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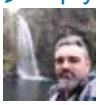

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with new data on its morphology, colouration, phylogenetic position and conservation in Ecuador

María José Sánchez-Carvajal[1,2], Grace C. Reyes-Ortega[1,2], Diego F. Cisneros-Heredia[3,4], H. Mauricio Ortega-Andrade[1,2,4]
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We report the rediscovery of Laura's Glassfrog, *Nymphargus laurae* Cisneros-Heredia & McDiarmid, 2007, based on two 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, 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.

Introduction

Nymphargus Cisneros-Heredia & McDiarmid, 2007 currently includes 42 describe species of glassfrogs (family Centrolenidae), and 21 of them occur in Ecuad (Guayasamin et al., 2020; Frost, 2021). Despite increasing efforts to better understand th diversity, natural history, ecology and distribution of glassfrogs in the tropical Ande several species of *Nymphargus* remain known only from their type localities or fe collected specimens (*e.g.*, *Nymphargus buenaventura* (Cisneros-Heredia & Yánez-Muño 2007; Yánez-Muñoz et al., 2014), *N. laurae* (Cisneros-Heredia & McDiarmid, 2007), *N lindae* Guayasamin in Guayasamin et al. (2020), *N. manduriacu* Guayasamin et al. (2019))

Laura's Glassfrog *Nymphargus laurae* was described based on a male specime collected in 1955 at Loreto, on the north-eastern foothills of the Andes of Ecuad (Cisneros-Heredia & McDiarmid, 2007). More than 60 years have passed since th collection of the holotype and single known specimen of *N. laurae*, and no addition individuals or information has become available for the species (Guayasamin et al., 202(Since its description, numerous herpetologists have searched for *N. laurae* along th eastern slopes of the Andes of Ecuador without success. Due to its apparent rarit restricted distribution, and extensive habitat change and loss at the type-locality, *N. laura* was classified as Endangered at the national level (Ortega-Andrade et al., 2021) ar Critically Endangered at the global level (Cisneros-Heredia, 2008).

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

Materials and methods

Study area and field surveys

The Colonso-Chalupas Biological Reserve is a national protected area located on th foothills of the north-eastern Andes of Ecuador, in the province of Napo. This biologic reserve protects 932.46 km^2, extending between 560–4,432 m elevation and being hom

cloud forests in Colonso-Chalupas are characterised by a great variety of trees of t families Melastomataceae, Solanaceae, Myrsinaceae, Aquifoliaceae, Araliacea Rubiaceae. Those trees reach up to 15–25 m in height, showing gnarled trunks ar branches, and dense and compact crowns covered by epiphytes, including orchid bromeliads, aroids, and ferns (Ministerio del Ambiente del Ecuador, 2012, 2013). Th ecosystem is usually covered by mist, either constantly or during the early morning ar late afternoon (Ramis, Álvarez-Solas & Peñuela, 2018). The average annual rainfall is 4,62 mm, and the average annual temperature is 28.7 °C. The rainy season extends betwee March and July, with 448 mm of monthly average rainfall and 23.5 °C of monthly averag temperature. The dry season is between August and January, with 353 mm of averag monthly precipitation and 23.9 °C of average monthly temperature (INAMHI, 201 Nocturnal surveys for collection of amphibians and reptiles were conducted at th Colonso-Chalupas Biological Reserve from 19h00–02h00, at a stream nearby to Ikiam Scientific Station, on 17 October 2016 (0.9348°S 77.9270°W, 1,506 m) and at the Narp stream, on 09 June 2018 (0.9353°S 77.9268°W, 1,440 m) (Fig. 1). Environmental Minist of Ecuador provided full approval for this research (MAE-DNB-CM-2017-0062 Specimens are deposited in the herpetological collection of the Instituto Nacional d Biodiversidad (Quito, Ecuador).

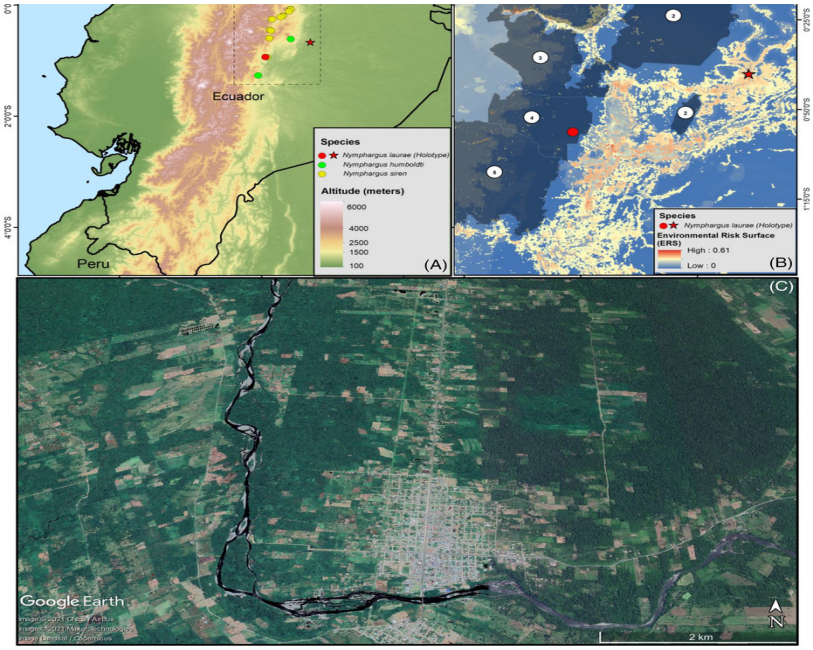

Figure 1: (A) Map of Ecuador showing the distribution ranges of *Nymphargus laurae*: type locality (red star) and new locality (specimens INABIO15383-84; red dot), and phylogenetic sister species of *N. laurae*: *N. siren* (yellow dots) and *N. humboldti* (green dots). Interlined rectangle in A delimit 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.

DOI: 10.7717/peerj.12644/fig-1

Morphological characteristics

Terminology, characters and measurements follow formats and definitions described b Cisneros-Heredia & McDiarmid (2007), Watters et al. (2016) and Guayasamin et al. (202(Examined frogs were photographed in life, anaesthetised with lidocaine 2%; specimer and tissues were fixed in 96% ethanol and preserved separately in 75% ethanol. Sex ar maturity were determined by directly examining gonads through dissections and notir secondary sexual characters (*i.e.*, vocal slits and nuptial pads). Colour patterns are base on photographs and annotations of living specimens taken in the field. Adjectiv "enamelled" is used to describe the shiny white colouration produced by th accumulation of iridophores (Lynch & Duellman, 1973; Cisneros-Heredia & McDiarmi 2007).

diameter (TD), upper eyelid width (UEW), forearm length (FLL), hand length (HAL), finger disk width (Fin4DW), thigh length (THL), tibia length (TL), foot length (FL).

Morphological data used for comparisons were obtained from direct examination specimens deposited in the following collections: The Natural History Museur Department of Zoology, London (BMNH); División de Herpetología, Instituto Nacional c Biodiversidad, Quito (DHMECN); Instituto de Ciencias Naturales, Universidad Nacional c Colombia, Bogotá (ICN); University of Kansas Natural History Museum, Lawrence, K (KU); Museum of Comparative Zoology, Harvard University, Cambridge, MA (MCZ); Muse de Zoología, Pontificia Universidad Católica del Ecuador, Quito (QCAZ); National Museu of Natural History, Smithsonian Institution, Washington, D.C. (USNM); Museo de Zoologí 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 fro specimen DHMECN 15383, using the "Isolation of Genomic DNA" protocol, Wiza Genomic DNA Purification Kit (Promega, 2019). Three mitochondrial gene fragments [12 ribosomal rRNA gene, 16S ribosomal rRNA gene, Cytochrome C Oxidase Subunit 1 (COI and one nuclear gene [Recombination Activating Protein 1 gene (RAG1)] were amplifie using the Polymerase Chain Reaction (Saiki et al., 1988). Primers for 12S gene (12S H1(FWR 5′-CACYTTCCRGTRCRYTTACCRTGTTACGACTT-3′/12S L4E-REV 5 TACACATGCAAGTYTCCGC-3′), 16S gene (16Sar-L-FWR 5′-CGCCTGTTTATCAAAAACA 3′/16Sbr-H-REV 5′-CCGGTCTGAACTCAGATCACGT-3′), COI (dgLCO-1490-FWR 5 GGTCAACAAATCATAAAGAYATYGG-3′/dgHCO-2198-REV 5 TAAACTTCAGGGTGACCAAARAAYCA-3′) and RAG1 (RAG1-R18 5′GCCATAACTGCTGGAGCATYAT3′/RAG1-R270 5′AGYAGATGTTGCCTGGGTCTTC3 were used (Heinicke, Duellman & Hedges, 2007). Each PCR reaction was composed of 25 µl reaction mix containing: 12.5 µl GoTaq Green Master Mix, 4.5 µl H_20, 1 µl on 10 µ of Forward primer and 1 µl on 10 µM Reverse primer, and 5 µl of genomic DNA. W perform amplifications on an Applied Biosystems GeneAmp PCR System 9,700 therm cycler. The amplification program for 12S primers was set with an initial denaturation of 9 °C (5 min) followed by 38 cycles of 94 °C (30 s), 49 °C (30 s), 72 °C (1 min), with a fin extension temperature of 72 °C (7 min) and 4 °C for an unlimited period; for 16S prime was set with an initial denaturation of 95 °C (5 min) followed by 30 cycles of 95 °C (30 s 57 °C (30 s), 72 °C (45 s), with a final extension temperature of 72 °C (5 min) and 4 °C f an unlimited period; for COI primers was set with an initial denaturation of 95 °C (5 mi followed by 30 cycles of 95 °C (30 s), 48 °C (30 s), 72 °C (45 s), with a final extensic temperature of 72 °C (5 min) and 4 °C for an unlimited period; and for RAG1 primers wa set with an initial denaturation of 94 °C (5 min) followed by 38 cycles of 94 °C (30 s), 52 ° (30 s), 72 °C (1 min), with a final extension temperature of 72 °C (7 min) and 4 °C for a unlimited period. Amplified DNA products were visualised by electrophoresis on a 2' agarose gel and post-staining with Tris/Borate/EDTA buffer (TBE) under blue light. PCF amplified products were purified using Illustra™ ExoProStar™ Enzymatic PCR ar Sequencing Clean-Up Kit. Sequencing was performed in both DNA strain directions ar undertaken by Macrogen, Seoul, South Korea (http://www.macrogen.com Chromatographs resulting from sequencing were revised and edited using Geneiou Prime v.2020.0.5 software (Kearse et al., 2012). New sequences were deposited GenBank with the following accession numbers: (12S: MZ820691; 16S: MZ831508; CO MZ828399; RAG1: MZ835991).

To infer the phylogenetic position of *Nymphargus laurae*, we included sequences for 11 species (Table S1) selected from Guayasamin et al. (2020), obtained from the NCI GenBank database (National Center for Biotechnology Information NCBI, 2020). W included two species for Allophrynidae, a sister lineage of Centrolenidae, as outgroup (Table S1) and rooted the phylogeny with *Allophryne ruthveni*. Alignments were reviewe and edited manually to remove regions with a high proportion of missing data at the edge and hypervariable regions with Geneious Prime v.2020.0.5 software (Kearse et al., 2012 Highly variable regions into the alignments are subject to the accumulation of gap caused by deletions, insertions and substitution mutation, commonly identified contributed to the inaccuracy of phylogenetic inference (Dwivedi & Gadagkar, 2009). W used Mesquite v3.61 software (Maddison & Maddison, 2019) to store the sequences ar to create a concatenated matrix of all genes (12S, 16S, COI and RAG1). Because o

GTR + I + G as the optimal model for nucleotide substitution for all partitions.

Phylogenetic analyses were conducted using Maximum Likelihood (ML) and Bayesia Methods (BA) on the aligned matrix in the CIPRES Science Gateway V.3.3. ML analyse were performed using Garli v2.0 [Genetic Algorithm for Rapid Likelihood Inferenc (Zwickl, 2006)]. We perform a total of 10 runs to reduce the probability of inferring suboptimal solution; all the other settings were set on default. Node support wa evaluated using 1,000 bootstrap pseudoreplicates. Bayesian phylogenetic analyses we performed in MrBayes v3.2.2 (Ronquist & Huelsenbeck, 2003), using five runs of th Monte Carlo Markov Chain (MCMC) algorithm for 20 million generations each, with fo heated chains (0.2 heating parameter). Trees were sampled every 20,000 generations, wi burning of 25% of the total trees. To evaluate the effective sampling size of the fiv independent, uncorrelated runs, we used the statistical number of effectively independe draws from the posterior (ESS > 200) visualised with Tracer v1.6. (Rambaut et al., 201 Phylogenetic trees were edited using FigTree v1.4.2. (Rambaut, 2014).

Conservation status

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

Results

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

Both specimens are very similar to the holotype in their anatomy and colouratio showing all diagnostic characters described for *Nymphargus laurae*: (1) vomerine tee absent; (2) snout truncated in dorsal and lateral views; (3) tympanic annulus evident; (dorsal skin shagreen with slightly elevated warts corresponding to ocelli and scattere spicules, (5) ventral skin granular, without cloacal ornamentation except for a pair of larg flat tubercles; (6) parietal peritoneum white, covering 2/3 of the abdomen; all oth peritonea clear; (7) liver lobed, hepatic peritoneum clear; (8) humeral spine absent; (basal webbing between fingers I, II and III, outer fingers III 2 ⅔–2 ⅓ IV; (10) webbing c feet similar to holotype (see below); (11) no dermal folds or tubercles on hands, arms, fe or tarsi; (12) unpigmented nuptial pad Type-I, concealed prepollex; (13) Finger II long than Finger I; (14) eye diameter larger than the width of disc on Finger III; (15) colouratic in life, green with ocelli (yellow spots surrounded by black), and in preservative, lavend with ocelli (cream-coloured centre surrounded by dark lavender).

The new specimens show no relevant differences with the holotype, and observe differences fall within known intraspecific variation found also between specimens of oth congeneric species. The male specimen (DHEMCN 15384) has SVL = 22.1 mm, slight larger than the male holotype (USNM 288453, 19.9 mm SVL), and both are smaller tha the subadult female (DHMECN 15383, 22.3 mm SVL). Differences in measurements ar proportions between these males are probably due to intraspecific variation (Table 1). Th male has a combination of large and small spicules (visible under magnification) on th head, dorsum, and flanks, but spicules on the lower part of dorsum and eyelids a smaller. The female has smaller spicules compared to the male on the head, dorsum ar flanks. A spicule is present in the centre of each ocellus, being more prominent ar pointed when compared to other body spicules. The female has the tympanic annulu

¾ III 1 ½–2 ¾ IV 2 ½–1 ¾ V in the male (Fig. 2B) (I 2 –2 II 1 ½–2 III 1 –2 ½ IV 2 ½–1 ½ V the male holotype). The male (DHMECN 15384) has two papillae on discs of Toe I and (Fig. 2C). The female (DHMECN 15383) lacks papillae on toes. The holotype of *N. laura* has two papillae on each toe disc, except for Toe V.

Table 1:

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

Character	Male (Holotype) USNM 288453	Male INABIO15384	Subadult 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

DOI: 10.7717/peerj.12644/table-1

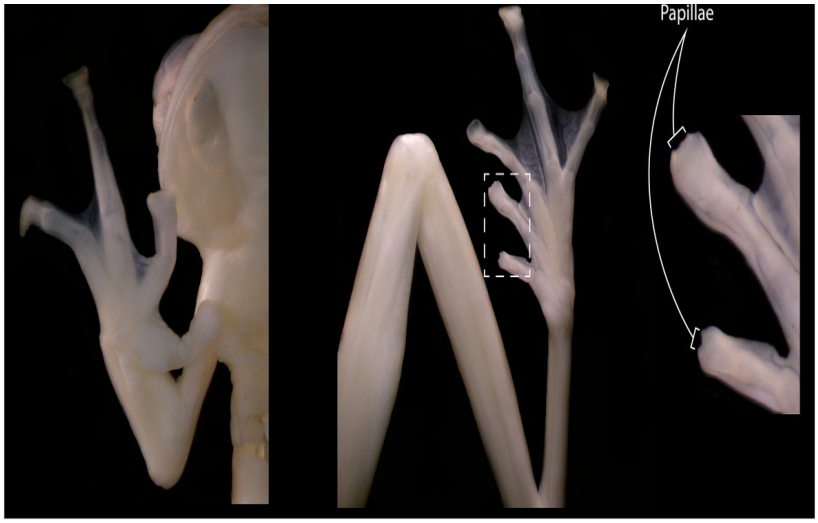

Figure 2: Hand (A), foot (B) and papillae (C) of *Nymphargus laurae* (INABIO15384). Tags and background color have been digitally removed.

DOI: 10.7717/peerj.12644/fig-2

In preservation, the new specimens show similar colourations to the holotype. Howeve the female shows a lavender dorsum, while the new male and the holotype have crea dorsum with lavender tones. The female has 19 ocelli on the body and eight on the hea (Fig. 3A), and the male has six ocelli on the body and three on the head (Fig. 3B) (14 c the body and five on the head of the holotype). Upper eyelids are dark lavender. Finge and toes lack melanophores. All ventral surfaces are cream. The parietal peritoneum ar sclera are white, covering 2/3 of the abdomen; pericardium, digestive peritonea, hepat peritoneum, and urogenital peritonea are clear.

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

DOI: 10.7717/peerj.12644/fig-3

The colouration in life of *Nymphargus laurae* remains known only from the bri description provided by Gustavo Orcés-Villagómez, Ecuadorian zoologists who donate the specimen to James A. Peters, USNM curator, and reported in the original descriptic of the species: “green with yellow spots surrounded by black” (Cisneros-Heredia McDiarmid, 2007). The new specimens allow for a complete description: Head gree darker than the body, lip greenish cream; dorsal surfaces of body, arms and legs gree ocelli on head and body having yellow spots surrounded by black; ocelli absent on arm and legs; upper flanks coloured as dorsum but lower flanks cream, with a sharp divisic between both; hands, finger, feet and toes yellowish-green, with yellow discs; nuptial pa cream (Fig. 4). Throat greenish-white, all other ventral surfaces cream white. Yello circumpupillary ring and whitish iris with thin dark reticulations and dark flecl concentrated towards the middle (Fig. 4). Nictitating membrane yellowish, witho reticulations. Green bones.

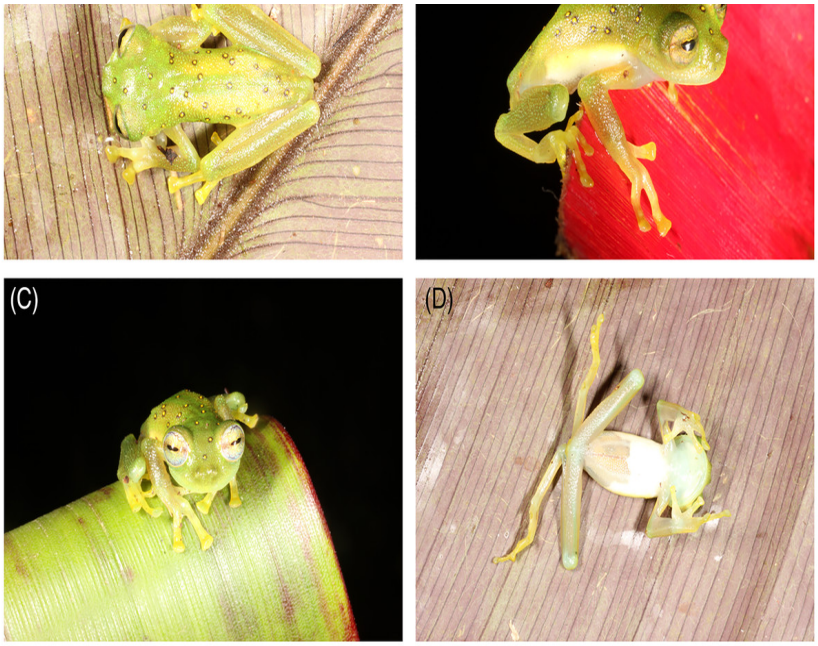

Figure 4: *Nymphargus laurae* (INABIO15383), (A) dorsal view, (B) side view, (C) front view and (D) ventral view.

DOI: 10.7717/peerj.12644/fig-4

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

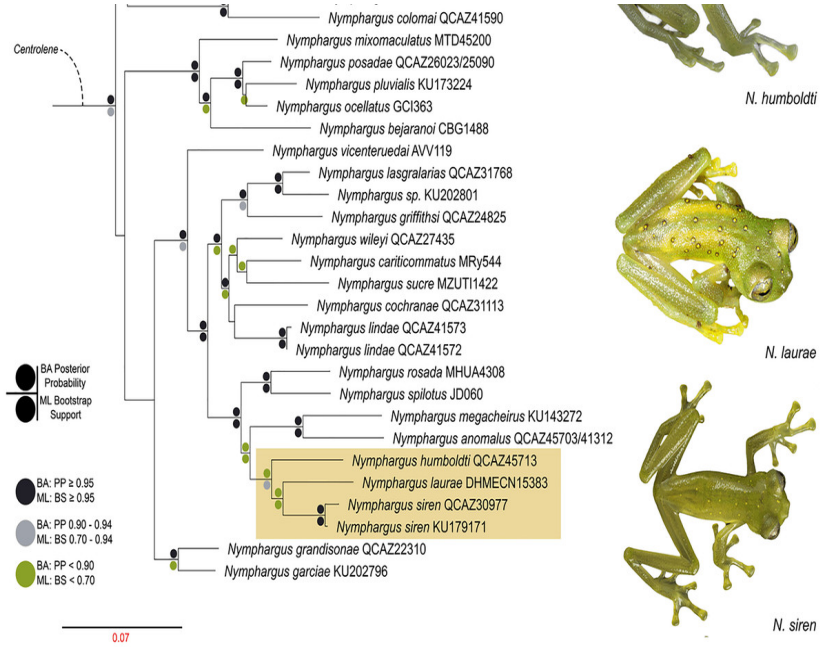

Figure 5: Optimal maximum likelihood tree (log likelihood = –28155.635) of *Nymphargus* (the clade including *N. laurae* is highlighted by an orange rectangle) inferred from concatenated DNA sequences of fragments of the mitochondrial genes 12S, 16S, and COI, and the nuclear gen RAG1, totaling 2,823 aligned base pairs. Circles indicate significant support values for clades recovered by Bayesian (BA) and Likelihood (ML) analyses.

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Nymphargus laurae is known from two localities in the province of Napo, on the nortl eastern flanks of the Andes of Ecuador, at elevations between 700–1,500 m (Fig. 1). Tl type locality, Loreto, was originally covered by lowland evergreen forests, and it is locate on the lower slopes of the Sumaco volcano, on the upper Napo valley. Satellite image (Fig. 1) show that less than 10% of the natural forests remains at the type locality. The ne locality, Colonso-Chalupas, is still covered by evergreen montane forest (Fig. 1). Tl environmental risk surface (ERS) results in threat values from 0 (Colonso-Chalupas) 0.37 (Loreto), due to habitat loss and fragmentation for cattle raising and agricultur deforestation, roads, oil pipelines, and stochastic events related with explosions of tl Sumaco Volcano.

Discussion

The records of *Nymphargus laurae* presented in this paper correspond to the first report the species after 66 years from its original collection. The Colonso-Chalupas Biologic Reserve is the second known locality of *N. laurae*, extending its geographic range in c 77 km SW from the type locality, at Loreto, province of Orellana, Ecuador (Cisnero Heredia & McDiarmid, 2007). These records also extend the altitudinal range of tl species from ca. 700 m (see comments on the elevation of Loreto by Urgilés, Sanche Nivicela & Cisneros-Heredia (2017) up to 1,500 m. Despite the altitudinal difference, bo localities are in the same biogeographic region and watershed, and no significa biogeographic barriers separate them. *Nymphargus laurae* maybe more widespread tha currently known, but possibly it is endemic to north-eastern Ecuador.

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 this species and *N. lindae* was weak and it was suggested 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*. Absence of papillae in the female and in some toes in the new male of *N. laurae* (Fig. 2B) suggest that papillae show intraspecific variation or is of external origin. The presence of papillae on toes discs was not used as a diagnostic character in the original description of *N. laurae*. Cisneros-Heredia & McDiarmid (2007) actually noted that intraspecific variation was observed in the presence/absence of papillae on toe discs of other congeneric species (*i.e*., *Nymphargus ignotus)*. We have seen similar papillae in some specimens of *Chimerella*, *Vitreorana* and *Espadarana*, showing intraspecific variation (*e.g*., *Espadarana prosoblepon*, *Vitreorana ritae*).

Phylogenetic analyses place *Nymphargus 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 small body size of *N. siren*. However, our phylogenetic information shows that, despite these 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 1,410–2,000 m; *N. humboldti*, is known from two localities on the eastern Andean slopes of central Ecuador, at elevations between 1,770–2,400 m (Guayasamin et al., 2020); and *N. laurae* is restricted to lowland and foothill forests along the Upper Napo River basin (Cisneros-Heredia & McDiarmid, 2007, this paper). Our results suggest that 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 *Nymphargus 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 habit

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

Supplemental Information

Additional Information and Declarations

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

María José Sánchez-Carvajal conceived and designed the experiments, performed th experiments, analyzed the data, prepared figures and/or tables, authored or reviewe drafts of the paper, and approved the final draft.
Grace C. Reyes-Ortega conceived and designed the experiments, analyzed the dat authored or reviewed drafts of the paper, logistics and organization of fieldwork ar expeditions, and approved the final draft.
Diego F. Cisneros-Heredia conceived and designed the experiments, performed th experiments, analyzed the data, authored or reviewed drafts of the paper, and approve the final draft.
H. Mauricio Ortega-Andrade conceived and designed the experiments, performed th experiments, analyzed the data, prepared figures and/or tables, authored or reviewe drafts of the paper, logistics and organization of fieldwork and expeditions, and approve the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (*i.e.*, approving boc and any reference numbers):
Environmental Ministry of Ecuador provided full approval for this research (MAE-DNB CM-2017-0062).

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:
The new sequences are available in GenBank: 12S MZ820691; 16S MZ831508; C(MZ828399; RAG1 MZ835991.

Data Availability

The following information was supplied regarding data availability:
The raw data is available in the Supplemental Files.

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