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A new glassfrog of the genus *Centrolene* (Amphibia, Centrolenidae) from the Subandean Kutukú Cordillera, eastern Ecuador

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Abstract

We describe a new species of *Centrolene* from the Subandean Cordillera of Kutukú in southeastern Ecuador. The new species differs from all other glassfrogs by the combination of the following characters: presence of processes of vomers but without vomerine teeth; humeral spines in males; dorsum green with light green dots and without dark marks; dorsal skin with abundant tubercles; all visceral peritonea translucent (except for pericardium); and small body size (snout-vent length 21.5–21.9 mm in adult males). The new species is sister to *Centrolene camposi* from the Western Cordillera of the Andes of southwestern Ecuador, and together they form a clade with *C. condor* from the Subandean Cóndor Cordillera in southeastern Ecuador. Our time tree suggests that the new species originated at the end of the Pliocene. In addition, we present new information for *C. zarza*, expanding its geographic range across the southeastern Andes and the Kutukú and Cóndor cordilleras, amending its definition and diagnosis, and offering new information on its natural history and extinction risk. We also discuss the taxonomic status of Ecuadorian populations reported as *C. huilensis* and conclude that they are *C. muelleri* based on their close phylogenetic relationships and morphological similarity to samples of *C. muelleri* from Peru. *Centrolene huilensis* is a valid species and not closely related to *C. muelleri*.

Resumen

Describimos una nueva especie de *Centrolene* de la cordillera subandina de Kutukú en el sureste de Ecuador. La nueva especie difiere de todas las demás ranas de cristal por la combinación de los siguientes caracteres: presencia de procesos vomerinos pero sin dientes vomerinos, espinas humerales en los machos, dorso verde con puntos verdes claros y sin marcas oscuras, piel dorsal con abundantes tubérculos, todos los peritoneos viscerales traslúcidos (excepto el pericardio) y un tamaño corporal pequeño (longitud rostro-cloacal de 21.5–21.9 mm en machos adultos). La nueva especie es hermana de *Centrolene camposi* de la Cordillera Occidental de los Andes del suroeste de Ecuador y juntas forman un clado con *C. condor* de la cordillera subandina del Cóndor en el sureste de Ecuador. Nuestro árbol de tiempo sugiere que la nueva especie se originó a finales del Plioceno. Adicionalmente, presentamos nueva información para *C. zarza*, expandiendo su rango geográfico a lo largo de los Andes del sureste y las cordilleras de Kutukú y Cóndor, modificando su definición y diagnóstico, y ofreciendo nueva información sobre su historia natural y riesgo de extinción. También discutimos el estado taxonómico de las poblaciones ecuatorianas reportadas como *C. huilensis* y concluimos que son *C. muelleri*, basados en sus estrechas relaciones filogenéticas y similitud morfológica con muestras de *C. muelleri* de Perú. *Centrolene huilensis* es una especie válida y no cercanamente relacionada con *C. muelleri*.

Key Words

Andes, Anura, *Centrolene kutuku* sp. nov., *Centrolene huilensis*, *Centrolene muelleri*, *Centrolene zarza*, Cóndor Cordillera, Kutukú Cordillera, new species, phylogenetic relationships, taxonomy

Palabras clave

Andes, Anura, *Centrolene kutuku* sp. nov., *Centrolene huilensis*, *Centrolene muelleri*, *Centrolene zarza*, Cordillera del Cóndor, Cordillera del Kutukú, nueva especie, relaciones filogenéticas, taxonomía

Introduction

The genus *Centrolene* Jiménez de la Espada, 1872 currently contains 33 described species of glassfrogs distributed across the Andes from Venezuela to Peru (AmphibiaWeb 2023; Cisneros-Heredia et al. 2023; Frost 2023). Frogs of the genus *Centrolene* are characterised by having humeral spines present in adult males (except *C. daidalea* Ruiz-Carranza & Lynch, 1991b, *C. savagei* Ruiz-Carranza & Lynch, 1991b, and *C. solitaria* Ruiz-Carranza & Lynch, 1991b), liver lobed and covered by a translucent hepatic peritoneum, ventral parietal peritoneum partially covered by iridophores, pericloacal warts enamelled, bones green in life, dorsal background lavender in preservative, and males with dorsal spicules during breeding season (Cisneros-Heredia and McDiarmid 2007; Guayasamin et al. 2009, 2020; Cisneros-Heredia et al. 2023). The monophyly of *Centrolene* has been supported by molecular evidence (Guayasamin et al. 2009; Twomey et al. 2014; Cisneros-Heredia et al. 2023), but the relationships of some species remain unexplored (Cisneros-Heredia and McDiarmid 2007; Guayasamin et al. 2020; Cisneros-Heredia et al. 2023).

Thirteen species of *Centrolene* are known in the Republic of Ecuador, all living in the Andes and adjacent Subandean Cordilleras (Lynch and Duellman 1973; Cisneros-Heredia and McDiarmid 2005, 2007; Guayasamin et al. 2006, 2020; Cisneros-Heredia 2007; Cisneros-Heredia and Yánez-Muñoz 2007; Cisneros-Heredia and Morales-Mite 2008; Almendáriz and Batallas 2012; Cisneros-Heredia et al. 2023; Székely et al. 2023). Four species of *Centrolene* are known to inhabit the Cóndor Cordillera: *C. charapita* Twomey, Delia, and Castroviejo-Fisher, 2014; *C. condor* Cisneros-Heredia and Morales-Mite, 2008; *C. sanchezi* Ruiz-Carranza & Lynch, 1991a; and *C. zarza* Székely, Córdova-Díaz, Hualpa-Vega, Hualpa-Vega & Székely, 2023, with *C. condor* and *C. zarza* being considered endemic to that cordillera (Cisneros-Heredia and Morales-Mite 2008; Guayasamin et al. 2020; Székely et al. 2023). However, there are no records of *Centrolene* in the Kutukú Cordillera.

During surveys in southeastern Ecuador, we found a new species of *Centrolene* at the Kutukú Cordillera, which we are pleased to describe herein based on morphological and molecular evidence. We also report new Subandean and Andean localities for *Centrolene zarza*, a recently described species known from a single locality at the Cóndor Cordillera. In addition, we discuss the status of the Ecuadorian population ascribed to *Centrolene huilensis* Ruiz-Carranza & Lynch, 1995.

Materials and methods

Ethics statement

Our study was authorised under research permits N° MAE-DNB-ARRGG-CM-2014-0002 (issued to PUCE), 003-17 IC-FAU-DNB/MA (PUCE), 011-2018-IC-FAU-DNB/MA (PUCE), and 028-2018-IC-FLO-FAU-DPAZCH-UPN-VS/MA (ZSFQ) and framework contracts for access to genetic resources MAE-DNB-CM-2015-0025 (PUCE) and MAE-DNB-CM-2018-0106 (USFQ) issued by the Ministerio del Ambiente, Agua y Transición Ecológica del Ecuador. We followed the standard guidelines for using live amphibians and reptiles in field research by Beaupre et al. (2004).

Species concept

We consider species as separately evolving metapopulation lineages, recognisable from an operational point of view to the extent that isolation from their putative sister lineages can be inferred (De Queiroz 2007).

Taxonomic sampling

Specimens from the following collections were examined: División de Herpetología, Instituto Nacional de Biodiversidad, Quito (DHMECN); Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá (ICN); University of Kansas Natural History Museum,

Lawrence (KU); Museum of Comparative Zoology, Harvard University, Cambridge, MA (MCZ); Museo de Zoología, Pontificia Universidad Católica del Ecuador, Quito (QCAZ); National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM); Museo de Zoología, Universidad San Francisco de Quito, Quito (ZSFQ).

The following specimens were examined for the diagnosis (H and P between square brackets stand for holotype and paratype, respectively): ***Centrolene camposi*** (2 specimens): ECUADOR: province of Azuay: La Enramada (DHMECN 11407 [H], DHMECN 11408 [P]). ***C. condor*** (7 specimens): ECUADOR: province of Zamora Chinchipe: Destacamento Militar Cóndor Mirador (QCAZ 37279 [H]), Paquisha Alto (DHMECN 11208–11210), Concesión Colibrí (DHMECN 12049), Concesión La Zarza (DHMECN 12053); province of Morona-Santiago: near Reserva Biológica El Quimi (QCAZ 72514). ***C. pipilata*** (3 specimens): ECUADOR: province of Napo: 14.7 km NE Salado River (ICN 23756 [P], USNM 286717, MCZ A-97803). ***C. sanchezi*** (15 specimens): COLOMBIA: department of Cauca: Guanacas River (ICN 11685 [H], ICN 11686 [H, *C. guanacarum*]); department of Caquetá: 3.1 km por carretera abajo del Alto Gabinete (ICN 24293 [H]); ECUADOR: province of Napo: Yanayacu Biological Station (QCAZ 16212, 17807, 22386–87); province of Morona-Santiago: 11.2 km WSW Plan de Milagro (KU 202803 [H, *C. bacata*], KU 202804, 202807-12 [P, *C. bacata*]). ***C. zarza***: (13 specimens): ECUADOR: province of Zamora Chinchipe: El Zarza (MUTPL-A 1051, 1022 [P]); Los Encuentros, Relaves, 3.7555°S, 78.4998°W, 1470 m (ZSFQ 2361–2363; ZSFQ 2361 tissue sample was sequenced under QCAZ 78311); Concesión Minera Kinross-Aurelian, Los Encuentros, 3.8121°S, 78.6147°W, 1530 m (DHMECN 10223); province of Morona Santiago: Sardinayacu, Parque Nacional Sangay, 2.0938°S, 78.1688°W, 1685 m (QCAZ 58685–88, 59066); Puchimi, Kutukú Cordillera, 2.7834°S, 78.1444°W, 1840 m (QCAZ 69116–18). Additional specimens examined during our studies in Centrolenidae are listed in Cisneros-Heredia & McDiarmid (2007), Guayasamin et al. (2020), and Cisneros-Heredia et al. (2023).

Fieldwork

Fieldwork was conducted at the following localities in Ecuador: province of Morona Santiago: Sardinayacu, Parque Nacional Sangay (2.0938°S, 78.1688°W, 1685 m elevation) in January 2015; Kutukú Cordillera, Puchimi, parish of San Francisco de Chinimbimi (2.7834°S, 78,1444°W, 1840 m) in September 2017 and January 2018, at both localities by expeditions of the Museo de Zoología, Pontificia Universidad Católica del Ecuador; and at the province of Zamora-Chinchipe, Los Encuentros, Relaves (3.7555°S, 78.4498°W, 1470 m) in February 2019 by an expedition affiliated to the Museo de Zoología, Universidad San Francisco de Quito. Specimens were found during visual encounter surveys for herpetological searches. Individuals were photographed alive and euthanised with benzocaine; a muscle tissue sample was extracted and preserved in 95% ethanol; and whole specimens were fixed in 10% formalin and preserved in 75% ethanol.

Morphology and colouration

Diagnosis, terminology, characters, and measurements follow the format and definitions proposed by Cisneros-Heredia & McDiarmid (2007) and Kok & Castroviejo-Fisher (2008) for fringes and folds. All characteristics reported in the description of the type series are from adult specimens. Sex and maturity were determined by directly examining gonads through dissections and noting the presence of secondary sexual characters (i.e., vocal slits and nuptial pads). All morphometric data were measured with a digital calliper (0.05 mm accuracy, rounded to the nearest 0.1 mm) under a stereomicroscope, reported as range (mean ± standard deviation), and included snout-vent length (SVL), head length (HL), head width (HW), interorbital distance (IOD), eye diameter (ED), internarial distance (IND), eye-nostril distance (EN), tympanum diameter (TD), tibia length (TL), foot length (FL), hand length (HAL), and Finger III disk width (F3DW). Digital X-ray images were obtained with the Thermo Kevex X-ray Imaging System at the QCAZ museum. Colour patterns are described based on photographs of live specimens taken in the field. The adjective "enamelled" describes the shiny white colouration produced by an accumulation of iridophores (Lynch and Duellman 1973; Cisneros-Heredia and McDiarmid 2007). Our species descriptions follow the recommendation by Vences (2020) to speed up species inventories by emphasising diagnosis over descriptions and images over words. To streamline the description of the new species, we present high-quality colour photographs of all relevant specimens instead of verbal descriptions, which have an inherently subjective component.

Phylogenetic analyses and genetic distances

We obtained DNA sequences for nuclear and mitochondrial genes to assess the phylogenetic relationships of the new species and additional populations of *C. zarza* within the genus *Centrolene*. DNA was extracted from muscle or liver tissue preserved in 95% ethanol or tissue storage buffer using standard phenol-chloroform extraction protocols (Sambrook et al. 1989). We used a polymerase chain reaction (PCR) to amplify DNA fragments for the mitochondrial genes 12S rRNA (12S), two overlapping fragments for the last ~320 bp of 16S rRNA (16S), NADH

dehydrogenase subunit 1 (ND1) and adjacent tRNAs ($tRNA^{Leu}$, $tRNA^{Ile}$, and $tRNA^{Gln}$), and the nuclear genes RAG1 and C-MYC 2. PCR amplification was performed under standard protocols and sequenced by the Macrogen Sequencing Team (Macrogen Inc., Seoul, Korea).

Our phylogeny also includes sequences from GenBank (Guayasamin et al. 2008, 2020; Castroviejo□Fisher et al. 2014; Twomey et al. 2014; Mendoza-Henao et al. 2023; Cisneros-Heredia et al. 2023). We obtained all available Genbank sequences of *Centrolene* and its sister clade, *Nymphargus*. We also included representative samples of *Allophryne*, *Celsiella*, *Chimerella*, *Cochranella*, *Espadarana*, *Hyalinobatrachium*, *Ikakogi*, *Rulyrana*, *Sachatamia*, *Teratohyla*, and *Vitreorana*. The phylogeny was rooted in *Allophryne ruthveni* (specimen MAD1857; outgroup choice based on Guayasamin et al. (2008, 2020) and Castroviejo□Fisher et al. (2014). Sample information for GenBank sequences is listed in the literature (Guayasamin et al. 2008; Castroviejo□Fisher et al. 2014; Mendoza-Henao et al. 2023; Cisneros-Heredia et al. 2023). GenBank accession numbers for newly generated sequences are listed in Table 1.

Table 1. Genbank accession numbers for DNA sequences included in the phylogenetic analysis.

Museum No.	Species	Genbank Accession No.				
		12S	16S	ND1	RAG1	C-MYC2
QCAZ 71386	*C. kutuku* sp. nov.	PP868294	PP868288	PP870124	–	–
DHMECN 10223 (QCAZ 59066)	*C. zarza*	PP868290	–	PP870119	PP886045	PP886042
QCAZ 58686	*C. zarza*	PP868291	PP868285	PP870120	PP886046	PP886043
QCAZ 58687	*C. zarza*	PP868292	PP868286	PP870121	PP886047	PP886044
QCAZ 58688	*C. zarza*	–	PP868287	PP870122	PP886048	–
QCAZ 69118	*C. zarza*	PP868293	–	PP870123	–	–
ZSFQ 2361 (QCAZ 78311)	*C. zarza*	–	PP868289	PP870125	–	–

We analysed the mitochondrial genes (12S rRNA, 16S rRNA, ND1) and the nuclear genes (BDNF, C-MYC 2, CXCR4, POMC, RAG1, SLC8A1, SLC8A3) for a total of 10 loci. The sequences were aligned in GeneiousPro 9.1.8 (Kearse et al. 2012) with the MAFFT plug-in (Katoh and Standley 2013). The alignment was manually corrected with Mesquite v.3.02 (Maddison and Maddison 2019). The aligned concatenated matrix had 6626 bp and 239 terminals (available at http://zenodo.org under doi: 10.5281/zenodo.11077755). Phylogenetic relationships were inferred for all genes concatenated using maximum likelihood (ML) as the optimality criterion. We partitioned the matrix by gene and codon position (the total number of partitions was 26). Each partition was analysed under model GTR + R + I in the software IQ-TREE multicore version 2.1.2 (Nguyen et al. 2015; Minh et al. 2020). We used the same software under default settings to find the best phylogeny. To estimate branch support, we made 1000 ultrafast non-parametric bootstrap searches (-bb 1000 command; Hoang et al. 2018) and 1000 replicates for the SH-like approximate likelihood ratio test with the -alrt 1000 command (Guindon et al. 2010). We considered that branches with bootstrap values > 94 and SH-aLRT values > 79 had strong support. We applied the least-squares dating method (To et al. 2016) to obtain a time tree in IQ-TREE. We used two calibration points based on the phylogeny of Hime et al. (2020): 23.1 Mya for the divergence between *Espadarana* and *Hyalinobatrachium* and 12.5 Mya for the divergence between *Espadarana* and *Teratohyla*. We prefer to rely on the estimates by Hime et al. (2020) because they were based on phylogenomic data instead of sequences from a few mitochondrial and nuclear genes, an improvement relative to previous time-tree estimates for Centrolenidae (e.g., Castroviejo-Fisher et al. 2014).

Extinction risk assessment

Extinction risk assessment was conducted following the system for classifying species at high risk of global extinction presented by the IUCN (2012) and the IUCN Standards and Petitions Committee (2022) guidelines. The extent of occurrence (EOO) and area of occupancy (AOO, with a cell size of 2 km) were calculated using GeoCAT (Bachman et al. 2011).

Nomenclatural acts

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Commission on Zoological Nomenclature (ICZN). Hence, the new names in the electronic version are effectively published under that code in the electronic edition alone. This published work and its nomenclatural acts have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved, and the associated information can be viewed through any standard web browser by appending the LSID to the prefix http://zoobank.org/. The LSID for this publication is urn:lsid:zoobank.org:pub:E07EDFEB-3798-490F-B676-C912AE951C92. The online version of this work is archived and available from the following digital repositories: Zenodo, CLOCKSS , and other international archives.

Results

Phylogenetic analysis

According to our time tree, *Centrolene* diverged from its sister clade, *Nymphargus*, during the early Miocene (~18 Mya), a value similar to the ~17 Mya estimated by Castroviejo□Fisher et al. (2014). This similarity is remarkable because our secondary calibration points are from a time tree with taxon and gene sampling strategies (Hime et al. 2020) that are extremely different from those of Castroviejo□Fisher et al. (2014). Our phylogeny (Fig. 1) is similar to recent *Centrolene* phylogenies (Guayasamin et al. 2020; Cisneros-Heredia et al. 2023). Two notable exceptions are (1) *C. charapita* as a sister species of all other species of *Centrolene* (embedded within *Centrolene* in Guayasamin et al. 2020) and (2) the paraphyly of samples of *C. muelleri* Duellman & Schulte, 1993, from Peru relative to a specimen previously reported as *C. huilensis* from Ecuador (*C. muelleri* monophyletic and sister to *C. huilensis* from Ecuador in Guayasamin et al. 2020). Our estimate of time divergence between the Ecuadorian sample of *C. huilensis* and *C. muelleri* from Peru (CORBIDI 14667, collected 15 km from the type locality of *C. muelleri*) is 0.45 Mya, a value too low for sister species within *Centrolene* (see Fig. 1 for comparisons). In addition, their genetic distance is low (0.6% for gene 16S), and both specimens are very similar to each other morpho-

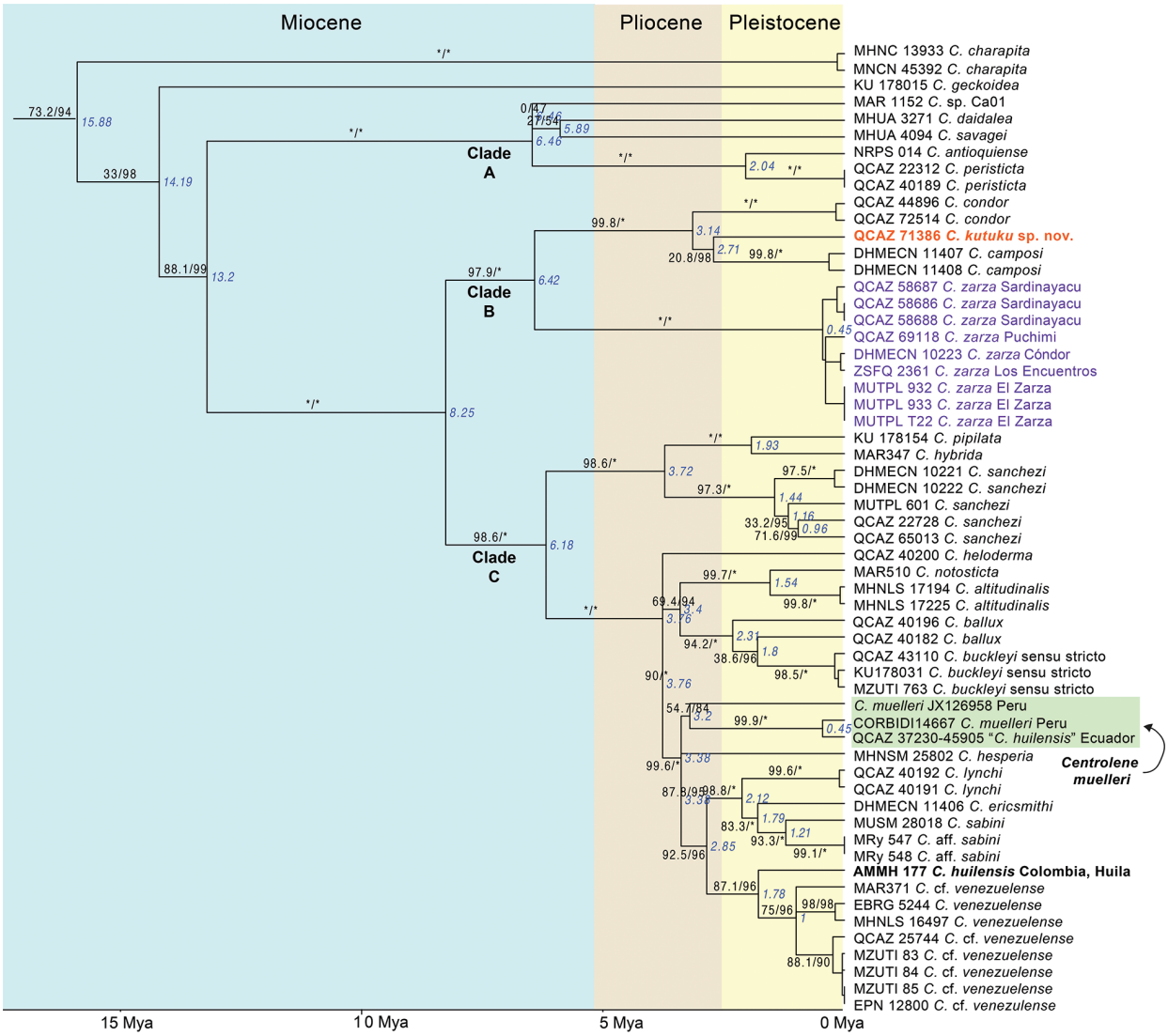

Figure 1. Maximum likelihood time-tree inferred from a partitioned analysis of 6626 aligned sites of the mitochondrial genes 12S rRNA, 16S rRNA, and ND1 and the nuclear genes BDNF, C-MYC 2, CXCR4, POMC, RAG1, SLC8A1, and SLC8A3 showing phylogenetic relationships of *Centrolene*. Support values are shown as percentages next to the branches: SH-aLRT before the slash and ultrafast bootstrap after. The "*" symbol indicates 100%. Node ages in Mya are shown in blue italics (ages lower than 0.5 Mya are not shown). Only the genus *Centrolene* is presented (a complete tree is available in the Suppl. material 1). Voucher catalogue numbers are displayed before species names. *Centrolene kutuku* sp. nov. and *C. zarza* are shown in orange and purple, respectively. The Colombian population of *C. huilensis* is shown in bold. The complete phylogeny is available as Suppl. material 1.

logically (Twomey et al. 2014). This evidence suggests that the identification of the Ecuadorian population as *C. huilensis* needs reconsideration. Moreover, the Colombian sample of *C. huilensis* (AMMH 177, from a locality 2 km N of the type locality of *C. huilensis*, Huila, Colombia) is not closely related to *C. muelleri* from Peru, nor to "*C. huilensis*" from Ecuador, but to the *C. venezuelense* species complex (see also Mendoza-Henao et al. 2023). The combined evidence allows us to confidently conclude that the Ecuadorian populations are not *C. huilensis*, but instead, they are *C. muelleri*.

Centrolene charapita and *C. geckoidea* Jiménez de la Espada, 1872 are unusual for having old divergence times (over 14 Mya; Fig. 1). The remaining species of *Centrolene* segregate into three younger and strongly supported clades (Clades A–C in Fig. 1). Clades A and B have few species (five and four, respectively) and are geographically restricted: Clade A in the Andes of northern Ecuador, Colombia, and western Venezuela; and Clade B in southern Ecuador. Clade C is more speciose, has younger species, and is geographically widespread. Within Clade B, a sample from the Kutukú Cordillera diverged from its closest relative, *C. camposi* Cisneros-Heredia, Yánez-Muñoz, Sánchez-Nivicela & Ron, 2023, approximately 2.7 Mya. Its time of divergence and morphological distinctiveness (see Systematic Account) indicate that it is a new species that we describe below. The uncorrected p-genetic distance, gene 12S, between the new species from Kutukú and *C. camposi* is 1.6%, and both are sister to specimens tentatively assigned to *C. condor*. The three species are sister to the recently described *C. zarza*, the oldest species of the clade (~6.4 Mya).

Samples previously ascribed to *C. buckleyi* (Boulenger, 1882) are non-monophyletic, as reported by Amador et al. (2018). Populations from the Eastern Cordillera of the Andes in northern Ecuador (e.g., MZUTI 83, QCAZ 25744) and Chingaza National Park in Colombia have a divergence time from *C. venezuelense* (Rivero, 1968) of ~ 1 Mya, suggesting they may be conspecific. We tentatively refer to them as "*C.* cf. *venezuelense*".

New data on *Centrolene zarza*

Centrolene zarza was recently described from a single locality, El Zarza Wildlife Refuge, in the Cóndor Cordillera (Székely et al. 2023). Samples collected during expeditions to the Subandean Cóndor and Kutukú cordilleras and the Sangay National Park in the Eastern Cordillera of the Andes allow us to add four new localities, increasing its known range 200 km to the north (Fig. 2). We examined two paratypes of *C. zarza*, an adult male (MUTPL-A 1022) and an adult female (MUTPL-A 1051), and eleven non-type specimens collected from these new localities. Contrary to what was reported in the original description, both paratypes have a thin layer of iridophores covering the pericardium (Fig. 3), slightly scalloped folds along the postaxial edge of Finger IV and Toe V, slightly scalloped metacarpal, ulnar, metatarsal, and tarsal folds, and abundant heel warts. To complement its original description, it can be mentioned that *C. zarza* has a distinct enamelled spot on the dorsal surfaces of upper arms and thighs; it shows abundant, elevated, rounded, enamelled warts on snout, infraocular and infratympanic areas, and flanks (sometimes low and flat due to preservation artefacts); the humeral spine is short, barely protruding through the skin; some specimens show a fairly distinct prepollex through the skin, giving the appearance of a slightly projecting prepollical spine; and adult males have nuptial excrescence Type VI (glandular clusters and individual glands distributed along the venter and flanks). Enamelled warts on infraocular and infratympanic areas are a characteristic otherwise present in five species not closely related to *C. zarza* or among themselves: *C. altitudinalis* (Rivero, 1968), *C. notosticta* Ruiz-Carranza & Lynch, 1991a, *C. pipilata* (Lynch & Duellman, 1973), *C. robledoi* Ruiz-Carranza & Lynch, 1995, and *C. sanchezi*. The presence of elevated warts on the snout of *C. zarza* is a distinctive condition, and few centrolenid frogs have dermal ornamentations on the snout (spiculated tubercles or warts, e.g., *Centrolene acanthidiocephalum* [Ruiz-Carranza & Lynch, 1989]).

Amended definition

We modified the definition of *C. zarza* as follows based on our findings (character states reported by Székely et al. are in square brackets and italics; character numbers follow Székely et al. 2023): **(2)** snout rounded with elevated warts at the tip in dorsal view and sloping in lateral view [snout rounded in dorsal view, sloping in profile]; **(4)** dorsal skin shagreen with scattered warts of varying size on head, dorsum, and limbs and abundant, elevated, enamelled warts on snout, infraocular and infratympanic areas, and flanks—reaching at least to the middle of flanks [dorsal skin shagreen with elevated, and some enamelled, warts corresponding to white spots]; **(6)** parietal peritoneum white—iridophores covering ½ of ventral parietal peritoneum (condition P3), pericardium covered by iridophores and all other visceral peritonea clear (condition V1, Fig. 3) [iridophores absent on all visceral peritonea, including pericardium, condition V0]; **(9)** absent or basal webbing between fingers I and II, II (2^{-}–2^{+}) –3^{-} III (2^{-}–2⅓) – (2^{-}–2^{+}) IV [webbing absent between Fingers I and II, basal between II and III, moderate between outer fingers: III 2^{+}–2 IV]; **(10)** toe webbing I (1^{-}–1 ½) – (2^{-}–2^{+}) II (1^{-}–1^{+}) – (2^{-}–2) III (1^{-}–1^{+}) – (1½–2) IV (1½–2) – (1–1^{+}) V [webbing between toes moderate: I 1−–2− II 1−–2 III 1−–2 IV 2–1+V]; **(11)** row of enamelled warts or scalloped dermal fringe along postaxial edge of Finger IV, enamelled metacarpal and ulnar folds, row of enamelled warts or scalloped dermal fringe along postaxial edge of Toe V, enamelled metatarsal and tarsal folds, and abundant enamelled heel warts [outer edge of forearms and tarsus with row of enamelled warts that often continue into the external edges of Finger IV and/or Toe V; fingers and toes with

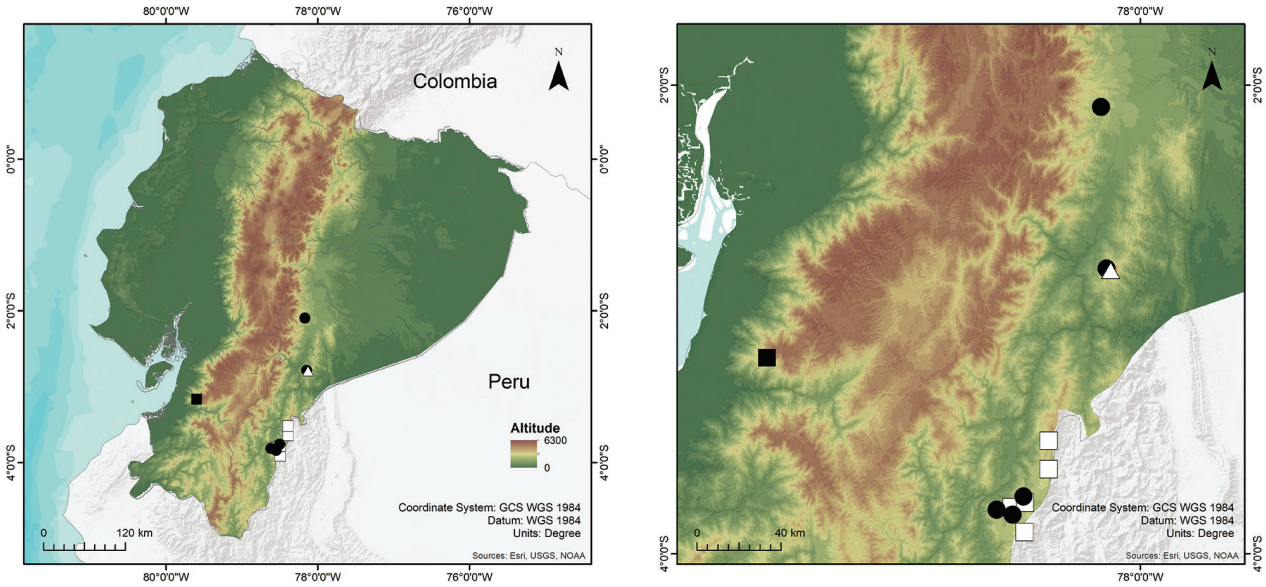

Figure 2. Map of Ecuador (left) and expanded area (right) showing known localities for *Centrolene camposi* (black square), *C. condor* (white square), *C. kutuku* sp. nov. (white triangle), and *C. zarza* (black circle) in southern Ecuador. Localities are based on specimens deposited at the DHMECN, QCAZ, and ZSFQ collections (see Materials and Methods for a list), Székely et al. (2023), and Cisneros-Heredia et al. (2023).

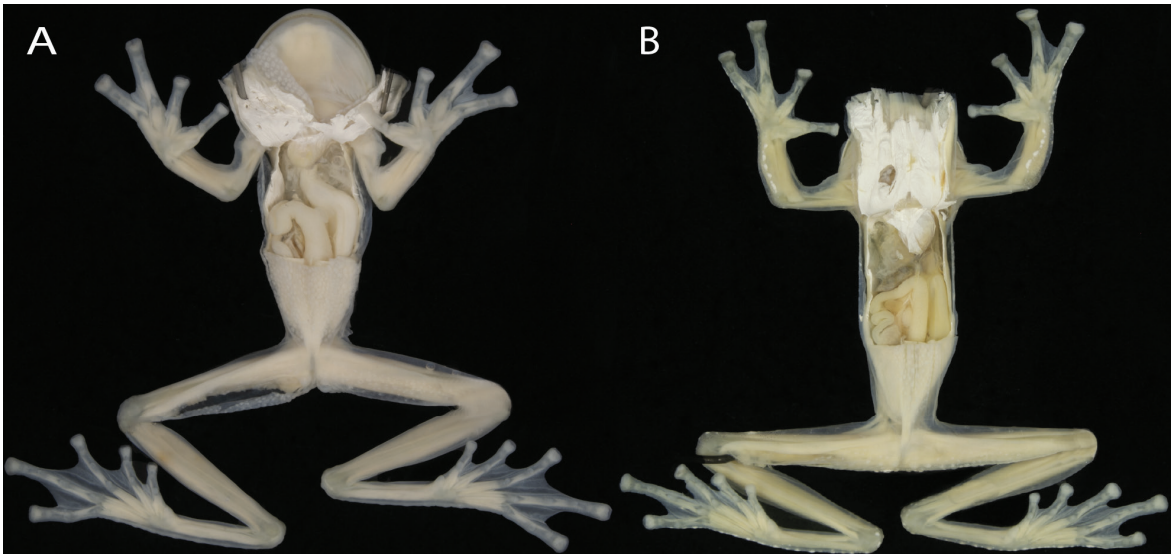

Figure 3. *Centrolene zarza* showing iridophores on the visceral peritonea, note pericardium covered by iridophores. **A.** MUTPL-A 1022 (paratype); **B.** QCAZ-A 58686.

broad lateral fringes]; **(12)** unpigmented nuptial pad Type I and Type VI; prepollex fairly distinct to concealed prepollex [unpigmented Type I nuptial pads present in males; concealed prepollex]; **(15)** color in life, all dorsal surfaces dark or light green with yellowish-green dots, enamelled warts on snout, infraocular and infratympanic areas, and flanks, a large enamelled spot on dorsal surfaces of each upper arm and thigh, enamelled flecks and dots on dorsal surfaces of hind and forelimbs, enamelled folds on hind and forelimbs, bones green (Fig. 4) [dorsum light green with many white or whitish, elevated, spots and flecks of various sizes; bones green]; **(16)** colour in preservative, dorsal surfaces dark grey to grey lavender with pale dots of varying size, enamelled warts on snout, infraocular and infratympanic areas, and flanks, a large enamelled spot on dorsal surfaces of each upper arm and thigh, enamelled flecks and dots on dorsal surfaces of hind and forelimbs, enamelled warts and folds on hind and forelimbs (Fig. 5) [dorsal surfaces greyish with white spots]; **(18)** abundant to scattered melanophores and small iridophore flecks present on Finger IV and towards the base of Finger III; abundant to scattered melanophores and small iridophore flecks on toes IV and V [fingers and toes yellowish, usually lacking melanophores except for Finger IV and Toes IV and V]; **(23)** SVL in adult females 25.5–30.0 [25.5–27.0 mm]. Differences are likely the result of interpopulation variation except for the presence of iridophores on the pericardium (verified in two paratypes of *C. zarza*, Fig. 3).

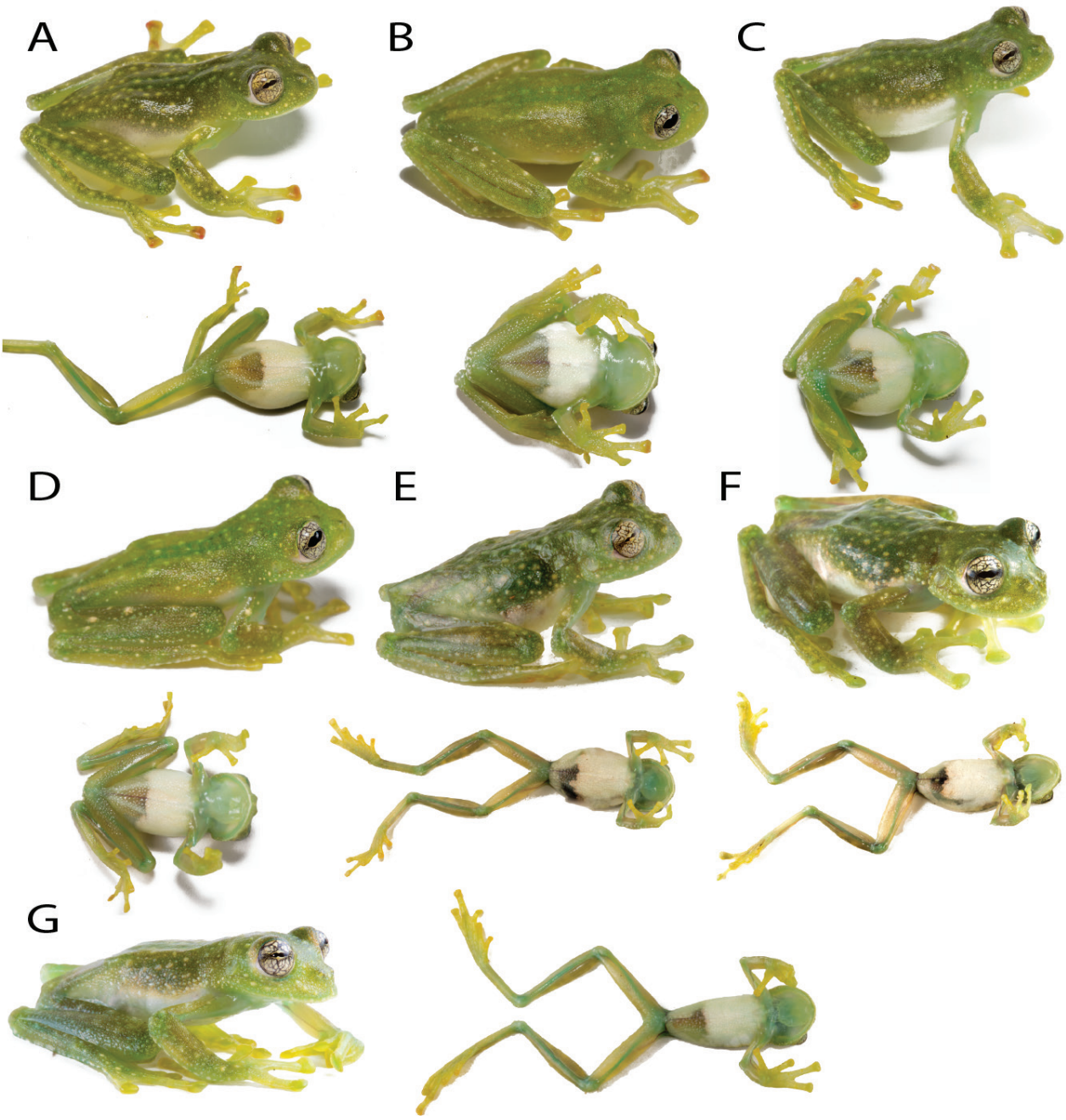

Figure 4. Dorsolateral and ventral views of live adult males of *Centrolene zarza*, corresponding to new records from Sardinayacu and Puchimi, Ecuador. **A.** QCAZ 58685, SVL = 23.8 mm; **B.** QCAZ 58688 (holotype), SVL = 23.8 mm; **C.** QCAZ 58687, SVL = 24.1 mm; **D.** QCAZ 58686, SVL = 23.9 mm; **E.** QCAZ 69116, SVL = 26.2 mm; **F.** QCAZ 69117, SVL = 29.4 mm; **G.** QCAZ 69118, SVL = 26.4 mm.

Distribution and Natural History

Centrolene zarza is now known from four localities in southeastern Ecuador: one on the eastern slopes of the Eastern Cordillera of the Andes at 1685 m elevation and three on the western slopes of the Subandean Cordilleras of Cóndor and Kutukú, between 1430–1905 m elevation (Fig. 2). The species inhabits Low Montane Evergreen Forest (Low Montane Evergreen Forest of the southern Eastern Cordillera of the Andes and Low Montane Evergreen Forest of the Cóndor-Kutukú Cordilleras, *sensu* MAE et al. 2013). All individuals have been found at night. At Sardinayacu, frogs were found next to a forested stream. All individuals were on the underside of leaves between 40 and 200 cm above the ground. At Puchimi, one adult male (QCAZ 69116) was calling 0.5 m above ground, next to a body of water at 20h59, and two individuals were found in an old-growth forest, 0.8–1.5 m above ground. At Los Encuentros, one individual was found on top of a leaf 0.5 m above ground, and two individuals were on top of leaves 0.3–2.8 m above a crystalline stream.

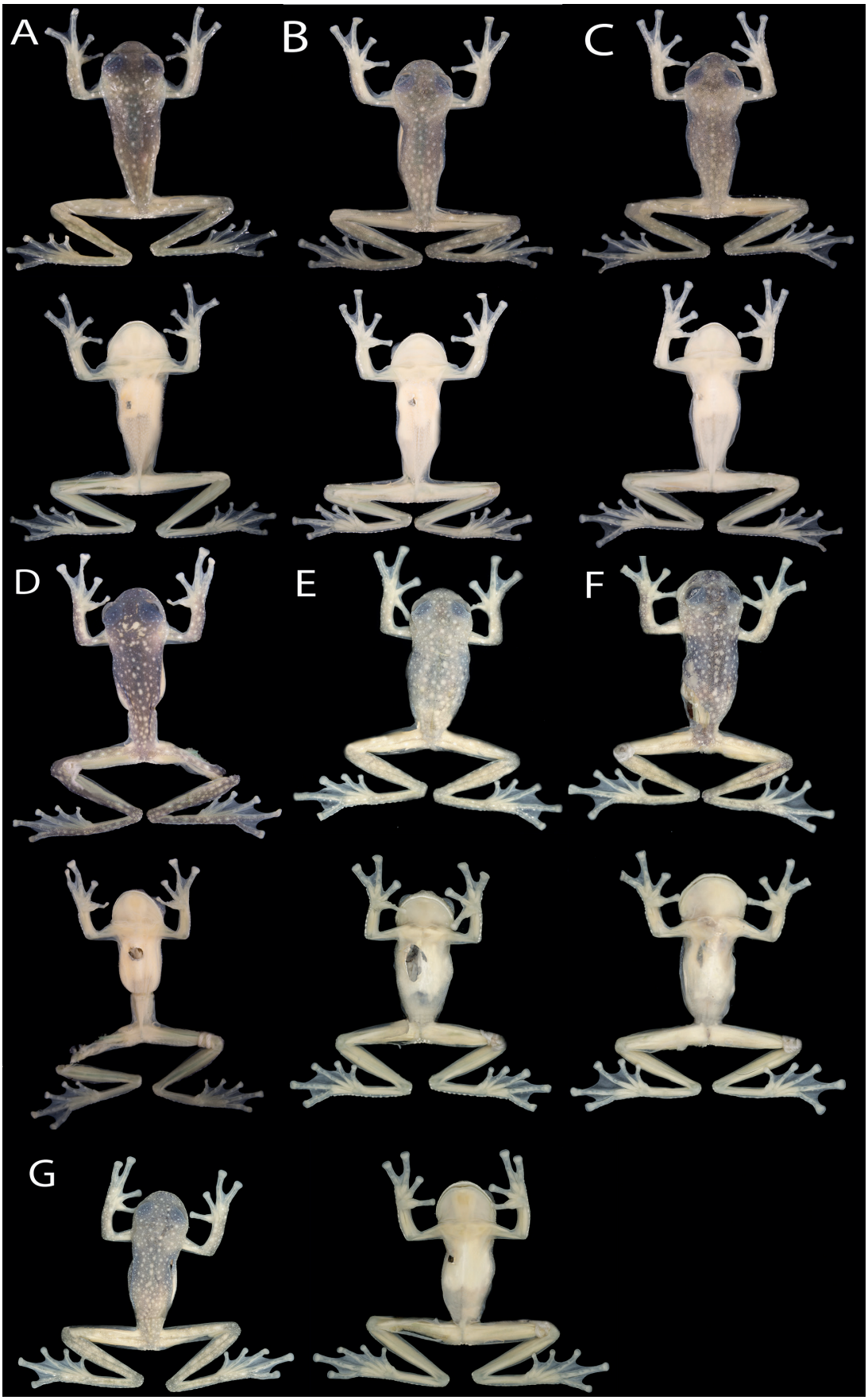

Figure 5. Dorsal and ventral views of preserved adult males of *Centrolene zarza* from Sardinayacu and Puchimi, Ecuador. **A.** QCAZ 58686, SVL = 23.9; **B.** QCAZ 58687, SVL = 24.1 mm; **C.** QCAZ 58688, SVL = 23.8 mm; **D.** QCAZ 58685, SVL = 23.8 mm; **E.** QCAZ 69116, SVL = 26.2; **F.** QCAZ 69117, SVL = 29.4; **G.** QCAZ 69118, SVL = 26.4 mm.

Conservation status

Székely et al. (2023) reported an extent of occurrence and area of occupancy for *C. zarza* of 7 km^2, based on a single known locality that could be affected by mining activities, thus categorising it as Critically Endangered [CR B1ab(i,ii,iii)+2ab(i,ii,iii)]. We increase the species' distribution range to four localities in three mountain ranges. Two localities are protected areas (Sangay National Park and La Zarza Wildlife Refuge), but localities at the Cóndor Cordillera are under intense mining pressure, and expansion of the agricultural frontier impacts the locality at the Kutukú Cordillera (Roy et al. 2018). The species has an extent of occurrence of 3026 km^2 and an area of occupancy of 24 km^2. We recommend categorising *C. zarza* as Endangered following the criteria B1ab(iii)+B2ab(iii).

***Centrolene kutuku* Ron, García, Brito-Zapata, Reyes-Puig, Figueroa-Coronel, & Cisneros-Heredia, sp. nov.**
https://zoobank.org/458CECC2-8C71-4390-8FD5-B4F1F6F42317

Proposed Spanish common name. Rana de Cristal de Kutukú.

Proposed English common name. Kutukú Glassfrog.

Type material. ***Holotype.*** (Figs 6–9) QCAZ-A 71386 (field number PUCE SC 61758), collected at República del Ecuador, provincia de Morona Santiago, cantón Santiago de Mendez, parroquia San Francisco de Chinimbimi, Cordillera del Kutukú, sector Puchimi, 22 km ESE Santiago de Mendez (2.7901°S, 78.1266°W, 2264 m elevation), by Diego Almeida, Diego Paucar, Darwin Núñez, Kunam Nusirquia, and Ricardo Gavilanes on 25 January 2018.

Paratypes. (1 specimen) QCAZ-A 71400, adult male, collected near the type locality (2.7903°S, 78.1265°W, 2255 m elevation) on 27 January 2018, same collectors as holotype.

Definition. *Centrolene kutuku* sp. nov. is distinguished from all other Centrolenidae by the following combination of characters: (1) dentigerous process of vomer present, without vomerine teeth; (2) snout slightly subacuminate to round in dorsal view and sloping in lateral view; (3) tympanic annulus evident, almost completely visible except for upper most border, tympanic membrane coloured as surrounding skin, supratympanic fold present; (4) dorsal skin microspiculate with abundant tubercles on head and body; (5) ventral skin granular, subcloacal area with two large subcloacal warts; elevated, abundant, enamelled, pericloacal warts, other cloacal ornamentation absent; (6) parietal peritoneum white—iridophores covering ½ of ventral parietal peritoneum (condition P2), pericardium covered by iridophores and all other visceral peritonea translucent (condition V1); (7) liver lobed and hepatic peritoneum translucent (condition H0); (8) adult males with short humeral spines, barely protruding through skin (Fig. 9); (9) absent webbing between fingers I and II, II (2$^-$– 2$^+$) –3$^-$ III (2$^+$) – (2½$^-$2 ⅓) IV; (10) toe webbing I (1–1 ½) –(2–2$^+$) II (1$^-$–1$^+$–2$^-$) III (1$^+$–1½) IV (1½–1) V; (11) dermal fringe along postaxial edge of Finger IV and hand, slightly enamelled ulnar fold; dermal fringe along postaxial edge of Toe V, slightly enamelled metatarsal and tarsal folds, warts on heel absent; (12) unpigmented nuptial pad Type I; prepollex concealed; (13) Finger II slightly longer than Finger I, (14) eye diameter larger than width of disc on Finger III; (15) colour in life, all dorsal surfaces green with light green dots, bones green; (16) colour in preservative, dorsal surfaces lavender with lighter tubercles, white supralabial stripe, flanks with contrasting dorsal-ventral transition in colour, dorsal surfaces of limbs lavender clearer than body with slightly lighter dots; two white spots on arm near shoulder; (17) in life, iris cream with grey reticulations; (18) melanophores on Finger IV absent; scattered melanophores along toes IV and V; (19) calling site of males unknown; call undescribed; (20, 21, 22) fighting behaviour, egg clutches, and tadpoles unknown; (23) SVL in adult males 21.5–21.9 mm (n = 2); females unknown.

Diagnosis. *Centrolene kutuku* sp. nov. differs from all other glassfrogs, except *C. heloderma*, by having humeral spines in males, dorsum green with light dots and without dark marks, dorsum with abundant tubercles, and visceral peritonea translucent (except for pericardium). *Centrolene kutuku* sp. nov. is similar to *C. camposi*, *C. ericsmithi*, *C. heloderma*, and *C. zarza* in having humeral spines in males and elevated dermal ornamentations (i.e., dorsal tubercles or warts) and lacking dorsal dark-coloured marks. *Centrolene camposi* differs from *C. kutuku* sp. nov. by having (characters of *C. kutuku* sp. nov. in parentheses) sloping snout in lateral view (rounded), tympanic annulus barely visible (completely visible), colouration in life green dorsum with thick yellowish-white labial stripe continuing into a faint yellowish lateral line and yellowish green flanks (green dorsum with light green dots), and larger body size in males (SVL 29.1–31.2 mm in *C. camposi* vs. 21.5–21.9 mm in *C. kutuku* sp. nov.). *Centrolene ericsmithi* differs from *C. kutuku* sp. nov. by having tympanic annulus barely visible (almost completely visible), dorsal skin with dispersed spicules (with abundant tubercles), colouration in life green dorsum with thin yellowish labial stripe continuing into a row of white lateral tubercles and yellowish lateral line (green dorsum with light green dots), and larger body size in males (SVL 27.3 mm in *C. ericsmithi* vs. 21.5–21.9 mm in *C. kutuku* sp. nov.). *Centrolene heloderma* differs by having pustular dorsal skin (with abundant tubercles), grey lavender dorsum in preservative (lavender), and outer tarsal fold with low white tubercles (enamelled fold without tubercles). *Centrolene zarza* differs by having snout rounded with elevated warts at the tip in dorsal view and sloping in lateral view (round, without elevated warts at the tip in dorsal view and rounded in lateral view), dorsal skin shagreen with scattered warts of varying size and abundant, elevated, enamelled warts on head and flanks (abundant tubercles, lacking enamelled warts), dorsum green with yellowish-green dots and enamelled warts (green dorsum with light green dots), and larger body size in males (SVL 23.2–26.2 mm in *C. zarza* vs. 21.5–21.9 mm in *C. kutuku* sp. nov.).

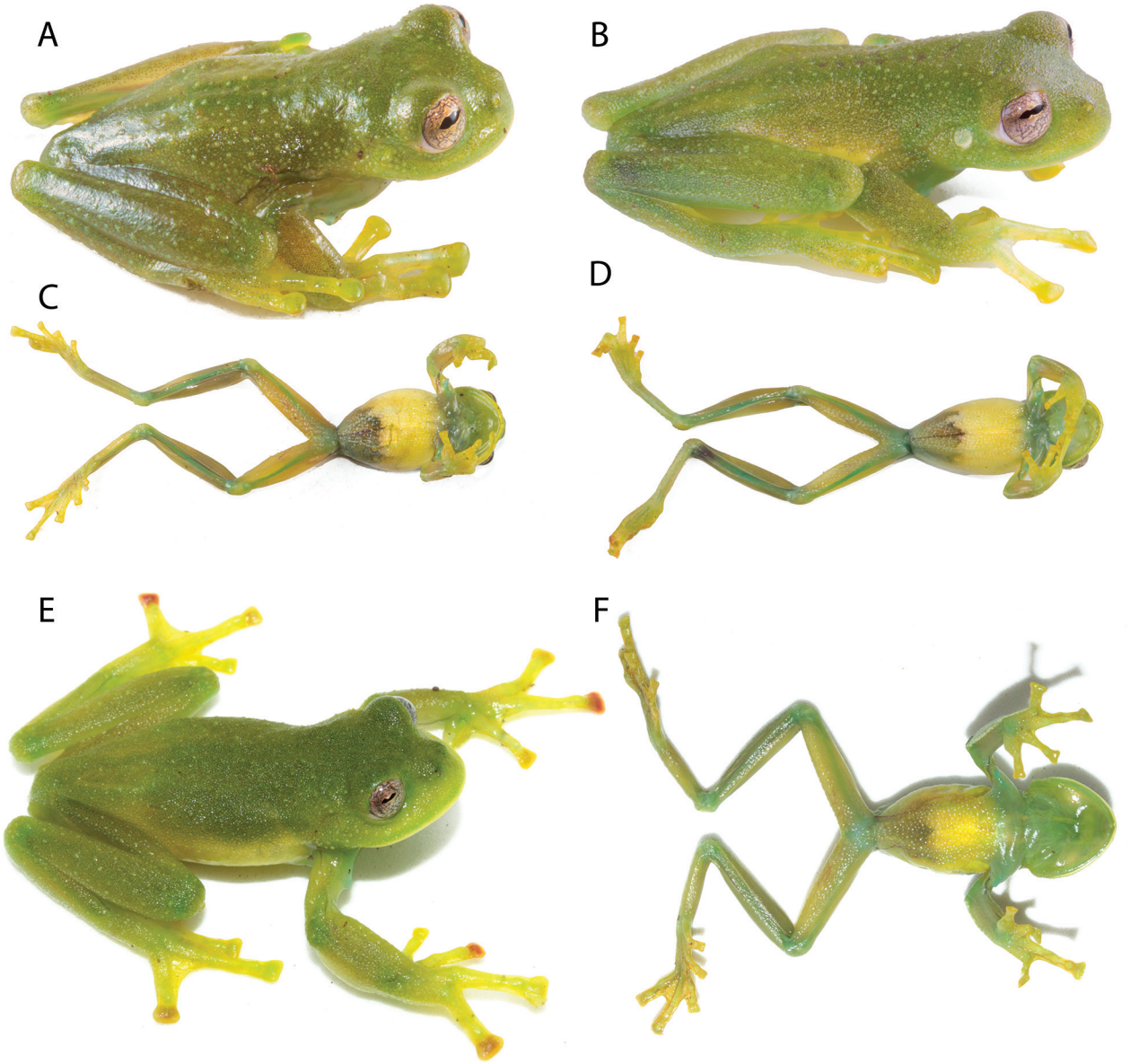

Figure 6. *Centrolene kutuku* sp. nov. and *C. camposi* in life. A, B, and E are dorsolateral views, and C, D, and F are ventral views. **A–C.** *Centrolene kutuku* sp. nov., holotype, male, QCAZ 71386, SVL = 21.9 mm; **B–D.** *C. kutuku* sp. nov., paratype, male, QCAZ 71400, SVL = 21.5 mm. **E–F.** *C. camposi*, holotype, DHMECN 11407, SVL = 29.1 mm. Photographs of *C. camposi* by Juan C. Sánchez-Nivicela.

Description of the holotype. (Figs 6–9) SVL = 21.9 mm, HW = 9.0, HL = 7.2, IOD = 4.1, ED = 2.8, EN = 1.4, IN = 1.9, TYD = 1.0, HAL = 8.6, FL = 10.8, TL = 13.2, F3DW = 1.5.

Adult male. Snout rounded in dorsal view, sloping in lateral view, EN/HL = 0.22; nostrils slightly elevated, producing a low depression in the internarial area, loreal region concave; canthus rostralis indistinct. Small-sized eyes, ED/HL = 0.38, directed anterolaterally at about 45° from midline, interorbital area wider than eye diameter, IOD/ED = 1.46, EN/ED = 0.59, EN/IOD = 0.34. Tympanic annulus evident and slightly oriented dorsolaterally, supratympanic fold above upper portion of tympanum and extending down to shoulder. Dentigerous processes of vomers present but vomerine teeth absent; choanae rounded, separated; tongue rounded; vocal slits present, extending from mid tongue to near jaw angle.

Skin of dorsal surfaces of body and limbs shagreen with scattered tubercles of varying size on head, dorsum and limbs. Skin of ventral surfaces of body granular. Cloacal opening directed posteriorly at upper level of thighs, no distinct cloacal sheath; subcloacal area coarsely granular with a pair of large, round, flat subcloacal warts on ventral surfaces of thighs below vent; pericloacal area with enamelled warts of similar size; other cloacal ornamentation absent.

Upper arm thinner than moderately robust forearm. Humeral spine present, short, and barely visible externally, not piercing the skin. Relative lengths of fingers III > IV > II > I; webbing formula between fingers absent

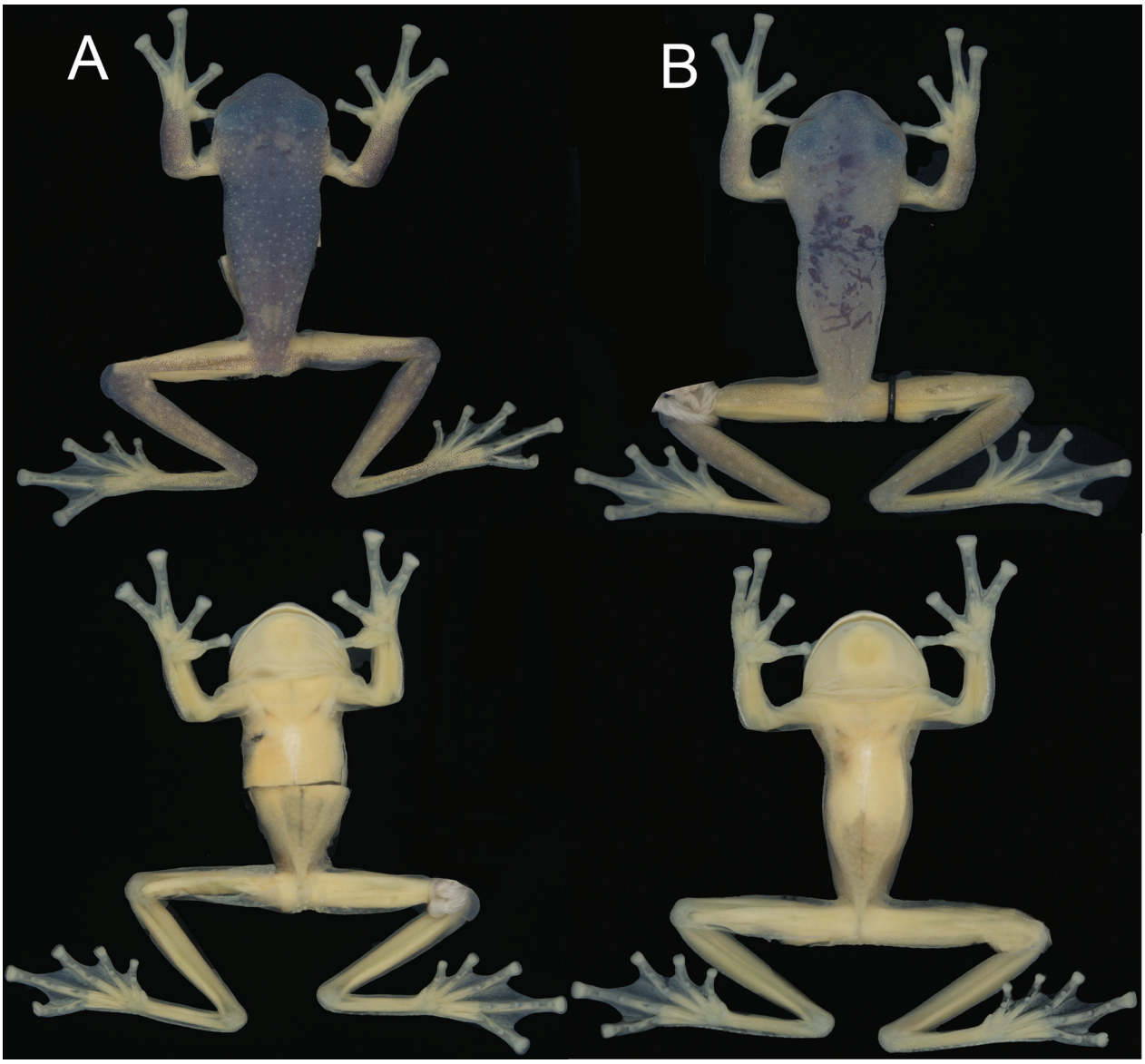

Figure 7. *Centrolene kutuku* sp. nov. in preservative. Upper row: dorsal view; lower row: ventral view. **A.** Holotype, male, QCAZ 71386, SVL = 21.9 mm; **B.** Paratype, male, QCAZ 71400, SVL = 21.5 mm.

between I and II, II basal III 2–2$^{1/3}$ IV; finger discs wider than the adjacent phalanx, nearly truncate; disc on third finger slightly larger than those on toes, ED/F3DW = 1.86; subarticular tubercles rounded and slightly elevated, supernumerary tubercles abundant and distinct; palmar tubercle prominent, rounded, elevated; thenar tubercle elliptic. Concealed prepollex, unpigmented nuptial excrescences present, Type I on dorsolateral side of thumbs.

Hind limbs slender; TL/SVL = 0.60, FL/SVL = 0.49. Inner metatarsal tubercle large and elliptical; outer metatarsal tubercle small and round. Subarticular tubercles rounded and low, supernumerary tubercles distinct but low. Webbing on feet I 1½–2 II 1–2 III 1–2 IV 2–1½ V; toe discs bluntly truncate. Papilla on tip of discs absent.

Colour of holotype. Colour in preservative is shown in Figs 7, 8. Pale dots on dorsum are of varying sizes. Iris silver with dark, fine reticulations. Parietal peritoneum white, iridophores covering 1/3 of ventral parietal peritoneum. Pericardium white (i.e., covered by iridophores), all other visceral peritonea translucent. Colour in life is shown in Fig. 6.

Variation. Measurements of QCAZ 71400 in mm: SVL = 21.5, HW = 8.5, HL = 7.0, IOD = 4.0, ED = 2.4, EN = 1.7, IN = 2.0, TYD = 0.9, HAL = 7.5, FL = 10.6, TL = 13.0, F3DW = 1.5. The single paratype differs from the holotype by having lower dorsal tubercles (wart-like), a dorsal lavender colour that is lighter, and more contrasting enamelled colouration on limbs.

Etymology. The specific name of this new taxon is a noun in apposition and refers to the Kutukú Cordillera, the Subandean Cordillera where the new species was discovered. The name originates from the Shuar language, meaning "paramo with stunted vegetation" (Pérez 1984).

Distribution and natural history. *Centrolene kutuku* sp. nov. is known from a single locality in southeastern

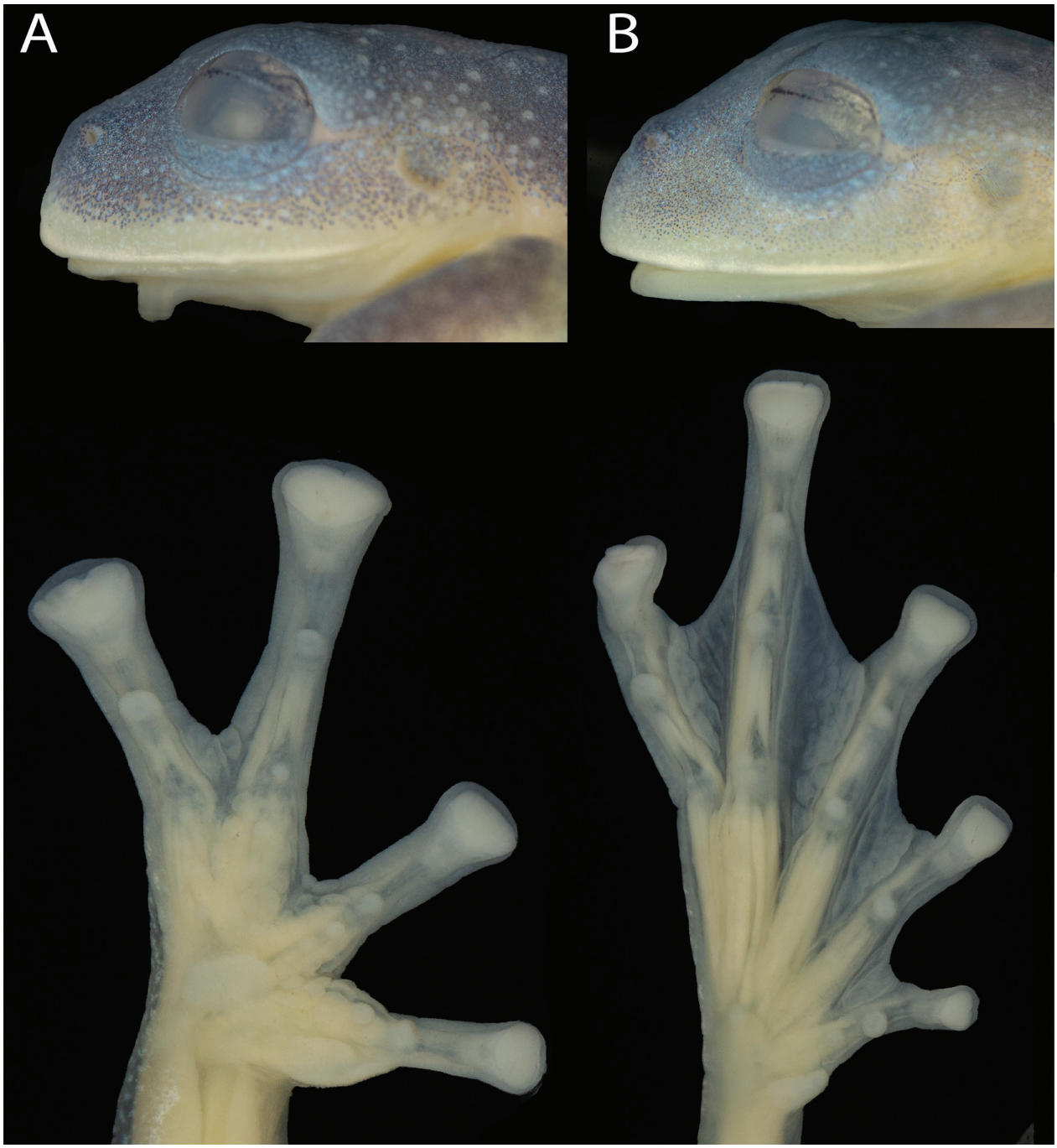

Figure 8. *Centrolene kutuku* sp. nov. head, hand, and foot in preservative. Upper row: lateral view of the head; lower row: ventral view of hand (left) and foot (right) of the holotype. **A.** Holotype, male, QCAZ 71386, SVL = 21.9 mm, head length = 7.2 mm; **B.** Paratype, male, QCAZ 71400, SVL = 21.5 mm, head length = 7.0 mm.

Ecuador, on the western slope of the Kutukú Subandean cordillera, between 2255–2264 m elevation (Fig. 2). The species inhabits Montane Evergreen Forest over the Sandstone Plateaus of the Cóndor-Kutukú Cordillera (*sensu* MAE et al. 2013). Sandstone plateaus in the Subandean Cordilleras have forest ground covered by mosses and roots, forming a false floor with large spaces between roots. The holotype was calling over a leaf 200 cm above the ground, on the edge of a small spring flowing into a black water ravine in a primary forest. The paratype was on a leaf, 300 cm above the ground, next to a black water stream. Both individuals were found in primary forest (QCAZ specimen database).

Conservation status. *Centrolene kutuku* sp. nov. is known from a single locality in the Kutukú cordillera, southeastern Ecuador. The locality is not in a protected area, and by 2020, there were agricultural lands at a distance of 2.7 km (MAATE 2022). Because collections in Kutukú have been limited, the distribution of the species is likely to be larger. The population status of this new species is unknown, and we recommend assigning it to the Data Deficient Red List category.

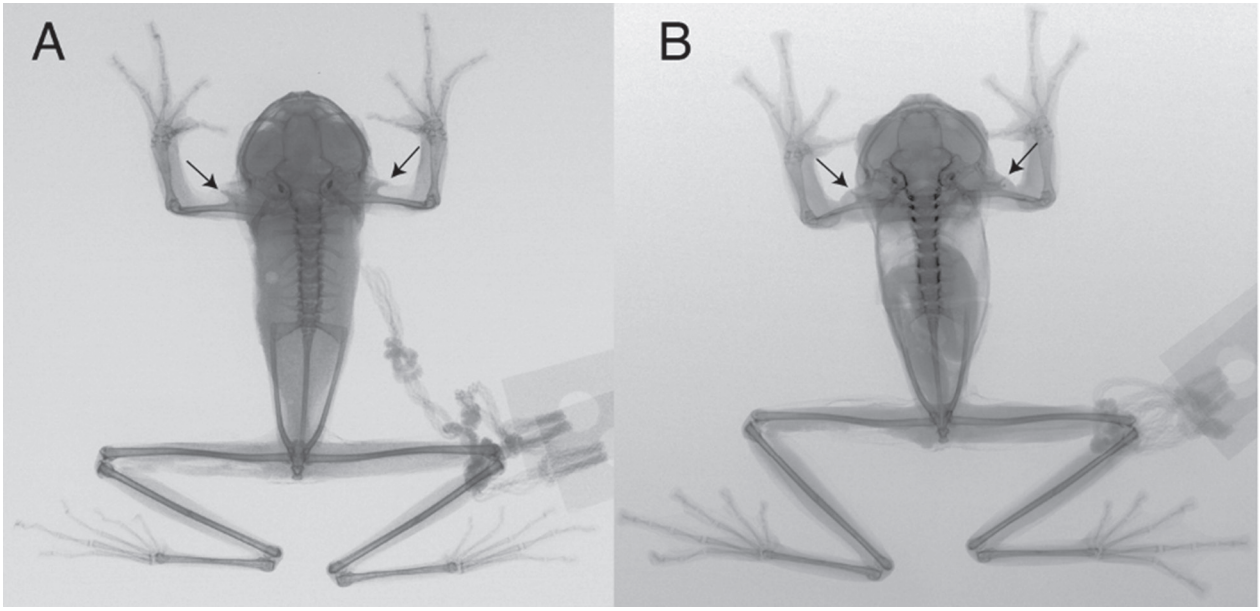

Figure 9. Dorsal X-ray views of *Centrolene zarza* and *Centrolene kutuku* sp. nov. **A.** *Centrolene zarza* QCAZ 58686, adult male, SVL = 23.9 mm; **B.** *Centrolene kutuku* sp. nov. QCAZ 71386, adult male (holotype), SVL = 21.9 mm. Black arrows point to humeral spines; notice the difference in angle orientation, curvature, and size.

Discussion

We present morphological and genetic evidence of the distinctiveness of *Centrolene kutuku* sp. nov., the 34th described species of *Centrolene* (AmphibiaWeb 2023). Of them, 29 have been included in molecular phylogenies (Fig. 1). The new species belongs to an old clade (origin ~8 Mya; clade B in Fig. 1) composed of four species, three of which have been described within the last two years (Cisneros-Heredia et al. 2023; Székely et al. 2023, and herein). Its sister clade (C in Fig. 1) has 15 species, almost four times more speciose. Because both clades have the same age, the diversification rate within clade C would appear much higher. However, we suspect this difference is partly a sampling artefact because clade B occurs in mountain regions of southern Ecuador and northern Peru, where amphibian inventories have been limited. Within the distribution range of clade B, the least sampled regions are the Subandean Cordilleras of Cóndor and Kutukú. *Centrolene kutuku* sp. nov. and *C. zarza* are the first *Centrolene* known from the Kutukú Cordillera. In addition to the Cóndor and Kutukú, the only other Subandean cordillera where species of *Centrolene* have been recorded is the Guacamayos Cordillera, with records of *Centrolene* cf. *venezuelense*, *C. muelleri*, and *C. sanchezi* (Guayasamin et al. 2020). Fieldwork in southwestern Ecuador and adjacent Peru should result in the discovery of additional species from that clade.

Centrolene kutuku sp. nov. is sister to *C. camposi*, a recently described species from the southwestern Andes of Ecuador (Cisneros-Heredia et al. 2023). This biogeographic pattern of sister species on opposite sides of the Andes is unusual, with only two other examples known in Centrolenidae: *Teratohyla amelie* (Cisneros-Heredia & Meza-Ramos, 2007)–*T. pulverata* (Peters, 1873) and *Cochranella granulosa* (Taylor, 1949)–*C. resplendens* (Lynch & Duellman, 1973) (Guayasamin et al. 2020). In both cases, divergence times are > 7 Mya, higher than the 2.7 Mya separating *C. camposi* and *C. kutuku* sp. nov. The topology and geographic distribution of Clade B suggest that its most recent common ancestor inhabited the Amazon basin of southern Ecuador. Therefore, *C. camposi* should result from recent dispersal across the Andes, from the Amazon basin to the Pacific basin, a putative example of peripatric speciation. Dispersal should have been possible due to the relatively low elevations of the Andes in southern Ecuador (Fig. 2). Cisneros-Heredia et al. (2023) discussed this pattern between *C. condor* and *C. camposi* because, at the time, they were considered sister species (*C. kutuku* sp. nov. was unknown).

Centrolene huilensis was described from the Huila department in southern Colombia and is currently known from a few localities in the southeastern slopes of the Central Cordillera of the Andes of Colombia (Ruiz-Carranza and Lynch 1995; Mendoza-Henao et al. 2020). It was reported from a single locality on the northeastern slopes of the Eastern Cordillera of the Andes of Ecuador, 320 km S of the type locality (Guayasamin et al. 2020). Our phylogeny shows that the Ecuadorian population is not closely related to *C. huilensis* from Colombia. Moreover, the Ecuadorian population has a low genetic distance from the Peruvian specimen of *C. muelleri* (CORBIDI 14667) collected near the species' type locality (0.6%, 16S gene). The colouration in life and external morphology of the Peruvian and Ecuadorian populations are very similar (see Fig. 9 in Twomey et al. 2014), reinforcing the hypothesis of conspecificity between them. The other Peruvian specimen (PV from

Gocta) probably represents a distinct species given its divergence time of 3.2 Mya relative to the other two populations. Our phylogeny and that of Twomey et al. (2014) are inconsistent with Guayasamin et al.'s (2020) phylogeny, which shows a monophyletic *C. muelleri* from Peru, sister to the Ecuadorian population. Our phylogeny shows, in contrast, that the Peruvian *C. muelleri* is paraphyletic relative to the Ecuadorian population (Fig. 1). This inconsistency is puzzling given that both topologies have strong support and, as far as we can tell, are based on the same set of sequences for both species. *Centrolene huilensis* and *C. muelleri* are morphologically very similar (Mendoza-Henao et al. 2020); nevertheless, both species are not closely related, indicating their phenotypic similarity is a remarkable case of morphological convergence within *Centrolene*.

Other samples with uncertain identification belong to populations of *Centrolene* from the eastern Andean slopes of Ecuador and Colombia (e.g., QCAZ 25744, MZUTI 85, MR371) that have been referred to either as "*C. buckleyi*" (e.g., Amador et al. 2018), "*C.* aff. *buckleyi*" (e.g., Castroviejo-Fisher et al. 2014), or "*Centrolene* sp." (Guayasamin et al. 2020). An additional closely related sample (QCAZ 47338) has been referred to as "*C. condor*" (e.g., Guayasamin et al. 2020; Székely et al. 2023). Our phylogeny indicates those samples are closely related to *C. venezuelense* (Fig. 1), with a divergence time of ~ 1 Mya. Because such a recent divergence is uncharacteristic for different species, we tentatively refer to them as "*C.* cf. *venezuelense*" until a thorough review is carried out for that clade. Similar to previous reviews (e.g., Castroviejo-Fisher et al. 2014; Cisneros-Heredia et al. 2023), we assign "*C. condor*" to a species belonging to clade B (Fig. 1) based on its resemblance to specimens of *C. condor* from the type locality.

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Supplementary material 1

Maximum likelihood time-tree of Centrolenidae

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Data type: pdf

Explanation note: Maximum likelihood time-tree inferred from a partitioned analysis of 6626 aligned sites of the mitochondrial genes 12S rRNA, 16S rRNA, ND1 and the nuclear genes BDNF, C-MYC 2, CXCR4, POMC, RAG1, SLC8A1, SLC8A3 showing phylogenetic relationships of Centrolenidae.

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