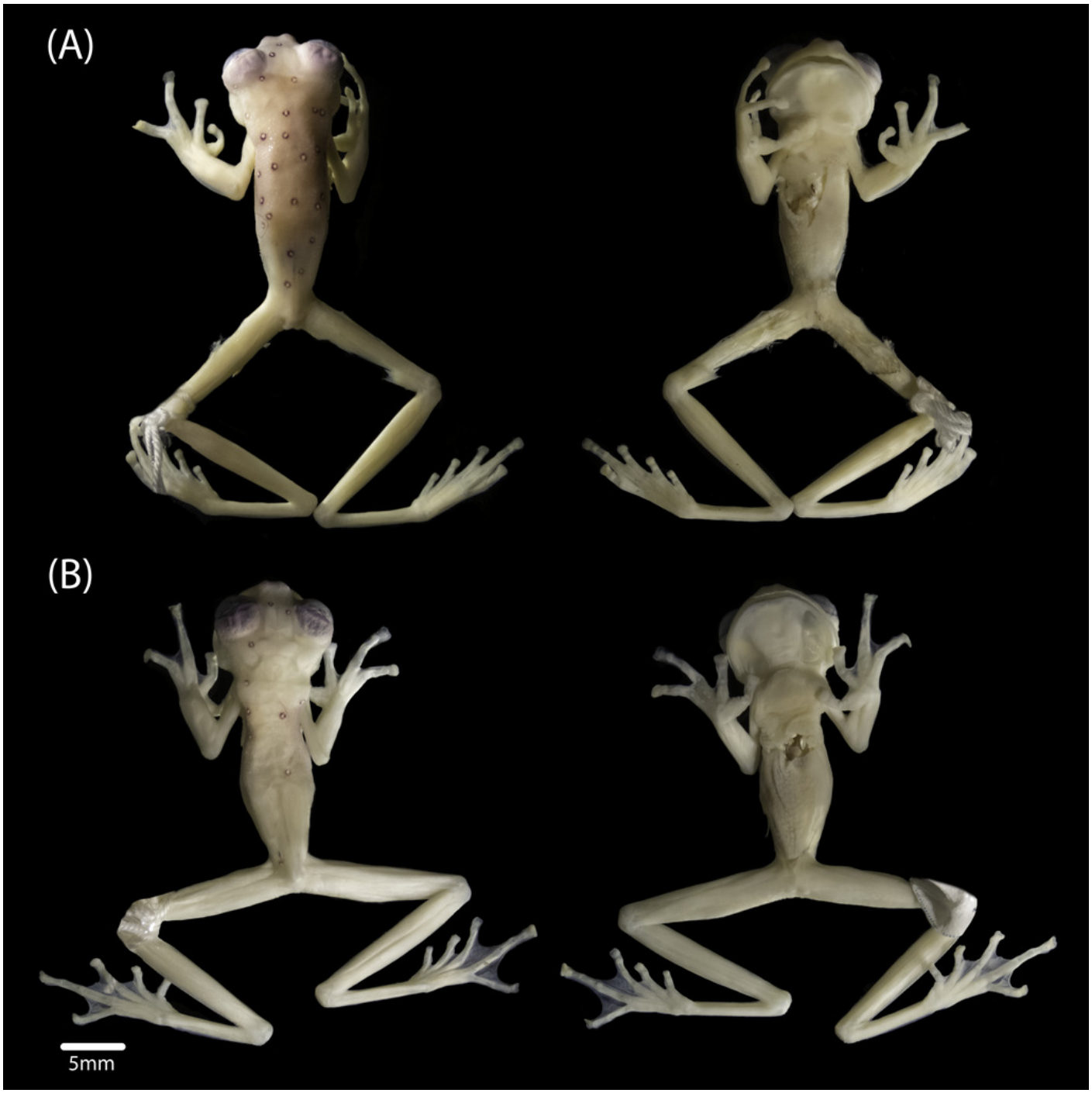
(A)
(B)
5mm

Figure 4

Figure 4

Nymphargus laurae (INABIO15383), (A) dorsal view, (B) side view, (C) front view and (D) ventral view.

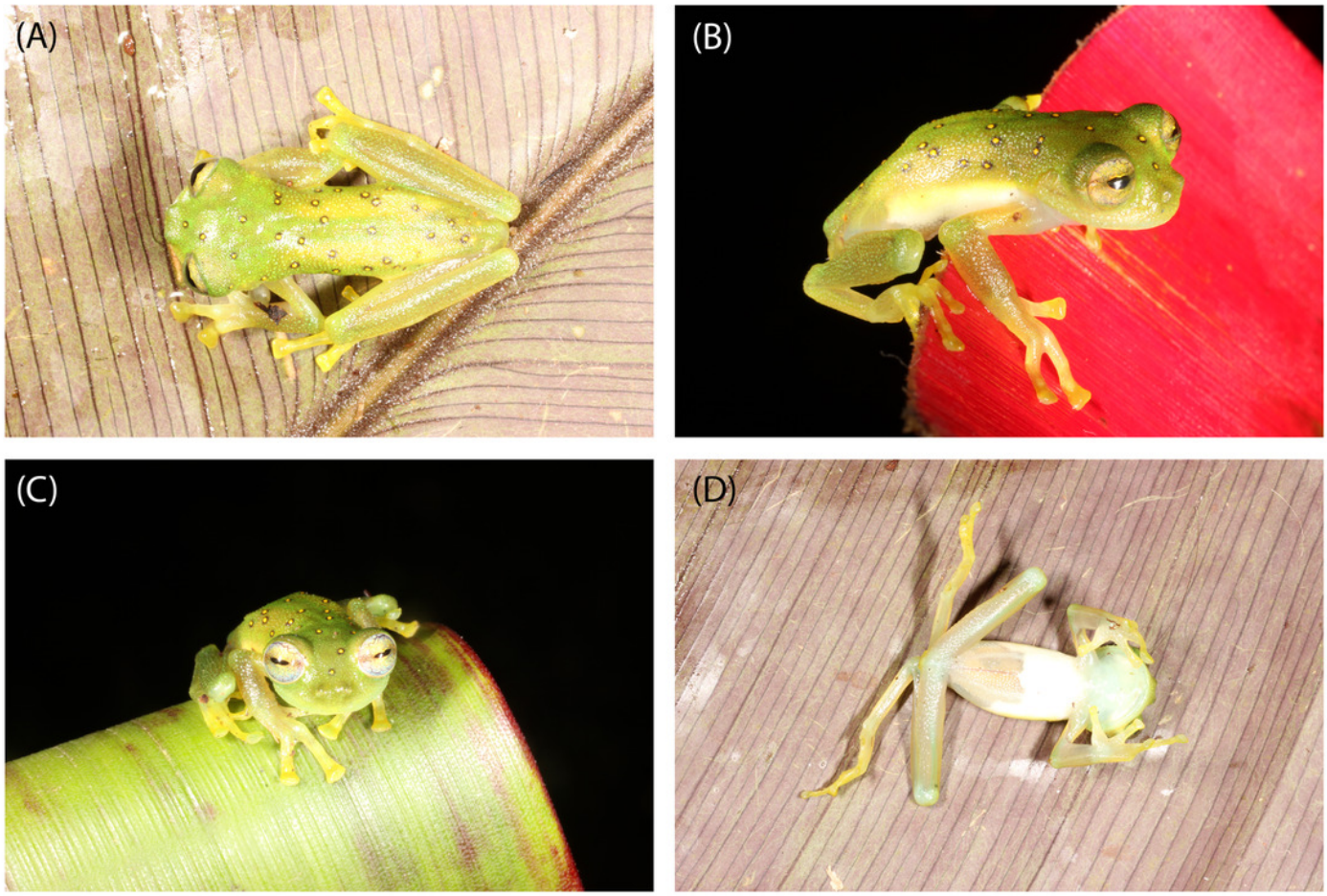

Figure 5

Figure 5

Optimal maximum likelihood tree (log likelihood= -28155.635) of clade Nymphargus (orange rectangle) inferred from a partitioned analysis of the 12s, 16s, COI and RAG-1 genes. Circles indicate significant support values for clades recovered by Bayesian (BA) and Likelihood (ML) analyses

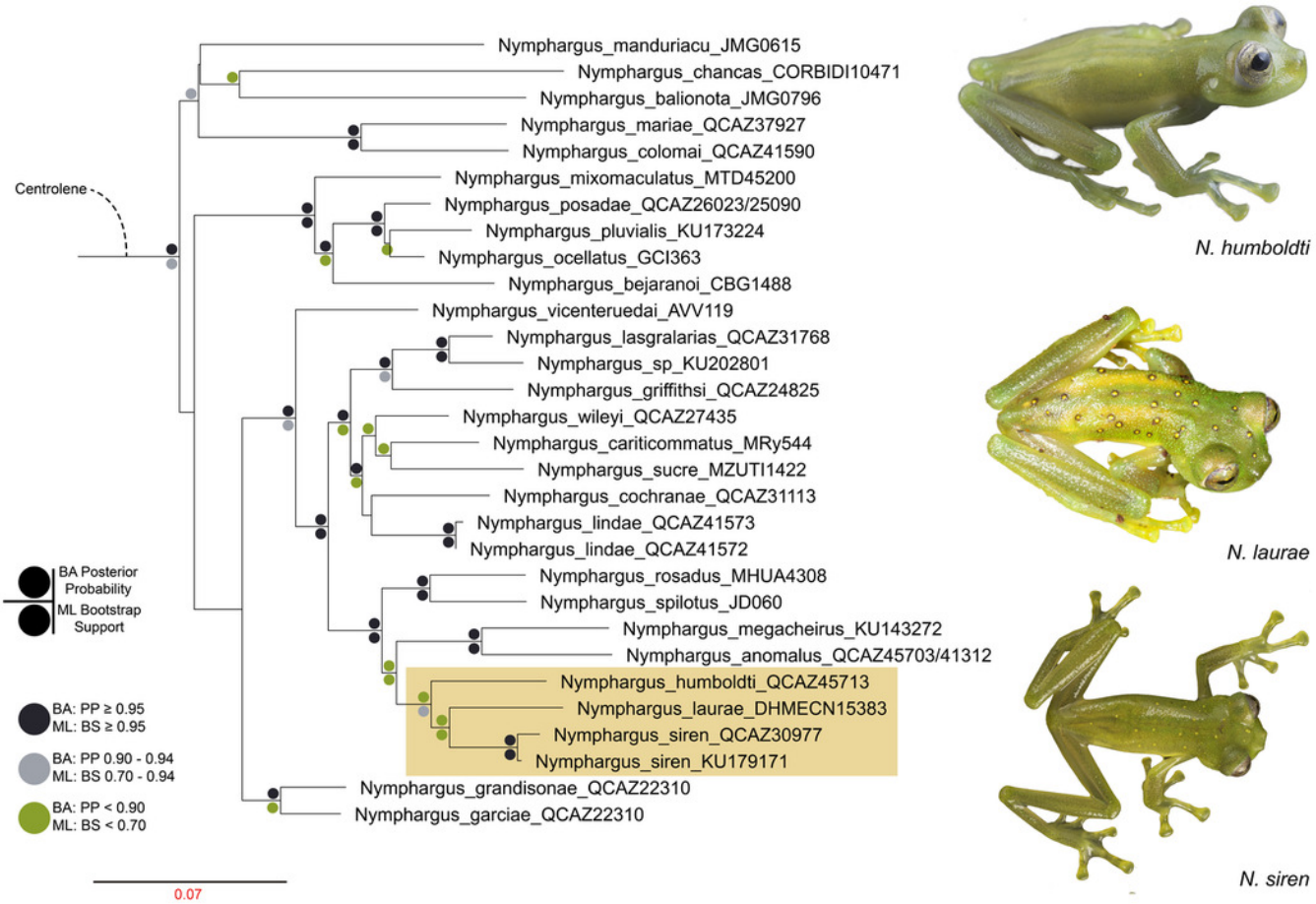

Table 1(on next page)

Table 1

Morphometric measurements (mm) in specimens of *Nymphargus laurae.*

Table 1:

Morphometric measurements (mm) in specimens of *Nymphargus laurae*.

Character	Male (Holotype) USNM 288453	Male **INABIO15384**	Female **INABIO15383**
HW	7.4	7.8	8.0
SVL	19.9	22.1	22.3
TL	11.7	13.8	13.6
IOD	3.8	3.1	2.8
HL	6.9	6.9	6.5
ED	2.9	3.4	3.6
IND	1.6	2.3	2.7
EN	1.7	2.1	3.0
FL	8.7	10.5	10.3
TD	-	0.9	0.7
THL	-	12.3	11.7
SL	-	3.1	2.8
FLL	-	5.0	4.4
UEW	-	2.1	2.9
HAL	-	7.8	7.4
Fin 4DW	-	1.5	2.0
HW/HL	1.1	1.1	1.2
HW/SVL	0.4	0.4	0.4
HL/SVL	0.4	0.3	0.3
EN/HL	0.3	0.3	0.5
ED/HL	0.4	0.5	0.5
IOD/ED	1.3	0.9	0.8
EN/ED	0.6	0.6	0.8
EN/IOD	0.5	0.7	1.1
TL/SVL	0.6	0.6	0.6
FL/SVL	0.4	0.5	0.5
HAL/SVL	-	0.4	0.3
FLL/SVL	-	0.2	0.2
ED/Fin 4DW	-	2.3	1.8