

## A new species of *Leucostethus* (Anura: Dendrobatidae) from the Cordillera Mache-Chindul in northwestern Ecuador, with comments on similar *Colostethus* and *Hyloxalus*

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### Abstract

We describe a new species of dendrobatid frog, *Leucostethus bilsa* sp. nov., using molecular, morphological, and acoustic evidence. We also comment on the taxonomic status of four similar *Hyloxalus* and *Colostethus*. We provide an updated phylogeny of *Leucostethus* that corroborates previous hypotheses of relationships of nine species. Phylogenetic analysis using mitochondrial (i.e., 7095 bp of combined data from NADH1, NAHD2, cytochrome c oxidase I, cytochrome b and 12S-16S rRNA) and seven nuclear genes (i.e., 4739 bp) indicate a close relationship of *L. bilsa* to an undescribed species from Gorgona Island, Colombia, both of which apparently diverged in the Pliocene about 3 million years ago with about 6.25% (i.e., 146/2335 bp) differences for the section of 12S-16S mitochondrial fragment. *Leucostethus bilsa* is diurnal and riparian, characterized by distinctive bright mustard-yellow flash marks in the axillar and groin regions, posterior belly, and in the hindlimbs, the presence of dark gray lower labial stripe or marks, sexual dimorphism in ventral pattern, and by having male uniparental care. We describe its osteology and the male advertisement call, which is a series of peep notes. Osteological microCT images of representatives of each of the Colostethinae genera reveal a number of intriguing characters that may prove to be useful in phylogenetic studies. In terms of its distribution, *Leucostethus bilsa* is currently known only from a very small area within the Reserva Biológica Bilsa, located within the Cordillera Mache-Chindul in the Chocoan region of northwestern Ecuador, which was a Pliocene-Pleistocene refugium. This region is highly threatened with habitat degradation and remains as the last surviving refuge for a forest community known for a high proportion of endemic species of both flora and fauna.

**Key words:** Chocoan, morphology, phylogeny, systematics, vocalization

### Resumen

Describimos una especie nueva de dendrobátido, *Leucostethus bilsa* sp. nov., utilizando evidencia molecular, morfológica y acústica. También comentamos sobre el estado taxonómico de cuatro *Hyloxalus* y *Colostethus* similares. Proveemos una filogenia actualizada de *Leucostethus*, la cual corrobora las hipótesis previas de las relaciones de nueve especies. Un análisis filogenético usando genes mitocondriales (ej. 7095 pb de datos combinados de NADH1, NAHD2, citocromo

c oxidasa I, citocromo b y 12S-16S rRNA) y siete genes nucleares (ej. 4739 pb) indica una relación cercana entre *L. bilsa* y una especie no descrita de la Isla Gorgona, Colombia. Ambas especies aparentemente divergieron en el Plioceno hace unos 3 millones de años con un 6.25% (ej. 146/2335 pb) de diferencias para la sección de fragmento mitocondrial 12S-16S. *Leucostethus bilsa* es diurno y ripario, caracterizado por marcas amarillo mostaza, brillantes y distintivas, en las regiones axilares e inguinales, abdomen posterior y patas, la presencia de una franja o marcas labiales inferiores de color gris oscuro, dimorfismo sexual en el patrón ventral, y por tener cuidado uniparental masculino. Describimos su osteología y el canto de anuncio del macho, el cual es una serie de notas breves. Las imágenes osteológicas microCT de representantes de cada uno de los géneros de Colostethinae revelan una serie de caracteres interesantes que pueden ser útiles en estudios filogenéticos. En cuanto a su distribución, *Leucostethus bilsa* se conoce sólo de una área muy pequeña dentro de la Reserva Biológica Bilsa, ubicada dentro de la Cordillera Mache-Chindul en la región chocoana del noroeste de Ecuador, la misma que fue un refugio del Plioceno-Pleistoceno. Esta región está severamente amenazada por la degradación de su hábitat y lo que resta sigue siendo el último refugio para una selva conocida por su alta proporción de especies endémicas tanto de flora como de fauna.

**Palabras claves:** Chocó, filogenia, *Leucostethus*, morfología, sistemática, vocalización

## Introduction

The Cordillera Mache-Chindul forms a narrow island of elevated landscape parallel to the northwestern Pacific coast of Ecuador, located within a region known for its overall rich biodiversity and high proportion of endemic species (Almendáriz & Carr 1992, Anderson & Jarrín 2002, Clay *et al.* 1995, Neill 1997, Cerón *et al.* 1999, Clark *et al.* 2006, Guayasamin & Bonaccorso 2004, Cuesta-Camacho *et al.* 2007, Cisneros-Heredia *et al.* 2010, Ortega-Andrade *et al.* 2010a). Deforestation and forest fragmentation within northwestern Ecuador has been rapid and ongoing since the 1960s, and the Reserva Biológica Bilsa (RBB) conserves some of the last remaining foothill evergreen forest within the Mache-Chindul range, and the region as a whole (Dodson & Gentry 1991, Sierra 1996, Ortega-Andrade *et al.* 2010b).

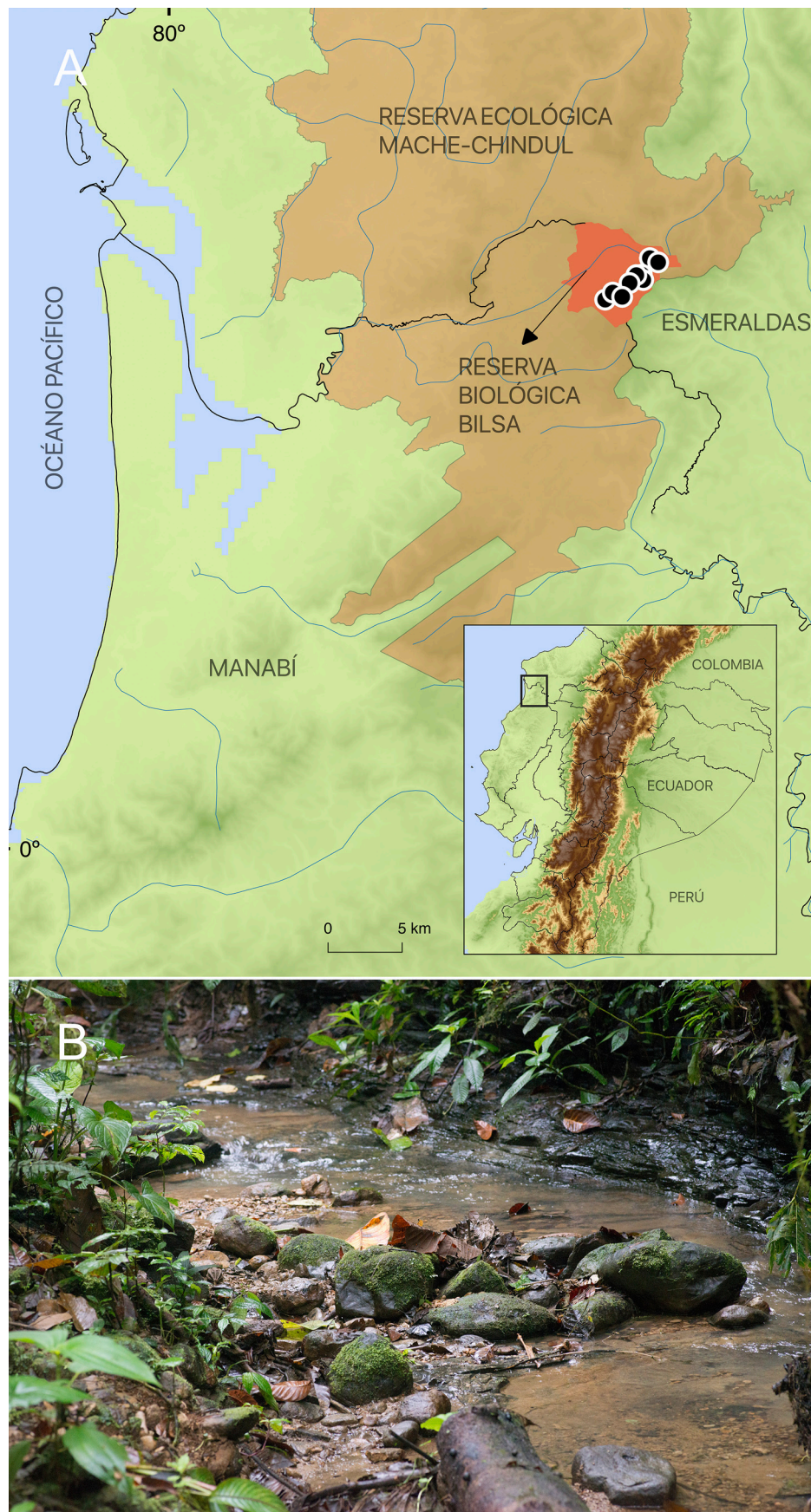
Dendrobatid frogs are prominent components of many lowland and montane anuran faunas in the Neotropics, and the number of described species has increased dramatically over the past three decades. In recent years, several molecular phylogenetic investigations have also addressed the diversity, phylogeny, and nomenclature of this species-rich family (Santos *et al.* 2003, 2009, 2014; Vences *et al.* 2003; Grant *et al.* 2006, 2017, Pyron and Wiens 2011). Grant *et al.* (2017) recognized five genera within the Colostethinae, among which they proposed the new genus *Leucostethus* to accommodate two species of *Colostethus*, *C. fugax* (Morales & Schulte 1993) and *C. argyrogaster* (Morales & Schulte 1993), from the lowlands of Amazonia in Ecuador and Peru. Marin *et al.* (2018) expanded the phylogeny of *Leucostethus* and recognized nine species (six described, three undescribed), distributed in the Andes, Amazonia in Ecuador and Peru, and Gorgona Island in the Pacific region of Colombia.

During field surveys at the RBB between 2000 and 2018 we collected and recorded a series of a relatively large new species (Ortega-Andrade *et al.* 2010b; a) that is here assigned to the genus *Leucostethus* based on a phylogenetic analysis of mitochondrial and nuclear gene sequences and a suite of morphological features. This new species is thus the second species of *Leucostethus* (*sensu* Marin *et al.* 2018) currently known from Ecuador and is, like the other species in this genus, a diurnal, terrestrial, and riparian frog that inhabits steep slopes and small valley streams. We herein describe this new taxon and briefly discuss its phylogenetic position with respect to similar *Colostethus* and *Hyloxalus*.

## Materials and methods

Taxon generic assignment follows Marin *et al.* (2018) that redefined *Leucostethus* from Grant *et al.* 2017. Species boundaries were assessed under the ‘General Lineage Concept of Species’ (de Queiroz 1998, 1999). We used morphological, acoustic, and genetic characters as sources of evidence to recognize the new species. Our description is enhanced by a phylogeny-based delimitation of species boundaries.

Specimens collected were euthanized using benzocaine. Those collected between 2000–2007 were fixed in 10% formalin and stored in 75% ethanol, whereas specimens collected in 2018 were fixed in 80% ethanol and preserved in 75% ethanol. The institutional abbreviations follow Sabaj Pérez (2014) and Frost (2020). Specimens



**FIGURE 1.**—(A) Map showing the collection sites of *Leucostethus bilsa* sp. nov.; (B) habitat of *Leucostethus bilsa* sp. nov. at Duchas stream. Reserva Biológica Bilsa, Provincia Esmeraldas, Ecuador. Map by Gustavo Pazmiño-Otamendi; photo by SH.



examined are housed at Centro Jambatu de Investigación y Conservación de Anfibios (CJ), Biodiversity Institute at The University of Kansas (KU), Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ), Instituto Nacional de Biodiversidad, División de Herpetología (INABIO), and the Museum of Comparative Zoology, Harvard University (MCZ).

**Study site.** Specimens were collected in 2000, 2002, 2004, 2006, 2007, and 2018 at the RBB, a private reserve of Fundación Jatun Sacha with an area of approximately 3300 hectares between 300 m and 800 m elevation (Ortega-Andrade *et al.* 2010b), in the Mache-Chindul mountains, ~27 km W [airline distance] of Quinindé (= Rosa Zárate), border of Esmeraldas and Manabí provinces in northwestern Ecuador (Fig. 1A). Within this range two forest types can be distinguished, coastal Foothill Evergreen Forest (~500–800 m) and Lowland Evergreen Forest (~350–500 m). The wet season occurs from January to May and the dry season from June to December, with an annual average rainfall of 1500–2000 mm (Ortega-Andrade *et al.* 2013). During the drier period, the reserve is often covered by fog at ground level (and dense cloud cover above) and subject to an undetermined but substantial amount of fog drip. Collections were done in primary and old secondary forest in the Green, Black, and White trails (near the Río Rompe Frente) and in the Duchas (Fig. 1B) and Aguacatal-Duchas streams. Topographically, the area is marked by steeply rolling terrain with alternating ridges and streams in narrow valleys. All records were obtained from 1–4 km SW of the RBB main station complex. A detailed map illustrating the location of all trails and streams at Bilsa is presented in Ortega-Andrade *et al.* (2010b).

**Sequencing of nuclear and mitochondrial genes.** New sequences for 15 taxa were generated for the purposes of this study (Table 1). Among them, a single individual (CJ 7211) of the new species from RBB was sequenced, using muscle and liver tissue samples. Samples were preserved in RNAlater solution (Ambion) that is adequate for nucleic acid preservation for Next-Generation Sequencing (NGS). From this preserved tissue, we isolated total RNA using a TRIzol protocol and the integrity, quality and quantity of RNA was determined using nanodrop, Bioanalyzer 2500 and Qubit RNA kits. If the sample showed high quality (i.e., RNA integrity number or RIN > 7.0), we sent it for further processing to Novogene Corp. which included mRNA isolation, directional RNA-Seq dUTP-based library preparation, cDNA libraries barcoding and enrichment using PCR. The final libraries were purified and 150 bp paired-end sequenced with an Illumina 2500 machine with a sequence depth per sample of at least 40 million reads. After completion, the quality of reads was checked with FastQC (Andrews 2010), barcodes were removed with trimmomatic (Bolger *et al.* 2014) and the reads assembled *de novo* with two assembly programs (i.e., MEGAHIT (Li *et al.* 2015) and SPAdes (Bankevich *et al.* 2012)) with a range 25–150 of kmer sizes.

**Retrieval of target nuclear and mitochondrial makers.** With these data, we further isolated known mitochondrial and nuclear markers commonly used across dendrobatids for systematics. These markers include a region of mitochondrial genome of ~7095 bp of combined data from NADH1, NAHD2, cytochrome c oxidase I (COX1), cytochrome b (CYTB) and 12S-16S rRNA genes (Santos *et al.* 2009; Santos *et al.* 2014; Santos & Cannatella 2011, and Grant *et al.* 2017). For nuclear genes with a total length of ~4739 bp, we used seven markers including: brain derived neurotrophic factor (BDNF), bone morphogenetic protein 2 (BMP2), sodium-calcium exchanger 1 (NCX1), 3'-nucleotidase (NT3), proopiomelanocortin (POMC), tyrosinase (TYR), and zinc Finger E-box binding homeobox 2 (ZEB2). Our protocol of sequence isolation using bioinformatics is a modification from a previous approach used in poison frogs (Santos *et al.* 2018): (1) a custom reference library for the selected 13 makers was constructed using the 'makeblastdb' application from the BLAST+ suite (Camacho *et al.* 2009) with nucleotide sequence type format; (2) the *de novo* transcriptome was annotated against this custom reference library using the 'blastn' application from the BLAST+ suite with an e-value < 0.0001; (3) only sequences that matched the references were isolated and further analyzed by performing a 'BLASTn' search at the NCBI website to further confirm that they match known *Leucostethus* and *Colostethus* homologous sequences; (4) any extraneous sequences at the 5' or 3' end from the isolated sequences were further trimmed to ensure that they accurately match and are homologous to the reference sequences; (5) for protein coding sequences (e.g., cytochrome b), the isolated sequences were translated to protein to verify if erroneous stop codons were introduced during the *de novo* reconstruction; (6) the verified sequences were aligned against the reference sequences derived from NCBI; information on voucher specimens, associated locality data, and GenBank accession numbers are summarized in Table 1; (7) we used majority consensus and most polymorphic sites were left with the corresponding ambiguity; (8) finally, we provide the number of unique changes between the new species described herein and its closest known relative, an unnamed species of *Leucostethus* from Gorgona, for the section of 12S-16S mitochondrial fragment.



**TABLE 1.** Taxa included in the phylogenetic analysis, with corresponding museum numbers, collecting localities, and Genbank accession codes (MW in the accession number are sequences generated in this study). Illumina raw reads used to reconstruct these genes can be provided upon request.

Genus	Species	Museum	Country	1216S	COX1	CYTB	ND1	ND2
<i>Ameerega</i>	<i>bilinguis</i>	QCAZ28835	Ecuador, Sucumbíos, Cuyabeno	HQ290996	MW042030	HQ290573	HQ290996	HQ290996
<i>Ameerega</i>	<i>hahneli</i>	QCAZ19240	Ecuador, Orellana, Yasuni	HQ290998	MW042031	HQ290575	HQ290998	HQ290998
<i>Ameerega</i>	<i>parvula</i>	QCAZ16584	Ecuador, Morona Santiago, Méndez	HQ290999	MW042032	HQ290576	HQ290999	HQ290999
<i>Colostethus</i>	<i>imbricolus</i>	MHNUC257_1229	Colombia, Chocó, Bahía Solano	DQ502178	--	--	--	--
<i>Colostethus</i>	<i>imbricolus</i> sp. 1	MAR696	Colombia, Chocó, El Afirmao	MF624213	MF614309	MF614207	--	--
<i>Colostethus</i>	<i>inguinalis</i>	MUJ3247_1348	Colombia, Caldas, Reserva Riomanso	DQ502265	DQ502927	--	--	--
<i>Colostethus</i>	<i>latinus</i>	CH6405	Panamá, Darién, Serranía de Pirre	KF807034	KF806995	--	--	--
<i>Colostethus</i>	<i>panamensis</i>	CH9042	Panamá, Darién, Cerro Sapo	KC129292	KC129185	--	--	--
<i>Colostethus</i>	<i>panamensis</i>	TNHCFS4810	Panamá, Colón, Fort Sherman	HQ290968	--	HQ290546	HQ290968	HQ290968
<i>Colostethus</i>	<i>pratti</i>	AJC1968	Panamá, Panamá, Cerro Brewster	KR863143	KR862888	--	--	--
<i>Colostethus</i>	<i>pratti</i>	TNHCFS4807	Panamá, Colón, PN Portobello	HQ290969	--	HQ290547	HQ290969	HQ290969
<i>Colostethus</i>	<i>pratti</i> sp. 1	TNHCFS4809	Panamá, Bocas del Toro, Cilico Creek	EU342591	--	--	--	--
<i>Colostethus</i>	<i>pratti</i> sp. 2	CH5524	Panamá, Darién, Cana	DQ502173	--	--	--	--
<i>Colostethus</i>	<i>pratti</i> sp. 2	CH5598	Panamá, Darién, Mina de Cana	EU342593	--	--	--	--
<i>Colostethus</i>	<i>pratti</i> sp. 2	MVUP1883	Panamá, Darién, Bahía Pina	MF624214	MF614310	MF614208	--	--
<i>Epipedobates</i>	<i>anthonyi</i>	QCAZ16597	Ecuador, Loja, Macará-Catacocha	HQ290995	MW042033	HQ290572	HQ290995	HQ290995
<i>Epipedobates</i>	<i>boulengeri</i>	TNHCFS4941	Colombia, Cauca, Isla Gorgona	EU342568	--	--	--	MW042040
<i>Epipedobates</i>	sp.	QCAZ16574	Ecuador, Esmeraldas, Durango	HQ290997	MW042034	HQ290574	HQ290997	HQ290997
<i>Epipedobates</i>	<i>darwinwallacei</i>	QCAZ16590	Ecuador, Pichincha, Unión del Toachi	HQ291000	MW042035	HQ290577	HQ291000	HQ291000
<i>Epipedobates</i>	<i>machalilla</i>	QCAZ16527	Ecuador, Manabí, Río Ayampe	HQ290964	MW042036	HQ290542	HQ290964	HQ290964
<i>Leucostethus</i>	<i>argyrogaster</i>	MHNSM22877	Perú, San Martín, Tarapoto-Yurimaguas	EU342606	--	MW042041	MW042042	MW042043

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TABLE 1 (Continued)

Genus	Species	Museum	Country	1216S	COX1	CYTB	ND1	ND2
<i>Leucostethus</i>	<i>brachistriatus</i>	CZPDUV4603	Colombia, Valle del Cauca, Cali	MF624204	MF614304	MF614198	--	--
<i>Leucostethus</i>	<i>brachistriatus</i>	TNHCFS4977	Colombia, Quindio, Lusitania	EU342603	--	MW042051	MW042052	MW042053
<i>Leucostethus</i>	<i>fraterdanieli</i>	MHUAA6242	Colombia	MH591049_ MH591020	MH590990	MH590975	--	--
<i>Leucostethus</i>	<i>fugax</i>	QCAZ16513	Ecuador, Morona Santiago, Santiago	HQ290958	MW042037	HQ290538	HQ290958	HQ290958
<i>Leucostethus</i>	<i>jota</i>	MHUAA8303	Colombia	MH591070_ MH591040	MH591010	MH590985	--	--
<i>Leucostethus</i>	<i>jota</i>	MHUAA9244	Colombia	MH591073_ MH591042	MH591013	MH590988	--	--
<i>Leucostethus</i>	<i>ramirezi</i>	MAR3312/ MAR3318	Colombia, Antioquia, El Almorzadero	MF624216	MF614311	MF614209	--	--
<i>Leucostethus</i>	<i>bilsa</i>	CJ7213	Ecuador, Esmeraldas, Bilsa	MW032695	MW042038	MW042061	MW042062	MW042063
<i>Leucostethus</i>	sp. C1	CZPDUV5015	Colombia, Valle del Cauca, Cerro Dapa	MF624208	--	MF614202	--	--
<i>Leucostethus</i>	sp. C2	I230	Colombia, Caldas, Cordillera Central (western slope)	DQ502179	--	--	--	--
<i>Leucostethus</i>	sp. C2	TG2130	Colombia, Caldas, Vereda Montano	MF624211	--	MF614205	--	--
<i>Leucostethus</i>	sp.	CZPDUV5013	Colombia, Cauca, Isla Gorgona	MF624217	MF614313	MF614211	--	--
<i>Paruwrobates</i>	<i>erythromos</i>	QCAZ37750	Ecuador, Los Rios, Palenque	KJ940458	--	MW042067	MW042068	MW042069
<i>Silverstoneia</i>	<i>flotator</i>	TNHCFS4804	Panamá, Cocle, PN Torrijos	HQ290957	MW042039	HQ290537	HQ290957	HQ290957

TABLE 1. Continued.

Genus	Species	BDNF_brain- derived neuro- trophic factor	BMP2_bone morphogenetic protein 2	NACA_sodium- calcium exchanger 1 NCX1	NT3_neuro- trophin-3 (NT3)	POMC_proopio- melanocortin A (POMC)	TYR_tyrosinase precursor (TYR)	ZFX_zinc finger E-box binding homeobox 2 (ZFX)
<i>Ameerega</i>	<i>bilinguis</i>	HQ290633	HQ291056	HQ290753	HQ290813	HQ290873	HQ290933	HQ290693
<i>Ameerega</i>	<i>hahneli</i>	HQ290635	HQ291058	HQ290755	HQ290815	HQ290875	HQ290935	HQ290695
<i>Ameerega</i>	<i>parvula</i>	HQ290636	HQ291059	HQ290756	HQ290816	HQ290876	HQ290936	HQ290696
<i>Colostethus</i>	<i>imbriculus</i>	--	--	--	--	--	--	--
<i>Colostethus</i>	<i>imbricolussp. 1</i>	--	--	--	--	--	--	--
<i>Colostethus</i>	<i>inguinalis</i>	--	--	--	--	--	--	--
<i>Colostethus</i>	<i>latinus</i>	--	--	--	--	--	--	--
<i>Colostethus</i>	<i>panamansis</i>	--	--	--	--	--	--	--
<i>Colostethus</i>	<i>panamansis</i>	HQ290606	HQ291029	HQ290726	HQ290786	HQ290846	HQ290906	HQ290666
<i>Colostethus</i>	<i>pratti</i>	--	--	--	--	--	--	--
<i>Colostethus</i>	<i>pratti</i>	HQ290607	HQ291030	HQ290727	HQ290787	HQ290847	HQ290907	HQ290667
<i>Colostethus</i>	<i>pratti</i> sp. 1	--	--	--	--	--	--	--
<i>Colostethus</i>	<i>pratti</i> sp. 2	--	--	--	--	--	--	--
<i>Colostethus</i>	<i>pratti</i> sp. 2	--	--	--	--	--	--	--
<i>Colostethus</i>	<i>pratti</i> sp. 2	--	--	--	--	--	--	--
<i>Epipedobates</i>	<i>anthonyi</i>	HQ290632	HQ291055	HQ290752	HQ290812	HQ290872	HQ290932	HQ290692
<i>Epipedobates</i>	<i>boulengeri</i>	--	--	--	--	--	--	--
<i>Epipedobates</i>	<i>sp.</i>	HQ290634	HQ291057	HQ290754	HQ290814	HQ290874	HQ290934	HQ290694
<i>Epipedobates</i>	<i>darwinwallacei</i>	HQ290637	HQ291060	HQ290757	HQ290817	HQ290877	HQ290937	HQ290697
<i>Epipedobates</i>	<i>machalilla</i>	HQ290602	HQ291025	HQ290722	HQ290782	HQ290842	HQ290902	HQ290662
<i>Leucostethus</i>	<i>argyrogastrer</i>	MW042044	MW042045	MW042046	MW042047	MW042048	MW042049	MW042050
<i>Leucostethus</i>	<i>brachistriatus</i>	--	--	--	--	--	MF624157	--
<i>Leucostethus</i>	<i>brachistriatus</i>	MW042054	MW042055	MW042056	MW042057	MW042058	MW042059	MW042060
<i>Leucostethus</i>	<i>fraterdanieli</i>	--	--	--	--	MH590950	MH590950	--

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TABLE 1. Continued.

Genus	Species	BDNF_brain- derived neuro-trophic factor	BMP2_bone morphogenetic protein 2	NACA_sodium- calcium exchanger 1 NCX1	NT3_neuro- trophin-3 (NT3)	POMC_proopio- melanocortin A (POMC)	TYR_tyrosinase precursor (TYR)	ZFX_zinc finger E-box binding homeobox 2 (ZFX)
<i>Leucostethus</i>	<i>fugax</i>	HQ290598	HQ291021	HQ290718	HQ290778	HQ290838	HQ290898	HQ290658
<i>Leucostethus</i>	<i>jota</i>	--	--	--	--	--	--	--
<i>Leucostethus</i>	<i>jota</i>	--	--	--	--	MH590968	MH590968	--
<i>Leucostethus</i>	<i>ramirezi</i>	--	--	--	--	--	MF624158	--
<i>Leucostethus</i>	<i>bilsa</i>	--	MW042064	MW042065	--	--	--	MW042066
<i>Leucostethus</i>	sp. C1	--	--	--	--	--	--	--
<i>Leucostethus</i>	sp. C2	--	--	--	--	--	--	--
<i>Leucostethus</i>	sp. C2	--	--	--	--	--	--	--
<i>Leucostethus</i>	sp.	--	--	--	--	--	MF624159	--
<i>Paruurobates</i>	<i>erythromos</i>	MW042070	MW042071	MW042072	MW042073	MW042074	MW042075	MW042076
<i>Silverstoneia</i>	<i>flotator</i>	HQ290597	HQ291020	HQ290717	HQ290777	HQ290837	HQ290897	HQ290657

**TABLE 2.** Summary of variation in osteological characters found in representative species of Colostethinae. \* = Inferred

	<i>Ameerega trivittata</i> UF 107200 doi:10.17602/M2/ M48873	<i>Colostethus panamansis</i> UF 106871 doi:10.17602/M2/ M140395	<i>Epipedobates anthonyi</i> KU 219763 doi:10.17602/M2/ M48494	<i>Leucostethus bilsa</i> CJ 8311, 7775 doi:10.17602/M2/ M114928	<i>Silverstoneia nubicola</i> KU 76833 doi:10.17602/M2/ M48500	<i>Silverstoneia flotator</i> YPM 020210 doi:10.17602/M2/M50177
Frontoparietals	Not fused	Partially fused	Partially fused	Not fused	Not fused	Fused
Teeth	Absent	Present	Present	Present	Present	Present
Maximum number of teeth in premaxilla	–	8	6	5	3	4
Maximum number of teeth cusps in maxilla	–	37	14	32	15	23
Size of teeth	–	Large	Small	Large	Very large	Very large
Columnella	Bony	Bony	Cartilaginous*	Bony	Bony	Bony
Style of Omosternum	Cartilaginous*	Bony	Cartilaginous*	Bony	Bony	Bony
Xyphisternum mineralization	Not calcified*	Calcified	Not calcified*	Calcified	Calcified	Calcified
Distal coccyx	Not expanded	Expanded	Not expanded	Not expanded	Not expanded	Expanded
Neural spines	Flat upper border	Keel upper border	Keel upper border	Keel upper border	Keel upper border	Keel upper border
Presacral I and II	Non fused	Non fused	Non fused	Non fused	Fused	Non fused
Sacral diapophyses	Oriented posteriorly	Oriented posteriorly	Oriented laterally	Oriented laterally	Oriented laterally	Oriented laterally

**Alignment, model of molecular evolution and phylogenetic estimation.** Sequence alignment of each gene was performed using SATE ver 2.2.7 (Liu *et al.* 2009) and sections with large missing data were excluded. Models of molecular evolution for the tRNAs, rRNAs, and protein-coding genes were determined using jModelTest v 0.1.1 (Posada 2008). The selected molecular models were GTR+G+I (12S, 16S, tRNA-Val), HKY+G+I (ZEB2), TPM1+G (TYR), TPM2uf+I (BDNF), TVM+G (BMP2), TVM+G+I (COX1), TIM2+G+I (CYTB, NCX1, NADH2), TIM2+I (POMC) and TIM3+G+I (NADH1). This matrix was used to estimate a maximum likelihood (ML) phylogeny using Garli ver 2.0 (Zwickl 2006) with 200 nonparametric bootstrap searches for nodal support. A chronogram of this clade was estimated using the best ML topology under a penalized likelihood rate smoothing (PLRS) approach with r8s ver 1.7 (Sanderson 2002). Nodal age for the calibration of the PLRS guide chronogram was derived from previous estimates of the chronogram of Dendrobatidae (*sensu* Santos *et al.* 2009). These time constraints included the crown *Ameerega* at 6.543–10.900 MYA and the split Hyloxinae and Colostethinae at 25.994–35.885 MYA. With these nodal boundaries, a final tree was estimated with the following options of r8s: after 20 random starts; with ‘checkgradient’ option activated; penalty function as additive; optimization parameters under TN routine; smoothing parameter set at  $10^4$  where  $t = 0$  from  $t, t + 1, \dots, t + 9$  for cross-validation; and local perturbation and fractional tolerance set to 0.01.

**Morphology.** Character descriptions mostly follow the format of Marin *et al.* (2018) except that we excluded from our diagnosis features that do not vary within known species of *Leucostethus*. The latter are the dorsolateral stripe (absent), median lingual process (absent), maxillary teeth (present), vomerine teeth (absent), and vocal slits (present) (characters 11, 15, 16, 17, and 18 of the diagnosis of Marin *et al.* (2018)). The absence of the median lingual process is useful to differentiate from *Ectopoglossus*. Also, we added five features that we consider might be relevant to distinguish among species of *Leucostethus*. Criteria for determining the sexual maturity (juvenile, sub-adult, or mature adult) for both sexes was done by examination of the gonadal characteristics and sex was determined by the presence of ovaries or testes, the ventral coloration pattern and presence of vocal slits. Coloration in life is based on notes and photos taken in the field, and under photo studio conditions. Photos are deposited in the CJ photo archives and are available at SapoPediaEcuador ([www.anfibiosecuador.ec/index.php?aw,2](http://www.anfibiosecuador.ec/index.php?aw,2)).

Osteological data of the male holotype (CJ 8311) were obtained via high-resolution X-ray microcomputed tomography (microCT). Scanning was performed at the University of Florida’s Nanoscale Research Facility, using a Phoenix v|tome|x M (GE Measurement & Control Solutions) with a 240kv x-ray tube containing a diamond-tungsten target, with the voltage set to 70 kV and current set to 200  $\mu$ A. Raw x-ray data were processed using GE’s proprietary datos|x software ver. 2.3 to produce a series of tomogram images and volumes, with a final voxel resolution of 15  $\mu$ m. The resulting microCT volume files were imported into VG StudioMax ver. 3.2.4 (Volume Graphics, Heidelberg, Germany), and the skeleton was isolated using VG StudioMax’s suite of segmentation tools. Computed tomography data (tiff stacks and mesh files) have been deposited in MorphoSource (<https://www.morphosource.org>). Their identifiers are given in Table 2. We also cleared and double stained (C&S) (with Alcian Blue and Alizarin Red) a laboratory raised female specimen (CJ 7775, female). The C&S specimen was prepared following Dingerkus & Uhler (1977), with minor modifications. Osteological nomenclature follows Duellman & Trueb (1986), Trueb (1993), and Fabrezi & Alberch (1996).

**Vocalizations.** Two recordings were analyzed. One from a single adult male that was recorded using a Sony TCD-8 DAT recorder with a Sony ECM-S959 microphone; obtained at ~11:35 AM on 5 January 2004 along the Green trail, ~1200m SW of the BBS main station complex; distance between the frog and the microphone was approximately one meter, and the frog was subsequently captured and photographed, but not collected. Another recording was obtained from an adult male (not collected) using an Olympus® DM-901 Digital Voice Recorder connected to a Sennheiser® ME-66 unidirectional microphone. The distance between the frog and microphone was approximately three meters. The call was recorded in an uncompressed format (WAV) with a sampling rate of 44.1 kHz/second and 16 bits of resolution. The sound was obtained on the White Trail on a slope adjacent to the Río Rompe Frente (0,347778N, 79,716389 W; 420 m) during a slight rain on 3 March 2018. The air temperature was 21.4°C. The oscilogram and spectrogram were produced using the software Raven 1.5.0. (Cornell Lab of Ornithology; [www.birds.cornell.edu/raven](http://www.birds.cornell.edu/raven)), with the Hann window at 50% of frame overlap. The size for the Fast Fourier Transformation (FFT) was set on 512, and the frequency grid resolution on 86.1 Hz.

The analyzed call recordings are deposited in full length in the Fonoteca Zoológica ([www.fonozoo.com](http://www.fonozoo.com)) of the Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain, and in the CJ sound archives.

**Red Assessment.** To determine the threatened category of new species, we followed Red List Criteria and



requirements proposed by IUCN (IUCN Species Survival Commission 2012; IUCN 2019). We calculated the Area of Occupancy (AOO) of the species based on the known records of its distribution. AOO is calculated as the area of all known or predicted cells for the species, with a resolution of 2 x 2 km as required by IUCN. Threats on the distribution of this species were assessed based on the Ecuadorian System for Continental Ecosystem proposed by Ministerio de Ambiente del Ecuador (2012), the National System of Protected Areas and deforestation maps from the Unique Environmental Information System- SUIA. (<http://ide.ambiente.gob.ec/mapainteractivo/>).

## Results

### *Leucostethus bilsa* sp. nov

*Colostethus* sp. Ortega-Andrade, Bermingham, Aulestia, and Paucar (2010b), Check List 6: 119–154.

**Proposed standard English name.** Bilsa white-chested frog

**Proposed standard Spanish name.** Rana pecho blanco de Bilsa

(Figs. 2–10, 12D)

**Holotype.** (Figs. 2, 5F, G, 8, 12D). CJ 8311, an adult male collected in a primary forest at the Brown Trail (0.358389 N, 79.708364 W; 478 m) in Reserva Biológica Bilsa (RBB), Quinindé, Provincia de Esmeraldas, Ecuador, 21 March 2018, by Sebastián Hernández-Nieto.

**Paratypes.** (Figs. 3B, 4A, C, D, F, 5A–E, 6A–D, 7A–C, 9) 22 preserved frogs (5 adult females, 4 adult males, 13 non-adults): QCAZ 14699 (gravid adult female), QCAZ 14700 (adult male) both collected between the small streams known locally as the Río Aguacatal and the Río Rompe Frente in the RBB, ~2 km SW of the Bilsa biological station (BBS) central facilities complex (0.355833 N, 79.703333 W, ~450 m elevation, Provincia de Esmeraldas, Ecuador, on the afternoon of 6 July 2000, by Russell G. Burge and William H. Schaedla; MCZ A-140205 (adult female), KU 327677 (sub-adult female) collected by day from areas ~3–4 km SW of the RBB on 15 July 2000, by a group of



**FIGURE 2.**—Live adult holotype (CJ 8311) of *Leucostethus bilsa* sp. nov., SVL = 24.3 mm. There is a skin lesion on the antero-dorsal left thigh. Photos by Diego Acosta-López.

A



B

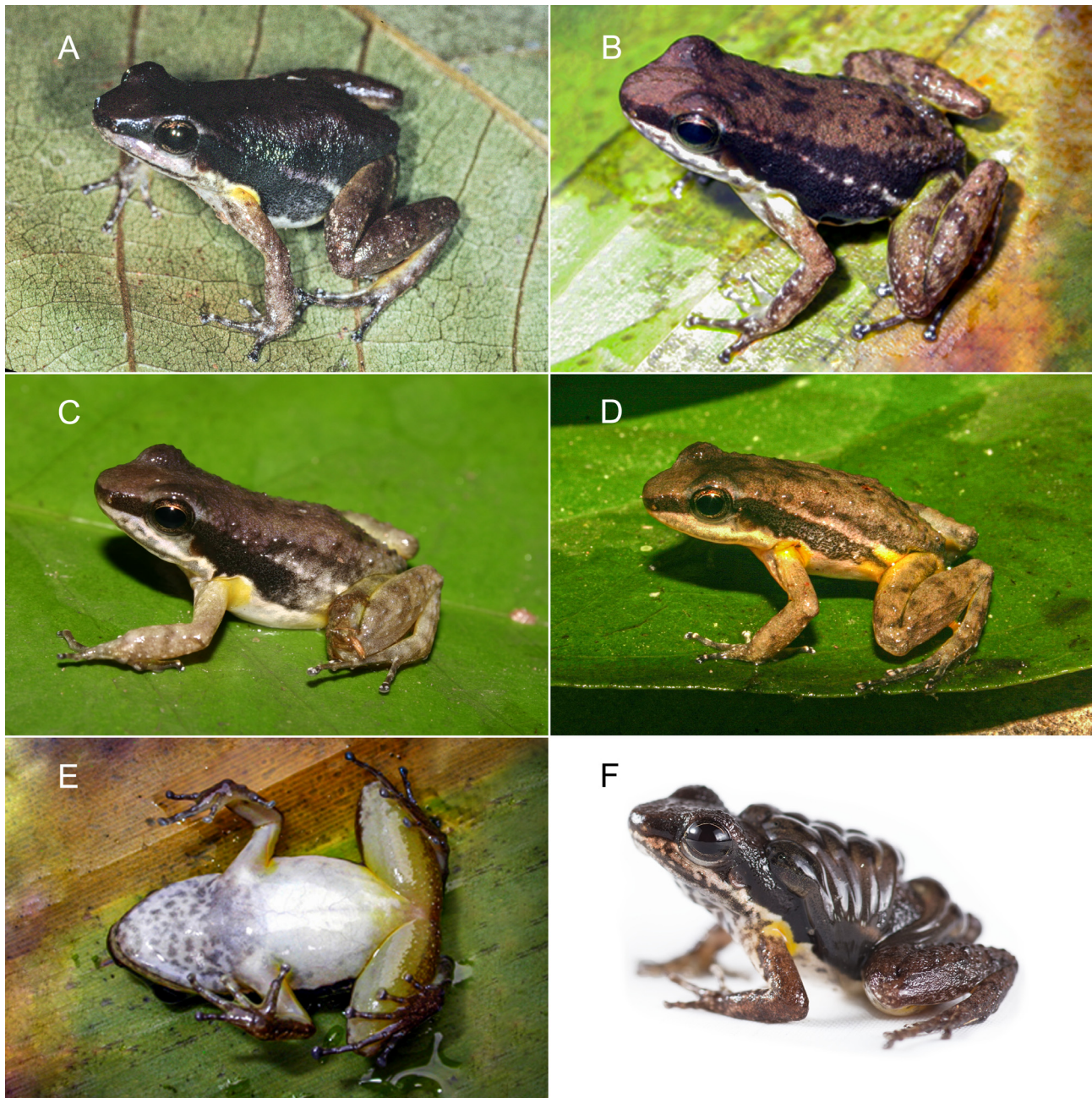


**FIGURE 3.**—Live adults of *Leucostethus bilsa* sp. nov. depicting variation in coloration: (A) CJ 8423, female, SVL = 25.8 mm); (B) CJ 7765, female, SVL = 25.0 mm). Photos by Diego Acosta-López.

Bilsa park guards (collected for Gregory O. Vigle); CJ 7765 (female, SVL = 22.5 mm), CJ 7775 (female, SVL = 25.6 mm, C&S specimen), collected as juveniles and raised to adult stage in the lab, from Río Rompe Frente in the



RBB on 20, 21 January 2018 by Sebastián Hernández-Nieto; CJ 8322 (female, SVL = 28.2 mm) collected from the RBB on 13 May 2018; CJ 8275 and CJ 8299 (post-metamorphs) collected from the Duchas stream (0.345083 N, 79.712347 W; 423 m) of the RBB on 22 February 2018, and 20 March 2018 respectively by Sebastián Hernández-Nieto; CJ 8416 (post-metamorph) collected from the White trail (0.348086 N, 79.716569 W; 426 m) of the RBB on 11 June 2018 by Sebastián Hernández-Nieto; CJ 8198, 8201, 8202, 8204, 8274, 8334, and 8413 (7 juveniles) collected from the RBB on 4, 5 December 2017, 22 February 2018, 15 March 2018 and 11 June 2018 by Sebastián Hernández-Nieto; KU 327679 (adult male carrying larvae) collected by day ~2.3 km SW of the BBS on 16 August 2002, by Scott D. Cashins; INABIO 3632 (adult male carrying larvae) collected ~1 km SW of the BBS on 11 December 2006; INABIO 3649 (sub-adult female) collected ~2 km SW of the BBS on 13 December 2006; INABIO 3785 (sub-adult female), and INABIO 3786 collected ~2 km SW of the BBS on 26 February 2007, by H. Mauricio Ortega-Andrade.



**FIGURE 4.**—Live adults of *Leucostethus bilsa* sp. nov. showing variation: (A) QCAZ 14699, gravid adult female, SVL = 27.4 mm; (B) Not collected, adult male; (C) INABIO 3786, adult male, SVL = 23.0 mm; (D) INABIO 3785, subadult female, SVL = 22.0 mm; (E) ventral view of male (call recording MR2004-1-12) showing gular-chest marks; (F) male (CJ 7213, SVL = 22.8 mm) carrying tadpoles. Photos by GOV (A), MR (B, E), HMO (C, D), SH (F).



**Etymology.** The specific epithet is used as a noun in apposition and refers to the type locality, the Reserva Biológica Bilsa; Bilsa is the name of a nearby river. RBB protects the headwaters of several rivers and belongs to the Chocó Darien-Tumbesino region, one of 25 global “hot spots” known for their high biodiversity and endemism of flora and fauna. This name is also intended to honor the conservation efforts of Fundación Jatun Sacha, which owns and administers the RBB and several other reserves in Ecuador.

**Definition.** *Leucostethus bilsa* is characterized by the following combination of characters: (1) large sized, snout-vent length (SVL) in females 27.4–28.2 mm (n = 3, wild caught), 22.5–28.0 (n = 3, lab-raised); SVL in males 21.7–24.2 mm (mean = 22.7; n = 4); (2) skin of dorsum posteriorly tubercular, smooth anteriorly, but with scattered low tubercles on the lateral sides of the dorsum anteriorly; (3) Finger I slightly longer than Finger II when adpressed; (4) length of Finger IV reaching distal half of distal subarticular tubercle of Finger III; (5) Finger III not swollen in adult males; (6) lateral fringes absent on fingers; (7) discs on fingers weakly to moderately expanded; (8) webbing absent between fingers; (9) outer tarsal fold absent, inner tarsal fold well-developed; (10) lateral fringe absent on Toe IV, but keels are present in toes II–IV; (11) discs on toes weakly to moderately expanded; (12) basal webbing between toes III–IV–IV, only trace webbing between toes II–III; (13) ventrolateral stripe absent, but junction of dark lateral with pale ventral surfaces abrupt, forming a sharply distinct linear border; (14) cream oblique lateral stripe present, usually incomplete; (15) in life, mustard-yellow paracloacal marks present; (16) in life, bright mustard-yellow flash marks in axillae, groin, and antero-ventral surfaces of hindlimbs; (17) in life, ventral surfaces of body white with yellow in posterior portion of belly, with gray mottling on chin; throat, and chest in adult males; (18) testis color polymorphic, white or brown-gray; (19) thin distinct dark stripe or flecks present along lower lip; (20) advertisement call composed of a series of peep notes emitted at a mean rate of 81 notes/minute, with dominant frequency of 3310–3722 Hz.

**Comparisons with other species.** *Leucostethus bilsa* differs from all *Leucostethus* (except *L. argyrogaster*) by lacking the Finger III swollen in adult males. *Leucostethus bilsa* is compared with all other *Leucostethus* described (*L. argyrogaster*, *L. brachistriatus* (Rivero & Serna 1986), *L. fraterdanieli* (Silverstone 1971), *L. fugax*, *L. jota* Marin *et al.* 2018, and *L. ramirezi* (Rivero & Serna 2000), and similar species of Dendrobatidae occurring in the lowlands of northwestern Ecuador (*Hyloxalus toachi* (Coloma 1995), *H. awa* (Coloma 1995), *H. infraguttatus* (Boulenger 1898)). *Leucostethus bilsa* is distinguished from *L. argyrogaster* by having bright mustard-yellow flash marks (absent in *L. argyrogaster*). *Leucostethus bilsa* differs from *L. fraterdanieli* (characters in parenthesis) in that the flash marks have diffuse borders (more defined), and form crescent-shaped arcs around arm insertions, extending to ventral surfaces (do not consistently form crescent-shaped arcs). Also, *L. bilsa* differs in having a complete to incomplete oblique lateral stripe (prominent and fully complete oblique lateral stripe), a dark line below oblique lateral stripe is absent (the oblique lateral stripe is bordered below by a distinct dark line). Ventrolateral patterns also differ; while both species lack a pale ventrolateral stripe, the border between the dark flanks and pale ventral surfaces is abrupt and linear in *L. bilsa*, forming a sharply defined straight or slightly sinuous line (fragmented). Dorsally (in life) both species are cryptically colored, with *L. bilsa* varying from brown to charcoal (dorsum may also have green, orange or golden tinges). *Leucostethus bilsa* further differs from *L. brachistriatus*, *L. fugax*, *L. jota*, and *L. ramirezi* by having an oblique lateral stripe that varies from complete to anteriorly faded or broken along its posterior length (pale oblique lateral stripe complete in *L. brachistriatus*, *L. fugax*, *L. jota*, and *L. ramirezi*). Additionally, it differs from *L. jota* by having mustard-yellow paracloacal marks (paracloacal marks absent in *L. jota*) and by lacking a ventrolateral stripe (ventrolateral stripe present in *L. jota*). *Leucostethus bilsa* is larger than *L. ramirezi* (maximum female SVL = 28.0 mm vs 22.8 mm in *L. ramirezi*).

*Leucostethus bilsa* and *Oophaga sylvatica* (Funkhouser 1956) are the largest species of dendrobatid frogs yet reported from western Ecuador; only in *Hyloxalus toachi* does the size of adults of either sex approach or exceed the sizes of adults in the type-series of *L. bilsa* (maximum sizes of adult males and females respectively for *H. toachi* are 23.1 and 28.2 mm SVL). *Leucostethus bilsa* may readily be distinguished from *H. toachi*, *H. awa*, and *H. infraguttatus* by having bright mustard-yellow axillary and groin flash marks, and presence of mustard-yellow paracloacal marks (absent in *H. toachi*, *H. awa*, and *H. infraguttatus*).

**Acoustic comparisons.** The call of *Leucostethus bilsa* (see description later under Vocalization) is compared to the calls of *L. fraterdanieli* and *L. jota* described by Marin *et al.* (2018). The calls of *L. brachistriatus* and *L. ramirezi* are unknown. The calls of all three species consist of a long string of roughly equally spaced notes, but the calls mostly differ in the call repetition rate of 98 notes per minute for *L. fraterdanieli*, 87 for *L. bilsa*, and 22 for *L. jota*. *Leucostethus bilsa* and *L. jota* produce much longer notes, note duration being 139 ms in *L. bilsa*, 140 ms in

*L. jota* vs. 90 ms in *L. fraterdanieli*. The dominant frequency in *L. fraterdanieli* is 3.1–3.5 KHz, whereas the *L. bilisa* (3.6–3.7 KHz) and of *L. jota* (3.7–4.9 KHz) are higher.

**Description of holotype.** CJ 8311, an adult male, 24.17 mm SVL; body robust; head longer than wide, head width 32% of SVL, head length 35.91% of SVL; snout short, truncate and acuminate at the mid-line in dorsal view, bluntly rounded and protruding slightly beyond lower jaw in profile. Loreal region slightly concave; canthus rostralis smoothly rounded, moderately well-defined; nostrils protuberant and directed laterally. Supra-tympanic fold evident but not pronounced, obscuring postero-dorsal portion of tympanic annulus; tympanum prominent, 49.26% of eye length, tympanic annulus prominent, most pronounced on antero-ventral 1/3; tympanum 0.56 mm from eye at closest point.

Forelimbs moderately slender, skin smooth, ulnar fold absent. Finger I slightly longer than finger II when adpressed; relative length of fingers III>I>II>IV. Webbing, lateral fringes, and keels absent on hands and fingers; Finger III not swollen. Palmar tubercle large, irregularly rounded; thenar tubercle elliptical, about 1/2 the size of palmar tubercle; supernumerary tubercles absent; subarticular tubercles large, slightly ovoid, subarticular tubercle on Finger I larger than all others. Terminal discs expanded about 1.3 times on Finger I, discs expanded about 1.5 times on fingers II and III, and 1.6 times the width of the distal end of the adjacent phalanx on finger IV; round, paired dorsal scutes on each disc.

Hind limbs moderately robust, tibia length 48.73% SVL. Outer tarsal fold not evident, inner tarsal fold well developed, extending from mid-point of tarsus and connected to inner metatarsal tubercle. Inner metatarsal tubercle elliptical, raised about two times the size of bluntly rounded conical outer metatarsal tubercle. Toes basally webbed between toes II and III and between toes III and IV; webbing between toes II and III barely evident; webbing between toes III and IV more developed but not extending to proximal edge of first subarticular tubercle on either toe III or toe IV. Webbing absent between other toes, lateral fringes absent on all toes. Subarticular tubercles small, round, largest on toes I and II; first subarticular tubercle present but small on toe IV; supernumerary tubercles absent. Terminal discs of toes I, III, and IV expanded approximately 1.6 times the width of the distal end of the adjacent phalanx, disc on toe II expanded 1.8 times, and disc of toe V 1.5 times wider than distal end of adjacent phalanx.

Skin of venter smooth, skin of dorsum posteriorly tubercular, smooth anteriorly, but with scattered low tubercles also evident on the lateral margins of the dorsum anteriorly (with skin smooth medially except for low rounded granules posteriorly, clustered just anterior to the anus). Anal sheath short; anal opening directed postero-ventrally at upper level of thighs, cloacal tubercles absent. Tongue elongated, free posteriorly for almost 3/4 of its length, not notched posteriorly; median lingual process absent. Choanae round, widely separated; vomerine odontophores absent. Vocal slits present.

**Coloration in life.** Dorsum of head and body brown to dark brown to charcoal with few, faintly evident, scattered irregular brown to black spots. Flanks brown to black, with a cream oblique lateral stripe extending from the groin anteriorly to about level of mid-body. The black flank surfaces end in a distinct linear border with the white ventral surface, and the mustard-yellow pigment around the arm and leg insertions; aside from this sharply delineated border, no distinct separate pale ventrolateral stripe is evident in life or after preservation. The flanks are dark brown-gray anteriorly and light gray washed with cream posteriorly. Lateral surfaces of head (from below *canthus rostralis* to the ventral margin of the eye) dark brown to black; upper lip (from ventral margin of eye to mouth) cream, with a faint wash of pale gray pigment. Dorsal surfaces of legs light brown with diffuse dark brown markings consisting of 3 transverse dark brown bands on dorsal surfaces of thighs, with a thin longitudinal stripe of the same color running posteriorly the length of each thigh; shanks with 4 transverse bands of the same color combination as bands on thighs; ankles also with dark brown transverse bands over brown dorsally. Ventral, and proximal antero-ventral surfaces of thighs bright mustard-yellow (remaining antero-ventral surfaces of thighs light brown); this mustard-yellow pigment also extends across the groin, and posterior belly. Light mustard-yellow paracloacal marks present. Ventral and antero-ventral surfaces of shanks also are bright yellow, yellow extending onto the anterior surface of the ankle and fading along the proximo-dorsal surface of the foot. Dorsal and anterior surfaces of arms light brown with dark brown transverse bands and irregular marks; ventral surfaces of arms and hands gray. Proximal 1/2 of the posterior and postero-ventral surfaces of upper arms bright mustard-yellow (same color as on thighs), extending in an arc around the dorsal, posterior, and ventral circumference of each arm. This arc of mustard-yellow pigment is narrowest on that portion of the flanks of the body immediately dorsal to the arm insertion (where it ends at a sharply-defined border with the black lateral surfaces), and then broadens posteriorly (forming a crescent-shaped mustard-yellow mark also defining a crescent-shaped border with the black lateral surfaces), and

ventrally (the ventral portion of this half-circle is the largest, with mustard-yellow pigment extending to a radius around the postero-ventral area of the arm insertion to a width of approximately 1/2 the length of upper arm). The iris was gold to copper/brown, with scattered small irregular black markings.

**Coloration in preservative.** Overall, the dark pigments on the dorsal and lateral surfaces of the body and limbs appear darker and less bright than in life, especially for pattern features that are narrow or small. All major features of pattern (pale or dark bands and stripes, ground color) remain nearly as distinct as in life despite this darkening. The white ventral ground color has faded to a dull cream, but darker pigments remain clearly visible aside from some fading, and all areas that were bright mustard-yellow in life have lost all trace of pigment, leaving only the underlying ground color (dull cream in ethanol).

**Variation.** Measurements of three adult female paratypes, and the ranges and means of measurements of the four adult male paratypes are presented in Table 3. We excluded from Table 3 two adult female paratypes that were lab-raised. The type series includes three subadult females, and ten unsexed juveniles; the three sub-adult females (INABIO 3649, INABIO 3785, and KU 327677) are 20.1, 22.0 and 22.9 mm SVL, respectively. The ten unsexed juveniles measure between 12.6–15.2 mm SVL.

**TABLE 3.** Measurements (in mm) of adults: male holotype, three female paratypes, and the range and means of four male paratypes (QCAZ 14700, KU 327679, INABIO 3632, 3786) of *Leucostethus bilsa* sp. nov.

Character	CJ 8311	Range and Means	CJ 8322	QCAZ 14699	MCZ A-140205
	♂	♂	♀	♀	♀
Snout vent length (SVL)	24.3	21.7–23.5 (22.7)	28.2	27.4	28.0
Head length (HL)	8.7	8.7–9.5 (9.3)	10.0	10.8	11.1
Head width (HW)	7.7	7.1–8.4 (7.7)	10.1	9.4	9.6
Femur length (FemL)	10.8	9.3–10.5 (9.9)	13.2	10.8	12.0
Tibia length (TibL)	11.8	10.9–11.2 (11.1)	12.6	13.1	13.4
Foot length (FootL)	10.1	9.6–11.0 (10.2)	11.2	11.8	12.0
Inter orbital distance (IOD)	2.9	2.7–3.0 (2.9)	3.6	3.5	3.7
Eye diameter (ED)	3.0	2.1–3.0 (2.8)	3.4	3.5	3.6
Tympanum diameter (TymD)	1.5	1.5–2.5 (1.7)	2.0	2.1	2.0
Eye to nostril distance (END)	2.6	2.0–3.0 (2.4)	2.4	2.7	2.8

In some individuals, the patches and spots of dorsum are more evident over the background brown color. The posterior flanks vary from cream to gray with the pattern from irregularly spotted to reticulated, gradually fading into the pale ventral pattern. In some specimens, the longitudinal stripe running the length of each thigh is bordered posteriorly by a lighter stripe. Bright mustard-yellow markings in the groin and upper arm areas were clearly visible in life in all specimens. The paracloacal mustard-yellow marks are also present varying from light (holotype) to more contrasting (e.g. CJ 7765, Fig. 5A). In all specimens, the bright mustard-yellow appeared to be a separate outer layer of pigment overlying the ground color of white, which faded very rapidly following preservation (within 24–48 hrs.), leaving only the white visible.

Black lip stripes occur along the lips. One in the upper lip and another along the dorsal margin of the lower lip (LLS). They occur in all specimens (with the line complete around the entire lower lip, and visible in preservative), but considerable variation is evident in the type series. In life, the LLS vary from a sinuous faint gray line visible only on close inspection of the dorsal margin of the lower lip, to a prominent straight black line that is obvious even from some distance (Fig. 4E). The LLS of paratype QCAZ 14699 was more prominent in life than any other specimen, as is the LLS in preservative of adult female MCZ A-14025. The LLS is generally darker and more obvious in larger individuals. Among the remaining adult and sub-adult preserved specimens, the LLS is clearly visible under magnification, with no indication of sexual dimorphism.

The pale oblique lateral stripe (OLS) of *Leucostethus bilsa* (among adults and sub-adults) varies in length and continuity, with some specimens exhibiting a complete OLS from groin to eye, and others exhibiting a faded, broken, or incomplete OLS (Figs. 3A, B, 4A–D). It fades to gray-brown anteriorly in some individuals; for example, in KU 327677 (sub-adult female) and KU 327679 (adult male) extending only from the groin to level of mid-body in the flank. The OLS is not present in three juveniles, but clearly evident in sub-adult specimens.





**FIGURE 5.**—Flash coloration, hands, and feet in *Leucostethus bilsa* sp. nov. (A–E) live adult female CJ 7765; (F–G) live adult male holotype CJ 8311. Note the mustard-yellow paracloacal marks on the dorsal thighs (A), and the mustard-yellow flash marks on axillae, groin, and hind limbs (B, C). Photos by Diego Acosta-López.

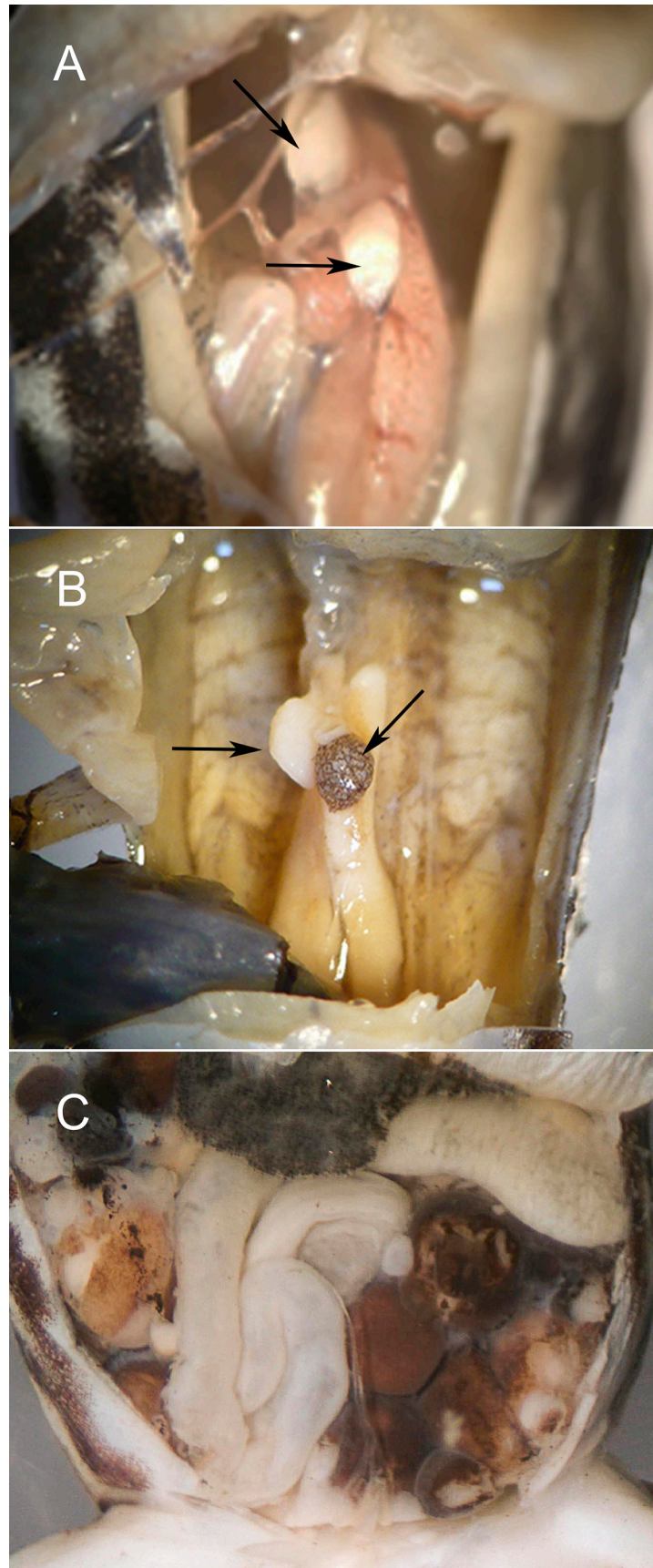
There is sexual dimorphism in ventral coloration pattern. In the adult male holotype CJ 8311 and all four-adult male paratypes, the anterior ventral surfaces (chin and throat) are spotted gray over cream to white ground color (in life and after preservation), with the dark pigment forming a reticulated pattern in some individuals; the dark spotting also continues on venter in both of the adult males found carrying larvae (INABIO 3632 and KU 327679),

extending to a point approximately one-half of the distance between arms and thighs. In both sub-adult and adult females, the anterior ventral surfaces (chin and throat) are cream to white with a faint and diffuse pattern of pale brown to gray flecks (in life and after preservation); in some individuals, these flecks coalesce to form a reticulated pattern, but this is not pronounced and can be readily seen only under magnification, and the entire abdomen is unmarked cream to white.



**FIGURE 6.**—Dorsal (upper row) and ventral (lower row) aspects of preserved paratype specimens of *Leucostethus bilsa* sp. nov. (A) INABIO 3632, adult male; (B) INABIO 3786, adult male; (C) INABIO 3649, juvenile female; (D) INABIO 3785, sub-adult female). Note dark spotting on the chin of adult males (INABIO 3632 and 3786), in contrast to pale unmarked ventral surfaces of females (INABIO 3649 and 3785), and dark spotting extending ventro-laterally on adult male INABIO 3632. Photos by HMOA.

No pronounced differences in color pattern among the three immature females and the six wild-caught adult specimens (2 females, 4 males) were noted, suggesting that ontogenetic color pattern changes are complete once the frogs reach ~20 mm SVL (although no sub-adult males are included in the type series). The three post-metamorphic unsexed juveniles were all nearly uniform brown or dark brown in life both dorsally and laterally, with oblique lateral stripes only very faintly evident under magnification. Despite the differences in dorsal, lateral, and oblique lateral stripe color as compared to sub-adults and adults, all three juveniles also exhibited obvious bright mustard-yellow flash marks in the axillary and groin regions. One noteworthy observation among these three juveniles pertains to their relative sizes in comparison to the respective lengths of their tail remnants; the two smallest juveniles (QCAZ 20137 and KU 327678) measure 12.8 and 13.4 mm SVL, with tail remnants of 1.7 and 0.7 mm, respectively. In contrast, the largest juvenile (QCAZ 14701) measures 14.0 mm SVL, yet has a tail remnant measuring 5.5 mm in length. There is no evidence of any ontogenetic change in ventral pattern.



**FIGURE 7.**—Testes (indicated by arrows) and eggs of *Leucostethus bilsa* sp. nov., showing differences in pigmentation: (A) both testes unpigmented white (INABIO 3632); (B) one testis unpigmented white, and one testis brown (QCAZ 14700); (C) eggs (CJ 7765). Photos (A, B) by Verónica Armas-Ortiz and HMOA and (C) by Diego Acosta-López and Andrea Terán-Valdez.



The color of the testis varies intraspecifically and intraindividually. Two adult males of *C. bilsa* (QCAZ 14700 and INABIO 3786) each have one white (right) and one brown (left) testis (see Fig. 7). However, both testes are white in the adult male holotype CJ 8311, and one adult male, INABIO 3632 (Fig. 7A), while both testes are brown in another (KU 327679). Oocytes in advanced stages are brown (Fig. 7C).

**Osteology.** General osteological features of the male holotype (CJ 8311) are depicted in Fig. 8, and the hyobranchium and pectoral girdle of a paratype (CJ 7775, female) are shown in Fig. 9.

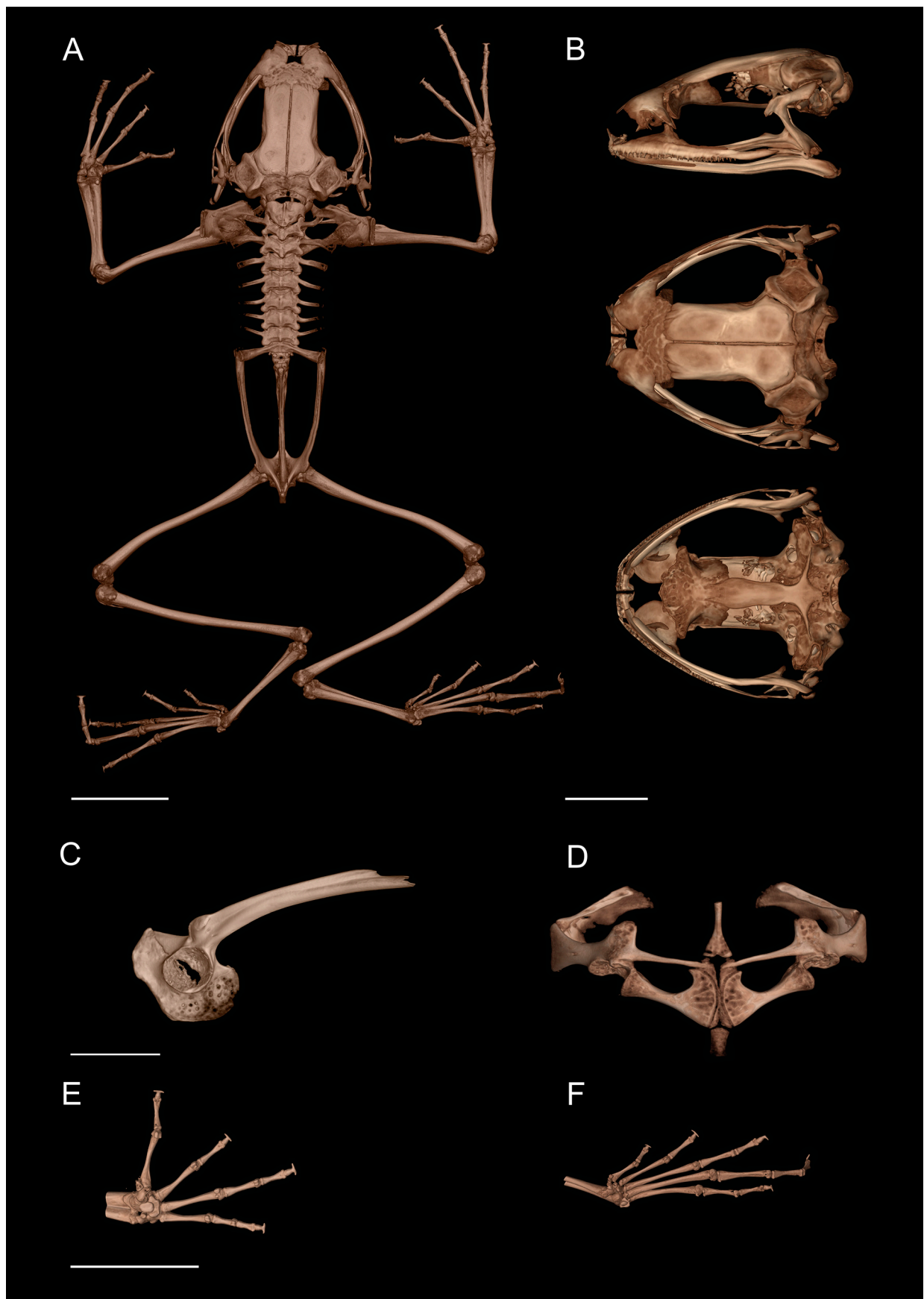
**Cranium.** The skull is longer than wide, about half as tall as long (skull length: 8.9 mm; skull width: 8.2 mm; skull height: 4.6 mm), lacks dermal ornamentation, but has rugose surfaces on some bones. The neurocranium is moderately ossified. The septomaxilla is small and round. The nasals are subcircular, bearing an acuminate posterolateral maxillary flange, but do not articulate with the pars facialis of the maxillary. The nasals are widely separated medially. The ossified sphenethmoid slightly underlies the posterior margins of nasals. The frontoparietals overlie the anteromedial prootic posterolaterally; the orbital edges of the frontoparietal are straight. The frontoparietals lack medial articulation. The crista parotica is well developed, long, and narrow; the occipital condyles are widely separated. The squamosal is moderately developed; the otic ramus articulates with the cartilaginous lateral margin of the crista parotica. The pterygoid is moderately developed, the medial ramus is not in contact with the ossified prootic; the anterior ramus extends to the anterior orbit and is in broad contact with the cartilaginous pterygoid process of the palatoquadrate adjacent to the pars dentalis of the maxilla. The quadratojugal is slender and articulates with the posterior end of the maxilla. The maxilla and premaxilla bear mostly monocuspid teeth, with a maximum number of 5 teeth cusps in the premaxillae and 32 in the maxillae. The maxillae are slender, lacking a postorbital process; the preorbital process is moderately well developed; the pars palatina of maxilla is narrow. The alary process of the premaxilla is narrow and curved posterolaterally; the pars palatina of the premaxilla is well-developed and broadly “U”-shaped in ventral aspect. The mentomeckelian bone is slightly constricted, about equal in width at medial and lateral ends. The angulosplenial extends posterior to the jaw articulation. The vomer is small, edentate, and bears a small pre- and a large postchoanal process bordering the anterior and lateral margins of the choana, respectively. The neopalatine is absent. The planum antorbitale is cartilaginous. The parasphenoid is “T”-shaped and lacks dermal modifications; the alae are acuminate, directed slightly posterolaterally; the cultriform process is about twice as wide as transverse length of alae; the anterior end of the cultriform processes bears large bifid projections and extends to the anterior region of the orbit. The pars media plectri of columella is slender. The tympanic annulus is sickle-shaped, with the open side upper. The tympanic region bears a cartilaginous round plate.

**Hyolaryngeal apparatus.** The hyoid plate is longer than wide (measured at midlines) and bears three holes at its anterior portion. The hyale are long, slender, angling posterolaterally to the otic region, wider in proximal than distal half, and bearing a slightly expanded region at the midline. The hyale bears one anterior process; it is straight and its distal end is rounded. The hyoglossal sinus is circumscribed “U-V”-shaped. The alary processes are basally stalked and relatively large. The posterolateral processes are slender and straight. The posteromedial processes are ossified. The cricoid ring is complete.

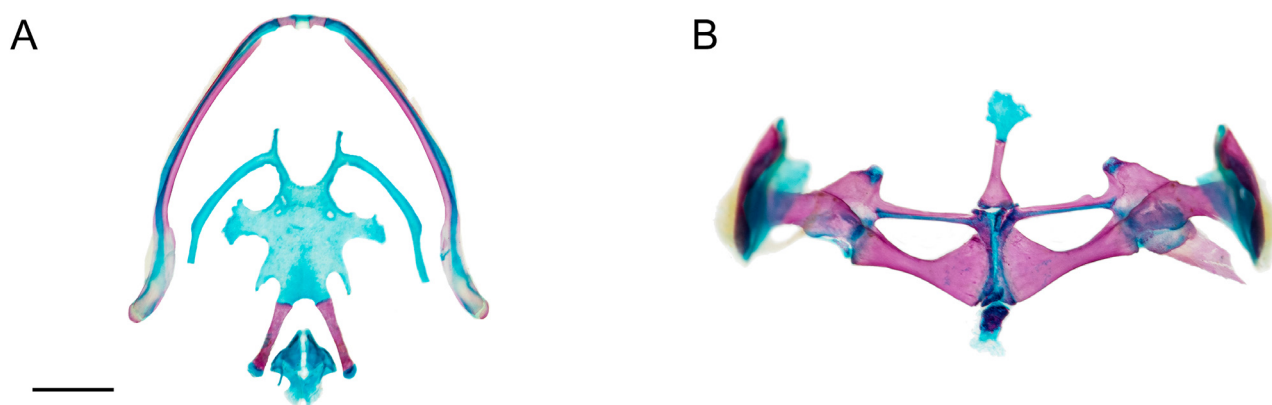
**Vertebral column.** There are eight procoelus presacral vertebrae with nonimbricate neural arches; the cervical cotyles are widely separated, the cotyles are stalked (cervical cotylar arrangement Type 1 of Lynch 1971). The neural spines decrease in height progressively from anterior to posterior and have a keeled upper border. The transverse processes of Presacral III are long; the transverse processes of the posterior presacral vertebra are short; and the transverse processes of Presacral II are the shortest. The transverse processes on Presacrals II–VIII bear cartilaginous distal ends, which are calcified in Presacrals III–VIII. The transverse processes of Presacrals II, VII and VIII are directed anteriorly, and those of Presacrals III–VI are directed nearly posteriorly. The sacral diapophyses are not dilated and directed slightly posteriorly. The sacrococcygeal articulation is bicondylar. The coccyx has a narrow dorsal ridge extending the entire length of coccyx; the coccyx is moderately wide anteriorly; the transverse processes of the coccyx are absent.

**Pectoral girdle.** The pectoral girdle is firmisternal; an omosternum is present. The style of the omosternum is ossified. The distal end of the style bears a short, broad cartilage. The xiphisternum is rectangular and basally calcified. The clavicles are oriented horizontally, forming an L with the outer lateral extreme. The coracoids are moderately robust, oriented so that the sternal end lays posteromedial to the glenoid end. The medial margin of the pectoral fenestra is formed by the coracoid; the calcified epicoracoids are abutting one another, narrowly separated anteriorly. The procoracoid cartilage is present, of uniform depth throughout transverse width. The cleithrum is distally bifurcate. The suprascapula lacks calcification in the margins.





**FIGURE 8.**—High-resolution computed tomography reconstructions of the skeleton of the male holotype (CJ 8311) of *Leucostethus bilsa* sp. nov. (A) dorsal view; (B) lateral, dorsal and ventral view of skull; (C) coccyx, ischia and pubes in lateral view; (D) ventral view of pectoral girdle; (E) ventral view of manus; (F) ventral view of pes. Scale = 5 mm.

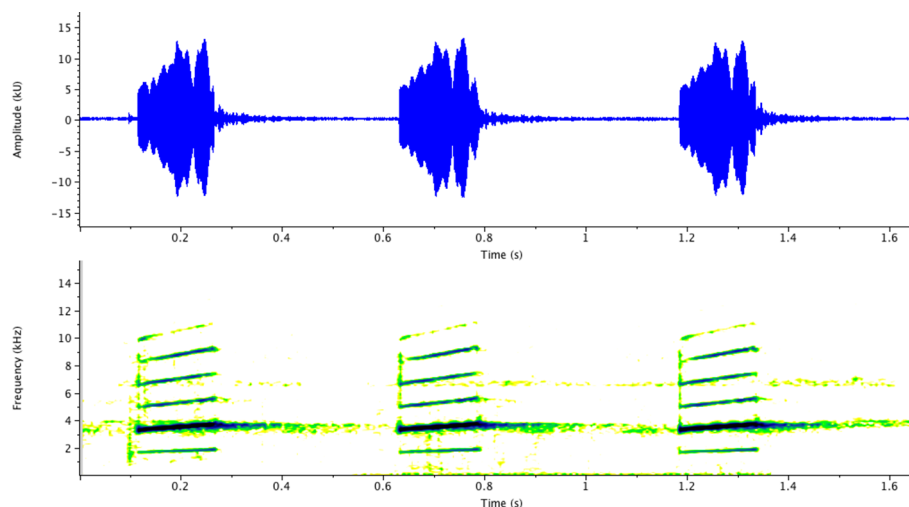


**FIGURE 9.**—Ventral view of hyoid plate and mandible (A) and scapular girdle (B) of the skeleton of *Leucostethus bilsa* sp. nov. (CJ 7775, female). Scale = 3 mm. Photos by Steven Guevara.

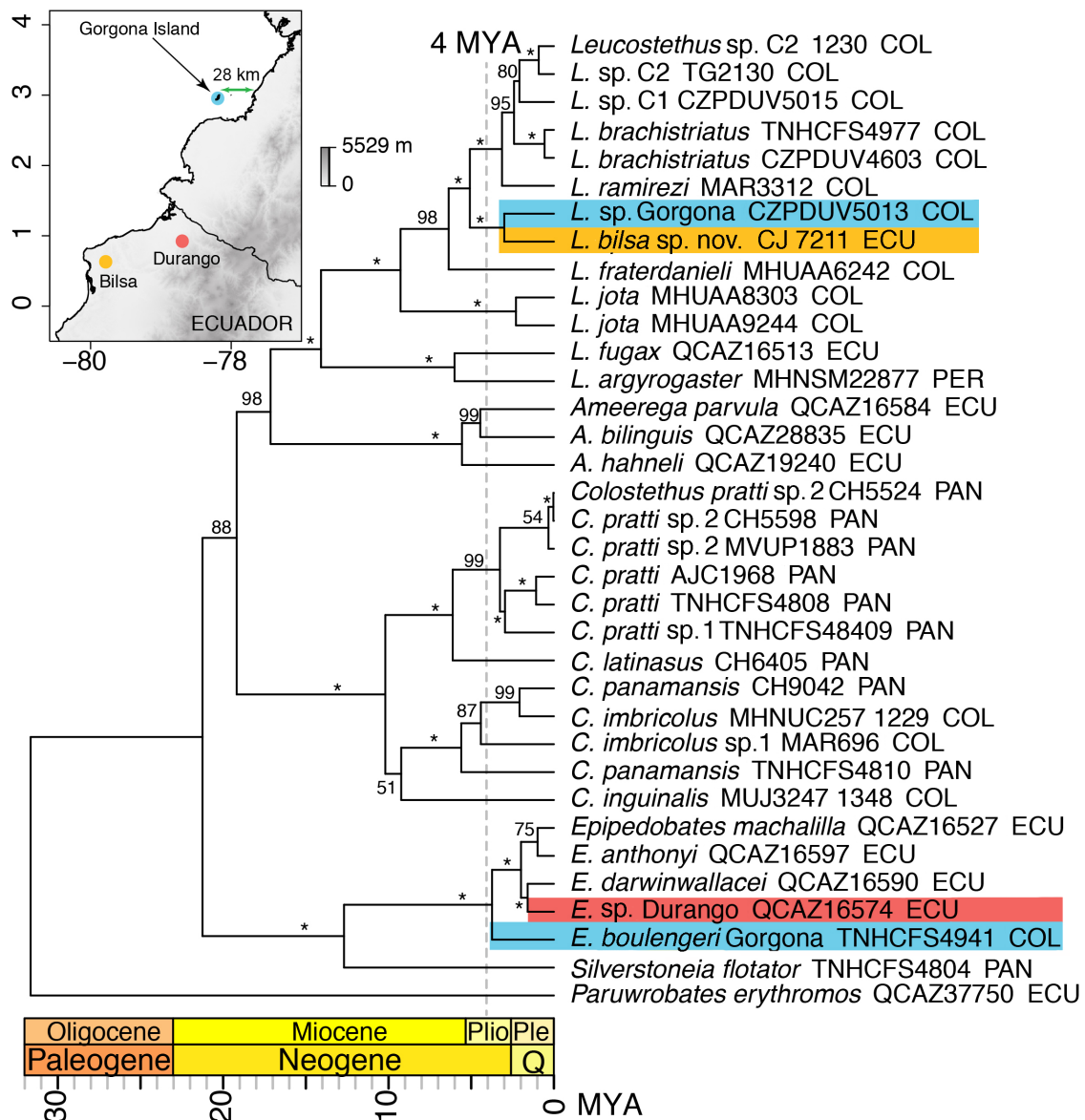
**Pelvic girdle.** The pelvic girdle is “V”-shaped in dorsal view; the ilial shafts extend anteriorly slightly anterior to the sacral articulation. The dorsal crest of the ilium is present. The preacetabular zone is not expanded ventrally; the postacetabular zone is not expanded. They are juxtaposed at ventral midline by a cartilage.

**Appendages.** The deltoid crests on the humerus are prominent. The phalangeal formula of the hand is 2-2-3-3. The prepollex is composed of two elements, with the basal element calcified. The distal transverse processes of the terminal phalanges on the fingers and toes are twice the width of the terminal phalange at midlength. The astragalus and calcaneum are fused proximally and distally. The phalangeal formula of the foot is 2-2-3-4-3. The terminal phalanges are “T”-shaped. The prehallux is composed of 2–3 cartilaginous elements; the basal element is calcified. There are three ossified tarsal bones and two tarsal sesamoid bones. There are one or two large sesamoid bones at the joints of phalanges of fingers, smaller and single ones on the toes.

**Vocalization.** A chorus of three or four individuals was heard calling from the forest floor in a relatively flat area of forest at the base of a steep slope beside the Green trail, ~1.2 km SW of the RBB main station complex on the morning of 5 January 2004. These individuals were calling within a relatively small area, spaced about ten meters apart. Typically, when one frog commenced calling, the other frogs would then start calling; between periods of calling continuing for only a few minutes, there would be long periods of silence. The individual recorded was perched in a prominent position on a dead leaf, raised a few centimeters above the rest of the leaf litter on the forest floor. Only a few individuals were heard calling elsewhere at Bilsa from 3–10 January 2004, and so *L. bilsa* was presumed to be uncommon at that time, but during the rainy season in February of 2007, calling males were more commonly heard in many areas, suggesting that *L. bilsa* may be fairly abundant at the RBB.

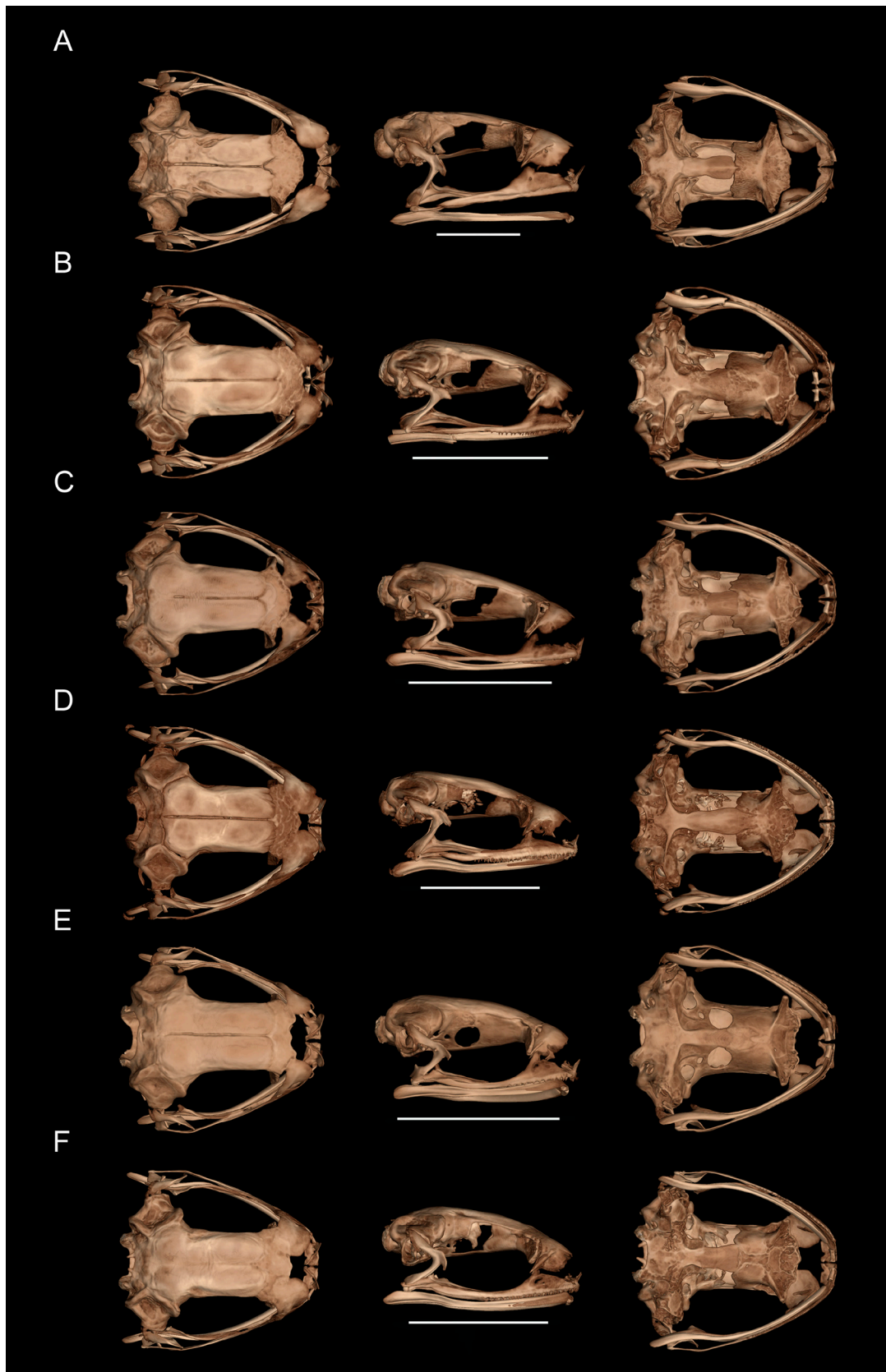


**FIGURE 10.**—Sonogram of the advertisement call of adult male *Leucostethus bilsa* sp. nov. recorded on 5 January 2004 (tape MR2004-1-12); specimen not collected but photographed in life (see Fig. 4B). Three consecutive notes selected from near the center of a call consisting of 84 notes.



**FIGURE 11.**—Maximum likelihood tree depicting evolutionary relationships within *Leucostethus* and Colostethinae inferred from mitochondrial and nuclear genes. Bayesian posterior probabilities > 95 and bootstrap support > 70% are indicated by an asterisk (\*). The inset map indicates the type locality of *Leucostethus bilsa* sp. nov. (yellow dot), and localities of *Leucostethus* sp., *Epipedobates boulengeri* and *E. sp. (aff. darwinwallacei)*. Colored species next to the clades are species occurring in the Isla Gorgona and the Pacific lowlands of Ecuador.

The call is a series of peep notes (Fig. 10) with a series of 65–88 peeping notes repeated at 427–739 ms intervals (mean = 504, SD = 92, N = 20), the entire sequence lasting from 46.8–59.3 s in duration. Two inter-call periods are 25 and 68 s. Overall note repetition rate was 1.35 notes per second, or 81.2 notes per minute. The notes were produced at regularly spaced intervals, (inter-note interval being 431–471 ms, mean = 443, SD = 16); however, the inter-note interval was shorter between some note pairs resulting in a double peep being heard. Only 12 double peeps were present in the entire sample of 237 notes and were never emitted more than three times in succession. In double peeps the inter-note interval was shorter (336–416 ms, mean = 363 ms N = 3), and between the pairs of double notes was longer (660–666 ms, mean = 662 ms, N = 3). Each peep was a weakly pulsatile frequency modulated note (rising from lower to higher) with a duration of 127–182 ms (mean = 153, SD = 17). The dominant frequency arose from 3310–3398 Hz (mean = 3340, SD = 28.9) to 3660–3722 Hz (mean = 3691, SD = 25.4). The fundamental frequency was about 1660 Hz rising to 1820 Hz. Four higher harmonics were present, their starting frequencies being about 4940, 6620, 8360, and 9890 Hz respectively.



**FIGURE 12.**—Representative frog skulls used in this study depicting morphological diversity of Colostethinae: (A) *Ameerega trivittata*, UF 107200; (B) *Colostethus panamansis*, UF 106871; (C) *Epipedobates anthonyi*, KU 219763; (D) *Leucostethus bilsa* CJ 8311; (E) *Silverstoneia nubicola*, KU 76833; (F) *Silverstoneia flotator*, YPM 020210. Columns indicate dorsal, lateral, and ventral views. Scale = 5 mm. Photos by DP.



**Distribution and natural history.** *Leucostethus bilsa* is only known from RBB, Provincia Esmeraldas, border-line between Esmeraldas and Manabí provinces, in the Chocoan region of Pacific northern coastal Ecuador, from elevations between 426 and 515 m, in an area of extent of occurrence of only about 6 km<sup>2</sup>.

This diurnal, riparian species inhabits primary or older secondary forest in the Evergreen Forest of Lowlands of Ecuadorial Chocó and the Evergreen Seasonal Montane Foothill Forest of Cordillera Costera of Chocó, where the average annual rainfall is 1759–2566 mm and the average annual temperature is 22.2–25.4 °C. All specimens of *Leucostethus bilsa* were found moving on surface leaf litter during the day along streams and adjacent slopes. The holotype CJ 8311 was collected along the Brown trail, approximately 1.3 km NE from the main station complex. Other specimens included as type series, here described, were collected along the Green and Black trail routes. Two juvenile paratypes were collected between rocks and leaf litter in the margins of the Duchas stream (Fig. 1B), close to a 10-meter waterfall. Other paratypes were collected in an old secondary forest along the White trail. One adult male (INABIO 3632, transporting larvae) was captured in primary forest on a large flat rock near the Aguacatal–Duchas stream.

Other dendrobatid frogs occurring sympatrically at Bilsa include *Epipedobates boulengeri* (Barbour 1905), *Hyloxalus awa* and *Oophaga sylvatica*.

Three adult males were transporting larvae when found. Two adult males (KU 327679: August, 2002, and INABIO 3632: December, 2006) were observed carrying larvae when encountered, although for both males all larvae were lost during capture; the former was found only ~2 meters from water in a streamside area strewn with rocks, and the latter was found on a rock, also very near a stream, carrying an estimated 5–6 larvae (dark brown to black in color). A third male (CJ 7213) was observed carrying 11 larvae (Fig. 4F) on 2 August 2017, under a rock covered by leaf litter in the Duchas stream. The latter tadpoles were preserved in RNAlater solution for molecular analyses. A lab-raised female had a maximum mass of 2.2 g. The juveniles (post-metamorphs) were found in December, February, March, May, and June. The presence of tadpoles and metamorphs from August to June is indicative that breeding occurs throughout the year.

**Phylogenetic analysis and systematics.** The new species was found to be nested in *Leucostethus* (Fig. 11) and it is the sister taxon to specimens of an undescribed species (*L. sp. Gorgona*) from Gorgona Island, located 28 km off the southwest of the Colombian Pacific coast (e.g., CZPDUV5013–14). There are 6.25% (i.e., 146/2335 bp) differences for the section of 12S-16S mitochondrial fragment, between *L. bilsa* and *L. sp.* from Gorgona. With the species described here, the taxa included in *Leucostethus* with molecular data are Amazonian species (i.e., *L. argyrogaster* and *L. fugax*), Chocoan and Northwestern Andean species (i.e., *L. brachistriatus*, *L. fraterdanieli*, *L. jota*, *L. ramirezi*, and *L. bilsa*) and several populations of undescribed species (i.e., *L. sp. C1*, *L. sp. C2* and *L. sp. Gorgona*). Overall, our estimated phylogenetic tree agrees with that of Marin *et al.* (2018) and supports their conclusions about *Leucostethus fraterdanieli* as a complex of species nested within the recently named *Leucostethus* (Grant *et al.*, 2017). This genus is well-supported (100 ML bootstrap) and sister to *Ameerega*. The clade *Ameerega* + *Leucostethus* is well-supported (98 ML bootstrap) and sister to *Colostethus* as redefined by Marin *et al.* (2018). Finally, the clade that includes *Ameerega* + *Leucostethus* + *Colostethus* is also well-supported (88 ML bootstrap).

## Discussion

**Taxonomy, phylogeny, and speciation.** Grant *et al.* (2017) proposed the genus *Leucostethus* to group two species (*L. fugax* and *L. argyrogaster*), which were the sister taxa of *Ameerega*. They erected *Leucostethus* to remedy the paraphyly of *Colostethus* with respect to *Ameerega*, excluding from *Leucostethus* the *C. fraterdanieli* Group, which was sister to the *C. latinasus* Group. They characterized and diagnosed the new genus based on the lack of a pale dorsolateral stripe and median lingual process, and the presence of the following features: teeth present on the maxillary arch, complete (groin–eye) pale oblique lateral stripes, ventrolateral stripes, entirely silver or white venters in both sexes, basal toe webbing between Toes III–IV, posteriorly granular dorsal skin, and Finger IV of adult males swollen (*L. fugax*) or not (*L. argyrogaster*). Subsequently, Marin *et al.* (2018) corroborated previous phylogenetic hypotheses proposed by other authors (Santos *et al.* 2009; Pyron & Wiens 2011; Pyron 2014; Santos *et al.* 2014), in which the *fraterdanieli*-like species were more closely related to *Leucostethus* than to the *Colostethus latinasus* Group (*contra* Grant *et al.* 2017). Also, Marin *et al.* (2018) presented sequence data from a number of closely related *Leucostethus* species, but *L. bilsa* was not sequenced as part of that study; they expanded *Leucostethus* to six

described species plus three putative new species. Marin *et al.* (2018) did not indicate phenetic synapomorphies for *Leucostethus*, but remarked that some external morphological traits such as the presence of maxillary teeth, basal toe webbing, absence of dorsolateral stripe and complete pale oblique lateral stripe are present in all species of the genus. Conversely, other traits such as the pattern of ventral coloration are not well conserved, since the cis-Andean species (*L. argyrogaster* and *L. fugax*) exhibit immaculate ventral pigmentation while the remaining trans-Andean species (*L. fraterdanieli* “complex” and *L. jota*) exhibit a pigmented pattern in at least one sex. Our phylogeny clearly supports the expanded *Leucostethus*. Nonetheless, a diagnosis of *Leucostethus* based on synapomorphic phenetic characters is not possible at this time given that unambiguous phenetic synapomorphies are not evident among the described characters and species.

**Comparative osteology of Colostethinae.** Osteological descriptions of most of the 67 species of Colostethinae are not yet available, thus we attempt to begin exploring relevant variation among genera. We present microCT scans (Figs. 8, 12) and character data (Table 2) of 6 species that belong to each of the five genera of Colostethinae: *Ameerega*, *Colostethus*, *Epipedobates*, *Leucostethus*, and *Silverstoneia*. Among them, there are differences mostly in skull shape and morphology, teeth number and size, pectoral and pelvic girdle architecture, vertebral column features, and degree of mineralization. Future, additional sampling would help to characterize this variation as either intra or interspecific, intra or intergeneric, continuous or discrete, polymorphic or monomorphic. Also, some of the character states provided in Table 2 may be either autapomorphic of the species analyzed or synapomorphic at other levels of universality (e.g. groups of species or genera). *Leucostethus* currently does not show any unique osteology character that could be a synapomorphy for the genus, although a more complete sampling of species and a phylogenetic analysis are needed. It is worth noting that *Ameerega trivittata* (Spix 1824) lacks maxillary teeth, a synapomorphy of the *A. trivittata* Group discussed by Grant *et al.* (2006) and Grant *et al.* (2017), whereas species of all other Colostethinae genera have dentition, as verified in our observations. Also, it is relevant to highlight the large number of maxillary teeth in *Colostethus panamansis* (Dunn 1933) (37) vs. a small number in *Epipedobates anthonyi* (Noble 1921) (14), and the very large size of premaxillary teeth in two species of *Silverstoneia* vs. smaller in other genera (see microCT scans, links in Table 2). The latter (a continuous character among Dendrobatidae), we might speculate, could be a synapomorphy of *Silverstoneia*. In any case, dentition characters need to be more closely studied, given the variation in size and number we report.

**Historical Biogeography.** *Leucostethus bilsa* is endemic to the Cordillera Mache-Chindul in the Chocoan region of northwestern Ecuador. Its closest relative is an undescribed species of *Leucostethus* found in Isla Gorgona. This island is about 26 km<sup>2</sup> in area and is 28 km from the continent, separated by a deep depression of about 250–300 meters. We emphasize that the 6.25% differences for the section of 12S-16S mitochondrial fragment, between *L. bilsa* and *L. sp.* from Gorgona is not a value used to determine species distinctiveness, but a relative distance with genetic markers available for *L. bilsa* and the Gorgona population. Thus, we provide a large volume of characters derived from morphology, bioacoustics, skeletal anatomy, and biogeography for such purpose; yet these are mostly unknown for the undescribed species from Gorgona. Only future work might determine if that population is a different species from *L. bilsa*. Our estimated time of divergence between *L. bilsa* and *L. sp.* from Isla Gorgona is about 3 MYA, which is younger than our estimate of the split of *Epipedobates boulengeri* from Isla Gorgona and the rest of *Epipedobates* at about 3.74 MYA (Fig. 11).

The evolutionary history of *Leucostethus* and *Epipedobates* species from Isla Gorgona and their continental relatives might trace events of the geological history of northwestern South America. Isla Gorgona has a complex geomorphology with geochemical data suggesting an age of about 86–90 MYA (Kerr 2005; Kerr & Tarney 2005). The flora and fauna of this island are predominantly Chocoan (Giraldo 2012). These taxonomic data suggest that Isla Gorgona and the continent were connected until recently, i.e. the late Miocene-early Pliocene, based on our estimates for dendrobatids. This evidence might suggest that the biodiversity of Isla Gorgona has been isolated in the last 3–5 MYA and has been an important Pliocene refugium.

The new taxon described, i.e., *Leucostethus bilsa*, occurs in a small mountainous patch of tropical forest in the Ecuadorian Chocoan with elevations between 420–515 m. During the Miocene and Pliocene, areas around Cordillera Mache-Chindul region experienced cyclical sea-level fluctuations resulting in repeated marine incursions (Vasquez-Velez 2014; Di Celma *et al.* 2010) and likely isolation, as evidenced by several marine invertebrate fossils and stratigraphic morphology (Cantalamessa *et al.* 2005). This evidence might suggest that Cordillera Mache-Chindul has been an important Pliocene-Pleistocene refugium during the significant climatic cycles. Therefore, the unique fauna and flora represent, like those from Isla Gorgona, important reservoirs of biodiversity and likely

house many more undescribed taxa related to mainland Chocoan species of frogs like *L. bilsa* of northwestern South America.

**Generic placement of similar *Colostethus* and *Hyloxalus*.** Although the phylogeny of *Leucostethus* is mostly resolved, there are pending species descriptions and taxonomic issues awaiting resolution. For example, several species of *Colostethus* and *Hyloxalus* that have not been included in molecular phylogenies are morphologically similar to species of *Leucostethus* and are suspected to belong to the *Leucostethus* clade. Among them, Grant et al. (2017) mentioned two nominal *Colostethus fraterdanieli*-like species that occupy the Cordillera Occidental of Colombia: *Colostethus yaguara* Rivero & Serna 1991 and *C. alacris* Rivero & Granados-Díaz 1990, but they expressed uncertainty regarding their generic placement as follows: “It is unclear if any available name can be applied to the Cordillera Occidental clade” because the genus *Leucostethus* at that time did not include *fraterdanieli*. Frost (2020) mentions a personal communication by Taran Grant indicating that *Colostethus yaguara* might be conspecific with *L. fraterdanieli*. The most recent analyses by Marin et al. (2018) and our research corroborated the previous hypothesis regarding the phylogenetic position of the *fraterdanieli* complex of populations within *Leucostethus*. So, it seems that “*yaguara*” and “*alacris*” probably are more related to *Leucostethus* instead of *Colostethus*, but placing them in the genus *Leucostethus* requires more evidence or data (e.g. molecular and/or morphological) from type series or topotypical samples.

Among the species of *Hyloxalus* occurring in Ecuador, for which molecular data is not available, the most similar to *Leucostethus* is *Hyloxalus maquipucuna* (Coloma 1995), and endemic species from the western versant of the Cordillera Occidental of the Andes. Duellman (field notes, 2 April 1984) described the color in life as having “legs orange, venter yellow”. Although the color description of *H. maquipucuna* is not detailed, the yellow venter adds to the overall similarity between *H. maquipucuna* and *L. bilsa*, both of which differ by having marks on the dorsum (reticulated pattern in *H. maquipucuna*, diffuse marks in *L. bilsa*) and gular patterns (absent in *H. maquipucuna*, gray marks in *L. bilsa*).

**Toxicity.** Although skin alkaloids in *Leucostethus bilsa* have not been investigated, *Leucostethus fugax* appear to be non-toxic and lack skin alkaloids. No evidence for alkaloids has been found based on TLC (thin layer chromatography) experiments on 5 samples of skins (Santos & Cannatella 2011). This lack of alkaloids in one species and the lack of information for other species of *Leucostethus* (including *L. bilsa*) contrasts with the evidence of chemical defenses in closely related taxa of the *Ameerega* clade and at least some species of *Colostethus* (e.g., *C. panamansis* with tetrodotoxin).

**Conservation.** Following the criteria by IUCN Species Survival Commission (2012) we propose this species as Critically Endangered, CR B1ab(i,iii,iv), based on: (B1) restricted distribution less than 100 km<sup>2</sup>, (a) known only from the type locality, and (b) by continuing decline observed, estimated, inferred or projected in (i) the extent of occurrence, (iii) area extent and/or quality of habitat and (iv) number of locations of subpopulations. Geographically, *Leucostethus bilsa* is presently known only from the type series reported herein, with all records obtained within an area of only approximately 6 km<sup>2</sup>. Given increased efforts over the past two decades in discovering and documenting previously unknown species of anurans in western Ecuador and Colombia, if *L. bilsa* also occurred in adjacent lowland regions to the north, south, and east; this species most likely would have already been recorded from these areas. The lack of records from elsewhere in the region suggests that *L. bilsa* may be endemic in the Mache-Chindul region. Riparian habitats are relatively well conserved in the Reserva Biológica Bilsa, but a wider distribution is expected at least into the Cordillera Mache Chindul. However, nearly 27% of the Mache Chindul Ecological Reserve (ca. 1200 km<sup>2</sup>) and areas surrounding the type locality have been lost to deforestation.

The discovery and study of new species from highly threatened biodiversity hot spot regions with a high concentration of endemics (such as the Choco and the Cordillera Mache-Chindul) is essential for conservation efforts; we must obtain comprehensive inventories of all component taxa in these communities to best determine management strategies and priorities for preserving remaining ecosystem remnants.

## Author contributions

GOV, LAC, and JCS conceived and designed research. Fieldwork was done by GOV, JCS, SH, HMOA, and MR. GOV, LAC, JCS, and SH analyzed data and wrote the paper. MR and SH described and discussed the calls. DP did the high-resolution X-ray computed tomography scanning. All authors contributed to manuscript editing and gave final approval for publication.

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