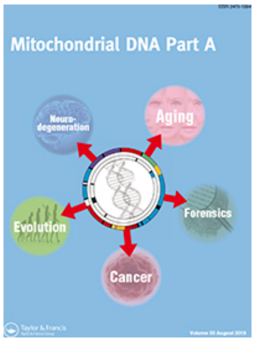

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REVIEW ARTICLE

Pristimantis in the Eastern Brazilian Amazon: DNA barcoding reveals underestimated diversity in a megadiverse genus

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ABSTRACT

The genus *Pristimantis* has the highest species diversity among all terrestrial vertebrates, with most species observed in the Andean region and the Guiana Shield. Even with the recent description of a new species, only *P. latro*, *P. dundeei* and *P. zimmermanae* occur in the south of the Amazon River. The lack of taxonomists specialized in the field leads to the propagation of dubious terminologies (e.g. *Pristimantis* sp1, *Pristimantis* sp2, *P.* aff. *Fenestratus* and *P.* gr. *conspicillatus*) or even misidentification of species, resulting in erroneous species distributions. In this study, we applied the Automatic Barcode Gap Discovery (ABGD) algorithm for the delimitation of candidate species and values of genetic distances using the mitochondrial marker Cytochrome Oxidase Subunit I (COI), proposed in the barcode methodology, where values greater than 10% are considered as indicative of different species. We found large genetic distances between *P. latro* and *Pristimantis* sp1 Unconfirmed Candidate Species – UCS1 (21%), and between *P. altamazonicus* and *Pristimantis* sp2 UCS2 (14%). The ABGD method recognized UCS1 and UCS2 as distinct species. *Pristimantis* sp. UCS1 and UCS2 in the east of the Brazilian Amazon are indicated as candidate species. We suggest greater sampling of *Pristimantis* sp. UCS1 and UCS2, integrating morphology and bioacoustics to solve the taxonomic status in the east of the Brazilian Amazon.

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KEYWORDS
ABGD; candidate species; COI; mitochondrial

Introduction

The genus *Pristimantis* Jimenez de la Espada 1870 is considered the most diverse among terrestrial vertebrates (Fouquet et al. 2013), with 529 described species (Frost 2018). New species are described every year, mainly for the Andes (Lehr 2010; Székely et al. 2016; Lehr and Moravec 2017; Valencia et al. 2017) and the Guiana Shield (Barrio-Amorós, Mesa, et al. 2010; Barrio-Amorós, Rojas-Runjaic, et al. 2010; Barrio-Amorós, et al. 2012; Barrio-Amorós, et al. 2013; Kok et al. 2011; Fouquet et al. 2013). On the other hand, for the Amazon basin, Xingu drainage, Tapajós, Teles-Pires and Araguaia rivers, studies on taxonomy and systematic of *Pristimantis* are scarce and restricted to records in faunistic inventories (Avila-Pires et al. 2010; Vaz-Silva et al. 2015), resulting in the propagation of taxonomic errors in a genus that presents high phenotypic conservation (Padial and De La Riva 2009; De Oliveira et al. 2017).

Pristimantis latro De Oliveira, Rodrigues, Kaefer, Pinto and Hernández-Ruz 2017), represents the last species described for the south of the Amazon River, in the Xingu and Tapajós region. New species of *Pristimantis* with great morphological similarity to *P. latro* can be found in the east of the Brazilian Amazon, so it is difficult to correctly identify those species based on rapid morphological observations (De Oliveira et al. 2017).

In the east of the Brazilian Amazon, species of *Pristimantis* with Finger I shorter than Finger II are known only as *P. inguinalis* (Parker, 1940) and *P. marmoratus* (Boulenger, 1900) (Avila-Pires et al. 2010) in the north, and *P. zimmermanae* (Heyer and Hardy, 1991) (IUCN SSC Amphibian Specialist Group, 2015) in the south of the Amazon River. To the west of the Amazon, *P. altamazonicus* (Barbour and Dunn, 1921), *P. diadematus* (Jiménez de la Espada, 1875), *P. ockendeni* (Boulenger, 1912), *P. reichlei* (Padial and De la Riva, 2009), *P. acuminatus* (Shreve, 1935) and *P. academicus* (Lehr, Moravec and Gagliardi-Urrutia, 2010) (Bernarde et al. 2011) can be found. Of these, *P. altamazonicus* is widely distributed in the highlands of the Amazon region (Colombian, Peruvian and Ecuadorian Amazon), resulting in several taxonomic problems with similar species, as in the case of *P. brevicrus* (Anderson, 1945), which is considered a junior synonym of *P. altamazonicus* by Lynch 1974. *Pristimantis brevicrus* was revalidated as a

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Supplemental data for this article can be accessed here.

distinct species of *P. altamazonicus* through molecular and morphological data (Ortega-Andrade et al. 2017).

The use of molecular data applied to the resolution of taxonomic problems in widely distributed species can be a powerful tool in the identification of lineages (Fouquet et al. 2007; Funk et al. 2012), supporting a review of the morphological and bioacoustic characters (Elmer and Cannatella 2008). The lineages identified by mitochondrial markers (16S rRNA and Cytochrome Oxidase Subunit I – COI) are considered as being Unconfirmed Candidate Species (Padial et al. 2010; Ortega-Andrade et al. 2017), pending a review of others characters (morphology, bioacoustics, distribution, ecology, among others), according to the proposal of integrative taxonomy (Dayrat 2005).

In this study, we found two candidate species of *Pristimantis*, which occur south of the Amazon River, using methods for molecular taxonomic delimitation such as the Automatic Barcode Gap Discovery, ABGD (Puillandre et al. 2012).

Materials and methods

Sampling

Sampling were carried out in two regions south of the Amazon river: 1) Serra do Pardo National Park (05° 46′ 40.38"S; 52° 38′ 52.04"W), a Conservation Unit (CU) with an area of 445,413.45 hectares, situated within the territory known as 'Terra do Meio', a mosaic of CU's located between two rivers, the Xingu to the east and the Iriri to the west, in the municipality of Altamira, state of Pará (MMA - Ministério do Meio Ambiente 2015); and 2) São Nicolau Farm in the municipality of Cotriguaçu (09° 59′ 57.16"S; 58° 26′ 28.08"O), state of Mato Grosso, on the left margin of the Juruena River, an important affluent of Tapajos river basin (Figure 1). The *Sistema de Autorização e Informação em Biodiversidade* was used for approving the licenses nr. 30034-1 in the state Mato Grosso, and nr. 32401 in the Conservation Unit Serra do Pardo in Terra do Meio.

Tissue samples were collected from the thigh and liver, and preserved in 96% ethyl alcohol for further DNA extraction. Individuals were fixed in 10% formalin and stored in 70% ethanol. *Pristimantis* sp1 was deposited in the collection of the Coleção Zoológica do Laboratório de Zoologia Adriano Giorgi (acronym LZA), da Universidade Federal do Pará Brazil and *Pristimantis* sp2 was deposited in the collection of the Acervo Biológico da Amazônia Meridional (acronym ABAM) at the Universidade Federal do Mato Grosso, Campus de Sinop, Brazil.

Terminology and morphological data

We use the term Unconfirmed Candidate Species (UCS) for a specimen that may represent a distinct species, pending a more complete morphological review (Vieites et al. 2009).

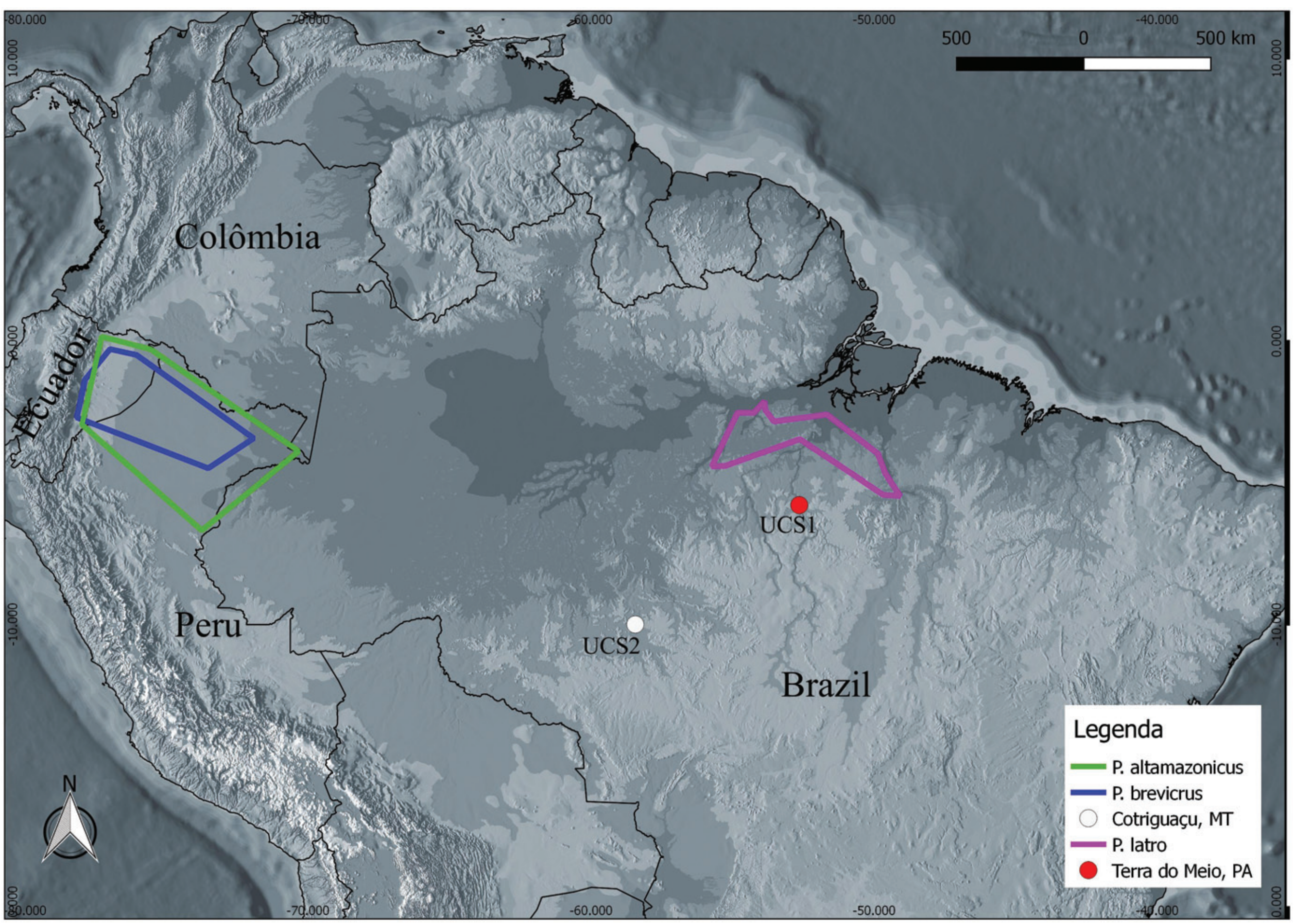

Figure 1. Distribution map of *P. altamazonicus* and *P. brevicrus* (proposed by Ortega-Andrade et al. 2017), *P. latro* (de Oliveira et al. 2017), *Pristimantis* sp. UCS1 and *Pristimantis* sp. UCS2.

Diagnosis was based on the definition of characters and terminologies from the literature (Duellman and Lehr 2009; Ortega-Andrade et al. 2017). Measurements were taken with a digital caliper to the nearest 0.01 mm and rounded to the nearest 0.1 mm as in Elmer and Cannatella (2008) and Heinicke et al. (2009). The measurements obtained are as follows: snout-vent length (tip of snout to posterior margin of vent), SVL; head length (posterior margin of lower jaw to tip of snout), HL; head width (measured at rictus level), HW; snout length (anterior corner of eye to tip of snout), SL; eye to nostril distance (anterior corner of eye to posterior margin of naris), DEN; internarial distance (taken between the median margins of the nares), ID; eye length (measured horizontally), EL; interorbital distance (taken between the inner margins of the orbits), IoD; eyelid width (the largest transverse width of the upper eyelid.), EW; tympanum length (the longest measure from the anterior margin to the posterior margin of the tympanum), TL; arm length (from the tip of the elbow to the proximal edge of the palmar tubercle), AL; hand length (from the proximal edge of the palmar tubercle to the tip of Finger III), HaL; thigh length (from vent to knee), ThL; tibia length (outer edge of flexed knee to heel), TiL; tarsus length (from the heel to the proximal edge of the inner metatarsal tubercle), TaL; foot length (proximal border of inner metatarsal tubercle to tip of fourth toe), FL and leg length (from the knee joint to the tip of Toe IV), LL. For *Pristimantis* sp2, *P. altamazonicus* and *P. brevicrus*, the following measures were added: disk width of Finger III, F3D; disk width of Toe IV, T4D; length of Finger I from inner edge of thenar tubercle to tip of disk, 1FiL; length of Finger II from the junction of Fingers I and III to the tip of finger disk, 2FiL. Sex and maturity were determined by direct examination of gonads through a lateral incision in the abdomen. In addition, we checked for secondary sexual characters in adult individuals, such as the presence or absence of vocal slits, vocal sac and nuptial pads in males.

Molecular data

Total genomic DNA was extracted using the 2% CTAB protocol (Doyle and Doyle 1987). A fragment of COI mtDNA was amplified by PCR using the primers dgLCO 1490 forward and dgHCO 2198 reverse (Meyer 2003).

The sequencing reaction was performed according to the manufacturer's recommendations for the sequencing mix ABI Big Dye Terminator, using the primers mentioned above (Applied Biosystems). Sequencing reactions were precipitated using the standard protocol EDTA/Ethanol, re-suspended with 10 μL of Hi-Di deionized formamide and sequenced by the automatic sequencer ABI 3500 (*Applied Biosystems*).

The sequences were aligned using the Clustal W algorithm (Thompson et al. 1994) implemented in BIOEDIT 7.2 software (Hall 1999). Phylogenetic trees of maximum likelihood (ML) were constructed in MEGA 6 software (Tamura et al. 2011). The same software was used to calculate uncorrected pairwise distances under a Kimura-2-Parameters (K2P) model. Hedges et al. (2008) suggest the genus *Oreobates* as basal group of *Pristimantis*; therefore, we use it as an outgroup. All sequences generated and/or analysed in this study are available from GenBank (accession numbers are listed in the Appendix 1).

To define the species limit, we used the Automatic Barcode Gap Discovery (ABGD) method, which automatically detects significant differences of intra- and inter-specific divergence (barcoding gap) and defines the distance limit based on pairwise patterns of genetic distance (Puillandre et al. 2012). In the ABGD, analyses were performed on the website http://wwwabi.snv.jussieu.fr/public/abgd/ using the K2P distance model. The threshold for intraspecific diversity was defined as a minimum of 0.001 and a maximum of 0.1. The default value of the barcoding gap was $X = 1.5$.

Results

Morphology

An adult female specimen labelled as *Pristimantis* sp1, collected from Terra do Meio, showed the same diagnostic characters of *P. latro* as described by De Oliveira et al. (2017). Diagnostic characters of *P. latro* have a phenotypic plasticity in several character states. For example, dorsal skin texture may be shagreened, strongly shagreened or smooth, and *Pristimantis* sp1 had strongly shagreen dorsal skin. The same pattern can be found when examining the character 'dorsal tubercle': most of the type series of *P. latro* do not have dorsal tubercles or those are scattered over the dorsum. *Pristimantis* sp1, however, has many scattered dorsal tubercles. *Pristimantis* sp1 has a divided palmar tubercle, a whitish cream coloured venter with black spots disposed randomly, and dark brown dorsum; *P. zeuctotylus* (Lynch and Hoogmoed, 1977) has a complete palmar tubercle, black venter and bronze dorsum (Lynch and Hoogmoed 1977). *Pristimantis* sp1 has dorsolateral folds and the presence of finger fringes, while in *P. dundeei* (Heyer and Muñoz, 1999) dorsolateral fold and finger fringe are absent (Maciel et al. 2012). *Pristimantis* sp1 presents basal webbing and fringe on the toes, and they are absent in *P. chiastonotus* (Lynch and Hoogmoed 1977). Morphometric measurements of *Pristimantis* sp1 and *P. latro* holotype are shown in Table 1.

A female sub-adult (Figure 2)-denominated *Pristimantis* sp2 was collected in the municipality of Cotriguaçu and differs from *P. altamazonicus* (characters in parentheses) by presenting shagreened dorsal skin texture with scattered tubercles (smooth with scattered tubercle), evident tympanic annulus and tympanic membrane (slightly apparent), venter strongly areolate (weakly areolate), basal webbing present between toes (absent), and occurs in east Brazilian Amazon (high in the Amazon Basin: Ecuador, Peru and Colombia) (Ortega-Andrade et al. 2017). It differs from *P. brevicrus* by presenting tympanum annulus (absent), basal webbing between toes (absent), and occurs in east Brazilian Amazon (high in the Amazon Basin: Ecuador, Peru and Colombia) (Ortega-Andrade et al. 2017); from *P. orcus*, by presenting strongly areolate venter (weakly areolate), dentigerous processes of vomer almost absent (prominent), and Finger V almost the same size as Finger III (Finger V much longer than Finger III) (Lehr et al. 2009); from

Table 1. Morphometric measurements (in mm) of females of *Pristimantis* sp. UCS1, UCS 2, *P. latro* (holotype LZATM 467), *P. altamazonicus* and *P. brevicrus* (Ortega-Andrade et al. 2017).

				P. altamazonicus		*P. brevicrus*	
Character	UCS1	UCS2	*P. latro*	Average ± SD	Range	Average ± SD	Range
SVL	38.1	15.6	40	29.2 ± 0.6	(28.4–30.1)	31.3 ± 2.6	(27.2–35)
TL	20.7	7.6	20,5	12.9 ± 0.6	(11.8–13.8)	13.4 ± 1.1	(11.4–15)
FL	20.2	6.3	18,9	18.4 ± 0.8	(17.1–19.7)	19.5 ± 1.3	(16.7–21.7)
HL	14.9	5.7	15,6	11.3 ± 0.7	(10.6–12.4)	11.7 ± 1.2	(9.8–13.9)
HW	14.4	5.2	14,5	12.8 ± 1.2	(11.3–14.8)	13.1 ± 1.6	(10.9–16.2)
IOD	3.5	1.6	3,9	3.7 ± 0.2	(3.4–3.9)	3.9 ± 0.5	(3.0–4.7)
EW	3.9	1.7	3,6	2.6 ± 0.2	(2.3–2.9)	2.9 ± 0.5	(2.3–4.0)
IND	3.1	1.5	3,1				
E-N	5.1	1.6	5,7	3.6 ± 0.2	(3.5–4)	3.6 ± 0.3	(3.2–4.1)
ED	4.6	2	4,5	3.8 ± 0.3	(3.4–4.1)	3.9 ± 0.4	(3.2–4.9)
TD	2.3	0.4	1,8	1.3 ± 0.0	(1.3–1.4)		
SL	23	8.6	22,8	13.1 ± 0.7	(12.2–13.8)	13.6 ± 0.9	(12–15)
H-L	10.3	4.1	10,5	7.3 ± 0.3	(6.9–7.6)	7.8 ± 0.6	(6.7–8.8)
S-L	7.6	2.6	7,9				
LEG	29.3	10.5	30,3				
LTA	10.6	4.2	11,9				
LA	9.5	3.1	8,9				
FIIID		0.8		1.6 ± 0.2	(1.4–1.8)	1.8 ± 0.2	(1.4–2.3)
T4D		0.8		1.6 ± 0.1	(1.4–1.8)	1.7 ± 0.2	(1.3–2.1)
1FiL		1.5					
2FiL		2					

Standard deviation represented by ±.

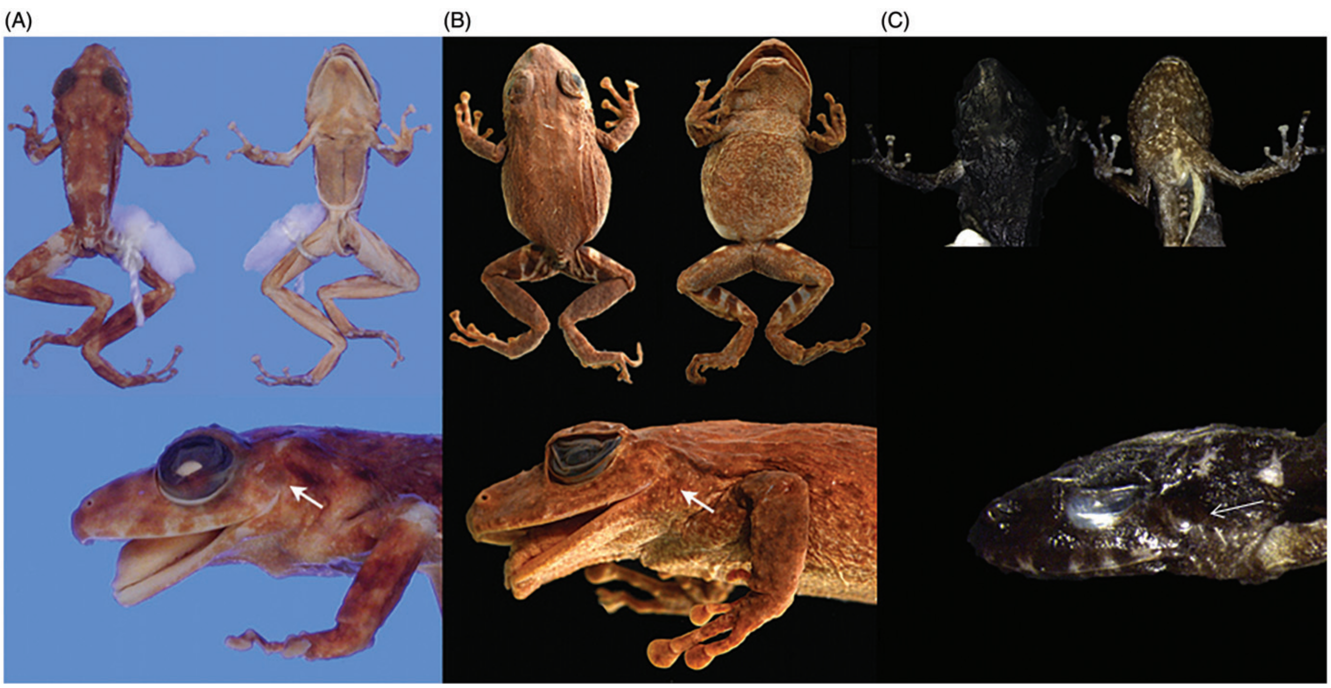

Figure 2. Dorsal, frontal and lateral view of the holotypes of A) *P. altamazonicus* (MCZ 2028, sub-adult female, SVL = 20.61 mm), photo by Sven Kullander, Swedish Museum of Natural History, removed from Ortega-Andrade et al. (2017); B) *P. brevicrus* (NRM 1920, gravid female, SVL = 33.0 mm), photo by Sven Kullander, Swedish Museum of Natural History, removed from Ortega-Andrade et al. (2017) and C) *Pristimantis* sp. UCS2 (ABAM 3286, sub-adult female, SVL = 15.6 mm). Note that the arrow points to the presence of the annulus tympanum in *P. altamazonicus*, the absence of this character in *P. brevicrus* and how both the tympanic membrane and the annulus tympanum are evident in *Pristimantis* sp. UCS2 (white arrow).

P. spedeus and *P. inguinalis*, by presenting almost absent dentigerous processes of vomer (prominent) (Barrio-Amorós et al. 2010b; Fouquet et al. 2013); from *P. marmoratus*, by presenting Finger V almost the same size as Finger III (Finger V longer than Finger III) (Kok et al. 2018). Morphometric measurements of *Pristimantis* sp2 and the holotypes of *P. altamazonicus* and *P. brevicrus* can be found in Table 1.

Phylogenetic relationship

Phylogenetic analysis of Maximum Likelihood (ML) (Figure 3) showed that *Pristimantis* sp1 represents a sister species of *P. latro*+*P.* aff. *fenestratus* (Marabá)+*P.* aff. *fenestratus* (Carolina), with a genetic distance of 21.2 and 19%, respectively (Table 2). According to ABGD criteria, using the initial partition with distribution of prior values of intraspecific divergence ($P = .001$–$.059$), the *Pristimantis* sp1 individual represents a

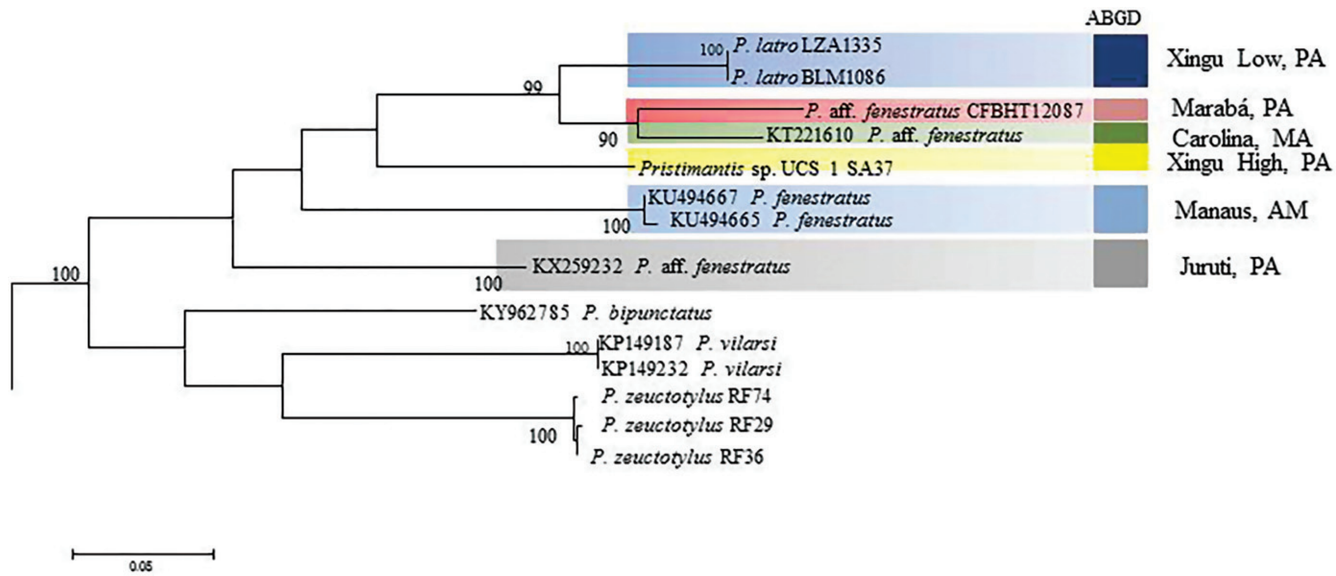

Figure 3. Maximum-likelihood phylogenetic tree based on a 531-bp COI mtDNA fragment from species of the group *conspicillatus* of *Pristimantis* genetically closer to the UCS1 in this study. Only bootstrap values >80 are shown (5000 pseudo-replicates). Vouchers' names appear after specimen names, and GenBank codes before the species name. Colours correspond to groups identified by the ABGD method. The outgroup *Oreobates quixensis* KU494611 has been removed from this figure.

Table 2. Genetic distance (K2P) in % from mitochondrial COI marker among species of the *Pristimantis conspicillatus* group which are morphologically and geographically closest to UCS1 in this study. 1) UCS1 Terra do Meio; 2) *P.* aff. *fenestratus* Carolina, MA; 3) *P.*aff. *fenestratus* Marabá, PA; 4) *P. altro* Altamira, PA; 5) *P. fenestratus* Manaus, AM; 6) *P.* aff. *fenestratus* Juruti, PA; 7) *P. zeuctotylus* Monte Alegre, PA; 8) *P. bipunctatus* (Peru); 9) *P. vilarsi* (Colômbia).

	1	2	3	4	5	6	7	8	9
1									
2	19								
3	20	10							
4	21	12	14						
5	21	23	24	24					
6	21	24	25	22	23				
7	22	24	28	25	25	25			
8	23	25	25	24	25	23	22		
9	25	26	27	28	24	24	22	21	

distinct species of *P. latro* that occurs in the right and left margins of low and middle Xingu River.

To *Pristimantis* sp2, the phylogenetic analysis of ML (Figure 4) revealed that it represents a sister species of *P. altamazonicus*+*P. brevicrus* from Ecuador, with genetic distance of 16% and 14%, respectively (Table 3). According to ABGD criteria, using the initial partition with distribution of prior values of intraspecific divergence (P=.001–.035), the *Pristimantis* sp2 individual represents a different species of *P. altamazonicus* and *P. brevicrus*.

Discussion

Genetic markers have been widely used for the identification of anuran lineages in recent years (Funk et al. 2012; Kieswetter and Schneider 2013; Moraes et al. 2017), revealing that the diversity of amphibian species in the Neotropics is underestimated (Gehara et al. 2014; Fouquet et al. 2016; Vacher et al. 2017). This approach, applied on UCS1 (*Pristimantis* sp1) and UCS2 (*Pristimantis* sp2), and supported by taxonomic revisions using morphology, bioacoustics and natural history, endorses the description of new species and, consequently, limits the distribution area of species previously considered widely distributed (de Oliveira et al. 2017).

To the east of the Brazilian Amazon, there are seven known species of *Pristimantis* (*P. latro*, *P. marmoratus*, *P. inguinalis*, *P. zeuctotylus, P. chiastonotus*, *P. dundeei* and *P. zimmermanae*). Of these, *P. latro*, *P. dundeei* and *P. zimmermanae* occur in the south of the Amazon River. *Pristimantis* sp. UCS1 presented greater genetic distance to all these species (>14%), while *Pristimantis* sp. UCS2 presented 16% for *P. altamazonicus* and 14% to *P. brevicrus*. These values are considered high enough to set different species apart by COI marker: generally, genetic distances of 10% (Vences et al. 2005) or 6% (Lyra et al. 2017) are recommended as the interspecific limit. The taxonomic status of many species of *Pristimantis* is unclear, especially those with large distributions throughout the Amazon (Duellman and Lehr 2009; Padial and De La Riva 2009; Siqueira et al. 2009; Moraes et al. 2017). Several authors use the name *P. fenestratus* (Steindachner, 1864) in herpetofauna surveys in the east of the Brazilian Amazon (Avila-Pires et al. 2010; Bernardo et al. 2012; Vaz-Silva et al. 2015), leading to taxonomic errors due to erroneous identification.

The UCS1 did not present diagnostic morphological differences to *P. latro*; however, the morphology preserved in anurans is strongly documented in the literature and is accompanied by deep genetic divergences (Kieswetter and Schneider 2013; Fouquet et al. 2016). This phenomenon suggests that the species may be genetically divergent without presenting phenotypic changes, leading to intraspecific recognition through other mechanisms, such as pheromones or vocalizations (Schonrogge et al. 2002; Bickford et al. 2007).

Although the UCS2 displayed diagnostic morphological differences to the genetically closest species, we believe that additional specimens are needed to solve their taxonomic status. Comparisons with other species from the old *unistrigatus* group (e.g. *P. orcus* Lehr et al. 2009, *P. ventrimarmoratus*

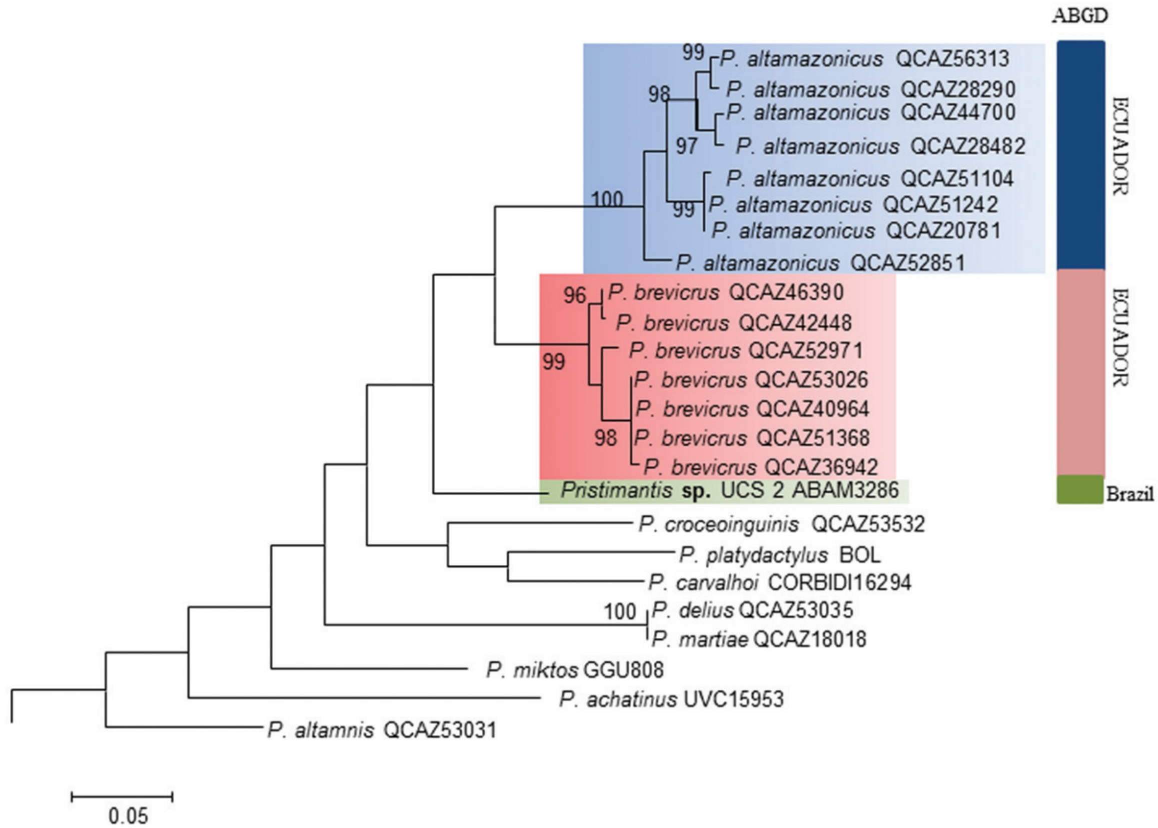

Figure 4. Maximum-likelihood phylogenetic tree based on a 531-bp fragment of COI mtDNA from species of the *Pristimantis unistrigatus* group, the closest to the UCS2 in this study. Only bootstrap values >80 are shown (5000 pseudo-replicates). Vouchers' names appear after the names of the specimens downloaded from GenBank. Colours correspond to groups identified by the ABGD method. Outgroup (*Oreobates quixensis* KU494611 has been removed from this figure.

Table 3. Genetic distance (K2P) in % from COI marker among species of *Pristimantis* which are genetically closest to UCS2: 1) UCS2 Cotriguaçu, MT; 2) *P. brevicrus* Ecuador; 3) *P. altamazonicus* Ecuador; 4) *P. carvalhoi* Peru; 5) *P. miktos* Peru; 6) *P. platydactylus* Bolivia; 7) *P. altamnis* Ecuador; 8) *P. croceoinguinis* Ecuador; 9) *P. delius* Ecuador; 10) *P. martiae* Ecuador; 11) *P. achatinus* Colombia.

	1	2	3	4	5	6	7	8	9	10	11
1											
2	14										
3	16	15									
4	18	19	20								
5	18	21	23	23							
6	19	18	22	15	19						
7	21	19	22	22	24	22					
8	21	20	21	17	20	18	22				
9	21	24	24	22	24	25	25	22			
10	21	24	24	22	24	25	25	22	00		
11	23	28	28	30	27	27	25	28	27	27	

(Boulenger, 1912), *P. inguinalis*, *P. marmoratus*, *P. diadematus*, *P. imitatrix* (Duellman, 1978)) is required for a more robust sampling. In the state of Mato Grosso, amphibian inventories have recorded species with uncertain taxonomic status, as *Pristimantis* sp.1 and sp.2 (Ávila and Kawashita-Ribeiro 2011), indicating the need for further studies. *Oreobates crepitans* (Bokermann, 1965), designated as '*P. crepitans*' (Meireles dos Santos et al. 2011; Strüssmann et al. 2011) was removed from the genus *Pristimantis* and allocated within *Oreobates* by Padial et al. (2012); thus, *P. dundeei* is the only species formally identified in the state of Mato Grosso.

In Brazil, the state of Acre presents eight species of *Pristimantis*: *P. acuminatus*, *P. altamazonicus*, *P. academicus*, *P. diadematus*, *P. fenestratus*, *P. ockendeni*, *P. peruvianus* (Melin, 1941) and *P. reichlei* (Bernarde et al. 2011). In the states of Pará, Maranhão, Tocantins and Mato Grosso, seven species are known: *Pristimantis inguinalis*, *P. marmoratus, P. latro*, *P. zeuctotylus*, *P. chiastonotus, P. zimmermanae* and *P. dundeei*. We removed *P. fenestratus* from this list, as we believe that populations under this name represent undescribed species (De Oliveira et al. 2017). Taking into consideration that the state of Acre has an area of 152,581 km^2, and the four states (Pará, Maranhão, Tocantins and Mato Grosso) cover an area of 2,760,961 km^2, it is expected that there will be a greater number of species in the latter part of the Brazilian Amazon, which includes areas of endemism such as Xingu, Tapajós, Belem and part of the Guiana Shield (Silva et al. 2002; Ribas et al. 2012). One possible explanation for this discrepancy is the lack of taxonomists in the region, and the adoption of such terminology as *Pristimantis* sp. or *P.* cf. *fenestratus* (Novais 2014; Vaz-Silva et al. 2015) reflects this deficiency. The territorial extension itself represents a challenge for institutional researchers, due to the inaccessibility of many locations requiring large logistic investment.

In this work, we conclude that the diversity of species of the genus *Pristimantis* in the east of the Brazilian Amazon is underestimated, requiring further fauna surveys in places of difficult access, supported by integrative taxonomy (morphological, molecular and behavioural approaches). *Pristimantis* sp1 (UCS1) and *Pristimantis* sp2 (UCS2) require more samples and taxonomic reviews (morphology and vocalization) for their correct identification.

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Appendix

Appendix 1. List of sequences used in the COI marker molecular analyses.

Species	Locality	N° GenBank	Voucher
Pristimantis **sp.** UCS1	Altamira, PA	MK689170	SA37
Pristimantis **sp.** UCS2	Cotriguaçu, MT	MK689174	ABAM 3286
P. latro	Altamira, PA	MK689168	LZA 1335
P. latro	Altamira, PA	MK689169	BLM 1086
P. fenestratus	Manaus, MA	KU494667	CFBHT05715
P. fenestratus	Manaus, MA	KU494665	CFBHT05708
Pristimantis sp.	Juruti, PA	KX259232	J330
P. zeuctotylus	Monte Alegre, PA	MK689171	RF 29
P. zeuctotylus	Monte Alegre, PA	MK689172	RF 36
P. zeuctotylus	Monte Alegre, PA	MK689173	RF 74
P. bipunctatus	Peru	KY962785	MUSM31179
P. vilarsi	Colombia	KP149187	AJC 2113
P. vilarsi	Colombia	KP149232	AJC 3945
P. fenestratus	Marabá, PA	KU494664	CFBHT 12087
P. fenestratus	Carolina	KT221610	
P. platydactylus	Bolívia	JN991394	MBH 5746
P. altamazonicus	Ecuador	MF118728	QCAZ56313
P. altamazonicus	Ecuador	MF118724	QCAZ52851
P. altamazonicus	Ecuador	MF118721	QCAZ51242
P. altamazonicus	Ecuador	MF118720	QCAZ51104
P. altamazonicus	Ecuador	MF118717	QCAZ44700
P. altamazonicus	Ecuador	MF118712	QCAZ28482
P. altamazonicus	Ecuador	MF118710	QCAZ28290
P. altamazonicus	Ecuador	MF118707	QCAZ20781
P. altamnis	Ecuador	KP064164	QCAZ53031
P. brevicrus	Ecuador	MF118722	QCAZ51368
P. brevicrus	Ecuador	MF118718	QCAZ46390
P. brevicrus	Ecuador	MF118716	QCAZ42448
P. brevicrus	Ecuador	MF118715	QCAZ40964
P. brevicrus	Ecuador	MF118713	QCAZ36942
P. brevicrus	Ecuador	MF118725	QCAZ52971
P. brevicrus	Ecuador	MF118727	QCAZ53026
P. carvalhoi	Peru	KY672983	CORBIDI16294
P. achatinus	Colombia	JN371122	UVC15953
P. croceoinguinis	Ecuador	KP064157	QCAZ53532
P. delius	Ecuador	KP064162	QCAZ53035
P. martiae	Ecuador	JN991381	QCAZ18018
P. miktos	Peru	KP064163	GGU808
Oreobates quixensis	Amazonas	KU494611	MTR_ALCX186P53

Legend: Suyana e Andressa (AS), Acervo Biológico da Amazônia Meridional (ABAM), Laboratório de Zoologia Adriano Giorgi (LZA), Belo Monte (BLM), Célio F. B. Haddad Tissue (CFBHT), Juruti (J), Raul Fróis (RF), Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru (MUSM), Andrew J. Crawford (AJC), Michael B. Harvey (MBH), Museo de Zoología of the Pontificia Universidad Católica del Ecuador (QCAZ), División de Herpetología, Centro de Ornitología y Biodiversidad (CORBIDI), Universidad Valle de Cauca, Cali (UVC), G. Gagliardi (GGU).