Molecular phylogeny of stream treefrogs (Hylidae: Hyloscirtus bogotensis Group), with a new species from the Andes of Ecuador

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RESEARCH ARTICLE

Molecular phylogeny of stream treefrogs (Hylidae: *Hyloscirtus bogotensis* Group), with a new species from the Andes of Ecuador

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We present a new molecular phylogeny of the stream treefrog genus *Hyloscirtus,* with an improved taxon sampling in the *Hyloscirtus bogotensis* group. The tree supports the existence of three clades within the genus (*Hyloscirtus armatus* group, *H. bogotensis* group and *Hyloscirtus larinopygion* group) in congruence with previous studies, and suggests the presence of at least three new species in the *H. bogotensis* group. Herein, we describe one of these species, *Hyloscirtus mashpi* n. sp. from the Pacific slope of the Ecuadorian Andes. The validity of the latter is supported by molecular, morphological and acoustic data. We also tested individuals of the new species for the chytrid fungus *Batrachochytrium dendrobatidis,* finding a prevalence of 17.6% (6 positives and 28 negatives). However, at sampled streams, frog densities were high, suggesting that *H. mashpi* n. sp. may be tolerant to the infection.

Keywords: amphibia; chytrid fungus; cryptic diversity; phylogeny; taxonomy

Se presenta una nueva filogenia de las ranas torrentícolas del género *Hyloscirtus,* la cual incluye un muestreo ampliado del grupo *H. bogotensis*. El árbol apoya la presencia de tres clados dentro del género (Grupo *H. armatus*, Grupo *H. bogotensis,* Grupo *H. larinopygion*) y sugiere la existencia de tres especies nuevas. En el presente estudio describimos una de estas especies, *Hyloscirtus mashpi* n. sp., distribuida en la vertiente pacífica de los Andes del Ecuador. La validez de la nueva especie se sustenta en datos moleculares, morfológicos y acústicos. Nuestro estudio también demuestra que la nueva especie está infectada por el quítrido *Batrachochytrium dendrobatidis*; sin embargo, las poblaciones son abundantes, sugiriendo que *H. mashpi* n. sp. tolera la infección.

Palabras claves: Amphibia; diversidad críptica; filogenia; hongo quítrido; taxonomía
http://zoobank.org/789AFE0F-261C-4B99-8B76-5872FD29E3C6

Introduction

The genus *Hyloscirtus* [1] is part of the diverse tree frog family Hylidae, and represents a conspicuous component of the anuran fauna in the Andean foothills and cloud forests. This genus currently contains 34 recognized, extant species,[2] all of which reproduce in streams.

Species of *Hyloscirtus* are diagnosed morphologically by the presence of wide dermal fringes on fingers and toes.[3] Monophyly of the group is also supported by molecular phylogenetic analyses.[3–6] In a recent comprehensive review of hylid taxonomy, Faivovich et al. [3] recognized three monophyletic species groups within *Hyloscirtus*: (i) *Hyloscirtus armatus* group, (ii) *Hyloscirtus bogotensis* group and (iii) *Hyloscirtus larinopygion* group. Herein, we focus on the *H. bogotensis* group, a clade diagnosed mainly by the presence of a mental gland in males [3,7] and numerous molecular

transformations.[3] The group is found in the Andes of Colombia, Ecuador and Venezuela, and the lowlands of Costa Rica, Panama, Colombia, Ecuador and Peru.[2]

Currently, the *bogotensis* group contains 16 species: *Hyloscirtus albopunctulatus*,[8] *H. alytolylax*,[7] *H. bogotensis*,[1] *H. callipeza*,[9] *H. colymba*,[10] *H. denticulentus*,[7] *H. estevesi*,[11] *H. jahni*,[12] *H. lascinius*,[13] *H. lynchi*,[14] *H. palmeri*,[15] *H. phyllognathus*, [16] *H. piceigularis*,[17] *H. platydactylus*,[18] *H. simmonsi*,[9] and *H. torrenticola*.[19]

Most of the species in the genus *Hyloscirtus* have relatively narrow distributions and are restricted to specific microhabitats (i.e. mountain streams) and climatic niches (the cool, moist environments near streams). As an example, in Ecuador, only 3 (*H. alytolylax*, *H. palmeri* *H. phyllognathus*) of the 17 species of *Hyloscirtus* have relatively large distributions.[2,7,20] Two of these

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species, *H. palmeri* and *H. phyllognathus*, are found in the lowlands, where geographic barriers are not as conspicuous as in mountains, and large distributions can be expected. However, the relatively large distribution of *H. alytolylax*, which inhabits the Pacific slopes of the Andes in Ecuador and Southern Colombia,[7,20] deserves particular examination, mainly because cryptic diversity is likely to be present when populations are under similar ecological conditions in topographically complex landscapes, such as the Andes.

In this study, we present a new molecular phylogeny for the *H. bogotensis* group, as well as morphological and acoustic data that support the validity of at least one new species, which has been previously confused with *H. alytolylax*. Two other potentially new species are also revealed by genetic data, but we refrain from describing them until complementary evidence becomes available. Finally, we present information on the prevalence of *Batrachochytrium dendrobatidis* in the new taxon.

Methods

Nomenclature

Generic names follow the taxonomy proposed by Faivovich et al. [3]. For an updated list of the species in the genus, see Frost [2] and AmphibiaWeb.[21]

Morphology

We examined comparative alcohol-preserved specimens from the herpetology collection at the Museo de Zoología of the Universidad Tecnológica Indoamérica (MZUTI), Instituto de Ciencias Naturales of the Universidad Nacional de Colombia (ICN) and the University of Kansas Biodiversity Institute (KU). See Appendix 1. Morphological characters studied follow the definitions provided by Duellman and Hillis [22] and Kizirian et al. [23]. Fingers are numbered from I to IV. Webbing formulae are described following Savage and Heyer,[24] with modifications by Myers and Duellman.[25] Morphological measurements were taken with Mitutoyo® digital caliper to the nearest 0.1 mm, as described by Guayasamin and Bonaccorso [26], except when noted, and are as follows: (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; and (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.

Molecular data

Taxon and gene sampling

We obtained mitochondrial DNA sequences (12S, tRNA^{val} and 16S) from GenBank (http://www.ncbi.nlm.nih.gov/genbank) of all available species in the genus *Hyloscirtus*; sequences were published primarily by Faivovich et al. [3], Crawford et al. [27], Coloma et al. [28] and Almendáriz et al. [29]. Additionally, we generated new sequences for 35 individuals (Appendix 2).

DNA extraction, amplification and sequencing

Genomic DNA was extracted from frozen tissue with a salt precipitation method (M. Fujita, unpubl.) based on the Puregene DNA purification kit (Gentra Systems). We amplified and sequenced the mitochondrial 12S and 16S gene regions. The 12S marker was amplified using the primers 12L29E-F (AAAGCRTAGCACTGAAAATGC-TAAGA) and 12H46E-R (GCTGCACYTTGACCTG-ACGT) developed by Heinicke et al. [30], whereas the 16S gene was obtained with the primers 16SC (GTRG-GCCTAAAAGCAGCCAC) and 16Sbr-H (CCGGTCTG-AACTCAGATCACGT) developed by Darst and Cannatella [31] and Palumbi et al. [32], respectively. Each polymerase chain reaction (PCR) reaction contained a final concentration of 3-mM $MgCl_2$, 0.2-mM dNTPs, 0.05 U/μL *Taq* DNA polymerase (Invitrogen) and 0.2-μM each primer, in a total volume of 25 μL. DNA amplification was achieved using the following touchdown protocol: 3-min denaturation at 94 °C; 10 cycles of 30 s at 93 °C, 30 s at 67 °C decreasing 1 °C/cycle and 1 min at 72 °C; 18–28 cycles (depending on initial DNA template amount) of 30 s at 93 °C, 30 s at 58 °C and 1 min at 72 °C; and final extensión of 7 min at 72 °C (this work). Single PCR products were visualized in 1.5% agarose gel, and unincorporated primers and DNTPs were removed from PCR products with ExoSap (ExoSap-it, Affimetrix). Cycle sequencing reactions were conducted by a commercial company Macrogen Inc. Data from heavy and light stands were compared to generate a consensus sequence for each DNA fragment using Geneious 6.05.[33]

Phylogenetic analyses

Sequences were aligned using MAFFT v. 7,[34] with the Q-INS-i strategy. Maximum likelihood (ML) trees were estimated using GARLI 2.01 (genetic algorithm for rapid likelihood inference [35]). GARLI uses a genetic algorithm that finds the tree topology, branch lengths and model parameters that maximize lnL simultaneously.[35] 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.[35] Bootstrap support was assessed via 1000 pseudoreplicates under the same settings used in tree search. GenBank accession numbers are listed in Appendix 2.

Vocalizations

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. Audio of KU specimens were recorded by W.E. Duellman with an Uhler 4000 S and microphone onto analogue tapes. The tapes were digitized at the Macaulay Library using their standard protocols (http://macaulaylibrary.org). 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 between them by a return to the background noise. Pulsed notes are considered 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.

Call parameter definitions follow Hutter et al. [36] (Table 1), and we chose the following relevant parameters: (1) note amplitude type (tonal or pulsed); (2) call duration (ms); (3) note interval (s); (4) number of notes per call; (5) note duration (ms); (6) note rate (/ms); (7) pulse rate (/ms); (8) peak of dominant frequency (Hz); (9) dominant and/or fundamental frequency (Hz) lower and upper bounds; (10) frequency modulation (Hz); and (11) first harmonic frequency (Hz). Measures are reported as the range followed by the mean ± two standard deviations from the mean.

The parameters above were measured using the R package SEEWAVE,[37] with a custom script to quickly batch analysed calls (available upon request). The analysis of calls was automated with the following routine: (1) audio file is normalized and filtered of background noise and other non-target frog sounds (insects, stream noise, rain, etc.), using a band-pass filter set to 2000–5000 Hz (removes sound that generates frequencies outside this range); (2) target calls are located in the filtered audio file by recording the start time of each amplitude increase above a 10% threshold; (3) each identified call was vetted to ensure that it was a call of the target species and not other sounds; (4) each individual call is normalized to a relative scale, which removes the effects of amplitude variation between calls; (5) using the call start times and the original recording, each call was saved as an unfiltered file; (6) recordings were analysed separately using various functions (for the call parameters above) available in SEEWAVE; and (7) significant outliers falling outside the 95% confidence intervals of the measurement data were manually inspected to ensure accuracy. Additionally, call durations and inter-note/call intervals were measured manually as it was difficult to detect the start and end of calls due to background noise. Recordings with excessive background noise (~25% amplitude) were carefully examined using frequency spectra and pulses above the background noise.

Table 1. Abundance of *H. mashpi* n. sp. at stream La Laguna, Reserva de Biodiversidad Mashpi, Ecuador.

Individual frog code	24 February 2015	25 February 2015	26 February 2015	27 February 2015
H1	1	0	0	0
H2	1	0	1	0
H3	1	0	0	0
H4	1	0	0	1
H5	1	1	0	0
H6	1	0	0	0
H7	0	1	0	1
H8	0	1	0	0
H9	0	1	0	0
H10	0	1	1	0
H11	0	1	0	1
H12	0	1	0	0
H13	0	0	1	0
H14	0	0	1	0
H15	0	0	1	0
H16	0	0	0	1
H17	0	0	0	1

Notes: The survey was carried out during four consecutive nights in a 200-m transect along the stream. 1 = presence of the individual. 0 = absence of the individual.

Finally, we evaluated the amount of bioacoustic differences between species using several criteria. We considered call variables influenced by body size (e.g. frequencies), motivation (e.g. call rate) and/or temperature (e.g. call rate) to be inadequate when left uncorrected for these factors. We considered differences to be important when the general call structure varied (pulsed vs. tonal notes, note arrangement and call arrangement) or when temporal variables varied independent of body size, temperature and motivation.

Population size

During four consecutive nights (24–27 February 2015; rainy season), we surveyed a 200 × 4 m transect along the stream La Laguna (0.1665° N, 78.8713° W; 1015 m). Each night, the stream was sampled by two people (LB and Frank Pichardo) for four hours starting at 20:00 h. All adult frogs were marked with standard procedures (toe-clipping); sex of marked individuals was assessed by external features (e.g. coloration, body size and presence/absence of vocal sac). Located frogs were swabbed for *B. dendrobatidis* (see below) and photographed. The population size of *Hyloscirtus mashpi* n. sp. was calculated using the program Mark v. 8.0.[38] We assumed that the population was closed, meaning that the model assumes that no births, deaths, immigration or emigration occur; given that the sampling period was only four days, this is a reasonable assumption. We used the *Full Likelihood p and c* model described in Otis et al. [39].

Diagnosis of **B. dendrobatidis** (Bd)

In the field, all captured individuals were swabbed, following the procedures described in Hyatt et al. [40]. Dry swabs were stored in −4 °C until analyses. Testing for *Bd* was carried out using end-point PCR. DNA extractions were obtained with a salt precipitation method (M. Fujita, unpubl.) based on the Puregene DNA purification kit (Gentra Systems). *Bd* presence was tested using the primers Bd1a (5′-CAGTGTGCCATATGTCACG-3′) and Bd2a (5′-CATGGTTCATATCTGTCCAG-3′) and PCR protocol described by Annis et al. [41]. PCR reaction was set up to a final concentration of 3-mM $MgCl_2$, 0.2-mM dNTPs, 0.05 U/µL *Taq* DNA polymerase (Invitrogen) and 0.5-µM of each primer in a 25 µL total volume. PCR protocol followed Annis et al. [41], except that 35 cycles were performed. When the PCR product retrieved was insufficient or dubious, an additional PCR was carried out, using a 1:50 dilution of the cleaned-up product from the first PCR as the template. The conditions of this second PCR were the same as described above, but fewer cycles were performed. A negative control, containing distilled water instead of DNA, was used each time. The presence/absence of *Bd* was determined by visualization of the amplified band in agarose gel electrophoresis.

Ethics statement

Animal research was performed under the approval and supervision of the Centro de Investigación de la Biodiversidad y Cambio Climático and Universidad Tecnológica Indoamérica. Research and collection permits were issued by the Ministerio del Ambiente de Ecuador (N°14-2011-IC-FAU-DPAP-MA, N°05-2013-IC-FAU-DPAP-MA, N°01-2014-AD-RIC-FAU-DPAP-MA).

Results

Phylogenetic analyses

The inferred topology (Figure 1) generally agrees with those presented in studies with similar taxon and gene sampling,[3,6,27,28] and differences are mostly explained by more complete taxon sampling in this study (i.e. sister relationship between *H. alytolylax* and new species).

Systematics

Hyloscirtus mashpi *n sp.*

http://zoobank.org/A943A79F-FACA-4B42-991E-FE6100 71461C

Suggested common name in English: Mashpi Stream treefrog

Suggested common name in Spanish: Rana torrentícola de Mashpi

Holotype. MZUTI 3747 (Figures 2 and 3), adult male, collected at Reserva de Biodiversidad Mashpi, Riachuelo Laguna (0.1631° N, 78.8678° W; 1022 m), Pichincha province, Ecuador, on 28 August 2014 by Jaime Culebras.

Paratypes (Figure 4). MZUTI 606, 609–614, adult males obtained from Cordillera de Chontilla, headwater of Sune Chico river (0.06803° N, 78.903° W; 908 m), Pichincha province, on 4 July 2011 by Ítalo G. Tapia. MZUTI 3096–98, adult males from Milpe (0.0324° N, 78.866° W; 1120 m), Pichincha province, Ecuador, on 10 April 2013 by Jaime Culebras and Alejandro Arteaga. MZUTI 3748, 3762–63, adult males, MZUTI 3760–61, adult females, obtained from Riachuelo Laguna (0.1631° N, 78.8678° W; 1022 m), Reserva de Biodiversidad Mashpi, Pichincha province, Ecuador, on 28–31 August 2014 by Jaime Culebras, Carlos Morochz and Juan M. Guayasamin.

Diagnosis. *Hyloscritus mashpi* n. sp. is characterized by the following combination of characters: (1) adult males
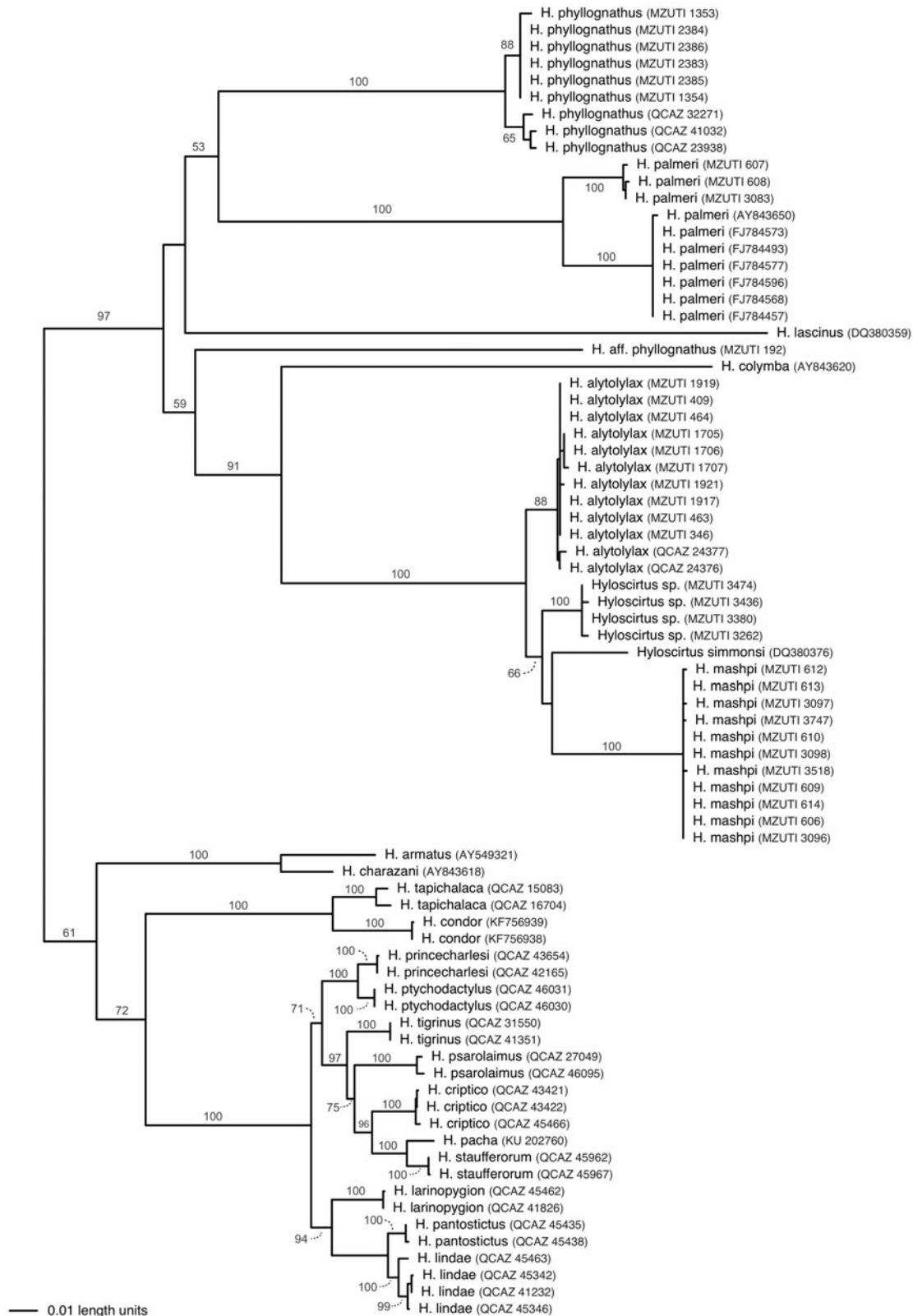
Figure 1.   Evolutionary relationships of species in the genus *Hyloscirtus* inferred with a ML criterion. Bootstrap supports are shown only for notes with values >50.

Figure 2.   *Hyloscirtus mashpi* n. sp. in life. Holotype, adult male, MZUTI 3747.

small   (SVL   28.7–33.8 mm,   mean = 31.5 ± 1.385, *n* = 14); females 37.0–38.5 (*n* = 2); (2) body relatively slender; (3) snout rounded in dorsal and lateral views; (4) in life, dorsum of males usually pale yellowish-green with brown mid-dorsal stripe; females with brown

dorsum with darker brown mid-dorsal stripe; (5) axillar and inguinal regions light yellowish-green; (6) mental gland in males present, mostly unpigmented; (7) upper lip without white stripe; (8) white parietal peritoneum; (9) iris brown to copper brown with thin black reticulation; (11) nuptial pad absent; (13) tympanum rounded, pigmented as surrounding skin; tympanic annulus only visible in females; (14) pale supratympanic and canthal stripes present; brown interorbital stripe present in most, but not all individuals; (15) ulnar fold and tarsal stripe cream-white; (16) calcar tubercle absent; (17) supracloacal fold low, with few iridophores, more conspicuous in males than in females; (18) low tubercles scattered around and below cloaca; (19) white bones in life; (20) elliptical prepollex, not modified as a projecting spine; (21) dentigerous processes of vomers, prominent, slightly curved, with discernible gap, with 10–14 teeth each; and (22) advertisement call with 2–3 notes, a call duration of 330.9–380.2 ms, and dominant frequency at 2842–2929 Hz.

Comparison with similar species. Only three species of the *H. bogotensis* group are known to occur on the Pacific lowlands and/or Andean Pacific slopes of Colombia and/or Ecuador; these species are: *H. alytolylax*, *H. palmeri* and *H. simmonsi*.

Individuals of *H. mashpi* n. sp. are almost identical to *H. alytolylax* and only two morphological traits are useful (although partially overlapping) to differentiate the two taxa; *H. mashpi* n. sp. has a smaller body size (*H. masphi* male SVL 28.7–33.8 mm; *H. alytolylax* male SVL 32.6–36.4 mm; Student's-*t* test, *p* < 0.001), and a dorsum that usually shows a conspicuous mid-dorsal stripe (stripe usually absent in *H. alytolylax*). The two species are readily differentiated in terms of vocalizations; *H. mashpi* n. sp. has a short call (331–380 ms, mean = 353 ± 11.16),

Figure 3.   Holotype of *Hyloscirtus mashpi* n. sp. in preservation, MZUTI 3747.

Figure 4.   Colour variation in ethanol of *Hyloscirtus mashpi* n. sp. *Top row:* MZUTI 613 (left) and MZUTI 611 (right). *Bottom row:* MZUTI 612 (left) and MZUTI 3096 (right).

with only 2 or 3 notes; in contrast, *H. alytolylax* has a longer call (264–811 ms, mean = 529 ± 187), with 5–8 notes (Table 2). Additionally, the two species seem to have allopatric distributions, with *H. mashpi* n. sp. being found at lower elevations (778–1279 m) than *H. alytolylax* (1510–1858 m). We acknowledge that an extensive revision of the latter is needed to determine if the distributional pattern observed in Ecuador is sustained.

H. mashpi n. sp. can be distinguished from *H. palmeri* (characters of the latter in parenthesis) mainly by having a noticeably smaller, non-overlapping body size (male SVL 34.9–41.4 mm, female SVL 35.7–50.0), mid-dorsal stripe usually present (absent), foot webbing pale yellowish-green (orange) and by lacking a heel calcar (present; Figure 5). In Ecuador, *H. mashpi* n. sp. and

H. palmeri have overlapping elevational ranges, and have been found in sympatry in one locality (Milpe).

Finally, when comparing *H. mashpi* n. sp. to its sister species (*H. simmonsi*; Figure 6C), *H. simmonsi* differs by having four notes in all recorded calls and longer, non-overlapping call duration than *H. mashpi* n. sp. (Table 2). Also, *H. simmonsi* is a larger species, with a male SVL of 35.0–37.0 mm, and is only known from the Pacific flank of Andes in Colombia at elevations of 1100–2000 m.[9]

Description of holotype. An adult male of 32.7 mm SVL. Body relatively slender. Head slightly longer than wide (Head length = 35% of SVL; Head width = 34% of SVL). Snout rounded in dorsal view and profile; *canthus rostralis* distinct, slightly concave; lips rounded, not

Table 2. Comparisons of call data recorded for *Hyloscirtus mashpi*, *H. alytolylax* and *H. simmonsi*.

Parameter	H. mashpi	H. alytolylax	H. simmonsi
Reference numbers	MZUTI 606, 1030–32	MZUTI 463–64, WED 818, 823, KU 164257, 164258, and 2 uncollected individuals	KU 169556 (paratype)
n individuals	4	9	1
n calls	83	105	12
n notes	162	1013	48
Notes/call	2–3	5–8	4
Note rate within call (notes/s)	0.177–0.496 (0.359 ± 0.983)	0.554–3.65 (1.01 ± 0.992)	0.101–0.169 (0.128 ± 0.656)
Note interval (ms)	115–162 (139 ± 13)	5–84 (26 ± 24)	103–125 (113 ± 19)
Call duration (ms)	330.9–380.2 (353 ± 11.155)	263.5–811.4 (529.2 ± 186.639)	515–542 (517 ± 13)
Peak of dominant frequency (Hz)	2842–2929 (2907 ± 37)	2239–3188 (2728 ± 248)	3000
Upper bound of dominant frequency (Hz)	3024–3065 (3043 ± 10)	2650–3697 (3085 ± 271)	3375
Lower bound of dominant frequency (Hz)	2721–2773 (2746 ± 13)	2375–2942 (2651 ± 138)	2625
First harmonic (Hz)	6091–6147 (6115 ± 15)	5933–6521 (6235 ± 165)	–

Notes: Calls were recorded from males calling at night. The dominant frequency falls within the fundamental frequency, and thus the range is reported as 'Upper' and 'Lower' frequency bounds. Data are the range and then the mean ± two standard deviations in parentheses. Statistics are summarized from pooling all of the calls from a given species.

flared. Pale canthal stripe present. Nostrils not protuberant, directed anterolaterally at the level of the anterior margin of lower jaw. Internarial region and top of head flat. Interorbital distance longer than upper eyelid. Eye prominent, its diameter about 11% of SVL. Tympanum visible, tympanic annulus inconspicuous, rounded and subtly discernible; its diameter is about 4% of SVL. Supratympanic fold developed, starting at posterior end of upper eyelid and reaching posterior margin of insertion of arm; supratympanic stripe present, lighter than the rest of the body. Mental gland present, diamond shaped, partially covering gular area and extending about half the length of throat.

Dentigerous processes of vomers conspicuous, straight, narrowly separated from each other; each process bears 11 (right) and 12 (left) teeth. Choanae large, elliptical, not concealed by palatal shelf of maxillary arch. Tongue cordiform, attached overall (narrowly free around lateral and posterior margin); vocal slits present, longitudinal, originating on sides of tongue and extending to posterolateral corner of mouth. Vocal sac moderately distensible, evident externally, single, median and subgular. Forearm moderately robust; axillary membrane absent. Outer ulnar fold present. Fingers relatively short, thick, bearing small, ovoid discs; each disc only slightly expands laterally, and with clearly defined circumferential groove; disc on finger III about same width as tympanum diameter. Relative lengths of fingers I < II < IV < III. Fingers with fleshy dermal fringes; webbing present only between outer fingers; webbing formula II 2^-–3 III $2^{1/2}$–2^- IV. Subarticular distal

tubercles large and elliptical. Supernumerary tubercles present, fleshy and small. Palmar tubercle poorly differentiated. Inner metacarpal tubercle large, thick, elliptical; broad elliptical prepollex, not modified as a spine. Nuptial excrescencies absent.

Hind limbs moderately robust; tibia length 51% SVL; foot length 44% SVL. Outer tarsal fold and calcar tubercle absent, but pale tarsal stripe is evident; inner tarsal fold indistinct. Toes relatively short, with thin lateral fringes, bearing discs slightly smaller than those on fingers. Relative length of toes I < II < V < III < IV; extensive toe webbing, formula: I 1–$1^{1/2}$ II $1^{1/3}$–$1^{1/2}$ III 1^+–$1^{2/3}$ IV 2–1V. Inner metatarsal tubercle elongate, elliptical, flat; subarticular tubercles small, round; outer metatarsal tubercle absent; supernumerary tubercles not distinctive. Cloacal opening directed posteroventrally at mid-level of thighs; supracloacal fold present; sheath short; low and small tubercles scattered around and below cloaca. Dorsal skin, gular and pectoral regions flanks and venter smooth. White parietal peritoneum present, covering all ventral areas of belly.

Colour in life of the holotype. Dorsum pale yellowish-green, with dark brown interorbital and mid-dorsal stripes. Yellowish-white line on canthus, external border of upper eyelid and supratympanic fold. Venter whitish-cream. Iris brown with thin black reticulation (Figure 2).

Colour in preservative of the holotype. Head cream with small brown blotch at tip of the snout and irregular dark brown interorbital stripe; whitish supratympanic fold that matches the coloration of the external border of upper

eyelid. Dorsum cream with a clearly defined dark brown mid-dorsal stripe and irregular patches on dorsolateral areas. Forelimbs and hindlimbs cream with dark brown blotches. Fingers and toes, cream, except for small brown melanophores on finger IV and toe V. Gular and ventral surfaces creamy white. Ulnar and supracloacal folds and ventrolateral border of tarsus with faint whitish coloration (Figure 3).

Measurements of the holotype (in mm). SVL 32.7; Tibia length 16.8; Foot length 14.3; Head length 11.4; Head width 11.0; Snout-to-eye distance 4.7; Interorbital
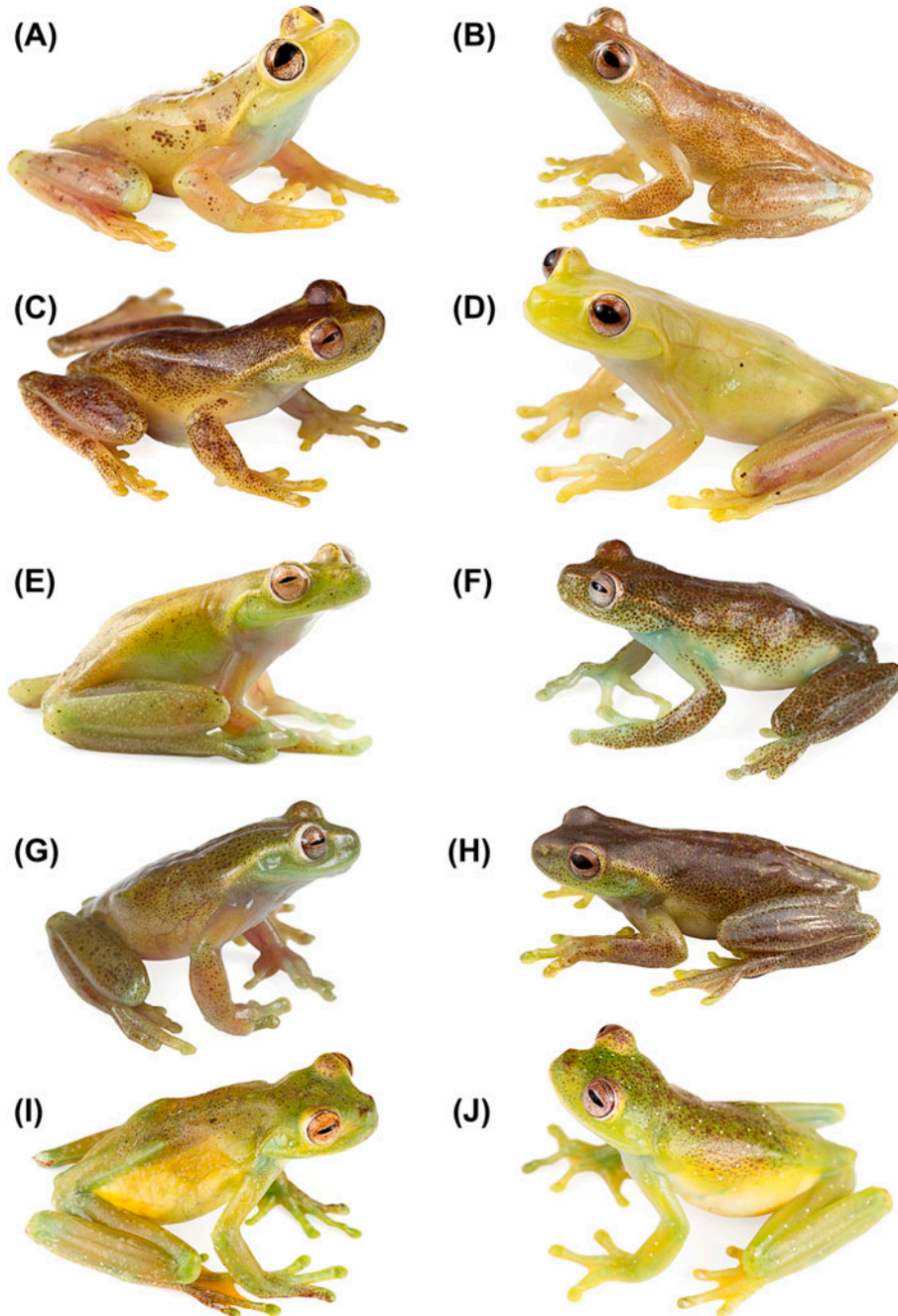

Figure 5. *Hyloscirtus* species, in life, from Ecuador. (A) *H. mashpi* n. sp., MZUTI 3097, from Milpe. (B) *H. mashpi* n. sp., MZUTI 3518, from Reserva de Biodiversidad Mashpi. (C) *H. mashpi* n. sp., MZUTI 3096, from Reserva de Biodiversidad Mashpi. (D) *H. mashpi* n. sp., not collected, from Reserva de Biodiversidad Mashpi. (E and F) *H. alytolylax*, not collected, from Mindo. (G) *H. alytolylax*, MZUTI 1921, from San Francisco de las Pampas. (H) *Hyloscirtus* sp., MZUTI 3262, from Reserva Buenaventura. (I) *H. palmeri*, not collected, from Milpe. (J) *H. phyllognathus*, not collected, from Maycu.

distance 3.7; Upper eyelid width 2.9; Eye diameter 3.7; Tympanum 1.3; Radioulna length 5.7; Hand length 9.8; Finger I length 7.0; and Finger II length 7.7.

Colour variation. The two observed females have a brown dorsal coloration, with a darker mid-dorsal stripe (Figure 7). In males, dorsal coloration varies from pale green with a faint brown mid-dorsal stripe to brown with a darker mid-dorsal stripe (Figures 5 and 7). In few individuals, the mid-dorsal stripe is nearly indistinguishable in life. All observed metamorphs and juveniles have a dark brown mid-dorsal stripe (Figure 7). Under stress conditions (e.g. handling), dorsal surfaces of individuals turn darker (Figure 5(C)).

Variation and sexual dimorphism. Females are larger than males (male SVL 28.7–33.8; female SVL 37.0–38.5).

Hand webbing variation is as follows: II $(2–2^-) − (3–3^-)$ III $(2^+–2^{1/2}) − (2^-–2^{1/3})$ IV. Foot webbing variation is as follows: I $(1–1^+) − (1^{1/2}–2^-)$ II $(1–1^{1/3}) − (1^-–1^{1/2})$ III $(1^+–1^{1/4}) − (1^{2/3}–2^-)$ IV $(2^-–2)–(1^-–1)$ V. Secondary sexual characters (mental gland, vocal slits and vocal sac) in females are absent.

Distribution. H. *mashpi* n. sp. is currently known only from localities on the western slopes of the Ecuadorian Andes, Pichincha province, at elevations between 778 and 1279 m (Figure 8). The localities are: Milpe (0.0324° N, 78.8660° W; 1120 m), Río Sune Chico (0.0680° N, 77.3973° W; 908 m) and Reserva de Biodiversidad Mashpi. Within Reserva de Biodiversidad Mashpi, the species has been found at the following localities: (i) Riachuelo Laguna (0.1631° N, 78.8678° W; 1022 m; Figure 9), (ii) Stream A (0.16243° N, 78.88125° W;
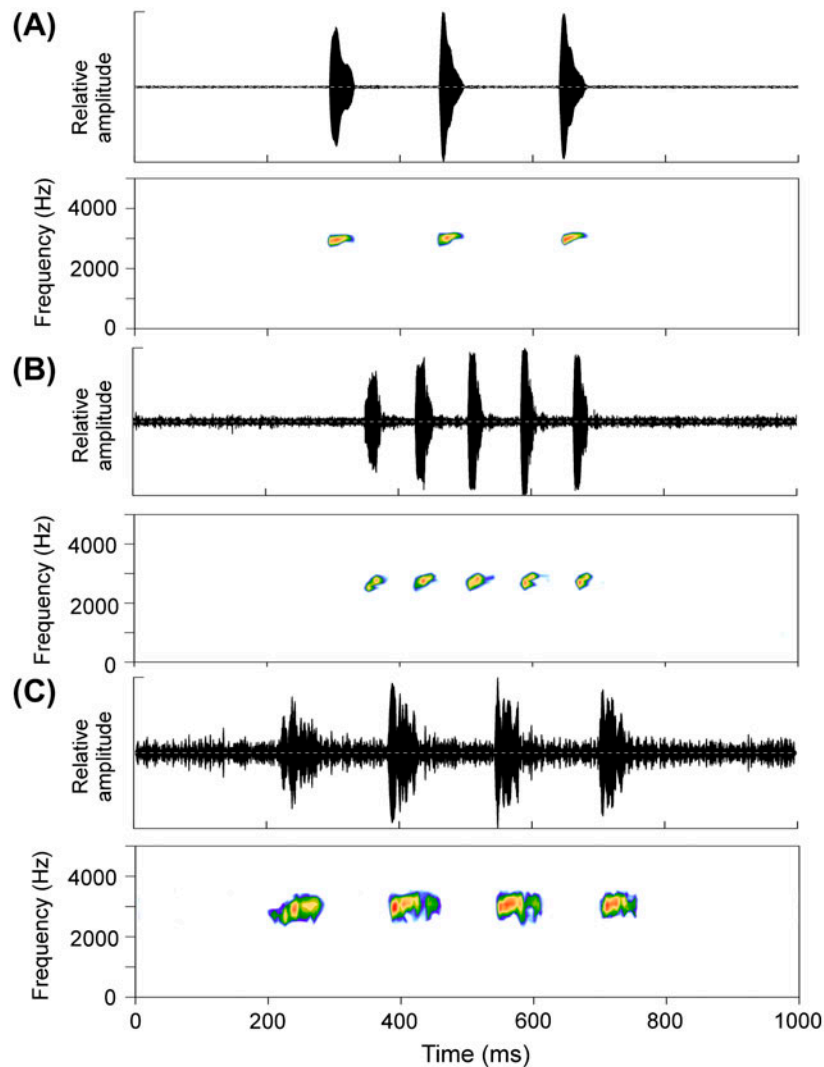

Figure 6. Oscillogram and spectrograms for (A) *Hyloscirtus mashpi* n. sp. (MZUTI 606), (B) *H. alytolylax* (MZUTI 463) and (C) *H. simmonsi* (KU 169556).
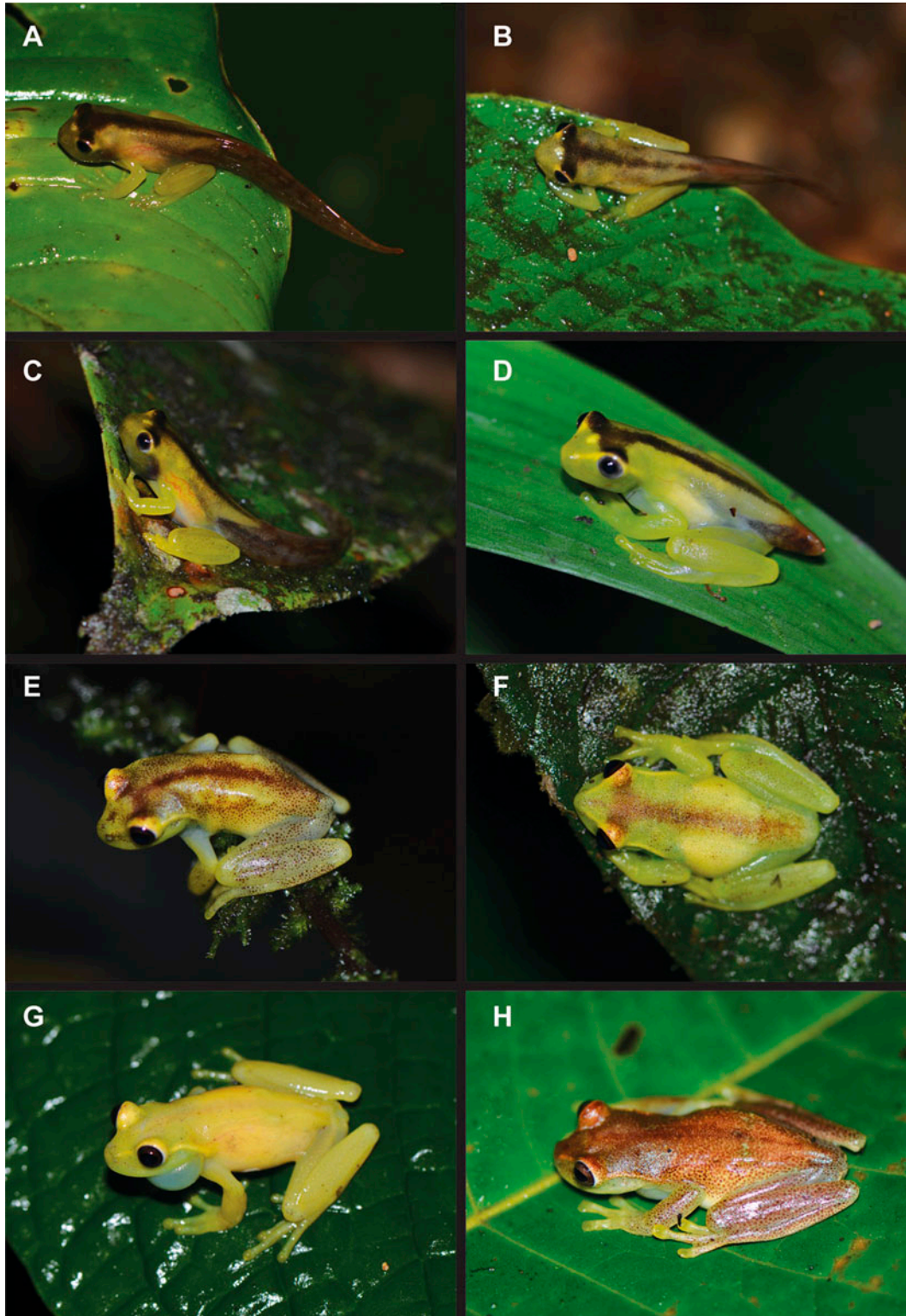
Figure 7.   Colour variation in life of *Hyloscirtus mashpi* n. sp. at Reserva de Biodiversidad Mashpi, Ecuador. (A–D) Metamorphs. (E–G) Adult males. (H) Adult female.
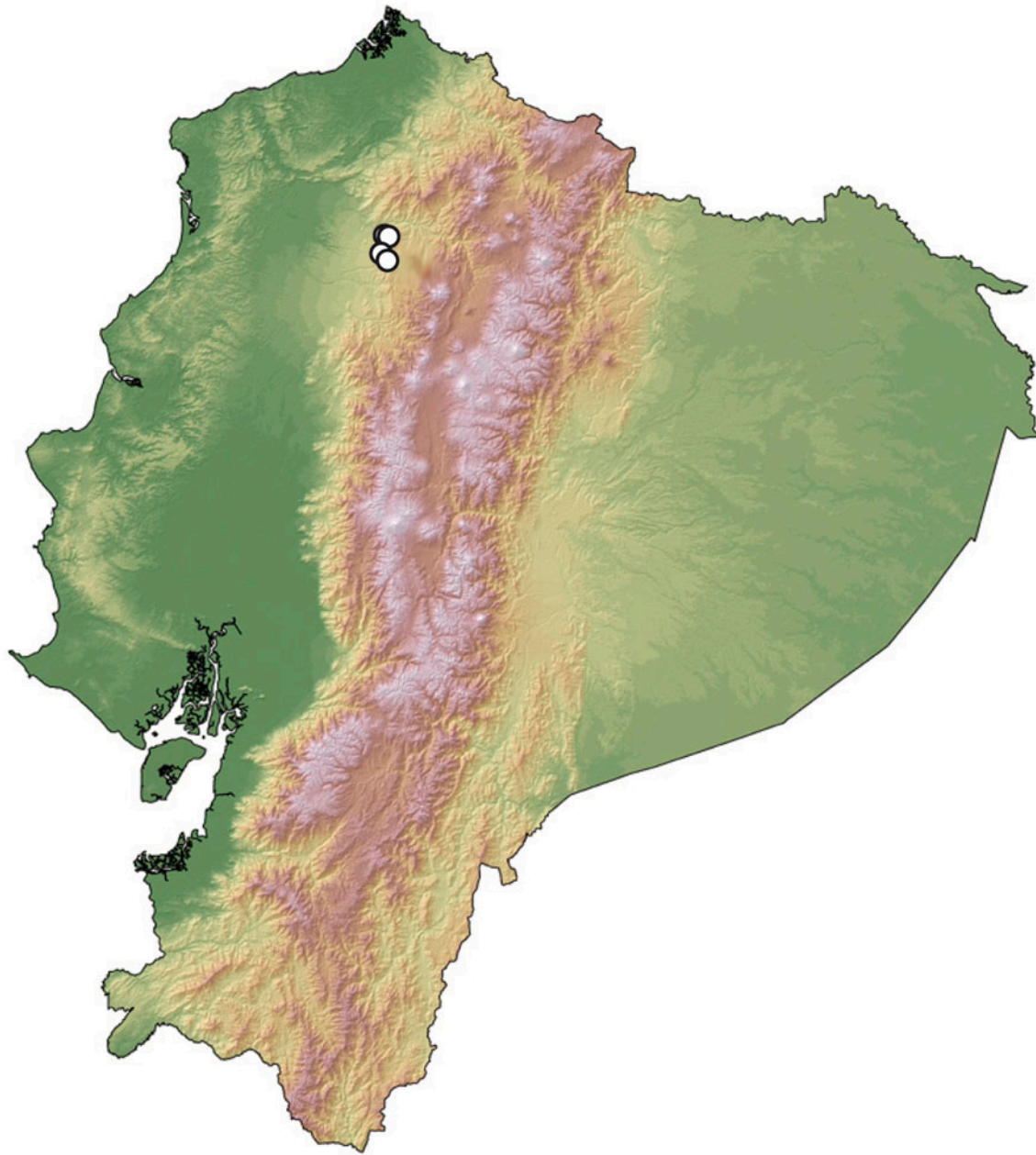
Figure 8. Distribution (white circles) of *Hyloscirtus mashpi* n. sp. in Ecuador.

778 m), Stream B (0.1572° N, 78.8624° W; 1176 m) and (iii) Stream C (0.1607° N, 78.8561° W; 1279 m).

Natural history. H. mashpi n. sp. is a nocturnal species restricted to riverine vegetation (Figure 9) in primary evergreen foothill forests. In this ecosystem, *H. mashpi* n. sp. perches on leaves and branches 30–400 cm above ground/stream level, and is active under a variety of climatic conditions. At Reserva de Biodiversidad Mashpi (Riachuelo Laguna), it is the most abundant stream amphibian; the sampled transect (300 × 4 m) along the

stream *Laguna* has an estimated population size of 68 individuals (Table 1). Abundance is patchy and can be as high as four adults per square meter; frogs have been found either hidden between leaves or exposed. The species seems to reproduce opportunistically during the whole year. At Riachuelo Laguna, tadpoles are abundant in flat areas with water accumulation, where they forage at the rocky bottom. At Reserva de Biodiversidad Mashpi, sympatric species include: (i) Riachuelo Laguna: *Espadarana prosoblepon*, *Hypsiboas picturatus*, *Pristimantis achatinus*, *P. labiosus*, *P. latidiscus* and

Figure 9. Habitat of *Hyloscirtus mashpi* n. sp. type locality (Riachuelo Laguna) at Reserva de Biodiversidad Mashpi, Ecuador. Tadpoles and metamorphs are usually found in areas with water accumulation (left). Reproductive adults are found on vegetation along fast-flowing streams (right).

P. luteolateralis; (ii) Stream A: *P. achatinus*, *P. labiosus*, *P. luteorateralis*, *P. latidiscus*, *P. muricatus*, *P. subsigillatus*, *E. prosoblepon*, *Sachatamia orejuela* and *Teratohyla spinosa; and* (iii) Stream C: *P. achatinus*, *P. crucifer*, *P. labiosus*, *P. mindo*, *P. luteolateralis*, *E. prosoblepon* and *Hyalinobatrachium valerioi*. At Río Sune Chico, sympatric species include: *E. prosoblepon*, *H. palmeri*, *H. picturatus*, *P. achatinus*, *P. labiosus*, *P. luteolateralis* and *S. orejuela*.

Vocalizations. We recorded 83 calls from four individuals of *H. mashpi* n. sp. (Table 2). The call of this species sounds like a rapid set of rings to the ear. *Hyloscirtus mashpi* n. sp. emits calls with two or three notes per call (Figure 7A). The call duration is 330.9–380.2 ms (353 ± 11.2), with a note interval of 115–162 (139 ± 13) ms. The fundamental frequency corresponds with the dominant frequency, which has a lower bound at 2721–2773 (2746 ± 13) Hz and an upper bound at 3024–3065 (3043 ± 10) Hz. The dominant frequency at peak amplitude is 2842–2929 (2907 ± 37) Hz, with no frequency modulation throughout the call. The first harmonic is 6091–6147 (6115 ± 15) Hz. See Table 2.

Conservation. The main threats to amphibian diversity are habitat destruction, climate change, infectious diseases and introduced species.[54] The known distribution of *H. mashpi* n. sp. lies within three protected areas, Reserva de Biodiversidad Mashpi, Área de Conservación y Uso Sustentable Mashpi–Guaycuyacu-Saguangal (ACUS) and Reserva Milpe. Thus, the habitat of the species at type localities is mostly protected. Also, as mentioned above, *H. mashpi* n. sp. is an abundant species with seemingly healthy populations, including reproductive adults, juveniles and tadpoles. We assessed the presence of the chytrid fungus *B. dendrobatidis* (*Bd*) in 34 adults of *H. mashpi* n. sp., 6 of which tested positive for the fungus. This means that the prevalence of *Bd* in this sample is 17.6%. None of the frogs that tested positive for the chytrid showed any obvious sign of sickness; on the contrary, all individuals were active and calling when found. Given the characteristics of the populations of *H. mashpi* n. sp., we speculate that this species is tolerant to *Bd*, as observed in other species.[42–44] We suggest that the most appropriate IUCN conservation category for *H. mashpi* n. sp. is Data Deficient, mainly because assessment of the distribution of *H. mashpi* n. sp. still

requires an extensive re-examination of collections of *H. alytolylax*.

Etymology. The specific epithet *mashpi* is used as a noun in apposition and refers to one of the localities where the species is found, Reserva de Biodiversidad Mashpi, a protected area where research and conservation efforts are carried out by Reserva de Biodiversidad Mashpi S.A., Universidad Tecnológica Indoamérica, Tropical Herping and other institutions. The word *mashpi* is a Yumbo word that means 'friend of water', which is a precise description of this treefrog, which is always found along pristine streams.

Discussion

Given that our study has a similar taxon and gene sampling than previous ones, it is not surprising that the recovered phylogenies are mostly congruent. As shown before,[3,4,6,7,27,28] the genus *Hyloscirtus* is divided into three clades (*H. armatus* group, *H. bogotensis* group and *H. larinopygion* group). The two species of the *H. armatus* group (*H. armatus* and *Hyloscirtus charazani*) form a well-supported clade that is sister to the species in the *H. larinopygion* group, although this relationship has weak support (bootstrap = 61). The close relationship between the *H. armatus* and *H. larinopygion* groups has been recovered in recent studies by Faivovich et al. [3] using parsimony, and Coloma et al. [28] using both parsimony and Bayesian criteria, although Bayesian support values in the later study were not significant. ML analysis by Coloma et al. [28] suggests, with low support, that the *H. armatus* group is sister to the *H. bogotensis* group. Thus, at the moment, it is premature to conclude about the relationships among the three groups in *Hyloscirtus*, mainly because most of the analyses show non-significant support values at this level and, as described above, even topological contradictions (e.g. Coloma et al. [28]: Figure 2). In contrast, the monophyly of each of these groups (i.e. *armatus*, *bogotensis* and *larinopygion*) has been recovered in all recent analyses.[3, 5, 28, this study]

Within the *Hyloscirtus bogotensis* group, genetic data support the existence of at least two cryptic species in what is currently recognized as *H. alytolylax*, as suggested by Arteaga et al. [20]. The undescribed species are: *Hyloscirtus* sp., from South-Western Ecuador (El Oro province), and *H. mashpi* n. sp., described above. We have decided not to describe the species from El Oro province until more data (such as specimens and calls) are obtained. Other noteworthy result includes the genetic separation between the populations of *H. phyllognathus* from Northern and Southern Ecuador and the existence of a potential new species (MZUTI 192, Napo province, Ecuador) that morphologically resembles *H. phyllognathus*. Within *H. palmeri*, the reciprocal

monophyly and genetic distance (uncorrected *p* distance = 5.7–6.3%) between Panamanian and Ecuadorian populations is not surprising since we lack sampling from Colombia, and thus the effect of isolation by distance is expected.

The diversity of neotropical frogs, particularly Andean groups such as *Hyloscirtus*, has been drastically underestimated, as revealed by the number of recent descriptions of new species.[28, 29, this work] This underestimation of diversity highlights the continued need for field expeditions to under-studied areas and habitats such as Andean torrents.[9] Additionally, the cryptic nature of the *Hyloscirtus* group emphasizes the necessity of detailed comparisons in morphologically and ecologically conserved groups that may harbour significant cryptic diversity. For organisms such as frogs, this will often require data from molecular markers, external morphology and advertisement calls to establish the presence of distinct species boundaries.[23,28] These data may also be useful in future comparative analyses to determine the influence of traits like advertisement call to speciation via sexual selection.

Speciation process in *Hyloscirtus* seems to be influenced mainly by a combination of the following factors:

(1) Linearity of the Andes [45]: the distribution patterns of three closely related species distributed on the western slopes of the Andes (*Hyloscirtus* sp., Southern Ecuador; *H. mashpi* n. sp., Northern Ecuador; and *H. simmonsi*, Central Colombia) are best explained by the presence of dispersal barriers transversal to the western slopes of the Andes, including the Jubones and the Guayllabamba river valleys.[46] On the Amazonian slopes of the Andes, a similar phenomenon explains the cladogenesis between *Hyloscirtus tapichalaca* and *Hyloscirtus condor*, where the Nangaritza, Zamora and Santiago river valleys separate the Cordillera del Cóndor (from which *H. condor* is endemic) from the main Andean mountain range, where *H. tapichalaca* is found.

(2) Elevational gradients [47]: it is evident that elevational gradients have provided opportunities for colonization and diversification in *Hyloscirtus*. The genus includes closely related species that have markedly different elevational ranges; for example, the two only members of the *H. armatus* group are found at distinct elevations (*H. charazani*: 1700–2400 m; *H. armatus*: 1700–2400 m). Similarly, *H. alytolylax* and *H. simmonsi* inhabit forests at higher elevations than their close relatives *H. mashpi* n. sp. and *Hyloscirtus* sp. Although recent studies suggest that elevational shifts are relatively rare in Andean frogs (i.e. glassfrogs), it is evident that

some species do colonize new environments where they eventually radiate.[48,49]

(3) Uplift of the Andes: with the current taxon and gene sampling, the uplift of the Andes is the most likely a vicariant event that separated the sister species *H. palmeri* and *H. phyllognathus.*

Finally, our data indicate that *Hyloscirtus mashpi* n. sp. is *Bd* positive, but maintains apparently healthy and robust populations at numerous sites. This suggests that *H. mashpi* n. sp. may exhibit total or partial tolerance to *Bd*, as do other amphibian species.[42,43,50,51] Within *Hyloscirtus*, *Bd* has been diagnosed in few additional species [44,52,53]; however, further studies are needed to determine the impact, if any, of the infection on population dynamics. In any case, monitoring of *H. mashpi* n. sp. is in place, and our preliminary results so far indicate that the populations are stable. This is heartening, as *Hyloscirtus* are generally considered rare and elusive species,[28] and would otherwise have been thought strongly susceptible to the general decline and extinction threatening other amphibians worldwide.[54–56]

Author contributions

JMG conceived and designed the study. JMG wrote the first draft of the manuscript. MR, AA, JC, LB, RAP, NP, CM and CRH reviewed and improved the manuscript. JMG, NP and CRH analysed the data. JMG, MR, AA, JC, LB, RAP, NP and CM collected specimens, calls and swabs.

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Appendix 1. Specimens examined

Hyloscirtus alytolylax

Ecuador

Provincia Pichincha: Mindo, Yellow House (0.04436° S, 78.753° W, 1510 m), MZUTI 346, 546. Mindo, Sachatamia Lodge (0.02336° S, 78.759° W, 1679 m), MZUTI 1394. Mindo, Séptimo Paraíso (0.02862° S, 78.7659° W, 1520 m), MZUTI 2195, 3177. Reserva Las Gralarias, Lucy's Creek (0.004° S, 78.740° W, 1850 m), MZUTI 464. Tandapi, creek along road to gazebo (0.4229° S, 78.796° W, 1550 m), MZUTI 1917–18. *Provincia Imbabura:* Reserva Los Cedros (0.2193° N, 77.475° W, 1620 m), MZUTI 1705–07. *Provincia Cotopaxi:* Creek near San Francisco de las Pampas–Peñas Coloradas road (0.4526° S, 78.981° W, 1765 m), MZUTI 1921, 1923.

Hyloscirtus palmeri

Ecuador

Provincia Pichincha: Headwaters of Río Sune Chico (0.068° N, 78.903° W, 908 m), MZUTI 607–08. Río Sune Chico (0.133° N, 78.903° W, 686 m), MZUTI 666, 681. Milpe (0.0349° N, 78.867° W; 1055 m), MZUTI 3750.

Hyloscirtus simmonsi

Colombia

Departamento del Cauca: El Tambo, Fundación Proselva, Hacienda El Tambito, ICN 32842; Puracé, Parque Natural Nacional Munchique, 46 km al NNW de La Uribe, ICN 25906. *Departamento del Valle:* Dagua, Cabeceras de La Quebrada La Seca, ICN 41304; Río Calima, 1.5 km (by road) W of Calima (4.00° N, 76.583° W; 1230 m), ICN 25906 (Holotype). *Departamento Valle del Cauca:* Municipio Frontino, Corregimiento Bitaco, ICN 33331–32. *Departamento Chocó:* ICN 41303, 41307.

Appendix 2.

Table A2. Taxon and gene sampling of species included in this study, with their corresponding Genbank numbers.

Species	Museum number	Locality	Genbank number	Source
Hyloscirtus alytolylax	MZUTI 346	Ecuador: Pichincha: Mindo, Yellow House trail (0.04436° S, 78.7536° W; 1510 m)	KT279509, KT279540	This study
Hyloscirtus alytolylax	MZUTI 409	Ecuador: Pichincha: Reserva Las Gralarias, Lucy´s Creek (0.004° S, 78.740° W; 1822–1858 m)	KT279504, KT279543	This study
Hyloscirtus alytolylax	MZUTI 463	Ecuador: Pichincha: Reserva Las Gralarias, Lucy´s Creek (0.004° S, 78.740° W; 1822–1858 m)	KT279505, KT279542	This study
Hyloscirtus alytolylax	MZUTI 464	Ecuador: Pichincha: Reserva Las Gralarias, Lucy´s Creek (0.004° S, 78.740° W; 1822–1858 m)	KT279541	This study
Hyloscirtus alytolylax	MZUTI 1705	Ecuador: Imbabura: Reserva Los Cedros (0.2193° N, 77.475° W; 1620 m)	KT279507, KT279537	This study
Hyloscirtus alytolylax	MZUTI 1706	Ecuador: Imbabura: Reserva Los Cedros (0.2193° N, 77.475° W; 1620 m)	KT279508, KT279538	This study
Hyloscirtus alytolylax	MZUTI 1707	Ecuador: Imbabura: Reserva Los Cedros (0.2193° N, 77.475° W; 1620 m)	KT279506, KT279536	This study
Hyloscirtus alytolylax	MZUTI 1917	Ecuador: Pichincha: Manuel Cornejo Astorga (Tandapi): Creek nearby town (0.4229° S, 78.7961° W; 1550 m)	KT279535	This study
Hyloscirtus alytolylax	MZUTI 1919	Ecuador: Pichincha: Manuel Cornejo Astorga (Tandapi): Creek nearby town (0.4229° S, 78.7961° W; 1550 m)	KT279539	This study
Hyloscirtus alytolylax	MZUTI 1921	Ecuador: Cotopaxi: Creek near the San Francisco de las Pampas–Peñas Coloradas road (0.4526° S, 78.9806° W; 1765 m)	KT279534	This study
Hyloscirtus alytolylax	QCAZ 24377	Ecuador: Cotopaxi: ca. San Francisco de las Pampas, 1760 m	JX155798 JX155825	Coloma et al. [28]
Hyloscirtus alytolylax	QCAZ 24376	Ecuador: Cotopaxi: ca. San Francisco de las Pampas, 1760 m	X155799 JX155826	Coloma et al. [28]
Hyloscirtus armatus	AMNH 1651632	Bolivia: Santacruz: Caballero: Cantón San Juan: Parque Nacional Amboró	AY549321	Faivovich et al. [3]
Hyloscirtus charazani	AMNH-A165132	Bolivia: La Paz, Bautista Saavedra, Canton Charazani, Stream 2	AY843618	Faivovich et al. [3]
Hyloscirtus colymba	SIUC H-7079	Panama: Cocle, Parque Nacional El Cope	AY843620	Faivovich et al. [3]
Hyloscirtus condor	MEPN 14758	Ecuador: Cordillera del Cóndor, Reserva Biológica Cerro Plateado, 2317	KF756938	Almendáriz et al. [29]
Hyloscirtus condor	MEPN 14754	Ecuador: Cordillera del Cóndor, Reserva Biológica Cerro Plateado, 2317	KF756939	Almendáriz et al. [29]
Hyloscirtus criptico	QCAZ 43421	Ecuador: Imbabura: ca. Cuellaje, 2560 m	JX155812 JX155839	Coloma et al. [28]
Hyloscirtus criptico	QCAZ 43422	Ecuador: Imbabura: ca. Cuellaje, 2560 m	JX155814 JX155841	Coloma et al. [28]
Hyloscirtus criptico	QCAZ 45466	Ecuador: Carchi: Road Tulcán-Maldonado. Quebrada Centella, 2806 m	JX155813 JX155840	Coloma et al. [28]
Hyloscirtus larinopygion	QCAZ 41826	Ecuador: Carchi: Morán, 2452 m	JX155817 JX155844	Coloma et al. [28]
Hyloscirtus larinopygion	QCAZ 45462	Ecuador: Carchi: Road Tulcán-Maldonado. Quebrada Centella, 2806 m	JX155818 JX155845	Coloma et al. [28]
Hyloscirtus lindae	QCAZ 41232	Ecuador: Napo: Parque Nacional Sumaco, Sumaco Lake, 2479 m	JX155821 JX155848	Coloma et al. [28]
Hyloscirtus lindae	QCAZ 45346	Ecuador: Napo: 11–12 km E Papallacta, 2600 m	JX155822 JX155849	Coloma et al. [28]
Hyloscirtus lindae	QCAZ 45463	Ecuador: Sucumbíos: ca. Santa Bárbara, 2341 m	JX155823 JX155850	Coloma et al. [28]
Hyloscirtus lindae	QCAZ 45342	Ecuador: Napo: 11–12 km E Papallacta, 2700 m	JX155824 JX155851	Coloma et al. [28]
Hyloscirtus mashpi	MZUTI 606	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.0680° N, 77.397° W; 908 m)	KT279533	This study
Hyloscirtus mashpi	MZUTI 609	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.0680° N, 77.397° W; 908 m)	KT279527	This study
Hyloscirtus mashpi	MZUTI 610	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.0680° N, 77.397° W; 908 m)	KT279510, KT279530	This study
Hyloscirtus mashpi	MZUTI 612	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.0680° N, 77.397° W; 908 m)	KT279514, KT279529	This study
Hyloscirtus mashpi	MZUTI 613	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.0680° N, 77.397° W; 908 m)	KT279523	This study

(*Continued*)

Table A2.   (*Continued*).

Species	Museum number	Locality	Genbank number	Source
Hyloscirtus mashpi	MZUTI 614	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.0680° N, 77.397° W; 908 m)	KT279526, KT279511	This study
Hyloscirtus mashpi	MZUTI 3747	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.1631° N, 78.8678° W; 1022 m)	KT279512, KT279532	This study
Hyloscirtus mashpi	MZUTI 3096	Ecuador: Pichincha: Milpe (0.03237° N, 78.86597° W; 1120 m)	KT279528	This study
Hyloscirtus mashpi	MZUTI 3098	Ecuador: Pichincha: Milpe (0.03237° N, 78.86597° W; 1120 m)	KT279531	This study
Hyloscirtus mashpi	MZUTI 3518	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.1631° N, 78.8678° W; 1022 m)	KT279525	This study
Hyloscirtus mashpi	MZUTI 3097	Ecuador: Pichincha: Milpe (0.03237° N, 78.86597° W; 1120 m)	KT279513, KT279524	This study
Hyloscirtus pacha	KU 202760	Ecuador: Azuay 2.0 km SSE Palmas, 2340 m	AY326057	Darst and Cannatella [31]
Hyloscirtus palmeri	MZUTI 607	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.0680° N, 77.397° W; 908 m)	KT279550	This study
Hyloscirtus palmeri	MZUTI 608	Ecuador: Pichincha: Reserva de Biodiversidad Mashpi (0.0680° N, 77.397° W; 908 m)	KT279549, KT279520	This study
Hyloscirtus palmeri	MZUTI 3083	Ecuador: Pichincha: Milpe (0.03237° N, 78.86597° W; 1120 m)	KT279551	This study
Hyloscirtus palmeri	KRL 1038	Panama: Parque Nacional Omar Torrijos, El Cope, 800 m	FJ784457	Crawford et al. [27]
Hyloscirtus palmeri	KRL 1692	Panama: Parque Nacional Omar Torrijos, El Cope, 800 m	FJ784568	Crawford et al. [27]
Hyloscirtus palmeri	TOE 141	Panama: Parque Nacional Omar Torrijos, El Cope, 800 m	FJ784596	Crawford et al. [27]
Hyloscirtus palmeri	KRL 1626	Panama: Parque Nacional Omar Torrijos, El Cope, 800 m	FJ784573	Crawford *et al.* [27]
Hyloscirtus palmeri	KRL 1216	Panama: Parque Nacional Omar Torrijos, El Cope, 800 m	FJ784493	Crawford et al. [27]
Hyloscirtus palmeri	KRL 1636	Panama: Parque Nacional Omar Torrijos, El Cope, 800 m	FJ784577	Crawford et al. [27]
Hyloscirtus palmeri	SIUC H-6924	Panama: El Cope, Parque Nacional Omar Torrijos	AY843650	Faivovich et al. [3]
Hyloscirtus pantostictus	QCAZ 45438	Ecuador: Sucumbíos: ca. Santa Bárbara, 2709 m	JX155819 JX155846	Coloma et al. [28]
Hyloscirtus pantostictus	QCAZ 45435	Ecuador: Sucumbíos: ca. Santa Bárbara, 2709 m	JX155820 JX155847	Coloma et al. [28]
Hyloscirtus phyllognathus	MZUTI 1353	Ecuador: Napo: Cordillera de los Guacamayos (0.93543° S, 77.79306° W; 1564 m)	KT279521	This study
Hyloscirtus phyllognathus	MZUTI 1354	Ecuador: Napo: Cordillera de los Guacamayos (0.93543° S, 77.79306° W; 1564 m)	KT279515	This study
Hyloscirtus phyllognathus	MZUTI 2383	Ecuador: Napo: Stream in the Chaco–Lago Agrio road (0.09954° S, 77°58408° W; 1243 m)	KT279518, KT279545	This study
Hyloscirtus phyllognathus	MZUTI 2384	Ecuador: Napo: Stream in the Chaco–Lago Agrio road (0.09954° S, 77°58408° W; 1243 m)	KT279519, KT279546	This study
Hyloscirtus phyllognathus	MZUTI 2385	Ecuador: Napo: Stream in the Chaco–Lago Agrio road (0.09954° S, 77°58408° W; 1243 m)	KT279517, KT279547	This study
Hyloscirtus phyllognathus	MZUTI 2386	Ecuador: Napo: Stream in the Chaco–Lago Agrio road (0.09954° S, 77°58408° W; 1243 m)	KT279516, KT279548	This study
Hyloscirtus aff. *phyllognathus*	MZUTI 192	Ecuador: Napo: Stream in the Chaco–Lago Agrio road (0.09954° S, 77°58408° W; 1243 m)	KT279522	This study
Hyloscirtus phyllognathus	QCAZ 23938	Ecuador: Morona Santiago: 16 km N El Ideal, 1600 m	JX155800 JX155827	Coloma et al. [28]
Hyloscirtus phyllognathus	QCAZ 41032	Ecuador: Zamora Chinchipe: ca. Miazi Alto, 1250 m	JX155801 JX155828	Coloma et al. [28]
Hyloscirtus phyllognathus	QCAZ 32271	Ecuador: Morona Santiago: ca. Nueve de Octubre, 1527 m	JX155802 JX155829	Coloma et al. [28]
Hyloscirtus princecharlesi	QCAZ 42165	Ecuador: Imbabura: ca. Cuellaje, 2720 m	JX155806 JX155833	Coloma et al. [28]
Hyloscirtus princecharlesi	QCAZ 43654	Ecuador: Imbabura: ca. Cuellaje, 2760 m	JX155807 JX155834	Coloma et al. [28]
Hyloscirtus psarolaimus	QCAZ 27049	Ecuador: Sucumbíos: ca. Santa Bárbara, 2600 m	JX155808 JX155835	Coloma et al. [28]
Hyloscirtus psarolaimus	QCAZ 46095	Ecuador: Napo: 60 km E Salcedo, 2748 m	JX155809 JX155836	Coloma et al. [28]
Hyloscirtus ptychodactylus	QCAZ 46030	Ecuador: Cotopaxi: ca. Pilaló, 2500 m	JX155804 JX155831	Coloma et al. [28]

(*Continued*)

Table A2. (*Continued*).

Species	Museum number	Locality	Genbank number	Source
Hyloscirtus ptychodactylus	QCAZ 46031	Ecuador: Cotopaxi: ca. Pilaló, 2500 m	JX155805 JX155832	Coloma et al. [28]
Hyloscirtus simmonsi	KU 181167	Colombia: Valle: Río Calima, 1.5 km W Lago Calima	DQ380376	Wiens et al. [57]
Hyloscirtus staufferorum	QCAZ 45967	Ecuador: Pastaza: ca. Santa Clara, 2250 m	JX155815 JX155842	Coloma et al. [28]
Hyloscirtus staufferorum	QCAZ 45962	Ecuador: Pastaza: ca. Santa Clara, 2250 m	JX155816 JX155843	Coloma et al. [28]
Hyloscirtus tapichalaca	QCAZ 15083	Ecuador: Zamora Chinchipe: Reserva Tapichalaca, 2625 m	JX155803 JX155830	Coloma et al. [28]
Hyloscirtus tapichalaca	QCAZ 16704	Ecuador: Zamora Chinchipe: Reserva Tapichalaca (4°29.049′S, 79°8.925′ W, 2697 m)	AY563625	Faivovich et al. [3]
Hyloscirtus tigrinus	QCAZ 41351	Ecuador: Sucumbíos: ca. Santa Bárbara, 2638 m	JX155810 JX155837	Coloma et al. [28]
Hyloscirtus tigrinus	QCAZ 31550	Ecuador: Napo: ca. Santa Bárbara, 2620 m	JX155811 JX155838	Coloma et al. [28]
Hyloscirtus sp.	MZUTI 3262	Ecuador: El Oro: Reserva Buenaventura (3.65317° S, 79.76314° W, 429 m)	KT279503, KT279544	This study
Hyloscirtus sp.	MZUTI 3380	Ecuador: El Oro: Reserva Buenaventura (3.66464° S, 79.7428° W, 1042 m)	KT279502	This study
Hyloscirtus sp.	MZUTI 3436	Ecuador: El Oro: Reserva Buenaventura (3.66464° S, 79.7428° W, 1073 m)	KT279501	This study
Hyloscirtus sp.	MZUTI 3474	Ecuador: El Oro: Reserva Buenaventura (3.66464° S, 79.7428° W, 1073 m)	KT279500	This study
Myersiohyla neblinaria	USNM 562071 (RWM 17688)	Venezuela: Amazonas, Cerro Neblina	AY843672	Faivovich et al. [3,6]