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New acoustic and molecular data shed light on the poorly known Amazonian frog *Adenomera simonstuarti* (Leptodactylidae): implications for distribution and conservation

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Abstract. *Adenomera simonstuarti* is a poorly known species complex inhabiting western Amazonia. Here we reevaluate the species diversity within this complex based on previously documented and newly acquired molecular and phenotypic data. We also redescribe the calling pattern of the nominal species based on the original recording (Peru) and a new recording (Brazil). Our results indicate eight geographically structured genetic lineages and the nominal species with a multi-note call pattern. This is the first association of calls and DNA sequence from a voucher specimen, thereby enabling the assignment of *A. simonstuarti* to one specific lineage within the complex. The multi-note call was not previously reported and represents an important additional diagnostic character within *Adenomera*. The geographic distribution of *A. simonstuarti* is substantially narrowed down to the southwestern portion of the entire geographic range recognized for the complex. The lack of taxonomic resolution in the complex is a major conservation concern by preventing us from evaluating the potential threats and extinction risks of each of the lineages. Future research should follow the protocol of combining calls and DNA sequences associated with voucher specimens as a means to address the taxonomic status of genetic lineages within the *A. simonstuarti* complex.

Keywords. Acoustic diagnosis, *Adenomera andreae* clade, Amazon Basin, cryptic diversity, western Amazonia.

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Introduction

Morphologically indistinguishable species (cryptic species) have challenged taxonomists and systematists across several taxonomic groups (Bickford *et al.* 2007). This evolutionary trend occurs when speciation generates distinction in one or more biological traits, for instance, molecular, acoustic or ecological characters, but there is no or very subtle morphological differentiation (Cherty *et al.* 1978). High levels of cryptic species have been documented for many Neotropical frog groups, especially over the past decades (e.g., Fouquet *et al.* 2007, 2016; Padial & De la Riva 2009; Simões *et al.* 2010; Jungfer *et al.* 2013). Understanding and conserving biodiversity levels, partly hidden in complexes of cryptic species, in such a megadiverse region strongly depends on the continued investigation of multiple sources of information to be compared with morphological variation (Padial *et al.* 2010).

A striking example of a Neotropical frog group with predominance of cryptic species is the genus *Adenomera* Steindachner, 1867 (e.g., Angulo & Reichle 2008; Carvalho & Giaretta 2013a; Carvalho *et al.* 2019a, 2019b). These small-sized leptodactylids (snout–vent length up to 34 mm; Kok *et al.* 2007) are widely distributed in South America east of the Andes, currently comprising 21 described species (Carvalho *et al.* 2019b). A phylogenetic study of the genus based on a comprehensive geographic sampling revealed many putative new species, reported as candidate species (Fouquet *et al.* 2014). Some of these nominal and candidate species of *Adenomera* exhibit marked genetic divergence among populations and some of them are also known to have distinct call patterns, suggesting extensive cryptic diversity within the genus (Fouquet *et al.* 2014; Carvalho *et al.* 2019c, 2019d). Fouquet *et al.* (2014) classified the species diversity of *Adenomera* into eight major clades, one of them being the Amazonian endemic *A. andreae* clade. This clade contains three described species, *A. andreae* (Müller, 1923), *A. chicomendesi* Carvalho, Angulo, Kokubum, Barrera, Souza, Haddad & Giaretta, 2019, and *A. simonstuarti* (Angulo & Icochea, 2010), plus three candidate species reported as *Adenomera* sp. C, *Adenomera* sp. D, and *Adenomera* sp. T (Fouquet *et al.* 2014; Carvalho *et al.* 2019b).

Adenomera simonstuarti was described from Camisea (Province of La Convención, District of Echarate, Region of Cusco), a region of lowland forest in southwestern Peruvian Amazonia, based on a series of four specimens, and two referred specimens from Pando, in northern Bolivia (Angulo & Icochea 2010). A few years later, Fouquet *et al.* (2014) showed, based on molecular evidence, that the species could actually be more widely distributed throughout lowland forests of western Amazonia and Andean montane forests, even though those authors also mentioned in their taxonomic considerations (see Fouquet *et al.* 2014: appendix S2a) that the deep genetic subdivisions within *A. simonstuarti* could suggest the existence of more than one species under the nominal species. A major limitation that holds back researchers to advance in the taxonomic resolution of this species complex is the lack of associated phenotypic and molecular data for the nominal species. Specimens have to date been identified as *A. simonstuarti* based on morphological and geographical data. Moreover, the only call description available is from the holotype in the original description, from which tissue samples were not collected (and neither were they collected from paratypes). It is important to highlight that species identification within *Adenomera* should be treated with caution in such cases which acoustic and/or molecular data

are not available in a frog genus having a notably high number of undescribed and/or cryptic species (Carvalho & Giaretta 2013a; Fouquet *et al.* 2014; Carvalho *et al.* 2019b; Cassini *et al.* 2020).

Here, we reevaluate the species diversity within the *Adenomera simonstuarti* complex by combining novel acoustic and molecular data, enabling for the first time that the nominal species could be linked to a specific genetic lineage within the complex. We also reinterpret the calling pattern of *A. simonstuarti* based on the original recording from the type locality in Peru and a new recording from the Brazilian Amazonia. Lastly, we discuss on the implications for distribution and conservation status of the genetic lineages subsumed under *A. simonstuarti* across their entire geographic range, resulting from the circumscription of the nominal species to one specific lineage.

Material and methods

Taxon sampling and identification

We collected five individuals of *Adenomera* that we associated with *A. simonstuarti* based on morphology, color patterns, and/or call characteristics, as follows: (1) tips of toes II–IV developed into discs (character state D; *sensu* Carvalho *et al.* 2019d); (2) presence of nearly solid, dark-colored stripe on the underside of forearm (*sensu* Angulo & Icochea 2010); (3) advertisement call consisting of short-lasting (< 80 ms), pulsed notes with the dominant frequency generally coinciding with the fundamental harmonic (Angulo & Icochea 2010). Specimens were euthanized using a topical solution of 10% lidocaine, fixed with 10% formalin and preserved in 70% ethanol. Voucher specimens were deposited in the Collection of Amphibians and Reptiles of the Instituto Nacional de Pesquisas da Amazônia (INPA-H) in Manaus (Amazonas, Brazil). These newly collected specimens were obtained from two localities along the Juruá River drainage in southwestern Brazilian Amazonia: (1) Unidade de Gestão Integrada (UGAI) Rio Acurauá, in the upper Juruá River, Tarauacá, Acre (7.792320º S, 71.013968º W; in all cases datum = WGS84; on 12 Jan. 2019), accession number: INPA-H 40967; and (2) Comunidade Cumaru, Reserva Extrativista (RESEX) do Baixo Juruá, on the east bank of the lower Juruá River, Juruá, Amazonas (3.756961–3.825022º S, 66.077231–66.083067º W; on 17–23 Jul. 2018), accession numbers: INPA-H 39792, 39796, 39813–39814. Specimens morphologically examined in this study are listed in Results. Institutional acronyms follow Sabaj (2019).

Molecular analysis

Genomic DNA was extracted from muscle and liver tissues preserved in 100% ethanol from three specimens (INPA-H 39792, 39814 and 40967) using standard protocols of a commercial kit (Wizard®, Promega, Madison, USA). We sequenced a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (*COI*; 657 bp), a widely used molecular marker for this frog group (Fouquet *et al.* 2014; Lyra *et al.* 2017). The primers CHmL4 (5-TYTCWACWAAYCAYAAAGAYATCGG-3) and CHmR4 (5-ACYTCRGGRTGRCCRAARAATCA-3) (Che *et al.* 2012) were used to perform amplification of the selected fragment via Polymerase Chain Reaction (PCR). The amplification reactions used a mix with 1.2 μL of 10 mM dNTPs, 3 μL of a 5X amplification buffer, 1.2 μL of 25 mM $MgCl_2$, 1.0 μL of DNA in a concentration of 50 ng/μL, 0.5 μL of each primer at 10 mM, 0.15 μL of Taq DNA polymerase and 7.45 μL of ddH_2O. Reaction conditions began with an initial heating step at 94°C for 60 s, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 50 s and extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min. PCR products were purified with polyethylene glycol 8000, submitted to a sequencing reaction following BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Waltham, USA) protocols, and sequenced with an ABI 3130 XL automated sequencer (Applied Biosystems, Waltham, USA). These laboratory procedures were conducted in the Thematic Laboratory of Molecular Biology of INPA. We used Geneious 7 (Kearse *et al.* 2012) for sequence editing.

Taxon sampling for the molecular analysis included each candidate new species and operational taxonomic units of nominal species from the eight major clades delimited by Fouquet *et al.* (2014), including all those identified therein as *A. simonstuarti*, as well as sequences from related genera to be used as outgroups (*Lithodytes* Fitzinger, 1843, *Hydrolaetare* Gallardo, 1963 and *Leptodactylus* Fitzinger, 1826). Besides the mtDNA gene *COI*, we downloaded additional sequences from another mtDNA gene (cytochrome b – *cytb*; 607 bp) and two nuclear genes (proopiomelanocortin A – *POMC* and recombination activating gene 1 – *RAG1*; 547 and 1422 bp, respectively) from the online repository GenBank (Clark *et al.* 2016). Accession numbers and other information of sequences included in the molecular analysis are provided in the Supplementary File SM.01. New sequences produced for this study were deposited in GenBank under the accession numbers MT472180–MT472182. We used MAFFT 7 online (Katoh & Standley 2013) to independently align the sequences of each gene under the G-INS-i strategy, more suited for protein coding genes (Katoh & Standley 2013). All genes were posteriorly concatenated, leading to a final aligned database containing 105 sequences and 3233 bp. We divided the dataset considering first, second, and third positions of the codon of each gene, and conducted the search for the best-fitting substitution models and partition schemes with PartitionFinder 2.1.1 (Lanfear *et al.* 2017) under the corrected Akaike information criterion (AICc; Hurvich & Tsai 1989). Best scheme indicated five partitions, with the general time-reversible model (GTR; Tavaré 1986) with a gamma distribution of rates across sites (+G) as the best-fitting nucleotide substitution model for the first and third position of *cytb*, and third position of *POMC* and *RAG1*, whereas the GTR+G with a proportion of invariant sites (+I) was indicated as the best-fitting nucleotide substitution model to remaining codon positions (all from *COI*, first and second of *POMC* and *RAG1*, and second and third of *cytb*).

We reconstructed phylogenetic trees using both Bayesian inference (BI) and maximum likelihood (ML) optimality criteria. For the Bayesian analysis we used two independent runs of 5.0×10^7 generations, starting with random trees and four Markov chains (one cold), sampled every 10000 generations in MrBayes 3.2.6 (Ronquist *et al.* 2012), discarding 25% of generations and trees as burn-in. We used the standard deviation of split frequencies (< 0.01) and estimated sample size (> 200) to assess run convergence with Tracer 1.7 (Rambaut *et al.* 2018). We conducted maximum likelihood analysis using RaxML 8.2.10 (Stamatakis 2014), searching the most likely tree 100 times and with 1000 non-parametric bootstrap replicates to assess support.

We used Mega 7 (Kumar *et al.* 2016) to compute the uncorrected and corrected (Jukes-Cantor model) pairwise genetic distances of the *COI* fragment among specimens of *Adenomera simonstuarti* – missing data removed using pairwise deletion option. Both uncorrected and corrected genetic distances were considered for our study in order to increase accuracy and comparability of results. With the Approximate Barcode Gap Discovery method (ABGD, Puillandre *et al.* 2012), we conducted an analysis to delimit lineages of *A. simonstuarti* based on comparisons of uncorrected intra- versus interspecific genetic distances in *COI*. The analysis were run at the ABGD online server using a prior of intraspecific divergence (P) between 0.001 and 0.1, a proxy for minimum relative gap width (X) of 0.5, and a number of bins (n) of 30. Based on an intraspecific divergence of 1%, a recognized threshold in delineation analysis among vertebrate groups and the end of a plateau for lineage number (Puillandre *et al.* 2012), we considered the 16th partition to delineate lineages.

Acoustic analysis

We recorded the advertisement call of one male *A. simonstuarti* from the upper Juruá River (see locality #1 earlier) using a Sony PCM-DC50 digital recorder (sampling rate = 44.1 kHz; bit depth = 16) and built-in microphones. The recording was stored as stereo-channel wave file (left channel was kept for the acoustic analysis). The sound recording was deposited in Fonoteca Neotropical Jacques Vielliard (Unicamp, Brazil) under the accession number FNJV 45412. Information on the recording

is as follows: individual recorded at 09.40 h in the morning; air temperature around 25°C. We also reanalyzed some of the original calls recorded from the type locality (Angulo & Icochea 2010) in order to allow direct and standardized comparisons (FNJV 45409–11). We analyzed calls using an interface built between an expanded version (0.9.6.1) of Soundruler (Gridi-Papp 2007) and Matlab 6.5.2 (Matlab 2004). Note rate was quantified manually in Audacity 2.1.1 (Audacity Team 2017). Acoustic definitions and terminology follow those of Carvalho *et al.* (2019b). Acoustic traits were quantified through automated analysis, for which we developed settings in the software to recognize and delimit the acoustic units both in the time and frequency domains. Data are presented as range (mean ± standard deviation). Ranges include the span of values from the raw dataset. In the case of pulse duration, given that acoustic signals analyzed had more than one pulse, we first averaged the duration of each pulse of a given note (call mean) and then obtained the averaged mean for each male analyzed from the mean duration of call pulses (individual mean), and lastly, we obtained the grand means and associated standard deviations by averaging individual means. We applied two bandpass (500-Hz high-pass and/or 5000-Hz low-pass) filters to some of the sound files in Soundruler prior to conducting the acoustic analysis to reduce background noise caused by wind and/or rain. Spectrogram parameters were set as follows: FFT size = 1024 points, FFT overlap = 90%, window type = Hanning, contrast = 70%; those for the automated analysis were (in sample sizes): detection (smoothing = 500, resolution = 1), delineation (smooth factor = 1, smoothing = 15 or 100, and resolution = 1); critical amplitude ratio = 0.8 or 1.0. We produced sound figures using seewave 2.1.0 (Sueur *et al.* 2008) and tuneR 1.3.2 (Ligges *et al.* 2017), in R 3.5.0 (R Core Team 2018). Spectrogram settings were: window Hanning, FFT size = 256 points, and FFT overlap = 90%; the level of frequency components was indicated by a relative 30-dB color scale (red = maximum energy).

Results

Order Anura Fischer von Waldhein, 1813
Family Leptodactylidae Werner, 1896
Subfamily Leptodactylinae Werner, 1896
Genus *Adenomera* Steindachner, 1867

Adenomera simonstuarti (Angulo & Icochea, 2010)

Material examined

Holotype

PERU • Cusco, La Convención, Echarate, Río Camisea; MUSM 18218.

Paratypes

PERU • 3 specs; same collection data as for holotype; MUSM 18220, 18221, 18229.

Other material

BRAZIL • 1 spec., Acre, Tarauacá; INPA-H 40967 • 5 specs; Amazonas, Juruá; INPA-H 5337, 39792, 39796, 39813, 39814.

Comparative material

Adenomera andreae (Müller, 1923)

BRAZIL • 3 specs; Amapá, Serra do Navio; AAG-UFU 5994, 6006, 6007 • 2 specs; same collection data as for preceding; CFBH 43259, 43265 • 11 specs; Amazonas, Manaus; INPA-H 34045, 34048, 34073, 34074, 34076, 34081, 34082, 34084 to 34086, 34090 • 5 specs; same collection data as for preceding; ZUEC 3937, 3969, 3973, 3974, 7799 • 2 specs; Pará, Belém; AAG-UFU 2797, 2798 • 7 specs; Nova Timboteua; AAG-UFU 2788 to 2794.

Adenomera chicomendesi Carvalho, Angulo, Kokubum, Barrera, Souza, Haddad & Giaretta, 2019
BRAZIL • holotype; Acre, Rio Branco, Parque Zoobotânico; CFBH 43562 • 3 specs, paratypes; same collection data as for holotype; AAG-UFU 5862 to 5864 • 1 spec., paratype; same collection data as for holotype; CFBH 43563 • 4 specs, paratypes; same collection data as for holotype; ZUEC 24528 to 245231.

PERU • 7 specs, paratypes; Madre de Dios, Reserva Nacional de Tambopata; MUSM 39462, 30463, 39467, 39468, 39472 to 39474.

Adenomera heyeri Boistel, Massary & Angulo, 2006
BRAZIL • 3 specs; Pará, Oriximiná, ESEC-Grão-Pará; MPEG 30099 to 30101.

Adenomera hylaedactyla (Cope, 1868)
BRAZIL • 5 specs; Acre, Cruzeiro do Sul; AAG-UFU 5907 to 5911 • 3 specs; Feijó; AAG-UFU 5895 to 5897 • 8 specs; Amazonas, Manaus; INPA-H 22410 to 22413, 26606 to 26609 • 8 specs; São Gabriel da Cachoeira; AAG-UFU 3859 to 3866 • 4 specs; Roraima, Cantá; AAG-UFU 5540 to 5443.

Adenomera lutzi Heyer, 1975
GUYANA • 6 specs; Potaro-Siparuni; MZUSP 150799 to 150804.

Adenomera phonotriccus Carvalho, Giaretta, Angulo, Haddad & Peloso, 2019
BRAZIL • holotype; Pará, Palestina do Pará; MPEG 41155 • 2 specs, paratypes; same collection data as for holotype; CFBH 43130, 43131 • 1 spec., paratype; same collection data as for holotype; MPEG 41156.

Adenomera **sp. (*A. andreae* clade)**
PERU • 1 spec.; Cusco, La Convención, Echarate, Río Camisea; MUSM 18219.

Phylogenetic relationships and genetic diversity

Both BI and ML phylogenetic reconstructions (Fig. 1) yielded similar results with regard to relationships in the *Adenomera andreae* clade and the monophyly of *A. simonstuarti*. All three new sequences from southwestern Brazilian Amazonia were recovered nested within *A. simonstuarti* (Fig. 1). The ABGD delimitation analysis recovered eight genetic lineages within *A. simonstuarti* (Fig. 2) with noticeable geographic structure (Figs 1–2). Mean genetic distances in *COI* among the lineages of *A. simonstuarti* (Table 1) range from 3.2−7.6% (uncorrected) and from 3.3−8.0% (corrected), whereas within-lineage genetic distances reach a maximum value of 1.7% (uncorrected and corrected).

Our genetic voucher INPA-H 40967 (Fig. 3) is the only specimen of *Adenomera simonstuarti* with associated acoustic data. Morphological and color features of the specimen fully agree with those presented in the original description of *A. simonstuarti* (Angulo & Icochea 2010). This voucher specimen from the upper Juruá River constitutes the lineage 3 together with other specimens from the upper Amazon basin in southwestern Amazonia (Figs 1–2). The lineage 3 is regarded hereinafter as conspecific with the nominal species. The other two new *COI* sequences (lower Juruá River) fell within the lineage 2. These two vouchers also have the recognized morphotype of *A. simonstuarti* (Fig. 4), but acoustic data for this lineage remain unknown. Mean genetic distances between the *COI* lineages 2 and 3 are noticeable, ranging from 5.0% (uncorrected) to 5.3% (corrected).

Advertisement call and acoustic diagnosis

The call of *Adenomera simonstuarti* (Fig. 5) is redescribed below based on combined values of calls recorded from southwestern Amazonia: the type locality (Camisea, Cusco, Peru) and the upper Juruá

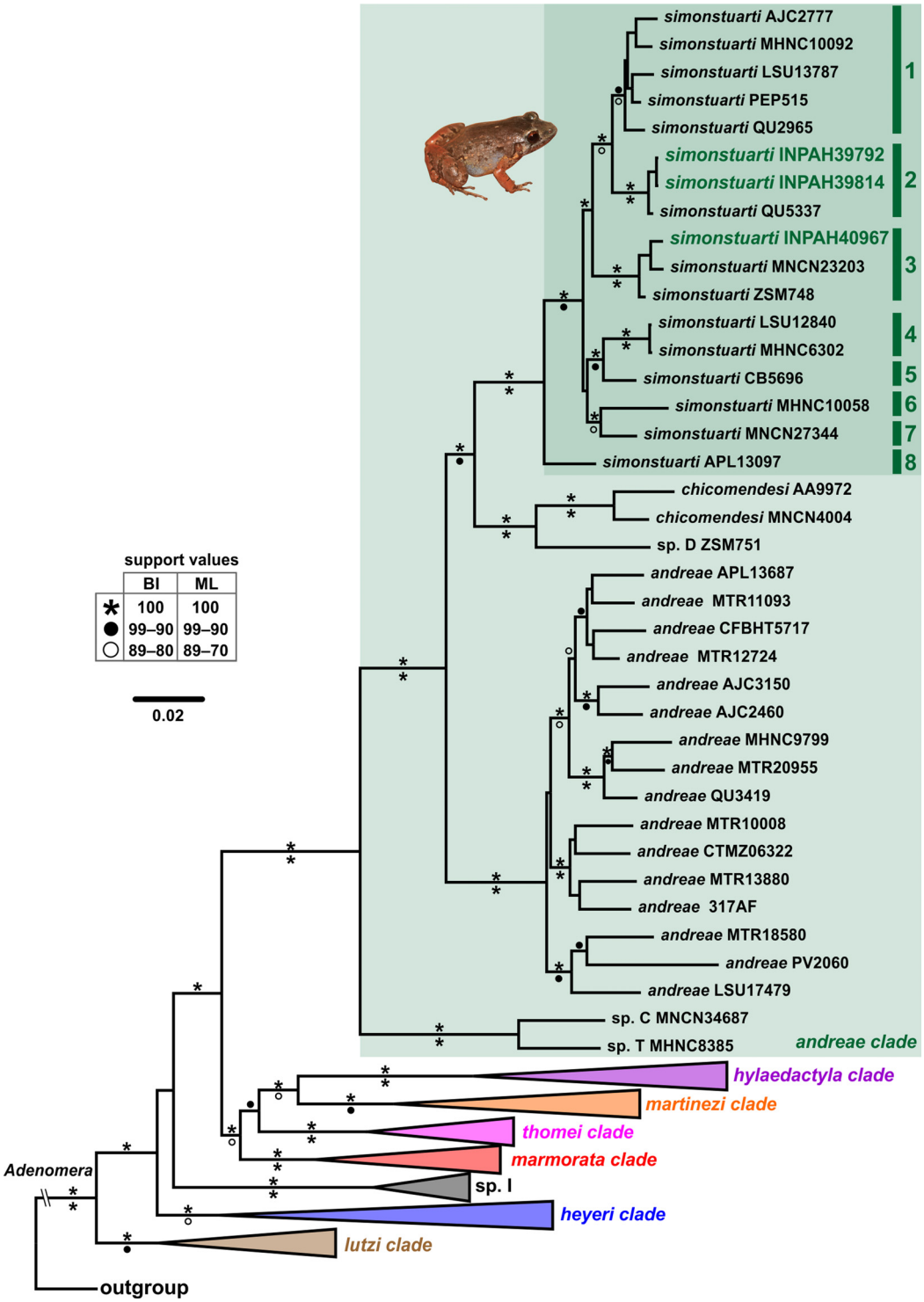

Fig. 1. Bayesian phylogenetic tree of *Adenomera* Steindachner, 1867 inferred from a concatenated dataset of four genes (two mitochondrial + two nuclear), showing the eight major clades delimited by colors. The emphasis on the diversification within the *A. simonstuarti* species complex shows a deep genetic divergence, with eight distinct lineages (see Fig. 2 for geographic distribution). Symbols above branches indicate posterior probabilities of Bayesian inference (BI) and those below branches indicate bootstraps of maximum likelihood inference (ML). Low support values (BI < 80 and ML < 70) were omitted. Branch scale is indicated in number of substitution per site.

Table 1. Uncorrected (lower diagonal) and corrected (Jukes-Cantor model; upper diagonal) pairwise genetic distances of a fragment from *COI* mtDNA gene among the lineages of the *Adenomera simonstuarti* species complex (named as sim1–8). Within-lineage distances (uncorrected / corrected), when applicable, were highlighted in bold across the central diagonal (upper left to lower right). The lineage 3 corresponds to the nominal species. Values are presented as means (in %).

	sim1	**sim2**	**sim3**	**sim4**	**sim5**	**sim6**	**sim7**	**sim8**
sim1	**1.7 / 1.7**	3.3	5.2	6.1	4.8	5.9	4.3	6.9
sim2	3.2	**0.2 / 0.2**	5.3	7.0	5.4	5.8	5.4	7.2
sim3	4.9	5.0	**0.7 / 0.7**	5.3	4.7	5.1	5.2	7.3
sim4	5.8	6.7	5.1	**0.0 / 0.0**	3.8	7.3	5.6	8.0
sim5	4.6	5.2	4.5	3.7	–	6.1	3.9	7.1
sim6	5.7	5.6	5.0	7.0	5.9	–	6.0	7.5
sim7	4.2	5.2	5.0	5.3	3.8	5.8	–	6.7
sim8	6.6	6.9	7.0	7.6	6.8	7.1	6.3	–

Table 2. Advertisement call traits of *Adenomera simonstuarti* (Angulo & Icochea, 2010) from southwestern Amazonia of Peru (type locality) and Brazil. One male was recorded from each locality. N = number of quantified notes and pulses, respectively. Values are presented as range (mean ± SD).

Acoustic traits	**Camisea (Cusco, Peru)** **N = 33 / 98**	**Tarauacá (Acre, Brazil)** **N = 24 / 55**
Call duration (s)	0.8–5.1 (2.1 ± 1.8)	1.5–6.5 (4.8 ± 1.7)
Notes per call	4–20 (8.6 ± 6.7)	8–30 (22.6 ± 7.6)
Note duration (ms)	57–71 (64.7 ± 3.4)	62–79 (68.5 ± 4.5)
Note rate per second	3.7–4.1 (3.8 ± 0.1)	4.5–4.9 (4.6 ± 0.1)
Note rise time (%)	13–73 (33.5 ± 14.0)	20–60 (38.1 ± 16.2)
Pulses per note	2–3 (3.0 ± 0.2)	2–3 (2.3 ± 0.5)
Pulse duration (ms)	10–31 (21.8 ± 2.3)	10–53 (30.9 ± 5.8)
Dominant frequency (Hz)	1873–2003 (1958.2 ± 20.1)	1873–2046 (1961.7 ± 56.7)
Frequency modulation (Hz)	43–301 (185.3 ± 55.4)	86–301 (202.8 ± 58.9)

River (Tarauacá, Acre, Brazil) (Table 2). The call consists of a multi-note signal given at a low repetition rate (< 10 per minute), lasting 0.8–6.5 (3.4 ± 1.9) s. Calls are formed by 4–30 (15.6 ± 9.9) notes. Call notes are given at a rate of 4–5 (4.2 ± 0.6) per second. Notes last 57–79 (66.6 ± 2.6) ms, and the rise time is at 13–73 (35.8 ± 3.2) % of note duration. Notes are formed by 2–3 (2.6 ± 0.5) partly fused pulses with duration varying from 10–53 (26.4 ± 6.4) ms. Notes have the dominant frequency coinciding almost always with the fundamental harmonic (1873–2046 Hz, 1959.9 ± 2.3), but coinciding with the second

harmonic (3596–4156 Hz, 3962.1 ± 254.8) in four notes given by the male from Brazil. The frequency modulation is upward, rising from 43–301 (194.0 ± 12.3) Hz.

The advertisement call of *Adenomera simonstuarti* (Fig. 5) recorded from the type locality (Camisea, Peru; Angulo & Icochea 2010) and from Brazil are given as multi-note calls. The multi-note call of *A. simonstuarti* represents a useful diagnostic character of the species by being unique among members of the *A. andreae* clade. The only other described species of *Adenomera* with multi-note call is the allopatric *A. cotuba* Carvalho & Giaretta, 2013, distributed in the Cerrado savannas and dry forests of north central Brazil (Carvalho & Giaretta 2013b). Additionally, the following morphological and color features, when combined with acoustic data, can help distinguish nominal *A. simonstuarti* from the seven Amazonian congeners [*A. andreae*, *A. chicomendesi*, *A. coca* (Angulo & Reichle, 2008), *A. heyeri*, *A. hylaedactyla*, *A. lutzi* and *A. phonotriccus*; see Boistel *et al.* 2006; Kok *et al.* 2007; Angulo & Reichle 2008; Carvalho *et al.* 2019b, 2019c, 2019d]: (1) a nearly solid, dark-colored stripe along the underside of the forearm; (2) absence of dorsolateral stripe; (3) toe tips fully expanded into discs; (4) absence of antebrachial tubercle on underside of forearm; and (5) multi-note advertisement call.

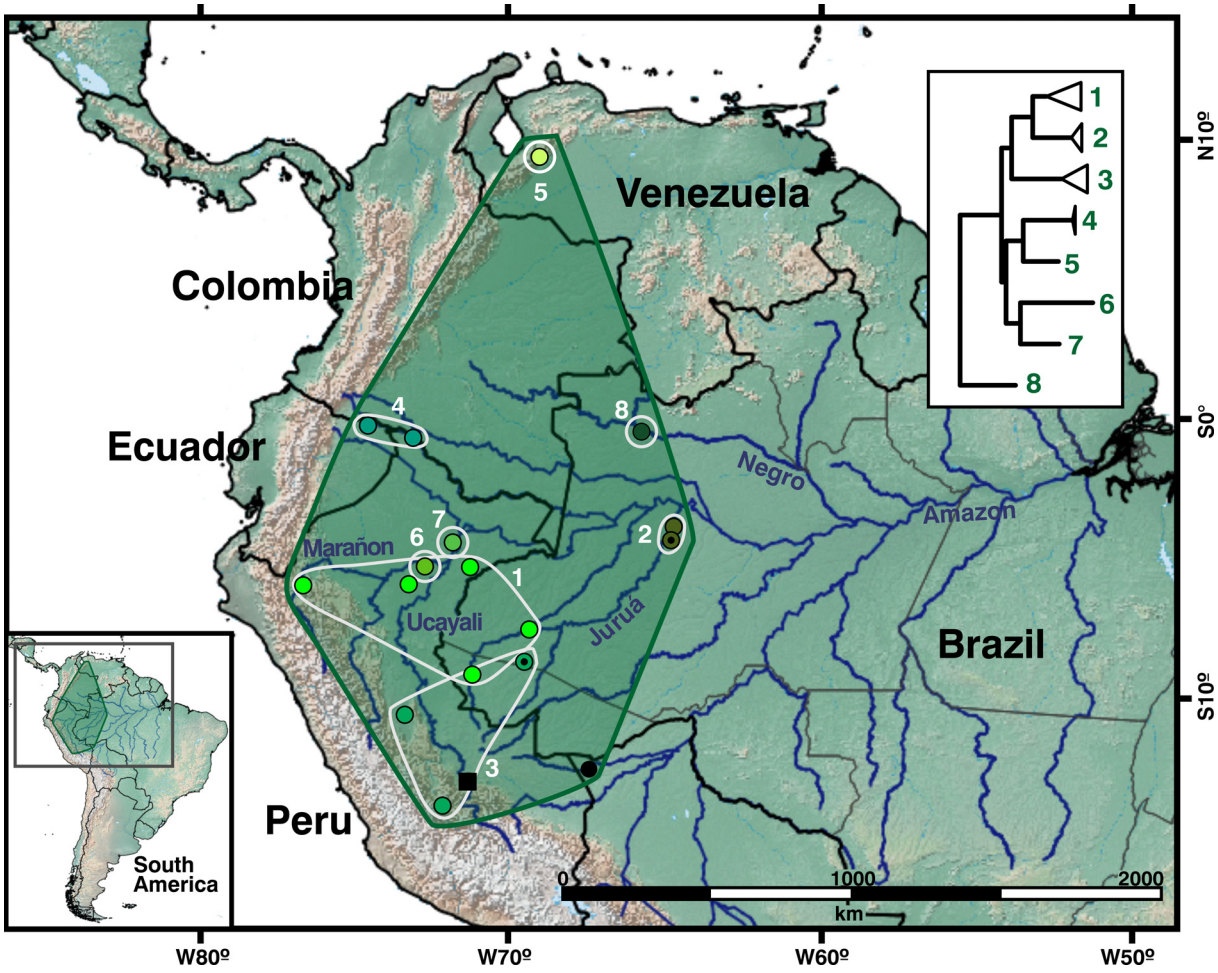

Fig. 2. Geographic distribution of the *Adenomera simonstuarti* species complex in northwestern South America; genetic lineage 3 corresponds to the nominal species. Phylogenetic relationships among the eight lineages are shown on the upper right. Black solid-filled symbols represent the localities reported in the original description (square = type locality at Camisea, Peru; circle = Pando, Bolivia). Black-dotted symbols indicate newly collected specimens from the Juruá River in the Brazilian Amazonia.

Habitat and natural history

The call voucher of *Adenomera simonstuarti* from the upper Juruá River (INPA-H 40967; Fig. 3), corresponding to the lineage 3, was collected from an open bamboo forest, approximately 2 km from BR-364 road. This individual and other two were heard calling from an old clearing surrounded by decomposing fallen logs. The three individuals called hidden underneath dense leaf litter, and only one of them (the call voucher) were found while surveying the area. *Adenomera simonstuarti* and *A. andreae* were found syntopically in this area.

The four specimens from the lower Juruá River (INPA-H 39792, 39796 and 39813–14; Fig. 4), corresponding to lineage 2, were collected in a non-flooded lowland forest (*terra firme* forest) with dense understory layer. Three specimens (INPA-H 39792 and 39813–14) were found in a forest affected by anthropogenic activities (i.e., logging), located close to Comunidade Cumaru village. This could indicate a certain degree of tolerance of the lower Juruá population to habitat disturbance, given that human occupation and activities in this region have begun during the late 1980s (ICMBio 2009). The specimen INPA-H 39796 (Fig. 4) was collected from a preserved forest, distant from that village. Specimens in the lower Juruá River were sympatric with *A. andreae* and *A. hylaedactyla*. *Adenomera*

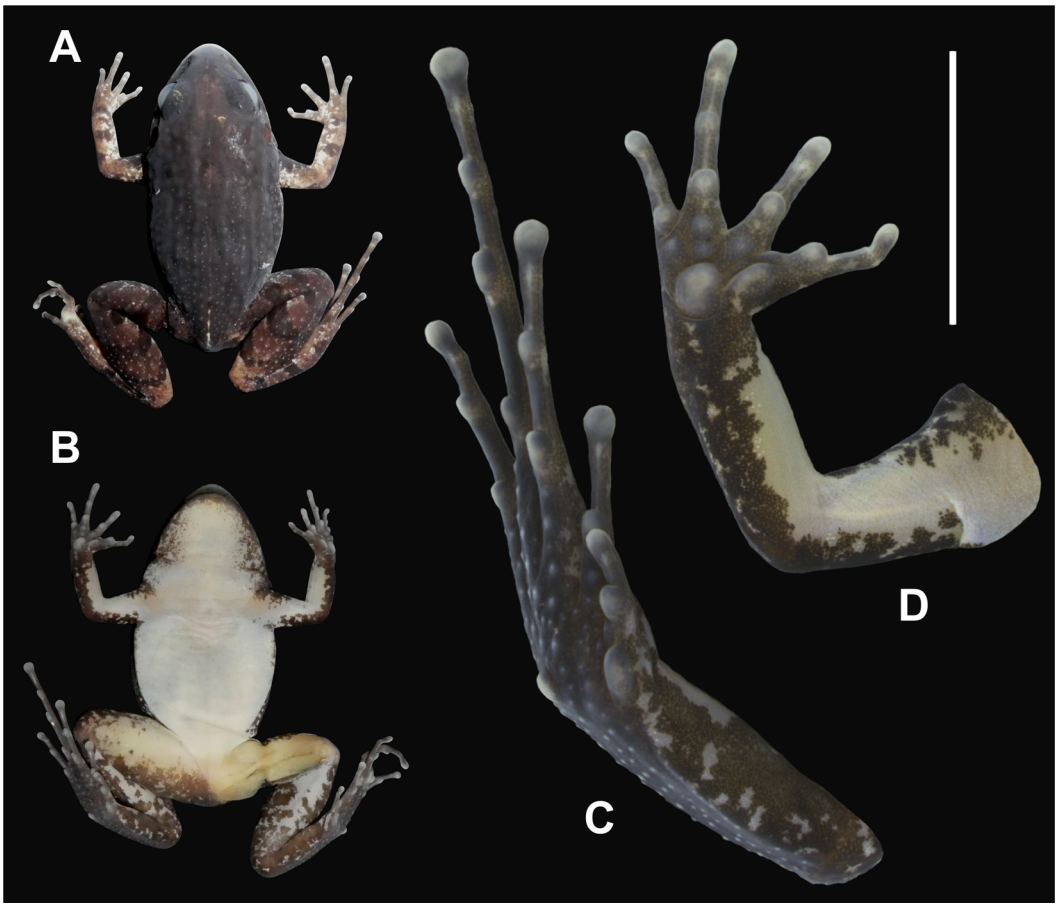

Fig. 3. Preserved male of nominal *Adenomera simonstuarti* (Angulo & Icochea, 2010) (= genetic lineage 3): call voucher INPA-H 40967 (SVL = 23.4 mm) from the upper Juruá River, in Tarauacá, Brazilian state of Acre. This specimen corresponds to a call voucher (see Fig. 5). **A**−**B**. Body in dorsal and ventral views, not to scale. **C**−**D**. Detail of the ventral surface of right foot and hand, respectively. Note the nearly solid, dark-colored stripe along the underside of the forearm. Photographs by J. Magnusson. Scale bar = 5 mm.

simonstuarti and *A. andreae* were found syntopically inside the forest, whereas *A. hylaedactyla* was only found along riverbanks.

Distribution patterns

Adenomera simonstuarti (= lineage 3) is distributed in the upper Amazon Basin of southwestern Brazilian and Peruvian Amazonia, and two locations in the eastern slopes of the Andes in south central Peru. Populations linked to the other seven lineages are in most cases allopatric among each other. Some lineages are widely distributed, such as lineage 1, from lowland and montane forests in the upper Amazon Basin of Peru and Brazil. Other lineages, such as lineage 2, may be narrowly distributed on the east bank of the lower Juruá River. Other distribution patterns include: lineage 4 in lowland Amazonia of northeastern Ecuador and extreme northern Peru; lineage 5 in Venezuelan Andes montane forests; lineage 6 in the Marañón-Ucayali interfluve; lineage 7 in the upper Amazon River; and lineage 8 in the upper Negro River. Based on the geographic patterns of each of the lineages, we could expect that some of them may have distributions associated with interfluve regions, such as lineages 4, 6 and 7 (Fig. 2). Another interesting pattern is that the nominal species (lineage 3) and other lineages (e.g., lineage 8) are distributed in the upper Amazon Basin, while some others are distributed in the middle-lower portions of major southern tributaries of the Amazon River (e.g., lineage 2; Fig. 2).

Discussion

We did not examine for the morphological analysis most of the genetic vouchers linked to the *Adenomera simonstuarti* lineages of Fouquet *et al*. (2014); see their appendix S1a. The only exception is the specimen

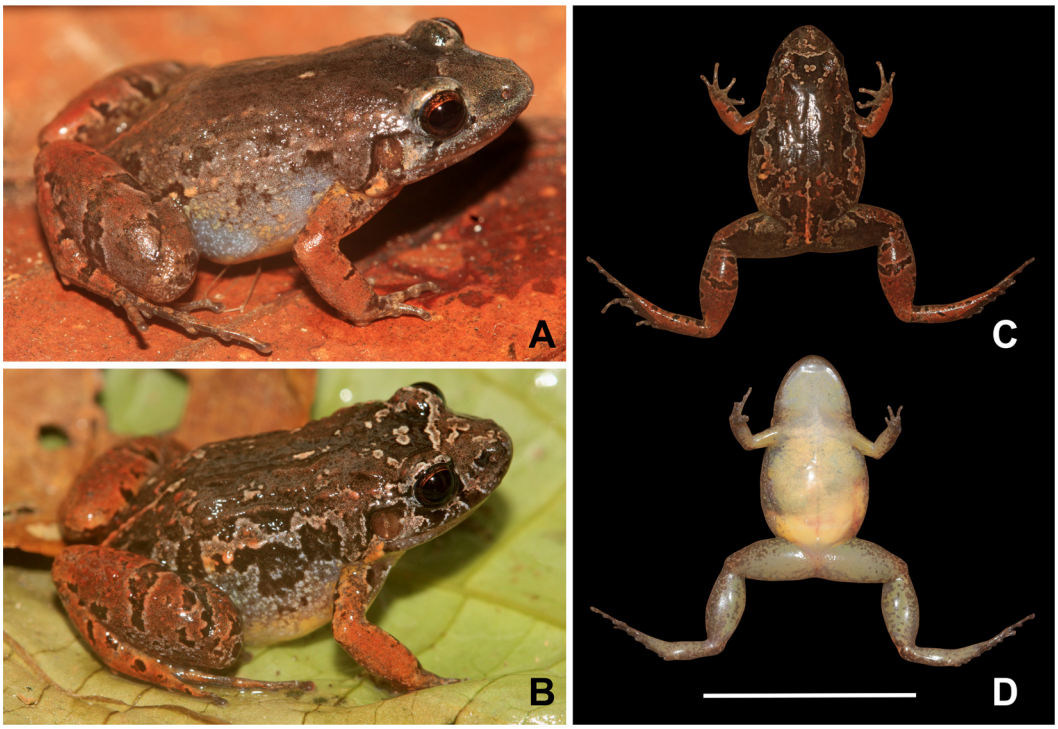

Fig. 4. Specimens of the genetic lineage 2 related to *Adenomera simonstuarti* (Angulo & Icochea, 2010) from the lower Juruá River, in Juruá, Brazilian state of Amazonas. **A**−**B**. Dorsolateral view of the adult male, SVL = 22.6 mm (INPA-H 39792) and the adult female, SVL = 26.1 mm (INPA-H 39814), respectively. **C**−**D**. Dorsal and ventral views of the female shown in B. Photographs by L.J.C.L. Moraes. Scale bar = 25 mm.

INPA-H 5337 (see Material examined) belonging to the lineage 2 from the lower Juruá River (previously reported as QU5337 by Fouquet *et al.* 2014). Likewise, acoustic data for the lineages other than the one containing the nominal species remain unknown. Nevertheless, by reinterpreting the calling pattern of *A. simonstuarti* (i.e., multi-note advertisement call) and linking the nominal species to a specific genetic lineage, our study contributes to the potential discrimination between the nominal species and closely related, putative new species within the *A. simonstuarti* complex. Due to the lack of acoustic and morphological data that could help to corroborate the existence of multiple, unnamed lineages within this species complex, a plausible alternative hypothesis would be the one of *A. simonstuarti* as a single species containing deep conspecific lineages across its geographic range in western Amazonia. Future studies should follow the protocol of combining calls and DNA sequences associated with voucher specimens as a means to fully address the taxonomic status of the other seven genetic lineages within the *A. simonstuarti* complex, regarded herein as putative new species.

Of special relevance is the acquisition of DNA sequences for the Amazonian *Adenomera* population from Camisea, in southeastern Peru, sympatric with *A. simonstuarti* in the type locality region. That population was originally reported as *Adenomera* cf. *andreae* by Angulo & Icochea (2003), but referred hereinafter to as *Adenomera* sp. from Camisea. Based on the few calls available (recording FNJV 45413; see Fig. 5C–D), we briefly and qualitatively characterized the advertisement call of *Adenomera* sp. from Camisea as nonpulsed and given as single notes, with the dominant frequency at the fundamental harmonic, and with negligible frequency modulation. These acoustic traits distinguish this taxon from the sympatric *A. simonstuarti* and all other Amazonian species of *Adenomera* (for acoustic comparisons in *Adenomera*, see Carvalho *et al.* 2019b, 2019c, 2019d). We also examined the recorded male of *Adenomera* sp. from Camisea (accession number: MUSM 18219), which differs from nominal *A. simonstuarti* by lacking the nearly solid, black-colored stripe along the underside of the forearm. In fact, the specimen is morphologically more similar to other members of the *A. andreae* clade, especially by the presence of toe tips fully expanded into small discs (Carvalho *et al.* 2019b, 2019d). Based on its distinctive call, however, *Adenomera* sp. from Camisea cannot be conspecific with nominal *A. andreae* or any other described species and candidate new species of the *A. andreae* clade with described calls

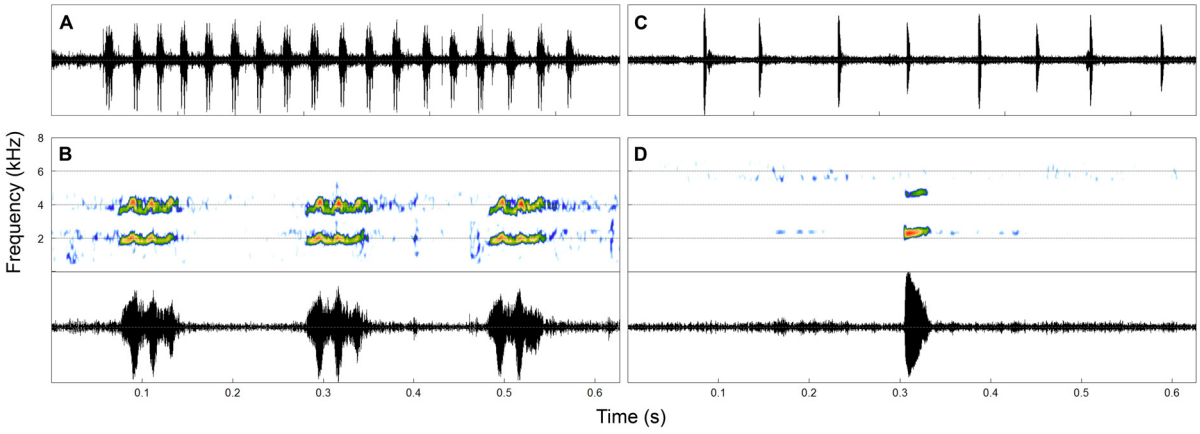

Fig. 5. Advertisement calls of (A–B) nominal *Adenomera simonstuarti* (Angulo & Icochea, 2010) (= genetic lineage 3) from the upper Juruá River, in southwestern Amazonia of the Brazilian state of Acre (voucher INPA-H 40976), and (C–D) the sympatric *Adenomera* sp. from Camisea, in the Region of Cusco, Peru (voucher MUSM 18219). **A**. Time-domain section containing a multi-note call (18 notes). **B**. Spectrogram and oscillogram of three notes (5th–7th) from the call in A. **C**. Time-domain section of two males in antiphonal calling (single-note calls). **D**. Spectrogram and oscillogram of the 4th note in C. Figures are equally scaled (ca 4.5 s on the x-axis of A and C, each marking corresponding to 1.0 s).

(Angulo *et al.* 2003; Carvalho *et al.* 2019b, 2019d), except for two Peruvian lineages (i.e., *Adenomera* sp. D and *Adenomera* sp. T; Fouquet *et al.* 2014) whose calls remain unknown (for further discussion, see Fouquet *et al.* 2014: appendix S2a). *Adenomera* sp. from Camisea should therefore correspond to an unnamed species that may be conspecific with one of the two candidate new species of the *A. andreae* clade with unknown calls (*Adenomera* sp. D and *Adenomera* sp. T), with one of the lineages under *A. simonstuarti* for which morphological data have not yet been assessed, or might even have not been genetically sampled to date.

The age of initial diversification of the *Adenomera simonstuarti* complex is unknown but likely during the Miocene, given that the divergence time between this species complex and its sister clade (*A. chicomendesi* + *Adenomera* sp. D) was estimated to have occurred during this geological period (8.0–8.5 Ma; Fouquet *et al.* 2014). The *A. simonstuarti* complex inhabits an Amazonian region that has been affected by several landscape changes and hydrological instability during the Miocene (Albert *et al.* 2018), which might have generated the deep genetic divergence that mirrors the allopatric distributions of lineages within the complex (Figs 1–2). Furthermore, the result of intense climatic variation in the region during the Pleistocene, which modified the extension and structure of the forest habitats in the region (Arruda *et al.* 2018), might also have contributed to the more recent divergences within this complex.

The southwestern lowland Amazonian forests consist of a heterogeneous mosaic of habitats. We surveyed for *Adenomera* in flooded forests (*várzeas*) and other riparian environments, but individuals of the *A. simonstuarti* complex were only found in non-flooded (*terra firme*) forest. These non-flooded forests are patchily distributed within the heterogeneous landscape, in some cases distantly located from the main course of rivers, which are the primary access route by researchers (Oliveira *et al.* 2016). For this reason, the limited access to the apparently preferred habitat may bias the understanding of the geographic range and patterns of distribution of lineages within the *A. simonstuarti* complex. The conservation status of *A. simonstuarti* was originally assessed as Data Deficient (DD), pending future surveys to confirm that it could also occur in between known localities of Peru and Bolivia (Angulo & Icochea 2010). With the inclusion of the new occurrence record in the Brazilian state of Acre (Fig. 2), the putative distribution of nominal *A. simonstuarti* is extended northward and the estimated Extent of Occurrence (EOO; IUCN 2012) is approximately 200 000 km² (*sensu* Bachman *et al.* 2011). Given the new estimated EOO, encompassing conservation units, and the fact that populations of *A. simonstuarti* appear to tolerate certain degree of habitat disturbance (Angulo & Icochea 2010; present study), we recommend that the extinction risk of nominal *A. simonstuarti* could be assessed as Least Concern (LC) following the IUCN Red List Categories and Criteria (IUCN 2012). The reassessment of extinction risk should only be taken into account, however, as long as the region does not suffer from severe impacts caused by human activities in the near future, including the indirect action of anti-environmental public policies, such as the recent fire crisis and illegal operations that have taken place across Amazonia (Ferrante & Fearnside 2019; Pereira & Viola 2019; Barlow *et al.* 2020).

Despite the recommendation of extinction risk of nominal *Adenomera simonstuarti* in the Least Concern category, it is important to highlight the potential threats and extinction risk to the other seven lineages subsumed within the *A. simonstuarti* complex, regarded as putative new species. This is especially relevant because conservation strategies are in many cases not feasible as long as the taxonomic status of unnamed lineages is not fully resolved; see Angulo & Icochea (2010) for a proposition on the impacts of cryptic species complexes on biodiversity and conservation assessments. By circumscribing *A. simonstuarti* to one of eight genetic lineages within the complex, its distribution is dramatically narrowed down to the southernmost portion of the entire range of the species complex (~2 million km² EOO; Fig. 2), corresponding to a decrease of 90% in EOO. Our current knowledge on species richness and distribution is still insufficient for accurate evaluations of conservation status and distribution patterns

of the *A. simonstuarti* complex. In the opposite scenario, by lumping the other seven lineages back into *A. simonstuarti* and considering the deep genetic divergence as intraspecific variation, the conservation of metapopulations displaying high genetic variability should also be taken into consideration as a significant component to safeguard the biological heritage (Crandall *et al.* 2000).

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Supplementary File

SM.01. GenBank accession numbers and locality data of genetic vouchers included in this study. New sequences are indicated in bold.