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Not just the river: genes, shapes, and sounds reveal population-structured diversification in the Amazonian frog *Allobates tapajos* (Dendrobatoidea)

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In the Amazon basin, the distribution of many vertebrate species is delimited by large rivers, which are frequently considered as biogeographical barriers strongly related to the origin and maintenance of the elevated biodiversity found in the region. In this study, we conducted a phylogeographical investigation of the effect of the Tapajós River on multiple classes of genotypic and phenotypic characters in a species of frog, *Allobates tapajos*. We sampled populations throughout the known distributional range of the species on both margins of the middle and lower sections of the river. We obtained fragments of mitochondrial (16S) and nuclear (RAG1) genes, as well as external morphometric measurements and advertisement call acoustic parameters of 48 individuals from six localities (populations). While the nuclear marker was monomorphic across the geographic distribution of *A. tapajos*, the mitochondrial fragment revealed low genetic distances accompanied by high spatial structuring, with restricted and absent haplotype sharing between populations and opposite river margins, respectively. Cladogenetic events were concentrated in the Pleistocene epoch, the time period corresponding to the establishment of the Tapajós River drainage. Acoustic parameters diverged between river margins, a pattern not observed in relation to the morphological markers analysed. There was no correlation in the variability pattern of the different classes of characters between them or in relation to linear geographic distance among populations. In addition, discriminant function analyses correctly assigned most of the individuals to their populations based on phenotypic characters. Our results show that the distribution of the variability within *A. tapajos* is affected not only by the transposition of a historical riverine barrier but also mostly by an elevated genotypic and phenotypic structure at the population level.

ADDITIONAL KEYWORDS: Amazon – Anura – bioacoustics – microevolution – phylogeography – Tapajós River.

INTRODUCTION

Evolutionary divergence between populations isolated by a geographical barrier is a frequently observed phenomenon and has been widely investigated in the context of allopatric differentiation (Mayr, 1947; Coyne & Orr, 2004). In isolation, such populations can respond to local interactions with the environment or with other species. This can result in local variation in the type and intensity of selective pressures (Schluter, 2001; Funk, Nosil & Etges, 2006). In addition, divergence between such populations may occur in the absence of selective factors due to the stochasticity of genetic drift (Avise, 2000). Thus, populations living in allopatry are good candidates for the study of genetic and phenotypic differences, since microevolutionary mechanisms may act directly and differentially on such characteristics. This may result in the evolution of differences in traits that contribute to reproductive isolation, with direct implications for the speciation process (Greenberg *et al.*, 2003; Coyne & Orr, 2004; Hoskin & Higgie, 2010).

Regardless of the evolutionary mechanisms promoting divergence, the longer groups have been separated, the greater should be the genotypic and phenotypic divergence between, likely due to the

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accumulation of mutations that become fixed over evolutionary time (Adams *et al.*, 2009). The magnitude of divergence of genetic and phenotypic characteristics between populations can be considered as indicators of their current stage in the speciation process (Coyne & Orr, 1997; Avise, 2000). Such characters can be used as markers in intraspecific studies designed to assess spatial patterns of variation at the population level, as well as investigations of the evolutionary mechanisms underlying such patterns (Habel *et al.*, 2015).

While the genotypic characters used as evolutionary markers in various taxa often focus on DNA nucleotide polymorphisms (Avise, 2004; Bar-Yaacov *et al.*, 2015; Toews *et al.*, 2016), the phenotypic characters that can be investigated often depend on particularities of the taxonomic group under study. In amphibians, morphological traits have served historically as the most commonly used markers in the study of between-population variations. This has resulted in great emphasis on morphology when proposing concepts and diagnoses at the species level (Cronquist, 1978; Coyne & Orr, 2004; Padial & De La Riva, 2010). The validity of this approach is supported by the fact that several studies have shown morphological differentiation, especially in relation to body shapes, among genetically divergent populations of Amphibia (Amézquita *et al.*, 2009; Kaefer *et al.*, 2013). Bioacoustic characters are another class of phenotypic characters that, in frogs, are commonly used for population-level studies. Vocalizations, especially the advertisement call, are the main form of communication in frogs, and bioacoustic characters carry a strong phylogenetic signal (Erdtmann & Amézquita, 2009; Goicoechea, De La Riva & Padial, 2010). Their importance in specific recognition means such calls may act as pre-zygotic reproductive isolation barriers, thereby leading to speciation via sexual selection (Gerhardt & Huber, 2002; Wells, 2007).

Many hypotheses about how species evolve in time and space are tested by phylogeographic approaches, which now integrate genetic markers to a great variety of other characters bearing phylogenetic signals, as well as physical and biological processes in historical time (Brusa *et al.*, 2013; Leite & Rogers, 2013; Maldonado-Coelho *et al.*, 2013; Brunes *et al.*, 2014; Fouquet *et al.*, 2014). Many studies in Amazonia focused on the rivers acting as barriers to gene flow leading to the diversification of terrestrial vertebrates (Peres, Patton & da Silva, 1996; Moritz *et al.*, 2000; Aleixo, 2004; Antonelli *et al.*, 2010; Ribas *et al.*, 2011). River barriers in Amazonia, especially when the river involved has few meanders, are considered to be one of the major factors affecting the spatial dynamics of species in historical time (Antonelli *et al.*, 2010; Hoorn *et al.*, 2010).

In Amazonia, some of the first studies on the diversity and distribution of the Amazonian flora and fauna were conducted by the British naturalist Alfred Russel Wallace (Wallace, 1852). The patterns he found indicated that several species of vertebrates, notably primates and birds, had their distribution determined by large rivers, with the River Madeira, Solimões/Amazonas and Negro rivers being the major ones to delimit and separate populations, thereby creating the main areas of endemism within the Amazonian fauna (Cracraft, 1985; Ron, 2000; Ribas *et al.*, 2011; Silva, 2013). Currently, based on the distribution of birds and primates, nine areas of endemism are recognized for the Amazon Basin (Peres *et al.*, 1996; van Roosmalen *et al.*, 1998): Guyana, Imeri, Napo, Inambari, Rondônia, Rio Negro, Tapajós, Xingu, and Belém (Silva, Rylands & Da Fonseca, 2005; Borges & Da Silva, 2012; Smith *et al.*, 2014).

Studies on the Madeira River, a southern bank tributary of the Amazon, found that it is congruent with a distribution limit for several species of primates, birds, and frogs (Ayres & Clutton-Brock, 1992; van Roosmalen *et al.*, 2000; Dias-Terceiro *et al.*, 2015). In contrast, studies conducted in Juruá River found no morphological or molecular differences, or composition differentiation in the frog and mammal species assemblages on either side of the river (Gascon, Lougheed & Bogart, 1996, 1998; Lougheed *et al.*, 1999; Gascon *et al.*, 2000), suggesting that this river does not act as a barrier to the movement of individuals from one shore to another. This therefore suggests that the particular factors of each river, such as age, width, flow rate, and channel dynamics can modify a river's effectiveness as a barrier to the dispersion of organisms over historical time (Ayres & Clutton-Brock, 1992; Leite & Rogers, 2013).

Amphibians of the genus *Allobates* (Anura: Dendrobatoidea) have been used as model in addressing evolutionary questions, especially those relating to geographic patterns of diversification (Lougheed *et al.*, 1999; Amézquita *et al.*, 2009; Tsuji-Nishikido *et al.*, 2012; Simões *et al.*, 2014). *Allobates tapajos* (Lima, Simões & Kaefer, 2015) is a newly described anuran with a type locality located on the west bank of the Tapajós River. However, Lima *et al.* (2015) suggested that this species has geographic distribution along both banks of the middle and lower portions of the river. As the Tapajós River delimits two areas of Amazonian endemism (Rondônia and Tapajós), it is possible that *A. tapajos* from both river margins may be experiencing a process of allopatric differentiation. In this context, we aimed, in the current study, to test the hypothesis that the biogeographical barrier represented by the Tapajós River, acts on the genetic, acoustic and morphological variability among six populations of *A. tapajos* (as currently defined). Based

on the magnitude and consistency of the effect of this barrier across the different classes of characters, we expected to gain an indication of the stage of differentiation occurring in this species.

METHODS

Studied species

Allobates tapajos is distributed in the tropical *terra firme* (never flooded) forests on both banks of the middle and lower portions of the Tapajós River, southern Pará State, Brazil (Lima *et al.*, 2015). They are diurnal frogs found on the forest floor close to small waterways. Reproduction occurs during the rainy season, and egg deposition happens on folded dead leaves within the leaf-litter. Later, tadpoles are transported by one of the parents to water bodies where they metamorphose into adults (Lima *et al.*, 2015).

Study area

The current study was conducted in the state of Pará, Brazil, at six different locations (hereafter considered separate populations) on both sides of the middle and lower Tapajós River, a major tributary of the Amazon (Fig. 1), between the municipalities of Santarém (2°26′22″S/54°41′55″W) and Itaituba (4°16′9″S/55°59′23″W). The average annual temperature in the region is 27.5 °C, and the average annual rainfall is 1950 mm. Rainfall is seasonal, with higher precipitation between the months of December and May (Miranda, 1993; Carvalho *et al.*, 2008).

Data collection

Collections were made where calling populations of *A. tapajos* were found. Field activities, including the acquisition of acoustic data and specimen collecting, took place from 12 January to 18 March 2015. All activities were conducted during the day (Table 1).

Acoustic data

Advertisement calls of *A. tapajos* males were recorded with a Marantz PMD660 digital recorder linked to an AKG 568 EB directional microphone, positioned 1.5 m from the calling animal. Each recording was composed of a minimum of three continuous minutes of vocalizations. Since air temperature may have an effect on acoustic properties of frog vocalizations (Gerhardt & Huber, 2002), this variable was measured after each recording using a thermometer.

Recordings of vocalizations were analysed using the Raven 1.4 program (Charif, Strickman & Waack, 2010). The *A. tapajos* advertisement call has the form of a continuous series of notes largely arranged in pairs or trios (Lima *et al.*, 2015). Spectrogram measurements

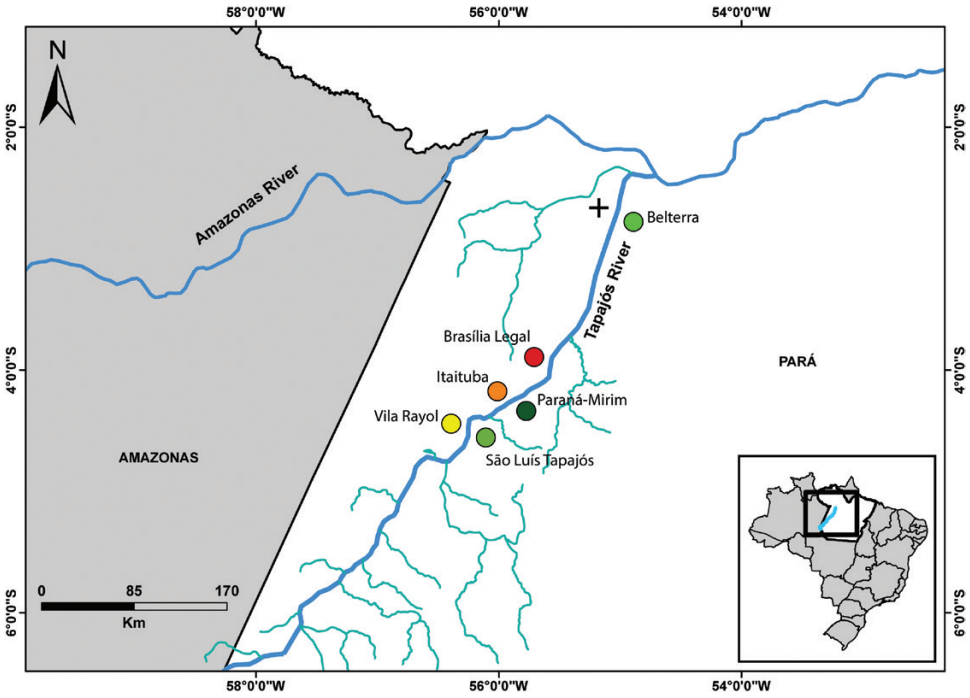

Figure 1. Geographical location of the six *A. tapajos* sampling points in the state of Pará. Point 1 (in yellow) is located near the type locality of the species. The sign (+) indicates that the species was not found at the site.

Table 1. Study sites in the state of Pará, with their geographical coordinates and size of the samples for each data class

Locality	Coordinates	Morphology	Acoustics	mtDNA	nuDNA
1. Vila Rayol	4°27′30.93″S 56°16′12.85″W	8	8	8	2
2. Itaituba	4°17′14.77″S 56°01′55.00″W	8	8	8	3
3. Brasília Legal	3°56′28.92″S 55°34′56.18″W	8	8	8	4
4. São Luís do Tapajós	4°27′24.645″S 56°14′53.213″W	8	8	8	2
5. Paraná-Mirim	4°19′23.33″S 56°00′39.42″W	7	7	8	2
6. Belterra	2°44′59.112″S 54°57′29.357″W	8	8	8	3
Total		**47**	**47**	**48**	**16**

were taken after a Fast Fourier Transform with a Blackman-type window at a resolution of 82 Hz and 2048 points. We measured the following temporal parameters: note duration (in minutes), note repetition rate (in notes/minutes), between-note interval (in seconds), and between-call interval (in seconds). The analysed spectral variables were obtained from selections made by applying the tool 'Selection spectrum' to the generated oscillograms: maximum (peak) frequency (in Hz, as the frequency of higher intensity calculated for the entire call by a power spectrum function), lowest frequency (in Hz), and highest frequency (in Hz). Lowest and highest frequencies were measured at 20 dB below the peak intensity, the value at which the signal energy could still be clearly distinguished from background noise (Kaefer & Lima, 2012). Ten calls were analysed per individual, and the arithmetic mean of the values obtained for each parameter was used as the final value for each specimen.

Morphological data

The collected animals were sacrificed by the application of a topical anesthetic cream (5% lidocaine), labelled, fixed in commercial formaldehyde diluted to 10%, then preserved in 70% ethanol. Eighteen external morphometric measurements (Table S1) were taken in the laboratory with the aid of optical stereomicroscope-coupled ocular micrometre. All measurements were taken from the left side of preserved individuals (Simões *et al.*, 2013). The specimens were preserved with the collection of amphibians and reptiles of the National Institute of Amazonian Research, Manaus, Brazil (INPA-H), as voucher numbers 021245–036785.

Molecular data

Muscle tissue was collected from individual *Allobates* and preserved in ethanol prior to the fixation of the donor animal in formaldehyde. Tissues were dissolved in proteinase K/SDS solution, and total genomic DNA was isolated from samples using the Genomic DNA Purification Kit (Promega, Madison, WI, USA), following the protocols provided by the manufacturer. Segments of 16S rRNA mitochondrial DNA and RAG1 nuclear DNA regions were amplified using the polymerase chain reaction (PCR) with universal primers (oligonucleotide initiators). These segments were chosen due to their ability to reveal phylogeographic patterns. In addition, the 16S region is widely used as a DNA barcode for amphibians (Vences *et al.*, 2005a; Fouquet *et al.*, 2007). A PCR was performed for both fragments in a final volume of 15 µL. For 16S, the reaction contained 6.2 µL of distilled and deionized water; 1.5 µL MgCl (25 mM); 1.5 µL of Tris-HCl buffer (10 mM); 1.5 l dNTPs (25 mM); 1.5 µL 16*sar* primer (5′-CGCCTGTTTATCAAAAACAT-3′) and 16*sbr* (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi, 1996) at 2 pmol/µL; 0.3 µL of taq DNA-polymerase (5 U/µL); and 1 uL of DNA (10 ng/µL). For the nuclear gene RAG1, the reaction contained 6.2 µL distilled and deionized water; 1.5 µL MgCl (50 mM); 1.5 µL of Tris-HCl buffer (10 mM); 1.5 l dNTPs (25 mM); 1.5 µL of each primer Amp_F2 (5′-ACNGGNMGICARATCTTYCARCC-3′); and Amp_R1 (5′-AACTACGCTGCATTKCCAATRTCACA-3′) (Chiari *et al.*, 2004) at 2 pmol/µL; 0.3 µL of Taq DNA-polymerase (5 U/µL); and 1 µL of DNA (10 ng/µL). For the amplification reaction of the 16S fragment, the following thermocycling process was used: 92 °C for 30 s for the initial denaturation, 35 cycles of denaturation at a temperature of 92 °C for 10 s, annealing at 50 °C for 35 s, and extension at 72 °C for 90 s; the final extension was performed at 72 °C for 10 min. For RAG1, 92 °C for 30 s for the initial denaturation, 35 cycles of denaturation at a temperature of 92 °C for 10 s, annealing between 55 °C and 57 °C for 35 s, and extension at 72 °C for 90 s; the final extension was performed at 72 °C for 10 min. A sample of 2 µL of each PCR product was analysed by electrophoresis of 1% agarose gel stained with ethidium bromide. PCR products were purified by reaction with EXO-SAP, following the protocol suggested by the manufacturer. Sequencing reactions were performed using the Big Dye kit (Applied Biosystems), according to the manufacturer's instructions. Sequencing products were

precipitated by EDTA/ethanol and analysed in an ABI 3130×l automatic capillary sequencer (Applied Biosystems). These procedures were performed in the molecular biology thematic laboratory of the National Institute for Amazonian Research, Manaus, Amazonas, Brazil.

Homologous regions of nucleotide sequences were automatically aligned using the MUSCLE algorithm (Edgar, 2004), the MEGA 6.6 program (Tamura *et al.*, 2013), which makes sequences available for visual inspection of errors and for coding any insertions or deletions. Sequences were manually checked in Geneious 5.3.4 (Kearse *et al.*, 2012). Final alignments had a length of 517 basepairs (bp) for the mtDNA 16S fragment and 555 bp for nuDNA RAG1. Sequences of representative haplotypes were deposited in the GenBank database according to Table S2 and S3.

DATA ANALYSIS

Population analysis

Genealogical relations between individual samples, taking into consideration the source populations, were estimated by means of a haplotype network using Haploview 4.2 program (Barrett *et al.*, 2005). A maximum likelihood tree (Table S4), necessary to estimate the network, was generated in RaxML 7 (Stamatakis, 2006), in accordance with a nucleotide substitution pattern preset in jModelTest 2 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012). The relative partitioning of the genetic variation (within populations, between populations, and between river banks) was established by standard analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) using the Arlequin Version 3.5 program (Excoffier & Lischer, 2010). The relative genetic structure between sampling locations was estimated by calculating distance-based fixation indexes (F_{ST}: Wright, 1951), which is equivalent to the N_{ST} of Lynch & Crease (1990). Statistically significant F_{ST} values were obtained after 1023 haplotype permutations in Arlequin. Pairwise genetic differentiation between sampling sites was estimated with the Kimura two-parameter distance (Kimura, 1980) in Mega 6 (Tamura *et al.*, 2013). The most probable number of genetic clusters formed by the sampled mtDNA sequences was inferred by Bayesian analysis of population structure using BAPS Version 6 (Corander *et al.*, 2008). Based on nucleotide frequencies, this model seeks to generate k groups of individuals, so that those assigned to the same group are as genetically similar to each other as possible. The upper limit for the number of groups was set from the number of sampling sites (n = 6). Log-likelihood values for the best models were used to select the arrangement of the most likely groups.

Divergence time estimation

Unique haplotypes were obtained from the population analyses carried out in Arlequin 3.5. Available 16S sequences of *A. gasconi* (GenBank KJ747333, GenBank KJ747334) were used as the outgroup since this is the most closely related species in relation to *A. tapajos* (Lima *et al.*, 2015). In addition, the co-sequence-specific 16S *A. tapajos* (GenBank KR047027, GenBank KR047028, type-location) and *Allobates* 'aff. *Marchesianus*' (GenBank EU342545, GenBank EU342546, Location Curuá-Una – which proved to be *A. tapajos*) were used as an internal group. Since there are no fossil *Allobates* or closely related anuran taxa, we used a calibration method based on mitochondrial DNA substitution rates to provide an idea about the relative divergence times. The time sequence of diversification was estimated via BEAST 1.7 (Drummond & Rambaut, 2007; Drummond *et al.*, 2012), using the settings "uncorrelated relaxed clock" and "Yule process prior" (Aldous, 2001). As a substitution rate, we used the 0.0069 site/million years proposed for amphibian mitochondrial DNA by Macey *et al.* (1998). An initial tree was generated randomly, with a chain length of 10 million generations, with samples taken every 10000 generations, discarding 10% of the trees as burn-in, resulting in 900 trees sampled in a Monte Carlo Markov Chain (MCMC). The stationarity of the posterior distributions for all model parameters, including medians and ranges from 95% (HPD) of the nodes, was assessed using Tracer 1.5 (Drummond & Rambaut, 2007). From the MCMC output, a maximum credibility tree was generated using Tree Annotator 1.6.2 (Drummond & Rambaut, 2007).

Phenotypic differentiation

Differentiation in acoustic and morphological characters on either side of the Tapajós River was tested using two generalized linear models (GLMs, one for each character class) using the first two components of each character class generated by a principal component analysis (PCA) as the dependent variables. Loadings of the acoustic and morphological characters on the first two components are provided in Table S5. In addition, river banks were considered as the independent variables. Discriminant function analysis (DFA) was used to test whether the sampled populations differed phenotypically, with acoustic and morphometric characters as predictor variables. Discriminant function was used to compute the probability of assigning each individual to its population (variable group) by a Jackknife classification matrix. This method included

all male specimens for which measurements were available. The adjustment of the bioacoustic data by linear regression was not needed as neither temperature (23.7–28.5 °C) nor body size (SVL, 14.3–18.3 mm) had an effect on the acoustic variables (Kaefer *et al.*, unpublished data). In order to minimize the effect of body size on morphometric measurements, a series of 12 morphological ratios was used as predictor variables (Verdade & Rodrigues, 2007): LL/SVL, HAND3/SVL, FL/SVL, HW/HL, EN/HL, EL/HL, TYM/HL, TYM/EL, IN/HW, HAND1/HAND2, HAND2/HAND3, and HAND4/HAND1 (See Table S1 for acronym meanings). These analyses were conducted on Systat Version 12 (Systat Software, San Jose, CA).

Correlation between geographic, phenotypic, and genetic differentiation

We tested the correlations between linear geographic (measured in kilometre from GPS coordinates), phenotypic, and genetic distances between populations by using Mantel tests with distance matrices (Mantel, 1967). We also performed partial Mantel tests to assess the correlation of genetic/phenotypic distances between populations by controlling the effects of the river transposition and geographical distance between sampling sites (Smouse, Long & Sokal, 1986; Telles *et al.*, 2001). A binary correlation matrix was constructed by assigning the values '0' and '1' to, respectively, pairs of locations within and between the opposing sides of the river.

A matrix of acoustic distances was obtained using Euclidean distances between all possible population pairs using the scores for the means of acoustic measurements of the first and second components produced by a PCA. The major components were obtained from the arithmetic mean of the advertisement call parameters between the sampled specimens from each sample location and were used to reduce the number of independent phenotypic variables. The same process was used to generate an array of morphological distances. Mantel tests were conducted using the ZT program (Bonnet & Van de Peer, 2002) via permutation of null models (Anderson & Legendre, 1999) with 10 000 randomizations.

RESULTS

Population analysis

We obtained 48 16S mtDNA sequences corresponding to 20 different haplotypes and 16 RAG1 nuDNA sequences corresponding to a single haplotype. Most of the mitochondrial DNA haplotypes were restricted to a single location, and there was no sharing of haplotypes between the two river banks (Fig. 2).

The AMOVA indicated that most of the total genetic variation was found between populations present on the same river bank (49.88%). River banks were responsible for 17.34% of the genetic variation (Table 2). F_{ST} values showed a high and significant level of general population structuring (Table 3). The highest genetic distances (1.3 and 1.2%) were observed between populations on opposite banks of the river. BAPS supported the partition of individuals of *A. tapajos* into three genetic groups, two of them restricted to

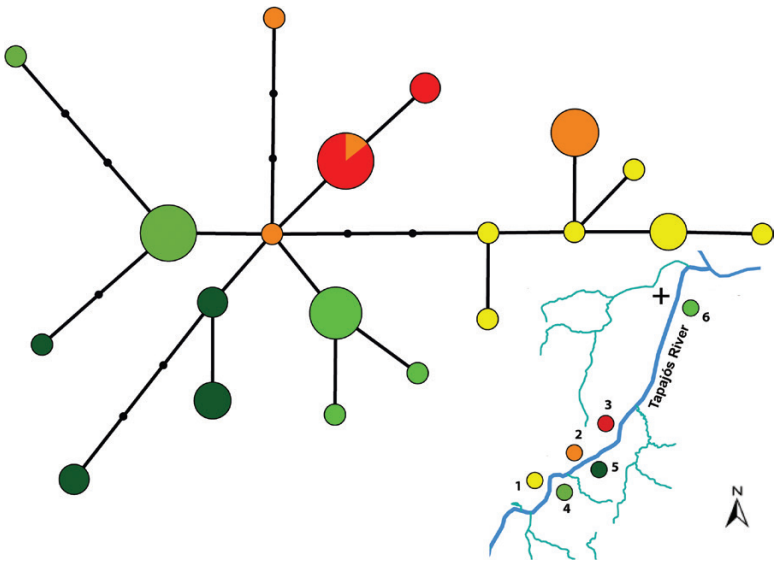

Figure 2. Haplotype network with 48 sequences of 16S mtDNA for *A. tapajos*. The size of each ellipse indicates the relative haplotype frequency, and the color indicates the origin of individuals according to the geographical map.

interfluvial regions (ML value = –280.7419; P = 0.95 or 0.04149). A co-occurrence of genetic groups within localities was observed (Fig. 3). Four locations (1, 3, 4, and 6) contained individuals from only one genetic group.

Divergence times estimation

There was weak support for intraspecific cladogenetic events in *A. tapajos*, with the exception of one clade composed exclusively of west bank haplotypes, which includes the type locality of the species. The most basal division in *A. tapajos* was assigned to the Pleistocene [median = 1.58 million years (mya) and HPD = 0.79, 2.42], with most of the diversification events occurring over the last million years (Fig. 4).

Phenotypic differentiation

Discriminant function analysis correctly identified 70.0% of the source locations of individual *A. tapajos* based on acoustic characters. When using morphological characters, 66% of *A. tapajos* individuals were allocated correctly to their populations of origin (Table S6–S8). For the acoustic data, the two main components together explained 70% of variability in *A. tapajos*, while the first two morphological components explained 43.34% of the data variability. The results of the GLMs showed significant difference in acoustic characteristics between the two river banks (F = 5.485 and P = 0.007, Fig. 5A), while individuals from different sides of the river did not differ in morphometric characters (F = 1.397 and P = 0.258, Fig. 5B).

Table 2. Analysis of molecular variance (AMOVA) based on the fragments of mitochondrial *16S* DNA. The relative distribution of genetic variability of *A. tapajos* is presented according to hierarchical levels

Source of variation	Percentage of variation
Between riversides	17.34
Among populations within riversides	49.88
Within populations	32.77

Correlation between geographical, phenotypic, and genetic distances

Mantel tests showed no correlation between the different sets of tested data, even when the effects of the river and distance were controlled via partial models; an exception was the correlation between the binary variable side of the river and acoustic distance (Table 4).

DISCUSSION

Overall, our results revealed a subtle but spatially structured differentiation process in the analysed character classes. While morphological and nuclear DNA characters exhibited, respectively, low and no variability over the study area, acoustic characters and mitochondrial DNA showed moderate spatially structured differentiation, both among populations and between the margins of the Tapajós River, the main geographical barrier in the study area.

The differing results obtained from nuclear and mitochondrial DNA markers reflect their different mutation rates and effective population sizes (slower and larger in nuclear DNA, respectively (Brown, George & Wilson, 1979; Moritz, Dowling & Brown, 1987). Mitochondrial DNA is widely used in phylogeographic studies and barcode approaches in amphibians (Vences *et al.*, 2005b) and, in this study, was somewhat variable despite the low degree of divergence among populations and between the river banks. High levels of genetic structure in mitochondrial DNA, such as observed within the distribution *A. tapajos*, have often been reported in studies involving frogs (Carnaval & Bates, 2007; Hurzaid *et al.*, 2014), particularly in

Table 3. Pairwise F_{ST} fixation indexes (lower left matrix) and average (%) Kimura 2-parameter genetic distances (upper right matrix).

Locality	Vila	Itaituba	Brasília	São Luís	Paraná	Belterra
1. Vila (W)	–	0.7	1.1	1.2	1.3	1.1
2. Itaituba (W)	0.231*	–	0.9	1.0	1.1	0.9
3. Brasília (W)	0.814*	0.555 *	–	0.5	0.7	0.5
4. São Luís (E)	0.791*	0.557*	0.775*	–	0.7	0.5
5. Paraná (E)	0.707*	0.472*	0.620*	0.561*	–	0.7
6. Belterra (E)	0.808*	0.571*	0.814 *	0.761*	0.610*	–

Note: Populations west (W) and east (E) from Tapajós river are indicated. Values calculated between the sampling localities for each *A. tapajos* population. Significant F_{ST} values are indicated with asterisks. Locality numbers are shown according to Fig. 1.

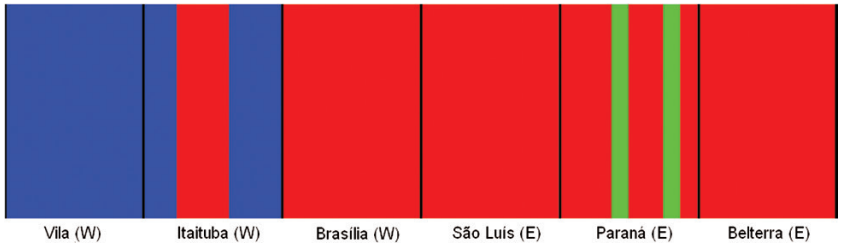

Figure 3. Bar plot from the Bayesian Analysis of population structure of 48 individuals of *A. tapajos*. Distinct colors represent each of the three estimated genetic clusters. Individuals are displayed according to sampling localities identified in the lower panel.

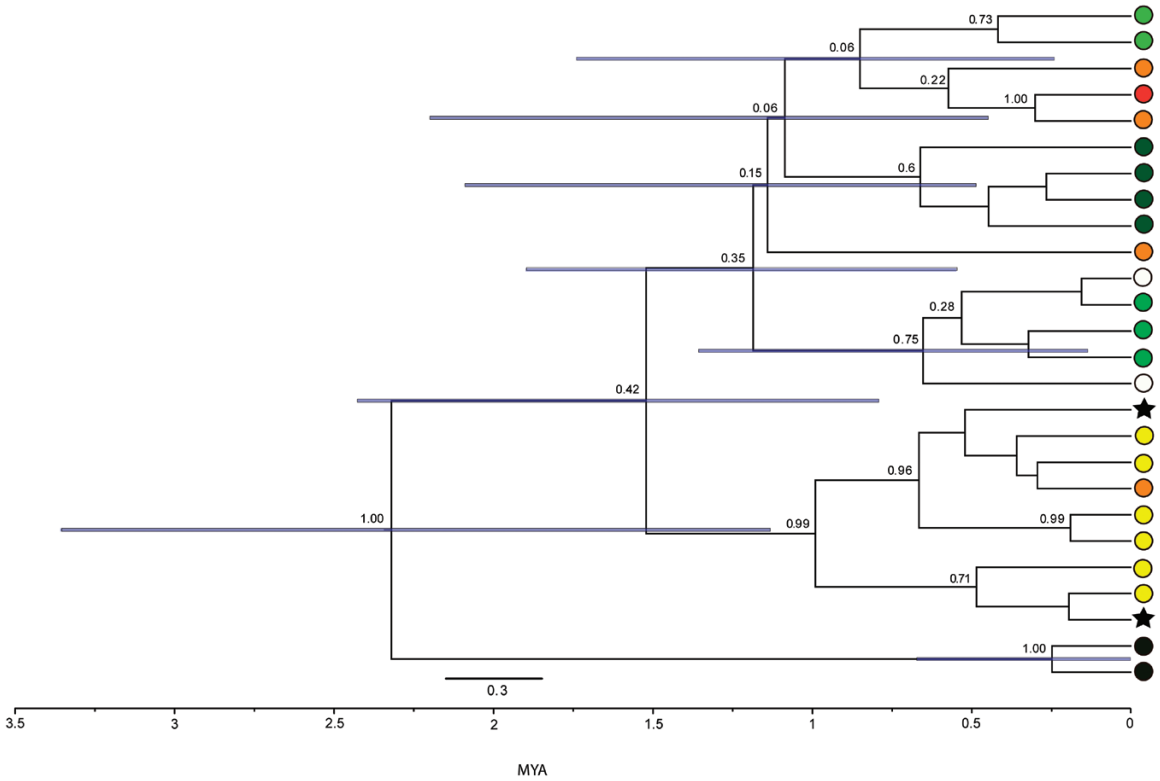

Figure 4. Timetree of *A. tapajos* based on fragments of the 16S rDNA gene. Only unique haplotypes were included. Age estimates and confidence intervals (horizontal bars) of the divergences were obtained via Bayesian Evolutionary Analysis Sampling Trees (BEAST). Posterior probabilities of the older nodes are presented. Terminals were colored according to their localities of origin, which are depicted in Fig. 1. White terminals represent Genbank sequences from the east margin of the river (Curuá-Una). The stars depict Genbank sequences from the type locality of the species in the west margin of the Tapajós River. Shades of green and orange represent the right and left margins, respectively.

small-sized and territorial Neotropical species, in which gene flow between sites is probably restricted due to low individual dispersion capacity (Kaefer *et al.*, 2013; Fouquet *et al.*, 2015). Our results, specifically (1) the significance levels of the F_{ST} values for all pairs of locations, (2) the haplotype network in which there was restricted and no gene sequence sharing between localities and between opposite banks of the Tapajós River, respectively, (3) the AMOVA results in which 49.8% of the genetic variability of the study system is distributed among populations, are all consistent with the hypothesis of low dispersal in *A. tapajos*. Besides the population structure found in molecular characters, the discriminant function analyses demonstrated that the phenotypic variability also mirrored our sampling design by the correct assignment of most of the individuals to their respective geographic locations. The accuracy rate based on acoustic (70%)

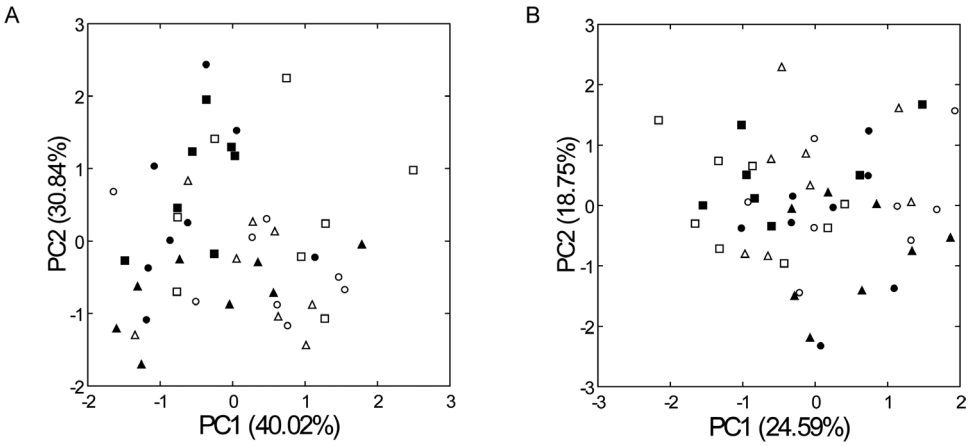

Figure 5. Distribution of individual scores of *A. tapajos* along the first and second principal components according to acoustic (A) and morphological (B) characters. Open and closed symbols indicate individuals from the west and east riverbanks, respectively. Each symbol indicates a sampling locality.

Table 4. Simple and partial Mantel tests evaluating correlations among geographic, phenotypic, and genetic distances of *A. tapajos* from the sampled localities

Model (Distance × Distance)	*r*	*P*
Genetic × Morphological	0.404	0.130
Genetic × Acoustic	0.434	0.055
Morphological × Acoustic	–0.167	0.216
Genetic × Geographic.River	0.483	0.083
Morphological × Geographic.River	0.108	0.350
Acoustic × Geographic.River	–0.180	0.358
Genetic × River.Geographic	0.393	0.095
Morphological × River.Geographic	0.003	0.433
Acoustic × River.Geographic	0.875	0.043*

Note: Simple Mantel tests are presented as 'Distance Matrix 1' × 'Distance Matrix 2', and partial Mantel tests are presented as 'Distance Matrix 1' × 'Distance Matrix 2'. 'Covariate matrix'. Significant correlations are indicated with asterisks.

and morphological (66%) traits demonstrates that phenotypes are also spatially structured according to populations even at the regional scale considered in this study.

The analysed phenotypic traits varied less between the sampled populations than did mtDNA. However, GLMs and the Mantel test showed variation in acoustic characters between the banks of the Tapajós River. This result may be related to the high levels of morphological conservatism reported in amphibians (Schonrogge *et al.*, 2002; Bickford *et al.*, 2007). When analysed in a single framework, it is often observed that acoustic characters show greater between-population variability than do morphological markers (Simões *et al.*, 2008; Tsuji-Nishikido *et al.*, 2012). In fact, acoustic characters are reported to promote speciation processes in anurans via mate recognition and sexual selection (Boul *et al.*, 2007; Guerra & Ron, 2008).

A possible role of the Tapajós River as barrier may be related to the greatest values of genetic divergence being observed between population pairs located on opposite banks. However, given the occurrence of a genetic cluster on both sides of the river, and because there is nearly no haplotype sharing between populations also on the same side of the river, this effect seems to be limited. The restriction of gene flow by Amazonian rivers is probably the biogeographic pattern most commonly reported in studies involving terrestrial vertebrates (Antonelli *et al.*, 2010; Ribas *et al.*, 2011; Leite & Rogers, 2013), including frogs (Kaefer *et al.*, 2013; Simões *et al.*, 2014; Fouquet *et al.*, 2015). The current study is the first to investigate the effect of the Tapajós River on genetic variability in a species of frog. Although the development of the Tapajós River drainage system is considered recent compared to others in the Amazon basin (1.3 – 0.8 mya; Ribas *et al.*, 2011), this river is widely known as a barrier that delimits areas of endemism in Amazonia (Cracraft, 1985; Borges & Da Silva, 2012), phylogeographic patterns at both genus and species levels (Ribas *et al.*, 2011; Simões *et al.*, 2014), and even the distribution of terrestrial animal assemblages (Moraes *et al.*, 2016).

Testing the correlation between different character classes and geographic factors can contribute to the understanding of the roles of stochastic (drift) and deterministic (selection) forces in differentiation processes. Often the correlation between the variation in any class of character and geographical distance (isolation by distance) is attributed to the effect of drift on the evolution of these characteristics (Hutchison & Templeton, 1999). In this study, unlike the findings of larger scale

investigations on Amazonian frogs (Amézquita *et al.*, 2009; Kaefer *et al.*, 2013), no studied markers showed variation patterns that correlated with the linear distance between geographic locations even when the effect of the river was considered as a covariate. This suggests that the chosen markers may be under locale-specific selective pressure, such as environmental (natural) or sexual selection. Similarly, Mantel tests detected no relationship between the variability of the analysed characters and the transposition of the Tapajós River. A notable exception was the significant effect of the riverine barrier on the acoustic interpopulation distances, which supports the idea that such characters have greater spatial variability than do morphological ones. Indeed, advertisement calls have been proposed as phylogeographical markers (Wycherley, Doran & Beebee, 2002a) for showing correlation with genetic distances between populations (Wycherley, Doran & Beebee, 2002b; Kaefer *et al.*, 2013). Failure to find a correlation between genetic and acoustic distances in *A. tapajos* is probably due to the low range of variation, especially of the mitochondrial marker, which showed a maximum distance of only 1.3%.

In turn, correlation tests between different classes of genotypic and phenotypic markers can be used to provide clues about the relative evolution of different attributes in study populations (Pröhl *et al.*, 2006). However, except for a marginally significant relationship between genetic and acoustic distances, this study did not find correlations of this nature. This result reinforces the utility of advertisement calls as phylogeographic markers and the great potential of this class of characters in studies addressing the role of sexual selection in evolutionary processes. Advertisement calls function as sexual signals and thus can act as pre-zygotic barriers, via reduced preference and recognition between individuals from different populations should they ever meet (Ryan, 1988).

With regard to the specific status of the different sampled populations, the extent of haplotype restriction, genetic groupings, and significant acoustic differentiation between the two banks of the river was not sufficient to delimit distinct taxonomic units under any operational concept of species requiring diagnostic character recognition (Goldstein & DeSalle, 2011). Even under theoretical species concepts, such as the lineage concept (de Queiroz, 1998, 2007), the low genetic differentiation between populations from opposite sides of the river and the non-monophyly of the clades that represent them suggest the occurrence of an incipient differentiation process in the study system. This assumption is supported by estimates of a recent establishment of the Tapajós River drainage (Ribas *et al.*, 2011), as well as by the divergence times estimated in this study between the clades now occurring on each side the river, which proved to be congruent. Thus, the characterization of the spatial distribution of genotypic and phenotypic variability in *A. tapajos* illustrates a stage within the process of divergence affected by the transposition of a riverine barrier, but also mostly by an elevated structure at the population level.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Morphometric variables measured from *A. tapajos*.
Table S2. Distribution of 16S rDNA haplotypes of *A. tapajos* among six sampled localities sampled in Brazilian Amazonia. Collection numbers of vouchers (INPA-H) and GenBank accession numbers are provided concerning representative sequences.
Table S3. Haplotype distribution RAG1 nuDNA of *A. tapajos* between six sampled locations.
Table S4. Maximum likelihood tree generated on RaxML 7, using 48 individual fragments of 16S mtDNA (517 bp) from *A. tapajos*.
Table S5. Loadings of the acoustic (above) and morphometric (below) measurements in the first (PC1) and second (PC2) axes generated via PCA.
Table S6. Morphometric measurements (in mm) of individuals of *A. tapajos* in each study locality in Brazilian Amazonia. Values are presented as 'mean ± SD'. Morphometric traits are described in SM1.
Table S7. Advertisement call measurements of individuals of *A. tapajos* in each study locality in Brazilian Amazonia. Values are presented as 'mean ± SD'.
Table S8. Classification matrix based on the discriminant function analysis, using phenotypic data from males of *A. tapajos* from the east and west banks of the Tapajos River, Brazil.