



A new species of *Amazophrynella* (Anura:Bufonidae) from the southwestern part of the Brazilian Guiana Shield

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Abstract

Amazophrynella is a genus of the family Bufonidae, currently represented by three species. The type species of the genus, *Amazophrynella minuta*, however, is a complex of species occurring throughout the Amazonian biome. This group remains problematic taxonomically; the difficulty lays principally in the lack of diagnostic characters in the original description of *A. minuta*, the lack of molecular data and refined taxonomic comparison of individuals through its wide distribution. We describe a new species of the genus *Amazophrynella*, distributed in the southwestern part of the Guiana Shield of Brazil based on a series of morphological and molecular characters. The new species differs from others of the genus by presenting a slightly truncated triangular snout, ventral texture covered by many fine granules, white belly covered with black spots and by fifteen molecular autapomorphies in the 16S rDNA fragment. Uncorrected p-distances of a fragment of the 16S mitochondrial rDNA gene revealed high divergence among other *Amazophrynella* species (9%–14%). Additionally we provide a new diagnosis of topotypic material of *Amazophrynella minuta* including molecular data. Our results show the existence of a subestimated diversity in the genus *Amazophrynella*.

Key words: Amphibian, Anura, Bufonidae, *Amazophrynella*, Brazil

Resumo

Amazophrynella é um gênero da família Bufonidae, atualmente representado por três espécies. *Amazophrynella minuta* é considerada como um complexo de espécies que se distribui por todo o bioma amazônico. Este grupo permanece taxonomicamente problemático, sendo as principais dificuldades a ausência de caracteres diagnósticos na descrição original de *A. minuta*, a inexistência de dados moleculares e comparações taxonômicas detalhadas dos indivíduos, através de sua extensa distribuição. Descrevemos uma nova espécie do gênero *Amazophrynella*, distribuída no sudoeste da região do escudo da Guiana baseados em caracteres morfológicos e moleculares. A nova espécie difere de outras do gênero por apresentar um focinho triangular ligeiramente truncado, textura ventral coberto por muitos grânulos finos, barriga branca coberta por manchas pretas e quinze automorfias moleculares no fragmento do 16S rDNA. As distâncias não corrigidas (p - distâncias) no fragmento 16S rDNA revelaram uma alta divergência entre as outras espécies de *Amazophrynella* (9%–14%). Adicionalmente providenciamos uma nova diagnose do material topotípico de *Amazophrynella minuta*, incluindo dados moleculares. Nossos resultados mostram a existência de uma diversidade subestimada dentro do gênero *Amazophrynella*.

Resumen

Amazophrynella es un género perteneciente a la familia Bufonidae representada actualmente por tres especies. *Amazophrynella minuta* es considerada como un complejo de especies que se distribuye por todo el bioma amazónico. Este grupo permanece taxonómicamente problemático, siendo la principal dificultad la ausencia de los caracteres diagnósticos en la descripción original de *A. minuta*, inexistencia de los datos moleculares y comparaciones taxonómicas refinadas a lo largo de su amplia distribución. Describimos una nueva especie del género *Amazophrynella*, distribuida en la parte suroeste de la región del escudo de Guyana basados en caracteres morfológicos y moleculares. La nueva especie difiere de otras del género por presentar una nariz triangular ligeramente truncada, textura ventral cubierta por muchos gránulos finos, vientre blanco cubierto por manchas negras y quince automorfias moleculares en el gen 16S rDNA (9%–14%). Adicionalmente proveemos una nueva diagnosis del material topotípico de *Amazophrynella minuta*, incluyendo datos moleculares. Nuestros resultados indican la existencia de una diversidad subestimada dentro del género *Amazophrynella*.

Introduction

Amazophrynella Fouquet, Recoder, Teixeira, Cassimiro, Amaro, Camacho, Damasceno, Carnaval, Moritz, & Rodrigues 2012, is a genus of small, early diverging bufonid frogs (Pramuk *et al.*, 2008; Bocxlaer *et al.*, 2010). The genus is currently represented by three nominal species: *Amazophrynella minuta* (Melin, 1941), *A. bokermanni* (Izecksohn, 1993) and *A. vote* Ávila, Carvalho, Gordo, Kawashita & Morais, 2012 (Frost, 2013). All species of *Amazophrynella* present a clear sexual size dimorphism, reproduce in seasonal puddles and share this characteristic only with *Dendrophryniscus leucomystax* (Izecksohn, 1968). Their exclusive habitat is leaf litter and they have a diurnal activity (Magnusson & Hero, 1991; Lima *et al.*, 2006; Ávila *et al.*, 2012). Currently there are no studies describing the bioacoustic patterns of any species of *Amazophrynella*.

Until 2012 species of the genus *Amazophrynella* were placed in the genus *Dendrophryniscus*. In 2012 Fouquet *et al.* (2012a) recognized that species of *Dendrophryniscus* from the Amazon and Atlantic rainforest represent morphologically and molecularly deeply divergent lineages, and thus the authors proposed a new genus: *Amazonella* for the Amazonian species, but because this name was already occupied by a genus of Acari, the name had to be changed to *Amazophrynella* (Fouquet *et al.*, 2012b). The type species of the genus is *Amazophrynella minuta* (Melin, 1941) described from the Taracú mission, on the right bank of the Uaupés River, near the Brazil-Colombia-Venezuela border. Melin (1941) collected only six individuals, all part of the type series, which are deposited in the Naturhistoriska Museet, Göteborg, Sweden (NHMG). Possibly due to lack of material other than the type series, and a relatively generalized diagnosis of the species, the name *Amazophrynella minuta* has been used for individuals from many different populations sampled throughout the Amazonian biome (e.g. Duellman, 1978; Zimmerman & Rodrigues, 1990; Magnusson & Hero, 1991; Rodrigues & Duellman, 1993; Lima *et al.*, 2006; Fouquet *et al.*, 2012a). However, it is unlikely that many or all of these publications refer to the true *A. minuta*; what is referred to as *A. minuta* in the literature likely represents a complex of species (Fouquet *et al.*, 2012a).

Herein, we describe a new species of the *Amazophrynella minuta* species complex using molecular and morphological data. The species is described from municipality of Manaus, state of Amazonas, Brazil. We also rediagnose *Amazophrynella minuta* based on newly collected specimens from its type locality.

Material and methods

We analyzed 57 specimens identified as *Amazophrynella minuta* (Melin, 1941) originating from six localities near Manaus, Brazil deposited in the Collection of Amphibians and Reptiles of the Instituto Nacional de Pesquisas da Amazônia—INPA (INPA-H). This material was compared with twenty specimens (fourteen males and six females, see appendix 1) of recently collected *A. minuta* from the type locality (Taracú mission, on the right bank of the Uaupés River, municipality of São Gabriel da Cachoeira, Brazil). Further comparisons were made with photographed material of the two syntypes deposited in the Naturhistoriska Museet, Göteborg, Sweden (NHMG), and the original description of the species (Melin, 1941).

In addition to this material we analyzed five specimens of *Amazophrynella bokermanni* (Izecksohn, 1993) from near the type locality deposited in the Collection of Amphibians and Reptiles of the Instituto Nacional de Pesquisas da Amazônia—INPA, Manaus, Amazonas, Brazil (INPA-H), the holotype of *Amazophrynella vote* Ávila, Carvalho, Gordo, Kawashita & Morais 2012, deposited in the Coleção Zoológica de Vertebrados of the

Universidade Federal de Mato Grosso—UFMT, Cuiabá, Mato Grosso, Brazil (UFMT-A) and seventeen *A. vote* paratypes deposited in the Collection of Amphibians and Reptiles of the Instituto Nacional de Pesquisas da Amazônia—INPA, Manaus, Amazonas, Brazil (INPA-H). List of specimens examined is found in Appendix 1.

Measurements were obtained with Mitotuyo® digital calliper (0.1 mm precision) with an ocular micrometer in a Zeiss stereomicroscope. Measurements were: snout-vent length (SVL); head length (HL); head width (HW), upper eyelid width (UEW); eye diameter (ED); snout length (SL); eye to nostril distance (END); internarial distance (IND); interorbital distance (IOD); hand length (HAL); upper arm length (UAL); thigh length (THL); tibial length (TL); tarsal length (TAL) and foot length (FL) following Cruz & Fussinato (2008). Sex was determined based on gonadal analysis.

Total DNA was extracted from muscle tissue using standard phenol/chloroform extraction (Sambrook *et al.*, 1989). A 480 bp fragment of the 16S rDNA was PCR amplified using the 16Sar and 16Sbr primers (Palumbi, 1996). Amplification was carried out under the following conditions: 60 s hot start at 92°C followed by 35 cycles of 92°C (60 sec), 50°C (50 sec) and 72°C (1.5 min). Final volume of the PCR reaction was 12 µL and contained 4.4 µL ddH₂O, 1.5 µL of 25 mM MgCl₂, 1.25 µL of 10 mM dNTPs (2.5mM each dNTP), 1.25 µL of 10x buffer (75 mM Tris HCl, 50 mM KCl, 20 mM (NH₄)₂SO₄), 1 µL of each 2 µM primer, 0.3 µL of 5 U/µL DNA Taq Polymerase (Biotools, Spain) and 1 µL of DNA (about 30 ng/µL). Sequencing reactions were carried out according to the manufacturer's recommendation for the ABI BigDye Terminator cycle sequencing mix, using 16Sa and an annealing temperature of 50°C. Sequencing reactions were precipitated using standard EDTA/EtOH protocol, and resolved in an ABI 3130xl automatic sequencer.

We obtained 16S rDNA sequence data from sixteen specimens of the new species (Genbank numbers: KF433954-69), including three topotypes and nine additional paratypes as well as five paratypes of *A. vote* (Genbank numbers: KF433970-74), two specimens of *A. bokermanni* (Genbank numbers: KF433975-76) and two specimens of *A. minuta* from its type locality (Genbank numbers: KF792834-35) (Table 1) deposited in the tissue collection of the Laboratório de Evolução e Genética Animal of the Universidade Federal do Amazonas (CTGA-ICB/UFAM). The dataset also included three sequences of *A. bokermanni* (Genbank numbers: JN867559, JN867561, JN867563) from Fouquet *et al.* (2012a) and three sequences of *A. aff. minuta* (Genbank numbers: AY326000, DQ158420, EU201057) from Darst and Canatella (2004), Pramuk (2006) and Fouquet *et al.* (2007). Sequences were aligned using the Clustal W algorithm (Thompson *et al.*, 1996) implemented in BioEdit (Hall, 1999) and alignment was adjusted as necessary against the secondary structure of the 16S rDNA. A second dataset was constructed via inclusion of sequences of three specimens of the new species and *A. vote*, and two specimens of *A. bokermanni* and *A. minuta* from its type locality, in the aligned dataset of Fouquet *et al.* (2012a) kindly provided by A. Fouquet. New sequences were aligned manually to conform to the existing 16S rDNA alignment used in Fouquet *et al.* (2012a).

Evolutionary lineages are diagnosed by discontinuities in character variation among lineages, and correspond to phylogenetic species. The existence of lineages is therefore a necessary and sufficient prerequisite for inferring the existence of a species under the different conceptualizations of the Phylogenetic Species Concept (PSC) (Cracraft, 1983; Baum & Donoghue, 1995). The existence of lineages in a non tree-based context (Cracraft, 1983) was inferred using Population Aggregation Analysis performed at the level of an individual (Davis & Nixon, 1992; Rach *et al.*, 2008) using the first dataset (*Amazophrynella* species only). The existence of lineages in a phylogenetic tree-based context (Baum & Donoghue, 1995) was performed using Maximum Likelihood analysis (Felsenstein, 1981) in the program Treefinder (Jobb, 2008) using the GTR+I+G model of substitution, selected via Akaike information criterion as implemented in Modeltest 3.7 (Posada & Crandall, 1998). Phylogenetic support was assessed via non-parametric bootstrap (Felsenstein, 1985). Additionally uncorrected pairwise genetic distances between species of *Amazophrynella* (first dataset) were calculated in MEGA 5.05 (Tamura *et al.*, 2007).

TABLE 1. Individuals used in the molecular analyses. Information includes classification (sample), collecting locality (locality), genbank accession number for the 16S rDNA fragment (Genbank accession number), INPA-H voucher number (museum number), whether individual is a topotype or is part of a type series (specimen status).

Sample	Locality	Genbank accession number	Museum number (INPA-H)	Specimen status
<i>A. manaos</i>	Campus of UFAM, Manaus, Amazonas, Brazil	KF433954	6983	paratopotype
<i>A. manaos</i>	Campus of UFAM, Manaus, Amazonas, Brazil	KF433955	6984	paratopotype

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TABLE 1. (Continued)

Sample	Locality	Genbank accession number	Museum number (INPA-H)	Specimen status
<i>A. manaos</i>	Campus of UFAM, Manaus, Amazonas, Brazil	KF433956	6987	paratopotype
<i>A. manaos</i>	Fazenda of UFAM, Manaus, Amazonas, Brazil	KF433957	7797	
<i>A. manaos</i>	Fazenda of UFAM, Manaus, Amazonas, Brazil	KF433958	7798	
<i>A. manaos</i>	Presidente Figueiredo, Amazonas, Brazil	KF433959	30577	paratype
<i>A. manaos</i>	Presidente Figueiredo, Amazonas, Brazil	KF433960	30576	paratype
<i>A. manaos</i>	Presidente Figueiredo, Amazonas, Brazil	KF433961	30573	paratype
<i>A. manaos</i>	Presidente Figueiredo, Amazonas, Brazil	KF433962	30571	
<i>A. manaos</i>	Presidente Figueiredo, Amazonas, Brazil	KF433963	30575	paratype
<i>A. manaos</i>	Mineração Taboca, Presidente Figueiredo municipality, Amazonas, Brazil	KF433964	29566	
<i>A. manaos</i>	Mineração Taboca, Presidente Figueiredo municipality, Amazonas, Brazil	KF433965	29567	
<i>A. manaos</i>	Mineração Taboca, Presidente Figueiredo municipality, Amazonas, Brazil	KF433966	29568	paratype
<i>A. manaos</i>	Mineração Taboca, Presidente Figueiredo municipality, Amazonas, Brazil	KF433967	29569	paratype
<i>A. manaos</i>	Mineração Taboca, Presidente Figueiredo municipality, Amazonas, Brazil	KF433968	29570	paratype
<i>A. manaos</i>	Parque Estadual Rio Negro Setor Sul, rio Cuieiras, Amazonas, Brazil	KF433969	1859	paratype
<i>A. vote</i>	Parque Nacional Nascentes do Lago Jari, Amazonas, Brazil	KF433970	28720	paratype
<i>A. vote</i>	Parque Nacional Nascentes do Lago Jari, Amazonas, Brazil	KF433971	28722	paratype
<i>A. vote</i>	Parque Nacional Nascentes do Lago Jari, Amazonas, Brazil	KF433972	28723	paratype
<i>A. vote</i>	Parque Nacional Nascentes do Lago Jari, Amazonas, Brazil	KF433973	28716	paratype
<i>A. vote</i>	Parque Nacional Nascentes do Lago Jari, Amazonas, Brazil	KF433974	28721	paratype
<i>A. bokermanni</i>	Juruti, Pará, Brazil	KF433975	31864	
<i>A. bokermanni</i>	Juruti, Pará, Brazil	KF433976	31861	
<i>A. minuta</i>	Taracá, Amazonas, Brazil	KF792834	32729	topotype
<i>A. minuta</i>	Taracá, Amazonas, Brazil	KF792835	32730	topotype

Results

Amazophrynella manaos sp. nov.

(Figure 1)

Dendrophryniscus minutus (McDiarmid, 1971; Zimmerman & Rodrigues, 1990; Magnusson & Hero, 1991; Lima *et al.*, 2006)

Amazonella minuta (Fouquet *et al.*, 2012a)

Amazophrynella minuta (Fouquet *et al.*, 2012b)

Holotype. INPA-H 31866, adult male, collected in forest remnants of the campus of the Universidade Federal do Amazonas—UFAM (03° 05' 56"S, 59°58' 09" W), municipality of Manaus, Amazonas State, by Vinicius Tadeu de Carvalho and Rommel Roberto Rojas, on June 26, 2012 at 16:35 h.

Paratypes. INPA-H 29568, INPA-H 29571, INPA-H 29570, INPA-H 29572, INPA-H 29569 (adult males), collected in Mineração Taboca (0° 42' 59" S, 60° 10' 24" W), municipality of Presidente Figueiredo, Amazonas State, by Vinicius Tadeu de Carvalho, on December 17, 2011; INPA-H 6983, INPA-H 6984, INPA-H 6987 (adult males), collected on the campus of the Universidade Federal do Amazonas—UFAM (03° 05' 37" S, 59° 58' 26"

W), Amazonas State, by Ana Cristina de Oliveira Cordeiro, on February 10, 2010; INPA-H 20986; INPA-H 21217 (adult females), INPA-H 21028, INPA-H 21170, INPA-H 21060 (adult males), collected in the Reserva Florestal Adolpho Ducke (02° 55' 37" S, 59° 58' 14" W), by Albertina P. Lima, in December 1992; INPA-H 30577, INPA-H 30575, INPA-H 30573, INPA-H 30572, INPA-H 30576 (adult females), collected at the municipality of Presidente Figueiredo (01° 56' 20" S, 60° 02' 14" W), Amazonas State, by André Luiz Ferreira da Silva, on January 5, 2012; INPA-H 21442, INPA-H 21400 (adult females), INPA-H 21451, INPA-H 21398, INPA-H 21821 (adult males), collected at Reserve ZF-2 (02°35' 20"S, 60°06' 55"W), municipality of Presidente Figueiredo, Amazonas State, by Albertina P. Lima, in December 1997; INPA-H 1859 (adult male), collected at Parque Estadual Rio Negro Setor Sul, Cuieiras River (02° 43' 27" S, 60° 24' 22" W), municipality of Manaus, Amazonas State, by Vinicius Tadeu de Carvalho and Lucécia Bonora, on January 10, 2007.

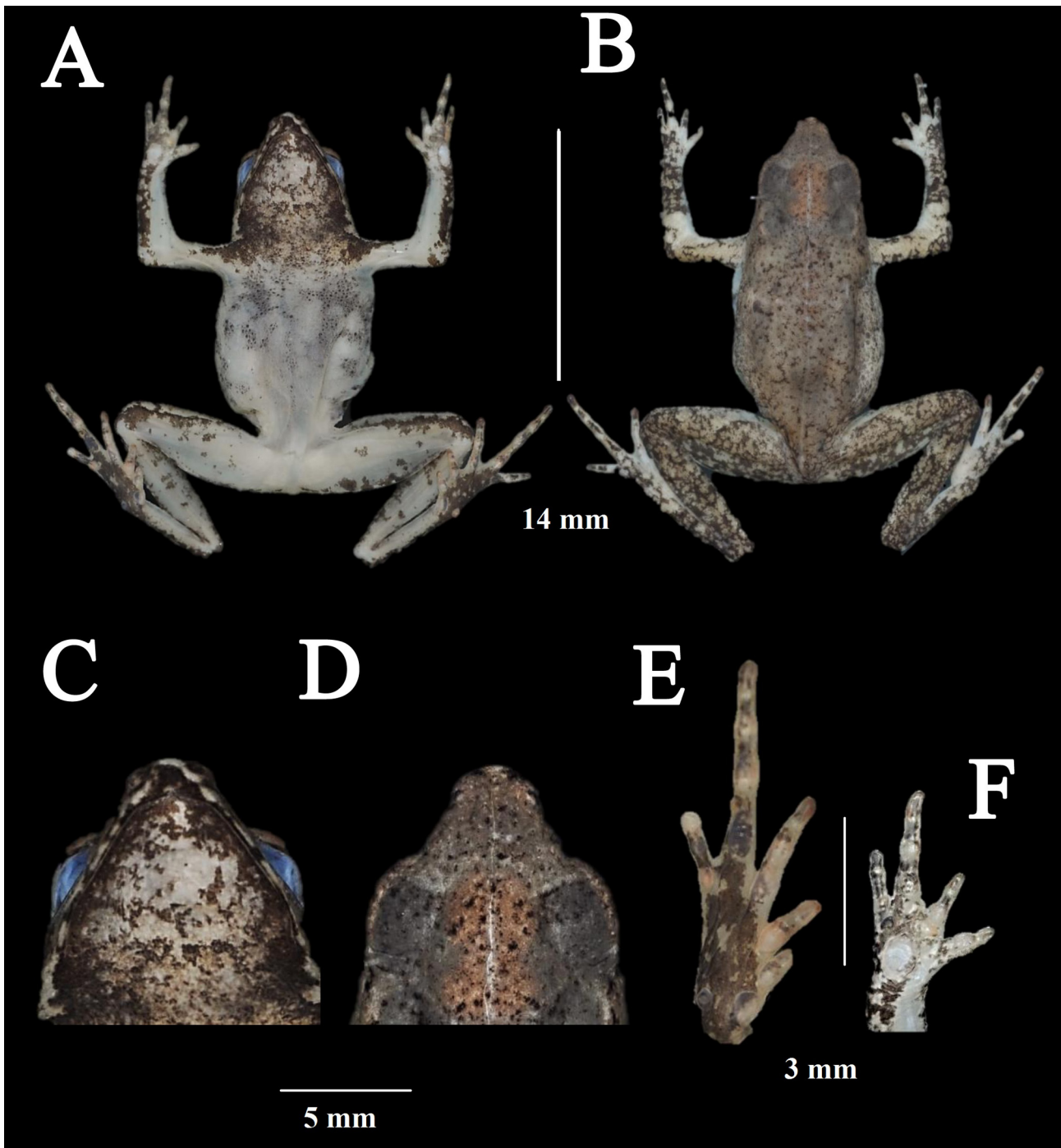


FIGURE 1. Holotype of *Amazophrynella manaos* sp. nov. (INPA-H 31866): A) Dorsal view, B) Ventral view, C) Dorsal view of head, D) Ventral view of head, E) Ventral view of the right foot, F) Ventral view of right hand.

Diagnosis. The new species is assigned to the genus *Amazophrynella* by the combination of the following generic characters: small size, depressed body, eardrum and parotid glands not visible, presence of granulate bodies, presence of subarticular tubercles on hands, elongated snout, and via phylogenetically nested position within the genus *Amazophrynella*. The new species is characterized by: 1) triangular snout with a slightly truncate culmination (in ventral view); 2) ventral surface covered with high density fine granules; 3) white abdomen; 4) black patches or stripes on the abdomen; 5) fifteen molecular autapomorphies in the 16S rDNA (Table 2). Divergence from its nominal sister taxon is 9%.

TABLE 2. Species level diagnostic characters observed in the 16S rDNA gene of *Amazophrynella manaos* sp. nov. and other species of *Amazophrynella*. First line indicates position of the character within the 16S rDNA gene (*Xenopus laevis* 16S rDNA gene was used as reference). (-) indicates a deletion.

Position (pb)	308	340	346	370	443	446	452	455	467	478	522	530	537	558	583
<i>A. manaos</i>	C	A	T	A	G	C	C	T	C	G	T	T	G	C	A
<i>A. vote</i>	T	C	C	C	A	T	A	A	T	A	C	A	A	A	G
<i>A. minuta</i>	T	C	C	T	A	T	A	A	T	A	C	G	A	A	G
<i>A. bokermanni</i>	T	C	C	G	T	T	-	A	T	A	C	G	A	T	G

Molecular phylogeny and genetic distance. Phylogenetic analysis of nominal and hypothetical taxa of the genus *Amazophrynella* indicates the existence of six lineages (Figure 2). Basal divergence is between the clade composed of an undescribed *Amazophrynella* species from western Amazônia and *A. minuta* specimens sampled from the type locality, and a clade comprising all other species and lineages. The *A. minuta* + *A. aff. minuta* “western Amazonia” clade is supported by 97% bootstrap value while the other clade is supported by 80% bootstrap value. The new species, *A. manaos* is sister to a possibly a new species of *Amazophrynella* from the Guiana Shield (bootstrap support 70%) and both are sister to *A. bokermanni* (bootstrap support 75%). The recently described *A. vote* (italicize) is sister to *A. bokermanni* + (*A. manaos* + *A. sp.* “Guianas”) with a bootstrap support of 80%. Smallest uncorrected 16S rDNA *p*-distances (4%) was observed between *A. manaos* sp. nov. and *A. sp.* “Guianas”. Greatest intrageneric distance (14%) was observed between *A. manaos* sp. nov. and *A. minuta*, and *A. sp.* “Guianas” and *A. minuta* (see Table 3).

TABLE 3. Uncorrected *p*-distances among *Amazophrynella* species and the sister genus *Dendrophryniscus*. Molecular distances are based on the 480 bp fragment the 16S rDNA. Included are *A. minuta* from its type locality and hypothetical species *Amazophrynella* sp. “Guianas” and *A. aff. minuta* “western Amazonia” from Fouquet *et al.* (2012a); both species pertain to the *A. minuta* species complex.

16S rDNA	1	2	3	4	5	6	7	8
1 <i>A. sp.</i> “Guianas”	-	-	-	-	-	-	-	-
2 <i>A. aff. minuta</i> “western Amazonian”	0.13	-	-	-	-	-	-	-
3 <i>D. brevipollicatus</i>	0.21	0.19	-	-	-	-	-	-
4 <i>D. berthaltutzae</i>	0.20	0.16	0.08	-	-	-	-	-
5 <i>D. krasuae</i>	0.19	0.16	0.08	0.00	-	-	-	-
6 <i>A. manaos</i>	0.04	0.12	0.20	0.18	0.18	-	-	-
7 <i>A. vote</i>	0.12	0.12	0.20	0.19	0.19	0.11	-	-
8 <i>A. minuta</i>	0.14	0.04	0.20	0.16	0.16	0.14	0.11	-
9 <i>A. bokermanni</i>	0.10	0.13	0.19	0.19	0.19	0.09	0.12	0.13

Characterization of topotypic material of *Amazophrynella minuta*. Topotypic material of *A. minuta* was collected in mission Taracua, right bank of the middle Uaupés River in the last week of August 2013. Adult males measured 13.5 ± 0.57 mm (12.4–14.2 mm), females 17.5 ± 0.61 mm (17.1–18.9 mm) in snout vent length. The specimens (Figure 3) were characterized by a pointed head (in ventral view); pointed snout (in lateral view); roughly granular dorsal and ventral skin; a scattering of abundant pointed spicules on the body; big warts on the

dorso-lateral region; dark gray brown throat and chest; intense yellow-orange coloration of the abdomen; and by irregular black blotches or ocelli on the abdomen. Fingers are unwebbed. Formula of the fingers: I<II<IV<III. Tips of the fingers are unexpanded. Webbing on the base of the foot. Formula of the toe: I<II<III<V<IV. Tips of the toes are unexpanded.

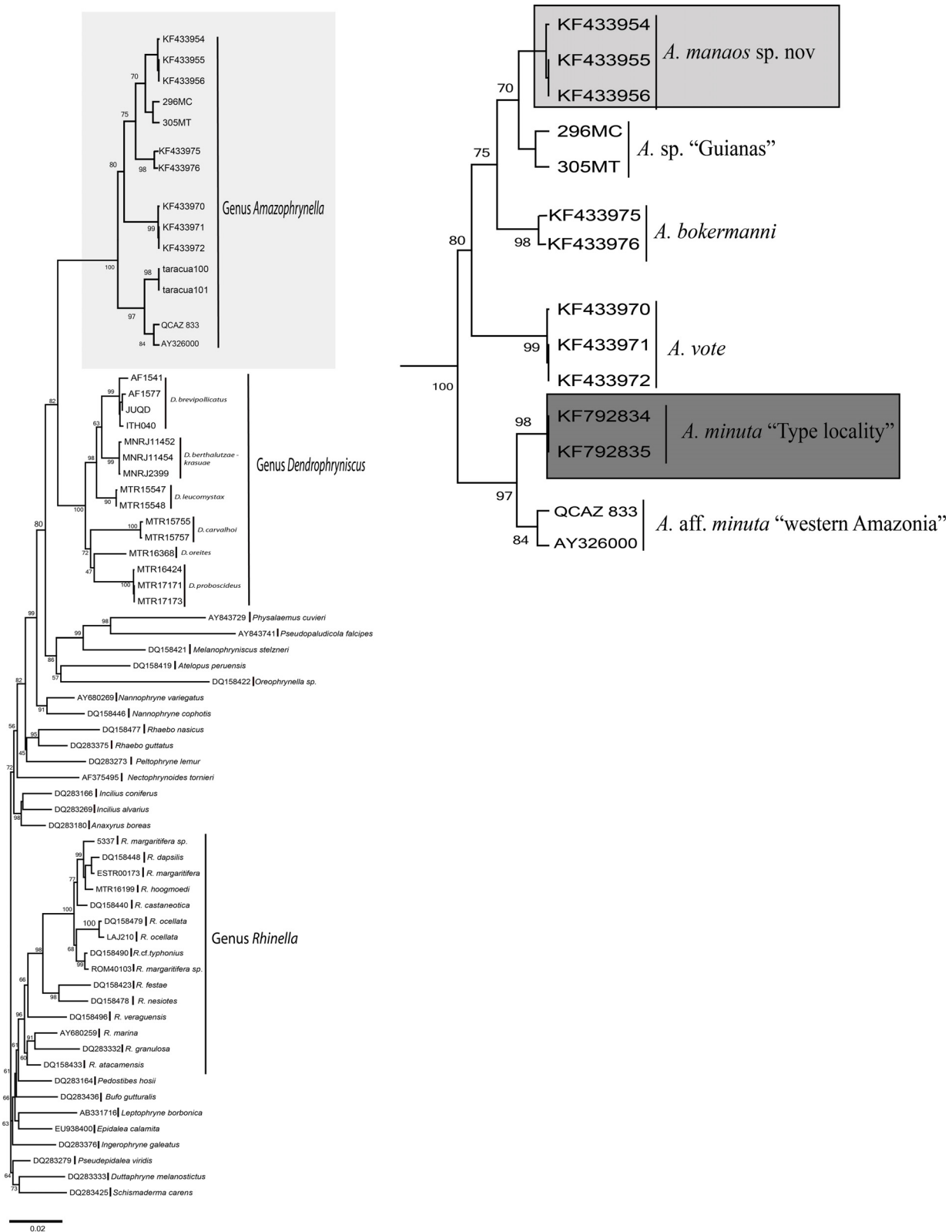


FIGURE 2. Phylogenetic relationship of *Amazophrynella* species inferred using the maximum-likelihood criterion. Analysis was performed with 480 bp of 16S rDNA under the GTR+I+G model of molecular evolution. Branch support values are based on 1000 bootstrap pseudoreplicates..



FIGURE 3. Specimens of *Amazophrynella minuta* from Taracua mission, Uaupés River, municipality of São Gabriel da Cachoeira, Amazonas, Brazil (INPA-H 32727 and INPA-H 32735).

Comparisons with other species. The new species can be differentiated from the three other nominal species of *Amazophrynella* by the following characters (characters of compared species in parentheses): a) from *A. minuta* (Melin, 1941) by the triangular snout with a slightly truncate culmination (snout pointed), absence of spiny tubercles on dorsum (prickly warty skin on dorsum), skin of axillary region and forearm finely granular (rough granular skin on forearm and axillary region, especially on the neck), venter white with black spots and dark-brown chest in life (venter bright reddish-orange, with small black blotches and throat light brown) (Figure 4A-C and Figure 5A, Table 4); b) from *A. bokermanni* (Izecksohn, 1993), by finger I shorter than finger II (finger I longer than finger II), smaller SVL, with males reaching 15.8 mm and females 24.7 mm (maximum SVL of males and females 22 mm and 28 mm, respectively) (Figure 4G-I, Figure 5B); and c) from *A. vote* Ávila, Carvalho, Gordo, Kawashita & Morais, 2012, by snout triangular with a slight culmination (rounded), slightly smaller SVL in males (14.7–15.8 mm vs. 16.4 mm—Ávila *et al.*, 2012), and white abdomen with black spots or stripes and blackish to dark-brown chest and throat in life (abdomen reddish-brown with small white dots and brown spots) and subrostral crest converging anteriorly (subrostral crest not converging anteriorly) (Figure 4D-F, Figure 5C).

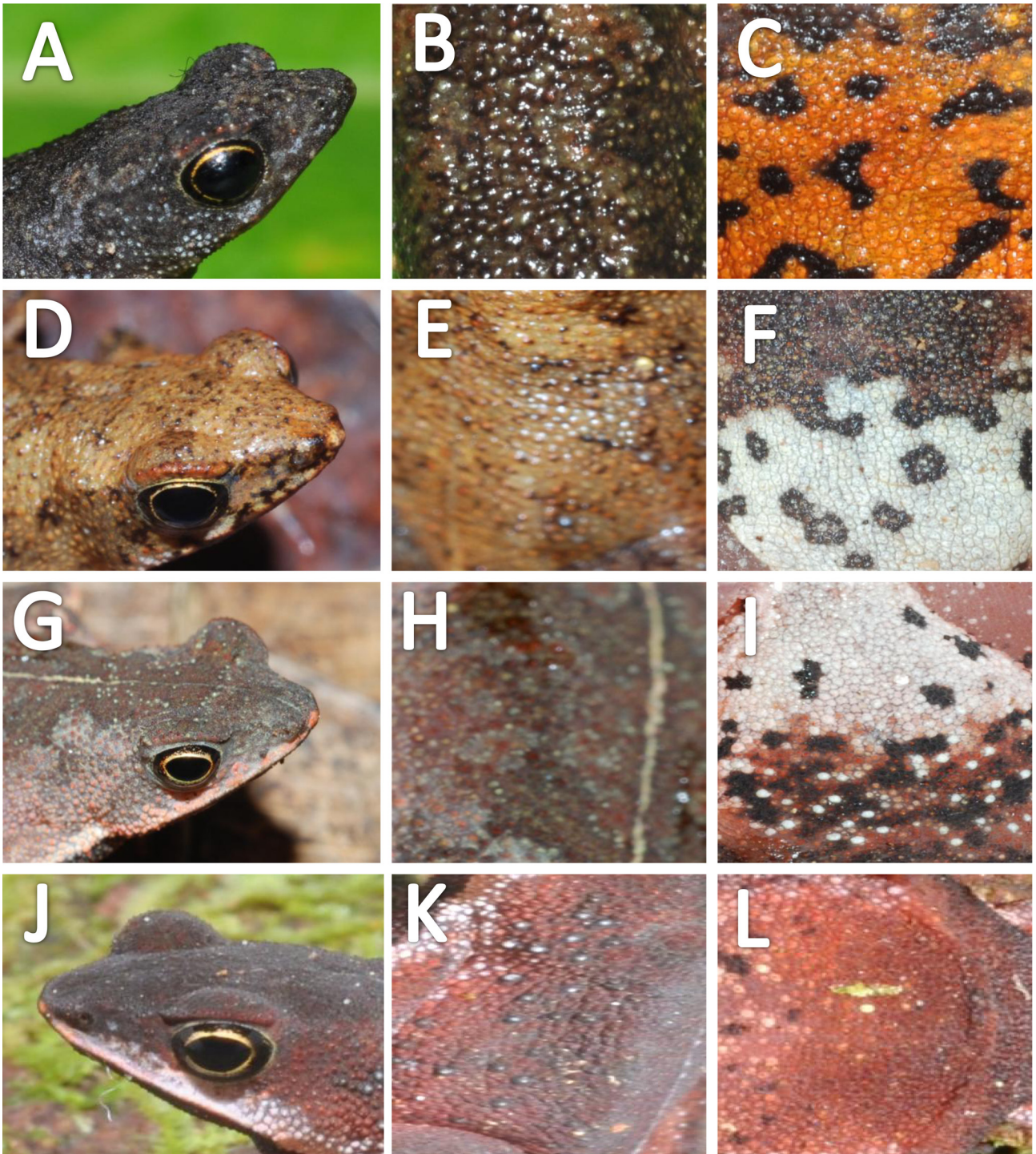


FIGURE 4. Some diagnostically morphological and color characters between the species of *Amazophrynella* (in life), type of snout (A,D,G,J); texture of the dorsal surface (B,E,H,K) and texture of ventral surface (C,F,I,L) of A–C) *A. minuta* (INPA-H 312725); D–F) *A. manaos* **sp. nov.** (unvoucher specimens); G–I) *A. bokermanni* (INPA-H 31863); J–L) *A. vote* (INPA-H 31870).

Description of the holotype. Small species; body slender; head longer than wide; head triangular; head length 36% SVL; upper eyelids close to 60% of interorbital distance; snout elongate; snout profile slightly truncated in lateral view; eyes prominent, 31% of head width; nostril is closer to the tip of the snout than to the eyes; tympanums not visible; parotid gland absent. Body covered by abundant rounded granules; texture of dorsal skin granular; abundant granules grouped in the axillar region; texture of the ventral skin granular. Forelimbs slender; upper arm covered by tiny conical tubercles. Fingers unwebbed; formula of the fingers: I<II<IV<III; palmar

tubercle rounded; supernumerary tubercle ovoid: one in finger I, two in the II and IV and three in the III; tip of the fingers unexpanded. Hind limbs slender; femur length 52% of SVL; conical tiny granules present on the thigh and tibia; tarsus length approximately 30% of SVL; foot length 68% of thigh length; webbing at the base of toes; formula of the toes: I<II<III<V<IV; elliptical inner metatarsal tubercle present; subarticular tubercles rounded and distinct, nearly the same size as fingertips: two in toe I, II and V , three in the III and IV; tiny triangular spines along rear edge of feet; tips of the toes unexpanded.

TABLE 4. Comparison of morphological and color characters of the *Amazophrynella* (Fouquet *et al.*, 2012a) species. For all species original descriptions and direct examination of specimens was used. We used adults specimens from all the species.

Species	<i>A. manaos</i>	<i>A. bokermanni</i>	<i>A. vote</i>	<i>A. minuta</i>
Source	This study	Izecksohn, 1993 + material studied	Ávila <i>et al.</i> , 2012 + material studied	Melin, 1941 + material studied
Males SVL in mm, mean ± SD (range)	15.0 ± 0.6 (14.0–15.8)	22.0 ± 3.7 (16.4–27.7)	16.4 ± 1.3 (15.2–19.3)	13.6 ± 0.8 (12.3–15.8)
Females SVL in mm, mean ± SD (range)	20.6 ± 1.8 (15.9–24.7)	20.1 ± 4.7 (19.4–30.6)	22.7 ± 1.6 (21.4–25.7)	17.9 ± 0.6 (17.9–18.9)
Dorsal skin texture	Fine granules, medium density	Fine granules, medium density	Fine granules, low density	Rough granules, high density
Ventral skin texture	Fine granules, high density	Fine granules, medium density	Fine granules, medium density	Rough granules, high density
Ventral coloration	White with black blotches	White with black spots	Red-brown with white dots	Yellow-orange with brown blotches
Snout profile	Slightly truncated	Pointed	Rounded	Pointed
Relative size finger I and II	I<II	I>II	I<II	I<II
Gular region	Blackish to dark-brown	Grayish-brown	Brown	Light-brown

Measurement of the holotype (in mm). SVL: 13.9; HL: 5.1; HW: 4.1; EW: 1.1; ED: 1.3; SL: 2.3; END: 1.4; IND: 1.0; IOD: 1.8; HAL: 3.0; UAL: 4.1; THL: 7.2; TL: 6.9; TAL: 4.4 and FL: 4.9.

Coloration of the holotype. In life, dorsal color pattern of the holotype dark-brown with transversal black and light-brown bars, more evident on limbs; thin cream medial line extending from head to cloaca, a white longitudinal stripe on upper jaw extending from nostril to forearm; arms white ventrally; throat and chest region blackish; belly white with large black spots, thighs and tibiae predominantly grey ventrally with few small black dots; palms and soles reddish (Figure 5D). Coloration of preserved specimens is nearly identical to that described in life but black spots on the belly are less evident.

Variation. Variation exists among individuals within the same locality as well as between localities. In some individuals the coloration of the throat extends onto the chest and the belly has few black spots; other specimens show a ventral pattern of black stripes that extend dorsally to the upper part of the lateral region. Juveniles have a less pronounced pattern on the belly, with few black spots. Individuals from some localities have a thin clear brown line that extends from the rostrum to the cloaca along the central dorsum (Figure 6).

Distribution and natural history. *Amazophrynella manaos* **sp. nov.** is distributed throughout the southwestern part of the Brazilian Guiana region. It was collected in eight localities in the state of Amazonas, Brazil: Mineração Taboca, Campus of the Universidade Federal do Amazonas, Reserva Florestal Adolpho Ducke, Presidente Figueiredo, Reserva ZF-2 , REBIO Uatumã, RDS Uatumã and Parque Estadual Rio Negro Setor Sul, Cuieiras River. To the south and west its distribution is likely to be delimited by the Amazon and Negro Rivers, respectively. It thus appears to be restricted to the south-central portion of the Guiana Shield (Figure 7). It occurs in primary forests and forest fragments, and is found in leaf litter—often fallen fronds of the buriti palm (*Mauritia flexuosa*)—generally close to creeks. The species is diurnal, is clearly sexually dimorphic in size (Figure 9, Table 5, online supplement 1) with females being distinctly larger than males. The amplexus is cephalic (Figure 9). Reproductive period is from November to April (Magnusson & Hero, 1991).

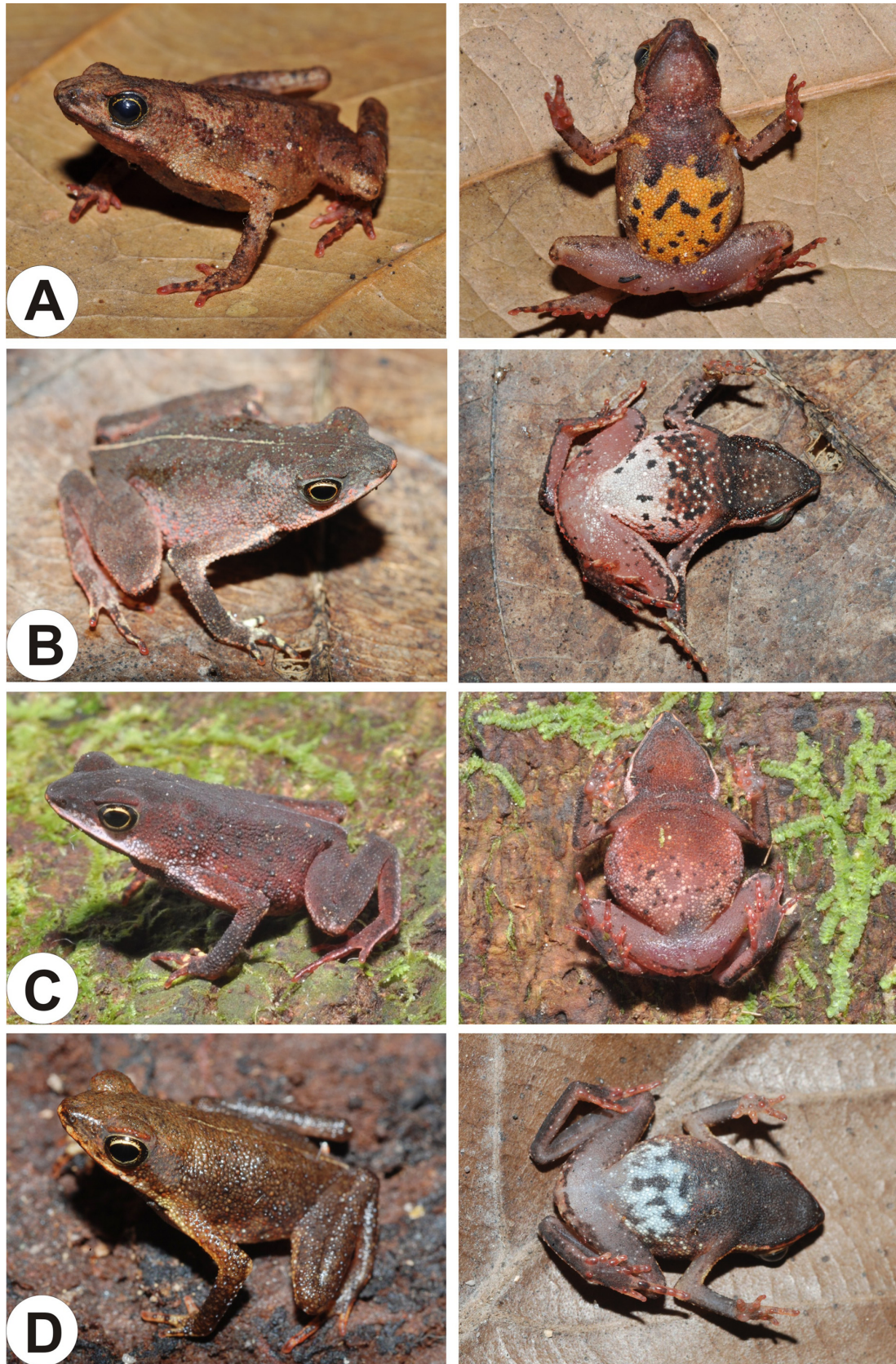


FIGURE 5. Dorsal and ventral view of male specimens of: A) Topotype of *Amazophrynella minuta*, Taracúá, Brazil (INPA-H 32732); B) *Amazophrynella bokermanni*, Juruti, State of Pará, Brazil (INPA-H 31861); C) *Amazophrynella vote*, Parque Estadual do Matupiri, State of Amazonas, Brazil (INPA-H 31870); D) Holotype of *Amazophrynella manaos* **sp. nov.** campus of the Universidade Federal do Amazonas-UFAM, State of Amazonas, Brazil (INPA-H 31866).

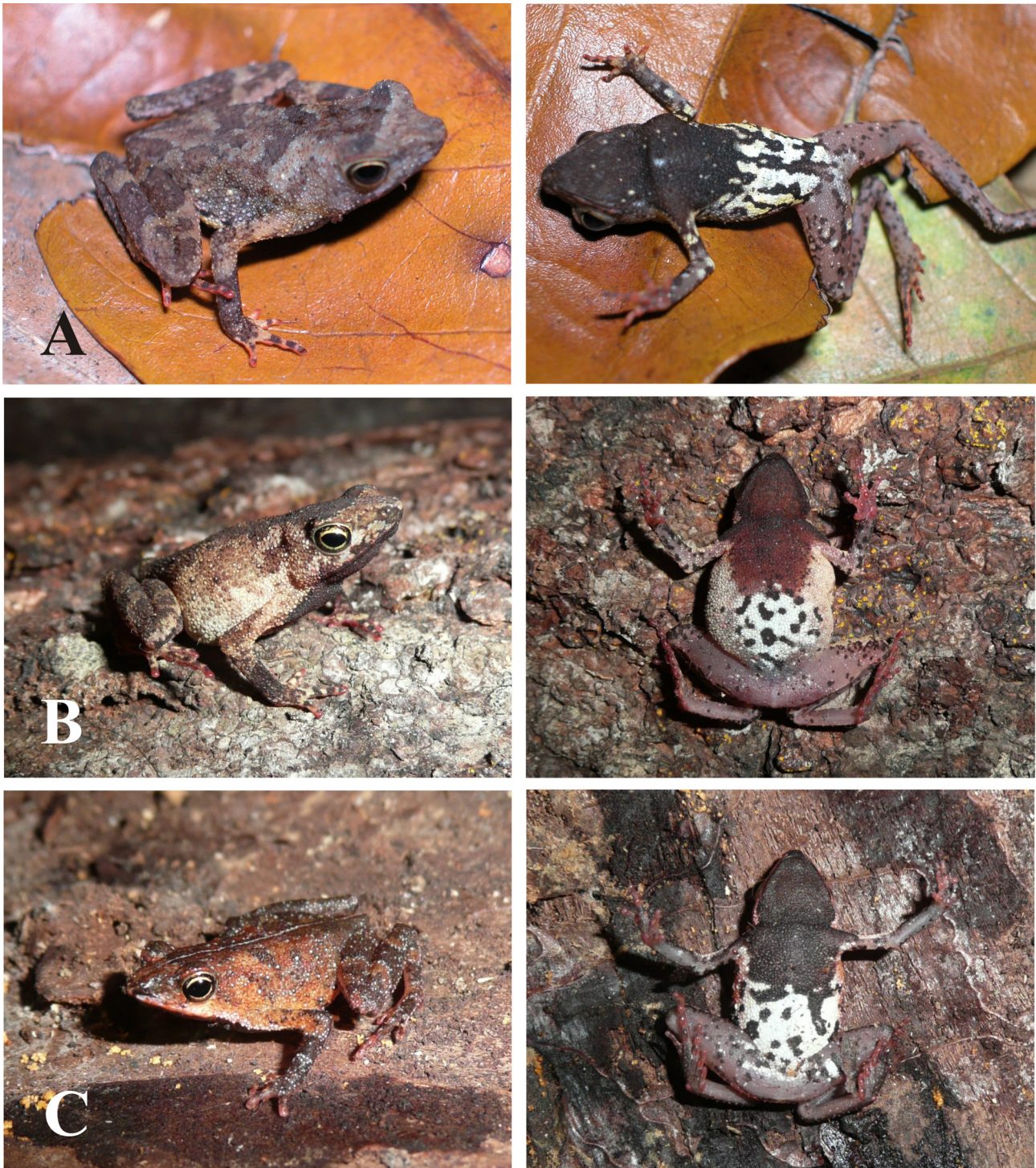


FIGURE 6. Patterns of variation in *Amazophrynella manaos* sp. nov., dorsal and ventral views.

Etymology. We name the species in honor of the Manaos Amerindian tribe that inhabited the region of the present day city of Manaus, Amazonas, Brazil, where the species is distributed.

Discussion

Amazophrynella minuta (Melin, 1941) was described as *Atelopus minutus*. Since the original description, all *Amazophrynella* specimens collected throughout the Amazonian biome including the Meta River llanos of

Colombia (Frost, 2013), were treated as the taxon described by Melin (1941) (see Duellman, 1978; Rodriguez & Duellman, 1994; Duellman & Mendelson, 1995; Lima *et al.*, 2006).

In 1993, Izecksohn recognized that populations from the eastern Amazon basin represent a different species, *Dendrophryniscus bokermanni*. More recently Fouquet *et al.* (2012a) recognized that species of *Dendrophryniscus* from the Amazon and Atlantic rainforest form deeply divergent, reciprocally monophyletic groups, and thus based on this observation and shared derived morphological characteristics of these two lineages, the authors proposed a new genus *Amazophrynella* for the Amazonia species. In the same year, Ávila *et al.* (2012) described a new species, *Amazophrynella vote*, from the upper Purus, Madeira and Tapajós River basins in state of Mato Grosso and the southern portion of the state of Amazonas, Brazil.

TABLE 5. Measurements (mm) of adult male specimens (including the holotype) and adult females in the type series *Amazophrynella manaos* **sp. nov.** Mean \pm standard deviation, ranges are in parentheses. Abbreviations are defined in Methods.

Character	Males (n=29)	Females (n=28)
SVL	15.0 \pm 0.6 (14.0–15.8)	20.6 \pm 1.8 (15.9–24.7)
HW	4.5 \pm 0.3 (4.2–4.9)	5.6 \pm 0.6 (4.8–6.9)
HL	5.5 \pm 0.3 (5.1–6.4)	6.8 \pm 0.5 (5.3–7.5)
SL	2.7 \pm 0.1 (2.4–2.7)	3.1 \pm 0.4 (2.1–3.6)
EW	1.2 \pm 0.1 (1.0–1.3)	1.4 \pm 0.2 (1.5–2.1)
ED	1.5 \pm 0.1 (1.3–1.7)	1.7 \pm 0.2 (1.8–2.3)
IOD	2.0 \pm 0.1 (1.8–2.3)	2.5 \pm 0.2 (1.8–2.9)
IND	1.2 \pm 0.1 (1.1–1.6)	1.9 \pm 0.1 (1.6–2.2)
END	2.0 \pm 0.4 (1.0–2.4)	2.6 \pm 0.4 (1.7–2.8)
HAL	3.1 \pm 0.3 (2.4–3.7)	4.2 \pm 0.5 (3.2–4.8)
UAL	4.0 \pm 0.4 (3.2–4.8)	5.3 \pm 0.4 (4.2–5.9)
THL	7.2 \pm 0.6 (6.3–10.5)	10.2 \pm 0.9 (6.1–11.9)
TL	6.8 \pm 0.7 (5.3–8.1)	10.2 \pm 0.7 (6.3–11.8)
TAL	5.9 \pm 0.5 (5.4–7.3)	6.5 \pm 0.6 (5.5–7.5)
FL	7.4 \pm 0.7 (6.6–8.8)	7.5 \pm 0.6 (5.3–8.9)

Amazophrynella manaos **sp. nov.** is one more species that was mistakenly called *A. minuta*, despite clear morphological differences (see Table 4). *Amazophrynella manaos* **sp. nov.** is diagnosable by at least four morphological and phenotypic characters, and by 15 molecular apomorphies in the 16S rDNA fragment which has become standard in analyses of amphibian biodiversity (Vences *et al.*, 2005). The phylogenetic analysis of the said 16S rDNA fragment also clearly support the sister taxon relationship of *Amazophrynella manaos* **sp. nov.** and *Amazophrynella bokermanni*, and its nested position within the genus *Amazophrynella*. The species status is further supported by a 9% uncorrected p-divergence from its nominal sister taxon (*A. bokermanni*), a divergence normally associated with interspecific divergences (Vences *et al.*, 2005; Fouquet *et al.* 2007).

Amazophrynella minuta its only know from its type locality and from the vicinity of the city of São Gabriel da Cacheoeria, approximately 110 km south-east of the type locality (observation of RRRZ and VTC). It is possible its distribution comprises the upper rio Negro basin, west of Santa Isabel do Rio Negro. The inclusion of additional localities in the rio Negro basin, and the compilation of additional lines of evidence (e.g. vocalizations, osteology and DNA sequence data) could better delimit the distribution of this species, making the identification more accurate.

The presented phylogenetic analysis together with uncorrected p-divergence estimates among lineages suggest the existence of additional taxa that previously have been referred to as *Amazophrynella minuta* or were considered part of the *Amazophrynella* spp. complex (sensu Fouquet *et al.*, 2012a). Given that species are lineages with independent evolutionary trajectories (de Queiroz, 2007), these lineages will represent species. However, one of the lineages, for example the geographically close *Amazophrynella* aff. *minuta* “western Amazonian” (sensu

Fouquet *et al.* 2012a) may be *A. minuta*. Without a detailed morphological analysis of representative individuals from the western Amazon and other lineages it is difficult to speculate if the western Amazon and other lineages are new nominal taxa.

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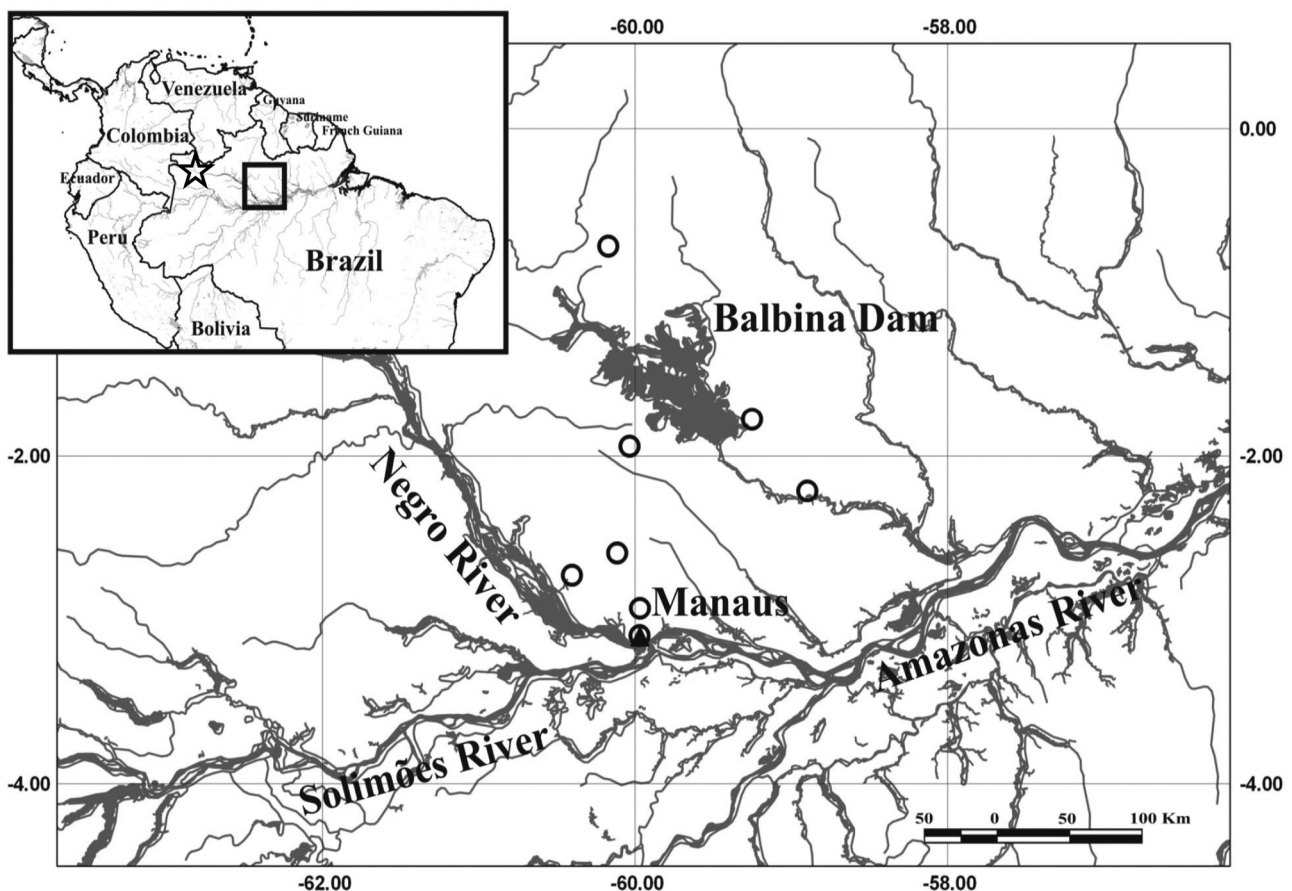


FIGURE 7. Geographical distribution of *Amazophrynella manaos* sp. nov. triangle marks the type locality and circles localities of paratypes. Star marks the type locality of *A. minuta*.



FIGURE 8. Type habitat of *Amazophrynella manaos* sp. nov. within a primary forest showing small temporary ponds near fallen logs used by the species.



FIGURE 9. Amplexus of *Amazophrynella manaos* sp. nov. (unvouchered specimens).

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APPENDIX 1. Specimens examined.

- Amazophrynella bokermanni*—INPA-H 31861, INPA-H 31864, INPA-H 31863, INPA-H 31862, INPA-H 31865, municipality of Juruti, Pará State, Brazil (50 km from type locality).
- Amazophrynella vote*—INPA-H 12256, 12331, 12255, 12342, 12343, 12366, 12267, Cachoerinha, Madeira River, municipality of Manicoré, INPA-H 12326, Lago do Açaí, Aripuanã River, municipality of Novo Aripuanã, INPA-H 21558, Parque Estadual do Guariba, municipality of Manicoré, INPA-H 27412, 27417-27419, 27421-27423, 27425-27426, Parque Nacional Nascentes do Lago Jari, Tapauá municipality, Amazonas State, Brazil. UFMT-A 11138, Fazenda São Nicolau, municipality of Cotriguaçu, Mato Grosso State, Brazil.
- Amazophrynella minuta*—INPA-H 32725, INPA-H 32723, INPA-H 32729, INPA-H32730, INPA-H32736, INPA-H32731 (females) and INPA-H 32724, INPA-H32728, INPA-H 32733, INPA-H 32735, INPA-H 32722, INPA-H 32738, INPA-H 32737, INPA-H 32739. INPA-H 32720, INPA-H 32732, INPA-H 32726, INPA-H 32730, INPA-H 32740, INPA-H 32734, INPA-H 32721 (males), Taracuá, right bank of the Uaupés River, Amazonas State, Brazil.

ONLINE SUPPLEMENT 1. Raw measurements of the 57 individuals of *Amazophrynella manaos* sp. nov. examined in this study.