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Genetic and morphological evidence reveal another new toad of the *Rhinella festae* species group (Anura: Bufonidae) from the Cordillera Azul in central Peru

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Abstract. We studied the status of toads of the genus *Rhinella* collected in the southern Cordillera Azul, central Peru. Molecular analysis of the mitochondrial 16S rRNA gene revealed them to be members of the recently proposed *Rhinella festae* species group, and sister to *R. lilyrodriguezae*, a species known from northern areas of the Cordillera Azul. The new specimens are differentiated from *R. lilyrodriguezae* and other species of *Rhinella* by substantial genetic divergence in the studied gene fragment (> 5% uncorrected pairwise distance) and several qualitative morphological characters, providing combined evidence for a divergent evolutionary lineage. We consequently describe the specimens from the southern part of the Cordillera Azul in Departamento Huánuco as a new species, *Rhinella chullachaki* sp. n. We briefly discuss the definition and content of species groups in *Rhinella* as well as the difficulties hampering taxonomic resolution within this species-rich genus.

Key words. Amphibia, *Rhinella*, new species, species groups, morphology, molecular genetics, systematics, taxonomy.

Introduction

The Neotropical bufonid genus *Rhinella* Fitzinger, 1826 currently comprises 96 recognized species (AmphibiaWeb 2021). Within *Rhinella* as currently defined, certain species groups have been proposed, initially mainly based on osteological and external morphological similarities, and subsequently refined and altered as per molecular phylogenetic evidence (e.g., Martin 1972, Duellman & Schulte 1992, Moravec et al. 2014). Currently recognized groups include the *Rhinella crucifer*, *R. festae*, *R. granulosa*, *R. margaritifera*, *R. marina*, *R. spinulosa*, and *R. veraguensis* species groups (Cusi et al. 2017). Most of these species groups have experienced a complex taxonomic history, partly because of numerous available names considered to represent synonyms, and an apparently high degree of both cryptic diversity and intra-specific variation, resulting in practical difficulties to assign populations encountered in the field or in recent collections to type specimens of taxa that often were named a long time ago (e.g., Hoogmoed 1990, Duellman & Schulte 1992). This situation still challenges the systematic research in *Rhinella,* and the application of molecular genetics indicates that many undescribed and/or unallocated phylogenetic lineages exist in various species groups (e.g., Fouquet et al. 2007, Acevedo et al. 2016, Murphy et al. 2017), and that hybridization and introgression may take place (Pereyra et al. 2015).

Among the currently recognized species groups, the *Rhinella festae* group was recently proposed by Moravec et al. (2014). It contains species formerly assigned to *Rhamphophryne* Trueb, 1971, a genus synonymized with *Rhinella* by Chaparro et al. (2007) based on results of previous molecular studies (Pauly et al. 2004, Frost et al. 2006, Pramuk 2006). The species of the former *Rhamphophryne* were placed in the *Rhinella acrolopha* group by Grant & Bolívar-G. (2014), but without providing arguments for its monophyly. In their analysis of *Rhinella*, Moravec et al. (2014) revealed a clade that was sister to the *R. margaritifera* species group, containing species formerly placed in *Rhamphophryne*, as well as species formerly allocated to the *Rhinella veraguensis* group (*R. chavin, R. manu, R. yanachaga*; Chaparro et al. 2007, Lehr et al.

2001, 2007); they consequently proposed a new *Rhinella festae* group. This species group might be assumed to possibly include further species of the former genus *Rhamphophryne* (and possibly also of the *Rhinella veraguensis* group) for which molecular data are so far unavailable. The recognition of a *Rhinella festae* species group was subsequently supported by Cusi et al. (2017) who described an additional species of it (*R. lilyrodriguezae*).

During fieldwork in November 2019 in different areas of central Peru, we discovered individuals of *Rhinella* in the southernmost region of the Cordillera Azul, which, based on their general morphology and strongly protruding snout, were immediately recognized as being similar to some species currently placed in the *R. festae* group (sensu Cusi et al. 2017). We here provide morphological and molecular data evidencing that these specimens represent an unrecognized taxon of the *Rhinella festae* group and describe them as a new species.

Materials and methods

Fieldwork and voucher specimens

Fieldwork was conducted in November 2019 in different areas of the departments Huánuco and Ucayali in central Peru. Specimens were observed and collected during opportunistic searching at night using torchlights and headlamps. Geographic positions were recorded using handheld GPS receivers set to WGS84 datum.

Collected specimens were euthanized with an overdose of 5% lidocaine gel applied on the ventral surfaces of individuals (McDiarmid 1994). Tissue samples (thigh muscle and tongue pieces) were taken prior to fixation and stored in 99% ethanol, while specimens were fixed using 96% ethanol and subsequently stored in 70% ethanol. Specimens were sexed by examination of gonads and secondary sex characters, and deposited in the herpetological collections of the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos (MUSM), Lima, Peru, and the Zoologische Staatssammlung München (ZSM), Germany. FGZC refers to Frank Glaw field numbers. Other museum abbreviations mentioned follow those listed by Frost (2020).

Morphology

Morphometric measurements (in millimetres) were taken by ECU with a digital calliper to the nearest 0.1 mm. Measurements taken and used throughout the text are: SVL, snout–vent length; HW, head width (at level of angle of jaws); HL, head length (diagonally from angle of jaw to tip of snout); ED, horizontal eye diameter; IOD, interorbital distance; EW, upper eyelid width; EL, eyelid length (upper eyelid length); IND, internarial distance; E–N, eye–nostril distance (straight line distance between anterior corner of orbit and posterior margin of external nares); NSD, nostril–snout distance; SL, snout length (between anterior corner of eye and tip of snout); FL, forearm length (between flexed elbow and proximal edge of palmar tubercle); HNDL, hand length (between proximal edge of palmar tubercle and tip of finger III); FEML, femur length (between vent and knee); TL, tibia length; FOOTL, foot length (distance from proximal margin of inner metatarsal tubercle to tip of toe IV); PL, parotoid gland length (horizontal).

Fingers and toes are numbered preaxially to postaxially from I–IV and I–V, respectively. We determined comparative lengths of toes III and V by adpressing both toes against toe IV; relative lengths of fingers I and II were determined by adpressing the fingers against each other. Condition of the middle ear was assessed by dissection and visual examination under a stereoscope. We consider the tympanum being absent if there is no externally visible tympanic membrane, following the terminology of Lynch & Duellman (1997) and the definitions of Wever (1985) and Pereyra et al. (2016). Cranial crest definitions follow Trueb (1971) and Pramuk (2006), using the term 'occipital crests' instead of 'parietal crests'. Webbing formulae follow Savage & Heyer (1997). For the character of a protuberant anteroventrally directed snout, we employ the term 'shark snout' as used by Cusi et al. (2017). Description scheme and diagnosis follow Cusi et al. (2017). Colouration in life was described based on digital photographs and field notes. X-ray radiographs were obtained using the digital radiography system Comed TITAN 2000 (45 kV, 300 mA, 30 mAs) and were taken of the dorsal side of the holotype.

Taxon sampling

Genetic analysis aimed at identifying lineage divergence among the focal lineages of *Rhinella*. For representative taxon sampling, we largely followed the approach of Cusi et al. (2017). We compared two of our collected specimens of the putative new species with available 16S rRNA sequences of *Rhinella*. For this, we first blasted the sequences of the new samples against GenBank and downloaded all sequences with an identity > 92%. Next, we manually searched for sequences of species that bear morphological similarities to the target species, plus a set of representatives of species formerly assigned to the *R. granulosa*, *R. margaritifera*, *R. marina*, and *R. veraguensis* species groups, plus *R. spinulosa*. For the *R. margaritifera* species group, we added four new samples recently collected by us at different localities of central Peru, one of it close to the locality of the putative new species in the Cordillera Azul. A sequence of *Bufo bufo* was included as outgroup.

We follow Cusi et al. (2017) in recognizing the GenBank sample AF375533 as representing *R. macrorhina*, and omitting sample AF375532, as this sequence is of poor quality and apparently originates from the same specimen (Cusi et al. 2017). Furthermore, we continue to refer to sample KT221613 of *Rhinella* cf. *acrolopha* (sensu Grant & Bolivar-G. 2014) as *Rhinella* sp. 'C' as was done by Machado et al. (2016) and Cusi et al. (2017). For details of used samples, see Supplementary Table S1.

Molecular analyses

We sequenced a DNA fragment of the mitochondrial 16S rRNA gene from tissue samples of newly collected specimens using standard protocols (e.g., Vences et al. 2003). In brief, DNA was extracted using a standard salt extraction protocol, Polymerase Chain Reaction (PCR) was carried out with primers 16SAr-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16SBr-H (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi et al. 1991), and the PCR products were then directly sequenced on automated DNA sequencers by LGC Genomics (Berlin, Germany). All new DNA sequences were submitted to GenBank (accession numbers MW493260–MW493265). MEGA7 (Kumar et al. 2016) was used to align sequences to reference sequences of other *Rhinella* spp. downloaded from GenBank using the Muscle algorithm, and to identify the GTR+G substitution model as best fitting the data set under the Bayesian Information Criterion. We used this substitution model to infer a Maximum Likelihood (ML) tree in MEGA7, assessing node support with 2000 non-parametric ML bootstrap replicates. To quantify genetic divergences we calculated uncorrected pairwise distances among the sequences (p-distances).

Nomenclatural acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The LSID (Life Science Identifier) for this publication is: urn:lsid:zoobank.org:pub: EA20A1AB-858F-4311-BD87-9BF47D2DE487. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: www.zenodo.org, www.salamandra-journal.com.

Results

Our morphological comparisons are based on specimens and literature data from species allocated to the *Rhinella festae* group (including those formerly placed in the genus *Rhamphophryne*), the *R. veraguensis* group, and, to a lesser extent, the *R. margaritifera* group. These comparisons (summarized in the Diagnosis below) revealed a unique combination of qualitative morphological characters in our newly collected specimens that distinguish them from all currently known species of *Rhinella*. The presence of a protruding, anteroventrally directed 'shark snout' placed our specimens among some similar species in the *R. festae* species group. However, the absence of a tympanic annulus and a tympanic membrane, and/or a combination of at least two other qualitative morphological characters differentiates our specimens from these.

The Maximum Likelihood tree based on an alignment of only 578 bp of the 16S rRNA gene (Fig. 1) is insufficient to establish a reliable hypothesis of phylogenetic relationships within *Rhinella*, and accordingly, the deepest nodes in the tree remained unsupported (bootstrap values < 50%; Fig. 1). However, the analysis was sufficient to recover several species groups and resolved relationships among closely related lineages. Both the *R. festae* group (98%) and the *R. margaritifera* group (98%) were supported as monophyletic, confirming the previous results of Moravec et al. (2014) and Cusi et al. (2017). The new focal specimens were placed within the *R. festae* group, sister to *R. lilyrodriguezae* (71%). Our focal specimens had uncorrected pairwise distances in the studied 16S fragment of 5.1% to *R. lilyrodriguezae,* 6.3–9.1% to the other species in the *R. festae* group, and 8.7–12.8% to the other *Rhinella* spp. included in our analysis. Within the *R. festae* group, minimal distances were found between the sequences assigned to *R. festae* and *R. macrorhina* (1.4%), whereas distances among other species varied between 5.2–9.4%.

Rhinella chullachaki sp. n.

ZooBank LSID: urn:lsid:zoobank.org:act: 7412B95C-EEBE-4398-AAF9-0AA9272BB866

Holotype. MUSM 40293 (FGZC 6248), adult male, from a point (coordinates: -9.20408, -75.82169; 1780 m above sea level) near to and slightly southeast of La Cumbre, the highest point where the national road 5N crosses the Cordillera Azul, Departamento Huánuco, Peru, collected on 7 November 2019, by E. Castillo-Urbina, F. Glaw, and J. Köhler.

Paratypes. MUSM 40292 (FGZC 6247), ZSM 237/2019 (FGZC 6249), two adult males, same locality and data as holotype.

Etymology. The specific epithet is derived from the Quechuan language. The Chullachaki is a mythical creature of the Peruvian and Brazilian Amazon region said to be living in the jungle, being able to turn into any creature, and guarding the forest by punishing people acting unwisely in the forest. Many depictive representations of the Chullachaki show it as having a large nose. We chose the name as a plea for a more respectful and sustainable treatment of Peruvian forests. The specific name is an invariable noun in apposition.

Proposed common names. Chullachaki Beaked Toad; Sapo picudo Chullachaki.

Diagnosis. A medium-sized species of the *Rhinella festae* species group, based on morphological similarities and phylogenetic relationships. The new species is characterized by (1) medium size, SVL 42.1–44.2 mm in adult males

(n = 3), females are unknown; (2) eight presacral vertebrae; (3) bicondylar articulation of sacral vertebrae and coccyx; (4) snout long, acuminate, pointed to rounded terminally in dorsal view; snout protuberant, directed slightly anteroventrally in profile as a 'shark snout'; nostrils at level or only slightly beyond anterior margin of lower jaw in lateral profile; (5) cranial crests moderately developed; (6) canthal, supraorbital, postorbital and supratympanic crests distinctly elevated and continuous; pretympanic crest present, occipital crest weakly developed; (7) tympanum and tympanic annulus absent, middle ear cavity and columella present; (8) mandibular angle protruding; (9) parotoid glands moderately large, roughly rectangular and rounded in outline, swollen laterally, incorporated into the lateral row of tubercles; (10) dorsolateral rows of large, conical tubercles extending from parotoid gland to groin; (11) hands and feet with long digits, fingers basally webbed and toes moderately webbed; (12) skin on dorsum tuberacular with scattered large tubercles in the lateral and lumbar regions in males; (13) subarticular tubercles distinct, round to ovoid; (14) supernumerary tubercles present, round and well developed; (15) cloacal sheath absent; (16) in life, dorsum yellowish green with irregular black and dark brown markings; venter pale cream to white with black blotches and spots; black spots and flecking on ventral surfaces of limbs and scattered black spots on throat; iris bronze with irregular black reticulation.

Comparison with other species. *Rhinella chullachaki* shares similarities with members of the *Rhinella festae* group (sen-

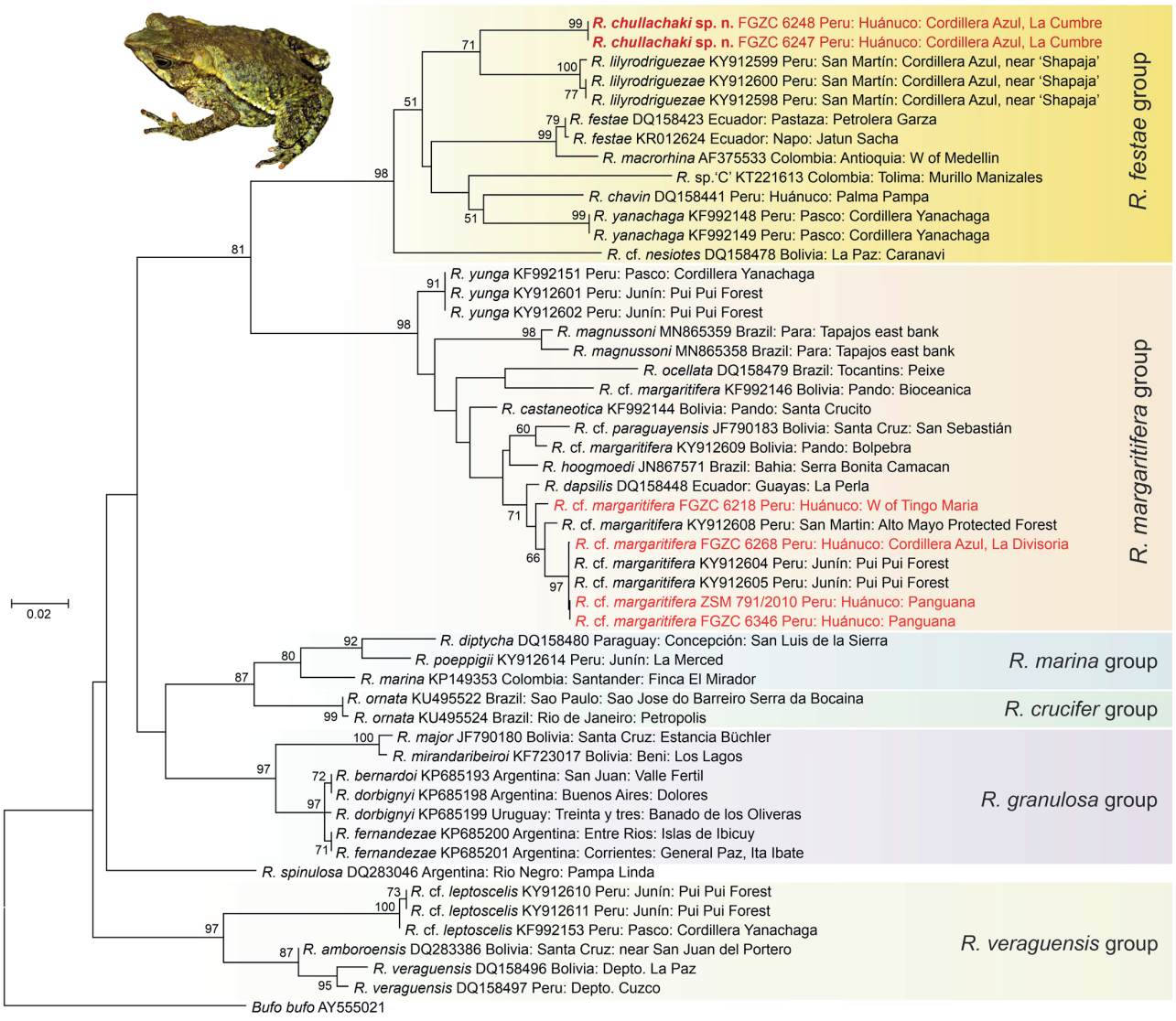

Figure 1. Maximum Likelihood tree inferred from a 578 bp alignment of the mitochondrial 16S rRNA gene of *Rhinella* species. Values at nodes are bootstrap proportions in percent (not shown if < 50%). Newly obtained sequences for this study are marked in red. *Bufo bufo* was used as outgroup. Inset photo depicts the holotype of *R. chullachaki* sp. n. in life (MUSM 40293, FGZC 6248).

su Moravec et al. 2014, Cusi et al. 2017). Seven species of the putative *R. festae* group from Panama, Colombia and Ecuador (*R. acrolopha*, *R. festae*, *R. macrorhina*, *R. paraguas, R. rostrata*, *R. ruizi* and *R. tenrec*) are morphologically most similar to *R. chullachaki*, with all of them having medium-sized adult males (maximum male SVL 41.9 mm in *R. acrolopha*, 42 mm in *R. festae*, 43.4 mm *R. macrorhina*, 41.7 mm in *R. paraguas*, 42 mm in *R. rostrata*, 39.4 mm in *R. ruizi*, 40.2 mm in *R. tenrec*, and 44.2 mm in *R. chullachaki*), a distinctly protuberant snout and the absence of a tympanum and tympanic annulus. *Rhinella chullachaki* is differentiated from *R. acrolopha*, *R. festae* and *R. macrorhina* by having the snout directed slightly anteroventrally, not bulbous at its tip (which is directed markedly anteroventrally in *R. acrolopha*, *R. festae* and *R. macrorhina,* and bulbous at the tip in *R. acrolopha* and *R. macrorhina*). Moreover, *R. chullachaki* differs from *R. acrolopha* by having eight presacral vertebrae (vs seven), moderately large parotoid glands (vs small), and hands basally webbed and feet moderately webbed (vs reduced webbing on hands and feet); from *R. festae* by having eight presacral vertebrae (vs seven), weakly developed, indistinct occipital crests (vs well developed), hands and feet with long digits (vs some shortened digits); and from *R. macrorhina* by having moderately developed cranial crests (vs well developed) and a dorsolateral row of large conical tubercles extending from parotoid gland to groin (vs row of small tubercles extending from posterior margin of the parotoid gland posteriorly to a point about half the distance between axilla and groin). *Rhinella chullachaki* is distinct from *R. tenrec* by having moderately developed cranial crests (vs poorly developed), pretympanic crests well defined (vs absent), dorsolateral row of tubercles extending from parotoid gland to groin (vs dorsolateral row of conical warts from posterior end of supratympanic fold to above groin), subarticular tubercles distinct, and supernumerary tubercles well developed (vs subarticular and supernumerary tubercles poorly developed). Additionally, *R. chullachaki* is distinguished from *R. macrorhina* and *R. tenrec* by having moderately large and evident parotoid glands (vs small, indistinct). The new species differs from *R. paraguas* and *R. ruizi* by having moderately developed cranial crests (vs very low and poorly defined cranial crests) and subarticular tubercles present, distinct (vs diffuse or indistinguishable); from *R. ruizi* and *R. rostrata* by having eight presacral vertebrae (vs seven) and snout directed slightly anteroventrally (vs directed straight anteriorly). Furthermore, *R. chullachaki* is distinguished from *R. ruizi* by its long digits (vs hands and feet with short digits); from *R. paraguas* by possessing basally webbed hands and moderately webbed feet (vs hands and feet extensively webbed); and from *R. rostrata* by males lacking vocal slits (vs present) (Trueb 1971, Lynch & Renjifo 1990, Grant 2000, Grant & Bolívar-G. 2014).

The remaining species possibly assignable to the *R. festae* group from Colombia are *R. nicefori*, *R. truebae*, and *R. lindae*. *Rhinella chullachaki* is distinguished from all these species by its snout being long, slightly directed anteroventrally as a 'shark snout' (vs short, directed slightly anteroventrally in *R. nicefori*; long, directed anteriorly in *R. truebae*; long, directed slightly upwards in *R. lindae*). Furthermore, *R. chullachaki* differs from *R. nicefori* by having larger males (maximum SVL 44.2 mm vs 32 mm), having eight presacral vertebrae (vs seven), postorbital crests (vs absent), well-defined supratympanic crests (vs barely evident), and moderately webbed toes (vs reduced webbing); from *R. truebae* and *R. lindae* by having moderately developed cranial crests (vs low), a dorsolateral row of spaced conical tubercles (vs dorsolateral folds formed by the fusion of tubercles), basally webbed hands and moderately webbed feet (vs both extensively webbed). Additionally, *R. chullachaki* differs from *R. truebae* by having eight presacral vertebrae (vs seven), tympanum and tympanic annulus absent (vs tympanum distinct and tympanic annulus evident ventrally), distinct subarticular tubercles (vs indistinct), and supernumerary tubercles present (vs absent) (see also Trueb 1971, Lynch & Renjifo 1990, Rivero & Castaño 1990).

Rhinella chullachaki is distinguished from all Peruvian species of the *R. festae* group (*R. chavin*, *R. manu*, *R. nesiotes, R. lilyrodriguezae*, and *R. yanachaga*) by the absence of a tympanum and tympanic annulus (vs tympanum and/or tympanic annulus present). Furthermore, *R. chullachaki* differs from *R. chavin* by having smaller males (maximum SVL 44.2 mm in *R. chullachaki* vs 52 mm in *R. chavin*), snout protuberant, directed slightly anteroventrally as a 'shark snout' (vs non-protuberant, rounded in lateral view), moderately large parotid glands, only slightly larger than ED (vs large, about twice the ED), dorsolateral row of conical tubercles extending from parotoid gland to groin (vs dorsolateral row of nearly round, elevated tubercles beginning above insertion of forelimb and extending to inguinal region), extremities without glands (vs elevated, elongate glands on forearm, tibia and outer dorsal margins of foot and hand) and hands and feet with long digits (vs short); from *R. nesiotes* by its larger males (maximum SVL 44.2 mm in *R. chullachaki* vs 29 mm in *R. nesiotes*), snout protuberant, directed slightly anteroventrally as a 'shark snout' (vs non-protuberant, rounded in lateral view), cranial crests moderately developed (vs absent), parotoid glands moderately large (vs smaller than ED), webbing of feet membranous (vs fleshy), and hands and feet with long digits (vs short); from *R. manu* by having larger males (maximum SVL 44.2 mm in *R. chullachaki* vs 32.3 mm in *R. manu*), snout protuberant, directed slightly anteroventrally as a 'shark snout' (vs snout pointed, non-protuberant), subrectangular to ovoid parotoid glands (vs swollen and oblong to the point of being nearly spherical), moderately developed cranial crests (vs inconspicuous, poorly developed), and its membranous webbing of the feet (vs fleshy); from *R. yanachaga* by possessing a distinctly protuberant, slightly anteroventrally directed 'shark snout' (vs slightly protruding), hands basally webbed and feet moderately webbed (well-developed webbing on hands and feet), and webbing of feet membranous (vs fleshy) (see also Lehr et al. 2001, 2017, Chaparro et al. 2007, Cusi et al. 2017).

The sister species *R. lilyrodriguezae* is rather similar to *R. chullachaki*, as both have a long, protuberant snout that is directed slightly anteroventrally as a 'shark snout', eight presacral vertebrae, moderately large parotoid glands, dorsolateral rows of conical tubercles extending from parotoid gland to groin, fingers and toes relatively long, hands basally webbed, feet moderately webbed, and long and slender extremities. *Rhinella chullachaki* differs from *R. lilyrodriguezae* by having smaller males (maximum SVL 44.2 mm vs 60.3 mm), tympanum and tympanic annulus absent (vs tympanum present and a weakly defined tympanic annulus), nostrils in lateral view at the level of or only slightly beyond the anterior margin of the lower jaw (vs distinctly beyond anterior margin of lower jaw; see Fig. 6) parotoid glands subrectangular to ovoid in outline (vs subtriangular), dorsolateral rows of large conical tubercles (vs dorsolateral row of small tubercles), occipital crest present, indistinct externally but distinct in X-ray images (vs absent; see Fig. 5 and Cusi et al. 2017), dorsal colouration in life green to yellowish green with predominantly brown and dark brown markings (vs uniformly dark brown to light brown with or without grey to whitish grey vertebral stripe), and ventral colouration pale cream to white with black spots and flecks, black spots on chest and limbs, dark markings on throat (vs cream yellow to brownish grey with minute light cream spots and darker mottling). More subtle differences between both species, which we are unable to confirm being consistent due to the small sample size, are: webbing of feet more fleshy in *R. lilyrodriguezae*; eyes less protruding laterally, and canthus rostralis only very slightly concave, almost straight, in dorsal view of head in *R. lilyrodriguezae* (vs a more concave outline in *R. chullachaki*; see Fig. 6).

From other species, formerly allocated to the *R. veraguensis* group (*R. amboroensis, R. arborescandens, R. fissipes, R. inca, R. justinianoi, R. leptoscelis, R. multiverrucosa, R. quechua, R. rumbolli, R. tacana, R. veraguensis*), of which some might actually represent members of the *R. festae* group (see Chaparro et al. 2007, Moravec et al. 2014), *R. chullachaki* is, among other characters, distinguished by the presence of a long, strongly protruding, anteroventrally directed 'shark snout', which is lacking in all the above-mentioned species (see Duellman & Schulte 1992, Harvey & Smith 1993, 1994, Köhler 2000, Lehr et al. 2005, Padial et al. 2006, 2009).

Rhinella chullachaki differs from species in the *R. margaritifera* group, several of which might exhibit a distinctly protruding snout, by genetic divergence and phylogenetic relationships, and (for the majority of recognized species) the lack of prominent orbital crests.

The new species furthermore differs from all other known species of *Rhinella* for which respective genetic data are available by a pronounced genetic divergence of at least 5% uncorrected p-distance in the analysed fragment of the mitochondrial 16S rRNA gene.

Description of the holotype. Adult male; body robust (Figs 2 and 3); SVL 44.2 mm; head triangular in dorsal view; head wider than long (HW 1.14 times HL), head width 34% of SVL; head length 29% of SVL; head narrower than body; snout acuminate, angular terminally in dorsal view; not bulbous at tip; distance from the nostril to the tip of the snout (2.9 mm) is noticeably less than the distance from the nostril to the eye (4.0 mm); snout long, protuberant, directed slightly anteroventrally as a 'shark snout', triangular at tip in profile (Fig. 3b); snout bearing a sagittal ridge ventrally between tip of snout and lip; canthus rostralis elevated posteriorly, angular in lateral view, distinctly concave in dorsal view; loreal region concave; nos-

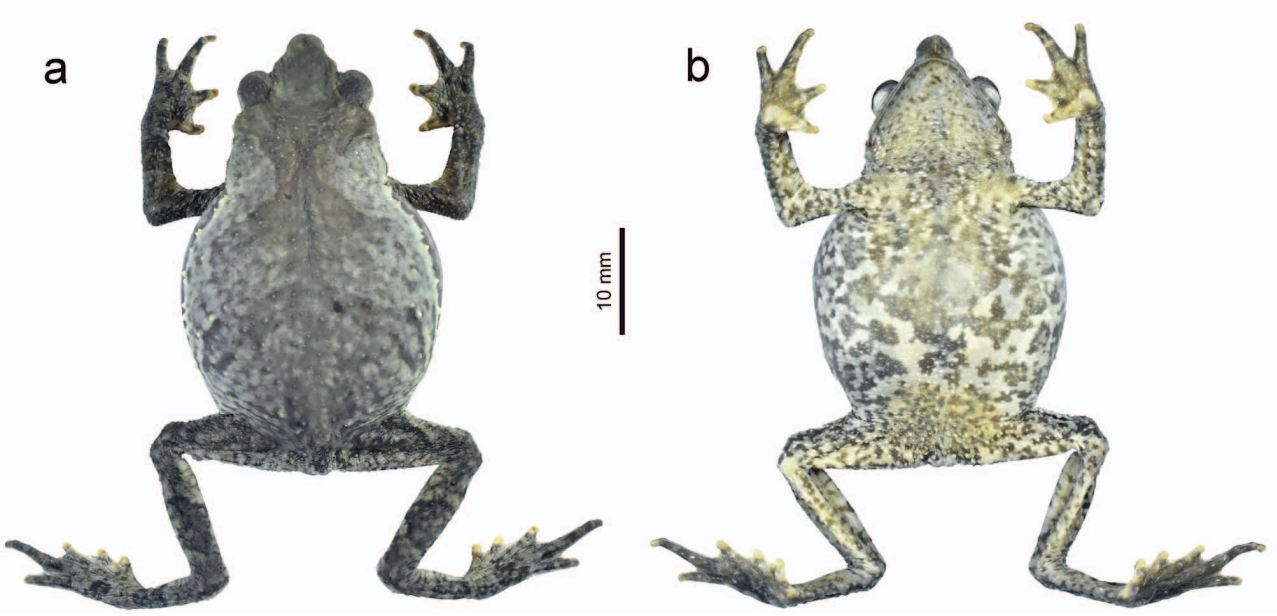

Figure 2. Preserved male holotype (MUSM 40293) of *Rhinella chullachaki* sp. n. in (a) dorsal and (b) ventral views.

trils small, rounded, slightly protuberant, directed laterally, slightly beyond anterior margin of lower jaw; dorsal internarial area concave; eye diameter slightly more than half the interorbital distance (ED/IOD = 0.58), ED shorter than E–N; canthal ridges angular and evident, expanded anteriorly at the tip of the snout to form an acuminate snout and slightly swollen posteriorly at the anterodorsal corner of the orbit; cephalic crests moderately developed; preorbital crest absent, supraorbital and postorbital crests distinct, continuous; occipital crest present, indistinct; supratympanic crest distinct, expanded laterally; pretympanic crests well defined; tympanum absent, tympanic annulus absent, eustachian tube and columella present; parotoid glands evident, subrectangular in dorsal and lateral views, moderately sized (slightly larger than ED), with roughly rounded corners in outline; skin on the upper eyelids granular, protruding and thickened laterally above the eye and bearing many, low, non-keratinized tubercles and some aggregated, low, keratinized tubercles on the flanks; forearms long, slender; forearm length 27% of SVL; dorsal surface of forelimbs spiculate, bearing densely scattered subconical tubercles; hand length 26% of SVL; mandibu-

Figure 3. Male holotype (MUSM 40293) of *Rhinella chullachaki* sp. n. in life (SVL 44.2 mm); (a) dorsolateral view; (b) lateral close-up view of head; (c) ventral view.

lar angle protruding in dorsal view, bearing some medium-sized subconical tubercles laterally and dorsally; hands with long, slender fingers; relative lengths of fingers I < II < IV < III; finger tips rounded; fingers basally webbed, webbing fleshy, with the following formula: I 2–2⁻ II 2⁻–3 III 3–3⁻ IV; all fingers bear well-defined lateral fringes; palmar tubercle prominent, round, larger than the oval thenar tubercle, about the same size as the palmar tubercle; subarticular tubercles evident, round to ovoid; supernumerary tubercles evident, well developed (Fig. 4a); fingers lacking nuptial pads; hindlimbs long, slender; tibia length 36% of SVL; tibia slightly longer than foot; dorsal surfaces of hindlimbs spiculate, with subconical tubercles; foot length 35% of SVL; toes long; relative lengths of toes I < II < III < V < IV; toe tips rounded; toes moderately webbed, webbing membranous, with the following formula: I 1–2⁻ II 0⁺–2 III 1–3$^{1/2}$ IV 3–2⁻ V; free portions of all toes bear well-defined lateral fringes; tarsal fold absent; inner metatarsal tubercle large, slightly elliptical, protuberant; outer metatarsal tubercle round, smaller than inner metatarsal tubercle, half the size of the inner metatarsal tubercle; subarticular tubercles distinct, round to ovoid (Fig. 4b); skin on dorsal surfaces of body with evenly distributed, numerous small, round, elevated tubercles, each bearing a single keratinized tip, large scattered subconical tubercles distributed in the lateral and lumbar regions; flanks with lower density of tubercles than dorsum; dorsolateral row of large, spaced, conical tubercles extending from parotoid gland to groin, not forming a continuous dorsolateral fold; skin of belly, throat, chest granular; cloacal opening slightly protruding, directed posteriorly at the mid-level of the thighs; tongue narrow, about 3.5 times as long as wide, notched anteriorly, posterior one half free; choanae small, ovoid, widely separated and partially concealed by the palatal shelf of the maxilla; maxillary, premaxillary and vomerine teeth absent; vocal slits absent; gonads white, with some scattered black reticulation. As inferred from X-ray radiographs, eight presacral vertebrae and a bicondylar articulation of the coccyx and sacral vertebrae (Fig. 5).

Measurements (in mm) of the holotype: SVL 44.2; HW 14.8; HL 13.0; ED 3.8; IOD 6.6; EW 3.5; EL 4.5; IND 3.6; E–N 4.0; NSD 2.9; SL 7.1; FL 12.0; HNDL 11.4; FEML 16.3; TL 15.9; FOOTL 15.3; parotoid gland length 4.6.

After app. 10 months in 70% ethanol (Fig. 2), mid-dorsal colouration grey with well distributed irregular dark grey and pale brown flecks and blotches; upper eyelids dark grey; white dorsolateral stripe, dorsolateral and parotoid colouration light grey; limbs with dark grey transverse bars and small, irregular, black spots; flanks grey with small, irregular, black spots and markings; tympanic region brown; upper lip cream without bars or spots; dorsolateral row of tubercles white, above a sharply contrasting black continuous ventrolateral band; throat, chest and posterior part of belly cream with irregular dark spots, belly white with black reticulations and black spots; ventral surfaces of limbs with black spots; palmar and plantar surfaces dark

Figure 4. Palmar and plantar surfaces of (a) left hand and (b) foot of the preserved holotype of *Rhinella chullachaki* sp. n. (MUSM 40293).

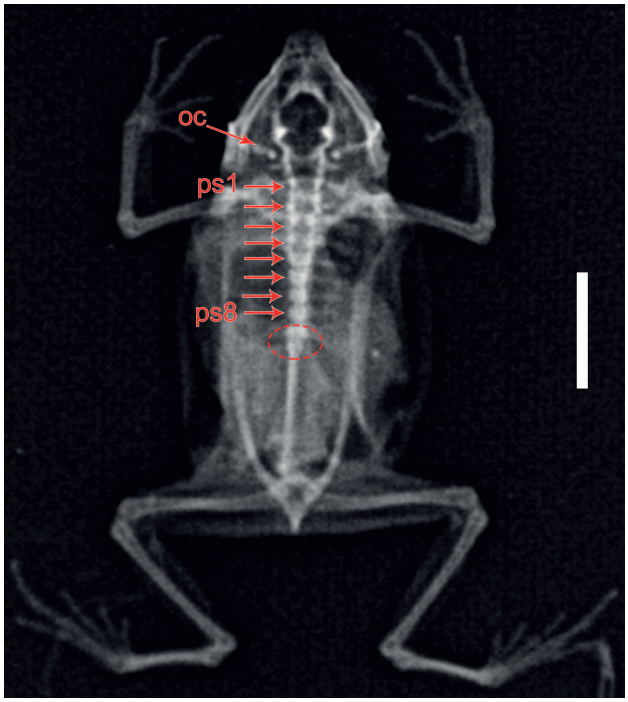

Figure 5. X-ray image of the preserved holotype of *Rhinella chullachaki* sp. n. (MUSM 40293) showing the number of presacral vertebrae (ps), the bicondylar articulation of sacral vertebrae and coccyx (red circle), and the occipital crest (oc). Scale bar equals 10 mm.

grey; subarticular and supernumerary tubercles on hand greyish white; tip of fingers and toes greyish cream.

In life (Fig. 3), the surfaces that appear grey in preservative used to be mostly green to yellowish green. This green colour generally covered all dorsal surfaces and the flanks. Darker markings were predominantly brown and dark brown on dorsum, and blackish on flanks and dorsal surfaces of limbs. Some conical dorsal tubercles brown to reddish brown. Enlarged, conical tubercles in dorsolateral row cream to white, some with orange tips. Dorsolateral row of tubercles bordered by a brown stripe below. Upper eyelid with reddish brown markings. Venter cream with black flecks and irregular blotches, throat with scattered black spotting. Palmar and plantar surfaces brown to reddish brown. Tips of fingers and toes orange brown. Iris bronze with irregular black reticulation.

Variation. With regard to measurements, the two paratypes vary as follows from the holotype (values in mm for MUSM 40292 followed by those for ZSM 237/2019 in parentheses): SVL 44.1 (42.1); HW 14.6 (14.1); HL 13.0 (12.1); ED 3.8 (3.9); IOD 6.2 (5.9); EW 3.1 (3.2); EL 4.4 (4.3); IND 3.5 (3.1); E–N 3.8 (3.8); NSD 2.4 (2.4); SL 6.9 (6.5); FL 12.0 (11.7); HNDL 10.8 (10.5); FEML 16.2 (15.7); TL 15.6 (15.3); FOOTL 15.2 (14.5); parotoid gland length 4.1 (3.7). With respect to colouration in life, MUSM 40292 was predominantly lemon green to yellowish green on all dorsal surfaces and exhibited fewer dark markings compared to the holotype (Fig. 7a). In ZSM 237/2019, the dorsal distribution of the green colour is similar to that of the holotype, but appeared lighter and more yellow in life (Fig. 7b). The same specimen exhibited rather sharply outlined black blotches and flecks on the belly, giving it a slightly different appearance in ventral view (Fig. 7d), whereas in MUSM 40292 the black flecks and blotches on the venter were somewhat more diffusely defined (Fig. 7c) compared to the holotype. All three specimens are very similar to identical with respect to all other morphological characters (e.g., crests, snout shape, tubercles, webbing), although in dorsal view the outline of the parotoid glands appears slightly more rounded posteriorly and ovoid in MUSM 40292, compared to the subrectangular outline in the holotype and ZSM 237/2019.

Distribution and threat status. The new species is reliably known only from the type locality (Fig. 8). Photos of an individual photographed close to the type locality of *R. chullachaki*, but apparently on the western flank of this mountain range (A. Sorokin pers. comm.), seem to confirm its presence in nearby areas of the southern Cordillera Azul (see the account of *R. lilyrodriguezae* at www.calphotos.berkeley.edu). Specimens found near Santa Rosa de

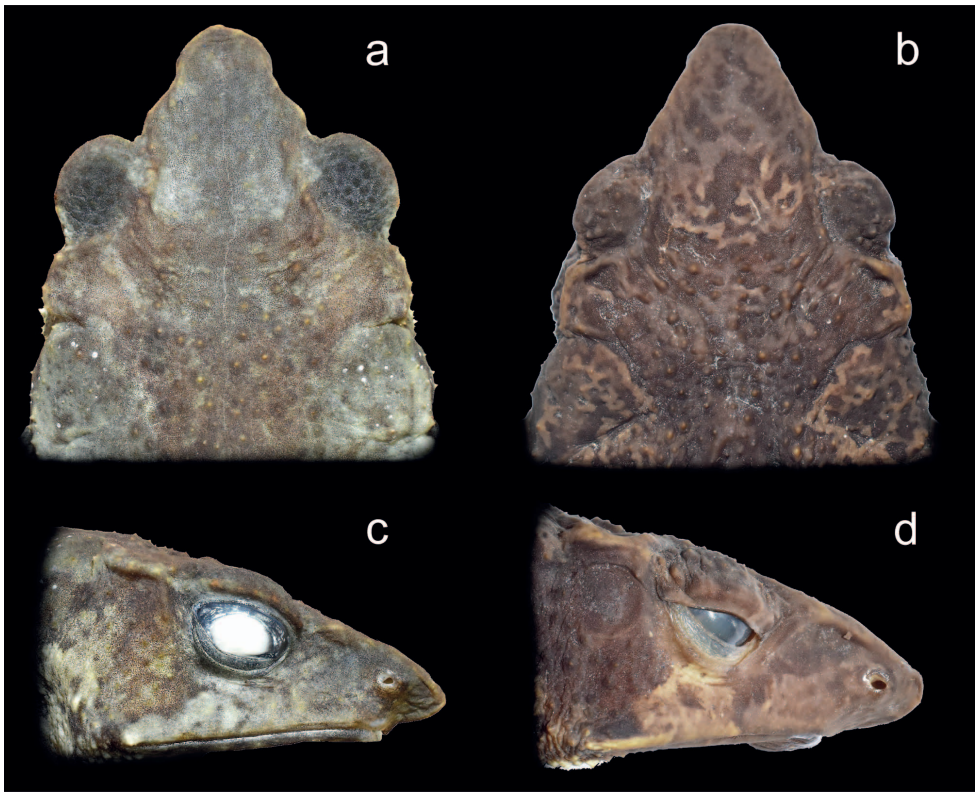

Figure 6. Comparative views of the preserved male holotype of *Rhinella chullachaki* sp. n. (MUSM 40293, left) and a male of *R. lilyrodriguezae* (MUSM 26511; right): (a, b) dorsal views of head (note differences in the outline of the canthus rostralis), and (c, d) head in lateral profile (note absence versus presence of a tympanic membrane and level of the nostril in relation to the anterior margin of the lower jaw). Not to scale.

la Cumbre and Chambirillo (Departamento San Martín) in the northern Cordillera Azul are identifiable as *R. lilyrodriguezae* based on morphological characters (S. Lötters pers. comm.). However, given the geographical distance of only app. 68 km air-line between the type locality of *R. chullachaki* and the locality of the male *R. lilyrodriguezae* (MUSM 26511), it seems plausible that both species may occur in sympatry in some areas of the Cordillera Azul, and it cannot be excluded that additional, still unnamed species of the *R. festae* species group occur in this mountain range.

Given the sparse data available and the fact that the possible occurrence of *R. chullachaki* in other areas of the Cordillera Azul still has to be confirmed, we propose to classify it as Data Deficient according to IUCN Red List criteria (IUCN 2017).

Natural history. The vegetation at the type locality constitutes evergreen montane rainforest on the eastern versant of the Cordillera Azul. The species was found upstream along a small creek, which crosses the main road 5N. At the date of collection, the rather steep slopes bordering the

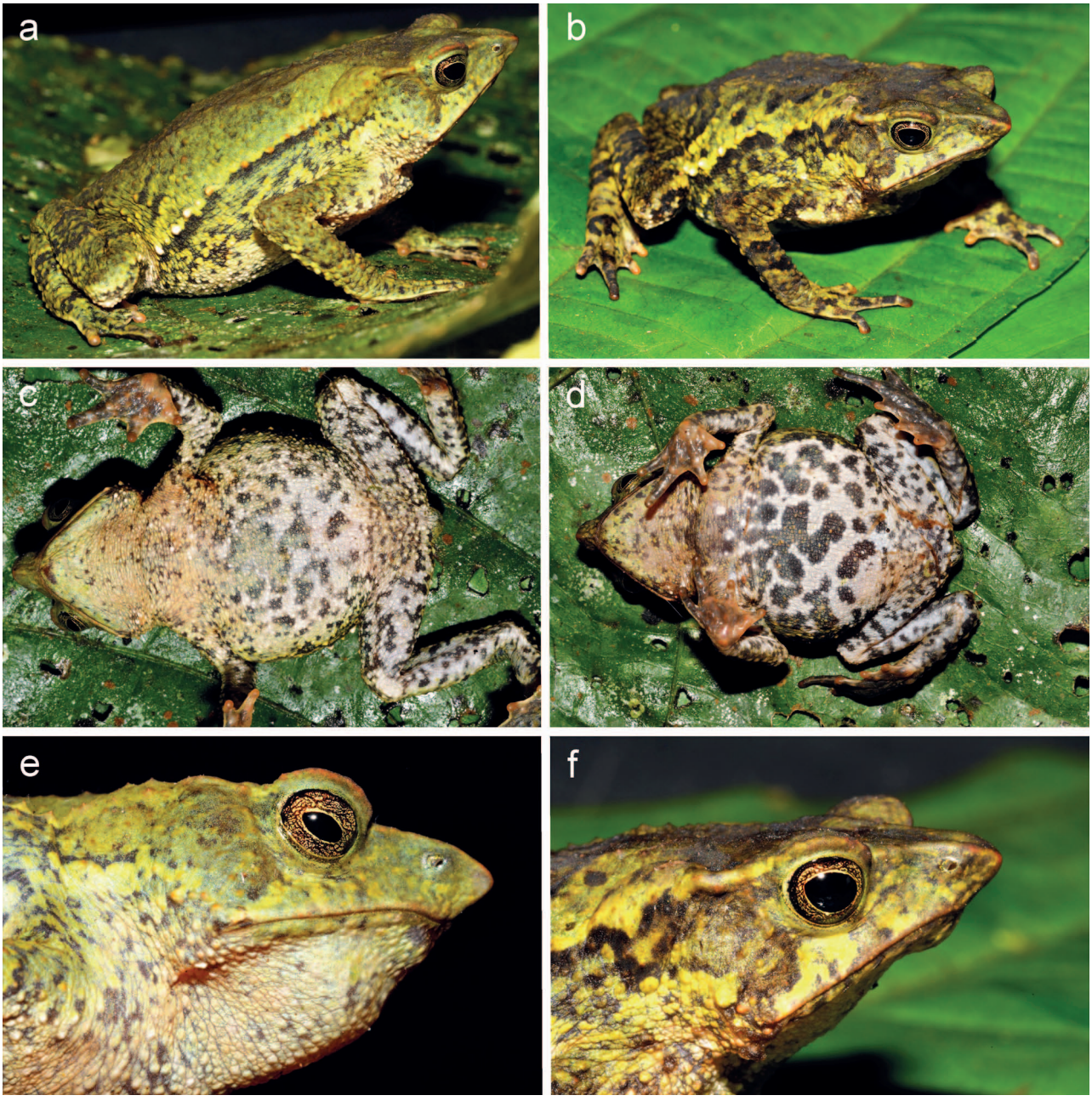

Figure 7. Male paratypes of *Rhinella chullachaki* sp. n. in life. Left column shows MUSM 40292, right column shows ZSM 237/2019, each in (a, b) dorsolateral, (c, d) ventral, and (e, f) close-up lateral head views.

creek were recently and largely cleared of vegetation, giving the habitat a heavily disturbed appearance. All specimens were found during a foggy and slightly rainy night, being perched on top of larger leaves of remaining plants approximately 1–2 m above the ground while inactive, suggesting diurnal and semiarboreal habits. Advertisement calls, larvae and reproductive mode are unknown. The only other anuran species found in sympatry at the type locality was an unidentified species of *Pristimantis* apparently distantly related to *P. rhabdocnemus*.

Discussion

Although not aimed at elucidating deeper phylogenetic relationships, our genetic analysis agrees with the former analyses of Moravec et al. (2014) and Cusi et al. (2017) in supporting the recognition of a *Rhinella festae* species group, being sister to the *R. margaritifera* species group. With the description of *R. chullachaki*, we add another species to this group, which seems to be most closely related to *R. lilyrodriguezae*, a species also occurring in the Cordillera Azul, Peru. A morphological comparison of both species was initially slightly impeded by the fact that the type series of *R. lilyrodriguezae* contained only females and juveniles (Cusi et al. 2017) and the known specimens of the new species are exclusively adult males. Sexual dimorphism in certain characters (e.g., tympanum condition) could thus not be completely excluded. The discovery of a male specimen in the MUSM collection (MUSM 26511; SVL 60.3 mm) originating from near the village of Consuelo (coordinates: -8.63056, -76.10778, ca. 650 m a.s.l.), on the western flank of the Cordillera Azul, Leoncio Prado, Huánuco, which we here identify as *R. lilyrodriguezae* based on its agreement in morphological characters (the only difference to the type specimens, probably due to sexual dimorphism, being its dorsal skin texture, which is more tubercular than in the females) confirmed the qualitative differences between both species provided in the comparison section above. Qualitative morphological and genetic differences thus provide conclusive evidence for their specific distinctness.

However, during the re-examination of the type specimens of *R. lilyrodriguezae* we noted that the condition of subarticular and supernumerary tubercles, which is listed as a diagnostic character (Cusi et al. 2017), is apparently variable between life and preserved stage, as observed by the comparison of specimens with photos of the same in life. In life, the tubercles are distinct and well-developed, but became diffuse and low in preservation. Consequently, we propose to regard such preservation-dependent characters with caution, as they may vary due to conservation period and method. We also observed that the holotype and the paratypes of *R. lilyrodriguezae* bear distinct lateral fringes on all fingers, in contrast with the condition "Finger IV bears well-defined lateral fringes" given in the

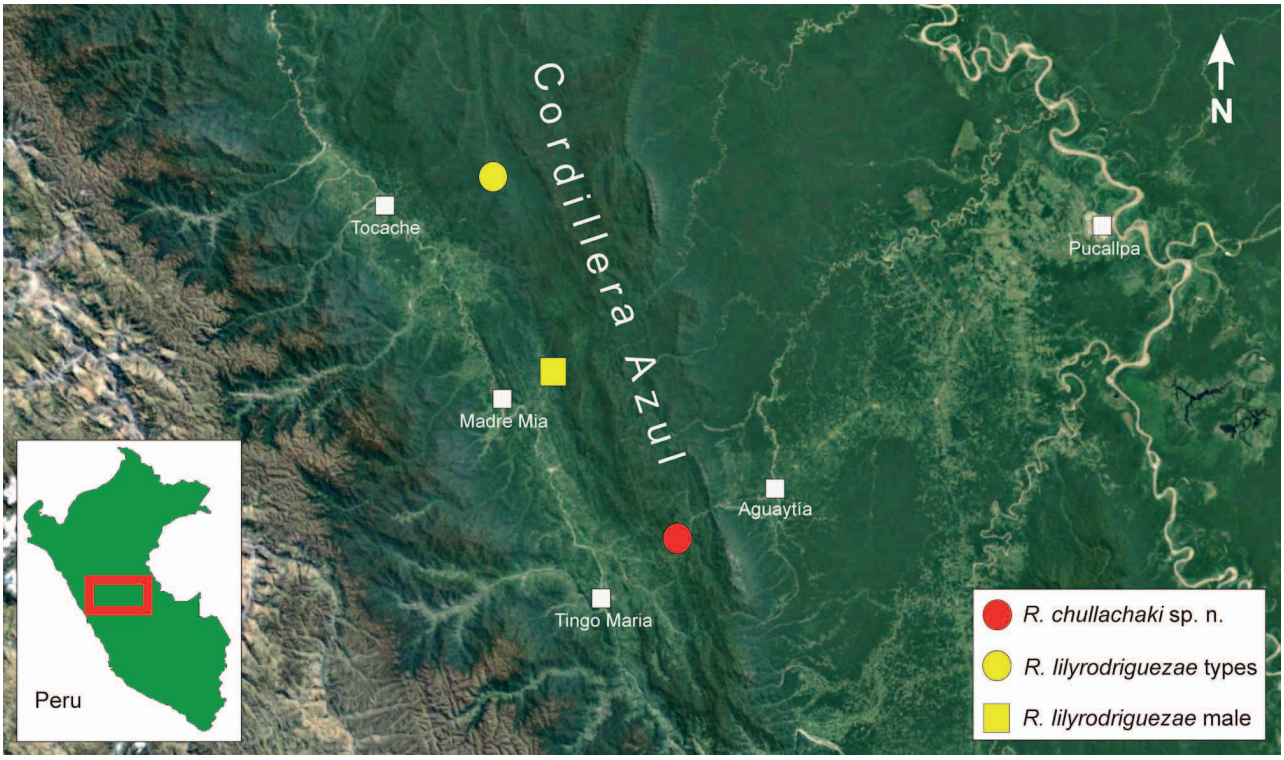

Figure 8. Satellite image of central Peru (from GoogleEarth) showing the known distribution of *Rhinella chullachaki* sp. n. and *R. lilyrodriguezae* in the Cordillera Azul.

original description (Cusi et al. 2017). Moreover, Cusi et al. (2017) state that in *R. lilyrodriguezae* the sacral vertebrae are fused with the coccyx. We found in *R. chullachaki* through X-rays and dissection that sacral vertebrae and coccyx (= urostyle) exhibit a bicondylar articulation as defined by Reilly & Jorgensen (2011). According to Pugener & Maglia (2009), the fusion of sacrum and urostyle is a common anomaly often identified in large adult specimens and likely the result of hyper-ossification. Thus, an apparent 'fusion' of sacral vertebrae and coccyx should be viewed with caution with regard to its use as a diagnostic character or putative synapomorphy.

A complete analysis of species in the *R. festae* group is hampered by the fact that genetic data are missing for several species supposed to represent members of it (mainly those formerly placed in the genus *Rhamphophryne*). In addition, the *R. festae* group may contain additional species without genetic data that were formerly allocated to the *R. veraguensis* group (Moravec et al. 2014), a phenetic group that several studies have demonstrated to be paraphyletic (Pramuk 2006, Chaparro et al. 2007, Van Bocxlaer et al. 2010, Pyron & Wiens 2011). For example, the GenBank sequence DQ158478 from Caranavi, Bolivia, we and former authors (Cusi et al. 2017) refer to as *R.* cf. *nesiotes*, is unlikely to be conspecific with *R. nesiotes* from the geographical distant type locality Serranía de Sira in central Peru. Although we were unable to examine the respective voucher specimen (UTA 53310), we speculate that this sample may actually represent a morphologically similar species, possibly *R. tacana* described from the nearby Madidi National Park and originally placed in the *R. veraguensis* group (Padial et al. 2006). Much broader sampling of taxa and genes is required to substantiate or disprove the monophyly of the proposed species groups and the allocation of certain populations and species to them. The current status of our knowledge is still rudimentary and the proposed species groups thus must be seen as preliminary hypotheses.

Furthermore, our analysis of samples again demonstrates the presence of an undescribed species diversity in the genus *Rhinella*. This became particularly evident in the *R. margaritifera* group (see also Fouquet et al. 2007, Moravec et al. 2014, Ferrão et al. 2020). Although we used a reduced dataset for this group, several apparently undescribed lineages were revealed. Interestingly, samples of *R.* cf. *margaritifera* from lowland Panguana seem to be conspecific with those from Pui Pui Protected Forest and La Divisoria (1650 m a.s.l.) in the Cordillera Azul, with the latter locality being rather close to the type locality of *R. chullachaki*. In contrast, our sample from west of Tingo Maria (coordinates: -9.29597, -76.12653, 783 m a.s.l.) is distinctly differentiated from the former. This demonstrates again that different species of this group may occur in close geographical proximity or even in sympatry (see Köhler & Lötters 1999, Moravec et al. 2014).

However, reaching taxonomic resolution in most species groups of *Rhinella* is hindered by the presence of available names considered to represent synonyms, historical taxa of uncertain status, or without precise locality data, in some cases by considerable intra-specific variation, morphological crypsis, and the occurrence of hybridisation and introgression (e.g., Hoogmoed 1990, Duellman & Schulte 1992, Padial et al. 2009, Pereyra et al. 2015). One example for an available historical name of uncertain status is *Bufo pleuropterus* Schmidt, 1857, a taxon probably originating from southern Peru and considered one of several synonyms of *Rhinella margaritifera* (Frost 2020). However, considering the external morphology of its type specimen (see Lötters & Köhler 2000), it seems more plausible that *Bufo pleuropterus* might be related to species placed in the *R. veraguensis* group, or even the recently proposed *R. festae* group. The newly established, albeit still challenging and costly, method of sequencing archival DNA from historical type specimens (see Hekkala et al. 2011, Rancilhac et al. 2020, Scherz et al. 2020) is probably the only possibility to overcome such taxonomic hurdles and shed light on the actual species diversity and relationships within *Rhinella*.

Bufonids were demonstrated to exhibit a remarkably high diversity with regard to the presence or absence of tympanic middle ear structures (Pereyra et al. 2016). The presence versus absence of a tympanic membrane and a tympanic annulus in certain species groups of *Rhinella* is noteworthy. A tympanum is absent in most species of the former genus *Rhamphophryne* (Trueb 1971, Grant 2000, Grant & Bolívar-G. 2014), with middle ear structures lacking at least in some of them (Pereyra et al. 2016). These species now are tentatively allocated to the *Rhinella festae* group, and the tympanum condition differs between the sister species *R. chullachaki* and *R. lilyrodriguezae* within this group. It appears that the absence of a tympanum and partly also the middle ear structures are a convergent condition that evolved independently multiple times in this clade. The same seems to be valid for the *R. margaritifera* species group (see Moravec et al. 2014). It has been demonstrated that bufonids with reduced external ear structures have a reduced sensitivity for high frequency reception and thus may rely mainly on low frequencies (< 900 Hz) in their hearing (Womack et al. 2017). A possible explanation for this phenomenon might be the use of extra-tympanic pathways to perceive sounds (Boistel et al. 2011, Pereyra et al. 2016), or the use of substrate vibrations by adults for intra-specific communication and in prey acquisition that may provide different selective pressures on ear structures. Vibration reception has been assumed for *R. paraguas* (see Grant & Bolívar-G. 2014), a putative member of the *R. festae* group, whereas the 'earless' *R. yunga* (*R. margaritifera* group) has been demonstrated to be equally sensitive to vibrations compared to bufonids that possess a tympanum (Womack et al. 2017). Ecological and evolutionary relevance of the tympanic condition in the species groups of *Rhinella* should be assessed in future studies focussing on bioacoustical behaviour to explain the phylogenetically widespread 'loss' (and 'regain') of this sensory structure (see Pereyra et al. 2016).

Note added in proof

While this article was in press, Pereyra et al. (2021) published a comprehensive phylogenetic analysis of toads of the genus *Rhinella*. The results supported the recognition of a *Rhinella festae* species group and are generally in agreement with our findings. The broader taxon sampling used by Pereyra et al. (2021) resulted in the inclusion of additional species in the *R. festae* group. Our assumption that the sample of *R.* cf. *nesiotes* from Bolivia (DQ158478) may actually correspond to *R. tacana* was supported by inclusion of Bolivian *R. tacana* in this species group. With respect to the samples and names used in our species description, we have to note that Pereyra et al. (2021) considered *R. yunga* a junior synonym of *R. iserni*, *R. paraguayensis* a junior synonym of *R. scitula*, *R. fernandezae* a junior synonym of *R. dorbignyi*, and *R. amboroensis* a junior synonym of *R. quechua*.

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Appendix
Specimens examined

Rhinella acutirostris: "flumen Amazonum" (= Amazon River, Brazil), ZSM 1147/0 (holotype).

Rhinella amboroensis: Bolivia: Cochabamba: 12.7 km by road E of Enpalme along road to Kara Huasi, 2150 m, MNK-A 953 (holotype).

Rhinella chavin: Peru: Huánuco: Palma Pampa, 20 km southeast of Chaglla, 3010 m, MUSM 20028 (holotype), MUSM 18439–18446; Peru: Huánuco: Carpish, MUSM 21473–21474.

Rhinella fissipes: Peru: Carabaya, Santo Domingo, 6000 feet, BMNH 1947.2.20.64 (holotype).

Rhinella inca: Peru: Huadquinia, 5000 feet, USNM 49557 (holotype).

Rhinella justinianoi: Bolivia: Santa Cruz: El Chape, 2050 m, MNK-A 950 (holotype); Bolivia: Cochabamba: old Chapare road, 1650 m, ZFMK 72600–72602; 2250 m, ZFMK 72621; Bolivia: Santa Cruz: Karahuasi, 1800 m, ZFMK 72657.

Rhinella leptoscelis: Peru: Carabaya, Santo Domingo, 6500 feet, BMNH 1907.5.7.32 (holotype); Bolivia: Cochabamba: old Chapare road, 1300 m, ZFMK 66985; 1400 m, ZFMK 72668–72671.

Rhinella lilyrodriguezae: Peru: San Martín: Bellavista: Alto Biavo: ca. 20 km from Park Rangers Center N° 53 "Shapaja" of the Cordillera Azul National Park, MUSM 32204 (holotype), MUSM 32201, 32205–32206, 32211, 32213 (paratypes); Peru: Huánuco: Leoncio Prado: Jose Crespo y Castillo: Consuelo village, MUSM 26511.

Rhinella manu: Peru: Cusco: Paucartambo, MUSM 21129, 26282, 27931–27932, 27929–27930, 27933, 30385.

Rhinella margaritifera: "Grenzgebiet von Bolivia gegen Peru, in etwa 3000 Höhe" [sic!] (= southern Peru; see Lötters & Köhler 2000), KM 1030 (holotype of *Bufo pleuropterus*).

Rhinella nesiotes: Peru: Huánuco: Yuyapichis: Comunidad El Sira, MUSM 29386–29387, 29390, 29404.

Rhinella quechua: Bolivia: Cochabamba: Parjacti, 83.2 km by road NE Cochabamba on road to Villa Tunari, USNM 257799 (holotype of *Bufo echinodes*); Bolivia: Cochabamba: Sehuencas, 2200 m, ZFMK 60255–60274, ZFMK 66835–66836; Bolivia: Cochabamba: Incachaca, 2300 m, ZFMK 66939–66941; Bolivia: Cochabamba: old Chapare road, 1400 m, ZFMK 72622.

Rhinella spinulosa: possibly Peru or Bolivia, NMW 16521 (syntype of *Bufo simus*); Peru: Puno: Arapa, 4500 m, MWNH 153/1 (lectotype of *Bufo spinulosus arapensis*).

Rhinella stanlaii: Bolivia: Cochabamba: road to San Onofre, 1900 m, CBF 3346 (holotype), USNM 257797–257798, ZFMK 67097 (paratypes); Bolivia: Cochabamba: km 96.7 on road from Cochabamba to Villa Tunari, 1967 m, USNM 257796 (paratype); Bolivia: Cochabamba: km 115 on road from Cochabamba to Villa Tunari, 1850 m, ZFMK 60464 (paratype); Bolivia: Cochabamba: km 36 on old Chapare road, 1600 m, ZFMK 67096 (paratype); Bolivia: Santa Cruz: La Hoyada, 1700 m, ZSM 144/1999 (paratype).

Rhinella veraguensis: Peru: Marcapata valley, BMNH 1947.2.21.23 (neotype; also lectotype of *Bufo ockendeni*); Bolivia: Santa Cruz: 29 km E of Guadalupe, 1600 m, ZFMK 66850–66851; Bolivia: Santa Cruz: La Yunga, 2300 m, ZFMK 66880; Bolivia: Cochabamba: old Chapare road, 1250 m, ZFMK 72555–72558; 1300–1500 m, ZFMK 72574–72575; 1650 m, ZFMK 72590–72592.

Rhinella yanachaga: Peru: Pasco: Oxapampa: Yanachaga-Chemillén National Park, W side of the Cordillera Yanachaga near Río San Alberto, 2600 m, MUSM 19994 (holotype).

Rhinella yunga: Peru: Pasco: Oxapampa: Yanachaga-Chemillén National Park, Quebrada San Alberto, 1950 m, MUSM 31097 (holotype).

Supplementary data

The following data are available online:

Supplementary Table S1. Species, GenBank accession numbers, voucher specimens, localities, and references for 16S rRNA sequences used in the genetic analysis.