A spectacular new species of *Hyloscirtus* (Anura: Hylidae) from the Cordillera de Los Llanganates in the eastern Andes of Ecuador

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Abstract

We have discovered a spectacular new species of frog in the genus *Hyloscirtus,* which according to the presence of characters like ……..it belongs to the *H. larinopygion* group (clade?). The adult female is characterized by a mostly black body with large bright red spots on the dorsal and ventral surface, extremities, and toe pads. Small juveniles are characterized by a yellow body with variable black markings on the flanks while one large juvenile displayed irregular orange or yellow marks on a black background color, with light orange or yellow toe pads. The color distribution of the adult male is yet unknown. Additional distinctive external morphological features such as ….(describe them in the abstract), and some osteological details are imaged and analyzed. The performed phylogeny places the new species as the sister to a clade of ten taxa, all of which are part of the *H. larinopygion* group. We estimate that the divergence of the new species from its known congeners pre-dates the Quaternary

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period (why?). The new species is currently only known from Cerro Mayordomo, in Fundación EcoMinga´s Machay Reserve, at 2900m in the eastern Andes of Tungurahua province, Ecuador, near the southern edge of Los Llanganates National Park, but its real distribution may be larger.

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KEY WORDS:
Hyloscirtus larinopygion group (clade?), Llanganates mountains, Upper Río Pastaza watershed, Machay, ?aposematic.

Introduction

Lynch & Duellman (1980) have identified the upper Rio Pastaza watershed as a center of endemism for amphibians, and subsequent investigations have tripled the number of species apparently endemic to this region, known as the Llanganates-Sangay Ecological Corridor (Reyes-Puig et al., 2010, 2014, 2015, 2019a, b; Reyes-Puig & Yánez-Muñoz, 2012; Reyes-Puig, 2013; Franco-Mena et al., 2019).

In the Llanganates-Sangay Ecological Corridor and the buffer zone of the Los Llanganates National Park, the Machay Reserve is a private reserve owned by the Ecuadorian NGO Fundación EcoMinga on Cerro Mayordomo. Investigators from Fundación EcoMinga and Instituto Nacional de Biodiversidad (INABIO) have been conducting botanical and herpetological expeditions there for two decades, which have led to the discovery of several dozen new species of plants, especially orchids (Jost, 2004) and more than ten new amphibian and reptile species (Reyes-Puig et al., 2010, 2014, 2015, 2019a, b; Reyes-Puig & Yánez-Muñoz, 2012; Reyes-Puig, 2013; Sheehy et al., 2014). During a botanical expedition in March 2018, one of the participants, Darwin Recalde, fortuitously found a striking black and red frog hiding in a leaf axil of a bromeliad at eye level. During the following years, herpetologists from Fundación EcoMinga and INABIO conducted additional expeditions to the site and found three juveniles of the same species just a few meters from the spot where the original female had been found. Further morphological and genetic comparisons identified these frogs as belonging to a new species of Stream Frog which we describe below, belonging to the genus *Hyloscirtus* Peters, 1882, in the *H. larinopygion* group.

Comentario [GP1]: Below you say that two individuals were found the first time.

The genus *Hyloscirtus,* in the family Hylidae, contains 38 species of arboreal frogs (Faivovich et al., 2005; Frost, 2021). The genus is characterized mainly by the synapomorphy of well-developed lateral fringes on the fingers and toes (Faivovich et al., 2005). All known species are thought to reproduce alongside rushing streams (Coloma et al., 2012). The genus is distributed from Costa Rica to the Andes of Venezuela, Colombia, Ecuador, Peru and Bolivia (Faivovich et al., 2005; Coloma et al., 2012; Frost, 2021). The *Hyloscirtus larinopygion* group is composed of 19 species (Frost, 2021), of which 13 are reported from Ecuador (Coloma et al., 2012; Ron et al., 2021). The group consists of two clades which correlate with latitude, with a small area of overlap in central Ecuador (Almendariz et al., 2014; Ron et al., 2018). Adults of this group are characterized by a snout vent length > 60 mm and dark skin color contrasting with bright patterns, especially on the arms and legs, and sometimes including the tips of the digits.

Materials and Methods

Ethics statement. Our study was authorized under research permits MAE-DNB-CM-2016-0045 and MAE-DNB-CM-2019-0120, issued by the Ministerio del Ambiente del Ecuador. We followed standard guidelines for use of live amphibians and reptiles in field research (Beaupre et al., 2004), compiled by the American Society of Ichthyologists and Herpetologists, the Herpetologists' League, and the Society for the Study of Amphibians and Reptiles.

Taxon sampling. We examined specimens deposited in the herpetological collections of the Instituto Nacional de Biodiversidad, Quito (DHMECN) and Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá (ICN) (Appendix 1). All museum acronyms follow Sabaj (2016). Our taxonomic description employs several lines of evidence, including external morphological characters, genetic divergence, monophyly and geographic data. Similar approaches have been useful in recognizing and identifying closely related species of anurans in the eastern Andes of Ecuador (Páez-Moscoso et al., 2011; Reyes-Puig et al., 2019a, b).

Comentario [GP2]: You have not enough data yet to determine the geographic distributuon. We have to hope that you do not eliminated two (or three) individuals of an extremely reduced population. In the next years you will have more information to define better the geographic extension of this speies.

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Field work. Two individuals were found fortuitously in the same spot during diurnal walks in botanical expeditions to the summit of Cerro Mayordomo (1.3702 S, 78.2679 W, 2970 m) on 16–20 March 2018 and 18–19 October 2018. Both were collected. A third individual, photographed in situ but not collected, was found in the same spot on December 11, 2019, and a fourth individual was found and collected in the same area

Comentario [GP3]: You said above that the first time was only found the black and red female. Please clarify

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on May 30,2022 Several additional expeditions to the same location failed to find individuals of this species.

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Laboratory work. The two original collected individuals of the new species were taken alive, in plastic containers, to INABIO, where they were photographed in life and euthanized with benzocaine. Tissue samples were then taken for DNA sampling. They were subsequently fixed in 10% formalin for twelve hours, and then preserved as voucher specimens in 70% ethanol following the recommendations of Heyer et al. (1994). These specimens were deposited in the herpetological collection (DHMECN) of INABIO as holotype and paratype. The third collected individual, an additional paratype, is being kept alive for observation and analysis at INABIO and will be deposited in the same collection.

External morphological data. Measurements and character descriptions were made according to the specialized literature treating the *H. larinopygion* group (Coloma et al., 2012; Almendáriz et al., 2014; Ron et al., 2018). Description of webbing formulae of the hands and follow Savage & Heyer (1967) as modified by Myers & Duellman (1982). We obtained morphological measurements of the two specimens preserved in 70% ethanol according to the methodology described in Duellman (1970), using digital calipers (± 0.01 mm). The following measurements were taken: snout-vent length (SVL), head length (HL), head width (HW), upper eyelid width (EW), interorbital distance (IOD), inter-nostril distance (IND), eye-nostril distance (END), eye diameter (ED), tympanum diameter (TD), hand length (HAL), tibia length (TL), femur length (FEL), and foot length (FL). Sex was determined by direct examination of gonads.

We also compared qualitative morphological characters between the new species and its closest relatives. Seven characters were evaluated: (1) dorsal coloration; (2) ventral coloration; (3) marks on flanks and hidden surfaces of thighs; (4) iris coloration; (5) prepollex condition; (6) in life, webbing coloration; and (7) cloacal ornamentation. Life coloration was obtained from live specimens and color photographs. Cloacal ornamentation condition was observed on preserved specimens.

Comentario [GP4]: Why a figure of the cloacal ornamentation in the four individuals putatively belonging to **H.** *sethmacfarlanei* is not provided? It is important to the comparison of morphological characters shared by the only known specimens.

Osteological data and analysis. The holotype (DHMECN 14416) of the new species, and one specimen of each of five closely related species (DHMECN 12483: *Hyloscirtus lindae*; DHMECN 12111: *H. pacha*; DHMECN 6493: *H. psarolaimus*; DHMECN 3799: *H. larinopygion*; DHMECN 9686: *H. tapichalaca*), were scanned using a high-resolution micro-computed tomography (micro-CT) desktop device (Bruker SkyScan 1173, Kontich, Belgium) at the Leibniz Institute for the Analysis of Biodiversity Change - Museum Koenig (LIB Bonn, Germany). To avoid movements during scanning, the specimens were placed in a small plastic container and mounted with styrofoam. The scans were conducted over 180 degrees with rotational steps of 0.3–0.4 degrees, with a source voltage of 35 kV and source current of 150 µA, without the use of a filter, at an image resolution of 39.3–50.0 µm. Scan duration was 30:01–45:37 min with an exposure time of 280 ms and average rate of 5 frames per second. The micro-CT datasets were reconstructed using N-Recon software (Bruker MicroCT, Kontich, Belgium) and rendered in three dimensions through the aid of CTVox for Windows 64 bits version 2.6 (Bruker MicroCT, Kontich, Belgium). Additionally, the skull of the holotype of the new species was rendered and segmented to separate and color individual bones in three dimensions using Amira visualization software (FEI, Thermo Fisher Scientific). Osteological terminology follows Trueb (1973), Duellman & Trueb (1994), Coloma et al. (2012), Kunisch et al. (2021), Reyes-Puig et al. (2021). For the description of the cranium and the osteology of the hand, we followed the proposal of Coloma et al. (2012). Cartilage structures were omitted from the osteological descriptions, because micro-CT does not render cartilage. To facilitate comparisons among skull bones, we added color to the micro-CT scan images using Adobe Photoshop.

Genetic sampling. We generated two new sequences (one from each of the individuals collected in 2018) for the mitochondrial 16S gene (see Fig. 1), following the primers and protocols described in Guayasamin et al. (2015). The new sequences (Genbank OM293945, OM293945) were aligned with all sequences available for *Hyloscirtus* in GenBank (http://www.ncbi.nlm. nih.gov/genbank), originally published by Faivovich et al. (2005), Coloma et al. (2012), Almendáriz et al. (2014), Guayasamin et al. (2015) and Ron et al. (2018). Genbank codes of downloaded species are shown in Figure 1.

Comentario [GP5]: Please indicate the collection data of these individuals.

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Phylogenetic analysis. Sequences were aligned using MAFFT v. 7 (Katoh & Standley, 2013) with the Q-INS-i strategy. Maximum likelihood (ML) trees were estimated using GARLI 2.01 (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006). GARLI uses a genetic algorithm that finds the tree topology, branch lengths and model parameters that maximize ln(L) simultaneously (Zwickl, 2006). In order to determine the outgroups for our analyzes, we conducted preliminary runs in GARLI and selected as outgroups those species that were inferred as most phylogenetically distant to the *Hyloscirtus larinopygion* group. With this information, we selected species in the *H. bogotensis* group as outgroups. During the ML analyses, individual solutions were selected after 10,000 generations with no significant improvement in likelihood, with the significant topological improvement level set at 0.01. The final solution was selected when the total improvement in likelihood score was lower than 0.05, compared to the last solution obtained. Default values were used for other GARLI settings, as per recommendations of the developer (Zwickl, 2006). Bootstrap support was assessed via 1000 pseudoreplicates under the same settings used in tree search. Genetic distances (uncorrected p) between the new species and its closest relatives were calculated using PAUP v.4.0a (Swofford, 2002).

Ecological niche modeling. We use Maxent (version 3.4.2) to obtain a model of the range of ecological niches for the northern clade of the *H. larinopygion* group. Localities for all species of the group were obtained from literature and museum collections. Recommended default values were used for convergence threshold, maximum number of iterations, and maximum background points; 25% of localities were randomly set aside as test points; regularization was set to 1. Selected format for representation of probabilities for models was logistic. Parametrization was based on WorldClim (version 2.1, Fick & Hijmans, 2017). Statistical analyses of variable contributions for data layers, including jackknife tests and correlation tests, were used to obtain more informative and less correlated variables. Models were evaluated through quantitative and qualitative tests, including threshold-independent test, threshold-dependent test, visual evaluations, and evaluation of variable importance and response curves. A geographical information system was developed based on grids from Maxent with ArcGis Desktop to analyze data and produce relevant maps.

Results

Phylogenetic relationships. Our phylogenetic analysis (Fig. 1) shows that the new species is sister to a clade containing ten *Hyloscirtus* species: *H. criptico* (Coloma et al., 2012)*, H. larinopygion* (Duellman, 1973)*, H. lindae* (Duellman & Altig,1978)*, H. pacha* (Duellman & Hillis, 1990), *H. pantostictus* (Duellman & Berger*,* 1982), *H. princecharlesi* Coloma et al. (2012)*, H. psarolaimus* (Duellman & Hillis, 1990), *H. ptychodactylus* (Duellman & Hillis, 1990)*, H. staufferorum* (Duellman & Coloma, 1993), and *H. tigrinus* (Mueses-Cisneros & Anganoy-Criollo 2008)*.* However, we note that support for the exact topology of this relationship is low (bootstrap = 54%).

Genetic distances (mitochondrial 16S percent differences calculated from uncorrected p values) between the new species and the most closely related *Hyloscirtus* are given in Table 1. The lowest values of genetic distances between the new species and its relatives were 2.2–2.9% to *H. tigrinus* and 2.6–2.8% to *H. ptychodactylus* (Table 1).

Most DNA sequences are publicly available (see GenBank codes in Fig. 1). The sequences of the new species are available as Supplemental Files.

Systematic account

***Hyloscirtus sethmacfarlanei* sp. nov.**

Proposed standard Spanish name: Rana de torrente de Seth MacFarlane
Proposed standard English name: Seth MacFarlane's torrent frog

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Holotype (Figs. 2–7). DHMECN 14416, adult female, collected in the Machay Reserve of Fundacion EcoMinga, Cerro Mayordomo (1.370204 S, 78.267943 W, 2970 m), Rio Verde parish, Baños township, Tungurahua province, Republic of Ecuador, on 17 March 2018, by Darwin Recalde, Fausto Recalde, Santiago Recalde, and Jordy Salazar.

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Paratypes (Figs. 4–7). DHMECN 14549, juvenile, collected at the type locality on 19 October 2018, by Fausto Recalde, Santiago Recalde, Darwin Recalde and Jordy Salazar; DHMECN 17554, juvenile, collected at the type locality on 30 May 2022, by Fausto Recalde, Luis Recalde, and Santiago Recalde.

Comentario [GP6]: You have considered this individual as a male in an early version of the manuscript and now as a juvenile. Thus, you have to describe the characters from which you based so different ontogenetic stages.

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Comentario [GP7]: The same as in commentary 6

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Generic placement. We assign the new species to the genus *Hyloscirtus* Peters, 1882, defined according to Faivovich et al. (2008) and Rojas-Runjaic et al. (2015), and to the *H. larinopygion* group (sensu Duellman & Hillis, 1990; Faivovich et al., 2005), according to its phylogenetic position (Fig. 1) and morphological traits such as wide dermal fringes on fingers and toes, hands and legs with large terminal discs and a reduced membrane, adults characterized by a snout vent length > 60 mm, and dark overall skin color contrasting with bright color patterns.

Diagnosis. *Hyloscirtus sethmacfarlanei* sp. nov. is a member of the *Hyloscirtus larinopygion* group as diagnosed by Duellman & Hillis (1990), Faivovich et al. (2005) and Weiens et al. (2005), and is characterized to a new species by the following combination of characters: discs of digits narrow; fleshy calcar present; cloacal ornamentation with two thick well-defined parallel paracloacal grooves; a well-defined supracloacal fold reaching the vent; skin surrounding cloaca strongly areolate and granular; anterior border of sphenethmoid not in contact with nasal; nasal not in contact with maxilla; frontoparietals rugose; vomers not in medial contact, and with 12–13 tooth loci; 54–56 maxillary tooth loci; 10–11 premaxillary tooth loci; zygomatic ramus of squamosal slightly longer than otic ramus, and otic ramus not in contact with prootic. Adult female is further characterized by black ground color covered with large bright red spots on both the dorsal and ventral surfaces, and red tips on all digits.

Comentario [GP8]: Based on which characters you placed the specimens in this group? Please include them here

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Comentario [GP9]: All these characters are diagnosing the new species or the H. larinopygion group? Please, this should be well delimited. Besides, you have to explain why you considered the black and red specimen as an adult female (by size, colour, cloacal area, digits, etc).

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Description of holotype (Figs. 2, 3). Adult female, SVL 72.0 mm. Body slender, head rounded in dorsal view, longer than wide (head length 113% of head width); width of upper eyelid 72% of the interorbital distance; texture of the dorsal surface of the head rough, including the eyelids; snout truncate in dorsal and lateral views; eye-nostril distance slightly less than the diameter of the eye; canthus rostralis short and slightly rounded, loreal region slightly concave; internarial region flat and slightly depressed; top of head slightly concave; nostrils oval and slightly protuberant, directed laterally; eyes large and protuberant, 25% of head length; interorbital region concave; eye diameter 1.8 times larger than the diameter of the tympanic ring; supratympanic fold well-defined, directed obliquely from the posterior border of the eye, covering the dorsal edge of the tympanum, extending back to the upper shoulder; tympanum and tympanic ring evident and round, 57% of eye diameter, separated from the eye by a distance 1.6 times larger than the diameter of the tympanum.

Anterior and posterior extremities slim. Relative length of fingers I < II < IV < III; fingers with large oval disks slightly wider than finger; subarticular tubercles simple and enlarged, round and prominent; multiple round and oval supernumerary tubercles present; thenar tubercle large and flat, oval and elongated; palmar tubercle asymmetric with a slightly heart-shaped outline; prepollex absent; glandular nuptal pad covering the outer margin of Finger I; fingers long with interdigital webbing basally and with fleshy lateral fringes on all fingers.

Hind limbs long and slender, tibia length 46% of SVL; foot length 46% of SVL; heel tubercles large and round in outline; inner tarsal fold absent; large rounded to slightly oval subarticular tubercles in all fingers, supernumerary foot tubercles rounded, low; toes long, narrower than the disc, discs not expanded; relative lengths of toes I < II < V< III < IV; inner metatarsal tubercle large, oval; outer metatarsal tubercle absent; toes with interdigital membrane, toe membrane formula: I 2-3 II 3- 2 III 3-2 IV 3-2 V (Fig. 3).

Body skin is finely granular, especially on flanks; inguinal glands absent; ventral skin densely areolate, less so towards the throat. Supracloacal flap transversal, well-defined, with supracloacal fold present, reaching the level of the vent, with two paracloacal folds; skin around the cloaca strongly areolate and granular (Fig. 8). Choana large and oval, notably separated from each other and perpendicular to the floor of the mouth; dentigerous processes of vomers transverse, with vomerine teeth numbering 9–10; tongue wide and rugose, slightly rounded, partially attached to the floor of the mouth.

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Coloration of holotype in life (Figs. 4–7, 9). Entire dorsal and ventral surfaces of the head, body, and limbs black with large bright red round to oval spots scattered over the whole body, including the tips of the digits; spots 3–4 mm in diameter on dorsal surface of body and 5–10 mm long on ventral surface and throat, more elongated on the extremities and flanks. Iris light grayish with fine dark reticulations, while the nictitating membrane, revealed in defense and at rest, is well-developed, black in color, with irregular red reticulations.

Coloration of holotype in preservative (~70% ethanol) (Fig. 2). Mainly black background; the red spots in life fade to yellowish-white or white; ventral surfaces and throat grayish black with scattered irregular white elongated spots; palms of hands and feet grayish.

Measurements of the holotype (in mm). SVL= 72.0; HL=22.9; HW =20.2; EW =6.0; IOD =8.3; IND=5.2; NED= 5.4 ; ED=5.7; TD=3.2; HAL=25.2; TL=33.3; FEL= 26.1; FL= 33.4.

Measurements of the paratypes. See Table 2.

Osteology of the preserved holotype (Figs. 10–13). Coloma et al. (2012) provide a detailed description of the osteology of the *H. larinopygion* group. In order to avoid redundancy, in the following we describe only those osteological features of the holotype of *H. sethmacfarlanei* sp. nov. where we found differences from the other species.

Skull (Figs. 10, 11). The anterior border of the sphenethmoid is not in contact with the nasal; the nasal is not in contact with the maxilla; the frontoparietals are rugose; the paired vomers bear 12–13 tooth loci and are not in contact medially; there are 54–56 tooth loci on each maxilla and 10–11 tooth loci on each premaxilla; the zygomatic ramus of the squamosal is slightly longer than the otic ramus, and the latter is not in contact with the prootic.

Posteromedial processes of the hyobranchium (Fig. 13). The posteromedial processes of the hyobranchium are paired ossified structures, longer than broad, the anterior portion with triangular “head-like" shape, and a posterior elongated stem.

Tadpole. Not known.

Advertising call. Not known.

Variation (Figs. 4, 5, 9). Standard measurements from the three collected individuals are shown in Table 2. The three known juveniles (DHMECN 14549, DHMECN 17554, and the uncollected individual) share the distinctive cloacal ornamentation and skin texture of the holotype, but differ from the female holotype as follows: sexual characters not clearly evident; in life the dorsal surface with irregular marks mustard yellow heavily stippled with black, especially on flanks and lower back (DHMENC 14549), or a variagated yellow-orange pattern (DHMECN 17554); nictitating membrane dotted with mustard yellow on a gray background (DHMENC 14549) or orange on a black background (DHMECN 17554); extremities orange banded (DHMENC 14549) or spotted (DHMECN 17554), on a grayish black to black ground; flanks black with orange reticulations and irregular spots; throat marbled with irregular orange or yellowish patches with orange tones on a grayish black or black ground; belly and ventral surfaces of the extremities grayish black with irregular sparse diffuse light orange or

Comentario [GP10]: Provide a figure

Con formato: Resaltar

Comentario [GP11]: Please, explain here that sexual assignation is based only in the observation of internal anatomy.

Comentario [GP12]: Yes, the two “juveniles” are very similar in coloration pattern, very different to the adult “female” and also to the tiny yellow featureless juvenile that you apparently do not collected. It was be good that you indicate if this ontogenetic changes in coloration are common among the components of the H. larinopygion group whitish-yellow patches (DHMENC 14549) or solid orange (DHMENC 17554); palms of hands and feet black with diffuse light orange spots. The uncollected juvenile had a mainly yellow dorsal coloration, with diffuse blackish spots scattered on the flanks and hidden surfaces of the arms and between the fingers, whose tips were yellow. The belly is light cream with diffuse blackish spots. We noted rapid temporal chromatic changes in the juvenile individuals, from dull yellow to orange tones. The juveniles all shared the same distinctive cloacal ornamentation as the adult.

Comentario [GP13]: Indeed, what you know is that juveniles are yellow at the first stages and at subadults they become orange and thus change again to black and red. Do you know if other of the species belonging to the H. larinopygion display such changes in skin coloration during ontogeny? Please add a paragraph on this condition at the discussion section.

Comparison with similar species (Figs. 7, 8, Table 3). The black and red pattern of the female of the new species is most similar to the patterns of *Hyloscirtus pantostictus* (Duellman & Berger*,* 1982), from extreme northeastern Ecuador, and *H. princecharlesi* Coloma et al. (2012), from the Pacific slopes of the Andes of northwestern Ecuador. The new species differs from these in having both the dorsal and ventral surfaces spotted with red (versus ventral surface without red spots in *H. pantostictus* and *H. princecharlesi*, Fig. 7), the cloacal ornamentation (Fig. 8) consisting of a well-defined supracloacal fold reaching next to the vent and the presence of a paracloacal fold (versus reduced supracloacal fold, without paracloacal fold, not contacting the side of the vent, in *H. pantostictus*, and supracloacal fold defined, reaching the border of the vent, with paracloacal fold thick, in *H. princecharlesi*), and strongly areolate skin texture (versus smooth in *H. pacha, H. staufferorum,* and *H. larinopygion*). The female of the new species also differs from these two species in having red discs on the tips of all digits (versus yellow discs in *H. pantostictus* and grayish discs in *H. princecharlesi*).

The new species' red discs are shared with *H. lindae* (Duellman & Altig, 1978) from the eastern Andes, but *H. lindae* does not have red spots on its dorsal surface and does not have a thick supracloacal fold close to the side of the vent (Fig. 8). Juveniles assigned to *H. sethmacfarlanei* sp. nov. have a pattern similar to those of *H. princecharlesi* and *H. larinopygion* (Duellman, 1973) from northwestern slopes of the Andes. They differ from juveniles of both species in having the dorsum mottled and stippled mustard-yellow and black (versus dorsum densely spotted orange-red in *H. princecharlesi,* and yellowish-brown with distinctive cream bars with black interspaces in *H. larinopygion*). The supracloacal fold is well-defined and reaches to the vent in *H. sethmacfarlanei* sp. nov. (versus faintly defined and distant from the side of the vent in *H. larinopygion*). *Hyloscirtus sarampiona* (Ruiz-Carranza & Lynch, 1982) from the western slopes of the Colombian Andes has dorsal surfaces orange spotted with pale olive, and further differs from the new species by having hidden areas of the limbs, flanks, palmar, plantar surfaces and discs black.

The skull of *H. sethmacfarlanei* sp. nov. (Figs. 10, 11) is generally consistent with those of the other species of the *H. larinopygion* group (Coloma et al., 2012). However, there were a few differences between the new species and the species of the group studied by us or by Coloma et al. (2012). In *H. sethmacfarlanei* sp. nov. and *H. ptychodactylus*, the sphenethmoid is not in contact with the nasal, whereas these two bones are in contact in *H. criptico* and in *H. staufferorum,* they are anteriorly in contact in *H. lindae* and *H. larinopygion,* they are in contact along most of their length in *H. psarolaimus,* they are in contact along their entire length but with still a visible suture in *H. pacha*, and they are completely fused without a visible suture in *H. tapichalaca.* In *H. sethmacfarlanei* sp. nov., *H. lindae*, *H. pacha*, and *H. larinopygion* the nasal is not in contact with the maxilla, whereas it is in contact with the maxilla in *H. criptico, H. pantostictus, H. ptychodactylus, H. staufferorum*, and *H. tapichalaca*. The frontoparietals of *H. sethmacfarlanei* sp. nov. are comparatively more rugose than in other species of the group (Fig. 11). In contrast to *H. pantostictus* and *H. staufferorum*, in *H. sethmacfarlanei* sp. nov., *H. criptico*, *H. lindae*, *H. pacha*, *H. psarolaimus*, *H. ptychodactylus*, *H. larinopygion*, and *H. tapichalaca* the otic ramus of the squamosal is not in contact with the prootic. In *H. sethmacfarlanei* sp. nov. the zygomatic ramus of the squamosal is only slightly longer than otic ramus, whereas it is moderately longer than the otic ramus in *H. pacha* and *H. staufferorum*, and distinctly longer than the otic ramus in *H. criptico, H. lindae, H. pantostictus, H. ptychodactylus, H. larinopygion*, and *H. tapichalaca*. In contrast to *H. criptico, H. larinopygion,* and *H. tapichalaca*, in *H. sethmacfarlanei* sp. nov., *H. lindae*, *H. pantostictus*, *H. ptychodactylus*, and *H. staufferorum* the vomers are not in medial contact. *Hyloscirtus sethmacfarlanei* sp. nov. has 12–13 vomerine tooth loci, 54–56 tooth loci on each maxilla, and 10–11 tooth loci on each premaxilla, whereas we counted 14 vomerian tooth loci, 59–60 maxillary tooth loci, and 11–12 premaxillary tooth loci in *H. lindae*, 14 vomerine tooth loci, 52–59 maxillary tooth loci, and 9 premaxillary tooth loci in *H. pacha*, 13–14 vomerine tooth loci, 52–53 maxillary tooth loci, and 9 premaxillary tooth loci in *H. psarolaimus*, 11–12 vomerine tooth loci, 56 maxillary tooth loci, and 12 premaxillary tooth loci in *H. larinopygion*, and only 5–6 vomerine tooth loci, 31–33 maxillary tooth loci, and 5–6 premaxillary tooth loci in *H. tapichalaca*.

In the new species the posteromedial processes of the hyobranchium possess a triangular shaped anterior portion, and a shorter posterior portion compared with the other species shown in Fig. 13, which have an external round border and an internal spine-like border. In *H. lindae, H. psarolaimus* and *H. pacha*, the anterior portions have rounded external and internal borders. In *H. tapichalaca* it is broad and “shell-like" in its anterior border.

There are no relevant differences between the forelimb bones of the new species and those of the other species in the group, with the exception of male specimens of *H. tapichalaca*, which have a greatly enlarged prepollex (Kizirian et al., 2003; this study) compared to the other species of the *H. larinopygion* group.

Distribution (Fig.14**).** *Hyloscirtus sethmacfarlanei* sp. nov. is known at the moment from the type locality in Fundación EcoMinga's Machay Reserve, Cerro Mayordomo, 2970 m altitude, in the eastern cordillera of the central Ecuadorian Andes, in the northern side of the upper Rio Pastaza watershed near the southern border of Llanganates National Park in the province of Tungurahua.

Natural history. The type locality consists of dwarf open mossy forest, covered with bryophytes and epiphytes, and saturated with humidity. All four known individuals of this species were found on a single narrow mountain ridge, in bromeliads of the genus *Guzmania* growing within 60-90 cm above the ground (Fig. 15). The holotype is an adult gravid female with a mass of eggs in early stage of development in March 2018. Adult male, tadpole and advertisement call remain unknown.

Comentario [GP14]: What a shame!

There is some evidence that the striking coloration of the adult female of *H. sethmacfarlanei* sp. nov. could be aposematic. The frog's discoverer (Darwin Recalde), after briefly handling the frog, noticed an unpleasant tingling sensation down his arm, not restricted to the area which had contacted the frog; the sensation lasted several hours. Fausto Recalde, who had shorter contact with the frog, developed similar but shorter-lasting symptoms. During handling of the holotype specimen in the museum, it emitted a white exudation in dorsal surfaces with a distinctive odor similar to diluted contact cement. Additionally, when tissue was taken from the liver, dark blackish-colored blood was observed.

Comentario [GP15]: And what happened to the collectors of the putative “juveniles”? It is possible that the toxins gradually increase with age?

The bright yellow uncollected juvenile slept during the day, and when disturbed, it adopted a defensive ball-like position, as observed in other species of the *H. larinopygion* group (Kizirian et al., 2003; Bejarano-Muñoz et al., 2015). Thus the juvenile coloration may also advertise its distastefulness.

Nocturnal surveys done by our team in the habitat of *Hyloscirtus sethmacfarlanei* sp. nov. revealed three sympatric anuran species: two undescribed *Pristimantis* species and one species of the *Pristimantis buckleyi* complex.

Conservation Status. All four known individuals are known only from the same few square meters of ridgeline, but the area is poorly studied and inaccessible because of steep topography. We suggest the IUCN category Data Deficient (DD) for this species.

Etymology. The specific epithet *sethmacfarlanei* is a patronym in honor of Seth MacFarlane, American writer, director, producer, actor, artist, musician and conservationist, with an outstanding passion for science, biodiversity and the natural world.

Discussion

Despite the low number of studied specimens (basicly limited to the type material) for defined a new species, the congruence of strong molecular and morphological evidence clearly shows that all specimens belong to a new monophyletic taxon (Fig. 1) that has been evolving independently from other named taxa, meeting the core criterion for species recognition (see Simpson, 1951, 1961; Wiley, 1978; de Queiroz, 2007). The collected juveniles and also the small uncollected young share the same cloacal ornamentation and skin texture as the adult female, and the performed genetic analyses confirmed that the adult female and one of the collected juveniles belong to the same species.

Though only one adult female specimen is known, we do not expect this color to vary much among other female individuals of the species, based on the lack of significant variation across individual adult female members of each of the other species in this group (Coloma et al., 2012). Some of the coloration differences between the female and the three observed juveniles may be related to ontogenetic changes, with larger, probably subadult individuals displaying color patterns more similar to the described female pattern, as seen in other species of the *Hyloscirtus larinopygion group* (Coloma et al., 2012).

The *Hyloscirtus larinopygion* group is characterized by overlapping morphological and morphometric characters. In many cases, the preserved and living coloration patterns continue to be the main trait used to discriminate externally between the species in this group (Duellman & Hillis, 1990; Duellman & Coloma, 1993; Coloma et al., 2012; Rivera-Correa & Faivovich, 2013; Rivera-Correa et al., 2016; Ron et al., 2018). Our analyses of micro-CT scan osteology, skin texture, and supracloacal folds show the importance of continuing to incorporate and explore additional evidence to help delimit lineages of the group, whose evolutionary radiation in the Ecuadorian Andes is apparently still underestimated.

Most osteological characters do not seem to vary greatly among the different species of the *H. larinopygion* group. Even though we were able to detect some differences that could be of diagnostic value, we only had the opportunity to osteologically examine one individual from each of the six species. In previous studies (Kizirian et al., 2003; Coloma et al., 2012), another 15 specimens from eight species could be examined, so that data for osteological comparisons are available for a total of eleven species, comprising three specimens each from four species, two specimens each from two species and only one specimen each from five species. Some of the differences found between species might be less clear with a larger sample size. In *H. pacha*, for example, the vomers are in medial contact in the two specimens studied by Coloma et al. (2012), while they were not in contact in our individual. The opposite is true for *H. psarolaimus*,

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Comentario [GP16]: You should use suggests here, instead of “clearly shows”

Comentario [GP17]: Do you are referring to the H. larinopygion clade? Please, clarify this sentence

Eliminado: juveniles

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Comentario [GP18]: Particularly because coloration can usually varies with chnges in environmental and climatic conditions, even it can occur annually (see Goedert, D., D. Clement, and R. Calsbeek 2021. Evolutionary trade-offs may interact with physi-ological constraints to maintain color variation. Ecological Monographs 91(1):e01430. 10.1002/ecm.1430)
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where the vomers are not in contact in the individual studied by Coloma et al. (2012) and are in contact in our individual. Furthermore, in Coloma's individual of *H. psarolaimus*, the nasal and maxilla are in contact, and the zygomatic ramus of the squamosal is approximately as long as the otic ramus. In our individual, however, the nasal and maxilla are not in contact and the zygomatic ramus is moderately longer than the otic ramus. On the other hand, we could not detect any osteological difference between the individual of *H. lindae* we examined and the two specimens of that species examined by Coloma et al. (2012), nor between the individual of *H. tapichalaca* we examined and the two specimens of that species examined by Kizirian et al. (2003). Fortunately, modern non-invasive techniques such as micro-CT scanning are now increasingly available to quickly visualize the skeletal anatomy of a specimen in three dimensions. Since dissection is not involved, multiple specimens of a species can be easily scanned and compared. In the future, many more individuals of the various species of the *H. larinopygion* group will hopefully be studied using this technique, so that we can get a more accurate picture of the osteological differences between the various species.

Comentario [GP19]: This could be an ontogenetic difference?

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Biogeographic interpretations of the evolutionary history of *H. sethmacfarlanei* sp. nov. would be too speculative, mainly because the sister relationship between the new species and other *Hyloscirtus* has low bootstrap support (Fig. 1). Our inferred phylogeny recovered two species (*H. armatus* and *H. charazani*) of the *H. armatus* group as part of the *H. larinopygion* (Fig. 1), but, again, with low bootstrap support. Other recent studies (Coloma et al., 2012; Ron et al., 2018) have found strong support for the monophyly of the *H. larinopygion* and *H. armatus* groups. Therefore, differences might be a consequence of different gene and taxon sampling schemes.

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Comentario [GP20]: Yes, if the specimens examined were all in the same ontogenetic stage.

The fossil-calibrated divergence times between some of the species in the *H. larinopygion* group were estimated by Coloma et al. (2012). While *H. sethmacfarlanei* sp.nov. was not known at the time of that study, the divergence times between pairs of previously known species are linearly correlated with our calculated genetic distances between those same pairs of species (R^2= 0.92-0.94; Fig. 16). We can therefore use our genetic distances to estimate the divergence times between the new species and its relatives. Based on the shortest genetic distance of 2.2% between the new species and its relatives, we estimate that the divergence time between these two species can be at least on the order of six to eight million years.

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The shortest genetic distances between *H. sethmacfarlanei* sp. nov. and its relatives (2.2–2.9%) are considerably greater than the genetic distances between some other clearly-defined species in the *H. larinopygion* group, such as the distance between *H. ptychodactylus* and *H. princecharlesi* (1.3%). Thus our taxonomic proposal is consistent (in terms of genetic distance and divergence times) with past taxonomic decisions in this group.

The two sequenced specimens of *H. sethmacfarlanei* sp. nov. show a genetic distance of 0.4%, although they come from exactly the same location. This degree of divergence within a population is about the average for sequenced conspecific members of the *H. larinopygion* group (0.2–0.9%; Coloma et al., 2012). With only two sequenced specimens, our conclusions are necessarily limited, but this level of heterozygosity between two randomly selected individuals would not be possible if the population were highly inbred, implying that the actual population is not exceptionally small (Jetz & Pyron, 2018). The forest at the type locality of the new species, at 2,900–3,000 m on Cerro Mayordomo, is continuous with similar forest on the Cerro Hermoso massif in the center of Los Llanganates National Park, 17 km to the north of the type locality. The new species probably occupies at least this range. During the Holocene glacial maximum this forest community would probably have moved down the mountains by 1000 m (Dodson, 2003), potentially connecting this population to many other nearby mountains.

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Ecological niche modeling is a powerful tool for biogeographic analyses. Bioclimatic modeling approaches have been applied beyond single species distribution models to identify the potential distribution of undiscovered taxa, understand the ecological niche of supra-specific taxa, or predict the community structure of multiple species assemblages (e.g., Larsen et al., 2012; Ihlow et al., 2016; Braun et al., 2019). A Maxent model was applied to the known species of the *H. larinopygion* clade to estimate its potential distribution (though it does not take into account the history of past connectivity between sites). Modeling the distribution of supra-specific taxa assumes that members of the taxon respond similarly to environmental conditions. This approach is considered appropriate for the northern clade of *H. larinopygion* group due to their occurrence in apparently similar ecosystems and habitats across their distribution (Duellman & Hillis, 1990; Kizirian et al., 2003; Coloma et al., 2012; Almendariz et al. 2014; Rivera-Correa et al., 2018; Ron et al., 2018, 2021).

It is remarkable that despite intensive research work in the upper Rio Pastaza watershed and in the *Hyloscirtus larinopygion* species group, researchers still continue to discover conspicuous new species in the group. Our Maxent model estimates the potential distribution of all members of the clade, showing areas where potential undiscovered species might occur. The Maxent model shows that the type locality of *H. sethmacfarlanei* sp. nov. is within the predicted range of niches of the northern clade of the *H. larinopygion* group. Many additional areas across the Andes of Colombia and Ecuador show high probability of occupation according to the model, but no species records, e.g., the Cordillera Oriental of Colombia, the southern Cordillera Occidental of

Colombia, and the extreme northern and central Cordillera Oriental of Ecuador (Figs. 14, 15).

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Conclusions

We present converging lines of evidence to show that a newly discovered population of *Hyloscirtus*, belonging to the *H. larinopygion* group, represents a distinctive new species. Our observations on its antipredatory behavior lead us to conclude that this species is almost certainly toxic and/or unpalatable, and that its bright colors are probably aposematic. Our genetic analysis leads us to conclude that *Hyloscirtus sethmacfarlanei* sp. nov. is an older species, not a product of Quaternary isolating mechanisms. Our study further confirms the importance of the Llanganates – Sangay Ecological Corridor, outside of Ecuador's national park system, as a center of endemism and diversity. Additionally, a distribution model for the *H. larinopygion* species group suggests many other potential areas of occurrence along the northern Andes for members of this group.

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Appendix 1. List of specimens examined of the Hyloscirtus larinopygion group (13). Hyloscirtus criptico (1): Ecuador: Carchi, Morán, DHMECN 15831; H. larinopygion (1): Ecuador: Carchi, Morán, DHMECN 3799; H. lindae (1): Ecuador: Napo, Guango Lodge, DHMECN 14483; H. pacha (4): Ecuador: Morona Santiago, Guabisai, DHMECN 12110–12113; H. psarolaimus (4): Ecuador: Sucumbíos, La Bonita, DHMECN 6493–6496; Morona Santiago; Zuñac, DHMECN 12114; H. sarampiona (1): Colombia: Valle del Cauca, Quebrada Sopladero, Holotipo ICN 7440; H. tapichalaca (1): Ecuador: Zamora Chinchipe, Reserva Tapichalaca, DHMECN 9686.

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