

Article

A New Genus of Terrestrial-Breeding Frogs (Holoadeninae, Strabomantidae, Terrarana) from Southern Peru

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Abstract: We propose to erect a new genus of terrestrial-breeding frogs of the Terrarana clade to accommodate three species from the Province La Convención, Department of Cusco, Peru previously assigned to *Bryophryne*: *B. flammiventris*, *B. gymnotis*, and *B. manco inca*. We examined types and specimens of most species, reviewed morphological and bioacoustic characteristics, and performed molecular analyses on the largest phylogeny of *Bryophryne* species to date. We performed phylogenetic analysis of a dataset of concatenated sequences from fragments of the 16S rRNA and 12S rRNA genes, the protein-coding gene cytochrome c oxidase subunit I (COI), the nuclear protein-coding gene recombination-activating protein 1 (RAG1), and the tyrosinase precursor (Tyr). The three species are immediately distinguishable from all other species of *Bryophryne* by the presence of a tympanic membrane and annulus, and by males having median subgular vocal sacs and emitting advertisement calls. Our molecular phylogeny confirms that the three species belong to a new, distinct clade, which we name *Qosqophryne*, and that they are reciprocally monophyletic with species of *Microkayla*. These two genera (*Qosqophryne* and *Microkayla*) are more closely related to species of *Noblella* and *Psychrophrynella* than to species of *Bryophryne*. Although there are no known morphological synapomorphies for either *Microkayla* or *Qosqophryne*, the high endemism of their species, and the disjoint geographic distribution of the two genera, with a gap region of ~310 km by airline where both genera are absent, provide further support for *Qosqophryne* having long diverged from *Microkayla*. The exploration of high elevation moss and leaf litter habitats in the tropical Andes will contribute to increase knowledge of the diversity and phylogenetic relationships within Terrarana.

Keywords: amphibian; Andes; Cusco; high elevation; Neotropical; *Qosqophryne*; tropical mountain; systematic; taxonomy

1. Introduction

Terrestrial-breeding frogs of the high Andes display an impressive degree of evolutionary convergence [1–4]. Such convergence is associated with life in the cloud forest and high-Andean grassland. Frogs in many genera of Terrarana have evolved strikingly similar body forms [4,5], typically a small, compact body with very short legs and feet, short arms and hands, loss of toe pads and discs, head wider than long, small eyes directed anterolaterally, and, in many groups, reduction or loss of tympanic structure and function [3]. The high similarity of body forms has delayed obtaining a

taxonomic arrangement that reflects the evolutionary history and phylogenetic relationships of most species of small, terrestrial-breeding frogs of the Andes [1,6,7].

Illustrating the complexity within Terrarana of identifying monophyletic groups in presence of ecological convergence, authors originally assigned frogs belonging to different evolutionary lineages to the genus *Phrynopus* [1,8,9]. Indeed, *Phrynopus* might still contain incorrectly classified species of *Pristimantis* that lack vocal sacs, external tympanic apparatus and toe pads [10]. Subsequent molecular analyses revealed a much greater diversity and deeper genetic structure, such that Hedges et al. [1] proposed to split *Phrynopus* into four genera, and to erect the new subfamily Holoadeninae to include the newly described genera *Bryophryne*, *Niceforonia*, and *Psychrophrynella*. Within Holoadeninae, the molecular phylogeny by Hedges et al. [1] recognized *Bryophryne* as a distinct clade on the basis of DNA sequences from a single species, *B. cophites* (formerly *Phrynopus cophites* Lynch, 1975). Hedges et al. [1] used morphological characters to assign to *Bryophryne* a second species, *Phrynopus bustamantei* Chaparro, De la Riva, Padial, Ochoa, and Lehr, 2007. The new genus *Byrophryne*, along with the other genera of Holoadeninae, was recognized using molecular data, despite the lack of morphological synapomorphies [1,2,5,11].

Since Hedges et al. [1] published their molecular phylogeny, researchers have continued discovering terrestrial-breeding frogs: the number of species of *Bryophryne* has increased from two to 14 species [12–17], and the number of species across all Holoadeninae genera from 36 to 151 species [8]. As far as we know, all species of *Bryophryne* have micro-endemic distribution, and are only known to occur at their respective type localities and immediate surroundings [2,12,14–16,18]. The most recent phylogeny included six of the 14 species of *Bryophryne*, and recovered *Bryophryne* as being the sister taxon to the clade containing *Barycholos*, “*Eleutherodactylus bilineatus*”, *Euparkerella*, *Holoaden*, and *Noblella* [2]. However, this phylogeny by De la Riva et al. [2] did not include sequences of the three species of *Bryophryne* having an external tympanum and males with subgular vocal sacs, because sequences were unavailable at the time. Additionally, De la Riva et al. [2] erected a new genus, *Microkayla*, to accommodate all species of *Psychrophrynella* from Bolivia (and one species of *Psychrophrynella* from Peru), as well as two new species from Peru. Because of these discoveries, the integration of molecular, acoustic and morphological approaches, and the ongoing revision of existing and new material, we have a better understanding of the diversity in this group of cryptic genera. As part of our ongoing work, we have become aware of (1) uncertainty regarding the evolutionary relationships of *Noblella* and *Psychrophrynella* [2,19,20], (2) an underestimated species richness and endemism in *Noblella* and *Psychrophrynella* [19–22], and (3) three species of *Bryophryne* (*B. flammiventris*, *B. gymnotis*, *B. mancoinca*; Figure 1) having traits not shared with any other species of *Bryophryne*, such as having an external tympanum and males with subgular vocal sacs and emitting advertisement calls. Here we address the latter of these findings, and propose a new genus for the only three species of *Bryophryne* known to produce vocalizations and possessing external tympanic membrane and annulus.



Figure 1. Holotypes of species of *Qosqophryne* gen. n. in dorsolateral and ventral views: (A,B) *Q. flammiventris* (MUSM 27613; SVL 19.8 mm); (C,D) *Q. gymnotis* (MUSM 25543; SVL 18.4 mm); (E,F) *Q. mancoinca* (MUBI 11152; SVL 26.5 mm). Photographs by E. Lehr (A,B), A. Catenazzi (C,D) and L. Mamani (E,F).

2. Materials and Methods

We are familiar with most described species of *Bryophryne*, which we have seen in the field or inspected in collections. We provide a complete list of examined specimens in Appendix A. We used the literature (i.e., original species descriptions) for species whose specimens we could not examine. We have described the advertisement calls of *B. gymnotis* and *B. mancoinca* [14,17], and have heard and provided a short description of the call of *B. flammiventris* [15]. We refer readers to the original publications for details on recording methods.

We combined DNA sequences available from GenBank with sequences from newly collected tissues to generate molecular phylogenies of *Bryophryne* and closely related Holoadeninae taxa (Table 1). We considered sequences for a fragment of the 16S rRNA gene (16S), a fragment of the 12S rRNA gene (12S), the protein-coding gene cytochrome c oxidase subunit I (COI), the nuclear protein-coding gene recombination-activating protein 1 (RAG1), and the tyrosinase precursor (Tyr). All taxa selected for our comparisons belong to the subfamily Holoadeninae [1,23,24].

Table 1. GenBank accession numbers for taxa and genes sampled in this study. Genbank accession codes of the new sequences are highlighted in bold font.

Taxon	16S	12S	COI	RAG1	Tyr	Voucher Nbr	Reference
<i>Barycholos pulcher</i>	EU186709	-	-	-	EU186765	KU 217781	[1]
<i>Barycholos ternetzi</i>	JX267466	-	-	JX267543	JX267680	CFBH 19426	[23]
<i>Bryophryne bakersfield</i>	KT276291	KT276283	-	-	-	MHNC 6007	[12]
<i>Bryophryne bakersfield</i>	MF186344	MF186287	-	KT276278	-	MHNC 6009	[12]
<i>Bryophryne bustamantei</i>	MT437052	-	-	MT431911	-	MUSM 24537	This study
<i>Bryophryne bustamantei</i>	CMT437053	-	-	MT431912	-	MUSM 24538	This study
<i>Bryophryne bustamantei</i>	KT276293	KT276286	-	KT276280	KT276296	MHNC 6019	[12]
<i>Bryophryne cf. zonalis</i>	MT437054	-	MT435518	-	-	CORBIDI 17475	This study
<i>Bryophryne cophites</i>	EF493537	-	-	EF493423	EF493508	KU173497	[9]
<i>Bryophryne cophites</i>	KY652641	-	KY672976	KY672961	KY681062	AC 270.07	[22]
<i>Bryophryne hanssaueri</i>	KY652642	-	KY672977	KY681084	KY681063	MUSM 27567	[22]
<i>Bryophryne nubilosus</i>	KY652643	-	KY672978	KY681085	KY681064	MUSM 27882	[22]
<i>Bryophryne phuyuhampatu</i>	MF419259	-	-	-	-	CORBIDI 18224	[16]
<i>Bryophryne phuyuhampatu</i>	MF419259	-	-	-	-	MUBI 14654	[16]
<i>Bryophryne quellokunka</i>	MT437061	-	-	-	-	MUSM 27571	This study
<i>Bryophryne quellokunka</i>	MF186387	MF186309	-	MF186526	-	MNCN 43780	[2]
<i>Bryophryne sp.</i>	MT437062	-	-	MT431916	-	MUSM 27961	This study
<i>Bryophryne sp.</i>	MT437063	-	-	MT431917	-	AC 41.09	This study
<i>Bryophryne totra</i>	MF186396	MF186315	-	MF186541	MF186583	MNCN 43786	[2]
<i>Bryophryne wilakunka</i>	MF186349	MF186291	-	-	-	MUBI 5425	[2]
<i>Bryophryne zonalis</i>	MT437064	-	-	-	-	MUSM 27939	This study
<i>Eleutherodactylus bilineatus</i>	JX267324	-	-	JX267556	JX267691	MNRJ 46476	[23]
<i>Euparkerella brasiliensis</i>	JX267468	-	-	JX267545	JX267682	-	[23]
<i>Holoaden bradei</i>	EF493366	EF493378	-	EF493449	EU186779	USNM 207945	[9]
<i>Holoaden luederwaldti</i>	EU186710	EU186728	-	EU186747	EU186768	MZUSP 131872	[1]
<i>Holoaden luederwaldti</i>	JX267470	-	-	-	-	CFBH 19552	[23]
<i>Lynchius flavomaculatus</i>	EU186667	EU186667	-	EU186745	EU186766	KU218210	[1]
<i>Lynchius nebulanastes</i>	EU186704	EU186704	-	-	-	KU 181408	[1]
<i>Lynchius oblitus</i>	KX470783	KX470776	-	KX470792	KX470799	MHNC 8614	[25]
<i>Lynchius parkeri</i>	EU186705	EU186705	-	-	-	KU 181307	[1]
<i>Lynchius simmonsii</i>	JF810004	JF809940	-	JF809915	JF809894	QZ 41639	[26]
<i>Microkayla adenopleura</i>	MF186339	-	-	-	-	MNCN 44809	[2]
<i>Microkayla adenopleura</i>	MF186340	MF186283	-	MF186537	MF186565	MNCN 44810	[2]
<i>Microkayla ankohuma</i>	-	MF186288	-	-	-	MNKA 7280	[2]
<i>Microkayla ankohuma</i>	-	MF186289	-	-	-	CBF 5982	[2]
<i>Microkayla boettgeri</i>	MF186352	MF186293	MF186456	-	-	MNCN 43778	[2]
<i>Microkayla boettgeri</i>	MF186353	MF186294	-	-	MF186559	MUBI 5363	[2]
<i>Microkayla boettgeri</i>	MF186354	-	-	-	-	MUBI 5364	[2]
<i>Microkayla cf. iatamasi</i>	MF186365	-	-	-	-	MNCN-DNA 20927	[2]
<i>Microkayla chacaltaya</i>	MF186357	-	-	MF186532	-	MNCN 42052	[2]
<i>Microkayla chapi</i>	MF186417	MF186328	-	MF186540	MF186562	MNCN 43762	[2]
<i>Microkayla chilina</i>	MF186411	-	-	-	-	MUBI 5350	[2]
<i>Microkayla chilina</i>	MF186414	MF186327	MF186457	MF186539	MF186561	MNCN 43772	[2]
<i>Microkayla condoriri</i>	MF186358	-	-	-	-	CBF 5988	[2]
<i>Microkayla guillei</i>	AY843720	AY843720	-	-	DQ282995	AMNH A165108	[9]
<i>Microkayla iatamasi</i>	AM039644	AM039712	-	-	-	MTD TD 1231	[9]
<i>Microkayla illampu</i>	MF186373	-	-	-	-	CBF 5999	[2]
<i>Microkayla kallawaya</i>	MF186379	-	-	-	-	MNCN 42509	[2]
<i>Microkayla katantika</i>	MF186380	-	MF186453	-	-	CBF 6012	[2]

Table 1. Cont.

Taxon	16S	12S	COI	RAG1	Tyr	Voucher Nbr	Reference
<i>Microkayla kempffi</i>	MF186384	-	-	-	-	MNCN 43646	[2]
<i>Microkayla quimsacruzis</i>	MF186407	-	-	-	-	MNCN 42039	[2]
<i>Microkayla saltator</i>	AM039642	AM039710	-	-	-	MTD TD 1229	[9]
<i>Microkayla</i> sp. Coscapa	MF186399	-	-	-	-	CBF 6564	[2]
<i>Microkayla</i> sp. Khatu River	MF186409	-	-	-	-	MNCN 42034	[2]
<i>Microkayla teqta</i>	MF186400	MF186318	-	-	MF186552	MNCN 45702	[2]
<i>Microkayla uturuuro</i>	MF186433	-	-	-	-	MNCN 46987	[2]
<i>Microkayla wettsteini</i>	MF186434	MF186338	-	MF186531	MF186551	CBF 6241	[2]
<i>Niceforonia brunnea</i>	EF493357	-	-	-	-	KU 178258	[9]
<i>Niceforonia dolops</i>	EF493394	-	-	-	-	-	[9]
<i>Noblella heyeri</i>	JX267541	JX267463	-	-	-	QCAZ 31471	[23]
<i>Noblella lochites</i>	EU186699	EU186699	-	EU186756	EU186777	KU 177356	[1]
<i>Noblella losamigos</i>	MN366392	-	MN356099	-	-	MVZ 292687	[27]
<i>Noblella losamigos</i>	KY652644	-	-	KY672962	KY681065	MUSA 6973	[22]
<i>Noblella losamigos</i>	MN056358	-	MN356098	-	-	MUBI 17413	[27]
<i>Noblella madreseiva</i>	MN064565	-	-	MN355547	-	CORBIDI 15769	[27]
<i>Noblella myrmecoides</i>	JX267542	JX267464	-	-	-	QCAZ 40180	[23]
<i>Noblella myrmecoides</i>	MN056357	-	-	-	-	CORBIDI PV45	[28]
<i>Noblella pygmaea</i>	KY652645	-	KY672979	KY681086	KY681066	MUSM 24536	[22]
<i>Noblella</i> sp.	AM039646	AM039714	-	-	-	MTD 45180	[29]
<i>Noblella</i> sp. R	KY652646	-	KY672980	KY681087	KY681067	MUSM 27582	[22]
<i>Noblella thiuni</i>	MK072732	-	-	-	-	CORBIDI 18723	[28]
<i>Oreobates amarakaeri</i>	JF809996	JF809934	-	JF809913	JF809891	MHNC 6975	[26]
<i>Oreobates ayacucho</i>	JF809970	JF809933	-	JF809912	JF809890	MNCN IDIR5024	[26]
<i>Oreobates cruralis</i>	EU186666	EU186666	-	EU186743	EU186764	KU 215462	[1]
<i>Oreobates gemcare</i>	JF809960	JF809930	-	JF809909	-	MHNC 6687	[26]
<i>Oreobates granulosis</i>	EU368897	JF809929	-	JF809908	JF809887	MHNC 3396	[30]
<i>Phrynopus auriculatus</i>	EF493708	EF493708	-	-	-	KU 291634	[9]
<i>Phrynopus barthlenae</i>	AM039653	AM039721	-	-	-	SMF 81720	[29]
<i>Phrynopus bracki</i>	EF493709	EF493709	-	EF493421	-	USNM 286919	[9]
<i>Phrynopus bufoides</i>	AM039645	AM039713	-	-	-	MHNSM 19860	[29]
<i>Phrynopus heimorum</i>	AM039635	AM039703	MF186462	MF186545	MF186580	MTD 45621	[29]
<i>Phrynopus horstpauli</i>	AM039651	AM039719	-	-	-	MTD 44333	[29]
<i>Phrynopus inti</i>	MF651902	MF651909	-	MF651917	-	MUSM 31968	[3]
<i>Phrynopus kauneorum</i>	AM039655	AM039723	-	-	-	MHNSM 20595	[29]
<i>Phrynopus peruanus</i>	MG896582	MG896605	MG896615	MG896626	MG896631	MUSM 38316	[3]
<i>Phrynopus pesantesi</i>	AM039656	AM039724	-	-	-	MTD 45072	[29]
<i>Phrynopus spI</i>	MG896589	MG896606	-	MG896629	-	MUSM 33261	[3]
<i>Phrynopus tautzorom</i>	AM039652	AM039720	-	-	-	MHNSM 20613	[29]
<i>Phrynopus tribulosus</i>	EU186725	EU186707	-	-	-	KU 291630	[1]
<i>Pristimantis attenboroughi</i>	KY594752	-	KY962779	KY962759	-	MUSM 31186	[10]
<i>Pristimantis pluviialis</i>	KX155577	-	-	KY962769	-	CORBIDI 11862	[31]
<i>Pristimantis reichlei</i>	EF493707	EF493707	-	EF493436	-	MHNSM 9267	[9]
<i>Pristimantis stictogaster</i>	EF493704	EF493704	-	EF493445	-	KU 291659	[9]
<i>Psychrophrynella chirihampatu</i>	KU884559	-	-	-	-	CORBIDI 16495	[19]
<i>Psychrophrynella chirihampatu</i>	KU884560	-	-	-	-	MHNC 14664	[19]
<i>Psychrophrynella glauca</i>	MG837565	-	-	-	-	CORBIDI 18729	[20]
<i>Psychrophrynella</i> sp.	MT437065	-	-	-	-	MUSM 27619	This study
<i>Psychrophrynella</i> sp.	MT437066	-	-	-	-	MTD 47488	This study

Table 1. Cont.

Taxon	16S	12S	COI	RAG1	Tyr	Voucher Nbr	Reference
<i>Psychrophrynella</i> sp. P	KY652660	-	KY672992	KY681089	KY681081	AC116.09	[22]
<i>Psychrophrynella</i> sp. R	KY652661	-	KY672993	KY681090	KY681082	AC148.07	[22]
<i>Psychrophrynella usurpator</i>	KY652662	-	KY672994	KY672975	KY681083	AC186.09	[22]
<i>Qosqophryne flammiventris</i>	MT437055	-	-	-	-	MTD 46890	This study
<i>Qosqophryne flammiventris</i>	MT437056	-	-	MT431913	-	MUSM 27615	This study
<i>Qosqophryne gymnotis</i>	MT437057	-	-	MT431914	-	MUSM 24546	This study
<i>Qosqophryne gymnotis</i>	MT437058	-	-	MT431915	-	MUSM 24543	This study
<i>Qosqophryne mancoince</i>	MT437059	-	MT435519	-	-	MUBI 16068	This study
<i>Qosqophryne mancoince</i>	MT437060	-	MT435520	-	-	MUBI 16069	This study

2.1. Laboratory Work

We followed protocols of extraction, amplification, and sequencing of DNA previously used for terrestrial-breeding frogs [1,20,22]. For the focal taxa (the three species members of the new genus), we extracted DNA from tissue samples obtained from six specimens collected in the field (two specimens per species). We also obtained DNA sequences from seven specimens in five other species of *Bryophryne*, and two specimens representing two species in other genera (*Noblella* and *Psychrophrynella*), and the remaining sequences are legacy data from GenBank.

We extracted DNA from liver tissue preserved in 70% ethanol by using a commercial extraction kit (IBI Scientific, Dubuque, IA, USA). We used selected primers (Table 2) to amplify DNA from each gene using the polymerase chain reaction (PCR) [22,32]. We obtained sequence data by running purified PCR products in an ABI 3730 Sequence Analyzer (Applied Biosystems), except sequences of *B. mancoince* and *B. phuyuhampatu*, which we shipped to MCLAB (San Francisco, CA) for sequencing. We deposited all new sequences in GenBank (Table 1). We provide updated names of 86 terminals included in the analysis for 314 GenBank sequences.

Table 2. Primers used in this study.

Locus	Primer		Sequence (5'-3')	Reference
16S	16SAR	F	CGCCTGTTTATCAAAAACAT	[33]
	16SBR	R	CCGGTCTGAACTCAGATCACGT	[33]
12S	L25195	F	AAACTGGGATTAGATACCCCACTA	[33]
	H2916	R	GAGGGTGACGGGCGGTGTGT	[33]
COI	dgLCO1490	F	GGTCAACAAATCATAAAGAYATYGG	[34]
	dgHCO2198	R	TAAACTTCAGGGT GACCAAARAAYCA	[34]
RAG1	R182	F	GCCATAACTGCTGGAGCATYAT	[9]
	R270	R	AGYAGATGTTGCCTGGGTCTTC	[9]
Tyr	Tyr1C	F	GGCAGAGGAWCRTGCCAAGATGT	[35]
	Tyr1G	R	TGCTGGGCRTCTCTCCARTCCCA	[35]

2.2. Molecular Phylogenetic Analyses

We inferred the phylogenetic relationships among taxa through analysis of concatenated DNA sequences of the five gene fragments (16S, 12S, COI, RAG1, Tyr). We used *Niceforonia dolops* to root the tree. We aligned sequences with Geneious R6, v. 6.1.8 (Biomatters 2013), using the built-in Geneious Aligner program. We then used PartitionFinder, v. 1.1.1 [36] to select the best partitioning scheme and substitution model for each gene using the Bayesian information criterion (BIC). The best partitioning scheme included the following six subsets (best fitting substitution models are in parentheses): partition subset 1 includes 12S and 16S sequences (GTR + I + G), partition 2 is the first codon position of COI (SYM + G), partition 3 is the second codon position of COI (F81), partition 4 is the third codon position

of COI (HKY + G), partition 5 includes the first and second codon positions of RAG together with the first and second codon positions of Tyr (HKY + I + G), and partition 6 includes the third codon position of RAG together with the third codon position of Tyr (K80 + G).

We used MrBayes, v. 3.2.0 [37] to infer a molecular phylogeny for the 106 terminals and 2632 bp concatenated partitioned dataset (16S, 12S, COI, RAG1, Tyr). We performed an MCMC Bayesian analysis that included two simultaneous runs of 10 million generations, sampled once every 1000 generations. Each run had one “cold” chain and three heated chains, and the burn-in was set to discard 25% samples from the cold chain. Upon completion of the MCMC Bayesian analysis, the average standard deviation of split frequencies was 0.003916. We used Tracer version 1.5 [38] to examine the effective sample sizes (ESS), to verify convergence, and to verify that the runs reached stationarity. The observed effective sample sizes were satisfactory for all parameters (ESS > 200). Lastly, we used FigTree v. 1.4.2 [39] to visualize the majority-rule consensus tree and assess node support (based on posterior probability values).

Our research was approved by the Institutional Animal Care and Use Committee of Florida International University (18-009). The Dirección General Forestal y de Fauna Silvestre, Ministerio de Agricultura y Riego issued the permit authorizing this research (collecting permits #292-2014-MINAGRI-DGFFS-DGEFFS, SERNANP-Machu Picchu 054-2012-SERNANP-JEF, Contrato de Acceso Marco a Recursos Genéticos, No 359-2013-MINAGRI-DGFFS-DGEFFS).

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3. Results

We recovered a phylogenetic tree (Figure 2) that was largely congruent with previous analyses [2,24]. However, our tree recovered three species of *Bryophryne* not previously included in phylogenetic analyses (*B. gymnotis*, *B. flammiventris*, and *B. mancoinca*) as a clade that is sister to the clade containing all species of *Microkayla*. Thus, species of *Microkayla*, instead of other species of *Bryophryne*, share the most common shared ancestor with *B. gymnotis*, *B. flammiventris*, and *B. mancoinca*. The presence of large, external tympanic membrane and annulus, and males with a median subgular vocal sac and production of vocalizations, immediately distinguishes the newly recognized genus from all other species of *Bryophryne*. At least four species of *Bryophryne* were described as having small, barely visible (under the skin surface) tympanic membranes and annuli (*B. bustamantei*, *B. quellokunka*, *B. tocra*, *B. wilakunka*), but their external appearance does not look that different from the other species of *Bryophryne* known to lack a visible tympanic membrane [2,14,18]. One of these species, *B. bustamantei* was described as producing a short whistle, but there is no recording of the call nor voucher associated with a call [18]. The distribution range of *B. bustamantei* overlaps with that of *B. gymnotis* in the cloud forest near Abra Málaga [14,18,40], and thus it is possible that the call of *B. gymnotis* was erroneously associated with males of *B. bustamantei*. There also seems to be some problems identifying specimens of this species, as shown by our phylogeny where specimens identified as *B. bustamantei* by one of us do not group with sequences from one of the paratypes of *B. bustamantei* (MHNC 6019).

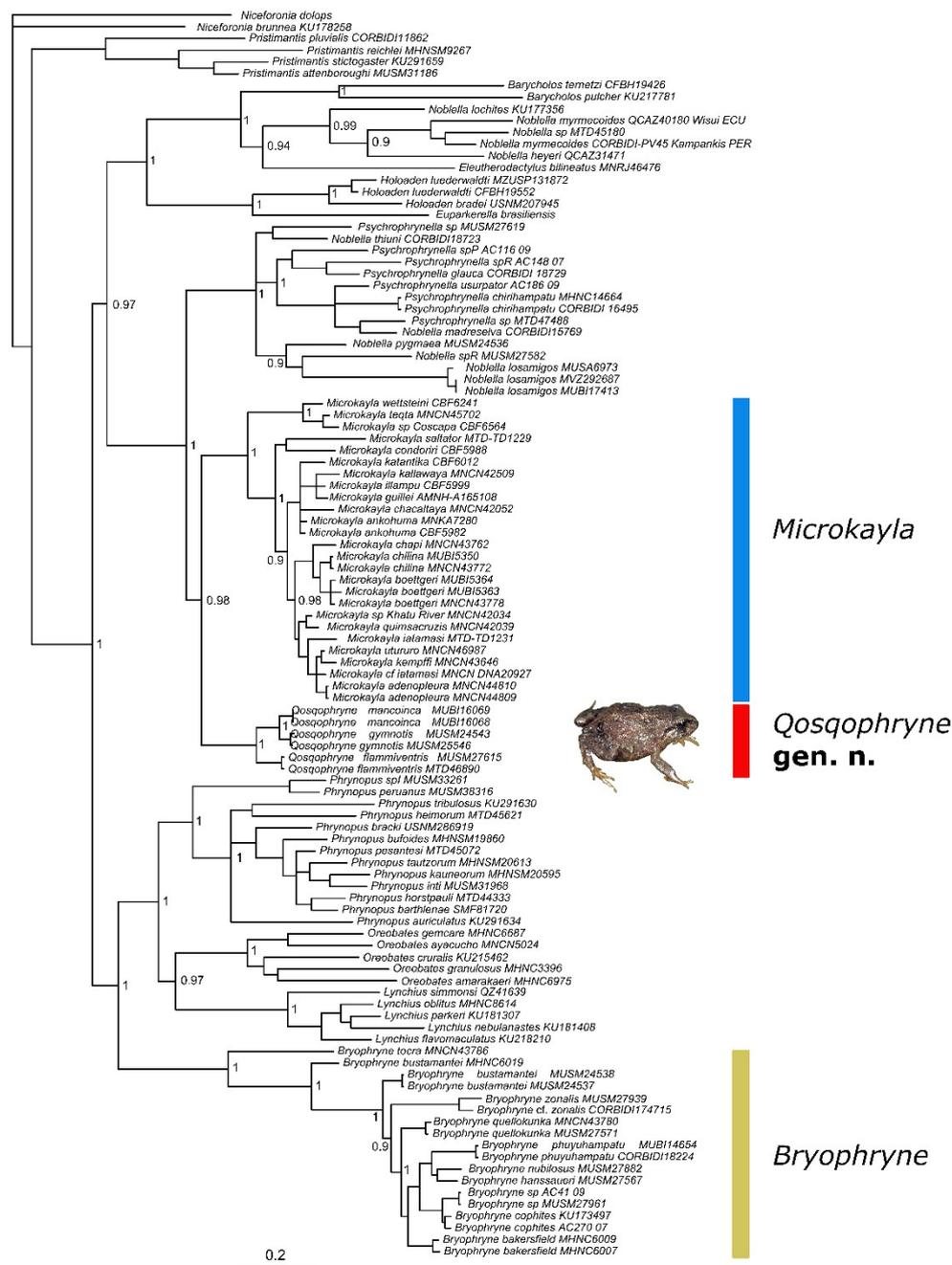


Figure 2. Bayesian maximum clade-credibility tree for 106 species of Holoadeninae (Terrarana) based on a 2646-bp concatenated partitioned dataset (fragments of genes 16S, 12S, COI, RAG1, and Tyr), highlighting the relationships of the three genera *Bryophryne*, *Microkayla* and *Qosqophryne* gen. n. Posterior probabilities are indicated at each node. The frog illustrated here is *Qosqophryne gymnnotis*, paratype MUSM 24542 (photograph by A. Catenazzi).

We propose to erect the new genus *Qosqophryne* gen. n. to accommodate *Bryophryne gymnnotis*, *B. flammiventris*, and *B. mancoincea*. Several lines of evidence support the idea that *Qosqophryne* is distinct from its sister genus *Microkayla*. The molecular phylogeny indicates there is a degree of divergence comparable to that observed between other genera of strabomantid frogs (Figure 2). Our molecular analyses show strong support for the divergence of *Microkayla* and *Qosqophryne* gen. n. The lack of geographic overlap between the two genera, with a gap region of ~320 km by airline where both genera are absent, further supports this divergence by preventing recent gene flow among species of both

genera (Figure 3). Furthermore, several glaciated peaks, including the massive Ausangate mountains and associates peaks of the Cordillera de Vilcanota, are interspersed along this gap region of 320 km.

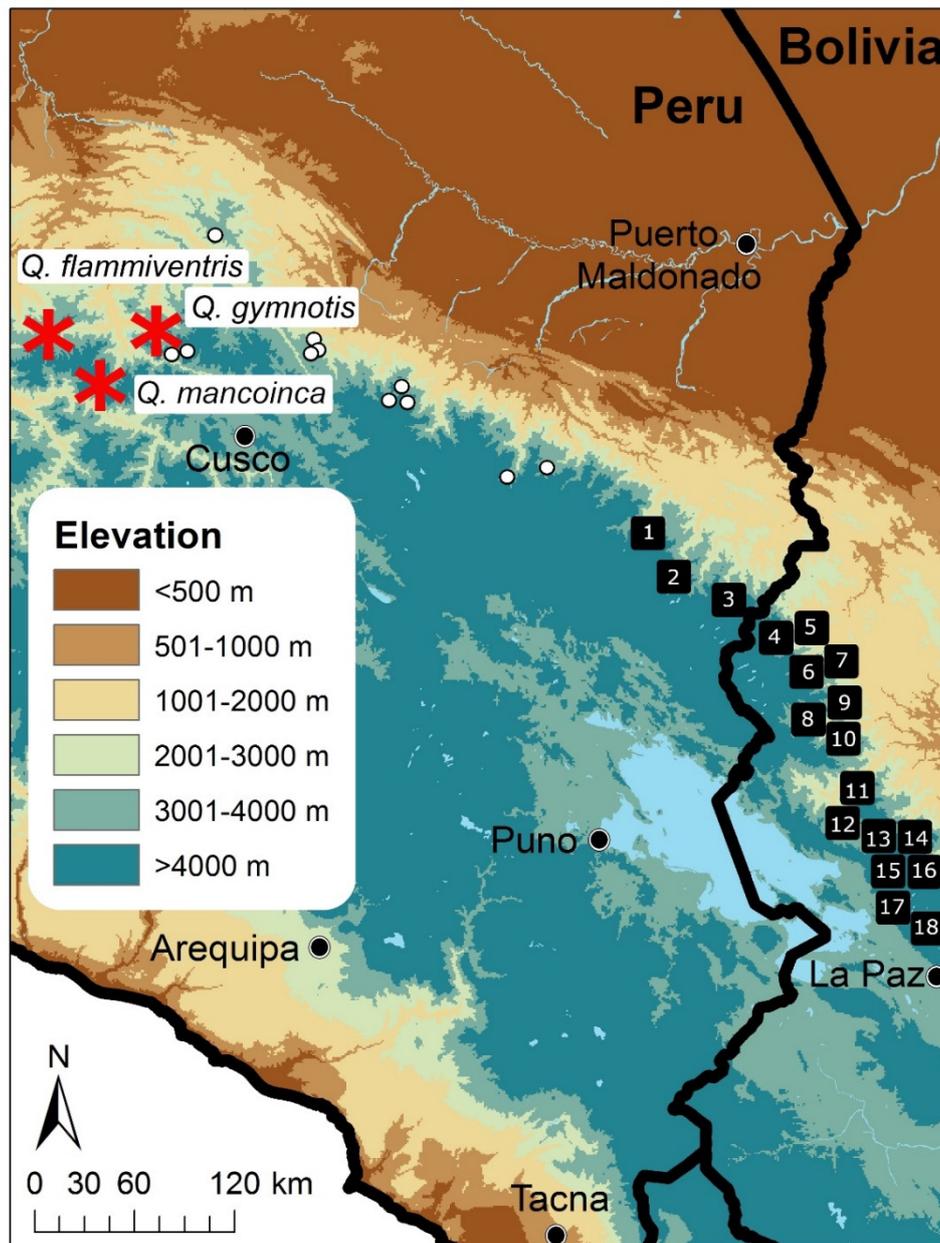


Figure 3. Type localities of frogs in the genera *Bryophryne* (white circles, species details not shown), *Microkayla* (squares) and *Qosqophryne* gen. n. (red asterisks) in southern Peru and northern Bolivia. The known distribution range of these frogs is limited to the type locality and immediate surroundings. For species of *Microkayla*: (1) *M. boettgeri*; (2) *M. chilina*; (3) *M. chapi*; (4) *M. katantika*; (5) *M. chaupi*; (6) *M. melanocheira*; (7) *M. colla*; (8) *M. kallawaya*; (9) *M. guillei*; (10) *M. saltator*; (11) *M. iani*; (12) *M. illampu*; (13) *M. ankohuma*; (14) *M. condoriri*; (15) *M. teqta*; (16) *M. huayna*; (17) *M. chacaltaya*; (18) *M. wettsteini*. The map does not include seven species of *Microkayla* distributed in central and southern Bolivia (type localities outside the limits of this map).

Similarly to recent phylogenies [28,41], we found that *Noblella* is not monophyletic: the species from southern Peru along with species of *Psychrophrynella* form a clade that is sister taxon to *Microkayla* + *Qosqophryne*, whereas the species of *Noblella* from northern Peru and Ecuador are closely related to “*Eleutherodactylus bilineatus*” and *Barycholos* (Figure 2). Because the type species *N. peruviana*

occurs in southern Peru, and the most similar species sequenced to date *N. thiuni* is part of the *Noblella/Psychrophrynella* clade [28], our findings support the hypothesis that *Noblella* occurs only in southern Peru and northern Bolivia, and that species from northern Peru and Ecuador belong to a different genus [28,41]. Furthermore, our tree suggests that species of *Noblella* and *Psychrophrynella* belong to the same lineage, as supported by the respective type genera sharing several morphological traits [2,5,20,28,42]. Therefore, the two possibilities are that some species of *Noblella* have been misidentified as *Psychrophrynella* (and vice versa), or that *Psychrophrynella* is a junior synonym of *Noblella*. We will not be able to resolve the taxonomic uncertainty associated with *Noblella* and *Psychrophrynella* until we obtain DNA sequences from the respective type species *N. peruviana* and *P. bagrecito* [2,19,20,28].

Finally, our inferred phylogeny suggests that there are at least seven additional putative new species of *Bryophryne*, *Noblella*, and *Psychrophrynella* (Figure 2), and confirms previous findings of cryptic species diversity particularly in leaf litter, cloud forest frogs in the *Noblella/Psychrophrynella* clade [22]. These putative new species, similarly to most known species of high-elevation Holoadeninae [4], are highly endemic and known from single localities (or, around those localities, from within a narrow elevational range in the same valley, [22]). Of special interest among the putative new species, *Psychrophrynella* MUSM 27619 is the first specimen of the *Noblella/Psychrophrynella* lineage known from the Vilcabamba range.

Taxonomy

Qosqophryne new genus

<http://zoobank.org/urn:lsid:zoobank.org:act:7DDB98AD-CCF9-4977-B814-285D25B3D1BF>

Type species. *Bryophryne gymnotis* Lehr and Catenazzi, 2009

Included species. *Qosqophryne flammiventris* (Lehr and Catenazzi, 2010), comb. nov.; *Q. mancoinca* (Mamani, Catenazzi, Tito, Mallqui, Chaparro, 2017), comb. nov.

Diagnosis. (1) Head wider than long, narrower than body, body robust, extremities short; (2) tympanic membrane and annulus present; (3) cranial crests absent; (4) prevomerine teeth and dentigerous process of vomers present (but absent in *Q. flammiventris*); (5) trips of digits narrow, rounded, circumferential grooves absent, terminal phalanges T-shaped to knobbed; (6) Finger I shorter than Finger II, nuptial pads absent; (7) Toe V shorter than Toe III; (8) fingers and toes with lateral fringes (but absent in *Q. flammiventris*); (9) subarticular tubercles small, rounded; (10) dorsolateral folds short, discontinuous or continuous; (11) discoidal fold absent (present in *Q. mancoinca*); (12) trigeminal nerve passing external to *m. adductor mandibulae externus* ('S' condition; Lynch, 1986); (13) snout-vent length from 16.7–19.3 mm in males and 16.0–22.2 mm in females of *Q. gymnotis*, to 19.6–22.9 mm in males and 23.6–26.5 mm in females of *Q. mancoinca*; (14) males with median subgular vocal sac and vocal slits, nuptial pads absent; (15) advertisement call whistle-like, composed of a single, tonal note in *Q. gymnotis*, 2–3 short notes in *Q. mancoinca*, and 3–4 short notes in *Q. flammiventris*.

There are no known morphological synapomorphies for *Qosqophryne*, but the three known species share the following traits (Table 3): (1) males with median subgular vocal sac produce whistle-like tonal calls composed of 1–4 short notes; (2) tongue ovate; (3) skin on venter smooth to weakly areolate (in *Q. flammiventris*); (4) inner tarsal fold absent. Four other genera of Holoadeninae occur south of the Apurimac canyon, a proposed biogeographic barrier for high-elevation terrestrial breeding frogs [13–15]. *Bryophryne* differs from *Qosqophryne* in lacking an externally visible tympanum, and having males without vocal sac and not emitting vocalizations [2,12,16]. *Oreobates* have head about the same width as body, smooth venter, subarticular and supernumerary tubercles large, conical or subconical, projecting, and range in snout-vent length from 20–63 mm [1,5]. *Noblella* and *Psychrophrynella* have smooth venter, elongated tongue, two prominent metatarsal tubercles, and in most species facial masks and/or a tarsal fold-like, sigmoid tubercle [2,19,20,28]. *Qosqophryne* is most similar to its sister genus *Microkayla*. Putative synapomorphies of *Microkayla* are a rounded tongue, areolate belly, and absence of prominent metatarsal tubercles [2]. It is presumed that all species of *Microkayla* vocalize, and known calls consist of a simple, short whistle-like tonal note [2,4]. *Qosqophryne* differs from

most *Microkayla* in having (except for *Q. flammiventris*) fingers and toes with lateral fringes (absent in *Microkayla*), and having (except *Q. flammiventris*) dentigerous processes of vomers (absent in *Microkayla*). Future examination of osteological characters, for example through computed tomography, might help identify such characters, and resolve the condition of the tympanic apparatus in the three genera *Bryophryne*, *Microkayla* and *Qosqophryne*.

Table 3. Meristic traits (+ = character present, - = character absent) for the three known species of *Qosqophryne* gen. n.

Characters	<i>Q. gymnotis</i>	<i>Q. flammiventris</i>	<i>Q. mancoinca</i>
Skin on dorsum	shagreen	Shagreen with small scattered tubercles	Shagreen with small conical tubercles
Skin on venter	smooth	Weakly areolate	smooth
Dorsolateral folds	Discontinuous, short	Discontinuous, short	Continuous, short
Tympanic membrane	+	+	+
Tympanic annulus	+	+	+
Dentigerous processes of vomers	+	-	+
Vocal sac	+	+	+
Vocal slits	+	+	+
Nuptial pads	-	-	-
Fingers with lateral fringes	+	-	+
Toes with lateral fringes	+	-	+
Inner tarsal fold	-	-	-
Dorsum coloration	Reddish, grayish or purplish brown or dark gray with narrow tan middorsal stripe	Grayish brown	Reddish brown or grayish brown with narrow tan middorsal stripe
Venter coloration	Dark brown, tan, or reddish brown with pale gray flecks	Blackish brown with yellow, orange or pink blotches	Gray or pale bluish gray with reddish-brown reticulation

Etymology. The name refers to the city of Cusco, using the spelling Qosqo which more closely reflects the name in Quechua. *Qosqo* is used in apposition with *phryne*, from the greek for “frog”. Thus, the name for the new genus alludes to the geographic distribution of the three known species in the Peruvian Department of Cusco.

Distribution, natural history, and conservation. The three species of *Qosqophryne* occur within a region of ~150 km² in the upper montane forests and grasslands of the Cordilleras de Urubamba and Cordillera de Vilcabamba, Provincia La Convención, Department Cusco, Peru. These frogs inhabit cloud forests, elfin forests, montane scrub and humid grasslands (puna) from 3270 to 3800 m a.s.l. Similar to other regions in the high Andes, these habitats and their amphibian communities are threatened by pasture burning, climate change and associated expansion of agricultural activities, deforestation, and the fungal disease chytridiomycosis [43,44]. Although chytridiomycosis has caused the collapse of montane frog communities at several sites in Departamento Cusco [45,46], terrestrial-breeding frogs have generally declined the least, and several species challenged in experimental infection trials appears to resist or tolerate infection [47]. Protection of natural habitats will benefit conservation of these frogs. Two of the three species occur within naturally protected areas: *Q. gymnotis* within the Área de Conservación Privada Abra Málaga, and *Q. mancoinca* within Machu Picchu Historic Sanctuary.

Remarks. The new genus is distinguished from all species of *Bryophryne* by the presence of tympanum and tympanic annulus, and median subgular vocal sacs in males. Furthermore, males of all three species of *Qosqophryne* are known to emit advertisement calls (unknown in all species of *Bryophryne*, except possibly for *B. bustamantei*). We have described the advertisement calls of *Q. gymnotis* and *Q. mancoinca* [14,17]. One of us (LM) has recorded the advertisement call of a male *Q. flammiventris* (MUBI 13365) at the type locality, and this call is composed of 3–4 short notes (~15–35 ms duration) at dominant frequency ~3000 Hz. Females of *Q. gymnotis* attend clutches of 14–16 eggs [39], but unattended clutches of up to 19 eggs have also been found [14].

The new genus *Qosqophryne* is supported by our molecular phylogeny, the most complete to date covering three mitochondrial and two nuclear gene fragments, as well as most described species of *Bryophryne* and *Microkayla*. Despite the absence of known synapomorphies for the sister clades *Microkayla* and *Qosqophryne*, we are confident that our proposed arrangement reflects the evolutionary history of these organisms, and yet still takes into consideration taxonomic stability [48]. There is strong support (bootstrap probabilities) at the node where *Microkayla* and *Qosqophryne* diverge, and the relative branch lengths leading to their respective living species is similar, or in some cases exceed the branch lengths separating other genera within Terrarana (e.g., *Euparkerella* and *Holoaden*, or *Barycholos* and the “northern clade” of *Noblella*).

4. Discussion

Our study integrating molecular, acoustic and morphological information justifies the erection of the new genus of strabomantid frog *Qosqophryne*. The molecular phylogeny we inferred, the most complete phylogeny to date in terms of terminal sampling for genera of Holoadeninae [2,24], provides strong support for this new genus forming a sister clade to *Microkayla*. Furthermore, our phylogeny confirms taxonomic uncertainty regarding the genera *Noblella* and *Psychrophrynella* [2,19,20], suggests the presence of several undescribed species of *Noblella* and *Psychrophrynella*, and generalizes the idea of high species endemism in high elevation Andean strabomantids [2,4,19–22,49].

Morphological synapomorphies for the new genus *Qosqophryne* have not been recognized, and there does not appear to be a unique combination of meristic traits to distinguish all species of *Microkayla* from species of *Qosqophryne*. However, there are some characteristics that help distinguish the two genera. Some of the traits present in *Qosqophryne* but absent in *Microkayla* are fingers and toes with lateral fringes, venter smooth (areolate in *Microkayla*), and presence of dentigerous processes of vomers (but absent in *Q. flammiventris*). The structure of the advertisement call, when known, appears to be similar in both genera, i.e., a whistle-like call, but composed of a single note in *Microkayla* vs. 2–4 notes in *Qosqophryne* (except for *Q. gymnotis*). There is limited information on parental care, but it appears that females attend clutches in *Q. gymnotis* [39], whereas males attend clutches in *M. illimani* and *M. teqta* [50,51]. Similarly to *Qosqophryne*, females attend clutches in *B. cophites* [52], *B. hanssaueri* and *B. nubilosus* (Catenazzi, pers. obs.). However, we lack natural history information from most species of strabomantid frogs, and thus any generalization on parental care is premature.

In support of our proposed new genus, there is a wide gap, both in terms of airline distance and the highly dissected topography, in the distribution range of species of *Microkayla* and *Qosqophryne*. These are all highly endemic, terrestrial-breeding frogs most likely characterized by extreme low vagility, as suggested by their patchy distribution in cloud forests and grasslands. All species of *Microkayla* occur from extreme southern Peru (Department Puno) to the western limits of department Santa Cruz in central Bolivia (Serranía Siberia), whereas the three species of *Qosqophryne* occur in the Vilcabamba mountain range in the Peruvian Department of Cusco. The gap of 320 km by airline between the southernmost locality of *Qosqophryne* (*Q. gymnotis*; $-13.07558, -72.38201$) and the northernmost locality of *Microkayla* (*M. boettgeri*) overlaps with the distribution range of *Bryophryne*. At the northern limit, *B. abramalagae* and *B. bustamantei* are marginally sympatric with *Q. gymnotis*, whereas at the southern limit, *B. wilakunka* (Ayapata, Puno, $-13.85294, -70.31450$) occurs ~80 km NW of the type locality of *M. boettgeri* (Phara, Puno, $-14.16247, -69.66250$). Although many species in these genera of Holoadeninae are likely “micro-endemic”, researchers have seldom invested much effort in documenting the distribution ranges of most species, and it is possible that some of these species occur more widely than presently known. Therefore, currently five genera of Holoadeninae occur in the tropical Andes south of the Apurimac canyon in Cusco, Puno and northern Bolivia: *Bryophryne*, *Psychrophrynella* and *Qosqophryne* in the Vilcabamba mountain range; *Bryophryne*, *Noblella* and *Psychrophrynella* in the Vilcanota range; *Bryophryne*, *Microkayala*, *Noblella* and *Psychrophrynella* in the Carabaya range, and *Microkayala* south of the Apolobamba range.

Author Contributions: Conceptualization, A.C., L.M., E.L. and R.v.M.; methodology, A.C., L.M., E.L. and R.v.M.; software, A.C. and R.v.M.; validation, A.C., L.M., E.L. and R.v.M.; formal analysis, A.C. and R.v.M.; investigation, A.C., L.M., E.L. and R.v.M.; resources, A.C. and R.v.M.; data curation, A.C., R.v.M.; writing—original draft preparation, A.C.; writing—review and editing, A.C., L.M., E.L. and R.v.M.; visualization, A.C., L.M., E.L. and R.v.M.; supervision, A.C.; project administration, A.C.; funding acquisition, A.C. All authors have read and agreed to the published version of the manuscript.

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Appendix A. Specimens Examined

Bryophryne abramalagae: PERU: CUSCO: Provincia La Convención: Distrito de Huayopata, Abra de Málaga (13°07'23.8" S, 72°20'51.2" W), 4000 m a.s.l., MUSM 27630–32, MTD 47489–91.

Bryophryne bakersfield: PERU: CUSCO: Provincia La Convención: Distrito de Echarate, Roquerío de Lorohuachana, 3620 m a.s.l. (12°29'43.8" S, 72°04'35.9" W), MHNC 7972.

Bryophryne bustamantei: PERU: CUSCO: Provincia La Convención: Abra de Málaga: MUSM 24537–38.

Bryophryne cophites: PERU: CUSCO: Provincia de Paucartambo: Distrito Kosñipata: S slope Abra Ac[j]anaco, 14 km NNE Paucartambo, 3400 m a.s.l.: KU 138884 (holotype); N slope Abra Ac[j]anaco, 27 km NNE Paucartambo, 3450 m a.s.l.: KU 138885–908, 138911–5 (all paratypes); 2 km NE of Abra Ac[j]anaco, 3280 m a.s.l.: MHNG 2698.24, 5.5 km N of Abra Acanacu [Acjanaco], 3523 m: MUSM 27895, Tres Cruces, 8.5 km N of Abra Ac[j]anaco, 3590 m a.s.l.: MUSM 20855–56, 26283–84, 26264, 26266–67, 26313, 26315, 27896, 30414–17, Pillco Grande, 3865 m a.s.l., near border of Manu NP: CORBIDI 11919.

Bryophryne flammiventris: PERU: CUSCO: Provincia de La Convención, Distrito de Vilcabamba, road between Vilcabamba and Pampaconas, 3800 m a.s.l.: MUSM 27613 (holotype), MUSM 27612, 27614–15, MTD 46890–92 (paratypes).

Bryophryne gymnotis: PERU: CUSCO: Provincia de La Convención, Distrito de Huayopata: 1 km east of San Luis, 3272–3354 m a.s.l.: MUSM 24543 (holotype), MHNG 2710.28, 2710.29, MTD 46860–64, 47288, 47291–92, 47297, MUSM 24541–42, 24544–45, 24546–56, MVZ 258407–10 (paratypes).

Bryophryne hanssaueri: PERU: CUSCO: Provincia de Paucartambo, Distrito de Kosñipata: Acjanaco, Manu National Park, 3266 m a.s.l.: MUSM 27567 (holotype); from near Acjanaco, Manu National Park, 3280–3430 m a.s.l.: MHNG 2698.25, MTD 46865–66, 46887–89, MUSM 24557, 27568–69, 27607–11, MVZ 258411–13 (all paratypes).

Bryophryne mancoinca: PERU: CUSCO: Provincia de La Convención, Hornopampa sector, near Salkantay Mountain, along the road to the Archeological Complex of Choquequirao, 3707 m a.s.l.: MUBI 11152 (holotype), MUBI 11147–11151, 11153, 11154, 11159, 16068, 16069, 16074, 16083 (paratypes).

Bryophryne nubilosus: PERU: CUSCO: Provincia de Paucartambo: Distrito de Kosñipata, 500 m NE of Esperanza, 2712 m a.s.l.: MUSM 26310 (holotype), MUSM 26311; near the type locality, 13°11'33.21" S, 71°35'25.17" W, 3065 m: MTD 47294; near Hito Pillahuata, 2600 m: MUSM 20970; Quebrada Toqoruyoc, 3097 m a.s.l.: MUSM 26312, MTD 47293; Esperanza, 2800 m: MHNSM 26316–17; 13°11'20.2" S, 71°35'07.3" W, 2900 m a.s.l.: MUSM 24539–40.

Bryophryne phuyuhampatu: PERU: CUSCO: Provincia de Paucartambo: Distrito de Paucartambo, Quispillomayo valley, Área de Conservación Privada (ACP) Ukumari Llaqta, 2795–2850 m a.s.l., 13°22'12.14" S; 71°6'49.82" W (WGS84; type locality), CORBIDI 18224–18226, MUBI 14654 and 14655.

Bryophryne quellokunka: PERU: CUSCO: Provincia de Quispicanchis: Distrito de Marcapata: Coline, 3672 m a.s.l.: MUSM 27571, 27573.

Bryophryne zonalis: PERU: CUSCO: Provincia de Quispicanchis, Distrito de Marcapata, Kusillochayoc at 3129 m a.s.l.: MUSM 27570 (holotype), MTD 46867, 46869–70, MUSM 27572, 27574–75, 27861, MVZ 258414 (paratypes); at Puente Colina, 3285 m a.s.l.: MVZ 258415 (paratype).

Microkayla boettgeri: PERU: PUNO: Provincia de Sandia, Distrito de Limbani, Phara, 3466 m a.s.l.: MHNSM 19966 (holotype), MHNSM 19967–76, MTD 46508–9, 46512–19 (paratypes).

Microkayla chapi: PERU: PUNO: Provincia de Sandia, Distrito de Limbani, 3.7 km from Sina, Hirigache River valley, 3466 m a.s.l.: MUBI 5326 (holotype), MUBI 5325, 5327, 5330, 5331, 5328, 5329 (paratypes).

Microkayla chilina: PERU: PUNO: Provincia de Sandia, Distrito de Limbani, 3.7 km from Sina, Hirigache River valley, 3466 m a.s.l.: MUBI 5355 (holotype), MUBI 5350, 5351, 5353, 5354 (paratypes).

Qosqophryne flammiventris: PERU: CUSCO: Provincia de La Convención, Distrito de Vilcabamba, road between Vilcabamba and Pampaconas, 3800 m a.s.l., MUBI 13365.

Qosqophryne gymnotis: PERU: CUSCO: Provincia de La Convención, Distrito de Huayopata: San Luis, MUBI 14315–14319.

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