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A candidate species currently classified as *Atelopus hoogmoedi* (Anura: Bufonidae) in the eastern Amazon, Pará, Brazil

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ABSTRACT. The genus *Atelopus* is one of the most diverse of the Bufonidae family; because of their bright color, they are referred to as harlequin frogs. They occur in mature tropical forest areas and in this region, these forests are under anthropic pressure and limited to fragments, which facilitates the action of pathogenic fungi. One of these toad species, *Atelopus hoogmoedi*, is only found to the north and south of the Amazon River. Based on genetic data this species name represents more than one evolutionary unit. To explore this premise, we compared individuals of *A. hoogmoedi* collected south and north of the Amazon river in the state of Pará, Brazil. The DNA was extracted by the phenol chloroform method from eight individuals, seven

from Monte Alegre (north of Amazon River) and one from Anapu (south of Amazon River) and then amplified via PCR using a mitochondrial 16S rRNA marker. Phylogenetic analysis of maximum likelihood for *A. hoogmoedi* revealed a paraphyletic group with three lines: French Guiana 1 and 2, Guyana and Monte Alegre, and Anapu. The genetic distance between Anapu and Monte Alegre was 2.9%. According to the Automatic Barcode Gap Discovery in both Simple Distance and Kimura 2 Parameters models, *A. hoogmoedi* collected in Anapu is recognized as a distinct species from those of the Guiana Shield. Thus *A. hoogmoedi* to the south of the Amazon River was classified as an unconfirmed candidate species, requiring more collections and access to individuals from other localities of its occurrence for confirmation.

Key words: ABGD; Candidate species; Endemic species; Guiana Shield; Xingu Basin

INTRODUCTION

The genus *Atelopus*, is one of the most diverse of the family Bufonidae, with 96 species, distributed from Costa Rica to Bolivia and French Guiana (Frost, 2018). The species of this genus are diurnal and have terrestrial activity in the litter of primary forests associated with water bodies (Lima et al., 2006; Lötters, 2007) and are referred to as harlequin frogs. Most species live in mountains above 1500m (Lötters, 1996). Reproduction occurs on the banks of streams (Lötters, 2007). Many areas of mature tropical forest areas are under anthropic pressure and limited to fragments, which facilitates the action of pathogenic fungi; consequently many species of harlequin frogs are at risk of extinction (Stuart et al., 2008), with populations drastically declining (La Marca et al., 2005).

Atelopus is divided into two main clades: Amazonia-Guiana (clade *flavescens-spumarius* + clado *tricolor*) and Central America-Andes-Chocó (clade *varius* + all other *Atelopus*) (Lötters et al., 2011). The first clade includes 12 species (*A. carbonerensis*, *A. chrysocorallus*, *A. cruciger*, *A. mucubajiensis*, *A. oxyrhynchus*, *A. pinangoi*, *A. sorianoi*, *A. tamaense* , *A. vogli*, *A. flavescens*, *A. spumarius* and *A. hoogmoedi*). Of these, only three occur in the Brazilian Amazon: *A. hoogmoedi*, *A. spumarius* and *A. flavescens* (Amphibia Web, 2018). Due to phenotypic conservation in species of this genus (Lötters et al., 2011), it is likely that other species are masked in the Amazon basin.

The species *A. hoogmoedi* is distributed in southern and western French Guiana, Suriname, southern Guiana, and in adjacent regions of Brazil in the states of Amapá, Pará and Roraima (Frost, 2018). Lötters et al. (2011) proposed the phylogeny of the genus *Atelopus* based on 12S and 16S markers and indicated the phylogenetic positioning of *A. hoogmoedi*, from the north of the Amazon River in the Guiana Shield. The population of *A. hoogmoedi* south of the Amazon River represents an isolated

group from those of the Guiana Shield, whose taxonomic status is uncertain, as are many other species of the genus (Noonan and Gaucher, 2005).

According to Lötters et al. (2011), *A. hoogmoedi* is a paraphyletic group with three strains (two in French Guiana and one in Guyana): the lineage of French Guiana 1 is the sister species of *A. spumarius barbotini*, French Guiana, Saul region, while the other two strains of French Guiana 2, Saul region and Guyana, Mabura Hill region, represent an external group of the former. According to Zink (1997), lineages grouped in a biological species do not share the same common ancestor (they are not monophyletic), which leads to the existence of paraphyletic species and to reproductive isolation.

The use of molecular data applied to solve taxonomic problems in widely distributed species can be a powerful tool in the identification of lineages (Funk et al., 2012), providing a means to compare with classifications based on morphological and bioacoustic characters (Elmer and Cannatella, 2008). The lineages identified with mitochondrial markers 16S and cytochrome oxidase subunit I (COI) are considered to be unconfirmed candidate species (Ortega-Andrade et al., 2017), awaiting a review of other characters (morphology, bioacoustics, distribution, ecology, among others), according to proposals of integrative taxonomy (Dayrat, 2005).

Consequently, we examined molecular heterogeneity in *A. hoogmoedi*, which probably represents more than one evolutionary unit, in which the objective was to compare the individuals of *A. hoogmoedi* to the south and north of the Amazon River. We used methods for molecular taxonomic delimitation such as Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012), to determine if the *A. hoogmoedi* south of the Amazon River is an unconfirmed candidate species.

MATERIAL AND METHODS

Study area

The work was carried out with individuals of *A. hoogmoedi* collected in two municipalities: Monte Alegre-PA (3°01'28.11" S; 53°15'26.17" W) in the region of the Guiana Shield which has vegetation coverage ranging from alluvial forests to the south (crossing the Amazon River), Amazonian Savannas (Campos de Monte Alegre) in the central part, submontane dense ombrophylous forest and rugged topography (Fróis et al., 2018). In the municipality of Anapu-PA (3°09'01.59" S; 51°29'37.08" W) the collections were carried out in the Virola-Jatobá Sustainable Development Project (PDS), a settlement of the Institute for Colonization of Agrarian Reform INCRA) with a territorial area of 41,153.31 hectares and about 180 settled families that live from extractive and family agriculture; this area has a dense ombrophylous forest with medium to large trees (Figure 1).

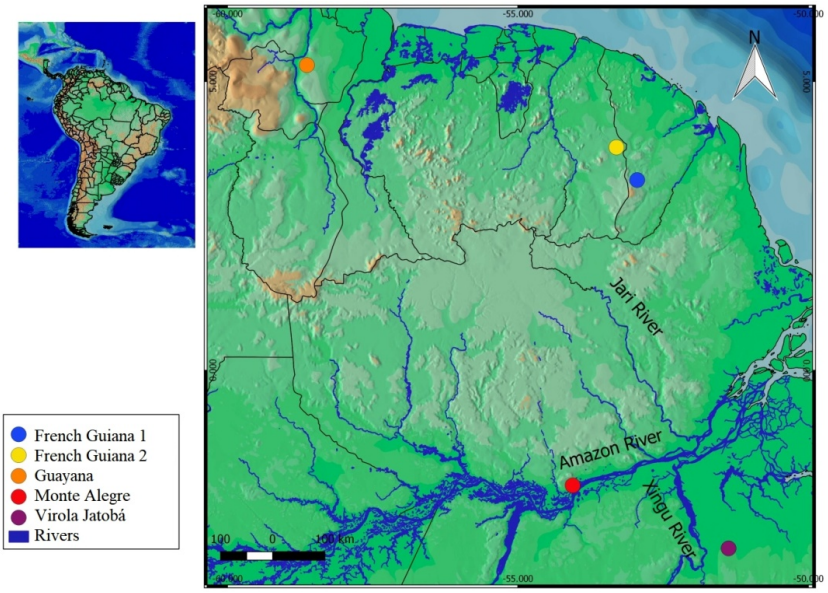

Figure 1. Localities where *Atelopus hoogmoedi* was sampled. The blue, yellow, orange and red circles represent localities to the north and the purple circle to the south of the Amazon River.

Extraction and amplification of DNA

DNA was extracted from eight individuals of *A. hoogmoedi*, seven from Monte Alegre and one from Anapu. Thigh muscle or liver tissue samples were stored in 95% alcohol and the specimens were deposited in the Laboratory of Zoology - Adriano Giorgi Collection of the Faculty of Biological Sciences at the Federal University of Pará - UFPA / Altamira.

The total DNA of each individual was extracted by the phenol chloroform method (Sambrook et al., 1989). The quality of the samples was observed by means of 1% agarose gel electrophoresis, visualized in a 302 nm UV trans illuminator, stained with GelRed.

The DNA was amplified via PCR using a primer pair (16Sa and 16Sb primers) reverse and forward to the 16S marker (Palumbi et al., 1991) with the following PCR conditions: 92°C (60 s), followed by 35 cycles of 92°C (60 s), 50°C (50 s) and 72°C (1.5 min), and final extension at 72°C for 7 min. The amplification was verified by means of 1% agarose gel electrophoresis.

Sample precipitation was purified following reading on an ABI 3500xL Genetic Analyzer (Life Technologies), using the manufacturer's protocol. After reading the sequences, they were aligned using the Clustal W algorithm (Thompson et al., 1994) implemented in the BioEdit program (Hall, 1999) and edited manually. The first eight sequences generated in this study are in Table 1; the other sequences that were analyzed are available from GenBank. The License of ICMBio No. 30034-1, contemplates the

ethical methods of euthanasia used in the animals of this study; the voucher specimens were fully euthanized with lidocaine hydrochloride 2%, fixed in 10% formaldehyde solution and then later transferred to 70% ethanol. Fixation was performed primarily by perforation with 10% formaldehyde solution and then covered with paper moistened with the same solution.

Table 1. List of toad specimens used for molecular analysis. The first eight specimens were collected in this study, the others are from GenBank.

Species	Locality	GenBank
Atelopus hoogmoedi	Anapu- PA	MK166205
A. hoogmoedi	Monte Alegre- PA	MK166206
A.hoogmoedi	Monte Alegre- PA	MK166207
A.hoogmoedi	Monte Alegre- PA	MK166208
A.hoogmoedi	Monte Alegre- PA	MK166209
A. hoogmoedi	Monte Alegre- PA	MK166210
A. hoogmoedi	Monte Alegre- PA	MK166211
A. hoogmoedi	Monte Alegre- PA	MK166212
A. hoogmoedi	French Guiana	EU672972
A. hoogmoedi	French Guiana	DQ283260
A. hoogmoedi	Guiana	EU672974
A. barbotini	French Guiana	EU672971
A. spumarius	Peru	EU672977
A. bomolochos	Ecuador	AF375508
A. bomolochos	Ecuador	GU252225
A. bomolochos	Ecuador	GU252226
A. bomolochos	Ecuador	GU252227
A. bomolochos	Ecuador	GU252231
A. bomolochos	Ecuador	GU252232
A. bomolochos	Ecuador	AF375509
A. varius	Costa Rica	AY325996
A. varius	Panama	U52779
A. peruensis	Peru	GU252229
A. peruensis	Peru	GU252230
A. halihelos	Ecuador	AF375510
A. longirostris	Ecuador	AF375511
A. zeteki	Panama	DQ283252
A. flavescens	French Guiana	EU672970
A. pulcher	Peru	EU672973
A. spurrelli	Colombia	EU672975
A. seminiferus	Peru	EU672976
A. tricolor	Bolivia	EU672978
A. oxapampae	Peru	EU672979
A. sp2	Peru	EU672980
A. nanay	Ecuador	GU252228
A. chiriquiensis	Panama	U52780
Melanophryniscus stelzneri	Brazil	U52782
Melanophryniscus sp.	Brazil	KM204371

Phylogenetic analysis

The evolutionary molecular model GTR + G was chosen using the software jModelTest (Darriba et al., 2012). The Maximum Likelihood (ML) tree was built in the software Treefinder (Jobb, 2011) with 20,000 replicas of bootstrap using *Melanophryniscus stelzneri* and *Melanophyniscus* sp., as external groups following

Lötters et al. (2011). The uncorrected peer-to-peer genetic distance (p-distance) between species was calculated with Mega 6.0 software (Tamura et al., 2011).

Delimitation of species

Automatic Barcode Gap Discovery or ABGD method (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) was used to identify clusters of sequences that may correspond to more than one species based on the distribution of genetic distances between aligned DNA sequences (Guarnizo et al., 2015; Vacher et al., 2017). This method statistically infers several gaps or potential bar code thresholds, thus partitioning the sequences so that the distance between two sequences taken from distinct clusters will be greater than the bar code gap (Puillandre et al., 2012).

The alignment of the 16S mitochondrial gene was processed in ABGD in two nucleotide substitution methods, Kimura 2 Parameters - K2P (Kimura, 1980) and Simple Distance, under the following configurations: Pmin: 0.001, Pmax: 0.1, steps: 10, Nb bins : 20 and (X) relative gap width: 1.5. K2P is the standard model of DNA substitution by bar code studies, performing well because it is similar to other more complex models in species identification (Collins and Cruickshank, 2013), although studies suggest that the success rate of identification of species is not affected by the model (Collins et al., 2012).

RESULTS

The phylogenetic analysis of ML of *A. hoogmoedi* revealed a paraphyletic group with three strains: French Guiana 1 and 2; Guyana and Monte Alegre; and Anapu. The first two are north of the Amazon while the last is south of the Amazon River (Figure 2). *A hoogmoedi*, *A. flavescens* and *A. barbotini* from French Guiana represent sister species of *A. hoogmoedi* from Guyana and Monte Alegre, with high bootstrap support 93; however, the phylogenetic relationship between *A. hoogmoedi*, *A. barbotini* and *A. flavescens* French Guiana appears poorly resolved, with present polytomies. The lineage of *A. hoogmoedi* of Anapu (south of the Amazon river) appears as a sister group of *A. hoogmoedi*, *A. flavescens*, *A. barbotini* of French Guiana and *A. hoogmoedi* of Guiana and Monte Alegre with bootstrap value 57.

The genetic distance between the lines of *A. hoogmoedi* of French Guiana 1 and 2 was 0%, the same value was found between Monte Alegre and Guyana. The genetic distance between the lines Monte Alegre/Guyana and French Guiana 1 and 2 was 0.5%. The Anapu line, from south of the Amazon River, presented a genetic distance of 2.3% from the lineage of French Guiana 1 and 2 and 2.9% from Monte Alegre / Guyana. Several valid species of *Atelopus* (*A. hoogmoedi* Guiana, *A. barbotini* French Guiana, *A. flavescens* French Guiana, *A. seminiferus* Peru, *A. pulcher* Peru, *A. spumariu*s Peru) had genetic distances varying from 0 - 2.6% while the same species presented values ranging from 2.3 - 3.2% for the Anapu line south of the Amazon River (Table 2).

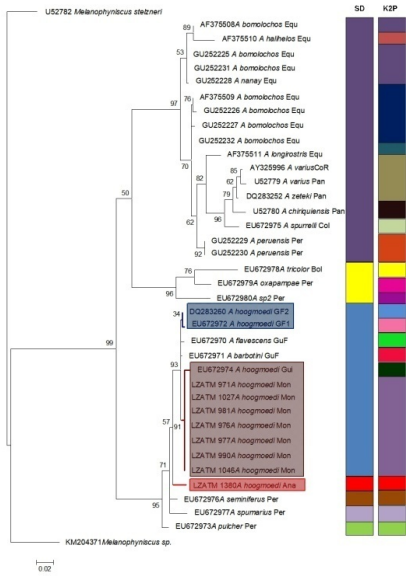

Figure 2. Tree ML with abbreviations: Equador (Equ); Costa Rica (CoR); Panama (Pan); Colombia (Col); Peru (Per); Bolivia (Bol); French Guiana 1 (GF1); French Guiana 2 (GF2); French Guiana (GuF); Guiana (Gui); Monte Alegre (Mon); Anapu (Ana); Each color in the bar on the right represents a species found by the ABGD method, based on Simple Distance and Kimura 2 Parameters.

Table 2. Genetic Distance based on the 16S rRNA marker.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1																							
2	2.9																						
3	2.3	0.5																					
4	2.3	0.5	0.0																				
5	2.9	0.0	0.5	0.5																			
6	2.3	0.5	0.0	0.0	0.5																		
7	2.3	0.5	0.0	0.0	0.5	0.0																	
8	2.6	2.6	2.0	2.0	2.6	2.0	2.0																
9	2.9	2.3	1.7	1.7	2.3	1.7	1.7	1.7															
10	3.2	2.0	1.4	1.4	2.0	1.4	1.4	1.7	1.7														
11	8.8	9.4	9.1	9.1	9.4	9.1	9.1	8.8	7.6	8.2													
12	9.1	9.8	9.5	9.5	9.8	9.5	9.5	8.9	8.1	8.7	0.9												
13	9.1	9.7	9.4	9.4	9.7	9.4	9.4	9.1	7.9	9.1	2.3	2.9											
14	9.4	10.2	10.0	10.0	10.2	10.0	10.0	9.1	9.1	9.1	2.6	2.2	3.8										
15	10.5	10.8	10.5	10.5	10.8	10.5	10.5	10.5	9.4	10.0	9.7	10.1	10.8	11.4									
16	10.5	9.1	9.4	9.4	9.1	9.4	9.4	9.4	7.6	8.8	8.8	9.2	10.0	11.1	4.4								
17	10.8	11.4	11.1	11.1	11.4	11.1	11.1	10.5	10.2	10.2	3.2	3.0	4.7	2.6	10.8	10.0							
18	10.8	10.5	10.2	10.2	10.5	10.2	10.2	9.7	8.5	10.2	8.5	8.9	8.8	10.8	6.4	5.5	8.8						
19	11.1	12.0	11.7	11.7	12.0	11.7	11.7	11.1	10.8	11.4	5.2	5.4	7.0	5.2	11.4	11.1	4.1	9.7					
20	12.0	12.9	12.6	12.6	12.9	12.6	12.6	12.0	11.7	12.3	5.5	5.7	7.3	5.5	12.3	12.0	4.4	10.5	0.8				
21	12.3	13.2	12.9	12.9	13.2	12.9	12.9	12.3	12.0	12.6	5.8	6.0	7.6	5.8	12.6	12.3	4.7	10.8	1.1	0.8			
22	12.6	12.9	12.6	12.6	12.9	12.6	12.6	12.3	11.7	11.7	4.7	4.6	5.8	4.4	12.0	11.1	2.9	9.7	3.2	3.5	3.8		
23	12.6	13.2	12.9	12.9	13.2	12.9	12.9	12.3	11.4	12.6	5.0	4.7	6.7	5.0	12.0	11.7	3.8	10.2	1.4	1.7	1.4	2.9	

1) *Atelopus hoogmoedi* (Anapu); 2) *A. hoogmoedi* (Monte Alegre); 3) *A. hoogmoedi* (French Guiana 1); 4) *A. hoogmoedi* (French Guiana 2); 5) *A. hoogmoedi* (Guiana); 6) *A. barbotini* (French Guiana); 7) *A. flavescens* (French Guiana); 8) *A. seminiferus* (Peru); 9) *A. pulcher* (Peru); 10) *A. spumarius* (Peru); 11) *A. nanay* (Ecuador); 12) *A. bomolochos* (Ecuador); 13) *A. halihelos* (Ecuador); 14) *A. peruensis* (Peru); 15) *A. tricolor* (Bolivia); 16) *A. oxapampae* (Peru); 17) *A. longirostris* (Ecuador); 18) *A. sp.2* (Peru); 19) *A. zeteki* (Panama); 20) *A. varius* (Costa Rica); 21) *A. varius* (Panama); 22) *A. spurrelli* (Colombia); 23) *A. chiriquiensis* (Panama).

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Based on ABGD, the simple distance model indicated that *A. hoogmoedi* of French Guiana 1, 2, Guyana, Monte Alegre, *A. flavescens* and *A. barbotini* represent a single species, whereas *A. hoogmoedi* of Anapu represents a distinct species. The K2P model showed that *A. hoogmoedi* of all localities mentioned above, *A. flavescens* and *A. barbotini* represent distinct species. Thus, in both models, *A. hoogmoedi* of Anapu (south of the Amazon river) is recognized as a species different from those of the Shield of the Guianas (Figure 2).

DISCUSSION

South of the Amazon River, *A. hoogmoedi* has been recorded in five localities: Tucuruí and Serra de Carajás (Xingu/Tocantíns-Araguaia Interfluvium), Itaituba and Santarém (Interfluvium Xingu/Tapajós) (Avila-Pires et al., 2010) of the lower Xingu River. These populations are separated from those of the Shield of Guianas by the Amazon River and between them by the Xingu River, delimiting two areas of endemism (Tapajós and Xingu) (Silva et al., 2002). Based on our data, we conclude that *A. hoogmoedi* of Anapu belongs to a different lineage than the Guiana Shield. Other studies indicate that *A. hoogmoedi* strains from south of the Amazon River are distinct from those of the Guiana Shield, as proposed by Noonan and Gaucher (2005).

In recent years, the 16S mitochondrial marker has been used to describe several species of Neotropical anurans (De Oliveira and Hernández-Ruz, 2017). The proposal of a limit value (>3%) to separate species with the 16S marker has been defended by several authors (Moraes et al., 2016; Lyra et al., 2017). However, some species are described with values lower than 3%, such as *Pseudopaludicola jaredi* (De Andrade et al., 2016) and *Proceratophrys ararype* (Mângia et al., 2018). Our results show that several valid *Atelopus* species present genetic distances ranging from 0 - 2.6% (*A. hoogmoedi*, *A. barbotini*, *A. flavescens*, *A. seminiferus*, *A. pulcher*, *A. spumarius*), whereas the same species presented genetic distances varying from 2.3 - 3.2% for *A. hoogmoedi* of Anapu. Values below 3% are observed in a complex of cryptic anurans, such as *Engystomops* and *Hypsiboas* (Funk et al., 2012) and *Ameerega* (Lötters et al., 2009).

In this work the proposed new *Atelopus hoogmoedi* line was verified to the south of the Amazon River, classified as an unconfirmed candidate species, requiring more collections and access to the individuals from other localities of its occurrence. The integration of morphological, bioacoustic and other molecular markers is strongly encouraged for integrative taxonomy (Padial and De La Riva, 2009), for the resolution of taxonomic status. Because the candidate species was found in a Sustainable Development Project, the need to establish priority areas for conservation is recommended until confirmation of occurrence in other locations.

CONCLUSIONS

Large genetic distances exist between the *A. hoogmoedi* populations to the south and north of the Amazon River; those found south of the Amazon River represent an unconfirmed candidate species. Thus, its conservation is of extreme importance due to the absence of studies of this population in relation to the Guiana Shield and species of this genus being in danger of extinction.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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