

Article

Out of Bound: A New Threatened Harlequin Toad (*Bufo*idae, *Atelopus*) from the Outer Borders of the Guiana Shield in Central Amazonia Described through Integrative Taxonomy

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Abstract: We used integrative taxonomy to describe a new species of *Atelopus* from the lowlands of Central Amazonia in the region of Manaus, Amazonas, Brazil. The new species is geographically isolated from the southernmost species of *Atelopus* of the Guiana Shield. *Atelopus manauensis* species nova (sp. nov.) is characterized by the combination of the following characteristics: male snout-vent length range (SVL = 19.1–26.4 mm; $n = 11$); dorsal and lateral skin smooth; ventral surface entirely white or white with cream-colored gular region; fingers and toes lacking subarticular tubercles and fringes. The advertisement call of the new species has a call duration of 689–840 ms, contains 15–26 pulses, is emitted at an average pulse rate of 25.5 pulses per second, and has a dominant frequency ranging 3088–3610 Hz. The genetic divergence between the new species and its morphologically most similar congeners (*A. spumarius* and *A. pulcher*) is greater than 4%. *Atelopus manauensis* sp. nov. is closely related to species of the *A. hoogmoedi* complex inhabiting the Guiana Shield. The new species has a small geographic distribution (approximately 4500 km²) in a landscape that is strongly threatened by the growth of Manaus, the largest city in Brazilian Amazonia. The new species is considered critically endangered and in need of urgent conservation measures.

Keywords: conservation; genetics; integrative taxonomy; morphology; vocalization

1. Introduction

The genus *Atelopus* comprises 96 [1] diurnal (except for *A. nocturnus*) and small-sized (17–50 mm) species known as harlequin frogs, which are distributed from Central America to northern South America [2]. Although few species have been included in recent phylogenetic studies, recent phylogenies show *Atelopus* to contain two major clades: the Andean–Chocó–Central American clade, and the Amazonian–Guianan clade. The latter comprises the *tricolor* subclade (pre-Andean region of Peru and adjacent Bolivia) and the *flavescens-spumarius* subclade [3]. The *flavescens-spumarius* subclade includes species distributed in the upper Amazon Basin (Ecuador, Peru, and Colombia), the Guiana Shield (Guyana, Suriname, French Guiana, and Brazil) and the lowlands of Central Amazonia (Brazil). The alpha taxonomy of the species distributed in the Guiana Shield and surrounding lowlands is complex due to morphological, bioacoustic, and genetic similarities [3,4], and different recognized

species may comprise a single polymorphic taxon [5]. Therefore, taxonomic studies integrating different traits (i.e., morphological, bioacoustic, and genetic) is needed to shed light on the taxonomy of *Atelopus* of the Guiana Shield and surrounding lowlands.

Atelopus spumarius was originally described from Iquitos, Peru [6], but due to the loss of the holotype, Lescure [7] assigned a neotype from Colonia, Peru. The forms that were initially treated as subspecies of *A. spumarius* distributed in the Andes (e.g., *A. s. andinus*; [8]) and in the Guiana Shield (*A. s. hoogmoedi* and *A. s. barbotini*; [7,9]) were latter considered different taxa by Lötters and De La Riva [10] and Lötters et al. (3,4). Additionally, *A. spumarius* sensu stricto (SS) exhibits considerable genetic differentiation (uncorrected p-distance > 3% from other species of the *A. spumarius* complex in the Guiana Shield, considering that *Atelopus* species within the Guiana Shield have less than 2% genetic differentiation [3]). Despite recent advances in understanding the phylogenetic relationships of the Amazonian–Guianan clade [4,11], there remain populations of *Atelopus* in areas of lowland forests adjacent to the Guiana Shield that require taxonomic review [3], such as the population in the region of Santarém (state of Pará) and the population in the region of Manaus (state of Amazonas), both of which are in Brazilian Amazonia.

Although occurring in the best studied portion of Brazilian Amazonia, the population of *Atelopus* in the region of Manaus and its surroundings has historically been assigned to different nominal species. The work of Zimmerman [12], Zimmerman and Bierregaard [13], and that of Zimmerman and Simberloff [14] assigned the name *Atelopus pulcher* to species collected from reserves in the region of Manaus. When describing the tadpole of an *Atelopus* collected as part of the Projeto Dinâmica Biológica de Fragmentos Florestais (PDBFF—80 km northeast of Manaus), Gascon [15] identified the population as *Atelopus pulcher* based mainly on the red color of the hands and feet of adults at this locality being similar to those of individuals from Peru and Ecuador, as described by Boulenger [16] and Peters [17]. However, the distribution of *A. pulcher* is restricted to the eastern slope of the Andes (600–900 m above sea level, or m a.s.l.) in these two countries [4]. In addition, both adults and tadpoles of *A. pulcher* in the eastern portion of the Andes are distinct from those of other species in the *A. spumarius* complex [4].

Although they did not analyze adult *Atelopus* from the region of Manaus, Lötters et al. [4] suggested that the species be treated as *Atelopus spumarius* sensu lato (SL) due to the red color of the ventral surface of the hands and feet. This nomenclature has been followed by several studies undertaken in this region (e.g., Lima et al. [18]; Rojas-Ahumada and Menin [19]; Menin et al. [20]; Siqueira et al. [21]; Jorge et al. [22]; Rößler et al. [23]). However, *A. spumarius* SS is restricted to the Upper Amazon Basin of Peru, Ecuador, and Colombia [4]. In addition, the tadpole of *Atelopus* in the region of Manaus (PDBFF) differs from that of *A. spumarius* described in Ecuador with regard to the position of the nostrils [24].

The present study employed integrative taxonomy to describe the taxon of *Atelopus* from the region of Manaus as a new species. This species is distinguished from others of the *A. spumarius* complex and of the Guiana Shield by morphology, color pattern, advertisement call, and phylogenetic position. Additionally, the new species is shown to be restricted to a small region of lowlands around Manaus that suffers from strong anthropic pressure. Thus, the formal description of this species will contribute to determining its conservation status and potentially help with the implementation of conservationist measures.

2. Materials and Methods

2.1. Sampling

Individuals of the new species were collected manually in the morning (07:00–10:00) and afternoon (15:00–17:00) at six locations in the municipality of Manaus (Amazonas, Brazil): (1) Reserva Florestal Adolpho Ducke (RFAD; 2°55'4.8" S, 59°53'52.8" W; 109 m a.s.l.); (2) training area of Centro de Instruções de Guerra na Selva do Exército Brasileiro (CIGS/EB; 2°54'52.4" S, 59°49'26.4" W; 61 m a.s.l.); (3) Cabo Frio camp (2°3'20.4" S, 59°55'51.6" W; 109 m a.s.l.); (4) Fazenda Dimona (2°19'43.39" S, 60°4'41.46" W; 102 m a.s.l.); (5) Fazenda Experimental da Universidade Federal do Amazonas (FEUFAM; 2°38'31.20" S,

60°5'45.6" W; 100 m a.s.l.); and (6) banks of a stream in a drainage system of middle Cuieiras River near LBA/ZF2 scientific station (LBA/ZF2; 2°33'32.40" S, 60°13'48.36" W; 110 m a.s.l.). Fazenda Dimona and Cabo Frio camp are located within the Área de Relevante Interesse Ecológico do Projeto Dinâmica Biológica de Fragmentos Florestais (ARIE PDBFF). Individuals collected at FEUFAM (2) and LBA/ZF2 (2) were used for genetic analyses.

For interspecific comparisons, eight adult males of *Atelopus hoogmoedi* were collected from two populations (four individuals of each population) with geographic distributions known to be the closest to the new species, both within the municipality of Presidente Figueiredo (state of Amazonas, Brazil): (1) Reserva Biológica do Uatumã (REBIO Uatumã; 1°46'48" S, 59°15'4.81" W; 80 m a.s.l.), 145 km straight-line distance from Manaus; and (2) Pitinga River drainage system (0°42'52.74" S–60°1'22.25" W; 177 m a.s.l.), 250 km straight-line distance from Manaus. Two individuals collected at REBIO Uatumã were used for genetic analyses.

The specimens were killed with a 5% lidocaine topical solution, fixed in 10% formaldehyde and preserved in 70% alcohol. Muscle tissue was extracted prior to fixation and kept in absolute alcohol. Specimens were deposited in the herpetology section of the zoological collection of the Instituto Nacional de Pesquisas da Amazônia (INPA-H), Manaus, Amazonas, Brazil. Specimens were collected under permit number 56,759 from Sistema de Autorização e Informação em Biodiversidade of Instituto Chico Mendes de Conservação da Biodiversidade (SISBIO/ICMBIO) and the study was approved by Comissão de Ética no Uso de Animais of INPA with registration number 002/2017 (CEUA/INPA).

Advertisement calls from two adult males of the new species were recorded in RFAD and one was recorded in Cabo Frio camp. The call of an uncollected male of the new species from RFAD presented by Lima et al. [18] was also used. Advertisement calls of seven males of *Atelopus hoogmoedi* were recorded: four males in REBIO Uatumã (INPA-H 041358, INPA-H 041359, INPA-H 041293, and INPA-H 041360) and three males on the banks of a stream of Pitinga River (INPA-H 041361, INPA-H 041362, and INPA-H 041363). Recordings were made with a Sennheiser K6/ME66 directional microphone coupled to a Marantz PMD660 digital recorder. The microphone was positioned approximately 150 cm from each individual, and recordings were made with a sampling rate of 44.1 kHz and sample size of 16 bits. Calls were recorded during the morning (08:00–11:00) and the average air temperature at the time of recording was approximately 26 °C.

In addition to the advertisement call (type 1 call), three other call types were recorded and analyzed: type 2, type 3, and type 4. Following Lötters et al. [25], the type 2 call represents the pure tone call; the type 3 call represents the short pure tone call; and the type 4 call represents the pulsed short call. A type 2 call was recorded from a male in the drainage system on the east bank of the middle Puraquequara River located in CIGS/EB (SVL = 20.3 mm; 26.5 °C; 2°51'50.4" S, 59°48'54.0" W; 108 m a.s.l.; INPA-H 041372). Type 3 calls were recorded from the holotype in RFAD and from a male at the headwaters of the upper Cuieiras River located in Fazenda Dimona (SVL = 20.9 mm; 26.5 °C; 2°19'43.3" S, 60°4'41.46" W; 95 m a.s.l.; INPA-H 041373). The type 4 call was emitted by the holotype and recorded in laboratory. Type 2 and type 3 calls emitted by the holotype were recorded with a Sennheiser K6/ME66 directional microphone coupled to a Marantz PMD660 digital recorder. Type 2 and type 3 calls of other males and type 4 calls were recorded with a SONY PX333 digital recorder (format mp3, 192 kbps) with an internal microphone at 30 cm from the individuals. Files in mp3 format were converted to wav format (sampling rate 44.1 kHz, sample size 16 bits) to obtain acoustic parameters.

2.2. Phylogenetic Analyses

To infer the phylogenetic relationships of the new species and its congeners, 28 16S ribosomal RNA gene (16S) sequences and seven cytochrome c oxidase subunit I gene (COI) sequences were selected from GenBank of the National Center for Biotechnology Information (NCBI). All sequences used in the current study were obtained from GenBank. The sequences correspond to 15 species of *Atelopus* [22 16S sequences of 13 described *Atelopus* species + one sequence of an undescribed species from the

south bank of the Amazon River (municipality of Anapu, state of Pará, Brazil) + four sequences from individuals of the new species, and four COI sequences from individuals of the new species + two from *A. hoogmoedi* from REBIO Uatumã + one from *A. spurrelli*, including two 16S sequences of *A. hoogmoedi* from Guyana, two from French Guiana, two from REBIO Uatumã, and three from the municipality of Monte Alegre, state of Pará, Brazil. A 16S sequence and another COI sequence of *Rhinella marina* were selected for rooting the phylogenetic trees. See Table 1 for detailed information on the specimens used in the phylogenetic analyses and accession numbers for all sequences in GenBank. After concatenating and aligning the total of 28 sequences in Geneious 4.8.2 using default parameters [26], a final matrix was obtained containing 1274 base-pairs (bp) (614 bp 16S and 660 bp COI). PartitionFinder2 [27] was used to infer the best partition scheme and its respective models of nucleotide evolution through PhyML 3.0 [28] and Bayesian Information Criterion (BIC). Suggested partitions and models were as follows: 16S = GTR + G; COI\1 = SYM; COI\2 = F81; COI\3 = HKY. We used for phylogenetic inference the same 20 16S and all COI sequences used in Jorge et al. [29], including those sequences of the new species, only adding eight new sequences, as follows: *A. hoogmoedi* (three sequences from the municipality of Monte Alegre, state of Pará, Brazil—IDs 8–10 in Table 1), *A. sp.* Anapu, *A. barbotini* “B”, *A. oxapampae*, *A. tricolor*, and *R. marina* (IDs 7; 14; 26–28 in Table 1, respectively). Overall, we obtained the same phylogenetic relationships and statistical support as obtained by Jorge et al. [29], with a slight difference regarding the relationships of the French Guiana species, which is likely because of the addition of new samples (see Results section, Section 3.1).

Table 1. Detailed information on the specimens used in the phylogenetic inferences: Species, GenBank accession number, voucher, origin of specimens and source reference for sequences.

ID	Species	GenBank 16S/COI	Voucher	Country	Source
1	<i>Atelopus manauensis</i> sp. nov.	MT176236/MT184269	INPA-H 041289	Brazil	[29]
2	<i>Atelopus manauensis</i> sp. nov.	MT176237/MT184270	INPA-H 041290	Brazil	[29]
3	<i>Atelopus manauensis</i> sp. nov.	MT176238/MT184271	INPA-H 041291	Brazil	[29]
4	<i>Atelopus manauensis</i> sp. nov.	MT176239/MT184272	INPA-H 041292	Brazil	[29]
5	<i>Atelopus hoogmoedi</i> REBIO Uatumã	MT176240/MT184273	INPA-H 041293	Brazil	[29]
6	<i>Atelopus hoogmoedi</i> REBIO Uatumã	MT176241/MT184274	INPA-H 041294	Brazil	[29]
7	<i>Atelopus</i> sp. Anapu	MK166205	KA14	Brazil	[30]
8	<i>Atelopus hoogmoedi</i> Monte Alegre	MK166206	LZA971	Brazil	[30]
9	<i>Atelopus hoogmoedi</i> Monte Alegre	MK166208	LZA990	Brazil	[30]
10	<i>Atelopus hoogmoedi</i> Monte Alegre	MK166211	LZA1046	Brazil	[30]
11	<i>Atelopus hoogmoedi</i>	JQ742148	IRSNB15781	Guyana	[31]
12	<i>Atelopus hoogmoedi</i>	JQ742149	IRSNB14477	Guyana	[31]
13	<i>Atelopus barbotini</i> “A”	EU672971	-	French Guiana	[32]
14	<i>Atelopus barbotini</i> “B”	GU183859	BPN1697	French Guiana	[33]
15	<i>Atelopus franciscus</i>	JQ742150	PK3306	French Guiana	[31]
16	<i>Atelopus (spumarius) hoogmoedi</i>	DQ283260	BPN754UTA	French Guiana	[34]
17	<i>Atelopus hoogmoedi</i>	EU672972	-	French Guiana	[32]
18	<i>Atelopus flavescens</i>	EU672970	-	French Guiana	[32]
19	<i>Atelopus seminiferus</i>	EU672976	-	Peru	[32]
20	<i>Atelopus spumarius</i>	EU672977	-	Peru	[32]
21	<i>Atelopus pulcher</i>	EU672973	KU211678	Peru	[32]
22	<i>Atelopus bomolochos</i>	GU252227	KU217468	Ecuador	[33]
23	<i>Atelopus peruensis</i>	GU252229	KU211631	Peru	[33]
24	<i>Atelopus spurrelli</i>	EU672975/DQ502895	MHNUC273	Colombia	[32, 34]
25	<i>Atelopus loettersi</i>	EU672980	-	Peru	[32]
26	<i>Atelopus oxapampae</i>	EU672979	MTD1276	Peru	[32]
27	<i>Atelopus tricolor</i>	EU672978	MNCN5885	Bolivia	[32]
28	<i>Rhinella marina</i>	KR012644/ KR012546	QCAZ50702	Ecuador	[35]

Phylogenetic relationships were estimated using Maximum Likelihood (ML) and Bayesian Inference (BI). The ML phylogenetic tree was estimated using IQ-TREE [36] with 10,000 ultrafast bootstraps; 10,000 iterations; a minimum correlation coefficient of 0.99; and 10,000 Shimodaira–Hasegawa approximate likelihood ratio replicates (SH-aLRT). The Bayesian phylogenetic tree was inferred using MrBayes 3.2.6 [37] through four runs of 10 million generations each using Monte Carlo

via Markov Chains (MCMC) with four chains each. Probabilities were sampled every 1000 generations. The tree was summarized after burning of 25% of the sampled trees. Uncorrected genetic distances (p-distance) and Kimura-2-parameters (K2P; [38]) were calculated in MEGA 6.0 [39] using the 16S alignment.

2.3. Morphological Analyses

Specimens were measured using a digital magnifying glass with a millimeter lens (measurements less than 10 mm) and a caliper with 0.01 mm precision (measurements greater than 10 mm). The sex of specimens was determined by the presence/absence of nuptial pads and vocal slits in males, or by the direct inspection of gonads. Thirteen morphometric measurements were taken following Gray and Cannatella [40] and Coloma et al. [41]: snout-vent length (SVL), sacrum width (SW), head width (HW), head length (HL), eye diameter (EYDM), eye-to-nostril distance (EYNO), interorbital distance (IOD), internarial distance (ITNA), length of flexed forearm (RDUL), hand length (HAND), thumb length (THBL), foot length (FOOT), and tibia length (TL). Formulae for interdigital webbing followed Savage and Heyer [42,43] and Myers and Duellman [44].

Populations of *Atelopus hoogmoedi* of REBIO Uatumã and Pitinga River are the southernmost populations of the genus of the Guiana Shield and the geographically closest populations to the new species described here. For this reason, we used morphometric measurements of these populations to distinguish the new species from *A. hoogmoedi*. Three cluster and classification analyses were used for this purpose: (1) Principal Component Analysis (PCA); (2) Discriminant Analysis of Principal Components (DAPC; [45]); and (3) Random Forest (RF; [46]). The analyses were conducted in R [47] using SVL and 12 morphometric ratios (HW/SVL, HL/SVL, EYDM/SVL, EYNO/SVL, TL/SVL, IOD/SVL, ITNA/SVL, RDUL/SVL, FOOT/SVL, HAND/SVL, THBL/SVL, and SW/SVL) of 11 males of the new species (holotype, type series, paratypes, and paratopotypes) and eight males of *A. hoogmoedi* collected in REBIO Uatumã (4) and at Pitinga River (4). Paratype and paratopotype specimens were included in the analyses so that the extreme minimum and maximum body sizes observed among individuals of the new species could be included, and for the same reason, they were not included in the “type series”. The PCA was performed using the “prcomp” function of the *stats* package using the parameters “scale = T” and “center = T” to scale and center the morphometric variables. A Multivariate Analysis of Variance (MANOVA) was performed to test whether the multidimensional morphometric space occupied by the new species differs significantly from that occupied by *A. hoogmoedi* in relation to the first two axes of the PCA. The DAPC was performed using the “dapc” function of the *adegenet* 2.1.2 package [45]. As recommended by Jombart et al. [45], all principal components (PCs) and discriminant analysis eigenvalues (DAE) were used in the DAPC. Random Forest is a learning algorithm that builds classification trees from random subsamples of a database to then aggregate results and classify samples (specimens) into groups (species) [45]. Random Forest was conducted with 4000 trees using the “randomForest” function of the *randomForest* 4.6-14 package [48]. Results are presented in the subsection “Morphological and Bioacoustic Analyses” of the section “Results”. Raw morphologic measurements can be found in Supplementary Materials Table S1.

2.4. Bioacoustics

Calls were analyzed in Raven 1.5 [49] and configured as follows: Blackman window; 3 dB Filter Bandwidth of 80 Hz; overlap of 80%; hop size of 4.1 ms; and discrete Fourier transform (DFT) size of 2048 samples. Temporal parameters were measured using oscillograms, and spectral parameters were measured using “power spectrum” graphs. Different sets of acoustic parameters were measured for each of the four call types.

Ten advertisement calls (type 1 call) from three males of the new species and 35 calls from four males of *Atelopus hoogmoedi* from REBIO Uatumã and three males from Pitinga River were analyzed. Eleven parameters were measured in the advertisement calls, as follows: (1) call duration (Call_dur); (2) number of pulses (N_pulses); (3) pulse duration of first pulse (Puls_dur_1); (4) pulse

duration of central pulse (Puls_dur_2), which usually precedes the last portion of the call when the pulses are emitted in a shorter time interval; (5) pulse duration of last pulse (Puls_dur_3); (6) pulse period of first three pulses, measured from the beginning of the first pulse to the end of the third pulse (Dur_3_first_puls); (7) pulse period of last three pulses, measured from the beginning of the antepenultimate pulse to the end of the last pulse (Dur_3_last_puls); (8) inter-pulse interval between first two pulses (Interpulse_1); (9) inter-pulse interval between central pulse and next pulse (Interpulse_2); (10) inter-pulse interval between last two pulses (Interpulse_3); (11) call dominant frequency measured using the “Peak Frequency” function (Domi_frequency); and (12) bandwidth. We also measured the advertisement calls of four males of *A. hoogmoedi* recorded by Costa-Campos and Carvalho [50] in Pedra Branca do Amapari (state of Amapá, Brazil), which is the closest population to the type locality of this species (Mont Atachi-Bacca, French Guiana) with data available to be re-analyzed in the same manner as the calls of the new species (recordings provided by the authors).

As with the morphological data, three cluster and classification analyses (PCA+MANOVA, DAPC, and RF) were used to test whether the new species can be distinguished from *Atelopus hoogmoedi* through the advertisement call. Analyses were performed using the average of each acoustic parameter for each male, three of the new species and 11 of *A. hoogmoedi*: four from REBIO Uatumã, three from Pitinga River, and four from Amapá recorded by Costa-Campos and Carvalho [50]. Results are presented in the subsection “Morphological and Bioacoustic Analyses” of the section “Results”.

Six parameters were measured in type 2–4 calls, as follows: call duration, intercall interval, low frequency, high frequency, dominant frequency, and bandwidth. Low and high frequencies, as well as the bandwidth, were measured 20 dB below the peak frequency in order to avoid background noise. Dominant frequency and bandwidth were measured using the functions “Peak frequency” and “Bandwidth 90%” in Raven, respectively. In addition, we measured the intercall interval within a series of type 2 calls, the number of pulses per call, and the pulse duration of the first and last pulse of type 4 calls. The nomenclature of bioacoustic traits followed Köhler [51].

2.5. Natural History

Information on the natural history of the new species was obtained through observations made during the rainy seasons (December–March) of 2012–2013 and 2016–2019, in every probable area of occurrence of the species, at different times of the day and under different climatic conditions.

3. Results

3.1. Phylogenetic Analyses

Both Bayesian Inference (BI) and Maximum Likelihood (ML) supported the *flavescens-spumarius* clade as monophyletic (Figure 1), with *Atelopus pulcher* being the sister species of all other species in the clade [ML support = 100, Posterior Probability (PP) = 1]. *Atelopus spumarius* SS (lowlands of western Amazonia, upper Amazon Basin) and *A. seminiferus* (eastern slope of the Andes) were inferred as sister species, forming a sister clade to the clade composed of the species distributed in the Guiana Shield and surrounding lowlands of Central Amazonia, and in the south bank of the Amazon River. Within this latter clade, the species from Anapu was recovered with low support (ML = 68, PP = 0.64) as a sister to the clade composed by the new species from Manaus and other species distributed in the Guiana Shield. In the latter clade, the new species of Manaus is inferred as the sister to the Guiana Shield clade (ML = 69, PP = 0.83). Samples of *A. hoogmoedi* from REBIO Uatumã formed a moderately-supported subclade (ML = 78, PP = 0.94) within the Guiana Shield clade with five more samples of *A. hoogmoedi* from different locations in Brazil and Guyana (Figure 1). The internal relationships among the other species of the Guiana Shield (French Guiana) were not well supported in any of our reconstructions.

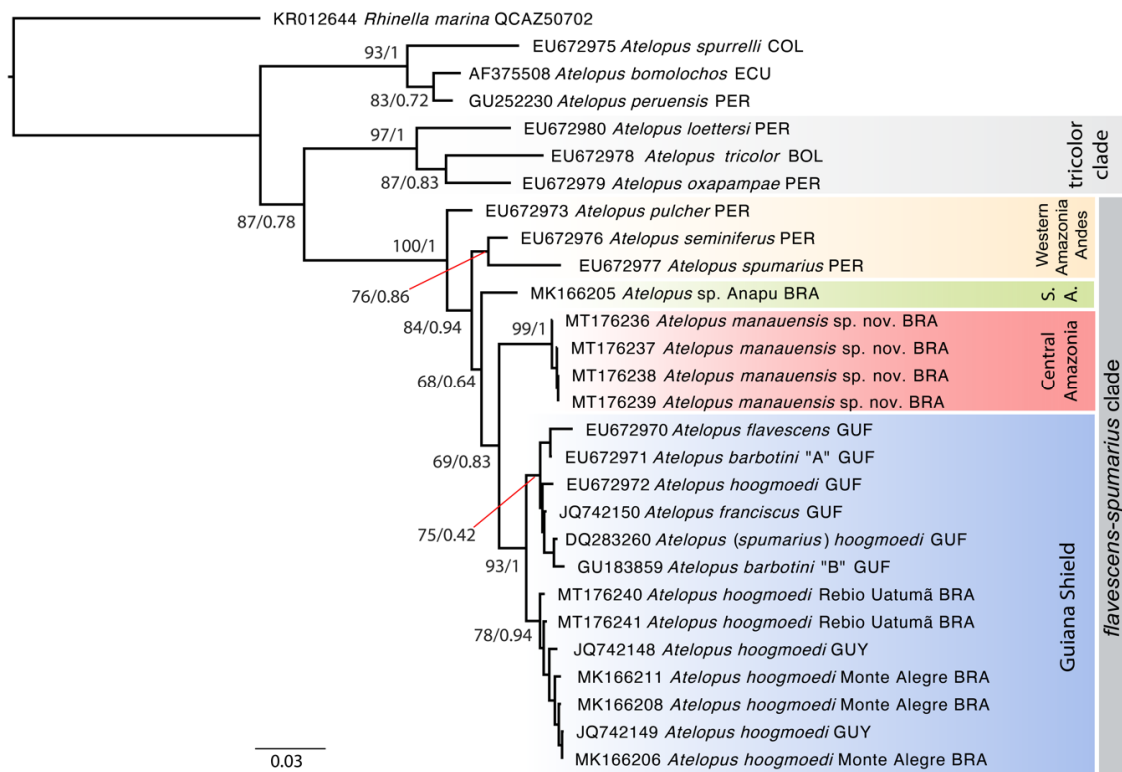


Figure 1. Phylogenetic reconstruction using Maximum Likelihood based on 614 base-pairs of 16S rRNA and 660 base-pairs of cytochrome c oxidase subunit I gene (COI). Bootstrap support is presented as bootstrap/posterior probability (PP). Horizontal yellow bar represents species distributed further west in Amazonia and on the eastern slope of the Andes; red bar represents the new species distributed around the city of Manaus (Brazil); blue bar denotes species distributed in the Guiana Shield. Abbreviations: (S.A.) south bank of the Amazon River—green bar.

Genetic distances between the new species and the other species of *Atelopus* included in the analyses (Table 2) ranged 2.3–11.6% (p-distance) and 2.3–12.7% (Kimura-2-parameters, K2P). Genetic distances were relatively high between the new species and those with which it has been confused in the past: *A. spumarius* SS (p-distance = 4.7%; K2P = 4.9%) and *A. pulcher* (p-distance = 3.9%; K2P = 4.0%). Although *A. hoogmoedi* at REBIO Uatumã is the geographically closest population to the new species, the genetic distances between them varied between 2.5% (p-distance) and 2.6% (K2P). Similar values differentiate the new species and *A. hoogmoedi* of French Guiana (p-distance = 2.6%; K2P = 2.7%). The lowest values of genetic distance were between species of the *A. hoogmoedi* complex occurring in the Guiana Shield, which varied between 0.2–1.7% (p-distance) and 0.2–1.7% (K2P).

Table 2. Uncorrected p-distances (upper diagonal) and Kimura 2-Parameter (lower diagonal) between *Atelopus manauensis* sp. nov. and species of the Amazonian–Guianan clade and of the Andean–Chocó–Central American clade. Genetic distances were calculated using 16S rRNA sequences and are presented as percentages. Abbreviations: (COL) Colombia, (BOL) Bolivia, (PER) Peru, (ECU) Ecuador, (SS) sensu stricto, (GUF) French Guiana, (BRA) Brazil, (GUY) Guyana, (REUA) REBIO Uatumã, (MA) Monte Alegre, state of Pará, Brazil.

ID	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	<i>A. spurrelli</i> COL		13.4	10.7	5.0	11.7	4.8	12.4	11.9	13.6	12.2	13.1	12.4	12.0	13.2	12.6	14.2	12.4	14.1	12.7
2	<i>A. tricolor</i> BOL	12.1		6.7	11.8	5.6	12.2	12.0	11.8	12.3	12.4	12.8	12.6	12.7	12.5	13.1	13.2	12.2	13.3	12.5
3	<i>A. loettersi</i> PER	9.9	6.3		10.7	6.1	11.1	13.7	10.7	12.3	11.6	13.1	12.0	11.2	12.0	12.3	12.7	11.7	13.1	11.9
4	<i>A. bomolochos</i> ECU	4.8	10.8	10.0		10.7	2.2	11.3	10.4	12.1	10.7	10.9	11.4	11.2	11.6	11.6	12.6	11.2	12.4	10.9
5	<i>A. oxapampae</i> PER	10.8	5.3	5.8	9.9		10.7	12.2	10.2	11.8	11.1	13.1	11.5	11.2	11.3	11.4	12.0	11.2	12.1	10.8
6	<i>A. peruensis</i> PER	4.6	11.1	10.3	2.2	9.9		11.1	10.3	11.6	10.4	10.8	10.9	10.7	11.3	11.1	12.1	10.7	12.3	10.5
7	<i>A. spumarius</i> SS PER	11.4	11.1	12.5	10.5	11.3	10.3		4.5	4.7	2.8	3.4	3.7	4.3	2.8	4.1	3.8	3.9	3.2	4.9
8	<i>A. pulcher</i> PER	11.0	10.9	9.9	9.6	9.5	9.6	4.3		3.4	2.6	3.3	2.7	3.4	2.7	3.1	2.8	2.9	3.1	4.0
9	<i>A. flavescens</i> GUF	12.4	11.3	11.3	11.2	10.9	10.7	4.6	3.3		3.2	2.6	1.1	1.7	0.4	1.5	1.1	1.3	0.8	3.0
10	<i>A. seminiferus</i> PER	11.2	11.4	10.7	9.9	10.3	9.7	2.7	2.6	3.1		2.4	2.2	2.8	2.2	2.5	2.7	2.4	2.6	3.2
11	<i>A. sp. Anapu</i> BRA	11.9	11.7	12.0	10.1	11.9	10.0	3.3	3.2	2.5	2.3		2.3	3.0	2.6	2.7	2.8	2.6	2.8	3.0
12	<i>A. franciscus</i> GUF	11.4	11.6	11.0	10.5	10.6	10.1	3.6	2.7	1.1	2.1	2.3		0.5	0.2	0.4	0.5	0.4	0.4	2.7
13	<i>A. barbotini</i> “B” GUF	11.0	11.7	10.4	10.4	10.3	9.9	4.2	3.3	1.7	2.7	2.9	0.5		0.8	0.7	1.0	1.3	1.1	3.0
14	<i>A. barbotini</i> “A” GUF	12.0	11.5	11.0	10.7	10.4	10.4	2.7	2.6	0.4	2.2	2.5	0.2	0.8		0.5	0.4	0.4	0.6	2.3
15	<i>A. hoogmoedi</i> GUF	11.6	11.9	11.2	10.7	10.5	10.2	4.0	3.0	1.4	2.5	2.7	0.4	0.7	0.5		0.4	0.6	0.3	2.9
16	<i>A. hoogmoedi</i> GUY	12.9	12.1	11.6	11.5	11.1	11.1	3.7	2.8	1.1	2.7	2.8	0.5	1.0	0.4	0.4		0.4	0.4	2.7
17	<i>A. hoogmoedi</i> REUA BRA	11.4	11.3	10.7	10.4	10.3	9.9	3.8	2.9	1.3	2.3	2.5	0.4	1.2	0.4	0.6	0.4		0.2	2.7
18	<i>A. hoogmoedi</i> MA BRA	12.8	12.1	12.0	11.3	11.1	11.3	3.1	3.0	0.8	2.5	2.7	0.4	1.0	0.6	0.3	0.4	0.2		2.4
19	<i>A. manauensis</i> sp. nov. BRA	11.6	11.5	11.0	10.1	10.0	9.7	4.7	3.9	3.0	3.1	2.9	2.7	2.9	2.3	2.8	2.7	2.6	2.3	

3.2. Morphological and Bioacoustic Analyses

The multidimensional morphometric (Figure 2A) and bioacoustic (Figure 2B) spaces occupied by *Atelopus manauensis* sp. nov. and the two geographically closest populations of *A. hoogmoedi* (REBIO Uatumã and Pitinga River) were distinct for the first two axes of the PCAs (MANOVA morphology: Pillai trace = 0.69, degrees of freedom (df) = 16, $p < 0.000001$; MANOVA bioacoustic: Pillai trace = 1.00, df = 22, $p < 0.002$). The DAPCs were congruent with the results of the PCAs and showed that the two species can be distinguished from each other with maximum values of posterior probability of membership through morphology (Figure 2C) and advertisement calls (Figure 2D).

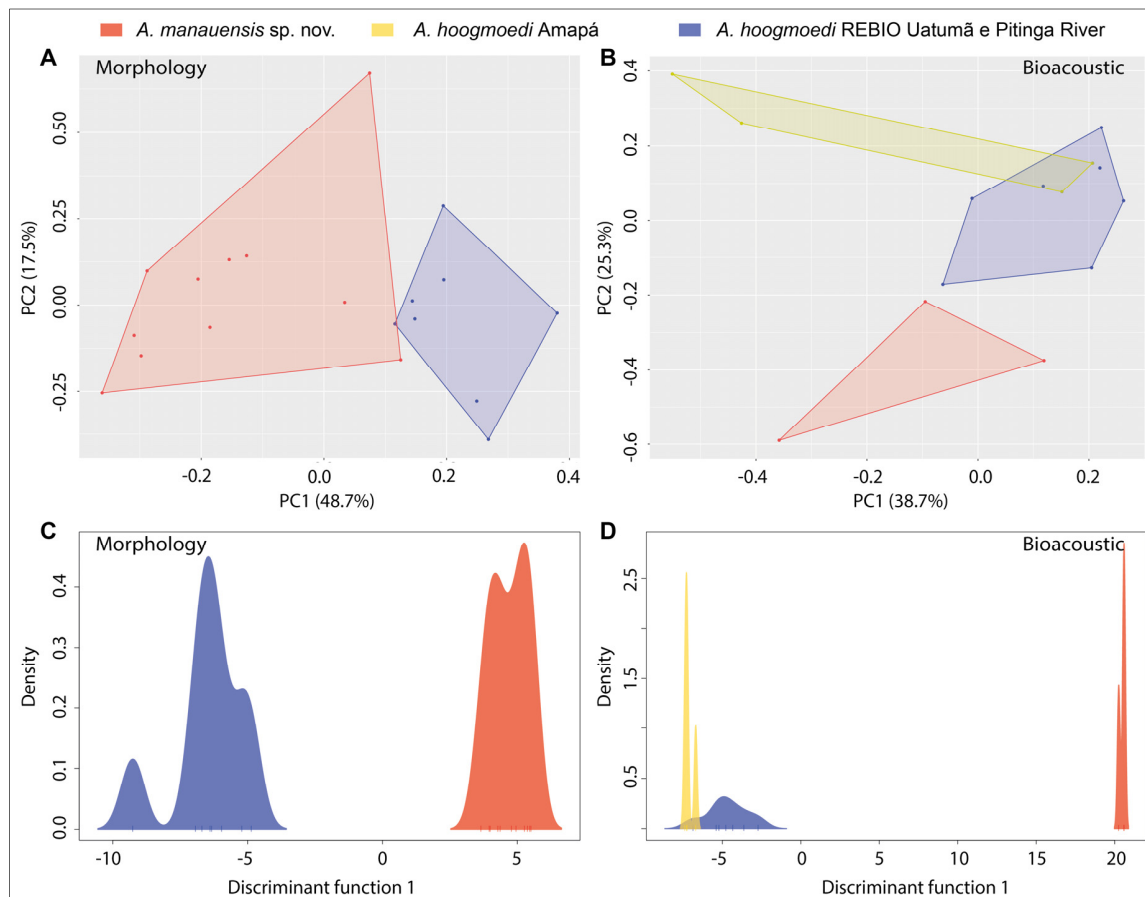


Figure 2. Principal Component Analysis (PCA) and Discriminant Analysis of Principal Components (DAPC) of *Atelopus manauensis* sp. nov. from Manaus (red) and *Atelopus hoogmoedi* from the state of Amapá (yellow) and REBIO Uatumã and Pitinga River (blue). All localities are in Brazil. (A) PCA for morphological variables (males); (B) PCA for advertisement call; (C) DAPC for morphological variables (males); and (D) DAPC for advertisement calls.

The variables that contributed most to the morphology DAPC were, in decreasing order, ITNA, THBL, and HW, while for the bioacoustic DAPC, they were the pulse duration of the central pulse, number of pulses, and pulse duration of the last pulse (Table 3). The multidimensional acoustic space occupied by *Atelopus manauensis* sp. nov. and that occupied by *A. hoogmoedi* of REBIO Uatumã, Pitinga River and Amapá in the first two axes of the PCA (Figure 2B) were distinct (MANOVA bioacoustic: Pillai trace = 1.00; df = 22; $p = 0.002$). The DAPC with advertisement call variables obtained 100% accuracy in attributing the calls of the analyzed individuals to their respective species (Figure 2D). The contribution of each acoustic variable and the explanation value of the first two PCs are provided in Table 3.

Table 3. Contribution of each variable to the first two axes of the PCAs in multidimensional morphological and acoustic spaces to explain the variation between *Atelopus manauensis* sp. nov. and *A. hoogmoedi* of REBIO Uatumã, Pitinga River and Amapá, Brazil. The total percentage of explanation of the variation between species and populations for each axis are provided at the bottom. Abbreviations: (SVL) snout-vent length, (SW) sacrum width, (HW) head width, (HL) head length, (EYDM) eye diameter, (EYNO) eye-to-nostril distance, (IOD) interorbital distance, (ITNA) internarial distance, (RDUL) length of flexed forearm, (HAND) hand length, (THBL) thumb length, (FOOT) foot length, (TL) tibia length. Bold numbers highlight five variables with highest contribution values for each Principal Component (PC).

Morphology	PC1	PC2	Call	PC1	PC2
SVL	0.3731	−0.0989	Call_dur	−0.2697	0.3854
HW	−0.3729	−0.0944	N_pulses	0.3058	0.3564
HL	−0.3726	−0.0171	Puls_dur_1	−0.3007	−0.0782
EYDM	−0.3512	−0.0744	Interpulse_1	−0.4138	−0.0042
ITNA	−0.3482	0.0172	Puls_dur_2	−0.2513	0.0300
IOD	−0.2834	−0.0745	Interpulse_2	−0.3626	−0.3038
EYNO	−0.0531	−0.0017	Puls_dur_3	0.0295	0.1371
SW	−0.3230	−0.1655	Interpulse_3	−0.2898	0.3132
FOOT	0.0910	−0.5572	Dur_3_first_puls	−0.4367	−0.0368
THBL	0.2361	−0.2680	Dur_3_last_puls	−0.2934	0.3254
HAND	0.0977	−0.5271	Domi_frequency	−0.1183	−0.4472
TL	−0.2663	−0.0910	bandwidth	0.0440	−0.4483
RDUL	−0.0798	−0.5233	–	–	–
Explanation (%)	48.7	17.5	Explanation (%)	38.7	25.3

3.3. Taxonomic Account

Atelopus manauensis sp. nov.

LSID: <http://zoobank.org/urn:lsid:zoobank.org:act:5D9F4EF7-74D2-4A48-AE50-F42282439C86>

Atelopus pulcher: Zimmerman [12]; Zimmerman and Bierregaard [13]; Zimmerman and Simberloff [14]; Gascon [15].

Atelopus spumarius sensu lato: Lötters et al. [4]; Rößler et al. [23].

Atelopus spumarius: Jorge et al. [22].

Atelopus sp.: Jorge et al. [29].

Examined material: Holotype. INPA-H 041378 (labeled “HOLOTYPE” in the field), adult male (Figure 3A,B) collected on 28th February 2019 by R.F. Jorge on the banks of a stream in the Tinga drainage system, Reserva Florestal Adolpho Ducke (RFAD; 2°55′4.8″ S, 59°53′52.8″ W), middle course on the west bank of Puraquequara River, tributary of the north bank of the Amazon River, municipality of Manaus, Amazonas, Brazil.

Paratopotypes. Eight adult specimens (six males and two females) collected at the same stream as the holotype. Five males collected on the same day as the holotype INPA-H 041365, INPA-H 041366, INPA-H 041367, INPA-H 041368, and INPA-H 041369 (field number ST1, ST2, ST3, ST4 and ST5, respectively) by R.F. Jorge; one female and one male INPA-H 041377 and INPA-H 041376 (field number 29RD and 31RD) collected on 20th February 2017 by R.F. Jorge; and a female INPA-H 011900 collected by D.J. Rodrigues on 29th April 2015. The female INPA-H 041377 (29RD) is designated the allotype (Figure 3C,D). Paratypes. Four adult specimens (all males), all from the state of Amazonas. Two males INPA-H 041370 and INPA-H 041371 (field number PTMP02-4 and PTMP02-5) collected by R.F. Jorge on 17th January 2019 on the margins of a stream in the middle course of the east bank of the Puraquequara River at CIGS/EB (2°54′52.4″ S, 59°49′26.4″ W) municipality of Manaus; a male INPA-H 041374 (field number 56DI) collected by R.F. Jorge and V.S. Pimentel on 1st December 2017 on the banks of a stream at the headwaters of the Cuieiras River, at Fazenda Dimona of PDBFF (2°19′43.39″ S, 60°4′41.46″ W), municipality of Manaus; a male INPA-H 041375 (field number 05ZF2)

collected by R.F. Jorge and S.S. Sales on 19th January 2017 on the banks of a stream in the middle course of the Cuieiras River near LBA/ZF2 scientific station (a tributary of the east bank of the Negro River— $2^{\circ}33'32.40''$ S, $60^{\circ}13'48.36''$ W), municipality of Manaus, Amazonas, Brazil.

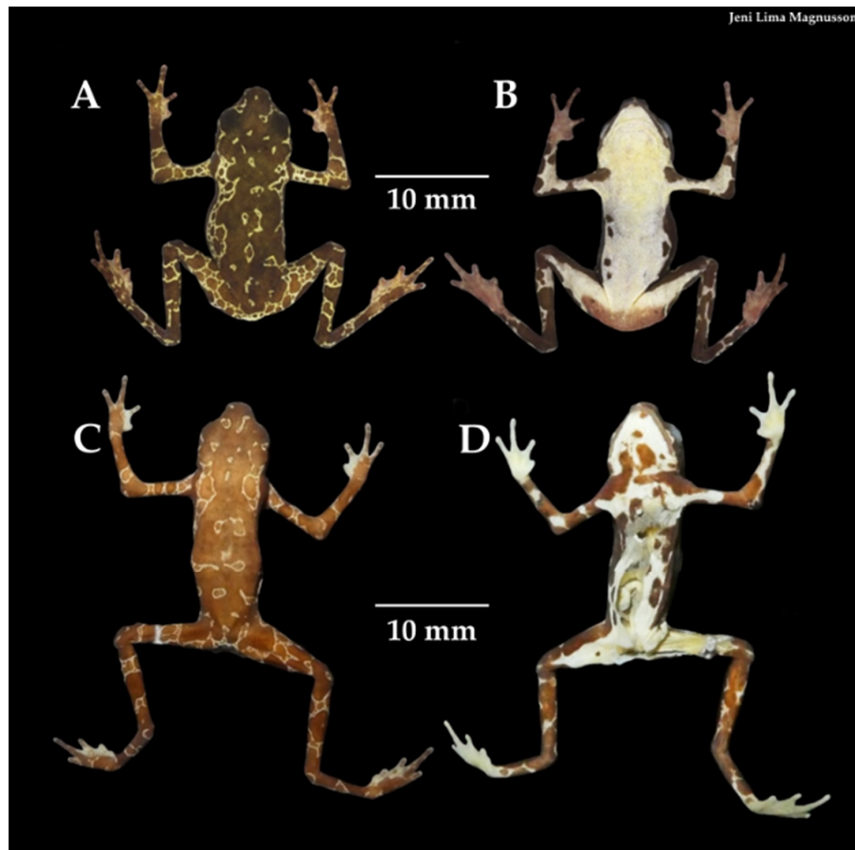


Figure 3. Specimens of *Atelopus manauensis* sp. nov. In preservative: (A) Dorsal and (B) Ventral view of the holotype (INPA-H 041378); (C) Dorsal and (D) Ventral view of the allotype (INPA-H 041377).

Referred specimens: Four adult specimens (all males), all from the state of Amazonas. Two males INPA-H 041289 and INPA-H 041290 (field number 02ZF2 and 03ZF2) collected by R.F. Jorge and S.S. Sales on 19th January 2017 on the banks of a stream in the middle course of the Cuieiras River near LBA/ZF2 scientific station ($2^{\circ}33'32.40''$ S, $60^{\circ}13'48.36''$ W), municipality of Manaus; two males INPA-H 041291 and INPA-H 041292 (field number 16FU and 17FU) collected by R.F. Jorge and S.S. Sales on 26th January 2017 on the margins of a stream in the headwaters of the Taruma-Açu River at Fazenda Experimental da Universidade Federal do Amazonas ($2^{\circ}38'31.20''$ S, $60^{\circ}5'45.6''$ W), municipality of Manaus. These specimens were used for genetic analyses. Two males INPA-H 041372 and INPA-H 041373 (field number: PTAP03-1 and 55DI) were used to record the type 2 and type 3 calls.

Etymology: The specific epithet *manauensis* refers to the location of the occurrence of the new species, municipality of Manaus, state of Amazonas, Brazil.

Diagnosis: A small species of *Atelopus*; adult males SVL 19.1–26.4 mm ($n = 11$), adult females 27.9–28.8 mm ($n = 2$); interdigital webbing covering all of Finger I and a phalange of Finger II and rudimentary between other fingers; absence of serrated fringe on the sides of Finger III (Figure 4A,B); first phalange of Toe I atrophied, completely hidden in skin of the foot (similar to a callus), with no visible phalanges (Figure 5A,B); subarticular tubercles absent from hand and foot; palmar tubercles round and visible and plantar tubercles oval and poorly defined; the interdigital webbing reaches half of the third phalange of Toe IV and half of the first phalange of Toe V; in life, dorsum light brown to reddish brown with light yellow or light green reticulation network (Figure 6A–D); ventral surface of

feet, hands, half of the posteroventral portion of thighs and half or all of the posteroventral portion of the cloacal region red (Figure 6E–H); throat, chest, and central portion of belly white or whitish cream without spots in males, spotted in females; advertisement call consisting of a single multipulsed note with a call duration of 689–840 ms consisting of 15–26 pulses, with a dominant frequency of 3088–3610 Hz and bandwidth of 633–915 Hz (Figure 7A,B). Morphometric measurements of the holotype and type series are shown in Table 4.

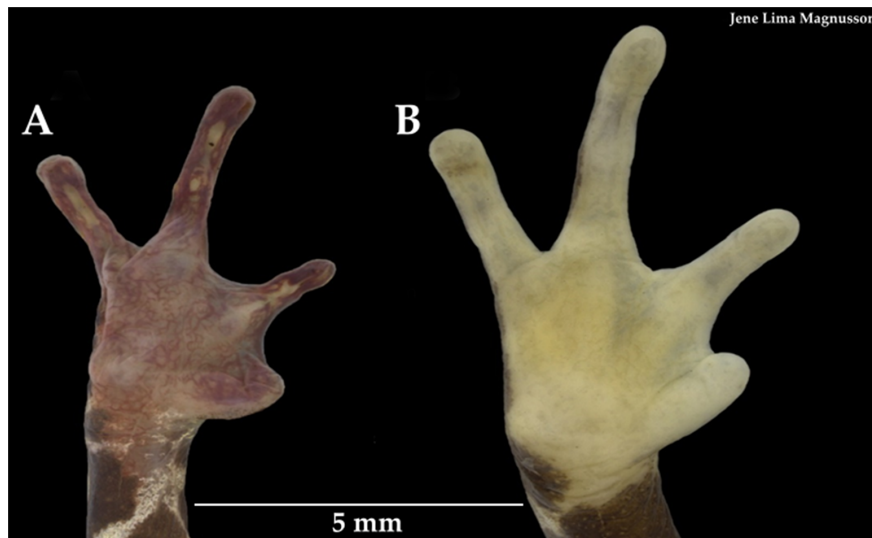


Figure 4. Ventral view of the hand of *Atelopus manauensis* sp. nov. In preservative: (A) holotype; (B) allotype.



Figure 5. Ventral view of the foot of *Atelopus manauensis* sp. nov. In preservative: (A) allotype; (B) holotype.

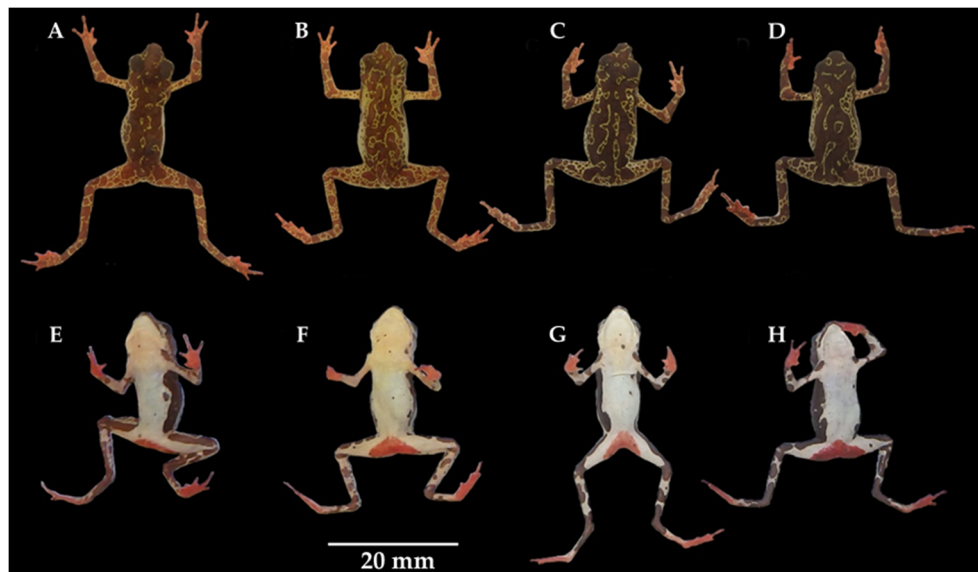


Figure 6. Coloration of the dorsal (upper images) and ventral (lower images) surfaces of the holotype and three males of type series of *Atelopus manauensis* sp. nov. recently euthanized: (A,E) INPA-H 041378, (B,F) INPA-H 041365, (C,G) INPA-H 041367, (D,H) INPA-H 041369. Note the reticulated network on the dorsum of each individual is unique; the variation in the distribution of the red spot on the thighs and posteroventral portion of the cloacal region; and the entirely white venter or white with a cream-colored gular region and head.

Table 4. Morphometric measurements of the holotype and type series of *Atelopus manauensis* sp. nov. Measurements of the holotype, followed by mean, standard deviation, and maximum–minimum in parentheses for 11 adult males. Measurements of the two females are presented in the last column. Abbreviations for morphometric measurements are described in Material and Methods. Abbreviation: (*n*) sample size.

Morphology	Holotype	Males (<i>n</i> = 11)	Females (<i>n</i> = 2)
SVL	21.2	21.8 ± 2.4 (19.1–26.4)	27.9–28.8
SW	6.5	7.1 ± 1.1 (5.7–8.6)	8.4–8.5
HW	7.3	7.4 ± 0.5 (6.5–8.3)	8.3–8.4
HL	7.5	7.8 ± 0.6 (6.8–8.7)	8.9–9.2
EYDM	2.4	2.4 ± 0.2 (2.1–2.7)	2.7–3.0
EYNO	2.4	2.5 ± 0.3 (2.1–3.0)	2.8–3.0
IOD	3.0	2.9 ± 0.3 (2.5–3.6)	3.1–3.1
ITNA	2.6	2.7 ± 0.2 (2.4–3.1)	3.1–3.1
RDUL	6.3	6.7 ± 0.8 (6.0–8.2)	8.7–10.0
HAND	5.0	5.2 ± 0.6 (4.9–6.6)	6.4–7.5
THBL	2.2	2.2 ± 0.2 (1.9–2.5)	2.5–3.0
FOOT	7.3	7.7 ± 1.1 (6.4–10.4)	10.4–10.9
TL	9.8	10.3 ± 0.8 (9.8–11.9)	12.1–12.9

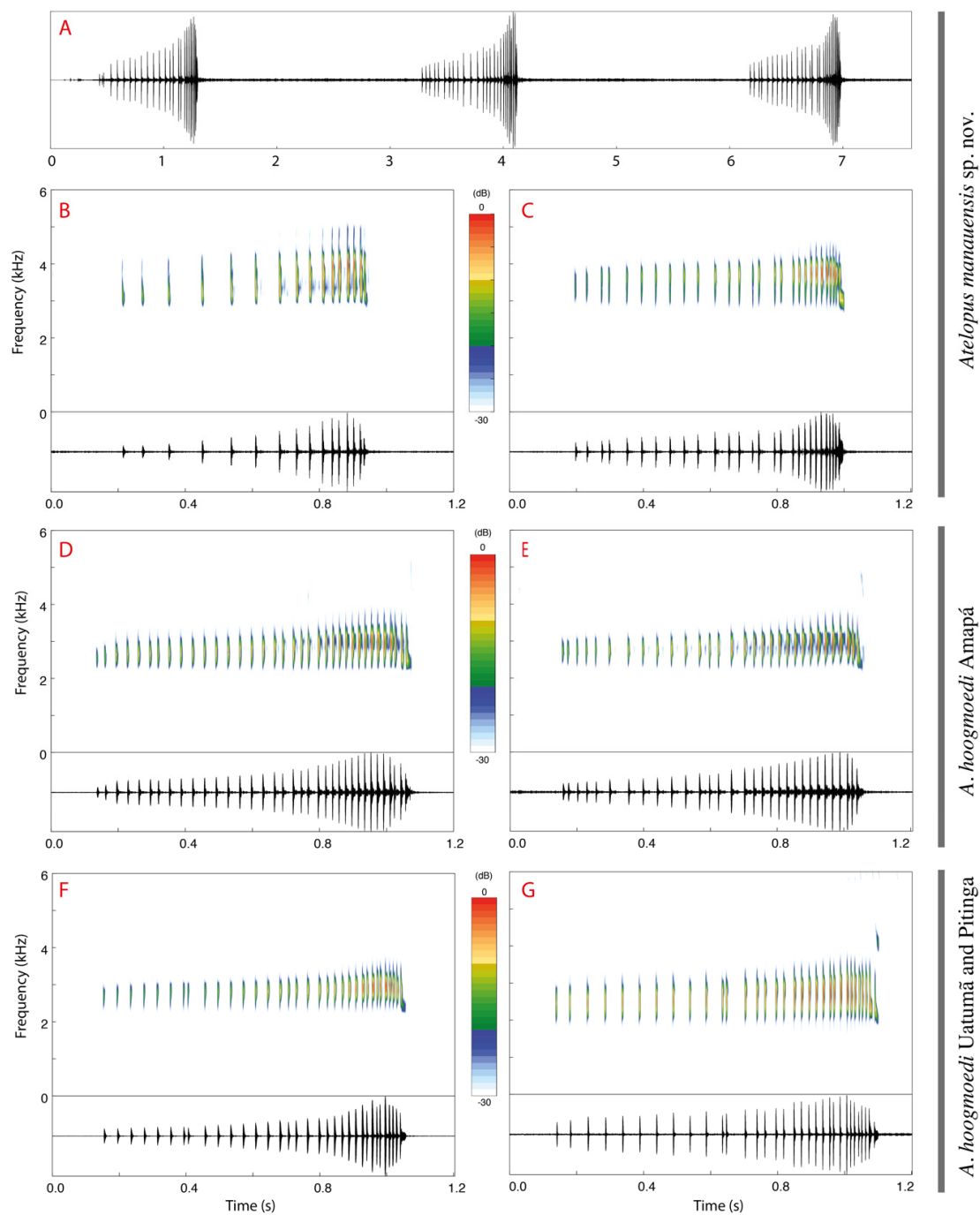


Figure 7. Advertisement call (type 1 call) of *Atelopus manauensis* sp. nov. and *A. hoogmoedi* of Amapá, of REBIO Uatumã, and of Pitinga River: (A) Oscillogram of three advertisement calls issued at regular intervals by an uncollected male of *A. manauensis* sp. nov. In Reserva Florestal Adolpho Ducke (RFAD), Manaus, Amazonas; (B) Spectrogram and oscillogram showing an advertisement call of the holotype and (C) another uncollected specimen of *A. manauensis* sp. nov. In RFAD; (D,E) Spectrograms and oscillograms of the advertisement calls of two specimens of *A. hoogmoedi* of Amapá described by Campos and Carvalho [50]; (F,G) Spectrograms and oscillograms of males of *A. hoogmoedi* of REBIO Uatumã (F) and Pitinga River (G), Amazonas. All localities are in Brazil.

3.3.1. Taxonomic Comparisons

Interspecific morphological and bioacoustic comparisons were made between *Atelopus manauensis* sp. nov. and the geographically and phylogenetically closest species. Characteristics of the compared species are presented in parentheses, unless specified.

Atelopus manauensis sp. nov. differs from the population of *A. hoogmoedi* of REBIO Uatumã and Pitinga River by lacking basal and subarticular tubercles on the hands [*A. hoogmoedi* basal tubercles present on hands (Figure 8A) and feet and two subarticular tubercles on Toe IV (Figure 8B)]. *Atelopus manauensis* sp. nov. is smaller than any individual from the populations of *A. hoogmoedi* of REBIO Uatumã and Pitinga River and is differentiated by a maximum SVL of 26.4 mm for adult males (minimum SVL for *A. hoogmoedi* of REBIO Uatumã and Pitinga River 32.9 mm); dorsum light brown to reddish brown with a light yellow or light green reticulation network (dorsum dark brown to black with yellow reticulation network); ventral surface of hand and feet red in life, cream or brown in preservative, without spots (cream-colored with dark brown spots in life and in preservative); venter all white or white with a cream-colored gular region and head (bright yellow); red spot restricted to half of the posteroventral portion of thighs and all or half of the posteroventral portion of cloacal region red (black spot limited to the cloacal region); advertisement call with a maximum duration of 840 ms (1071 ms) consisting of up to 26 pulses (37 pulses) and a dominant frequency of 3088–3610 Hz (2498–3058 Hz) (Figure 7F,G).

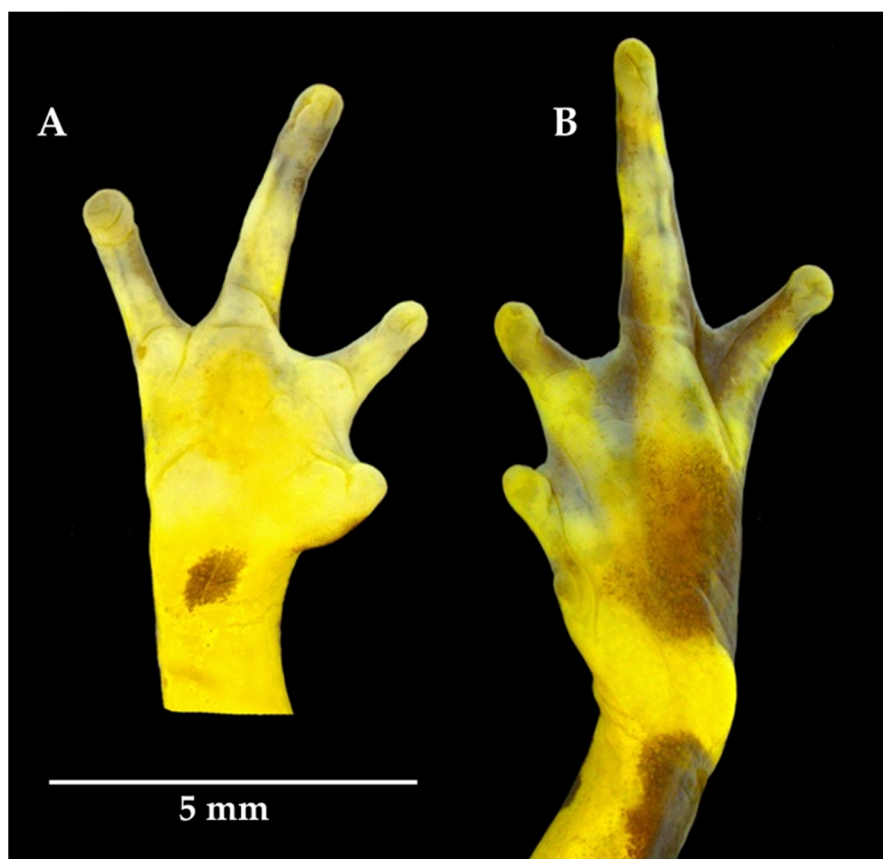


Figure 8. Ventral region of the hand (A) and foot (B) of *Atelopus hoogmoedi* from REBIO Uatumã (INPA-H 041293).

It differs from *Atelopus hoogmoedi* of Amapá (Brazil) by having a light brown to reddish brown dorsum with a light yellow or light green reticulation network (dark brown to black dorsum with yellow, orange, or pink reticulation network); venter all white or white with cream-colored gular region and head (bright yellow); red spot restricted to half of the posteroventral region of thighs

and half or all of the posteroventral portion of the cloacal region red (red spot covering the entire posteroventral portion of the cloacal region extending to near the knee and to the initial portion of the belly; photos C.E. Costa-Campos at AmphibiaWeb/*Atelopus hoogmoedi*); advertisement call duration of 689–840 ms (873–1710 ms; [50]) consisting of up to 26 pulses (35 pulses; [50]), dominant frequency of 3088–3610 Hz (2812–2838 Hz; [50]), and a bandwidth of 633–915 Hz (280–568 Hz; [50]) (Figure 7D,E).

Atelopus manauensis sp. nov. can be differentiated from *A. hoogmoedi* SS of French Guiana by a maximum SVL of 28.8 mm for females (minimum SVL 31.2 mm for females; [9]), tibia 47% of SVL (tibia varying from 43% to 45% of SVL among populations; [9]); dorsum light brown to reddish brown with light yellow or light green reticulation network (dorsum dark brown to black with yellow, orange, or pink reticulation network; [52,53]); and advertisement call duration of 689–840 ms (1190–1200 ms; [7]) consisting of 15–26 pulses (40–42 pulses; [7]).

It differs from *Atelopus hoogmoedi* of Guyana by having a light brown to reddish brown dorsum with a light yellow or light green reticulation network (dark brown to black dorsum with yellow dorsolateral bands and marks with black spots; [54]); venter all white or white with a cream-colored gular region and head (venter, throat, and head yellow, pink, or orange with irregular black marks; [54]); interdigital webbing covering all of Finger I and a phalange of Finger II and rudimentary between other fingers (fingers unwebbed; [54]); longer interdigital webbing between toes IV and V (toes moderately webbed; [54]); and Toe I reduced (Toe I and Toe II much reduced; [54]).

The new species is distinguished from *Atelopus franciscus* by the larger size of females, a minimum SVL of 27.9 mm (maximum SVL of 26.5 mm; [9]); venter all white or white with a cream-colored gular region and head (venter and thighs red; [9]); and advertisement call duration of 689–840 ms (1340–1640 ms; [7]) consisting of 15–26 pulses (31–39 pulses; [7]).

Atelopus manauensis sp. nov. differs from *A. flavescens* by the smaller size of females, maximum SVL of 28.8 mm (minimum SVL of 31.5 mm; [9]), and of males, maximum SVL of 26.4 mm (minimum SVL of 27 mm; [9]); shorter tibia in females of 12.1–12.9 mm (minimum tibia length 13.0–16.0 mm; [9]); dorsum light brown to reddish brown with light yellow or light green reticulation network (dorsum varies in light yellow tones with small brown or light red vermiculation; [9]); venter all white or white with cream-colored gular region and head in males and white with rounded spots in females (venter pink-salmon in females and pink in males; [8]); and advertisement call duration of 689–840 ms (1340–1820 ms; [7]) consisting of 15–26 pulses (45–58 pulses; [7]).

The new species is distinguished from *A. barbotini* by having on average a longer tibia in males (TL/SVL = 0.47 in *A. manauensis* sp. nov.; TL/SVL = 0.44–0.45 in *A. barbotini*; [7]); dorsum light brown to reddish brown with light yellow or light green reticulation network (dorsum black with scattered sinuous lines of opaque red colors; Lescure [7]); and advertisement call duration of 689–840 ms (1300–1680 ms; [7]) consisting of 15–26 pulses (41–53 pulses; [7]).

Atelopus manauensis sp. nov. differs from *A. spumarius* SS by possessing a longer tibia, 47% of SVL (43% of SVL; [7]), although female sizes are similar; absence of small warts behind the eyes (warts present; [55]); and advertisement call consisting of up to 26 pulses (37 pulses; [7]).

The new species differs from *A. pulcher* by its smaller size, with a maximum SVL for females of 28.8 mm (minimum SVL of 32.0 mm; [4]); longer interdigital webbing between toes IV and V (shorter interdigital webbing; [4]); no subarticular tubercles on the hand or foot (ill-defined subarticular tubercles on fingers II, III, and IV and toes II, III, IV, and V; [4]); venter all white or white with a cream-colored gular region and head in males and white with dark brown rounded spots in females (light red venter; [4]); red spot restricted to half of the posteroventral region of thighs and all or half of the posteroventral portion of the cloacal region red in males and females (red spot covering the entire ventral region of the thighs and all of the posteroventral portion of the cloacal region extending to the belly in males, and all of the venter, thighs, and head red with black spots in females; [4]); advertisement call duration 689–840 ms (1100–1300 ms; [4]) consisting of up to 26 pulses (47 pulses; [4]) and a dominant frequency of 3088–3610 Hz (2034–2824 Hz; [4]).

Finally, *Atelopus manauensis* sp. nov. differs from *A. seminiferus* by the smaller size of males and females: SVL 19.1–26.4 mm in males, 27.9–28.8 mm in females (SVL 33.8–35.2 mm in males, 40 mm in females; [56]); interdigital webbing covers two phalanges of Toe IV (interdigital webbing covers all of Toe IV; [57]); dorsum light brown to reddish brown with light yellow or light green reticulation network (dorsum uniformly dark brown or black with small yellow spots; [56,57]); venter all white or white with a cream-colored gular region and head (venter dark brown streaked with white and orange; [56]); and smooth sides (sides with tubercles; [57]).

Description of the Holotype: Body slender; neural spines not evident externally; head slightly longer than wide (HW/HL = 0.97); snout acuminate, with oval tip, dorsally concave; head length 30% of SVL; maxilla projected slightly over mandible; nostrils lateral and not visible from above; canthus rostralis concave between nostril and tip of snout and moderately straight between eye and nostril; loreal region concave; nostril closer to tip of snout than to eye; distance between nostrils and eye equal to eye diameter; distance between nostrils greater than eye diameter; tibia 51% of SVL; foot shorter than tibia (FOOT/TL = 0.74); relative sizes of fingers I < II < IV < III and toes I < II < III < V < IV, with first phalange of Toe I being atrophied and the toe hidden in the skin, similar to a callus; hand webbing rudimentary, present only between Finger I and Finger II; foot webbing, disregarding the Toe I in the form of a callus: II 0–2⁻, III 1^{1/2}–2^{-1/2}, IV 2^{-1/3}–1⁺ V; palmar tubercle well defined and rounded (Figure 4A) and plantar tubercle poorly defined and oval (Figure 5A); subarticular tubercles absent; thumb 44% of hand length (THBL/HAND = 0.44), covered by tiny brown keratinized spikes (nuptial pads); dorsal skin smooth; belly with tiny black keratinized dots; gular region smooth. In life, dorsal and lateral surfaces of the body with a lime-green reticulated network spread irregularly on a chocolate brown background. Ventral region white with a cream-colored gular region and head; lower part of limbs with brown bands on the sides. Red spot restricted to half of the posteroventral region of thighs and half of the posteroventral portion of the cloacal region (Figure 6E). In preservative, the dorsal surface was brown and the reticulated network was pale yellow. Ventral region colored similar as in life, only the red spots on the hands, feet, posteroventral region of thighs, and posteroventral portion of the cloacal region turn reddish brown (Figure 3A–D).

Vocalization: The advertisement call of *Atelopus manauensis* sp. nov. consists of a single multi-pulsed note issued at regular time intervals (Figure 7A). The mean call duration is 744 ± 84 ms (689–840 ms) and consists of 19 ± 6 pulses (15–26 pulses) (Figure 7B). Based on the inter-pulse interval, the song can be temporally divided into two portions, with the pulses being emitted more widely during the first two-thirds of the call than during the last third. The first pulse and central pulse have similar average pulse durations of 7 ± 1 ms (6–8 ms) and 6 ± 1 ms (5–7 ms), respectively. On the other hand, the last pulse is on average longer than all the other pulses in the song with a pulse duration of 14 ± 6 ms (8–19 ms), with downward frequency modulation (Figure 7C). The inter-pulse interval at the beginning and center of the song lasts for 54 ± 16 ms (36–67 ms) and 50 ± 13 ms (40–65 ms), respectively, while the inter-pulse interval between the last two pulses lasts for 7 ± 3 ms (4–9 ms). The total pulse period duration of the first three pulses [132 ± 40 ms (92–172 ms)] is approximately three times longer than the pulse period duration of the last three pulses of the call [43 ± 6 ms (38–49 ms)]. The call has a dominant frequency of 3334 ± 263 Hz (3088–3610 Hz) and a bandwidth of 743 ± 151 Hz (633–915 Hz). The temporal and spectral parameters of the advertisement call of *A. manauensis* sp. nov., *A. hoogmoedi* of REBIO Uatumã, of Pitinga River, and of Amapá are provided in Table 5.

Type 2 call (pure tone call) of *Atelopus manauensis* sp. nov. consists of an unpulsed short note (Figure 9A–C) with a call duration of 137–217 ms (174 ± 24 ms, *n* = 15) and shows an upward frequency modulation from the onset until the central portion of the call, and a downward frequency modulation from the central until the final portion (Figure 9C). Calls are commonly emitted singly (*n* = 12) and in a series of two (*n* = 5) or three (*n* = 2) calls. The average inter-call interval within a series is 499 ± 39 ms (434–541 ms, *n* = 6) while the inter-call interval between single calls is 2793 ± 1441 ms (963–4766 ms, *n* = 10). Type 2 calls have a dominant frequency of 2928–3143 Hz (3059 ± 75 Hz, *n* = 15), a low frequency

of 2630–2692 Hz (2676 ± 19 Hz, $n = 15$), and a high frequency of 3052–3252 Hz (3171 ± 72 Hz, $n = 15$). The average bandwidth is 303 ± 55 Hz (215–388 Hz, $n = 15$).

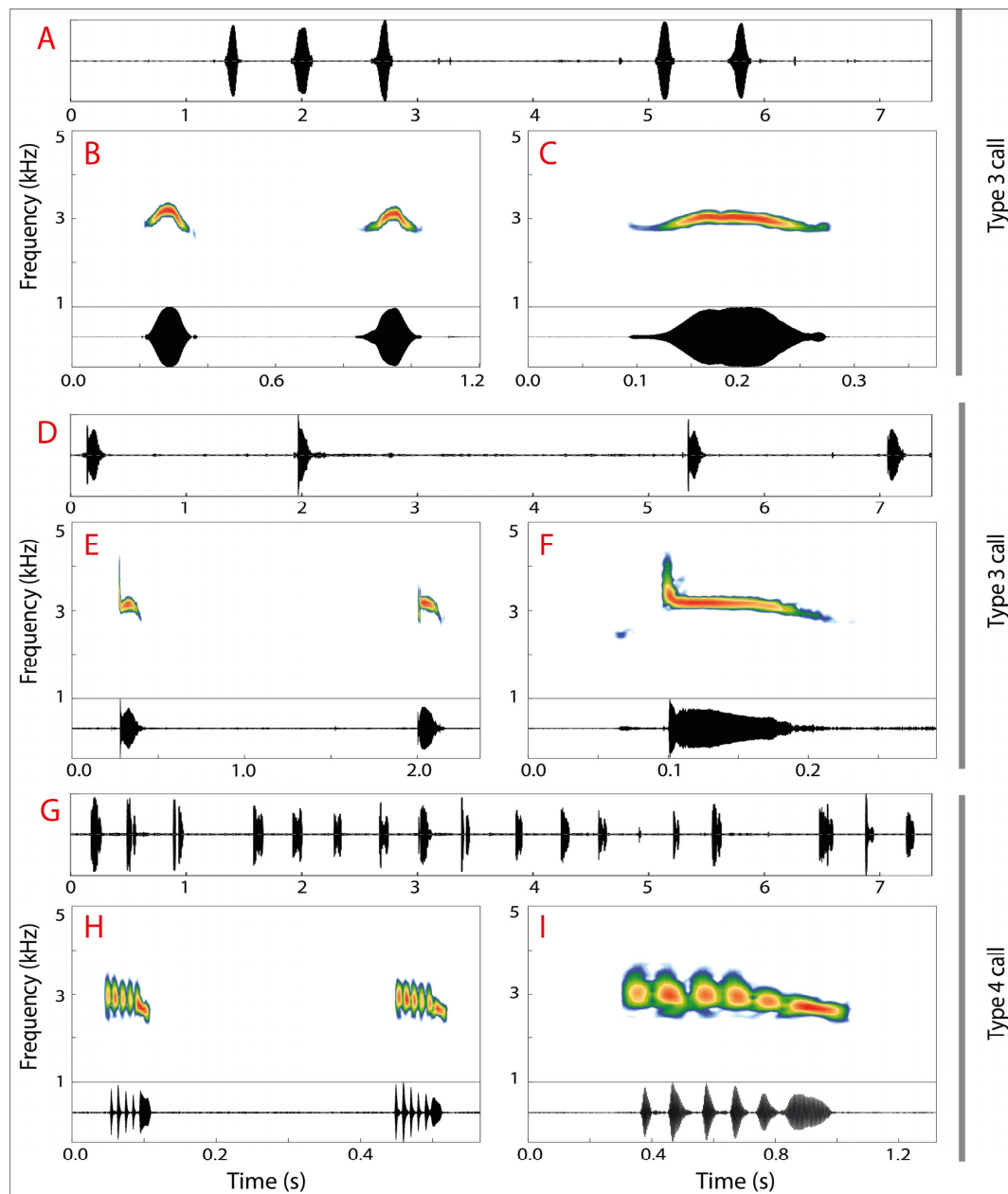


Figure 9. Type 2, type 3, and type 4 calls of *Atelopus manauensis* sp. nov.: (A) Oscillogram showing type 2 calls emitted within approximately 7.5 s; (B) Spectrogram and oscillogram showing two calls of the type 2 call; (C) A detailed view of one type 2 call; (D) Oscillogram showing type 3 calls emitted within approximately 7.5 s; (E) Spectrogram and oscillogram depicting two calls of the type 3 call; (F) A detailed view of one call of type 3 call; (G) Oscillogram showing type 4 calls emitted within approximately 7.5 s.; (H) Spectrogram and oscillogram showing two calls of the type 4 call; (I) A detailed view of one call of type 4 call. Note the difference of frequency modulation among type 2–4 calls: (A–C) recorded at the training area of the Centro de Instruções de Guerra na Selva do Exército Brasileiro (CIGS/EB; SVL = 20.3 mm; air temperature = 26.5 °C; INPA-H 041372); (D–F) recorded at Fazenda Dimona (SVL = 20.9 mm; air temperature = 26.5 °C; INPA-H 041373); and (G–I) recorded from the holotype at Reserva Florestal Adolpho Ducke (SVL = 21.2 mm; INPA-H 041378).

The type 3 call (short pure tone call) of *Atelopus manauensis* sp. nov. is characterized by an unpulsed short note (Figure 9D–F) with a call duration of 57–165 ms (129 ± 31 ms, $n = 15$). The frequency modulation of type 3 calls rapidly descends during the first fifth portion, after which it declines slowly until the end of the call (Figure 9F). Type 3 calls are irregularly emitted and have an inter-call interval of 493–9264 ms (2295 ± 2812 ms, $n = 10$). Type 3 calls are characterized by having a dominant frequency of 2907–3100 Hz (3020 ± 66 Hz, $n = 15$), a low frequency of 2722–2907 Hz (2800 ± 69 Hz, $n = 15$), and a high frequency of 3031–3304 Hz (3174 ± 78 Hz, $n = 15$). Bandwidth ranges from 129 to 366 Hz (182 ± 75 Hz, $n = 15$).

Different from type 2 and type 3 calls, the type 4 call (short pulsed call) of *Atelopus manauensis* sp. nov. (Figure 9G–I) consists of a short-pulsed note with a downward frequency modulation. Type 4 calls have a call duration of 53–93 ms (77 ± 10 ms, $n = 15$), 4–8 pulses (6 ± 1 pulses, $n = 15$), and inter-call interval of 285–1394 ms (437 ± 285 ms, $n = 15$). The last pulse is always longer than the others (Figure 9I); the first pulse has a pulse duration of 4–10 ms (5 ± 2 ms, $n = 15$), while the last pulse has a pulse duration of 13–25 ms (17 ± 4 ms, $n = 15$). Type 4 calls are emitted with a dominant frequency of 2585–2842 Hz (2721 ± 80 Hz, $n = 15$), a low frequency of 2358–2482 Hz (2421 ± 46 Hz, $n = 15$), and a high frequency of 3181–3421 Hz (3319 ± 77 Hz, $n = 15$). The bandwidth of the type 4 call is 409–538 Hz (476 ± 42 Hz, $n = 15$).

Table 5. Advertisement call parameters for *Atelopus manauensis* sp. nov., two Brazilian populations of *A. hoogmoedi* of the Guiana Shield that are the geographically closest to the area of occurrence of *A. manauensis* sp. nov. (REBIO Uatumã and Pitinga River, Amazonas), and a Brazilian population of *A. hoogmoedi* (Pedra Branca do Amapari, Amapá) that is the geographically closest to the occurrence area of *A. hoogmoedi* SS in French Guiana. Abbreviations of the acoustic parameters are defined in Material and Methods. Abbreviation: (n) sample size.

Acoustic Traits	<i>Atelopus manauensis</i> sp. nov. ($n = 3$)	<i>Atelopus hoogmoedi</i> Amapá ($n = 4$)	<i>Atelopus hoogmoedi</i> REBIO Uatumã and Pitinga River ($n = 7$)
Call_dur (ms)	744 ± 84 (689–840)	1164 ± 387 (873–1710)	858 ± 89 (733–1071)
N_pulses	19 ± 6 (15–26)	29 ± 5 (23–35)	29 ± 5 (21–37)
Puls_dur_1 (ms)	7 ± 1 (6–8)	7 ± 1 (6–9)	6 ± 2 (3–11)
Puls_dur_2 (ms)	6 ± 1 (5–7)	8 ± 1 (7–9)	7 ± 2 (4–12)
Puls_dur_3 (ms)	14 ± 6 (8–19)	14 ± 5 (10–22)	15 ± 4 (7–27)
Interpulse_1 (ms)	54 ± 16 (36–67)	48 ± 33 (15–78)	35 ± 9 (19–50)
Interpulse_2 (ms)	50 ± 13 (40–65)	37 ± 11 (23–47)	32 ± 9 (15–54)
Interpulse_3 (ms)	7 ± 3 (4–9)	10 ± 3 (7–13)	7 ± 2 (4–13)
Dur_3_first_puls (ms)	132 ± 40 (92–172)	120 ± 66 (61–178)	88 ± 16 (56–116)
Dur_3_last_puls (ms)	43 ± 6 (38–49)	54 ± 5 (49–59)	42 ± 7 (30–59)
Domi_frequency (Hz)	3334 ± 263 (3088–3610)	2825 ± 13 (2812–2838)	2741 ± 161 (2498–3058)
Bandwidth (Hz)	743 ± 151 (633–915)	442 ± 127 (280–568)	555 ± 94 (388–754)

3.3.2. Variability

Male INPA-H 041376 is 4.6 mm larger than average in relation to total body size (SVL = 26.4 mm); its nuptial callus is a little evident; and the spot on the lower ventral portion was light red and changed to cream-color in preservative, similar to the two females. In general, the other males are similar to the holotype: nine individuals have an all-white venter and two have a white belly and cream-colored gular region and head; all males have the hands, feet, and half of the posteroventral portion of the thighs colored red, while seven individuals had this spot covering the entire posteroventral portion of the cloacal region and four had it covering only half of this region. There is a variation in the light yellow or light green reticulation network on a background of different shades of brown on the dorsum; each individual has its own unique pattern, as if it were an individual fingerprint (Figure 6A–D). In preservative, red spots on the hands, feet, posteroventral region of the thighs, and posteroventral portion of the cloacal region turn reddish brown in males and cream in females.

Some males ($n = 3$) have an eye diameter greater than the distance from the eye to the nostril, but in general, it is the same or slightly smaller ($ED/EYNO = 0.87\text{--}0.96$). Females are considerably larger than males. Nuptial pads and vocal slits are present in males. Females have a wrinkled gular region, whereas this region is smooth and white or cream-colored in males; and females have a venter without spicules and with brown spots, unlike males, who possess a venter with spicules and is rarely spotted.

3.4. Distribution and Natural History

The distribution of *Atelopus manauensis* sp. nov. is limited to the interfluvium between the Negro and the Uatumã Rivers in the Alter do Chão formation (absent from the Guiana Shield and the Trombetas Group), within a narrow altitudinal range (61–125 m a.s.l.), and restricted to the micro-interfluvium between the east bank of the Cuieiras River and the west bank of the Urubu River (Figure 10), which are tributaries of the Negro and the Amazon Rivers, respectively [29]. In this region, *Atelopus manauensis* sp. nov. inhabits the banks of small streams in dense ombrophilous forests of lowlands not flooded by large rivers [22]. Most of the individuals can be found within a four-meter strip along the banks of these streams, but some can be seen up to 10 m from the bank.

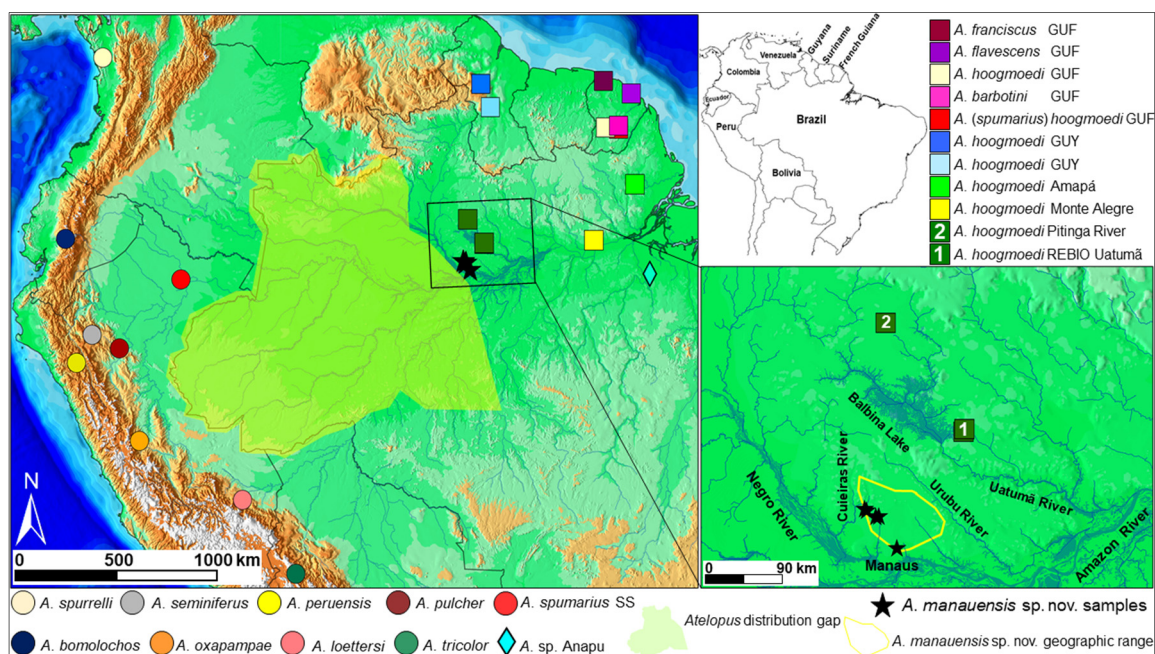


Figure 10. Distribution map for species of *Atelopus* used in the calculations of genetic distances and phylogenetic inferences, with colored circles representing species distributed to the west of Amazonia, on eastern and western versants of the Andes and Andean highlands; the squares representing species distributed in the Guiana Shield, and the green diamond a candidate species distributed on the south bank of the Amazon River, municipality of Anapu, state of Pará, Brazil [30]. The shaded polygon represents the portion of the Amazonian plain where there has been no record yet of a species of *Atelopus*, indicating a distribution gap for the genus (adapted from Lötters et al. [32]). Insert in the lower right shows the limits of the geographical distribution of *Atelopus manauensis* sp. nov. as represented by the yellow polygon between the Urubu and the Cuieiras Rivers; black stars refer to the type of locality where the holotype, allotype, and paratypes (RFAD) and individuals used for the genetics analyses (FEUFAM and LBA/ZF2) of *Atelopus manauensis* sp. nov. were collected; numbered dark green squares represent populations of *Atelopus hoogmoedi* of REBIO Uatumã (1) and of Pitinga River (2), on the east bank of the Uatumã River, the geographically closest *Atelopus* populations to the new species. Two specimens of *A. hoogmoedi* from REBIO Uatumã were used for genetic analyses.

Reproductive activity likely occurs from the beginning of the rainy season in the region (November) until March. On cloudy days, males are less exposed, but when the sun's rays start to warm the forest floor, they look for sun spots and then start vocalizing at any time of the day. They vocalize and forage on roots, accumulated leaves, fallen tree trunks on the bank, or over streams, at the base of herbaceous plants or stemless palms and even climbing inclined trunks of small shrubs, usually during the day (07:00–10:00 and 15:00–17:00) with milder temperatures. During the night, it is common to see individuals resting more than 1 m high on leaves of herbaceous plants on the banks of streams. The variety of calls emitted by *Atelopus* species is related to intra- and interspecific communication, but the functions of these calls have been poorly studied [25]. Type 1 calls (advertisement call) are used, as with other anurans, in the context of attracting reproductive mates, whereas type 2 calls (pure tone call) are likely used in an aggressive context such as in intraspecific interaction. Type 3 calls (pure tone short calls) are mostly of unknown function but may have a function between aggressive and release calls in the context of close-range interactions between conspecifics males with physical contact. Type 4 calls (pulsed short calls) clearly represent release calls [25]. Information on eggs and tadpoles of *Atelopus manauensis* sp. nov. can be found in Gascon [23]. There is no information on the eggs or tadpoles of closely related species. Lötters et al. [4] recorded a female and a male of *A. pulcher* in auxiliary amplexus, and after 2–3 weeks, c.a. 600 unpigmented eggs were deposited in the water arranged in a single chain or string-like fashion [4].

4. Discussion

We used integrative taxonomy to describe a new species of *Atelopus* with a distribution mostly restricted to the municipality of Manaus, Brazil, including two of the most intensively studied forests in Amazonia (RFAD and PDBFF). *Atelopus manauensis* is the first species of the genus to be formally described for Brazil. Another *Atelopus* species known from Brazil is *A. hoogmoedi*, which is distributed in the states of Amapá and Pará, but its type locality is in French Guiana. As is the case for several of its congeners, *A. manauensis* has a small area of occurrence (approximately 4500 km²) and is critically threatened by urban expansion of the largest city in Brazilian Amazonia—Manaus [29].

Atelopus manauensis has ecological, morphological, and genetic similarities with species of *Atelopus* distributed c.a. 1500 km straight-line distance from Manaus, so the threats for extinctions identified for those species are likely to be relevant to *A. manauensis* as well. For example, the pathogenic fungus *Batrachochytrium dendrobatidis* (BD) has caused the extinction of numerous species of *Atelopus* [58], and the deforestation that is reaching Central Amazonia via highway BR-174 (Manaus-Boa Vista) could very likely facilitate its invasion into the area of occurrence of the new species. Although BD has not been found in populations of *A. manauensis* [29], an ongoing study indicates that BD infection would cause high mortality rates for the new species [59]. In addition to the threat of BD, the urban expansion of Manaus and other nearby cities is fragmenting areas with aggregations of individuals of *A. manauensis*. The conservation status of *A. manauensis* was recently assessed as “Endangered” according to the criteria established by the International Union for Conservation of Nature, which are as follows: reduction of area of occurrence, area of occupation, and habitat quality; projected decline in the area of occurrence, area of occupation, and habitat quality; and area of occurrence less than 5000 km² (approximately 4500 km²; [29]).

The interspecific phylogenetic relationships reconstructed in this study are in agreement with those of previous studies with regard to the monophyly of the clade distributed in the Guiana Shield [3,11,29]. Our phylogenetic analysis and that of Jorge et al. [29] were the first to show that *Atelopus manauensis* is sister to the clade containing the species of *Atelopus* distributed in the Guiana Shield. The genetic distances inferred between these species and *A. manauensis* vary between 2.3–2.8% (K2P) and 2.3–2.7% (p-distance) and are close to the average observed for anurans using 16S markers [60,61]. On the other hand, the genetic distances among species distributed in the Guiana Shield (*A. franciscus*, *A. flavescens*, *A. barbotini*, and *A. hoogmoedi*) were very low, ranging between 0.2–1.5% (p-distance) and 0.2–1.3% (K2P). This finding, together with the lack of bioacoustic divergences among the species of the Guiana

Shield as reported by Costa-Campos and Carvalho [50], corroborate the suggestion of Kok [5] and Lötters et al. [3] that these species actually represent a single polymorphic species and that they should be synonymized.

In the past, *Atelopus manauensis* has also been mistaken for *A. pulcher* and *A. spumarius*. However, *A. spumarius* SS from Peru is the sister of *A. seminiferus*, which together form a clade positioned as the sister of the clade containing the species of the Guiana Shield and *A. manauensis*. *Atelopus pulcher* was finally allocated as a basal species to the aforementioned clade. The genetic distances between the new species and *A. pulcher* and *A. spumarius* SS were moderately high at 3.9% and 4.0% (p-distance) and 4.0% and 3.9% (K2P), respectively. In addition, species distributed in western Amazonia (i.e., *A. pulcher* and *A. spumarius* SS) and in the Guiana Shield (i.e., *A. hoogmoedi*) are geographically and ecologically isolated from each other and from *A. manauensis* due to historical biogeographical barriers, such as large rivers and mountains, and ecological barriers, such extensive areas of open, floodable forests that contain acidic water drainage systems to which the tadpole of *A. manauensis* is probably sensitive [22], preventing any possible contact of the new species with the genetically closest species of the *A. hoogmoedi* complex or with the morphologically most similar species of the *A. spumarius* complex.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1424-2818/12/8/310/s1>, Table S1: Morphologic measurements of *Atelopus manauensis* sp. nov. and *Atelopus hoogmoedi* of REBIO Uatumã (REUA) and Pitinga River. Abbreviations: (INPA-H) Herpetological section of the zoological collection of Instituto Nacional de Pesquisas da Amazônia, (SEX) sexes, (M) male, (F) female, (RFAD) Reserva Florestal Adolpho Ducke, (CIGS/EB) Centro de Instruções de Guerra na Selva do Exército Brasileiro, (FD) Fazenda Dimona, (REUA) Reserva Biológica do Uatumã, (Pitinga) Pitinga River, (SVL) snout-vent length, (SW) sacrum width, (HW) head width, (HL) head length, (EYDM) eye diameter, (EYNO) eye to nostril distance, (IOD) interorbital distance, (ITNA) internarial distance, (RDUL) length of flexed forearm, (HAND) hand length, (THBL) thumb length, (FOOT) foot length, (TL) tibia length.

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