A new glassfrog (Centrolenidae) from the Chocó-Andean Río Manduriacu Reserve, Ecuador, endangered by mining

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We describe a new glassfrog from Río Manduriacu Reserve, Imbabura Province, on the Pacific slopes of the Ecuadorian Andes. The new species can be distinguished from most other glassfrogs by having numerous yellow spots on the dorsum and lacking membranes among fingers. Both morphological and molecular data support the placement of the species in the genus Nymphargus. We present a new mitochondrial phylogeny of Nymphargus and discuss the speciation patterns of this genus; most importantly, recent speciation events seem to result from the effect of the linearity of the Andes. Finally, although the new species occurs within a private reserve, it is seriously endangered by mining activities; thus, following IUCN criteria, we consider the new species as Critically Endangered.

RESUMEN

Se describe una nueva rana de cristal de la Reserva Río Manduriacu, provincia de Imbabura, en la vertiente pacífica de los Andes del Ecuador. La nueva especie se diferencia de sus congenéricos principalmente por tener un dorso con numerosos puntos amarillos y por carecer de membranas entre los dedos de la mano. Tanto los datos morfológicos como los moleculares apoyan la inclusión de la especie en el género Nymphargus. Se presenta una nueva filogenia mitocondrial de Nymphargus y discutimos los patrones de especiación de este género. El resultado biogeográfico más relevante es que la especiación reciente dentro del género parece ser el resultado de la linearidad de los Andes. Finalmente, a pesar de que la nueva especie se encuentra dentro de una reserva privada, está seriamente amenazada por actividades mineras, por lo que, siguiendo los criterios de la UICN, la consideramos como Criticamente Amenazada.
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INTRODUCTION

Glassfrogs represent one of the most charismatic Neotropical radiations (see Guayasamin et al., 2009; Hutter et al., 2013; Castroviejo-Fisher et al., 2014). This clade contains more than 150 species, with an amazing variety of morphology (Cisneros-Heredia & McDiarmid, 2007; Guayasamin et al., 2009), behavior (Delia et al., 2017, 2018), and unresolved taxonomic problems.

Within the tropical Andes, the most diverse glassfrog genus is *Nymphargus* (Cisneros-Heredia & McDiarmid, 2007, as modified by Guayasamin et al., 2009), a taxon previously defined as the *Cochranella ocellata* Group (Ruiz-Carranza & Lynch, 1991, 1995). *Nymphargus* is a monophyletic taxon containing 36 species (Frost, 2018). This Andean genus is primarily composed of species with restricted distributions and, therefore, it is not unusual to find new taxa in previously unexplored areas (e.g., N. caucanus Rada, Ospina-Sarria, Guayasamin, 2017; N. sucre Guayasamin, 2013; N. buena ventura Cisneros-Heredia & Yánez-Muñoz, 2007).

Recent fieldwork at Rio Manduricu Reserve (RMR, hereafter), Imbabura Province, Ecuador, has revealed a striking new *Nymphargus* that we describe below. The new species, and two other critically endangered anurans, *Nymphargus balionotus* (Duellman, 1981) and *Rhaebo olallai* (Hoogmoed, 1985) are threatened by illegal mining activities within the reserve (see Discussion; Lynch et al., 2014).

Although our work presents several novel components (i.e., new phylogeny of *Nymphargus*, discussion of biogeographic patterns), we would like to stress the discussion on the conservation problems that the Chocó-Andean forests of Ecuador are currently facing. Ecuador is a megadiverse country (Mittermeier et al., 1999), part of the most diverse hotspot on Earth (the Tropical Andes; Myers et al., 2000), and the country with the highest amphibian species richness
per unit of area of the world (600 species in an area of 256,423 km²). However, ecosystems are
under heavy pressure from agriculture, wood extraction, oil palm plantation, and, most recently,
mining (Roy et al., 2018; Lessmann et al., 2016). Our study, thus, is an attempt to draw attention
from the government, NGOs, local communities, scientists, and the general public towards the
conservations of the few Chocó-Andean forests still remaining in Ecuador. We also think that
taxonomists should play a more active role in conservation biology, mainly because the results of
our work (i.e., new species with limited distributions) are powerful tools to justify habitat
conservation, specially through partnerships with environmental NGOs.

MATERIALS & METHODS

Ethics statement: Research was conducted under permits No MAE-DNB-CM-2015-2017 and
018-2017-IC-FAU-DNB/MAE, issued by the Ministerio del Ambiente del Ecuador. The study
was carried out in accordance with the guidelines for use of live amphibians and reptiles in field
research (Beaupre et al. 2004), compiled by the American Society of Ichthyologists and
Herpetologists (ASIH), the Herpetologists’ League (HL) and the Society for the Study of
Amphibians and Reptiles (SSAR).

Taxonomy and species concept: Glassfrog generic and family names follow the taxonomy
proposed by Guayasamin et al. (2009). For recognizing species, we adhere to the General
Species Concept (de Queiroz 2005, 2007). Under this concept, the only necessary property for an
entity to be a recognized as a species is that it corresponds to a temporal segment of a
metapopulation lineage evolving separately from other lineages (de Queiroz, 2005, 2007).
Independent evolution generates traits that can be used to diagnose the species, such as morphology, monophyly, vocalizations, among others.

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone. This published work, and the nomenclatural acts it contains, have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix http://zoobank.org/. The LSID for this publication is: urn:lsid:zoobank.org:pub:E5C0E7E4-9C69-4830-A514-AD1F4B80311C. The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central and CLOCKSS.

**Morphological data:** Morphological characterization follows Cisneros-Heredia & McDiarmid (2007). Webbing nomenclature follows Savage & Heyer (1967), as modified by Guayasamin *et al.* (2006). We examined alcohol-preserved specimens mostly from the collection at the Instituto de Ciencias Naturales of the Universidad Nacional de Colombia (ICN), Museo de Zoología of the Universidad Tecnológica Indoamérica (MZUTI), Natural History Museum and Biodiversity Research Center of the University of Kansas (KU), and Museo de Zoología of the Universidad San Francisco de Quito (ZSFQ); all examined specimens are listed below. Morphological measurements were taken with Mitutoyo® digital caliper to the nearest 0.1 mm, as described by Guayasamin & Bonaccorso (2004) and Cisneros-Heredia & McDiarmid (2007) except when
noted, and are as follow: (1) snout–vent length (SVL); (2) tibia length; (3) foot length; (4) head length; (5) head width; (6) interorbital distance; (7) upper eyelid width; (8) internarial distance; (10) eye diameter; (11) tympanum diameter; (12) radioulna length; (13) hand length; (14) Finger I length; (15) Finger II length = distance from outer margin of palmar tubercle to tip of Finger II; (16) width of disc of Finger III. Sexual maturity was determined by the presence of vocal slits in males and by the presence of eggs or convoluted oviducts in females. Color patterns are described based on photographs of live specimens taken in the field. The adjective “enamelled” is used to describe the shiny white coloration produced by accumulation of iridophores (Lynch & Duellman, 1973; Cisneros-Heredia & McDiarmid, 2007). Examined material is listed in Appendix 1.

Study site: The RMR (0.31°N, 78.85°W, 1200–2000 m; Fig. 1) is located at the juncture of the Chocó and Tropical Andes bioregions, near the following Important Bird Areas and Key Biodiversity Areas: Reserva Ecológica Cotacachi Cayapas (EC037), Intag-Toisán (EC038), Bosque Protector Los Cedros (EC039), and Mashpi-Pachijal (EC108; Freile & Santander, 2005).

Bioacoustics: Sound recordings were made with an Olympus LS-10 Linear PCM Field Recorder and a Sennheiser K6–ME 66 unidirectional microphone. The calls were recorded in WAV format with a sampling rate of 44.1 kHz / second with 16 bits / sample. All calls are stored at the Laboratorio de Biología Evolutiva at Universidad San Francisco de Quito (LBE). Measurements of acoustic variables were obtained as described in Hutter et al. (2013). A call is defined as the collection of acoustic signals emitted in sequence and produced in a single exhalation of air. A note is a temporally distinct segment within a call; notes are separated by a silent interval.
Pulsed notes are those having one or more clear amplitude peaks while tonal notes have relatively constant amplitude throughout the call. A call series is defined as a sequence of calls that are separated by a consistent time interval of background noise between calls (see Köhler et al., 2017).

Fieldwork: Sampling at RMR was conducted during the following dates: 7–8 November 2012 (RL, SK), 13–15 May 2013, 21–22 February 2014 (Fernando Ayala, RL, SK, Santiago Ron), 8–11 April 2018 (Jaime Culebras, Jorge Brito, SK), 17–30 October 2016 (PH, RJM, RL, and 3 assistants), 20–30 January 2018 (PH, RJM, Amanda Northrup, Nathalie Aall, Bill Langworthy, and 2 assistants), 6–14 February 2018 (JV, PH, RJM, RL, SK, Jo Bowman, Bill Langworthy, and 2 assistants). Visual encounter surveys (VES) were conducted along transects of various lengths within primary forest, secondary and riparian forest, and along streams of various sizes. For smaller streams that had thick vegetation and were too narrow to perform linear transects, we performed general searches of the habitat. During the February 2018 trip (the only survey period when the new species was abundant), surveys consisted of walks along different streams starting at a 1900h until 0200h for nine nights.

Evolutionary relationships: We generated mitochondrial sequences (12S, 16S) for three individuals (ZSFQ 462, 463, 466) of the new species and several other Nymphargus species (Table S1). Extraction, amplification, and sequencing protocols are as described in Guayasamin et al. (2008). The newly obtained sequences (Table S1) were compared with those of all other available species of Nymphargus (see Fig. 2) and all other glassfrog genera, downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/); sequence information and GenBank codes
of the outgroups are listed in Guayasamin et al. (2008), Castroviejo-Fisher et al. (2014), and Twomey, Delia & Castroviejo-Fisher (2014). Sequences were aligned using MAFFT v.7 (Multiple Alignment Program for Amino Acid or Nucleotide Sequences: http://mafft.cbrc.jp/alignment/software/), with the Q-INS-i strategy (Katoh & Standley 2013). MacClade 4.07 (Maddison & Maddison, 2010) was used to visualize the alignment (no modifications were necessary). Maximum likelihood was run in the IQ-TREE 1.5.5 software (Nguyen et al., 2015). The best-fitting nucleotide substitution model was implemented using ModelFinder within IQ-TREE (Kalyaanamoorthy et al., 2017), which groups partitions with the same model and similar rates and simultaneously searches model and tree space; since only mitochondrial sequences were analyzed, they were considered as a single gene (i.e., they evolve as unit—maternal inheritance and no recombination). Node support was assessed via 1000 ultra-fast bootstrap replicates, a method that shows less bias that other support estimates (Minh et al., 2013). Ultra-fast bootstrapping also leads to straightforward interpretation of the support values (e.g., support of ≥ 95% should be interpreted as significant; Minh et al., 2013).

RESULTS

Phylogenetic relationships of Nymphargus: Based on the Bayesian Information Criterion, the best-fit model for our dataset was TIM2+F+R5. Rate parameters were estimated as follows: A-C: 5.14658, A-G: 16.73402, A-T: 5.14658, C-G: 1.00000, C-T: 49.89941, G-T: 1.00000. Base frequencies were: A: 0.341, C: 0.255, G: 0.186, T: 0.218. We generated 31 new sequences (Table S1), including species that have never been part of centrolenid phylogenies.

The inferred phylogeny (Fig. 2) confirms the placement of the following species within the genus Nymphargus (sensu Guayasamin et al. 2009): N. balionotus (Duellman, 1981), N. cariticommatus (Wild, 1994), N. lasgralarias Hutter & Guayasamin, 2012, N. spilotus (Ruiz-
Carranza & Lynch, 1997), and *N. sucre* Guayasamin, 2013. The new species, described below, is also part of the genus *Nymphargus*. Relationships among *Nymphargus* species are similar to those reported in previous studies (Guayasamin *et al.*, 2008; Castroviejo-Fisher *et al.*, 2014; Twomey, Delia & Castroviejo-Fisher, 2014), but some novel relationships are revealed because of our increased taxon sampling (Fig. 2).

**Species description**

*Nymphargus manduriacu* **new species**

LSID urn:lsid:zoobank.org:pub:E5C0E7E4-9C69-4830-A514-AD1F4B80311C

**Common names:** English: Manduriacu glassfrog. Spanish: Rana de Cristal de Manduriacu.

**Holotype:** ZSFQ 0466 (Fig. 3), adult male from Reserva Río Manduriacu (0.310755°N, 78.8569°W; 1215 m), Provincia de Imbabura, República del Ecuador, collected by JV and RJM on February 7th, 2018.

**Paratypes:** ZSFQ 0465, adult male, with same data as holotype. ZSFQ 0462, adult female, and ZSFQ 0463 (Fig. 3), adult male, with same data as holotype, but collected at a different stream (0.310818°N, 78.857°W; 1230 m) on February 6th, 2018.

**Referred material:** ZSFQ 0464 (Fig. 4), metamorph, with same data as holotype.
**Generic placement:** The new species is placed in the genus *Nymphargus* Cisneros-Heredia & McDiarmid 2007, as modified by Guayasamin *et al.* (2009), based on morphological and genetic data. All species in *Nymphargus* share an absence of webbing among Fingers I–III and absence or reduced webbing between Fingers III and IV; additionally, males lack humeral spines (except *N. grandisonae*; *N. armatus* and some populations of *N. griffithsi* have an enlarged ventral crest on their humeri that can resemble a humeral spine). *Nymphargus manduriacu* sp. nov. presents all the aforementioned traits and its placement within *Nymphargus* is unambiguous. Phylogenetic analyses of mitochondrial genes also place *N. manduriacu* sp. nov. in the genus *Nymphargus* (Fig. 2).

**Diagnosis:** *Nymphargus manduriacu* sp. nov. is distinguished from most glassfrogs by lacking webbing between inner fingers and having, in life, a grayish green dorsum with numerous yellow spots, which sometimes are surrounded by an ill-defined black ring (i.e., false ocelli). On the Pacific slopes of the Ecuadorian and Colombian Andes, there are very few species that share the two aforementioned traits with *N. manduriacu* sp. nov.; these species are: *N. buenaventura*, *N. ignotus*, *N. spilotus*, and *N. luminosus*. Differences among these species are summarized in Table 1 and Figures 5 and 6. The sister species of *N. manduriacu* sp. nov. is *N. balionotus*, which is easily differentiated by its unique dorsal color pattern, a green dorsum with several black and occasionally yellow to cinnamon blotches (Duellman, 1981; Arteaga *et al.*, 2013). Also, the uncorrected *p* genetic distance between *N. manduriacu* sp. nov. and *N. balionotus* is 6.4–6.7% for the 12S and 16S concatenated matrix.
Definition: The new species is distinguished from all other Centrolenidae by the following combination of characters: (1) dentigerous process of vomer low or absent, without vomerine teeth; (2) snout truncate in dorsal and truncate to slight rounded in lateral view; (3) tympanic annulus barely evident, lower ¼ visible, tympanic membrane colored as dorsal skin, supratympanic fold present; (4) dorsal skin shagreen, with microspicules in adult males; (5) ventral skin granular, subcloacal area with two large subcloacal warts; (6) parietal peritoneum white, iridophores covering 1/3 to 1/2 parietal peritoneum (conditions P2 or P3); pericardium white (i.e., covered by iridophores), all other visceral peritonea clear (condition V1); (7) liver lobed and hepatic peritoneum clear (lacking iridophore layer, condition H0); (8) adult males without projecting humeral spine; (9) webbing between fingers I, II and III absent, basal between fingers III and IV; (10) toe webbing basal between toes I and II, III \(1^{1/6} - (2^{1/6} - 3^-)\) III \(1^{1/6} - 1^{1/6} - (3^-)\) IV \(3 - 3^- - (1^{1/6} - 2^-)\) V; (11) lacking dermal ornamentations in the form of tubercles, folds, or fringes on hands, arms, feet, or legs; (12) nuptial excrescences Type I and VI; concealed prepollex; (13) Finger I slightly longer than Finger II; (14) diameter of eye larger than width of disc on Finger III; (15) color in life, grayish green to olive green with yellow spots, which, sometimes, are surrounded an ill-defined black ring (i.e., false ocelli); bones green; (16) color in preservative, lavender dorsum with cream spots; (17) iris coloration in life: light grey with thin grey reticulations and pale yellow hue around pupil; (18) melanophores present and abundant along Fingers III and IV, less dense on Finger II, and rarely present on Finger I, present and abundant along Toes IV and V, less dense on Toe III, only at the base of Toes I and II; (19) males call from upper side of leaves; advertisement call is a high-pitched “chirp”, with a single, pulsed note with a duration of 0.093–0.118 s \(\bar{x} = 0.10 \pm 0.007; n = 10\) and a dominant frequency at 4052–4447 Hz \(\bar{x} = 4267.7 \pm 118.3\); (20) fighting behavior unknown; (21) egg
masses deposited on upper side of leaves, clutch size 15–32 ($n = 4$); males do not attend or get in
contact with clutches; (22) tadpoles undescribed; (23) snout-vent length (SVL) in adult males
24.0–25.7 mm ($n = 3$), and in an adult female 28.8 mm.

**Description of holotype:** Adult male (ZSFQ-0466; Figs. 3–7). Head wider than long (head
length 90% of head width); snout truncate in dorsal view and slightly rounded in lateral profile;
canthus rostralis indistinct, slightly concave; loreal region concave; lips not flared; nostrils
protuberant, closer to tip of snout than to eye, directed frontolaterally; internarial area barely
depressed. Eyes large, directed anterolaterally at an angle $\sim 45^\circ$; transverse diameter of disc of
Finger III 53% eye diameter. Supratympanic fold low, obscuring upper edge of tympanic
annulus; tympanic annulus small and almost indistinct, oriented mostly vertically; tympanic
membrane colored as surrounding skin. Dentigerous process of vomer absent; choanae rounded;
tongue ovoid and unnotched, with ventral posterior fourth not attached to mouth floor; vocal slits
extending posterolaterally from about the lateral margin of tongue (at about half the length of
tongue) to angle of jaws.

Humeral spine absent. Hand and ulnar folds absent; relative lengths of fingers: III > IV > II > I;
webbing absent between Fingers I–III, basal between Fingers III and IV, webbing formula III
$2^{3/4}–2^{3/4}$ IV; discs expanded, nearly elliptical; disc pads with triangular shape; subarticular
tubercles small, round, simple; supernumerary tubercles numerous, fleshy, giving the palm a
warty texture; palmar tubercle elliptical, simple; nuptial pad Type I present but faint, extending
from ventrolateral base to dorsal surface of Finger I, covering the proximal half of Finger I.
Length of tibia 59% SVL; tarsal folds absent; two-thirds webbed foot; toe webbing basal between toes I and II, III 1½–3– III 1½–3– IV 3–1½ V; discs on toes elliptical; disc on Toe IV narrower that disc on Finger III; disc pads triangular; inner metatarsal tubercle large, ovoid; outer metatarsal not evident; subarticular tubercles small, round; supernumerary tubercles absent. Skin on dorsal surfaces of head, body, and lateral surface of head and flanks shagreen with numerous minute spinules; throat smooth; belly and lower flanks granular; cloacal opening directed posteriorly at upper level of thighs; cloacal ornamentation absent except for pair of enlarged subcloacal tubercles.

**Color in life:** Grayish green to olive green dorsum with yellow spots. Melanophores concentrated around yellow spots, sometimes looking like false ocelli. Upper lip unpigmented. Inner fingers and toes yellowish. Anterior half of ventral parietal peritoneum white, posterior portion translucent. Color of bones green. Iris light grey with thin, dark grey reticulations and pale yellow hue around pupil (Figs. 3, 5).

**Color in ethanol:** Dorsal surfaces gray lavender with small white spots. Parietal peritoneum white, iridophores covering 1/3 to 1/2 parietal peritoneum. Heart covered by white pericardium; all other visceral peritonea unpigmented.

**Variation:** The only known female is larger than males and lacks microspicules (Fig. 1). Male holotype was slightly greener than female. Metamorph was uniformly green showing faint light spots. Meristic variation is reported in Table 2.
Natural history: Although RMR has been visited numerous times during the past years (see Methods), the new species was only regularly detected during February 2018. This was during the wet season, with the site experiencing particularly heavy rains on a daily basis during this time frame. During all previous surveys, only a single individual was observed along a transect in mature secondary forest (0.3144°N, 78.855°W; 1238 m; 22 October 2016 at 2036 h). The point where the individual was found was along a narrow, sloping ridge, with the Manduriacu River ca. 20 m east, and a smaller stream (ca. 3–4 m wide) about 15 m west; the individual, uncollected, was perched on a leaf with a perch-height of 350 cm when found.

In February 2018, even though sampling targeted numerous large rivers (ca. 4–7 m wide) and smaller streams (ca. 0.5–2 m wide), all individuals of the new species were found in only two places. The first location is a narrow stream (ca. 0.75 m wide, 0.310818°N, 78.857°W; 1230 m) with dense vegetation, where on 6 February 2018 at 2033 h, a male (ZSFQ-0463) was calling and perched on a leaf above the water 280 cm high, and a female (ZSFQ-0462) on a leaf below the male, with a perch height of 170 cm. Conditions were wet, with light rain during the time of capture. The following night, three additional individuals were collected along the same stream. The first was an adult male (ZSFQ-0465) collected at 1930 h, found moving on a leaf 220 cm above the ground, and located 5 m from the stream (i.e. not directly above water). The second individual was a metamorph (ZSFQ-0464; 16.4 mm SVL) collected at 2000 h, perched on a leaf 100 cm directly above the stream. The third specimen was a male (ZSFQ-0466) collected at 2200 h, perched on a leaf 400 cm directly above water. This male was 15–30 cm from four egg clutches; these clutches, placed on the upper surfaces of leaves (Fig. 4), contained 26–32 embryos ($\overline{x} = 25.75 \pm 7.5883$). Finally, an uncollected adult individual was found on 12 February 2018 at 2311 h along a slightly larger stream (2–3 m wide) immediately adjacent to camp on the
This uncollected individual was perched in herbaceous vegetation in an area where a large treefall was lying across the stream; the individual was found 0.5 m from the stream, with a perch high of 65 cm.

**Call (Fig. 7):** The following description is based on the recording of two males (ZSFQ 0465-0466) obtained by JV on 05 February 2018 at the type locality. Each call is a high-pitched “chirp” that consists of a single note and has a duration of 0.093–0.118 s ($\bar{x} = 0.10 \pm 0.007$; $n = 10$). Notes are pulsed (8–12 pulses per note; $\bar{x} = 10.33 \pm 1.366$). In each call, there is a very slight increase in the dominant frequency with time; the dominant frequency is at 4052–4447 Hz ($\bar{x} = 4267.7 \pm 118.3$). Time between calls is 3.9–8.6 s ($\bar{x} = 5.72 \pm 1.82$). Among closely related species to the new taxon (Fig. 2), only the call of *N. grandisonae* is described (Hutter *et al.* 2013). The call of *N. manduriacu* sp. nov. is differentiated mainly by having a higher dominant frequency (4052–4447 Hz in *N. manduriacu* sp. nov., 3100–4048 Hz in *N. grandisonae*).

**Evolutionary relationships of the new species:** Given the gene and taxon sampling of our study, *Nymphargus manduriacu* sp. nov. is sister to *N. balionotus*. The latter taxon was considered to be as incertae sedis within the subfamily Centroleninae (Guayasamin *et al.* 2009). Here we formally place *Centrolenella balionota* Duellman 1981 in the genus *Nymphargus* sensu Guayasamin *et al.* 2009. *Nymphargus manduriacu* sp. nov. and *N. balionotus* are endemic to the Pacific slopes of the northern Andes, and are found syntopically at RMR.

**Distribution:** *Nymphargus manduriacu* is only known from a few nearby streams within the Río Manduriacu Reserve (0.31°N, 78.85°W), Imbabura province, on the Pacific slopes of the Andes.
of Ecuador (Fig. 1). Based on these limited records, the species occupies a narrow elevational range of 1215–1242 m.

**Conservation status:** We recommend that *Nymphargus manduriacu* should be considered as Critically Endangered, following IUCN (2001) criteria B2a (known to exist from a single locality) and B2biii (continuing decline, observed, inferred or projected, in area, extent and/or quality of habitat). The main threats for the species are habitat destruction and contamination associated with cattle ranching, agriculture and, most seriously, mining activities (see Discussion; Fig. 8). Although RMR is still poorly surveyed, northwestern Ecuador has been the target of intense herpetological research (e.g., Lynch & Duellman, 1973, 1997; Arteaga, Bustamente & Guayasamin, 2013; Arteaga *et al.*, 2016), including areas nearby RMR (i.e., Reserva Los Cedros; Hutter & Guayasamin, 2015); thus, the restricted distribution of the new species is, most likely, real.

**Etymology:** The specific epithet “manduriacu” is a noun in apposition and refers to the type locality of the species, Río Manduriacu Reserve, a conservation area managed by Fundación EcoMinga ([https://ecomingafoundation.wordpress.com/](https://ecomingafoundation.wordpress.com/)).

**DISCUSSION**

**Biogeographic patterns within *Nymphargus*:** The phylogeny we present (Figure 2) reveals a number of biogeographic patterns that are worth highlighting. Given the current taxon sampling (60% of the described diversity of the genus), all sister species within *Nymphargus* are geographic neighbors, including some sister taxa that, in some areas, are sympatric (e.g. *N.*
manduriacu/N. balionotus, N. griffithsi/N. lasgralarias). On the Amazonian slope of the Andes, most sister species have allopatric distributions: N. aff. chancas/N. mariae, N. cariticommatus/N. sucre, N. cochranae/N. sp, N. siren/N. sp, N. pluvialis/N. posadae. The only two sampled Nymphargus species distributed on the eastern slope of the Cordillera Central of Colombia (N. spilotus/N. rosadus) are also sister species. These biogeographic patterns are congruent with the hypothesis that recent speciation in Nymphargus is mediated by the linearity of the Andes, which results in elongate geographical ranges and reduces potential contact and gene flow among parapatric populations (Remsen, 1984; Graves, 1988). It is also possible that dry river-valleys play a role as barriers (see Krabbe, 2008; Guayasamin et al., 2010; Arteaga et al., 2016; Prieto-Torres et al., 2018; Winger & Bates, 2015). Examples of dry Andean river valleys in Ecuador include the Mira, Guayllabamba, Jubones, Girón, and Paute. To date, however, there are no explicit studies designed to test the effect of the mentioned valleys on diversification processes.

**Biodiversity value of Río Manduriacu Reserve:** In Ecuador, RMR is a very atypical site for vertebrate diversity. It is the only known place that houses an extant reproducing population of the long lost and presumed-extinct Tandayapa Andean Toad (*Rhaebo olallai*; Lynch et al., 2014), and the Mindo Cochran frog (*Nymphargus balionotus*; R. Maynard, personal observation, 2016–2018). Both species had not been seen in the country for over three decades prior to their rediscovery at RMR. Additionally, there is a large number of other threatened amphibians, including the critically endangered *Centrolene ballux* and the endangered *Pristimantis crenunguis, P. pteridophilus, and P. scolodiscus*, as well as several undescribed anurans (e.g., *Noblella* sp, *Pristimantis* spp). The reserve also serves as a stronghold for other Critically Endangered animals, such as the brown-headed Spider Monkey (*Ateles fusciceps fusciceps*),
included on the list of the 25 most endangered primates in the world (Schwitzer et al. 2017).

Other mammals recorded at RMR include the Spectacled Bear (*Tremarctos ornatus*), Pacarana (*Dinomys branickii*), and Oncilla (*Leopardus tigrinus*), all classified as Vulnerable (IUCN VU) (S. Kohn, personal observation, 2015–2018). Also notable is the presence of jaguars (*Panthera onca*; S. Kohn, personal observation, 2016), as their populations along the western slopes of the Andes are considered Critically Endangered, despite having a global status of Near Threatened (Espinoza et al. 2011).

**Threats to the Río Manduriacu Reserve**

*Mining:* Immediate threats to the forests at RMR make the conservation of this newly described species, and the biodiversity in the area, a difficult challenge. The primary threat comes from mining concessions (Fig. 8) given by the government to Cerro Quebrado, a subsidiary to the Australian BHP Hillinton, the world’s largest mining company. Ecuador’s legislation requires that any mining operation must consult with local communities and landowners prior to any mining activity (Article 398, Constitución del Ecuador, 2008). Despite the fact that Cerro Quebrado did not consult the local population or local landowners, the Ecuadorian government granted the company a concession to extract gold and copper through an open pit mine (see mining cadaster: [http://geo.controlminero.gob.ec:1026/geo_visor/](http://geo.controlminero.gob.ec:1026/geo_visor/)). Thus, with this documentation in hand, it is clear that the mining concession in RMR and nearby areas are void and that mining activities should be prohibited until Cerro Quebrado and the Ecuadorian government abide by the Ecuadorian constitution.

*Logging:* Illegal and uncontrolled logging also pose a grave threat. Local communities have relied historically on logging as one of their main sources of income. Nonetheless, this logging is
mostly illegal (e.g., without government permits or using a permit for one area to extract wood from a different area) and remains poorly regulated. As a result, forest cover in the area surrounding RMR has been reduced drastically in the last two decades (http://mapainteractivo.ambiente.gob.ec/portal/). This problem is exacerbated by the generalized lack of legal land ownership titles.

Conservation actions: Numerous local landowners have protected the forest of RMR for several decades. In 2010 several tracts of forest were incorporated into the Socio Bosque program, a conservation initiative by the Ecuadorian government (http://sociobosque.ambiente.gob.ec/). Through Socio Bosque, landowners and communities that are willing to conserve their forests get financial incentives if they maintain the original forest cover. Starting in 2015 Fundación EcoMinga signed an agreement with the owners of several lots to control, protect and manage the reserve. Through this agreement all funds from Socio Bosque are directed to fund salaries of park rangers and reserve managers. IUCN Netherlands has supported EcoMinga in purchasing new plots in order to expand the reserve. With this new purchase the land belonging to RMR has grown to almost 600 ha, while the plots affiliated to Socio Bosque, but outside the limits of the reserve, cover an additional 350 ha. EcoMinga’s medium and long term goal is to purchase nearby land that is not under any conservation program.

In early 2017, EcoMinga began a community project to promote sustainable alternatives (e.g. ecotourism, revenues from academic projects, sustainable agriculture, vanilla farming) to wood extraction (training locals as guides, mainly for birdwatching tourism). The aim is to generate a model for community work that is appropriate for the country and that can later be replicated in other reserves. This program has faced difficulties since recently local community members were
hired by Cerro Quebrado to illegally enter into RMR. This has created constant tension within
the communities and with stakeholders.

CONCLUSIONS

We provide morphological, genetic, and acoustic evidence that support the validity of a new
species, *Nymphargus manduriacu*. Also, we infer a new mitochondrial phylogeny of the genus
*Nymphargus* that allows us to reveal speciation patterns in this taxon, mainly that recent
speciation events in this genus seem to be heavily influenced by the linearity of the Andes and
dry river-valleys that are run transversal to this mountain range. Finally, the new species is
considered as Critically Endangered because of its restricted distribution, habitat destruction and
contamination associated with cattle ranching, agriculture and, most seriously, mining activities.

At Río Manduriacu Reserve, mining has become one the most dangerous threats to biodiversity,
specially to species with very restricted distributions.

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Appendix I: Examined specimens.

_Nymphargus buenaventura:_ **Ecuador:** Provincia de El Oro: Cantón El Guabo: Cascadas de Manuel, 800 m, DHMECN 10982; Cantón Piñas: Reserva Buenaventura (03°38’S, 79°45’W, 1200 m), DHMECN 3563 (holotype), 2524, 3561–62 (paratypes).

_Nymphargus balionotus:_ **Ecuador:** Provincia de Imbabura: Río Manduríacu Reserve (0.31°N, 78.85°W; 1215–1238 m), ZSFQ 0531–533.

_Nymphargus griffithsi:_ **Ecuador:** Provincia de Pichincha: Río Saloya, 1219 meters, BMNH 1940.2.20.4 (holotype), BMNH 1940.2.20.3 (paratype); Reserva Las Gralarias, MZUTI 100, 102 and 099, “Hercules Giant Tree Frog Creek”, (0°01.529’ S, 78°42.243’ W; 2175 m); MZUTI 101, “Five Frog Creek”, (0°01.870’ S, 78°42.358’ W; 2150 m); MZUTI 098, “Heloderma Creek” (0°01.245’ S, 78°42.370’ W; 2200 m).

_Nymphargus ignotus:_ **Colombia:** Departamento del Valle del Cauca: Municipio de La Cumbre, Corregimiento de Bitaco, Vereda Chicoral, tributary of Río Bitaco (03°34’09.9’’N, 76°35’42.7’’W, 1950 m), ICN 55799–800, ICN 21524–5; Peñas Blancas, Farallones de Cali, ca. 6 km by road SW of Pichindé (04°53’05.2’’N, 76°08’52.5’’W, 1900 m), ICN 14748, holotype, ICN 14749–77; Municipio de Dagua, Finca San Pedro, headwater of the Quebrada La Seca, 8 km S of Queremal (03°28’30.1’’N, 76°42’10.8’’W, 1940–2050 m), ICN 41333–41. **Departamento de Chocó:** Municipio de San José del Palmar, 12–12.6 km on the San José del Palmar-Cartago road (03°24’59.8’’N, 76°37’12.6’’W, 1850 m), ICN 19641. **Departamento de Risaralda:** Municipio de Mistrató, km 10–11 carretera Mistrató- San Antonio del Chami, quebrada Mampay (05°21’N, 75°52’W, 1760 m), ICN 30040–8, ICN 30056.
**Nymphargus lasgralarias**: Ecuador: Provincia de Pichincha: Reserva Las Gralarias, MZUTI 096 (holotype), MZUTI 091–095, 097 (paratypes).

**Nymphargus luminosus**: Colombia: Departamento de Antioquia: Municipio de Frontino: Corregimiento Nutibara: Km 23–27 on the Nutibara-La Blanquita road, 1000–1430 m, ICN 15930 (holotype), ICN 15918–20, 15922–29, 15931–33 (paratypes).

**Nymphargus spilotus**: Colombia: Departamento de Caldas: Municipio de Samaná, Corregimiento Florencia: sitio “Rancho Quemado”, 1940 m, ICN 35155 (holotype); sitio El Estadero, 1850 m, ICN 35157–58 (paratypes); zona “El Estadero” (o “Rancho Quemado”), ICN 38073 (paratype).
Table 1 (on next page)

Differences between *N. manduriacu* sp. nov. and similar species from the Pacific Andes of Ecuador and Pacific and Central Andes of Colombia.
|                      | N. manduriacu sp. nov. | N. buenaventura | N. ignotus | N. luminosus | N. spilotus |
|----------------------|------------------------|-----------------|------------|--------------|------------|
| **Distribution**     | 1215–1238 m            | 800–1200 m      | 1280–2050 m| 1140–1430 m  | 1850–1940 m|
| **SVL (adult males)**| 24.0–25.7 (n = 3)      | 20.9–22.4 (n = 4)| 22.2–25.4 (n = 61)| 27.7–30.0 (n = 15)| 25.7–26.6 (n = 2) |
| **Dorsal coloration in life** | Grayish green with yellow spots, which are sometimes surrounded by ill-defined rings | Light green with diffuse pale yellow spots | Dorsum pale tan to olive brown with black ocelli surrounding orange or yellow spots | Green with numerous yellow spots (95–217 spots; n = 16) | Olive green with small yellow spots |
| **Webbing between Fingers III and IV** | Basal (Fig. 6A) | Basal | Basal | Basal, but more extended than in other *Nymphargus* (Fig. 6B) | Basal (Fig. 6D) |
| **Source**           | This study             | Cisneros-Heredia & Yánez-Muñoz (2007); this study | Rada *et al.* (2017) | Ruiz-Carranza & Lynch (1995); this study | Ruiz-Carranza & Lynch (1997); this study |
Table 2 (on next page)

Morphological measurements (in mm) of the type series of *N. manduriacu* sp. nov.
|                  | ZSFQ-0466 | ZSFQ-0463 | ZSFQ-0465 | ZSFQ-0462 |
|------------------|-----------|-----------|-----------|-----------|
|                  | male      | male      | male      | female    |
| SVL              | 25.3      | 25.7      | 24.0      | 28.8      |
| Tibia length     | 15.0      | 15.0      | 14.2      | 18.0      |
| Foot length      | 12.1      | 11.6      | 11.0      | 13.8      |
| Head length      | 8.2       | 8.3       | 8.1       | 8.7       |
| Head width       | 9.1       | 8.8       | 8.7       | 10.2      |
| Snout to eye distance | 2.8      | 2.8       | 3.2       | 3.7       |
| Interorbital distance | 3.7      | 3.9       | 3.9       | 4.0       |
| Upper eyelid width | 2.2      | 2.1       | 2.2       | 2.6       |
| Internarinal distance | 2.3      | 2.4       | 2.1       | 2.4       |
| Eye diameter     | 3.4       | 3.2       | 3.1       | 3.8       |
| Tympanum diameter | 0.8      | 0.9       | 1.0       | 1.2       |
| Radioulna length | 5.7       | 5.6       | 5.5       | 6.9       |
| Hand             | 8.9       | 9.1       | 8.5       | 10.0      |
| Finger I         | 4.1       | 4.4       | 4.2       | 5.4       |
| Finger II        | 5.5       | 5.2       | 4.8       | 6.1       |
| Disc of Finger III width | 1.8     | 1.9       | 1.7       | 1.8       |
Figure 1

Map of Ecuador showing the location of Río Manduricu Reserve, the type locality of *Nymphargus manduricu* sp. nov.
Figure 2

Inferred mitochondrial phylogeny of the genus *Nymphargus*, with the positioning of the new species, *Nymphargus manduriacu* sp. nov.

Clade support values (bootstraps) were obtained as described in Minh et al. (2013). Taxa in blue correspond to sequences added in this study.
Figure 3

*Nymphargus manduriacu* sp. nov. in life.

**(A-C)** Adult male, holotype, ZSFQ 0466. **(D-F)** Adult female, paratype, ZSFQ 0462.
Figure 4

Life stages of *Nymphargus manduriacu* sp. nov.

(A) Egg clutch (ZSFQ 467). (B) Metamorph (ZSFQ 618).
Figure 5

*Nymphargus manduriacu* sp. nov. and similar species.

(A) *N. manduriacu*, Reserva Río Manduriacu, Ecuador, uncollected. (B) *N. buenaventura*, Cascadas de Manuel, Cantón El Guabo, Provincia El Oro, 800 m, Ecuador, DHMECN 10982, photo by Juan Carlos Sánchez. (C) *N. luminosus*, Quebrada la Honda y La Amarill, Vereda Venados Arriba, Municipio de Frontino, Departamento de Antioquia, Colombia, MAR 3576, photo by Marco Rada. (D) *N. spilotus*, Parque Nacional Natural Selva de Florencia, Colombia, JD 060, photo by Jesse Delia.
Figure 6

Hand webbing *Nymphargus manduriacu* sp. nov. and similar species.

(A) *N. manduriacu*, ZSFQ 0463, adult male, paratype. (B) *N. luminosus*, ICN 15930, adult female, holotype. (C) *N. spilotus*, ICN 35255, adult female, holotype.
Figure 7

Call of the holotype of *Nymphargus manduriacu* sp. nov.

**(A)** Oscillogram. **(B)** Audiospectrogram. File number: LBE-C-042.
Figure 8

Map of Río Manduriacu Reserve (Imbabura province, Ecuador), with conservation plans and mining threats.