Article

A new species of *Engystomops* (Anura: Leiuperidae) from southwestern Ecuador

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Abstract

We describe *Engystomops puyango* **sp. nov.** from the lowlands of southwestern Ecuador. The new species is closely related to *E. pustulatus* from which it differs in skin texture, advertisement call, and karyotype features. The new species also differs from *E. pustulatus* at genes 12S and 16S of mitochondrial DNA (6.5%–6.8% of sequence divergence). A phylogeny based on mtDNA shows that *E.* **sp. nov.** is part of a clade of *Engystomops* distributed below 1300 m in western Ecuador and northwestern Peru. The new species occurs in Evergreen Lower Montane Forest and Foothill Semideciduous Costa Forest. The karyotype of the new species has $2n = 20$ chromosomes which represents the first known reduction in chromosome number in Leiuperidae ($2n = 22$). Its advertisement calls as well as those of *E. pustulatus* have a facultative component that resembles the chuck in calls of *E. pustulosus* but that are less discrete and have lower acoustic complexity.

Key words: Anura, Advertisement call, Karyotype, Morphology, Phylogeny, New species, *Engystomops pustulatus*, *Physalaemus*

Resumen

[Una nueva especie de *Engystomops* (Anura: Leiuperidae) del suroccidente de Ecuador]
Describimos *Engystomops puyango* **sp. nov.** de las tierras bajas del suroccidente de Ecuador. La nueva especie está cercanamente relacionada a *E. pustulatus* de la que difiere por la textura de su piel, su canto de anuncio, y características del cariotipo. La nueva especie también se diferencia de *E. pustulatus* en los genes 12S y 16S de ADNmt (6.5%–6.8% de divergencia en la secuencia). Una filogenia basada en ADNmt muestra que *E.* **sp. nov.** es parte de Douvox, un clado distribuido por debajo de 1300 m en el occidente de Ecuador y el noroccidente de Perú. La nueva especie se encuentra en Bosque Siempreverde Montano Bajo y Bosque Semideciduo Piemontano de la Costa. El cariotipo de la nueva especie tiene $2n = 20$ cromosomas lo cual representa la primera reducción conocida en el número de cromosomas de Leiuperidae ($2n = 22$). Sus cantos así como los de *E. pustulatus* presentan un componente facultativo que se parece al "chuck" del canto de *E. pustulosus* pero que es menos discreto y tiene menor complejidad acústica.

Palabras clave: Anura, Canto de anuncio, Cariotipo, *Engystomops pustulatus*, Filogenia, Morfología, Nuevas especies, *Physalaemus*.

Introduction

Engystomops is a genus of leiuperid frogs that contains eight described species distributed in Central America, northwestern South America and the Amazon Basin. Phylogenetic analyses based on mitochondrial DNA (mtDNA) revealed two basal clades within *Engystomops.* They received that unranked names of Duovox and Edentulus (Ron *et al*. 2006). The same phylogeny suggested the existence of one undescribed species from southern Ecuador, closely related and morphologically similar to *E. pustulatus* (Ron, *et al.*, 2006). The putative new species (referred as *E.* sp. D by Ron, *et al.*, 2006 and Ron, 2008) was discovered in 2004 at

Bosque Protector Puyango, a tropical deciduous forest. Fieldwork between 2005 and 2008 resulted in findings of additional populations.

The genetic data also suggested the existence of an undescribed species in Peru, referred as *E.* sp. B, sister to *E.* sp. D (Ron*, et al.*, 2006). The proposal of *E.* sp. D and *E.* sp. B as separate species was merely based on genetic divergence in mtDNA. Analyses of additional characters were needed to confirm those taxonomic proposals. Herein we analyze variation in genetic, acoustic, cytogenetic, and morphological characters and confirm that *E.* sp. D is an undescribed species. We describe the new species including its advertisement call and karyotype. The new species belongs to Duovox, a clade distributed in the lowlands of northwestern South America.

Methods

Morphological terminology follows Lynch and Duellman (1997). Sex was determined by the presence of nuptial pads, vocal sac folds, and/or by gonadal inspection. Snout-vent length is abbreviated as SVL throughout. Examined specimens (listed in the type-series and Appendix I) are housed in California Academy of sciences (CAS), Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ), Museum of Comparative Zoology, Harvard University (MCZ), National Museum of Natural History (USNM), and Natural History Museum, University of Kansas (KU). Geographic coordinates for QCAZ specimens are based on geodetic datum WGS 84. Coordinates were measured with a Garmin™ 12CX GPS or Garmin™ eTrex Legend® receivers.

Sound recordings were made with a Sennheiser™ ME-67 directional microphone with analog tape recorder Sony™ WM-D6C or digital recorder SONY™ MZ-NH1. Calls were analyzed using software Raven 1.2.1 (Charif*, et al.*, 2004) at a sampling frequency of 22.1 kHz and a frequency resolution of 21.5 Hz. Measured call variables are defined in Table 1. Eight of those variables (listed in Table 2) were used to run a Principal Components Analysis (PCA) and a Discriminant Function Analysis (DFA) to assess the degree of acoustic differentiation between calls from 10 males of *Engystomops* **sp. nov.** (from Puyango) and 39 males of *E. pustulatus* (from Patricia Pilar, Puerto Rico, El Empalme, Arenillas, and Huaquillas). Original recordings are deposited in the audio archive of the QCAZ and the Texas Natural History Collection of the Texas Memorial Museum (University of Texas at Austin). *Engystomops* **sp. nov.** can add a facultative high frequency suffix to their calls (referred as "third component"; see Results). We conducted an experiment in captivity to determine the influence of the proximity of other males to the production of the third component. A male was placed on a 50 cm x 50 cm artificial arena and recorded before and after another individual (male or female) approached. For six males, we compared the proportion of calls on which the third component was present when the male was calling alone vs. the proportion when the male was calling next to other male. The proportions were compared with Wilcoxon's signed rank sum test.

All well-preserved specimens (Simmons, 2002) were measured for the following morphometric variables: (1) snout-vent length; (2) head length; (3) head width; (4) eye-nostril distance; (5) femur length; (6) tibia length; and (7) arm length. Measurements were made according to the methodology described in Duellman (1970) and Ron *et al*. (2004) with digital calipers (nearest 0.01 mm) from specimens fixed in 10% formalin and preserved in 70% ethanol. Because of small sample sizes for preserved female specimens, most morphometric comparisons were made only among adult males. Female SVL data (n = 72) was obtained during a behavioral study at Bosque Protector Puyango from females found in amplexus and released after the experiment. Comparisons of SVL and call properties between populations were performed with Tukey-Kramer Honestly Significant Difference (HSD) test to control for multiple comparisons.

Multivariate analyses were used to assess the degree of morphometric differentiation among the new species and *E. pustulatus*. To remove the effect of covariation in size, the PCA and DFA were applied to the residuals from the linear regressions between the six morphometric variables and SVL. All variables were log transformed prior to the analysis. For the PCA, only components with eigenvalues > 1 were retained. The number of specimens included were 30 *E.* **sp. nov.** (from Catamayo, Mangaurquillo, and Puyango), 51 *E. pustulatus* (Arenillas, Huaquillas, Patricia Pilar, and Puerto Rico), and 11 *E.* sp. B (Chongoyape, Morropón,

and Motupe). See type-series lists and Appendix I for detailed locality information. Multivariate analyses were carried out in JMP® 8.0.1 (SAS Institute, 2008).

TABLE 1. Call traits analyzed in this study. See text for details.

Character	Description
Call duration	Time from the beginning to the end of the call
Duration of the first component	Time from beginning of the call to point of maximum amplitude
Duration of the second component	Time from end of the first component to end of the call or to increase of amplitude of third component (if present)
Duration of the third component	Time from end of second component to end of call
Frequency of the 1st spectral peak of the call	Frequency of the 1st spectral peak in power spectrum of the entire call (fundamental frequency)
Frequency of the 2nd spectral peak of the call	Frequency of the 2nd spectral peak in power spectrum of the entire call
Frequency of the 1st spectral peak of the first component	Frequency of the 1st spectral peak in power spectrum of the first component (fundamental frequency). This peak mostly corresponds to the first harmonic.
Frequency of the 2nd spectral peak of the first component	Frequency of the 2nd spectral peak in power spectrum of the first component. This peak mostly corresponds to the second harmonic.
Frequency of the 1st spectral peak of the second component	Frequency of the 1st spectral peak in power spectrum of the entire whine (fundamental frequency)
Frequency of the 2nd spectral peak of the second component	Frequency of the 2nd spectral peak in power spectrum of the entire whine
Fundamental frequency of the 3rd component	Frequency of the first harmonic of the 3rd component (fundamental frequency)
Dominant frequency of the 3rd component	Frequency of the harmonic with the most energy in the 3rd component. Corresponds to the 5th or 6th harmonic.

TABLE 2. Character loading and percentage of explained variance for Principal Components (PC) I–II for eight acoustic variables from the advertisement call of *Engystomops puyango* and *E. pustulatus*. Bold figures indicate highest loadings.

Variable	Character Loading	
	PC I	PC II
Call duration	–0.125	**0.623**
Duration of the 1st component	–0.219	0.424
Frequency of the 1st spectral peak of the 1st component	0.414	0.045
Frequency of the 1st spectral peak of the 2nd component	0.282	0.485
Frequency of the 2nd spectral peak of the 1st component	**0.422**	–0.198
Frequency of the 2nd spectral peak of the call	**0.451**	–0.148
Frequency of the 1st spectral peak of the call	0.336	0.351
Frequency of the 2nd spectral peak of the 2nd component	**0.437**	0.110
Eigenvalue	4.106	2.026
%	51.3	25.3

Cytogenetics. Chromosomal analyses of *E. puyango* were carried out on three females and three males from Bosque Protector Puyango. Mitotic metaphases were obtained from cellular suspensions of the specimens' intestinal epithelium inoculated with colchicine (2%) for at least four hours (modified from

Schmid, 1978 and Schmid, *et al.*, 1979) before being sacrificed by hypothermia. Chromosomal preparations were stained with a 10% Giemsa solution and then submitted to C-banding (King, 1980) and Ag-NOR impregnation (Howell & Black, 1980). The slides were examined using an Axioscope Zeiss microscope and the chromosome morphometric analysis was done using Axio Vision 4.0 software (Carl Zeizz, 2005). The chromosomes were classified according to Green & Sessions (1991) criteria.

Phylogenetic analyses. Phylogenetic analyses are an expansion of those presented by Ron *et al.* (2006) to include three additional populations of *E. puyango* and *E. pustulatus* (Arenillas, Huaquillas, and Catamayo). The same matrix and alignment of mitochondrial DNA sequence data (genes12S rRNA, valine-tRNA, and 16S rRNA; ~2.4 kb) was employed except that *Physalaemus* and most populations of *E. petersi* and *E. pustulosus* were excluded. Laboratory procedures for DNA extraction and PCR were the same described by Ron *et al.* (2006). Amplified products of the new samples were sequenced by the Macrogen Sequencing Team (Macrogen Inc., Seoul, Korea).

Phylogenetic relationships were assessed under maximum likelihood with a genetic algorithm using GARLI version 0.96 (Zwickl, 2006). The model of character evolution for the analyses was the same employed in Ron *et al.* (2006). Five stochastic likelihood searches from different starting trees were conducted to ensure recovery of the best tree. Nodal support was assessed with non-parametric bootstrapping from 500 maximum likelihood searches. *Engystomops pustulosus*, *E. freibergi*, and *E. petersi* were used as outgroup based on the topology recovered by Ron *et al.* (2006). Locality of new specimens and GenBank accession numbers are shown in Appendix II.

Engystomops puyango **sp. nov.**

Holotype. (Fig. 1) QCAZ 26978 (field no. PUCE 14586), adult male from Ecuador, Provincia El Oro, Bosque Protector Puyango, near the bridge over the Puyango River on the road Alamor-Arenillas (3.87159° S, 80.03770° W), 321 m above sea level, collected by S. R. Ron, E. E. Tapia, I. G. Tapia on 19 February 2004.

Paratopotypes. QCAZ 26983–84, 26986, 26991, 26996, 26999, 27000–01, 27004, 27006, 27008–14, 27016, adult males, 26987–88, 26990, 27005, adult females, collected by S. R. Ron, I. G. Tapia, and E. E. Tapia on 19 February 2004; QCAZ 26969, 26971–72, 26974–75, 26977–78, adult males, 26967, adult female collected by G. Romero, E. E. Tapia, and S. R. Ron on 7 January 2005; 28717, adult male, 28716, adult female collected by C. Proaño, M. Guerra and S. R. Ron between 13 and 20 February 2005.

Paratypes. Provincia Loja: 10 km north from Zapotillo along the road to Arenillas (4.31198° S, 80.21690° W), 231 m above sea level, QCAZ 26959, adult male, collected by S. R. Ron, I. G. Tapia, E. E. Tapia on 18 February 2004; old road Alamor-Puyango (3.93548° S, 80.10865° W), 650 m above sea level, QCAZ 28758–62, adult males, collected by C. Proaño, M. Guerra and S. R. Ron between 20 and 23 February 2005; Catamayo (3.97004° S, 79.36876° W), 1291 m above sea level, QCAZ 31506–08, adult males, collected by I. G. Tapia and G. Onore on 30 December 2005; Mangaurquillo, FHGO 3372, 3374–76, adult males, collected by F. Nogales.

Diagnosis. A member of *Engystomops*, clade Duovox. The assignment to *Engystomops* is based on the molecular phylogeny published by Ron *et al.* (2006) which shows high support for the inclusion of *E. puyango* (referred as "*Engystomops* sp. D") within *Engystomops*. The following morphological synapomorphies (Cannatella, *et al.*, 1998) also support this assignment: (1) presence of flank glands; (2) presence of parotoid glands; and (3) warty skin.

Engystomops puyango (Fig. 2) is characterized by: (1) mean SVL 27.59 mm in males (range 23.78–30.48; $n = 45$), 28.68 mm in females (range 25.41–32.68; $n = 72$); (2) skin on dorsum bearing a mixture of scattered pustules and minute tubercles; (3) snout varying between rounded and protruding in dorsal view and rounded to subacuminate in lateral view; (4) vomerine teeth and odontophores absent; (5) maxillary and premaxillary teeth present; (6) parotoid and flank glands present, usually fused, mean length = 16.63 mm (SD = 1.05; $n = 28$; 56.9–66.2% of SVL); (7) tarsal tubercle absent; (8) nuptial pads present; (9) Finger I shorter than II; (10) tympanic annulus evident, concealed dorsally and posteriorly; (11) tympanic membrane almost always not tuberculate.
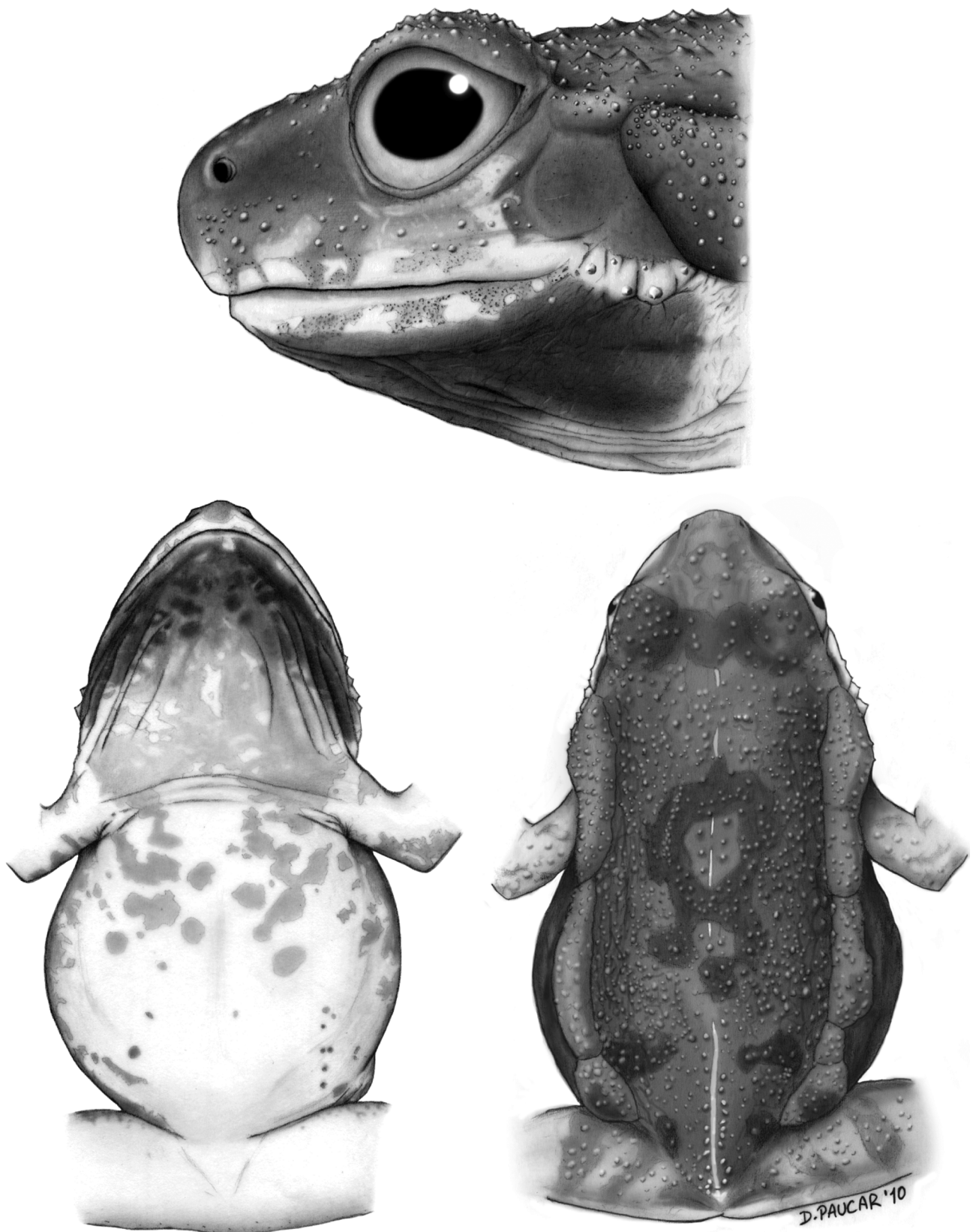
FIGURE 1. Lateral (top), ventral (left), and dorsal (right) views of the holotype of *Engystomops puyango* (QCAZ 26978). Not drawn to scale.
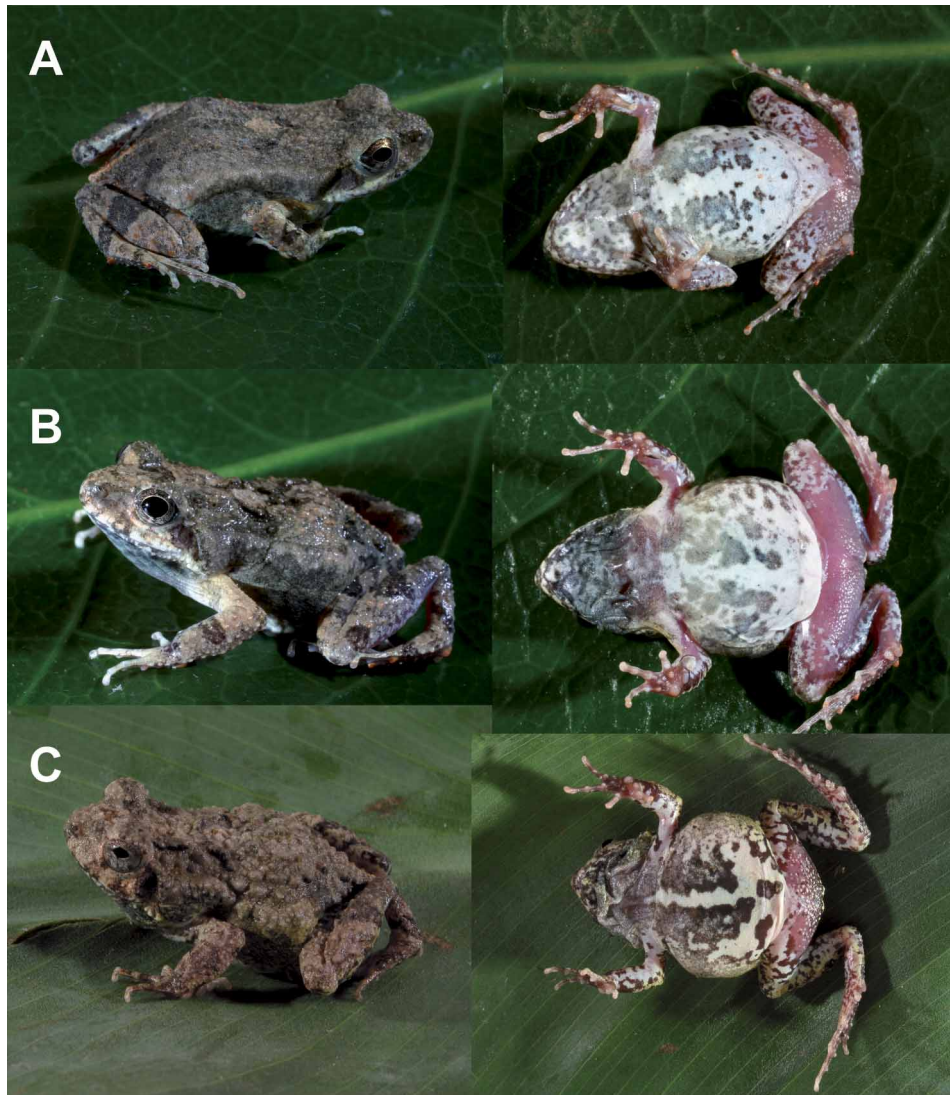
FIGURE 2. Dorsolateral and ventral views of (A) *Engystomops puyango*, QCAZ 26988, adult female, SVL = 26.54; (B) *E. puyango*, QCAZ 26987, adult male, SVL = 28.19, and (C) *E. pustulatus*, QCAZ 26114, adult male, SVL = 28.5. *Engystomops puyango* from Bosque Protector Puyango (Provincia El Oro, Ecuador); *E. pustulatus*, from La Maná (Provincia Cotopaxi, Ecuador).
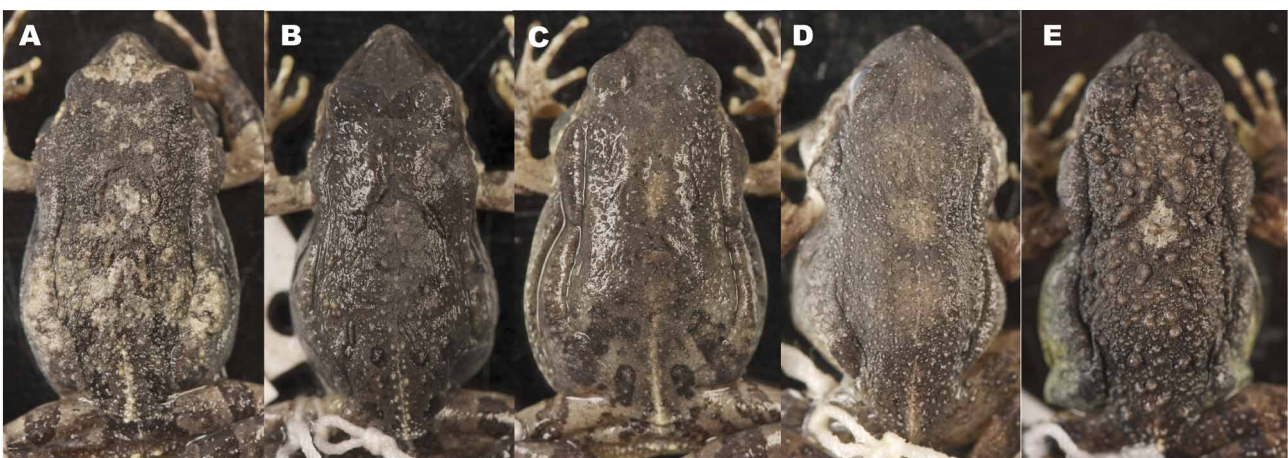
FIGURE 3. Dorsal photographs of adult *Engystomops puyango* (A) QCAZ 26980, (B) QCAZ 31506, (C) QCAZ 26998, (D) QCAZ 2880; and *E. pustulatus* (E) QCAZ 26736, showing differences in skin texture. Note that *E. puyango* has smaller tubercles.

TABLE 3. Morphometry of karyotypes of *Engystomops puyango* and *E. pustulatus.* Means and standard deviations are from 25 metaphases from three males (QCAZ 47296, 46932, 47295) and five females (QCAZ 35690–91, 46934, 47297, SC 26360) of *E. puyango* from Bosque Protector Puyango, Ecuador; and four of *E. pustulatus* (from Cerro Blanco, Ecuador; QCAZ 47031–33, QCAZ 46931). Abbreviations are: m = metacentric; sm = submetacentric.

Chromosome number	*Engystomops puyango*				*Engystomops pustulatus*		
	Relative Length ± SD	Arm Radio ± SD	Centromeric Index ± SD	Type	Relative Length ± SD	Centromeric Index ± SD	Type
1	14.08 (±0.08)	1.36 (±0.02)	0.42 (±0.01)	m	15.45 (±0.10)	0.43 (±0.03)	m
2	12.80 (±0.12)	1.81 (±0.05)	0.36 (±0.06)	sm	12.54 (±0.09)	0.37 (±0.02)	sm
3	11.66 (±0.01)	1.93 (±0.05)	0.34 (±0.04)	sm	10.9 (±0.04)	0.34 (±0.04)	sm
4	10.53 (±0.17)	2.70 (±0.06)	0.27 (±0.07)	sm	10.77 (±0.11)	0.45 (±0.05)	m
5	9.25 (±0.22)	1.32 (±0.04)	0.43 (±0.06)	m	10.44 (±0.28)	0.34 (±0.03)	sm
6	9.25 (±0.09)	1.60 (±0.03)	0.38 (±0.03)	m	10.03 (±0.17)	0.31 (±0.03)	sm
7	9.10 (±0.07)	1.29 (±0.04)	0.44 (±0.05)	m	9.02 (±0.12)	0.42 (±0.07)	m
8	8.82 (±0.026)	2.10 (±0.03)	0.32 (±0.02)	sm	8.62 (±0.015)	0.37 (±0.04)	sm
9a, 9b	8.61 (±0.32)	1.63 (±0.07) 1.77 (±0.06)	0.38 (±0.04) 0.36 (±0.03)	m sm	6.52 (±0.23)	0.44 (±0.08)	m
10	5.90 (±0.19)	1.37 (±0.02)	0.42 (±0.01)	m	5.69 (±0.22)	0.43 (±0.07)	m

Engystomops puyango is most similar to *E. pustulatus*. They differ in dorsal skin texture (less tuberculate in *E. puyango*; Fig. 3), chromosomes shape and size (Table 3), and advertisement call (shorter and with higher frequency in *E. puyango*; Fig. 4). Calls are significantly different in total duration ($t = 9.68$, df = 26, $P < 0.001$), duration of the first component ($t = 4.01$, df = 24, $P < 0.001$), frequency of the 2nd spectral peak of the call ($t = 910.47$, df = 43, $P < 0.001$), and frequency of the 2nd spectral peak of the first component ($t = 10.65$, df = 45, $P < 0.001$; Table 4). Genetically, large distances, typical of species pairs in *Engystomops*, separate both species. Pairwise uncorrected *p*-distances between *E. pustulatus* and *E. puyango* (range 0.065–0.068) are higher than distances between sister species *E. montubio* and *E. randi* (0.027–0.032) or even between non-sister species like *E. guayaco* and *E. montubio* (0.059), and *E. montubio* and *E. coloradorum* (0.057–0.060). However, genetic distances should not be used as the only evidence for species delimitation (Vences & Wake, 2007). In our dataset, nevertheless, the genetic data is corroborated by the pattern of differentiation in skin texture, advertisement calls, and chromosome morphology and is consistent with the recognition of *E. puyango* as a separate species from *E. pustulatus*.

Engystomops puyango is larger than *E. randi*, *E. montubio*, and *E. guayaco* (non overlapping SVL in adult males) and has less extensive to absent lateral fringes on the toes (fringes are prominent in *E. randi*, *E. montubio*, and *E guayaco*; Ron*, et al.*, 2004; Ron*, et al.*, 2005). Furthermore, the advertisement call of *E. puyango* is longer and lacks well-defined pulses at the beginning as in *E. randi*, *E. montubio*, and *E. guayaco* (Ron, 2008; Table 4, Fig. 5). The absence of a tarsal tubercle and the presence of teeth in the maxilla and premaxilla distinguish *E. puyango* from *E. petersi*, *E. freibergi*, and *E. pustulosus. Engystomops coloradorum* has a shorter advertisement call (Ryan & Rand, 2001) and more prominent dorsal tubercles (tubercles are smaller and scattered in *E. puyango*; Fig. 3). *Engystomops coloradorum* further differs from *E. puyango* in having a vertical loreal region (oblique in *E. puyango*).

Description of holotype. Adult male, 27.33 mm SVL, tibia length 12.43 mm, femur length 11.84 mm, arm length 6.17 mm, head length 8.82 mm, head width 9.02 mm, eye-nostril distance 2.7 mm, head narrower than body except in scapular region; diameter of eye 2.2 times diameter of tympanic annulus; tympanic membrane and tympanic annulus barely evident; tympanic annulus ovoid, longer dorsoventrally; few small tubercles on anterior margin of tympanic annulus, absent from tympanic membrane; supratympanic fold absent; head between the orbits and intercanthal region flat except for scattered tubercles; snout protruding in profile and slightly truncated in dorsal view; nostrils slightly elevated, internarial region convex; canthus rostralis rounded; loreal region concave**.**

TABLE 4. Descriptive statistics for call parameters of *Engystomops puyango* (from Bosque Protector Puyango), *E. pustulatus*, and *E.* sp. B (from Motupe). Mean ± SD is given with range below. The call of *E. puyango* and *E. pustulatus* consists of two obligatory (First and Second) and one facultative (Third) components. Except for calls from La Maná, all *E. pustulatus* calls lack the third component. Sample sizes are number of males. Average water temperature for recordings was 25.9 C (SD = 0.41, n = 9) for *E. puyango* and 27.1 (SD = 1.13, n = 36) for *E. pustulatus*. Temporal characters are shown in seconds; spectral characters in Hertz.

| Population | *E. pustulatus* | | | | | | | *E. puyango* (n = 10) | *E.* sp. B (n = 12) |
	Combined (n = 39)	Arenillas (n = 7)	El Empalme (n = 3)	Huaquillas (n = 10)	La Maná (n = 10)	Patricia Pilar (n = 3)	Puerto Rico (n = 6)	Bosque Puyango	Motupe
Call Duration	0.572 ± 0.089 (0.384–0.750)	0.574 ± 0.070 (0.490–0.688)	0.499 ± 0.042 (0.463–0.546)	0.658 ± 0.063 (0.584–0.750)	0.592 ± 0.051 (0.481–0.652)	0.500 ± 0.054 (0.468–0.563)	0.463 ± 0.070 (0.384–0.560)	0.366 ± 0.049 (0.287–0.438)	0.397 ± 0.043 (0.324–0.477)
Duration First Component	0.150 ± 0.024 (0.099–0.207)	0.133 ± 0.021 (0.099–0.159)	0.143 ± 0.023 (0.117–0.163)	0.153 ± 0.016 (0.130–0.182)	0.170 ± 0.023 (0.136–0.207)	0.147 ± 0.023 (0.129–0.173)	0.134 ± 0.022 (0.110–0.172)	0.125 ± 0.014 (0.102–0.142)	0.117 ± 0.021 (0.075–0.144)
Duration Second Component	0.381 ± 0.088 (0.150–0.555)	0.441 ± 0.058 (0.383–0.528)	0.319 ± 0.030 (0.286–0.345)	0.468 ± 0.059 (0.361–0.555)	0.310 ± 0.070 (0.150–0.415)	0.353 ± 0.031 (0.331–0.389)	0.328 ± 0.058 (0.252–0.390)	0.129 ± 0.015 (0.102–0.149)	0.150 ± 0.038 (0.092–0.211)
Duration Third Component	0.116 ± 0.079 (0.046–0.325)				0.116 ± 0.079 (0.046–0.325)			0.111 ± 0.038 (0.050–0.186)	0.130 ± 0.034 (0.071–0.175)
Frequency of the First Spectral Peak of the Call	870 ± 55 (747–988)	888 ± 26 (855–926)	838 ± 33 (801–859)	920 ± 45 (859–988)	816 ± 46 (747–883)	873 ± 53 (823–928)	867 ± 40 (797–911)	859 ± 106 (683–990)	858 ± 84 (624–947)
Frequency of the Second Spectral Peak of the Call	1790 ± 113 (1572–2067)	1828 ± 75 (1745–1981)	1774 ± 106 (1654–1860)	1900 ± 90 (1809–2067)	1669 ± 65 (1572–1798)	1803 ± 105 (1688–1895)	1766 ± 53 (1723–1860)	2019 ± 38 (1970–2093)	1850 ± 141 (1670–2204)

TABLE 4. (continued)

Population	E. pustulatus							E. puyango (n = 10)	E. sp. B (n = 12)
	Combined (n = 39)	Arenillas (n = 7)	El Empalme (n = 3)	Huaquillas (n = 10)	La Maná (n = 10)	Patricia Pilar (n = 3)	Puerto Rico (n = 6)	Bosque Puyango	Motupe
Frequency of the First Spectral Peak of the First Component	925 ± 60 (797–1076)	943 ± 35 (900–992)	888 ± 40 (842–917)	972 ± 41 (930–1057)	862 ± 39 (797–915)	916 ± 44 (872–960)	955 ± 64 (896–1076)	968 ± 18 (939–995)	909 ± 39 (831–964)
Frequency of the Second Spectral Peak of the First Component	1767 ± 125 (1552–2080)	1804 ± 88 (1675–1959)	1705 ± 118 (1596–1830)	1891 ± 102 (1697–2080)	1659 ± 62 (1581–1776)	1787 ± 139 (1628–1884)	1720 ± 102 (1553–1837)	2017 ± 38 (1950–2097)	1809 ± 106 (1658–1950)
Frequency of the First Spectral Peak of the Second Component	824 ± 57 (700–973)	840 ± 39 (775–875)	819 ± 37 (779–853)	874 ± 40 (827–973)	766 ± 50 (700–861)	819 ± 53 (777–878)	826 ± 39 (777–878)	774 ± 46 (677–831)	814 ± 65 (624–869)
Frequency of the Second Spectral Peak of the Second Component	1763 ± 115 (1492–2024)	1794 ± 43 (1725–1830)	1744 ± 108 (1634–1850)	1880 ± 75 (1809–2024)	1643 ± 54 (1550–1729)	1789 ± 102 (1677–1875)	1728 ± 122 (1492–1839)	1807 ± 204 (1267–1959)	1816 ± 79 (1636–1924)
Fundamental Frequency of the Third Component	458 ± 28 (422–506)				458 ± 28 (422–506)			509 ± 25 (474–549)	548 ± 24 (517–581)
Dominant Frequency of the Third Component	2544 ± 133 (2250–2695)				2544 ± 133 (2250–2695)			2851 ± 135 (2605–3083)	2693 ± 240 (2118–3014)
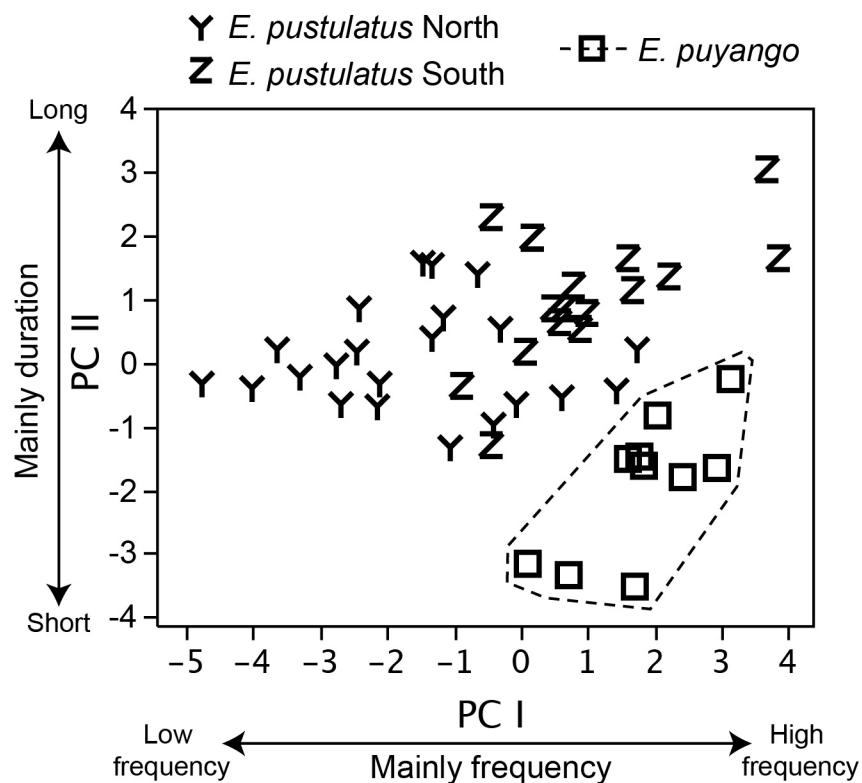
FIGURE 4. Axes I and II from Principal Components Analysis based on eight acoustic variables from the advertisement calls of *Engystomops puyango* (10 males) and *E. pustulatus* (39). See Table 2 for character loadings on each component.

Fingers without expanded discs; nuptial pad present, keratinized, brown, divided in two portions, one covering posterior half of thenar tubercle, other covering Finger I dorsally and posteriorly along its proximal half and posteriorly to distal edge of first phalanx. Base of thenar tubercle ovoid, that of palmar tubercle nearly round; palmar tubercle less prominent than thenar tubercle; subarticular tubercles with round base, all conical; second subarticular tubercle on Finger IV present; few supernumerary palmar tubercles present. Webbing between fingers absent; relative lengths of adpressed fingers III > IV > II > I. Toes without expanded discs; base of inner metatarsal tubercle ovoid, larger than round base of outer metatarsal tubercle; inner metatarsal tubercle more prominent than outer metatarsal tubercle; subarticular tubercles with round base except for proximal tubercles of Toe III and IV (ovoid base), all conical except for subconical distal subarticular tubercles of Toe V; sparse and minute conical and subconical plantar supernumerary tubercles; tarsal tubercle absent. Lateral fringes on toes absent; webbing between toes absent; relative lengths of adpressed toes IV > III > V > II > I.

Skin on dorsum bearing a mixture of pustules and minute, round to subconical tubercles. Some tubercles are arranged in longitudinal rows; skin on venter smooth. Tongue longer than wide; vomerine teeth and odontophores absent. Maxillary and premaxillary teeth present. Vocal slits present, parallel to margins of mandible. Deflated vocal sac forming folds on gular region, extending posteriorly to base of arm.

Color of holotype in preservative. Dorsum grayish brown with lighter gray on the snout; dark gray transversal bar between orbits and irregular dark marks on posterior half of dorsum; light gray middorsal blotch bordered by irregular dark bands at arm insertion axis; dorsal tubercles and pustules light gray; dorsal surfaces of forearms and hindlimbs light gray with dark gray transversal bands. Venter cream yellowish with few minute light gray marks along posterior one-third of the body; Dark-gray blotches on chest and anterior half of abdomen; minute dark gray spots abundant between arms and on posterior half of gular region; anterior half of gular region dark gray with light gray irregular marks close to midline; ventral surfaces of hindlimbs and forelimbs cream to cream yellowish, becoming light gray or cream towards outer and inner edges; some dark blotches present on shanks; outer half of ventral surfaces of forearms dark gray; sides of

head gray with ill-defined dark gray marks on the snout and a cream area below the orbit and tympanum, extending posteriorly above deflated vocal folds and anteriorly as a labial stripe; flanks dark gray dorsally, light gray ventrally.

Etymology. The specific name *puyango* is a noun in apposition, in reference to the type locality, the Puyango Protected Forest. The 2658 ha reserve has one of the world's largest deposits of petrified tree trunks. It is also one of the largest reserves of tropical dry forest in Ecuador.

Variation. Variation in dorsal coloration of preserved specimens is extensive (Fig. 6). Background dorsal coloration varies from light gray (QCAZ 27005, 27009) to dark gray (QCAZ 31508) or dark brown (QCAZ 35691). Irregular dark marks may be present in diverse patterns (Fig. 6). A clear mid-dorsal line extends along the posterior half of the body (QCAZ 36511) but in some specimens (QCAZ 26988, 26997) it only extends along the posterior one fifth. There is variation in the abundance and arrangement of tubercles (all lighter than the background; Figs. 3 and 6). Tubercles coalesce into small ridges that partly enclose a mid-dorsal light blotch in QCAZ 26984, 27005, 27012. The parotoid glands are fused with the flank glands in 28 out of 32 specimens (average gland length is 16.63 mm, SD = 1.05; 56.9–66.2% of SVL). In QCAZ 27013, the glands are fused on the left side but separated on the right side. In QCAZ 26983 and 26989, the flank glands are separated from the parotoid glands on both sides.

Ventral surfaces of preserved specimens have a cream to yellowish-cream background color with light gray (QCAZ 26991, 28762) to dark gray markings (QCAZ 26874, 26984). Marks are arranged in diverse patterns (Fig. 6) and vary from being restricted to the anterior half of the body (darker on folded vocal sacs; QCAZ 26968) to being present over the entire venter (less abundant posteriorly, QCAZ 27008). In a few specimens, the ventral marks are arranged in well-defined large spots (QCAZ 27002, 26983). A midventral cream stripe can be present from near the tip of the snout to the gular region (QCAZ 26994) or continue to the mid venter (QCAZ 26988). In most individuals the stripe is absent (QCAZ 27008) or ill defined (QCAZ 26986). The arrangement of dark spots and tubercles on the ventral surfaces of the feet and hands of QCAZ 26975 is shown in Figure 7.

Head shape varies between rounded (QCAZ 34511) and subacuminate (QCAZ 28760) in dorsal view; in lateral view it varies between rounded (QCAZ 26972) and protruding (QCAZ 31506, 26974). Lateral head coloration varies between light gray and dark gray. The area below the eye and tympanum is cream in most specimens (e.g., QCAZ 26987–88) but can be restricted to a thin longitudinal light stripe from the jaw articulation to the parotoid gland (QCAZ 26976). Two or three vertical dark bars can be present in the loreal region and below the orbit (e.g., QCAZ 26968, 26973, 26982). The tympanic annulus is concealed dorsally and posteriorly. With few exceptions (e.g., QCAZ 26984, 34511) the tympanic annulus has tubercles; the tympanic membrane has tubercles in only five specimens of the type series (e.g., QCAZ 26989, 28758).

The following morphometric data pertain to adults. In the type series, the largest male has a SVL of 30.48 mm, and the largest female 32.62 mm; mean male SVL = 27.59 mm (n = 45; SD = 1.17), mean female SVL = 27.98 mm (n = 6; SD = 2.38). Amplectant pairs measured during a behavioral study at Bosque Protector Puyango (not collected) had mean male SVL = 26.76 (n = 72; SD = 1.62) and mean female SVL = 28.68 (n = 72; SD = 1.49). Females were significantly larger than males (t = 6.19, df = 100, $P < 0.001$). Snout-vent length was positively correlated between amplectant males and females (ANOVA F = 19.93, df = 71, $P < 0.001$).

Among the type specimens, Catamayo has the highest male SVL (mean = 29.31 mm, SD = 0.51) followed by Puyango (27.50, SD = 0.95) and Mangaurquillo (26.17, SD = 1.75; Table 5). Differences in SVL are significant between Catamayo and Mangaurquillo (Tukey's HSD $P < 0.001$), and Catamayo and Puyango (Tukey's HSD $P = 0.020$). All other pairwise comparisons were non-significant (P values > 0.071).

Call. Males call while floating on standing water. Acoustic parameters of the advertisement call of *E. puyango* are shown in Table 4. The call consists of two obligatory and one facultative components with harmonic structure. The first component is characterized by a slight increase in frequency (mean = 48.0 Hz, SD = 67.24, n = 10) with decreasing amplitude modulation. Sidebands (from amplitude modulation) are evident in each harmonic but they merge at the end of the component (as a result, amplitude modulation ends; Fig. 5). The power spectrum along all the call shows three spectral peaks (Fig. 5C). Dominant frequency of the call was on the second spectral peak in calls from nine out of ten males (mean = 2022.3 Hz, SD = 30.8).

Dominant frequency was 835.5 Hz in calls from the only male on which the dominant frequency was on the first spectral peak. The third spectral peak has high energy only in calls with the third component (see below). The dominant frequency of the first component was on the second spectral peak in calls of nine out of ten males (mean = 2017.6, SD = 40.5). Dominant frequency was 994 Hz in the only male on which the dominant was on the first spectral peak.

TABLE 5. Descriptive statistics for morphometric measurements of male *Engystomops puyango* and *E. pustulatus* used for Principal Component Analysis. Mean ± SD is given with range below. Bold figures are combined for males of all populations. Abbreviations are: SVL = snout-vent length; TL = tibia length; FL = femur length; AL = arm length; HL = head length; HW = head width; EN = eye-nostril distance. All measurements are in mm.

| Species	SVL	TL	FL	AL	HL	HW	EN
E. pustulatus (*n* = 51)	**27.08 ± 1.36**	**11.42 ± 0.59**	**11.50 ± 1.06**	**6.26 ± 0.36**	**8.73 ± 0.42**	**9.11 ± 0.80**	**2.75 ± 0.23**
Arenillas (*n* = 10)	28.33 ± 0.80 26.84–29.34	11.78 ±0.39 11.24–12.37	11.35 ± 0.41 10.85–12.28	6.50 ±0.24 6.22–6.99	8.99 ± 0.19 8.76–9.37	8.72 ± 0.22 8.34–9.21	2.68 ± 0.13 2.37–2.85
Huaquillas (*n* = 10)	25.53 ± 1.13 23.90–28.14	10.64 ± 0.48 9.98–11.44	9.82 ± 0.50 9.02–10.73	5.98 ± 0.33 5.25–6.46	8.54 ± 0.37 8.08–9.33	7.92 ± 0.23 7.56–8.25	2.58 ± 0.07 2.49–2.72
Patricia Pilar (*n* = 17)	27.43 ± 1.24 25.17–29.88	11.59 ± 0.53 10.65–12.72	12.09 ± 0.71 10.45–13.01	6.24 ± 0.41 5.69–6.84	8.73 ± 0.54 7.68–9.6	9.60 ± 0.47 8.63–10.28	2.84 ± 0.27 2.36–3.42
Puerto Rico (*n* = 14)	26.86 ± 0.77 25.85–27.96	11.50 ± 0.33 10.94–12.14	12.08 ± 0.64 10.44–13.03	6.32 ± 0.26 5.76–6.71	8.70 ± 0.32 8.15–9.29	9.65 ± 0.53 8.76–10.52	2.82 ± 0.23 2.31–3.17
E. puyango (*n* = 30)	**27.31 ± 1.16**	**11.88 ± 0.58**	11.10 ± 0.64	**6.36 ± 0.31**	**8.49 ± 0.44**	8.81 ± 0.61	**2.65 ± 0.16**
Catamayo (*n* = 3)	29.31 ± 0.51 28.76–29.79	12.31 ± 0.61 11.86–13.01	11.84 ± 0.82 11.36–12.80	6.80 ± 0.32 6.58–7.17	9.11 ± 0.12 9.02–9.26	8.50 ± 0.08 8.42–8.59	2.65 ± 0.05 2.61–2.71
Mangaurquillo (*n* = 4)	26.17 ± 1.75 23.78–27.67	11.47 ± 0.19 11.21–11.68	10.28 ± 0.45 9.79–10.83	6.04 ± 0.23 5.81–6.36	8.35 ± 0.52 7.74–8.97	9.36 ± 0.37 9.15–9.92	2.54 ± 0.21 2.33–2.81
Puyango (*n* = 23)	27.43 ± 0.83 25.98–29.29	12.03 ± 0.47 11.33–12.77	11.29 ± 0.46 10.38–12.19	6.42 ± 0.25 5.90–6.84	8.50 ± 0.36 7.91–9.45	8.80 ± 0.57 7.54–9.8	2.68 ± 0.15 2.36–2.92

The second component follows immediately and is a whine-like note. Each harmonic is a nearly pure tone with descending frequency (Fig. 5). The average decrease in frequency of the first harmonic is 448 Hz (SD = 45.6, *n* = 10; measured to the end of the call). The average frequency at the beginning of the second component is 937.7 Hz (SD = 41.0, *n* = 10) for the first harmonic and 1924 Hz (SD = 67.15, *n* = 10) for the second. The first spectral peak had more energy than the second in calls of 8 males out of 10 males. The number of visible harmonics in the spectrogram varies between 6 and 12.

A facultative third component can be added after the whine and is characterized by a gradual increase in amplitude and a switch in dominant frequency from the first to the fifth or sixth harmonic (Fig. 5). Average fundamental frequency of the third component is 509 Hz (SD = 24.9, *n* = 10; Table 4); average dominant frequency is 2851 Hz (SD = 135.5; *n* = 10). Infrequently, the third component exhibits a doubling in the number of harmonics (as shown in Fig. 5B). The majority of males may be capable of doubling the harmonics because in captivity we recorded repeatedly calls from eight males and all of them produced at least once a call with doubling. This type of call, however, was produced infrequently: out of 12 recordings of third components from seven captive males, only two presented doubling in the number of harmonics; out of ten males recorded at Bosque Protector Puyango, only one did.

Observations at Bosque Protector Puyango suggest that the third component is produced when another individual approaches the calling male. Our experiment to test the influence of the proximity of other individuals in the production of the third component shown a significant positive effect. The average proportion of calls with third component for accompanied males was 0.682 (SD = 0.1943, *n* = 6) while the proportion for lonely males was 0.085 (SD = 0.140, *n* = 6). Differences between proportions were significant (Wilcoxon's *z* = -2.207; *P* = 0.027).
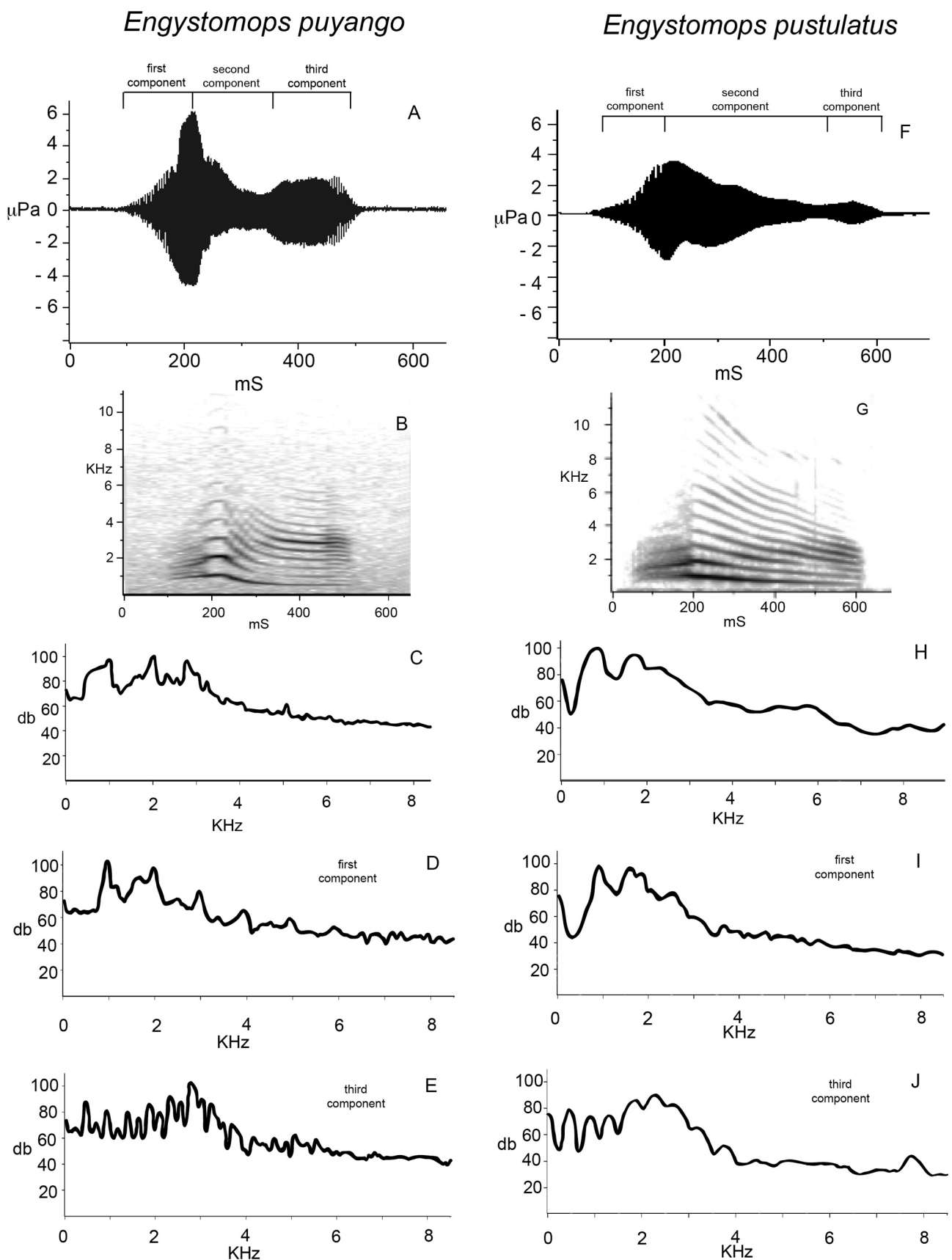
FIGURE 5. Calls of *Engystomops* from western Ecuador. A–E: *E. puyango* (QCAZ 26968) from Bosque Protector Puyango, Provincia El Oro, Ecuador; F–J: *E. pustulatus* (QCAZ 26852) from El Empalme, Provincia Guayas, Ecuador. A and F are oscilograms; B and G spectrograms; C and H power spectra of complete call, D and I power spectra of the first component, E and J power spectra of the third component.
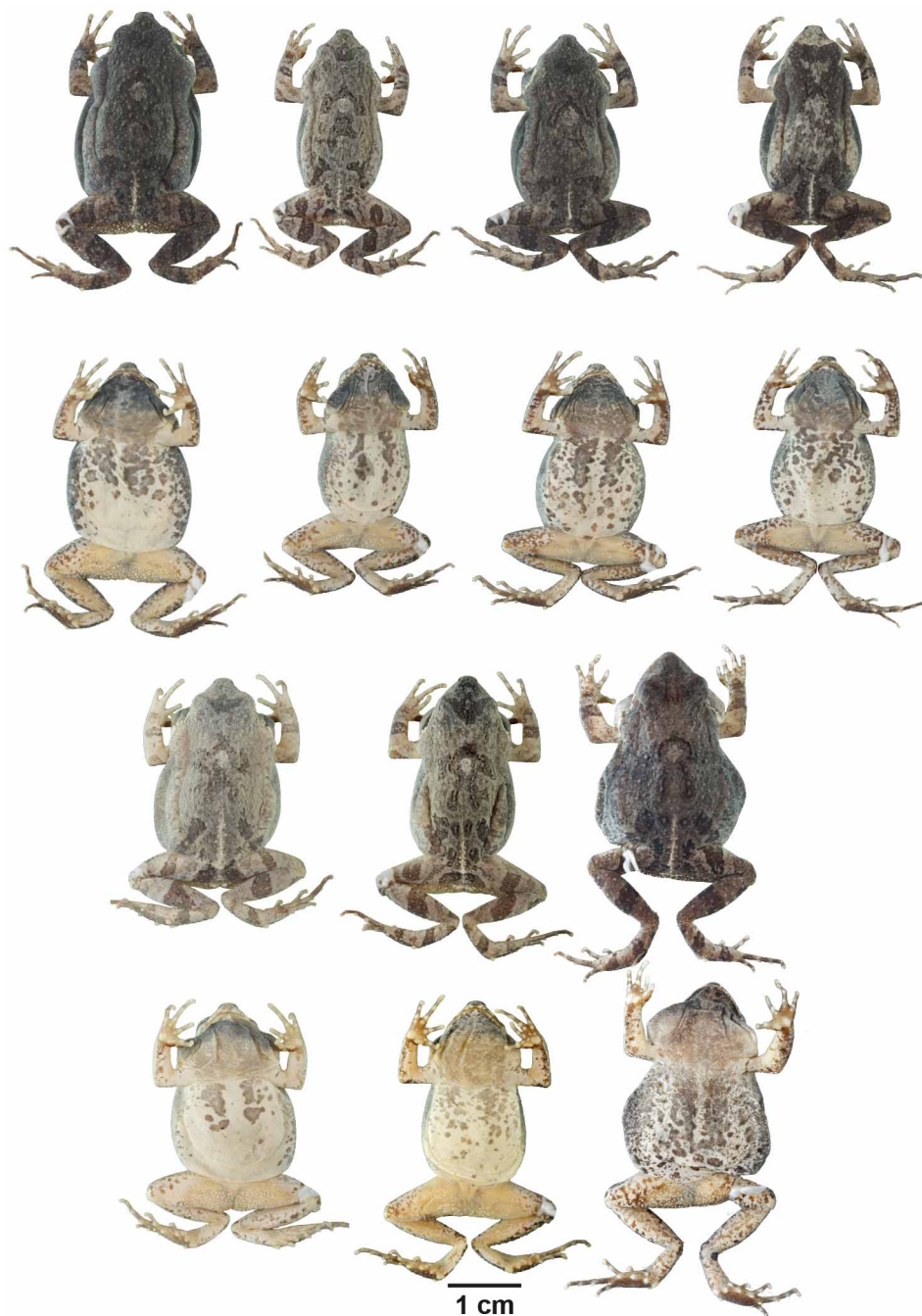
FIGURE 6. Dorsal and ventral views of adult *Engystomops puyango* showing variation in dorsal and ventral patterns. From left to right, above: QCAZ 26976, 26982–83, 26986; below: QCAZ 27009, 27013, 37276 (all males). Provincia Loja and El Oro, Ecuador (See Appendix I for locality data).

Two components with eigenvalues > 1.0 were extracted from the PCA of calls from 49 males of *E. puyango* and *E. pustulatus*. The two PCs accounted for 76.6% of the total variation. Principal Component I loads mostly on frequency variables while PC II on call duration (Table 2). The acoustic space of *E. puyango* is distinctive from that of *E. pustulatus* as shown by significant differences between both species in scores for PC I ($t = 5.34$, $df = 32$, $P < 0.001$) and PC II ($t = 6.34$, $df = 13$, $P < 0.001$; Fig. 4).

In the DFA classification procedure, calls from all 10 males of *E. puyango* were correctly classified as *E. puyango* and calls from all 29 males of *E. pustulatus* were classified as *E. pustulatus*. The absence of incorrect assignments confirms the high acoustic distinctiveness between the calls of both species.

Morphometric comparisons. Three components with eigenvalues > 1.0 were extracted from the PCA of 30 specimens of *E. puyango*, 51 of *E. pustulatus*, and 11 of *E.* sp. B. The three PCs accounted for 76.1% of the

total variation. The highest loadings were head width and femur length for PC I, arm length for PC II, and head length for PC III (Table 6). The morphometric space of *E. puyango* overlaps with *E. pustulatus* populations from Arenillas and Huaquillas (Fig. 8) but only slightly with *E. pustulatus* from Puerto Rico and Patricia Pilar. In contrast, *E.* sp. B overlaps widely in morphometric space with *E. puyango* as shown by the lack of significant differences along both PCs (PC I: $t = 0.44$, df = 23, $P = 0.661$; PC II: $t = 0.55$, df = 15, $P = 0.587$). Comparisons between *E. puyango* and *E. pustulatus* of the variables with the highest loadings show significant differences in residual head width ($t = 2.20$, df = 65, $P = 0.03$) and residual femur length ($t = 2.45$, df = 78, $P = 0.016$) but not in residual arm length ($t = 1.38$, df = 65, $P = 0.17$).

In the DFA classification procedure, 16 out of 30 specimens of *E. puyango* were classified correctly. The misclassified specimens were assigned to *E. pustulatus* (four specimens) and *E.* sp. B (10 specimens). Six out of 11 *E.* sp. B were correctly classified; all misclassified specimens were assigned *E. puyango*. Overall, the multivariate analyses indicate some morphometric differentiation between *E. puyango* and *E. pustulatus* but not between *E. puyango* and *E.* sp. B.

FIGURE 7. Ventral views of the right hand and foot of *Engystomops puyango*, QCAZ 26975 (adult male from Bosque Protector Puyango, Ecuador; SVL = 28.16).

TABLE 6. Character loading and percentage of explained variance for Principal Components (PC) I–II for six morphometric variables. Bold figures indicate highest loadings.

| Variable	Size-free morphology	
	PC I	PC II
Tibia length	0.413	0.409
Femur length	**0.526**	–0.128
Arm length	0.293	**0.664**
Head length	0.249	0.073
Head width	**0.543**	–0.242
Eye-nostril distance	0.328	**–0.557**
Eigenvalue	2.233	1.315
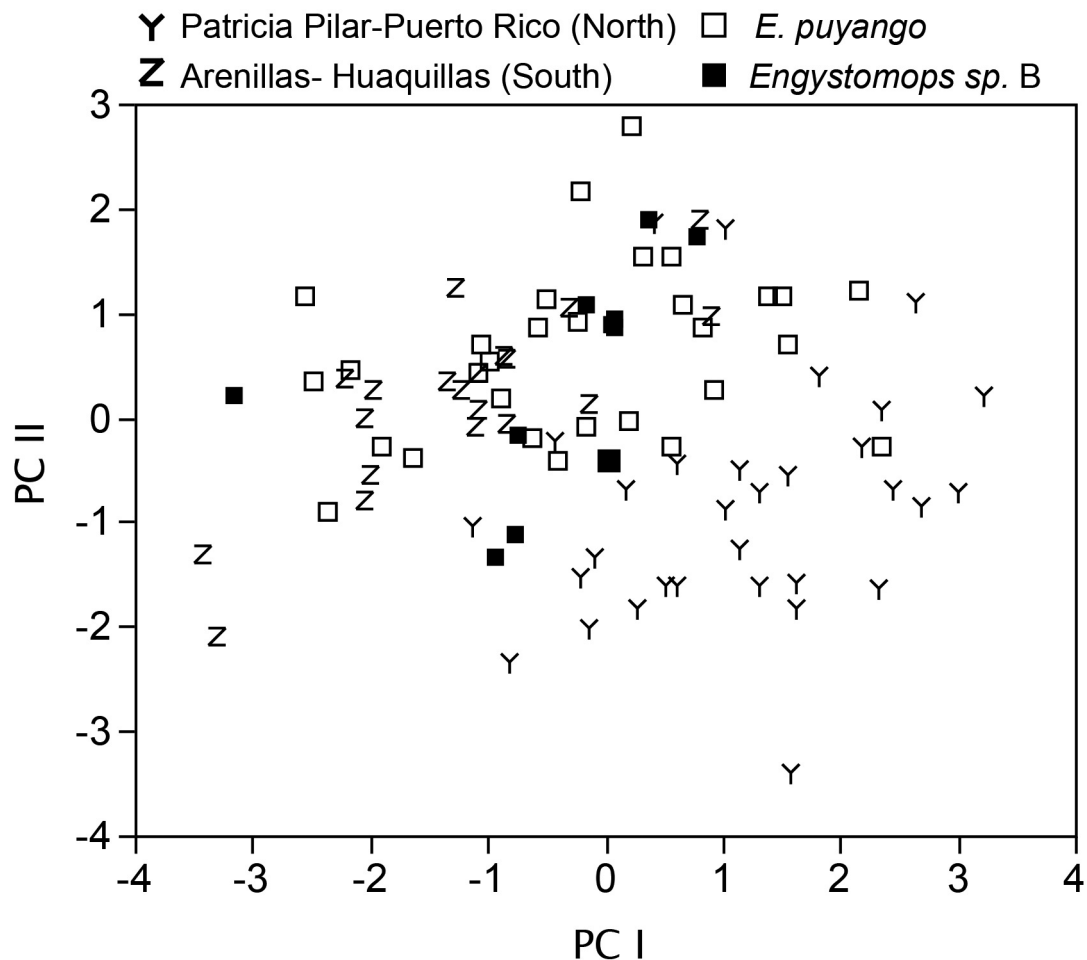
FIGURE 8. Axes I and II from Principal Components Analysis based on six size-corrected morphological variables for *Engystomops puyango* (30 specimens), *E. pustulatus* (51), and *E.* sp. B (11). See Table 6 for character loadings on each component.

Distribution and ecology. *Engystomops puyango* has been recorded in southwestern Ecuador (Provincia Loja and Provincia El Oro) between 320–1291 m above sea level (Fig. 9). Maximum straight-line distance between localities is 120 km. The occurrence of *E. puyango* in Peru is highly probable because Bosque Protector Puyango and Zapotillo are in the border with Peru.

The geographic range of *E. puyango* is characterized by scant and highly seasonal precipitation. The rainy season lasts between February and April (Lynch & Duellman, 1997). Localities are in Evergreen Lower

Montane Forest of the Western Andes (Catamayo) and Foothill Semideciduous Costa Forest (Puyango, Mangaurquillo, and Zapotillo; vegetation types are as defined by Sierra, *et al.*, 1999 and Cerón, *et al.*, 1999). The Evergreen Lower Montane Forest is a transitional form between humid forest and the dry forests that predominates in the Andes of southern Ecuador. The Foothill Semideciduous Costa Forest has scattered trees, less than 20 m tall, and a dense understory dominated by herbaceous plants.
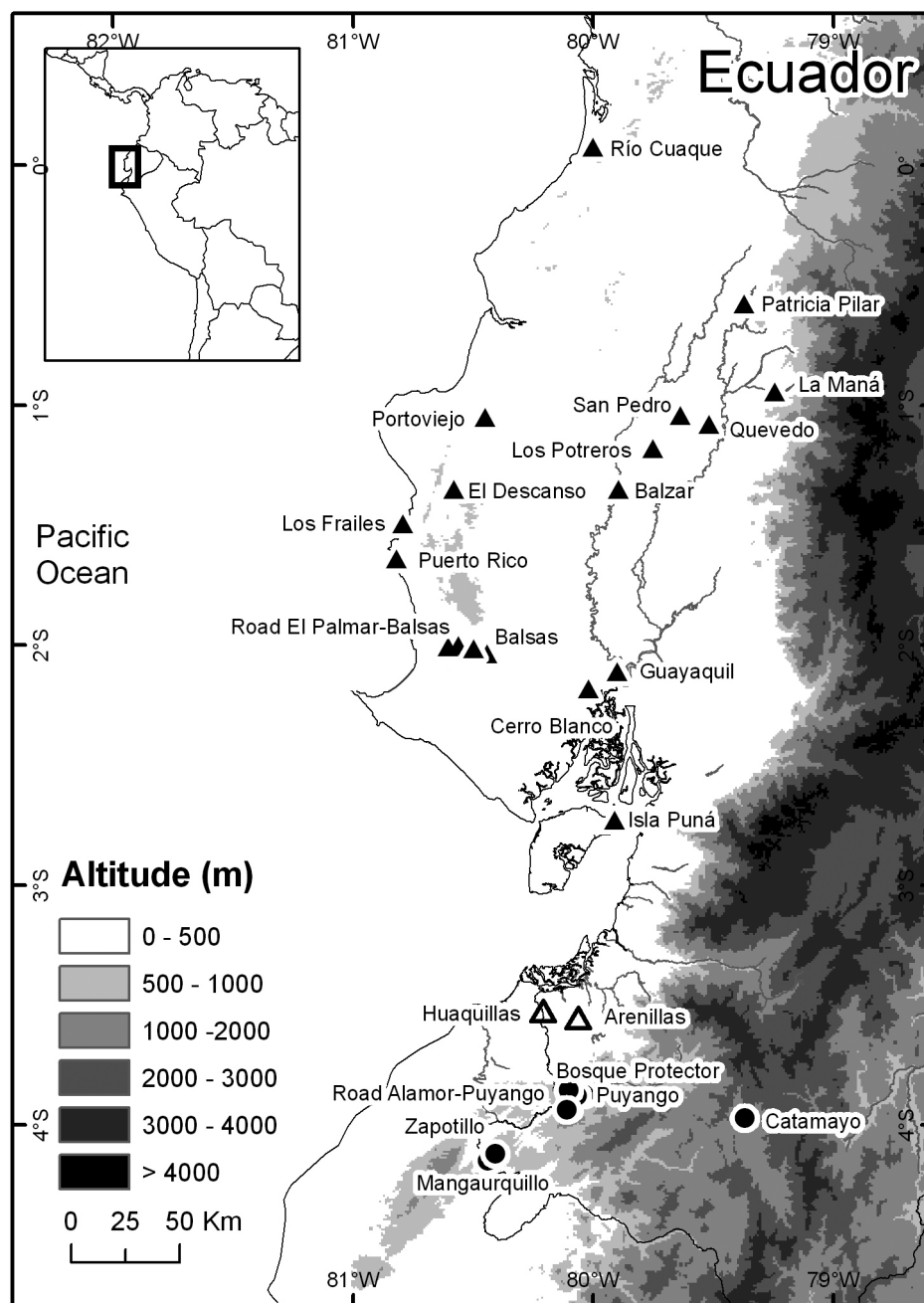

FIGURE 9. Known records of *Engystomops puyango* (circles) and *E. pustulatus* (solid triangles for northern range, hollow triangles for the southern range). Locality data are based on specimens deposited in, California Academy of Sciences, Museo de Zoología de la Pontificia Universidad Católica del Ecuador, and Museum of Comparative Zoology Harvard University (Appendix I).

All individuals were found in open to sparsely forested areas where the original natural vegetation has been partly or completely removed by humans. At Bosque Protector Puyango, choruses were found in February 2004 and February 2005. Males were calling from water in ponds and ditches. Amplexus and egg deposition take place at the same sites where choruses call. *Engystomops puyango* constructs floating foam

nests during amplexus. While the female discharges the egg masses, the male beats them with his legs to produce the foam.

Males *E. puyango* and the smaller *E. randi* were frequently calling at the same ponds, sometimes at distances less than 20 cm. At a dense chorus of both species at Bosque Protector Puyango, on February 19 2005, we found a male (QCAZ 28801) with an unusual advertisement call. Average call duration (0.222 s, SD = 0.006, $n = 10$) was below the range for *E. puyango* (Table 4) and close to the average of *E. randi* (0.206 s, SD = 0.013, based on recordings from 7 males from the same locality). Call interval was closer to that of *E. randi* (QCAZ 28801 mean = 0.203, SD = 0.011, $n = 10$ calls; *E. randi* = 0.324, SD = 0.091, $n = 7$ males; *E. puyango* = 1.083, SD = 0.146, $n = 10$ males). However, similarly to *E. puyango*, the first component of the call lacked the well-defined pulses present in calls of *E. randi* (Ron *et al.* 2004) and its SVL (28.31 mm) was outside the range of adult males of *E. randi* (20.09–24.00 mm, $n = 24$). Because the characteristics of the advertisement call of QCAZ 28801 are intermediate between *E. puyango* and *E. randi*, we hypothesize that it is a hybrid individual. A sequence of 750 bases of mtDNA, gene 16S, is identical to that of *E. randi* QCAZ 23768 from the same population. Thus, the hybrid could be the product of the cross of a female *E. randi* x male *E. puyango*. Other species calling in syntopy with *E. puyango* were *Scinax quinquefasciatus* and *Phrynohyas venulosa*.
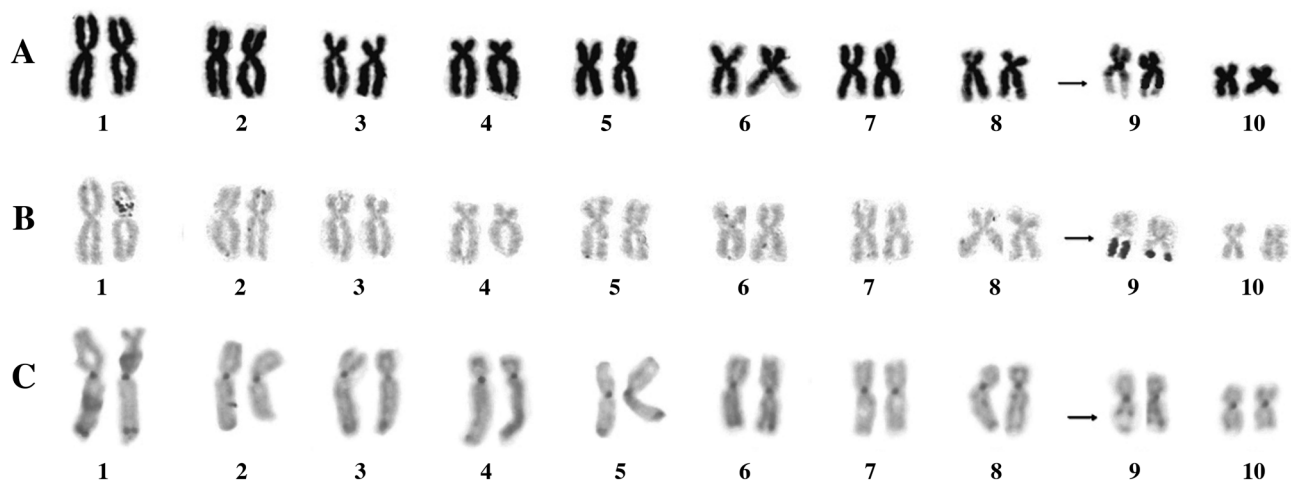
FIGURE 10. Karyotype of *Engystomops puyango*. (A) Giemsa stained, (B) Ag-NOR stained, (C) C-banded. Arrows indicate the secondary constriction (A), Ag-NOR (B), C-banded in pair 9 (C).

Cytogenetics. All eight analyzed individuals had a chromosome number of $2n = 20$. This is the first known departure from the $2n = 22$ karyotype characteristic of Leiuperidae. All species analyzed of *Eladorhina*, *Eupemphix*, *Pleurodema*, *Pseudopaludicola*, *Physalaemus*, and *Engystomops* have a karyotype $2n = 22$ (e.g., Ananias, *et al.*, 2007; Duellman, 1967; Lourenço, *et al.*, 2000; Lourenço, *et al.*, 2006; Quindere, *et al.*, 2009; Tomatis, *et al.*, 2009). Chromosome pairs 1, 5, 6, 7 and 10 were metacentric while pairs 2, 3, 4 and 8 were submetacentric (Table 3, Fig. 10). Pair 9 was heteromorphic with one chromosome being submetacentric and the other metacentric and slightly smaller. An extended secondary constriction was detected with Giemsa staining in the long arm of two chromosomes 9 (Fig. 10A); using the Ag-NOR method, it was determined that the constriction corresponds to the nucleolar organizing region (NOR) (Fig. 10B). The difference in size and morphology of pair 9 is a result of the size of NOR: in the submetacentric chromosome 9 the NOR is a large block that doubles the size of the NOR of the metacentric chromosome 9. This heteromorphism in the size of the NOR may be due to varying amounts of its transcription units associated with events of duplication and/or deletion of NOR constituents; NORs are made of highly repetitive ribosomal genes that allow such genetic events. It is also possible that the NOR heteromorphism could result from other mechanisms such as unequal sister chromatid exchange or non-peer linkage in meiosis (Kasahara, 2009). It could also be the result of differential activity of rDNA transcription, which could explain the existence of inter-cellular polymorphism present in some specimens.

C-banding revealed small blocks of heterochromatin in the centromere of all chromosomes. C-positive bands were observed in the telomeres of the long and short arms of pairs 1 and 5, in the long arm of pairs 3, 6 and 8. Faint intercalated C-bands were observed in the long arms of pairs 1 and 8 (Fig. 10C). Pair 9 had C bands in the internal and external borders of the NOR (Fig. 10C).

The karyotype of *Engystomops puyango* shares with *E. pustulatus* the same number of chromosomes and the position of the NOR in pair 9 (M. Rivera & J. P. Falconí, unpublished). However, there are morphological differences in chromosome pairs 4, 5, and 6 (Table 3).

Taxonomic remarks

The genus *Engystomops* was described by Jimenez de la Espada (1872) with *E. petersi* as type species. Lynch (1970) synonymized it with *Physalaemus* based on incongruent distribution of what he considered to be "generic characters". Lynch (1970) also proposed the "*Physalaemus pustulosus* species group" based on morphological characters. Subsequent reviews (Cannatella, *et al.*, 1998; Ron, *et al.*, 2006) confirmed the monophyly of the group and its morphological distinctiveness. Nascimento *et al.*, (2005) resurrected *Engystomops* for the *pustulosus* species group and this proposal has been followed by several authors (e.g, del Pino, *et al.*, 2007; Fleming, *et al.*, 2009; Ron, *et al.*, 2006). Funk *et al.* (2008; 2007), however, considered that the use of *Engystomops* was unjustified. The available evidence (Tárano & Ryan, 2002) suggests that the validation of *Engystomops* does not generate paraphyletic genera. Thus, the choice of either taxonomic alternative is a matter of preference between taxonomic stability and taxonomic informativeness. We opt to continue the use of *Engystomops* given its morphological distinctiveness and the need of providing taxonomic information among the over 50 species included in *Physalaemus* and *Engystomops*.

Status of the populations of Engystomops *from northwestern Peru.* The available data is insufficient to determine unequivocally the status of the populations of *Engystomops* sp. B. Dorsal skin texture is similar to that of *E. puyango*; a genetic sample (from Olmos, near Motupe) suggests that they are either conspecific or sister species to *E. puyango*. *Engystomops* sp. B overlaps in morphometric space with both *E. puyango* and *E. pustulatus* (Fig. 8). Similarly to *E. pustulatus* and *E. puyango*, the advertisement call has three components. However, call duration is closer to values observed in *E. puyango* (Table 4). A DFA classification including calls from 12 males from Morropón and Olmos, classified all calls as either *E.* sp. B (11 individuals) or *E. puyango* (1); none was assigned to *E. pustulatus*. Similarly to the mtDNA phylogeny, the morphologic and acoustic data suggests a closer relationship with *E. puyango* than *E. pustulatus*. Additional populations need to be sampled to determine if they should be considered a separate species from *E. puyango*. The only evidence suggesting that they are separate is their relatively large genetic distance (uncorrected *p*-distance range 0.052–0.054).

Description of the advertisement call of *E. pustulatus*

Males call while floating on standing water. Acoustic parameters of the advertisement call of *E. pustulatus* are shown in Table 4. The power spectrum along all the call shows two or three spectral peaks. At La Maná, the dominant frequency of the call was always in the first spectral peak in calls from ten males (mean = 816.5, SD = 48.3).

The basic structure of the call is similar to that of other congeners (Ron, 2008). The call has two obligatory and one facultative components with harmonic structure. The first component is characterized by weak amplitude modulation (Fig. 5). Modulation sidebands are not well defined and disappear towards the end of the component. At La Maná, the dominant frequency of the first component was in the first spectral peak in all calls from ten males (mean = 861.9, SD = 38.9).

The second component is a whine-like note without amplitude modulation (Fig. 5). Each harmonic is a nearly pure tone with descending frequency. At La Maná, the average decrease in frequency of the first

harmonic from the beginning of the second component to the end of the call is 405.7 Hz (SD = 34.9, *n* = 10). The average frequency at the beginning of the second component is 833.7 Hz (SD = 53.7, *n* = 10) for the first harmonic and 1690 Hz (SD = 82.8, *n* = 10) for the second. The first spectral peak had more energy than the second in all ten calls from La Maná. The number of harmonics visible in spectrograms varies between 6 and 14.

As in *E. puyango*, a facultative third component, characterized by a gradual increase in amplitude and a switch in dominant frequency from the first to the fifth or sixth harmonic, can be present (Fig. 5). At La Maná, average fundamental frequency of the third component is 457.7 Hz (SD = 28.4, *n* = 10; Table 4); average dominant frequency is 2543.8 Hz (SD = 132.8; *n* = 10). Infrequently, the third component exhibits a doubling in the number of harmonics. As in *E. puyango*, the transition between the second and third component is gradual. Observations at La Maná suggest that the third component is produced when the calling male is approached by other individual (Narváez, 2010).

Comparisons were carried out between populations for which there were at least six recorded males: Puerto Rico, La Maná, Arenillas and Huaquillas. We found significant differences for PC I between La Maná and all populations (all Tukey's HSD *P* < 0.004). All other pairwise comparisons were not significantly different (all HSD *P* > 0.09). Along PC II, there were significant differences between Huaquillas and Puerto Rico (HSD *P* = 0.005) and Huaquillas and La Maná (HSD *P* = 0.046). All other pairwise comparisons were not significantly different (all HSD *P* > 0.154). A DFA classification for the four populations, shows that calls from all 26 males from Huaquillas, La Maná, and Puerto Rico were correctly classified. Out of 7 calls from Arenillas, four were correctly classified, two assigned to Huaquillas and one to Puerto Rico. The low frequency of incorrect assignments shows high acoustic distinctiveness between populations of *E. pustulatus*.

Calls described by Ryan & Rand (2001) as belonging to *E. pustulatus*, actually belong to *E. randi* (from Pasaje, Provincia El Oro). Ron *et al*. (2004) described a single call of *E. pustulatus* from Puerto Rico.
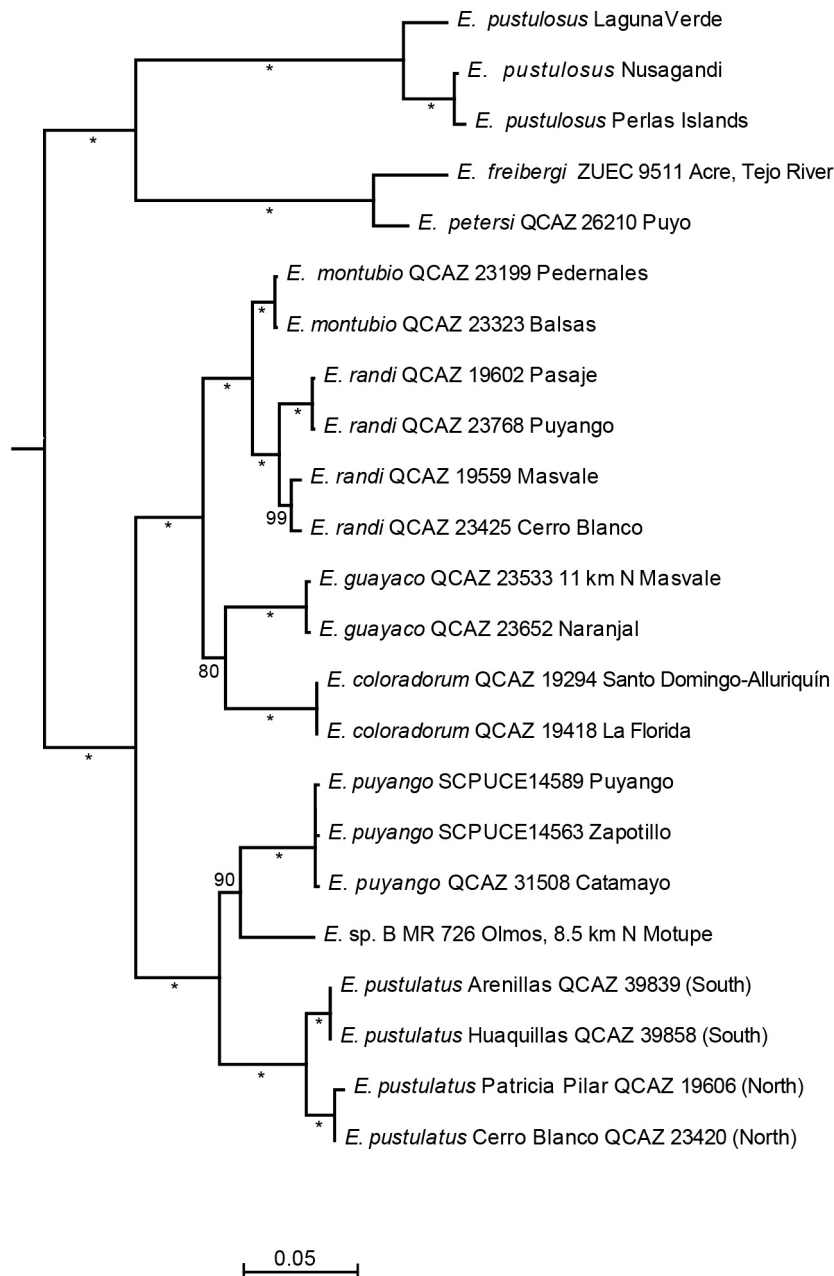
Phylogenetic relationships

The phylogeny is shown in Figure 11. The topology is consistent with the phylogenies reported by Ron *et al*. (2006). Nodal support is strong (bootstrap values ≥ 90) for all but one node. *Engystomops puyango* is sister to *Engystomops* sp. B, from northwestern Peru (Departamento Lambayeque, Olmos); *E. puyango* + *E.* sp. B are sister to *E. pustulatus*. There are large genetic distances between populations of *E. pustulatus* and *E. puyango* that are separated by only 30 km (Arenillas vs. Bosque Protector Puyango uncorrected *p*-distance = 0.066). Within *E. puyango*, the three sampled populations are almost identical (*p*-distance < 0.004) despite geographic distances of up to 115 km.

Within *E. pustulatus* two clades are strongly supported, one is distributed north of the central Costa Region (Cerro Blanco and Patricia Pilar) and the other is in the southern Costa Region (Huaquillas + Arenillas). Pairwise uncorrected *p*-distance between both clades range from 0.019 to 0.021. This genetic differentiation is paralleled by differentiation in morphometric and acoustic space between clades (Figs. 4 and 8).

Discussion

Evolution of call complexity in Engystomops. Our call analyses show that the new species as well as its sister species, *E. pustulatus*, have a previously unknown call component that has some structural similarity to the chuck in the calls of *E. pustulosus* (Ryan, 1985) and the squawk in the calls of *E. petersi* and *E. freibergi* (Funk*, et al.*, 2008). The chuck and the squawk have been used as models for the study of the evolution of complex traits under sexual selection for over three decades (e.g., Boul*, et al.*, 2007; Rand & Ryan, 1981). Our findings of a similar but less discrete element in closely related species shed light on the evolutionary origin of call complexity in this system.

FIGURE 11. Phylogenetic relationships of *Engystomops*. Maximum likelihood tree obtained under the GTR + Γ + I model of character evolution using GARLI (ln$L$ = -9674.4). Numbers at branches are nonparametric bootstrap values (from 500 maximum likelihood pseudoreplicates); asterisks represent 100 values.

The third component shares with the chuck and the squawk its temporal position and a high dominant frequency (increase of over 2000 Hz relative to the whine in *E. puyango*). Our laboratory and field observations show that *E. puyango* and *E. pustulatus* add the third component when other individuals approach; this tendency parallels observations for the production of the chuck and squawk which are also facultative elements (Rand & Ryan, 1981; Ryan, 1985).

The chuck differs from the third component in *E. puyango* and *E. pustulatus* in having a lower fundamental frequency (half the fundamental of the whine) and obligatory doubling in the number of harmonics (doubling is infrequent in the third component of *E. puyango* and *E. pustulatus*). The transition between the whine and the chuck is abrupt and several chucks (up to six) can be added as seemingly discrete and well defined units (Ryan, 1985). In contrast, in *E. puyango* and *E. pustulatus* the third component varies

from barely evident to well defined and the transition with the whine is gradual. Overall, the third component has less structural complexity than the chuck or the squawk in *E. pustulosus*, *E. freibergi*, and *E. petersi*.

The chuck, the squawk, and the third component should be homologous according to Remane (1956) set of criteria to identify homologous traits. The criteria are (1) similarity of position in an organ system (which for advertisement calls can be equated to temporal position), (2) special quality (e.g., shared fine structure), and (3) continuity through intermediate forms. The facultative components of all four species have the same temporal position and similar acoustic structure. Continuity through intermediate forms is suggested by an experiment in which surgical elimination of a fibrous mass attached to the larynx resulted in the production of chucks without intermediate harmonics, similar to *E. puyango* and *E. pustulosus* (Gridi-Papp, *et al.*, 2006).

If in fact the facultative components are homologous, they likely originated in the most recent common ancestor of *Engystomops* (i.e., the most recent common ancestor of the five species) because, as far as we know, this trait is absent in *Physalaemus* (*Engystomops* sister clade). This scenario is supported by the presence of a structurally similar third component in *E. coloradorum* (S. R. Ron, unpublished data). The origin of additional complexity observed in the chuck and squawk in the *E. pustulosus-E. petersi* clade (probably linked to the appearance of enlarged fibrous masses attached to the larynx) should be a more recent event, taking place after the divergence of the two basal clades within *Engystomops*, Edentulus and Duovox (Ron, 2008).

Interestingly, the absence of a third component in the smaller *E. randi*, *E. montubio*, and *E. guayaco* could be the result of a secondary loss. Loss of complexity in sexually selected traits is puzzling given the general tendency of females to prefer complex and/or energetically expensive calls (Ryan & Keddy-Hector, 1992; Wells, 2007). Schluter & Price (1993) proposed a sexual selection model in which females switch their preferences from one male trait to another. It is plausible that this type of process could explain the loss of the third component and the gain of a pulsed first component characteristic of the calls of *E. randi*, *E. montubio*, and *E. guayaco*.

Acknowledgments

Literature cited

Ananias, F., Bombeiro, A.L., Silva, C.D., Silva, P., Haddad, C.F.B. & Kasahara, S. (2007) Cytogenetics of *Eupemphix natterei* Steindachner, 1863 (Anura: Leiuperidae) and karyotypic similarity with species of related genera: taxonomic implications. *Acta Zoologica Sinica*, 53, 285–293.

Boul, K.E., Funk, W.C., Darst, C.R., Cannatella, D.C. & Ryan, M.J. (2007) Sexual selection drives speciation in an Amazonina frog. *Proceedings of the Royal Society B-Biological Sciences*, 274, 399–406.

Cannatella, D.C., Hillis, D.M., Chippindale, P.T., Weigt, L., Rand, A.S. & Ryan, M.J. (1998) Phylogeny of frogs of the *Physalaemus pustulosus* species group, with an examination of data incongruence. *Systematic Biology*, 47, 311–335.

Carl Zeizz. (2005) Axio Vision 4.0, Göttingen.

Cerón, C., Palacios, W., Valencia, R. & Sierra, R. (1999) Las formaciones naturales de la Costa del Ecuador. *In:* Sierra, R. (Ed.) *Propuesta preliminar de un sistema de clasificación de vegetación para el Ecuador continental*. Proyecto INEFAN/GERF-BIRF y Ecociencia, Quito.

Charif, R.A., Clark, C.W. & Fristrup, K.M. (2004) *Raven 1.2 User's Manual*. Cornell Laboratory of Ornitology, Ithaca,

NY, USA.

del Pino, E.M., Venegas-Ferrin, M., Romero-Carvajal, A., Montenegro-Larrea, P., Saenz-Ponce, N., Moya, I.M., Alarcon, I., Sudou, N., Yamamoto, S. & Taira, M. (2007) A comparative analysis of frog early development. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 11882–11888.

Duellman, W.E. (1967) Additional studies of chromosomes of anuran amphibians. *Systematic Zoology*, 16, 38–43.

Duellman, W.E. (1970) Hylid frogs of Middle America. *Monograph of the Museum of Natural History University of Kansas*, 1, 1–753.

Fleming, R.I., Mackenzie, C.D., Cooper, A. & Kennedy, M.W. (2009) Foam nest components of the tungara frog: a cocktail of proteins conferring physical and biological resilience. *Proc Biol Sci*, 276, 1787–1795.

Funk, W.C., Angulo, A., Caldwell, J.P., Ryan, M.J. & Cannatella, D.C. (2008) Comparison of morphology and calls of two cryptic species of *Physalaemus* (Anura: Leiuperidae). *Herpetologica*, 64, 290–304.

Funk, W.C., Caldwell, J.P., Peden, C.E., Padial, J.M., De la Riva, I. & Cannatella, D.C. (2007) Tests of biogeographic hypotheses for diversification in the Amazonian forest frog, *Physalaemus petersi*. *Molecular Phylogenetics and Evolution*, 44, 825–837.

Green, D.M. & Sessions, S.K. (1991) Nomenclature for chromosomes. *In:* Green, D.M. & Sessions, S.K. (Eds.) *Amphibian Cytogenetics and Evolution*. Acacemic Press, San Diego.

Gridi-Papp, M., Rand, A.S. & Ryan, M.J. (2006) Complex call production in the túngara frog. *Nature*, 441, 38.

Howell, W.M. & Black, D.A. (1980) Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Amino Acids/*analysis*, 36, 1014–1015.

Jiménez de la Espada, M. (1872) Nuevos batracios americanos. *Anales de la Sociedad Española de Historia Natural, Madrid*, 1, 85–88.

Kasahara, S. (2009) *Introdução à pesquisa em citogenética de Vertebrados*. Sociedade Brasileira de Genética, Ribeirão Preto.

King, M. (1980) C-banding studies on Australian hylid frogs: secondary constriction structure and the concept of euchromatin transformation. *Alligators and Crocodiles/genetics*, 80, 191–217.

Lourenço, L.B., Cardoso, A.J. & Recco-Pimentel, S.M. (2000) Cytogenetics of *Edalorhina perezi* (Anura, Leptodactylidae). *Cytologia*, 65, 359–363.

Lourenço, L.B., Nascimento, A.A., Andrade, G.V., Rossa-Feres, D.C. & Recco-Pimentel, S.M. (2006) Chromosomal analysis of the leptodactylids *Pleurodema diplolistris* and *Physalaemus nattereri*. *Amphibia-Reptilia*, 27, 481–489.

Lynch, J.D. & Duellman, W.E. (1997) Frogs of the genus *Eleutherodactylus* in Western Ecuador. *Special Publication The University of Kansas Natural History Museum*, 23, 1–236.

Narváez, A.E. (2010) Comportamiento reproductivo y análisis de dieta en *Engystomops pustulatus* (Anura: Leiuperidae). Unpublished Licenciatura Thesis. Pontificia Universidad Católica del Ecuador, Quito.

Nascimento, L.B., Caramaschi, U. & Goncalvez-Cruz, C.A. (2005) Taxonomic review of the species groups of the genus *Physalaemus* Fitzinger, 1862 with revalidation of the genus *Engystomops* Jiménez-de-la-Espada, 1872 and *Eupemphix* Stendachner, 1863 (Amphibia, Anura, Leptodactylidae). *Arquivos do Museu Nacional, Rio de Janeiro*, 63, 297–320.

Quindere, Y.R., Lourenço, L.B., Andrade, G.V., Tomatis, C., Baldo, D. & Recco-Pimentel, S.M. (2009) Polytypic and polymorphic cytogenetic variations in the widespread anuran *Physalaemus cuvieri* (Anura, Leiuperidae) with emphasis on nucleolar organizing regions. *Biological Research*, 42, 79–92.

Rand, A.S. & Ryan, M.J. (1981) The adaptive significance of a complex vocal repertoire in a Neotropical frog. *Zeitschrift für Tierpsychologie*, 57, 209–214.

Remane, A. (1956) *Die Grundlagen des natfirlichen Systems, der vergleichenden Anatomie und der Phylogenetik. Theoretische Morphologie und Systematik I, second ed.* Geest & Portig, Leipzig.

Ron, S.R. (2008) The evolution of female mate choice for complex calls in túngara frogs. *Animal Behaviour*, 76, 1783–1794.

Ron, S.R., Cannatella, D.C. & Coloma, L.A. (2004) Two new species of *Physalaemus* (Anura : Leptodactylidae) from western Ecuador. *Herpetologica*, 60, 261–275.

Ron, S.R., Coloma, L.A. & Cannatella, D.C. (2005) A new, cryptic species of *Physalaemus* (Anura: Leptodactylidae) from western Ecuador with comments on the call structure of the *P. pustulosus* species group. *Herpetologica*, 61, 178–198.

Ron, S.R., Santos, J.C. & Cannatella, D.C. (2006) Phylogeny of the túngara frog genus *Engystomops* (= *Physalaemus pustulosus* species group; Anura; Leptodactylidae). *Molecular Phylogenetics and Evolution*, 39, 392–403.

Ryan, M.J. (1985) *The Tungara Frog: a study in sexual selection and communication*. The University of Chicago Press, Chicago.

Ryan, M.J. & Keddy-Hector, A. (1992) Directional patterns of female mate choice and the role of sensory biases. *American Naturalist*, 139, S4–S35.

Ryan, M.J. & Rand, A.S. (2001) Feature weighting in signal recognition and discrimination by túngara frogs. *In:* Ryan, M.J. (Ed.) *Anuran Communication*. Smithsonian Institution Press, Washington.

SAS Institute. (2008) *User guide. Version 8.01*. SAS Institute, Cary.

Schluter, D. & Price, T. (1993) Honesty, perception and population divergence in sexually selected traits. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 253, 117–122.

Schmid, M. (1978) Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. *Alligators and Crocodiles/genetics*, 66, 361–388.

Schmid, M., Olert, J. & Klett, C. (1979) Chromosome binding in Amphibia. III. Sex chromosomes in *Triturus*. *Alligators and Crocodiles/genetics*, 71,

Sierra, R., Cerón, C., Palacios, W. & Valencia, R. (1999) Mapa de vegetación del Ecuador Continental 1:1'000.000. Proyecto INEFAN/GEF-BIRF, Wildlife Conservation Society y Ecociencia, Quito.

Simmons, J.E. (2002) Herpetological collecting and collection management. *Herpetological Circular*, 31, 1–153.

Tárano, Z. & Ryan, M.J. (2002) No pre-existing biases for heterospecific call traits in the frog *Physalaemus enesefae*. *Animal Behaviour*, 64, 599–607.

Tomatis, C., Baldo, D., Kolenc, F. & Borteiro, C. (2009) Chromosomal variation in the species of the *Physalaemus henseii* group (Anura: Leiuperidae) *Journal of Herpetology*, 43, 555–560.

Vences, M. & Wake, D.B. (2007) Speciation, species boundaries and phylogeography of amphibians. *In:* Heatwole, H. & Tyler, M.J. (Eds.) *Amphibian Biology: Systematics*. Surrey Beatty & Sons Pty. Limited, Chipping Norton.

Wells, K.D. (2007) *The Ecology and Behavior of Amphibians*. The University of Chicago Press, London.

Zwickl, D.L. (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence dataset under the maximum likelihood criterion University of Texas, Austin.

Appendix I. Examined specimens.

Engystomops coloradorum: ECUADOR: PROVINCIA SANTO DOMINGO DE LOS TSÁCHILAS: Tinalandia (QCAZ 35696–97, 36057–58); Río Toachi near to Parador Colorado, 649 m (QCAZ 28508, 28517, 28519–20, 28523, 28525–26); 1–5 km NW from La Florida, 1003 m (QCAZ 2975, 19289,19373–74, 19417–18, 19439–41); Near Alluriquín (QCAZ 19336, 35692–95); on road between Alluriquín and Santo Domingo de los Tsáchilas (QCAZ 29291, 19294); 6 km E Santo Domingo de los Tsáchilas (USNM 212256); Río Baba, 5–10 km SSW Santo Domingo de los Tsáchilas (KU 146194); Río Cupa (USNM 196869); near to Finca La Gloria, 800 m (QCAZ 19287, 19436, 19438).

Engystomops montubio: ECUADOR: PROVINCIA MANABÍ: Pedernales, 85 m (QCAZ 23197–3201); Estero Ancho, 329 m (QCAZ 23188–192); Río Chico, 24 m (QCAZ 23252–59); road between Canoas and San Vicente, 10–20 m (QCAZ 23204–05, 23207–09, 23231–36); San Vicente, 10 m (QCAZ 23210, 23212, 23214–18, 23220–24, 23226–30); Portoviejo, 56 m (QCAZ 23237–247, 23249); Puerto Rico, 30 m (QCAZ 19366, 19375, 19377–380, 19511, 19515–17, 19519–522, 19524 [holotype], 19526–527, 19530–33, 19549–550, 19552, 19555–57). PROVINCIA GUAYAS: Montañita, 20 m (QCAZ 23271–282); on road between El Palmar and Balsas, 5–110 m (QCAZ 23323, 23325, 23330–31, 23373–77, 23379–81, 23386–392, 23397).

Engystomops petersi: ECUADOR: PROVINCIA ORELLANA: Estación Científica de la Universidad Católica del Ecuador, Parque Nacional Yasuní, 240 m (QCAZ 14733–38).

Engystomops pustulatus: ECUADOR: PROVINCIA MANABÍ: Los Frailes; Parque Nacional Machalilla, 5 m (QCAZ 15938–40); Portoviejo, 50 m (QCAZ 3211–12, 3214–19); El Descanso (QCAZ 3156); Puerto Rico, 10 m (QCAZ 19355, 19513–14, 19518, 19523, 19541–42, 19545–48, 19551, 19553–54); Pedernales, minas del río Quaque, 50m (QCAZ 37455–57, 37459–60, 37462–65, 37469). PROVINCIA LOS RÍOS: Quevedo, 81 m (QCAZ 26837–39); San Pedro, 72 m (QCAZ 26840–41, 26844–50); Patricia Pilar, 200 m (QCAZ 19538–40, 19605–14, 19745–47). PROVINCIA GUAYAS: Balzar, 66 m (QCAZ 26914–18); Potreros, 75 m (QCAZ 26852–54); Road El Palmar-Balsas, 50 m (QCAZ 23394–97, 23399–40); Balsas, 107 m (QCAZ 23312–18, 23320–22); Road Las Palmas–Balsas, (QCAZ 23393); Guayaquil (MCZ 7666 [holotype]); Isla Puná (CAS 5408); Cerro Blanco (QCAZ 23427, 38360, 38362, 38365–66, 38370–71, 38373–76, 47031–32). PROVINCIA COTOPAXI: La Maná, 200–250 m (QCAZ 26293, 26520, 26569, 26581, 26682, 26741, 26747, 26761, 26770), 165 m (QCAZ 26071, 26121–22, 26138–65, 26289, 26291–93, 26408, 26413, 26462–67, 26501–02, 26511, 26518–33, 26566–81, 26588–94, 26596, 26598, 26600, 26638, 26646–51, 26653–56, 26658, 26660–64, 26671–78, 26681–83, 26690–96, 26698–709, 26708, 26711, 26713–17, 26721–24, 26726–27, 26729–31, 26733–43, 26745–48, 26759–61, 26763–72, 26775–79, 26786, 26788–89, 26791, 26825). PROVINCIA EL ORO: Huaquillas, campamento Hidalgo & Hidalgo 3 km from Huaquillas, on the road Arenillas-Huaquillas (3.52368° S, 80.20694 ° W), 23 m, QCAZ 39847–66, adult males; Arenillas, Reserva Ecológica Arenillas (3.55374° S, 80.06171° W), 45 m, QCAZ 37671–76, 37677–82, adult males; close to Arenillas, on the road to Guayaquil (3.56232° S, 80.06984° W), 24 m, QCAZ 39826–46, adult males.

Engystomops randi: ECUADOR: PROVINCIA GUAYAS: 11 km N Cerro Masvale, 40 m (QCAZ 23461, 23523); Cerro Masvale, 92 m (QCAZ 19558–560, 19563 [holotype], 19564–66, 19569, 19571, 19573–75, 19577–78, 19585–88, 19590–91, 19597, 19752–55); Surrondings of Estación Cerro Masvale (QCAZ 23470, 23473–74, 23579); Road El Guabo–Machala, 9 km E from Machala (QCAZ 23666–69); on road between Huaquillas and La Cuca, Arenillas, 53 m (QCAZ 23685–91); Puerto Inca, 23489–493. PROVINCIA EL ORO: Entrance to Bosque Protector Puyango, 295m (QCAZ 23762, 23767, 28532, 28691–92, 28706–07, 28741, 28743, 28751–52, 28755).

Engystomops sp. B: PERÚ: DEPARTAMENTO DE CAJAMARCA: road Chiclayo-Cutervo, 14 km to Chongoyape, 364 m (SC 16066, 16070–72). DEPARTAMENTO DE LAMBAYAQUE: road Motupe-Chonchope-Salas, 342 m (SC 16073–76, 16079, 16083–90, 16092). DEPARTAMENTO DE PIURA: road Carrasquillos-Morropón, 3.5 km from Morropón, 125 m (SC 16041–51, 16053, 16055–56, 16062–64).

Appendix II.

Locality data and GenBank accession numbers for *Engystomops* samples added to the Ron *et al.* (2006) matrix of DNA mitochondrial sequences for phylogenetic analysis. All specimens were collected in Ecuador and are deposited at the collection of the Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ).

Species	QCAZ Museum No.	Locality	GenBank Accession No.
E. pustulatus	QCAZ 39839	Provincia El Oro: Arenillas	HQ111349
E. pustulatus	QCAZ 39858	Provincia El Oro: Huaquillas	HQ111348
E. puyango	QCAZ 31508	Provincia Loja: Catamayo	HQ111350