Acoustic and Morphological Differentiation in the Frog *Allobates femoralis*: Relationships with the Upper Madeira River and Other Potential Geological Barriers

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**ABSTRACT**

We studied patterns of call acoustics and external morphological differentiation in populations of the dart-poison frog *Allobates femoralis* occurring in forested areas along a 250-km stretch of the upper Madeira River, Brazil. Multivariate analyses of variance using principal components representing shared acoustic and morphological parameters distinguished three groups in relation to call structure and external morphology: (1) populations belonging to a two-note call morphotype; (2) populations with four-note calls inhabiting the left riverbank; and (3) populations with four-note calls inhabiting the right riverbank. Our results report a case of Amazonian anuran diversity hidden by current taxonomy and provide evidence for the upper Madeira River being a boundary between distinct populations of *A. femoralis*, and suggest a new taxonomic interpretation for these groups. Samples that did not fit into the general differentiation pattern and the existence of a well-defined contact zone between two morphotypes on the left riverbank indicate that mechanisms complementary to river-barrier hypotheses are necessary to explain the phenotypic differentiation between populations. Our study shows that at least one anuran species shows congruence between population differentiation and separation by a large Amazonian river, as documented for birds and mammals. Conservation efforts should not consider the taxon now known as *A. femoralis* as a homogeneous entity. There is much within-taxon variability, which can be probably explained partly by the existence of cryptic species, partly by geological barriers and part of which currently has no obvious explanation.


Key words: bioacoustics; Brazil; Dendrobatididae; external morphology; Madeira River; phenotypic differentiation; population distribution patterns.

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distressing calling neighbors (Hödl 1987; Roithmair 1992, 1994; Rodriguez & Duellman 1994).

The study of interpopulational variation on phenotypic traits, such as external morphology and call characteristics, is useful for evaluating divergence patterns, because populations of a single species presumably developed unique characters in relatively recent times. A more recent differentiation between groups of interest reduces the probability that groups showing intermediate phenotypes have become extinct, and it is even possible that those populations still inhabit the location where the divergence process took place, providing unique opportunities for the study of the evolutionary mechanisms that triggered divergence (Foster 1999, Moritz et al. 2000).

Considering their apparent restriction to forested environments, and consequently reduced interpopulation gene flow across floodplains (Gascon et al. 1998, Lougheed et al. 1999), populations of *A. femoralis* are potential models to test the role of the upper Madeira River in differentiation of anuran populations.

**METHODS**

**STUDY AREA.—**We sampled forests along the upper Madeira River, from the city of Porto Velho (8°43′34″ S, 63°55′24″ W) to the mouth of the Abunã River, a western tributary (9°40′41″ S, 65°25′58″ W), in the State of Rondônia, Brazil. Along this stretch, the muddy Madeira River is not meandering and flows between narrow floodplains, limited by pronounced slopes. On the right bank, the landscape is a continuous flat plateau, originated from Pliopleistocene sediments. On the left bank, the landscape is represented by tabular formations of the same plateau and by fragments of a largely eroded plateau associated with pre-Cambrian sediments. The drainage system constituting this part of the Madeira River basin runs primarily through tectonic valleys. Slope ruptures attributed to tectonic faults along the river cause large rapids across the riverbed. Climate is characterized by well-defined wet and dry seasons, which directly influence water levels. On both riverbanks, vegetation is constituted by low rain forest, with open pioneer vegetation on the river margins (DNPM 1978, Souza Filho et al. 1999). It is not known if plant composition and forest structure differ between geological domains or between riverbanks in the study area, but large deforested gaps are found on the right riverbank, near populated localities (Porto Velho, Jaci-Paraná, Mutum-Paraná).

**SAMPLING DESIGN.—**Initially, we established 19 sampling sites in areas of terra firme forest along the upper Madeira River, ten on the left bank and nine on the right bank (Fig. 1). Populations of *A. femoralis* occur in patches in continuous-forest areas, so each sampling site was searched for vocally active populations. We found no populations of *A. femoralis* in two of the sampling sites established on the right bank (corresponding to the localities of Abunã and Jirau), both of which had sites apparently appropriate for the species, but no individuals. Therefore, we used samples from 17 sites. Minimum and maximum distances between effective sampling sites were of 1.3 and 168.0 km, respectively. We also searched for contact zones along the left riverbank, where at least two mor-
68 EB directional microphone (2003, AKG Acoustics GMBH, Austria) positioned 1.0 m from calling males. For each individual recorded, air temperature at the calling site at the time of recording was registered. For each recording session, we selected the three call samples that showed the least interference by environmental noise, starting from the most central calls. Thus, we avoided selecting warm-up calls at the beginning of the calling bout, that vary greatly in duration and frequency, and calls at the end of the bout, which could show temporal and spectral variations due to fatigue (Gerhardt & Huber 2002).

For each of the three call samples, we obtained temporal and spectral variables using sonograms generated by Raven 1.2, software (Charif et al. 2004). Spectral analysis was done by a fast Fourier transform with a frequency resolution of 82 Hz and 2048 points. Because two acoustic morphotypes were found in the study area, the number of variables obtained for each group depended on the number of notes that constituted its advertisement calls. Means, standard deviations, and maximum and minimum values of all acoustic variables for each sampling site are given in Table S1.

We used a principal component analysis (PCA) to reduce dimensionality and produce a smaller number of independent acoustic variables. The first and second components generated by the analysis were used as dependent variables in a multivariate analysis of variance (MANOVA) model, to test whether three groups defined by the number of notes of advertisement calls and origin (i.e., right or left bank) were significantly different from each other in terms of acoustic characteristics. Body weight was included as a covariate in the MANOVA, as body size can affect spectral features of anuran acoustic characteristics. Body weight was included as a covariate in the MANOVA, as body size can affect spectral features of anuran calls (Ryan 1988, Keddy-Hector et al. 1992). Air temperature at the time of recording was included as another covariate, as variation in temperature can influence temporal features of advertisement calls (Ryan 1988, Keddy-Hector et al. 1992, Gerhardt & Huber 2002). Sampling-site co-ordinates in UTM units were also included as covariates, to represent distance between sites (Legendre et al. 2002), because morphological and behavioral characteristics could vary with geographic distance, independent of physical barriers. Early components produced by PCA usually recover important patterns of similarity between samples, while later components tend to accumulate variation related to noise (Gauch 1982). For the present analysis and for other analyses described below, we used the minimum number of components necessary to explain at least 50 percent of the total variation in our data sets.

Because two acoustic morphotypes were found in the study area, the analysis described above was done twice. First, we used a set of nine variables obtained only from the first and second notes of the advertisement calls of all individuals sampled. In both acoustic morphotypes, the first and second notes showed similar spectrographic patterns, with first note always narrowly modulated in frequency and short in duration, followed by a longer, broadly modulated second note. Therefore, we assumed first and second notes to be homologous in calls of both morphotypes. A second analysis was carried out using 24 acoustic variables obtained from the four notes constituting the advertisement calls of one of the morphotypes that inhabited both riverbanks, to test the acoustic divergence between populations of a single acoustic morphotype living on opposite sides of the river.

MORPHOLOGICAL DATA COLLECTION AND ANALYSES.—Males were collected after recording procedures, euthanized, and weighed after the removal of the digestive tracts, because gut content represented approximately 10 percent of total body mass in our samples (mean gut content mass was 0.16 ± 0.08 g for an average body mass of 1.68 ± 0.27 g, considering all individuals collected). Our measure of body mass therefore reflects long-term differences in morphology and not short-term differences due to the time an individual encountered a prey item. For each of the 209 individuals used, 19 external morphological variables were measured using a digital caliper and a stereoscopic microscope with a graduated ocular lens (precisions 0.01 and 0.10 mm). All variables were obtained from the left side of the specimen. Means, standard deviations, and maximum and minimum values of all morphological variables for each sampling site are presented in Table S2. Voucher specimens were deposited in the Herpetology Collections of the Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil, accession numbers INPA-H16541 to INPA-H16823.

The 19 morphological variables were log_{10} transformed for scale adjustment. The first and second components generated by a PCA were included in a MANOVA model as dependent variables to test whether the three groups identified in the acoustic analysis presented significant morphological differences.

Principal component analysis on external morphological variables usually produces a first principal component carrying heavy size effects while the remaining components contain more information on body shape. Therefore, because direct measurements usually vary continuously with body size, individual body weight was included in the MANOVA model as a covariate representing body size (Reis et al. 1990, Strauss & Bond 1990). All morphological and acoustic statistical procedures were done in SYSTAT 8.0 (Wilkinson 1990).

RESULTS

Two distinct morphotypes of *A. femoralis* were found in the study area. One has reddish posterior ventral coloration and advertisement calls constituted by two notes (Fig. 2A). This morphotype occurs only on the upper left riverbank, and has a contact zone with the other morphotype on the same bank, where there are no apparent barriers to dispersal of individuals at the present time. The second morphotype has black and white ventral coloration and advertisement calls constituted by four notes (Fig. 2B,C). The distribution of this morphotype is split by the Madeira River (Fig. 1).

ACOUSTIC ANALYSES.—Principal component analysis using six spectral variables and two temporal variables present in the first and second notes produced a first and a second component explaining together 82.2 percent of the acoustic variation. The first component (PC1) explained 58.9 percent of the total acoustic variation, and spectral variables had high loadings on that component. The second component (PC 2) explained 23.3 percent of the acoustic variation, and temporal variables had high loadings on that component (Table S3). Both components were used as dependent variables in a MANOVA to test if acoustic characters were statistically different
among the three groups defined a priori: (1) two-note advertisement call morphotype; (2) four-note advertisement call morphotype from the left riverbank; and (3) four-note advertisement call morphotype from the right riverbank.

MANOVA indicated that body mass (Pillai trace = 0.225; \( P < 0.001, df = 192 \)) and air temperature (Pillai trace = 0.043; \( P = 0.015, df = 192 \)) significantly affected the acoustic features of the two notes. However, the three groups defined a priori were significantly different from each other in relation to acoustic characteristics, independent of covariate effects (Pillai trace = 0.354; \( P < 0.001, df = 192 \)). Geographic distance between sampling sites did not influence call features (Pillai trace = 0.035; \( P = 0.144, df = 192 \)).

We plotted the variable means per site on PC1 and PC2, which represented spectral and temporal characters, respectively. Populations from the right and left bank formed distinct groups along PC1 (Fig. 3) as a result of the lower frequencies of calls and notes of the populations inhabiting the right bank (Table S1). Sampling site No. 17 (St. Antonio-L), on the left bank, was closer in multidimensional space to sampling sites on the right bank, showing low values for spectral characteristics. This site was located ca 30 km from another sampling site on the left bank, where individuals showed acoustic characteristics typical of that riverbank (Fig. 3). Two-note morphotype populations (sites from no. 1–5) tended to be different in temporal characteristics of calls, presenting shorter notes in relation to four-note call populations from the left riverbank.

It was expected that the two-note call morphotype on the left side of the river would have sufficiently different acoustic features to form a vocally distinct group. Thus, a second PCA was carried out, using only samples of the four-note call morphotype. Besides providing a larger number of acoustic variables (15 spectral and 9 temporal), the four-note call morphotype was present on both riverbanks, so the river effect on acoustic traits could be tested for a single morphotype.

The first and second components explained 51.2 and 18.0 percent of the acoustic variation, respectively. Again, spectral variables had high loadings on PC 1 and temporal variables had high loadings on PC 2 (Table S4). MANOVA showed that the covariates body mass (Pillai trace = 0.293; \( P < 0.001, df = 140 \)) and air temperature (Pillai trace = 0.227; \( P < 0.001, df = 140 \)) significantly affected acoustic features of the four notes. Nonetheless, acoustic characteristics differed statistically between riverbanks, independent of covariate effects (Pillai trace = 0.099; \( P = 0.001, df = 140 \)). Distances between sampling sites did not influence call features (Pillai trace = 0.028; \( P = 0.410, df = 140 \)). Distribution of sampling sites along the first and second PCA axes showed that samples from opposite riverbanks were separated mainly along PC1, which described the spectral variation of calls (Fig. 3), reflecting lower frequencies of calls recorded on the right bank (Table S1). A PC3 was related to the variation in silent interval duration, but variation in this acoustic trait occurred between individual sampling sites, and not between groups of sites defined by riverbanks.

Variation explained by other components was never greater than expected for components constituted by random variables, and inclusion increases the chance of type II errors. However, analyses using more components, not reported here, produced similar qualitative results to analyses with two components.

**MORPHOLOGICAL ANALYSES.**—A principal component analysis of 19 transformed morphological variables generated a first component explaining 43.9 percent of the external morphological variation. All variables used had positive loadings on that component. The second principal component explained only 6.8 percent of the morphological variation.

First and second principal components were used as dependent variables in a MANOVA to test for morphological differences between the three groups as defined in the acoustic analysis. Body mass (Pillai trace = 0.507; \( P < 0.001, df = 199 \)) and geographic distance between sites (Pillai trace = 0.086; \( P = 0.002, df = 199 \)) had significant effects on morphological traits, but there were statistically significant differences among groups (Pillai trace = 0.208; \( P < 0.001, df = 199 \)), after accounting for the effects of covariates.

Groups were generally separated along the principal components, especially along PC1. As this component relates mostly to body size, separation of populations on this axis is probably due to larger individuals inhabiting the right riverbank (Table S2). Considering only the sampling sites on the left riverbank, samples of the two-note call morphotype tended to form a group distinct from the two-note morphotype along PC 2, which accounted mostly...
FIGURE 3. Distribution of population means along the original principal components scores generated from acoustic variables of the two notes present in calls of both morphotypes (A) and variables obtained from the four notes of a single morphotype (B). Spectral divergence between populations of the right (R) and left (L) riverbanks were evident in both analyses. On the left riverbank, populations of the two-note call morphotype (in gray) significantly differed in call characteristics from populations of the four-note call morphotype. See text for statistical comparisons between groups.

FIGURE 4. Distribution of population means along the original scores of the first and second principal components using external morphological variables. PC1 accounts for great part of the morphological variation related to size. Individuals taken from the right bank populations (R) were larger, on average, than individuals from the left riverbank (L).

DISCUSSION

Many studies suggested that large Amazonian rivers represent distribution boundaries for species and distinct populations of primates (Wallace 1852; Ayres & Clutton-Brock 1992; Peres et al. 1996; Roosmalen et al. 1998, 2000) and birds (Hellmayr 1910, Capparella 1987, Hayes & Sewlal 2004). However, the only studies evaluating river boundaries on anurans in Amazonian lowlands were restricted to the Juruá River, in Brazil (Gascon et al. 1996, 1998, 2000; Lougheed et al. 1999). Those studies found no evidence of differentiation between populations or communities of anurans on opposite sides of the river, but some authors argue that shifts in the river course and sediment deposit dynamics, typical of Amazonian meandering rivers, such as the Juruá, can cause terrain to move from one riverbank to another, passively transporting individuals between riverbanks from time to time and homogenizing populations via interbreeding (Ayres & Clutton-Brock 1992, Moritz et al. 2000).

Our results clearly suggest that the upper Madeira River limits distinct populations of the poison-dart frog *A. femoralis*. The two-note call morphotype is restricted to the left riverbank and there is significant differentiation in acoustic and morphological traits between populations of the four-note morphotype inhabiting opposite riverbanks. In the study area, the Madeira River has an average width of 1.0 km and certainly represents a considerable barrier to forest-specialist anurans. The river runs mostly along an incisive tectonic valley, predominantly crystalline and geologically ancient (DNPM 1978), so it is improbable that changes of river-course orientation occurred in geologically recent times. A system of successive tectonic faults (altitude decreases from west to east) also results in a system of rapids along this section of the river, causing water velocity to be particularly fast. Passive transportation of individuals from one riverbank to another by means of sedimentary island dynamics is unlikely due to slow rates of sediment deposit.

Although populations of the four-note call on opposite banks of the Madeira River had statistically significant differences, those were greatly influenced by size-related characteristics of morphology and calls. Body size affects calls due to physical relationships between frequency wavelength and the dimensions of primary and secondary oscillators (*i.e.*, larynx, vocal cords, nostrils and vocal sacs; Gerhardt...
potential vicariant events associated with past tectonic phenomena. Thus, at this time, it is not possible to determine whether there was an effective vicariant barrier at the contact zone between *A. femoralis* morphotypes or their divergence occurred in sympathy.

Tectonic events have been previously suggested as possible factors generating genetic population divergence in *A. femoralis*. Lougheed et al. (1999) suggested that factors other than the riverbed could have generated patterns of genetic divergence in populations along the Juruá River. The tectonic arch of Iquitos (a rocky crest formed during the genesis of the Andes), was found to cross the Juruá River at a right angle and coincides with sampling sites showing high genetic divergence. The contact zone between *A. femoralis* morphotypes on the left bank of the Madeira River is not coincident with any of the proposed locations of tectonic arches (Räsänen 1990). Also, if a tectonic arch was responsible for the differentiation, evidence of a contact zone between different groups of populations should be present on the right riverbank, where only relatively uniform populations of the four-note morphotype occur.

Haffer (1997a) described the occurrence of at least 20 contact zones between 40 bird taxa in Amazonia. Those zones occur in continuous rain forest areas and generally cross large Amazonian rivers at right angles, providing a similar pattern to what is observed for *A. femoralis*. He considered them as suture zones between taxa that diverged in forest refugia during the Pleistocene, supporting his refuge theory as an explanation for the existence of such lines, and discarding ecological factors as determinants of those zones. As we lack detailed information on microhabitat structure and vegetation composition in our study area, we cannot reject at this point that an ecological mechanism of adaptation to a discrete environmental gradient across geological domains (*sensu* Endler 1982) is the driving force that originated or maintains the *A. femoralis* contact zone.

Our results provide scope for a taxonomic reinterpretation of two- and four-note call morphotypes based on their phenotypic differences, as it is highly probable that each morphotype represents a well-delimited evolutionary lineage. Ongoing phylogenetic and population genetic approaches will help to clarify their evolutionary relationships and evaluate current gene flow. Information provided by those analyses will also be used to test alternative diversification hypotheses (river-barrier, forest refugia, tectonic arches, among others) that can explain the current distribution pattern of phenotypic differentiation in *A. femoralis* along the Madeira River.

Establishing the taxonomic limits between species is a key step for planning conservation strategies based on beta diversity. Cryptic anuran species are thought to be frequent among widespread taxa in the Amazon region, and lack of detailed data on variation within species is still a great impediment to the recognition of diversity at this level (Azevedo-Ramos & Gallati 2002). Mapping population variability within-species is also an important conservation tool to maximize potential adaptation and persistence of species in face of contemporary environmental changes. Our study reports that populations included in what is thought to be a single anuran species (*Allobates femoralis*) present significant phenotypic differences, part of which is concordant with their division by a large Amazonian river. These findings have implications for both the planning of

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**FIGURE 5.** The contact zone between *Allobates femoralis* morphotypes on the left riverbank meets the riverbed almost at a right angle and coincides with the boundary between two geomorphological domains, represented by plateaus of different origins.
amphibian conservation strategies in the Amazon and the management of conservation units along the Madeira River.

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SUPPLEMENTARY MATERIAL

The following supplementary material for this article is available online at: www.blackwell-synergy.com/loi/btp

Table S1. Means, standard deviations and amplitude of acoustic variables obtained from advertisement calls of Allobates femoralis.

Table S2. Means, standard deviations and amplitude of body weight, snout-to-vent length and 19 other morphological variables obtained from Allobates femoralis males.

Table S3. Results of PCA using three temporal and six spectral acoustic variables from the first and second notes present in the advertisement calls of both Allobates femoralis morphotypes.

Table S4. Results of PCA using nine temporal and 18 spectral variables obtained from the 4-note calls of the morphotype present on both riverbanks.

LITERATURE CITED


