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PRIMARY RESEARCH PAPER

Analysis of the mitochondrial D-Loop reveals that neither river boundaries nor geographic distance structure the fine-scale genetic variation of an Amazonian treefrog

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Abstract While most anurans have limited vagility and local fidelity, there are some exceptions. In the present study, we used *Boana boans*, a large treefrog found throughout most of the Amazon basin, as a model organism. We investigated the possible isolation of the *B. boans* demes located on opposite margins of the Juruena River and their population structure. We sampled 14 individuals of *B. boans* and analyzed the mitochondrial D-Loop to verify whether the river or Euclidean distance is acting as barrier to the dispersal of this frog. The sequencing revealed 12 haplotypes, with global *Fst* values of − 0.079, K2P values ranging from − 0.187 to 0.054, and primarily intrapopulation (81.78%) genetic diversity, with only 18.22% of the variation being found among populations. Analysis of molecular variance and Bayesian cluster analysis detected a lack of genetic structuring within the study area. The model species presented a capacity for dispersal over long distances in comparison with most other amphibians, which, together with its resistance to desiccation and reproductive mode, enable this treefrog to disperse across rivers and overland. In the specific case of Juruena River, many fluvial islands present within the study area may also be favorable to the dispersal of the species.

Keywords Anura · *Boana boans* · D-Loop · Landscape genetics · River barriers

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Introduction

The limited vagility and local fidelity of most amphibians have led researchers to consider anurans and caudates (less vagile) to be poor dispersers, given their morphological and metabolic constraints (Coster et al., 2015; Nowakowski et al., 2015). This designation of amphibians as poor dispersers generates a degree of inconsistency, especially considering that many species are widely distributed, in particular in the Neotropics (Reading et al., 1991; Gascon et al., 1998). In fact, anurans may often cover distances of up

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to 10 km (Smith & Green, 2005). At this spatial scale, gene flow may be hampered primarily by major barriers such as rivers (Angelone et al., 2011) which play a fundamental role in the maintenance of species diversity in the tropics, by impeding gene flow between populations on their opposite margins, and reinforcing possible allopatric speciation (Gascon et al., 1998).

Two theories have been proposed to account for the role of rivers in the zoogeography of vertebrates: Wallace's (1854) river barrier hypothesis and the river refuge hypothesis of Ayres & Clutton-Brock (1992). The latter hypothesis assumes that the Amazon rainforest contracted during the glaciations but did not disappear. This shrinkage reduced forest cover at the headwaters of Amazonian rivers, isolating populations at river mouths. The river barrier hypothesis has been tested in multiple vertebrate taxa over the past 160 years (Wallace, 1854; Gascon et al., 1998; Bates et al., 2004; Souza et al., 2013; Duarte et al., 2014).

The displacement and gene flow of terrestrial animals are influenced by a series of barriers, from anthropic or natural origin. An example of natural barriers is rivers, which exert a barrier function in all *taxa* (Waits et al., 2015). The Amazonian rivers are able to isolate populations and species of anurans, shaping the intraspecific population structure and contributing to the biogeographic regionalization of the Anura group (Godinho & Da Silva, 2018; Ortiz et al., 2018). These rivers can act as barriers to dispersal, where body size of anurans is a determining factor in the isolation and distribution of amphibian species (Moraes et al., 2016).

Studies of river barriers in amphibians based on molecular markers have revealed a number of different scenarios, ranging from highly structured populations forming well-defined clusters (Fouquet et al., 2012; Kaefer et al., 2013; Maia et al., 2017), to reduced structuring and limited genetic distance between demes (Gascon et al., 1998; Lougheed et al., 1999; Funk et al., 2007), and even panmixia (Crawford, 2003; Zeisset & Beebee, 2008). Rivers may play a relevant role in the genetic structuring of many anuran species, although the degree of permeability of these barriers depends fundamentally on the specific characteristics of each species (Fouquet et al., 2015). The application of molecular markers as an analytical marker is a relatively new approach that may help to answer many unanswered or poorly resolved questions.

Amphibians are considered to be valuable models for investigating the processes that shape the genetic structure of populations (Zeisset & Beebee, 2008). In the Amazon region, most of the studies have focused on dendrobatidis models, and there has been little work on hylids (Amézquita et al., 2009; Kaefer et al., 2013; Maia et al., 2017). In contrast with these models (Simões et al., 2014; Maia et al., 2017), *Boana boans* (Linnaeus, 1758) is a species of large frog and its males have the habit of vocalizing on the banks of the Amazonian rivers in their reproductive period; however, these males are also territorialist and philopatric to the reproductive site (Magnusson et al., 1999).

Among the mitochondrial molecular markers is the D-Loop or Control Region (CR), this region evolves much faster than the rest of the mitochondrial gene (Brown et al., 1986). This rapid change capacity (changeability) makes the D-Loop segment an appropriate marker to address genetic issues at the population level (Hoelzel et al., 1991), such as population diversity, (Chen et al., 2012; Kawabe et al., 2014), besides (as well as) being useful to test evolutionary relations and biodiversity (Arif & Khan, 2009). Although genetic studies of landscape and diversity are essential, there are few studies that use the D-Loop in anuran amphibians, which would reveal important information about intra- and interpopulation genetic diversity (Segelbacher et al., 2010). Genetic diversity among individuals of the same population is an important factor for fitness to environmental conditions (Takahashi et al., 2018). Therefore, knowledge of the genetic variability status and its spatial–temporal distribution are fundamental for a correct analysis of the situation and detection of possible threats to a species (Escudero et al., 2003). In the present study, we investigated the possible genetic isolation of demes in a local population of *B. boans* through the analysis of molecular diversity and population structure.

Materials and methods

Sampling

We captured the *Boana boans* specimens in the municipality of Cotriguaçu (09°49′09.0″ S,

58°15′31.1″ W), in northwestern Mato Grosso, Brazil. The study area encompassed a stretch of approximately 6 km of the Juruena River, a third-order tributary of the Amazon, which is 2,700–3,100 m wide at this point (Fig. 1). The level of the Juruena varies by up to 5 m between the rainy and the dry seasons. The margins of the river in this area are covered with well-preserved riparian forest with large trees, but few streams. This stretch of the rivers also has a number of rapids and rocky outcrops, and many islands, some of which are relatively large, with an area of over one hectare. Although we used the sample size of 14 individuals (from three to five at each point), this is usual among researches with mitochondrial *D-loop* DNA. For review, see Shaffer & McKnight (1996), Zhong et al. (2008) and Gvoždík et al. (2010) (one or two, three to seven and two to three individuals per population, respectively). Chen et al. (2012) used a sample size of three to nine specimens and considered the variation in his results as somewhat experimental. Tao et al. (2005) sequenced the *D-Loop* of 28 *Andrias davidianus* (Blanchard, 1871) salamander to investigate the patterns of genetic structure of four sites.

We conducted nocturnal visits to both left (LM) and right (RM1 and RM2) margins of the Juruena, and an island in the middle of the river, during which we located *B. boans* specimens through visual and auditory searches. While Vitt & Caldwell (2014) reported the aggregation of choirs of *B. boans* during the mating season, on the margins of the Juruena River, individual frogs were separated by distances exceeding 200 m. We collected 14 specimens of *B. boans* (13 males and one females), four from the island, three from the LM, four from RM1, and three from RM2 (the female was captured at the last mentioned location). Specimen collection was authorized by SISBIO permanent license 18573-1. We extracted a sample of liver tissue from each specimen and preserved it in 100% ethanol for the subsequent extraction of the mitochondrial DNA. We fixed all the specimens and deposited them as vouchers in the herpetological sector of the Biological Collection of Southern Amazonia (ABAM: *Acervo Biológico da Amazônia Meridional*) in Sinop, Mato Grosso (Brazil).

Extraction of the DNA

We extracted the total DNA using the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, Buchs, SG, Switzerland), following the manufacturer's recommendations. We quantified the DNA in a NanoK (Kasvi) spectrophotometer.

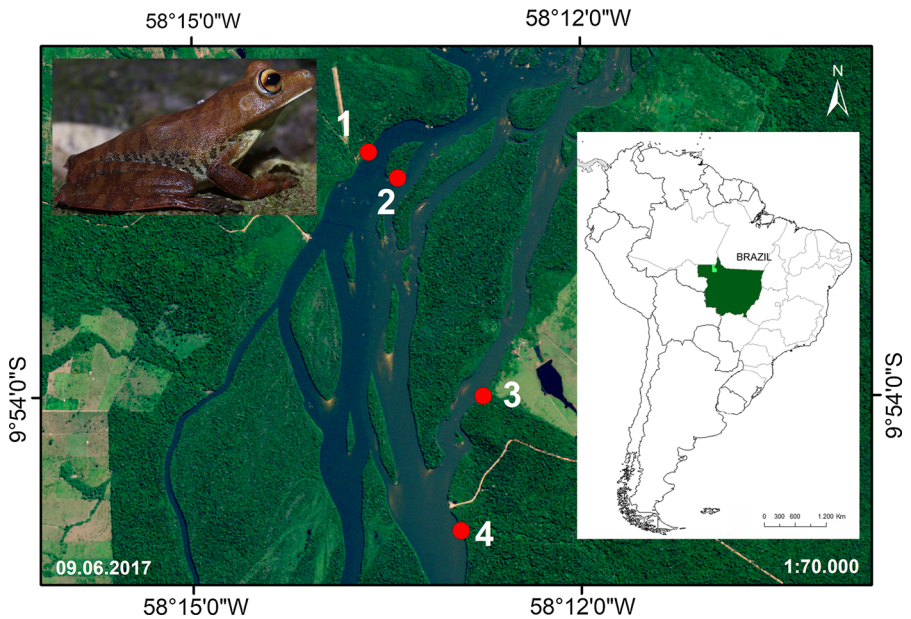

Fig. 1 Map of the study area located in the Juruena River domains located in the municipality of Cotriguaçu, Mato Grosso, Brazil. Collection points were identified with red circles. In upper left corner, a male individual of *B. boans*

Sequencing the mitochondrial DNA

We used the IP-H Control and Wrev-L Control primers—both described by Goebel et al. (1999)—to amplify the mitochondrial D-Loop. The reaction solution contained 1 × PCR buffer (Promega), 3 mM of $MgCl_2$, 4.6 mM of dNTPs, 0.6 mM of each primer, 2 U of Taq DNA polymerase (Promega), and 10 ng of the DNA. We ran the PCR in a thermocycler under the following conditions: 1 min at 94°C, followed by 36 cycles of 94°C (1 min), 48°C (40 s), and 72°C (1 min and 30 s), and then a final extension of 7 min at 72°C. We loaded the final PCR product into a 2.5% agarose gel stained with ethidium bromide. We purified the PCR products using the Wizard® SV Gel and PCR Clean-Up system (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. We then sent the samples to the Human Genome Research Center at the University of São Paulo, in São Paulo, Brazil, where they were sequenced with the same primers used in the amplification. We deposit the sequences on the GenBank—NCBI platform (Accession numbers: MK690361–MK690374).

Analysis of the mitochondrial D-Loop sequences

We obtained the consensus sequence for each specimen in the Electropherogram Quality Analysis software (Togawa et al., 2006). We used BIOEDIT (Hall, 1999) to edit the sequences, and MEGA 7 (Kumar et al., 2016) to align them and confirm the polymorphic sites and haplotype affinities.

We verified the saturation of substitutions in DAMBE (Xia, 2013) and selected the best nucleotide substitution model in MEGA 7 (Kumar et al., 2016), based on the Akaike Information Criterion (AIC). The software selected the Hasegawa, Kishino, and Yano model with a discrete Gamma- distribution (HKY+G), and the phylogenetic analyses were based on this model, using the Neighbor-Joining (NJ) algorithm (Saitou & Nei, 1987) in PAUP 4.0 and the Maximum Likelihood (ML) algorithm in PHYML (Guindon & Gascuel, 2003). The support for the NJ and ML analyses was based on 1,000 replicates. We analyzed the molecular fixation index (*Fst*) in ARLEQUIN v3.5.2.2 (Excoffier & Lischer, 2010) with the significance being tested by 20,000 permutations. We divided the genetic variation into intra- and interpopulation levels for the AMOVA, also run in ARLEQUIN v3.5.2.2 (Excoffier & Lischer, 2010). We estimated the pairwise genetic differentiation between sites based on the Kimura 2-parameter model (Kimura, 1980) in the Mega 7 program (Kumar et al., 2016).

We estimated the genetic relationships between individual samples in relation to their source populations, through a TCS haplotype network produced by the PopART program (Clement et al., 2000). We used BAPS v 6.0 (Corander et al., 2013) to identify discrete genetic clusters within the dataset, with the most probable number of genetic groups formed by the sequences being inferred by a Bayesian analysis of the population structure. Bayesian statistics provide an inference framework that calculates probability distributions for the parameters of interest, using previous distributions of these parameters, updated according to the empirical data (Segelbacher et al., 2010).

Results

After the editing and alignment of the sequences, we obtained a consensus D-Loop sequence of 656 base pairs (bps), of which only three bps were not useful for analysis. We found no evidence of saturation found in any of the sequences and, overall, we detected 55 polymorphic traits in the 656 bps. Thymine (38.31%) and adenine (31.04%) were the most common nucleotides, followed by guanine (18.47%) and cytosine (12.18%).

Nucleotide diversity was low in all demes, being 0.010 ± 0.007 on the island, 0.041 ± 0.031 on the LM, 0.036 ± 0.024 at RM1 and 0.016 ± 0.013 at RM2. Overall, 81.78% of this diversity was derived from intrapopulation variation and 18.22% from interpopulation variation. The overall F_{ST} was -0.079, indicating a lack of genetic structure.

The evolutionary history inferred by using the Maximum Likelihood method based on the Tamura-Nei model generate a tree involved the 14 nucleotide sequences (14 individuals). The tree with the highest log likelihood ($-1269{,}11$) is shown (Fig. 2).

The 14 individuals had 12 haplotypes (Hap), and haplotype diversity (Hd) was 0.978. Two haplotypes were shared, Hap-4 was shared by one individual from the RM1 and one from the island, while Hap-6 was

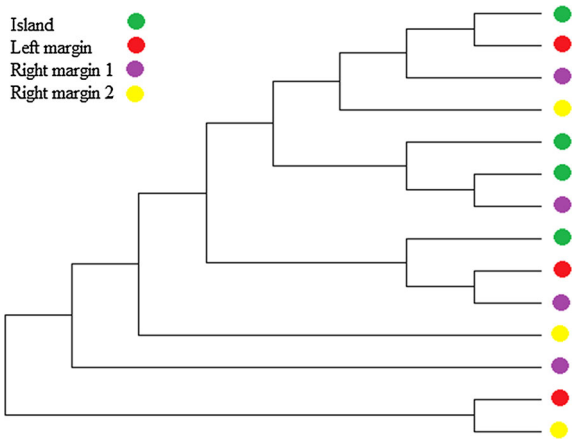

Fig. 2 Molecular phylogenetic analysis of *B. boans* by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (– 1269,11) is shown. The analysis involved 14 nucleotide sequences. Evolutionary analyses were conducted in MEGA7

shared by individuals from the RM1 and the LM, as shown in the haplotype network (Fig. 3).

The most genetically distant haplotypes (9 and 11) were separated by a Euclidean distance of 2,870 m, although both were collected on the same margin, while Hap-1 (Island) and Hap-7 (LM) were separated by a distance of 2,050 m (930 m over water), and Hap-3 and Hap-4 were collected at the same site. These findings reflect considerable dispersal over both land and water, given that the genetically most distant pair was found on the same margin, while individuals with the same haplotypes were separated by a large body of water. Haplotype 4 was shared by one specimen from the island and another from the RM1, at point separated by a Euclidean distance of 4,000 m, including 1,750 m of water. The other pair of specimens that shared a haplotype were collected on the LM and the RM1, at sites separated by a Euclidean distance of 4,500 m, including 2,900 m of water.

Neither the NJ nor the ML algorithm identified genetic structure in the haplotypes. The low F_{ST} values, which were close to zero and negative in all cases, further confirmed the absence of population structure, indicating a lack of any significant genetic differentiation in the proposed demes (Table 1).

The Bayesian analysis generated three groups which did not correspond to the geographic localities (Fig. 4). Most individuals were assigned to one group, while the second group contained two individuals from opposite margins, and the third group, a single specimen.

Discussion

Most of the studies that have recorded negative molecular variance in the analysis of population structure have shown an absence of genetic structure and high connectivity between populations (Vásquez et al., 2013; Coster et al., 2015). When the molecular

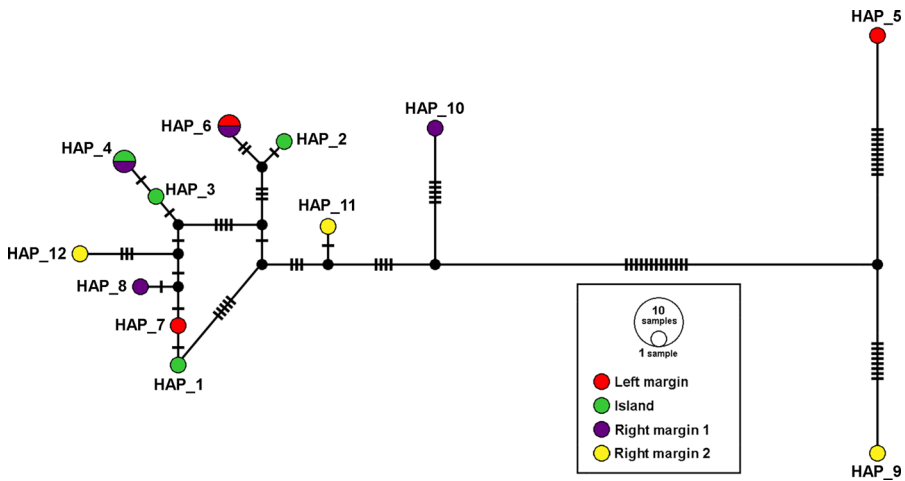

Fig. 3 Haplotype network for *Boana boans*. The network was built from 14 D-Loop sequences. The size and color of each ellipse indicate the frequency and geographical origin of individuals with this haplotype. The black dots and the crossbars represent the intermediate haplotypes and the mutational processes, respectively.

Table 1 Fixation indexes of F_{ST} (left inferior matrix) and Kimura average genetic distances of two parameters (right upper matrix)

Demes	Island	Left margin	Right margin 1	Right margin 2
Island	–	0.025	0.026	0.012
Left margin	0.0005	–	0.034	0.025
Right margin 1	0.0542	− 0.1811	–	0.025
Right margin 2	− 0.1319	− 0.1853	− 0.1036	–

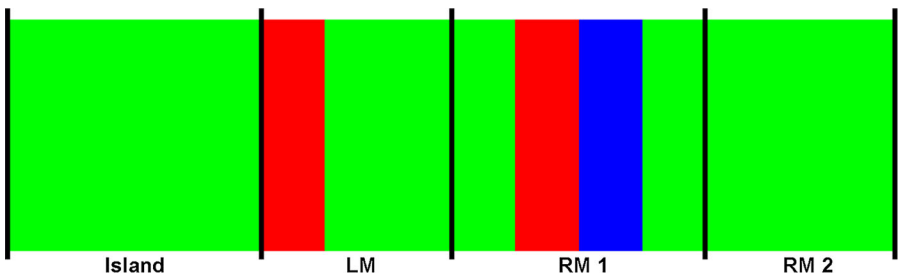

Fig. 4 Graph of the Bayesian analysis of population structure in 14 sequences of *Boana boans* D-Loop mtDNA on both banks of the river and an island where LM = left margin, RM 1 = right margin 1, and RM 2 = right margin 2

variance is only slightly positive or negative, the estimator can effectively be considered to be equal to zero (Excoffier & Lischer, 2010). This appears to be the case in the *B. boans* populations analyzed in the present study, with genes from different populations being more closely related than those from the same population. The results of the AMOVA showed that most of the variation was found within each deme, indicating the occurrence of gene flow among demes, and that the distance between the margins of the river does not represent a barrier to the dispersal of individuals, which are able to move freely between margins. This was further reinforced by the sharing of haplotypes (4 and 6) across the river. Fouquet et al. (2015) also found that *B. boans* had dispersed across a smaller river (200–500 m wide) in the Amazon basin, based on the analysis of a much more conserved molecular marker (the 16S gene) in only four specimens.

Tao et al. (2005) sequenced the mitochondrial D-Loop to assess the genetic structure of Chinese giant salamanders (*Andrias davidianus*), and found a similar lack of population structure in relation to the presence of rivers, with the AMOVA indicating that less than 1% of the genetic variation was found between groups. In a study of Kaiser's spotted newt (*Neurergus kaiseri* Schmidt, 1952), however, also based on the D-Loop, Farasat et al. (2016) found that 94.03% of the variation was distributed among the populations, and only 5.97% within populations. In the toad *Rhinella arunco* (Molina, 1782), neither the limits of the hydrographic basin nor the rivers within the geographic distribution of the species represented geographic barriers to the dispersal of individuals, as indicated by a combined Geneland, AMOVA, and haplotype network analysis, which indicated low levels of phylogeographic structure in this species (Vásquez et al., 2013). Degner et al. (2010) used a combination of mitochondrial sequences and seven nuclear microsatellite markers to assess the genetic structure of the ornate chorus frog, *Pseudacris ornate* (Holbrook, 1836), and found that the haplogroups of this species were not determined by physical barriers (i.e., major rivers or mountain ranges), although the observed pattern of genetic variation was associated with the geographic distance among sites.

Extremely low genetic distances indicate the sharing of alleles, and reduced differentiation between populations, with ample variation within populations. In the present study, the differentiation and distance values did not point to a significant pattern of population structure, given that the F_{ST} values between demes were close to zero, and the K2P values were negative, indicating that the genetic distances

among populations were not consistent with their geographical locations, further supporting the conclusion that the species is panmictic in this region. Other types of barrier may influence the dispersal of anurans more effectively. Funk et al. (2005) demonstrated the effects of isolation by mountain ridges on the Columbia spotted frog, *Rana luteiventris* Thompson (1913), based on the pairwise F_{ST} values between sites in adjacent basins with those recorded within each basin. The analysis of microsatellite markers indicated that mountain peaks and the variation in elevation were reflected in genetic divergence between sites. In this case, a model of landscape resistance indicated that different features of the landscape influence the genetic patterns observed in *Rana sylvatica* LeConte (1825). In the analysis of the top ten models based on the F_{ST}, isolation by distance was the best, and all the other nine were associated with roads, indicating that both the presence of roads and geographic distance shape the spatial genetic structure of these frogs (Richardson, 2012).

In a landscape genetic analysis of the European treefrog, *Hyla arborea* Linnaeus (1758), based on pairwise F_{ST} values for 11 microsatellite loci, Angelone et al. (2011) found that, at distances of less than 2 km, only one large river acted as a barrier to gene flow, but at distances over 2 km, geographical distance, as well as forests and roads, all had a negative effect on gene flow. While we did not aim to analyze isolation by geographic distance, the *B. boans* specimens separated by a distance of approximately 6 km, which included the river, presented a F_{ST} value effectively equal to zero. In *Andrias davidianus*, Tao et al. (2005) found very low levels of F_{ST} (< 0.01) overall, but significant levels of population differentiation between individuals from the Pearl river and the Yellow and Yangtze rivers (although no difference was found between the Yellow and Yangtze rivers).

In recent years, molecular analyses have increasingly applied Bayesian clustering techniques to provide a more objective approach to landscape genetics (Storfer et al., 2010) and phylogeography (Fouquet et al., 2012; Brunes et al., 2015). Bayesian techniques can be used to identify discontinuities that may reflect the presence of major barriers or historical effects within the genetic clusters (Born et al., 2008). In the present study, the results of the Bayesian analysis indicated no genetic structuring related to either Euclidian distances or the presence of the river.

The absence of genetic structure found in the present study may be reflecting recent genetic exchange, as indicated by the distribution of haplotypes between the demes within and between the sampling points. The lack of significant correlation between geographic and genetic distances refutes role of riverine barriers or geographic divergence in the formulation of the genetic variation in these anurans. Fouquet et al. (2015) found less genetic variation between river margins in tree-dwelling anuran species in comparison with litter-dwelling species. In a study of 26 amphibian species in the Amazon basin, Moraes et al. (2016) found that the Tapajós River, a major Amazon tributary, was the principal barrier, whereas the much smaller Jamanxin River played only a minor role. The functional groups most affected by these barriers were small, terrestrial, diurnal anurans, and the assemblage most affected was that of the non-riparian amphibians. The abundance of some species increased in proximity to the bodies of water, while *B. boans* and its congeners, *Boana multifasciata* (Günther, 1859) and *Boana leucochelia* (Caramaschi & Niemeyer, 2003), occurred exclusively in these areas.

Boana boans is considered to be territorial, with males exhibiting high fidelity to spawning sites, due to the construction of nests in the clay or sand (Magnusson et al., 1999). The results of the present study nevertheless suggest that this species is a good disperser, which may range over substantial areas. As de Oliveira et al. (2016) recorded a similar pattern in *Boana faber* (Wied-Neuwied, 1821), the evidence indicates clearly that some species tropical and subtropical anurans may not be sedentary. In a 15-year study of *B. boans*, however, Magnusson et al. (1999) rarely found specimens more than 100 m from the monitoring site. The dispersal capacity of a species may be especially important when it is vulnerable to local extinction, with more vagile taxa being able to recolonise an area from a source population more easily (Magnusson et al., 1999).

In a review of landscape genetic studies of terrestrial animals, Waits et al. (2015) identified a set of natural and anthropogenic barriers to dispersal and gene flow, with rivers being identified as barriers in all taxonomic groups. In the case of the principal rivers of the Amazon basin, however, the available studies are limited to the analysis of isolation and dispersal in leaf-litter anurans with direct development driven by a

combination of life history traits (small size, no larval dispersion, territoriality, low resistance to desiccation), attributes that intuitively reflect reduced dispersal capacity (Van Bocxlaer et al., 2010). Rivers are no longer considered to be geographic barriers, although their margins may be refuges of biodiversity. In a review of the data on 1952 species representing 14 taxonomic groups found in the basin of the Madeira River, Santorelli et al. (2018) found evidence that the river barrier hypothesis accounted for less than 1% of the diversity of species found in the region. These findings were corroborated by our results, including the low pairwise *Fst* values, the lack of influence of the Juruena River on the haplotype network or the sharing of haplotypes, and less than a fifth (18.22%) of the genetic variability being distributed among demes. These data are contrary the hypotheses of a lack of anuran vagility and the role of one the main Amazonian rivers as barrier to amphibians dispersal.

The lack of genetic structuring found in the present study may be related to the capacity of *B. boans* to climb, swim, and jump long distances (pers. obs.), its tolerance of desiccation, and its reproductive mode (spawning directly into the river, with large tadpoles). Trees falling into the river, with frogs attached, may also to their dispersal between margins. Intrinsic features of the Juruena may also facilitate river crossings, including its slow currents and many islands, including relatively large islands that effectively reduce the course of the river to a number of small channels. In this landscape, *B. boans* may form a single, panmictic population. Considering the idiosyncrasies of the model organism and the intrinsic features of the Juruena River, the findings of this study of *B. boans* provide an important insight into the river barrier hypothesis for amphibians.

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