

# Significant but not diagnostic: Differentiation through morphology and calls in the Amazonian frogs *Allobates nidicola* and *A. masniger*

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Acoustic parameters often usefully supplement the classic morphological approaches used to discriminate between similar species of frogs, and thus provide better assessments about their taxonomy at the species level. *Allobates nidicola* and *A. masniger* are known only from their respective type localities in Brazilian Amazonia, and the two species share all the available qualitative diagnostic traits. This study describes and compares a set of quantitative morphometric (external measurements) and acoustic (temporal and spectral parameters) characters of these species in a sampling design that included their type localities, in order to assess whether they are phenotypically distinguishable. The advertisement call of *A. masniger* is described here for the first time. Both acoustic (including temporal and spectral properties) and morphometric characters overlapped with those found in *A. nidicola*. The two species differed statistically in acoustic and morphometric characters when we compared between individuals from their type localities, and also when assuming that they are allopatric by the transposition of the Madeira River, the main biogeographic barrier in the study area. However, multivariate analyses failed to prove unambiguous distributional boundaries of these species based on the analysed phenotypic traits. Therefore, further lines of evidence - such as behavioural, developmental and molecular data - are required in order to detect diagnostic characters and thus clarify the taxonomic status of these phenotypically similar species.

*Key words:* advertisement call, Anura, Aromobatidae, biogeography, Madeira River

## INTRODUCTION

Several Neotropical anuran species formerly considered to be part of a widely-distributed taxon have recently become recognised (e.g., Funk et al., 2008; Angulo & Icochea, 2010; Simões et al., 2010). Conversely, the synonymization of pairs or even groups of species into one taxonomic entity may be needed when previously described species do not find support in empirical evaluations of phenotypic variability (e.g., Caramaschi & Pombal Jr., 2001; Verdade & Rodrigues, 2007). These two pathways by which taxonomy reveals anuran species richness are enabled through integrated analyses of different classes of characters (e.g. acoustic parameters supplementing classic morphological approaches, Dayrat, 2005; Padiá et al., 2010; Schlick-Steiner et al., 2010).

Advances in alpha taxonomy have led to revised definitions of the geographic ranges of many species, and researchers have become interested in revealing the biogeographic factors that determine the novel distributional patterns emerging from these discoveries. Since the observations of Wallace in 1852, studies have shown the effect of large Amazonian rivers on the establishment and maintenance of geographic barriers to

gene flow and consequent diversification in vertebrates (Antonelli et al., 2010). However, few studies have evaluated the performance of non-meandering rivers as vicariant barriers in anurans, although this hypothesis has found support in both genetic and phenotypic data (e.g., Funk et al., 2007; Simões et al., 2008). Such evidence suggests that interfluves act as areas of endemism in anurans, and that studies on geographic variation of frogs should consider them as potential barriers to dispersal.

Amazonia is home to several species of frogs with wide geographic distributions (Azevedo-Ramos & Galatti, 2002; Angulo & Icochea, 2010). However, recent detailed approaches to the study of geographic variation of pan-Amazonian species have suggested that they actually consist of a mosaic distribution of different taxa (e.g., Funk et al., 2007; Amézquita et al., 2009; Angulo & Icochea, 2010); most of the original taxa are now confined to their type localities or to particular regions such as interfluves. *Allobates* (Aromobatidae), the most speciose genus of Dendrobatoidea (Frost, 2011), is a current example of this phenomenon, excelling in the number of morphologically similar species recognised (e.g., Lima et al., 2007; Barrio-Amorós & Santos, 2009; Lima et al., 2010; Simões et al., 2010). This suggests that

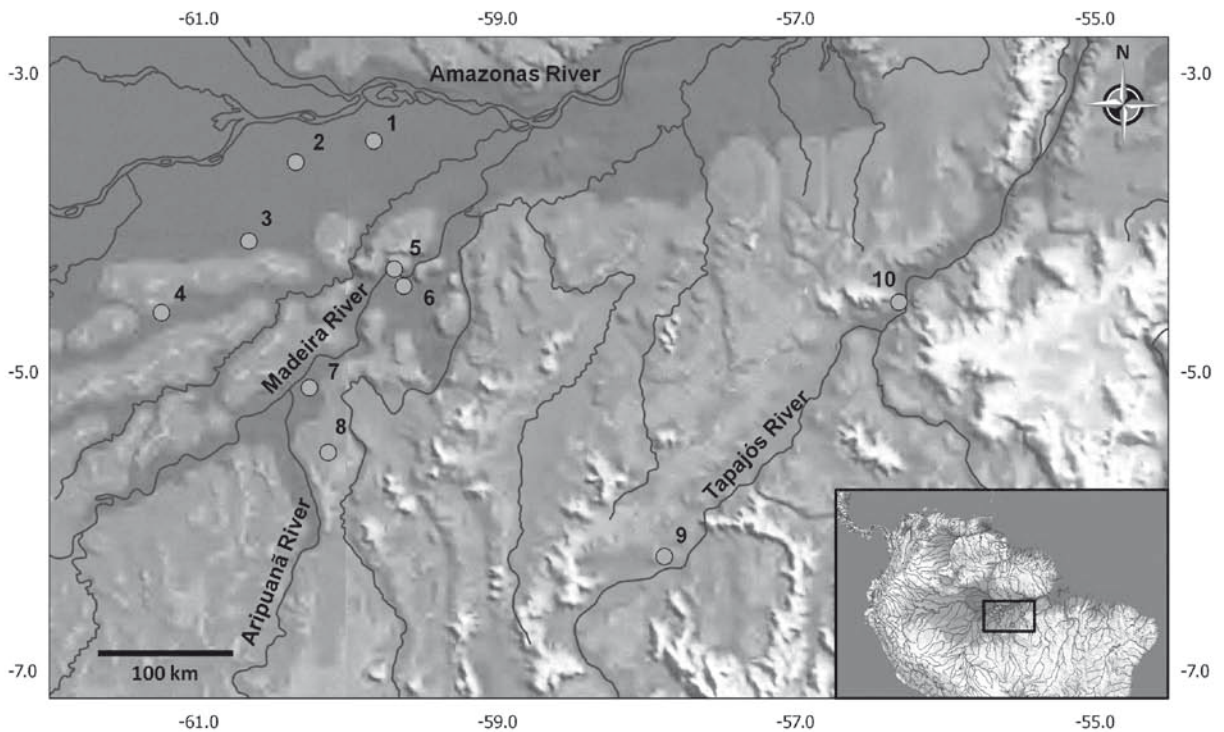
the species richness and consequently the distributional ranges of frogs in the Amazon are poorly known, which has serious implications for the understanding of the conservation status of many species (Peloso, 2010).

*Allobates nidicola* (Caldwell & Lima, 2003) and *A. masniger* (Morales, 2002) are known only from their type localities in Brazil (see Fig. 1), and share all the available diagnostic traits. Whereas for *A. nidicola*, larval characters, bioacoustics and behaviour have been described, such information is unknown for *A. masniger*. Recently, field studies conducted at the type locality of *A. masniger* found that reproductive (breeding sites and direct larval development) and acoustic (advertisement call) characteristics of both species are similar (Lima, A.P., personal observation). This study describes and compares a set of quantitative acoustic and morphometric characters of *A. nidicola* and *A. masniger*. The sampling design included the type localities of both species, in order to assess whether these taxa are phenotypically distinguishable. The advertisement call of *A. masniger* is described for the first time. In addition, we aimed to determine the geographic distribution of both species, and its relation with the transposition of the Madeira River, an important biogeographic barrier located in the study area.

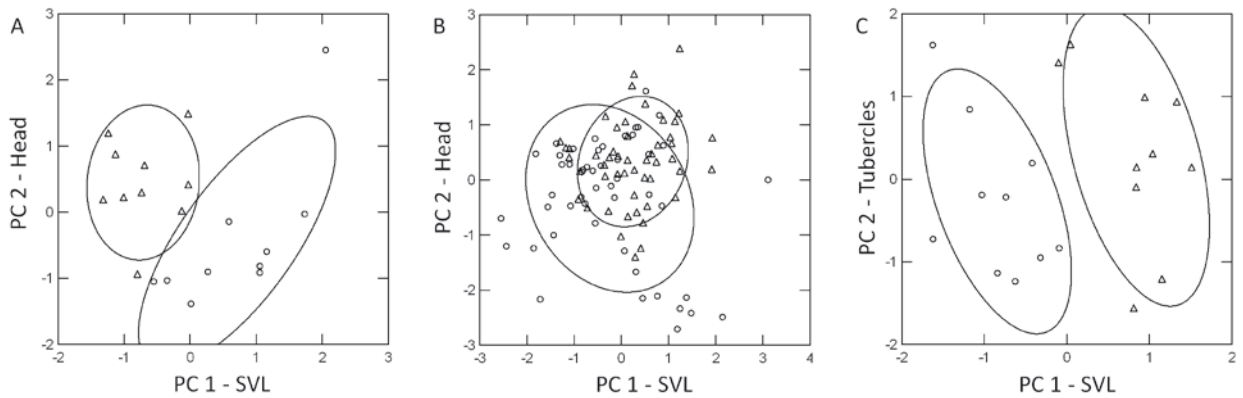
## MATERIALS AND METHODS

Fieldwork was conducted at ten sites located in the states of Amazonas and Pará, Brazil, including the type localities of *A. nidicola* and *A. masniger* (Fig. 1). Data were collected during the rainy season, between December 2009 and

March 2010. This period encompassed the breeding seasons of both species. Individuals were found within the *terra firme* (non-flooded) forests, mainly between 0530 and 0700 hours. We also recorded continuous activity of these animals during cloudy and rainy days. The following procedures were adopted for each male (total sample size:  $n=100$  individuals; ten individuals per locality) found in calling activity: 1) recording of its advertisement call (identified by the social context in which it was emitted), 2) measurement of the air temperature at its calling site, 3) capture of the individual, 4) anesthesia and euthanasia with a topical anesthetic ointment (benzocaine) in order to obtain tissue samples for later molecular analyses, 5) fixing the individual in 10% formalin and storage in 70% ethanol. The collection of specimens constituted the first assessment of the taxonomic identity of individuals through the distributional ranges of the two species. *Allobates nidicola* and *A. masniger* are broadly distributed in Brazilian Amazonia (Kaefer I.L., unpubl. data; this study), and a four-year mark-recapture study showed that *A. nidicola* occurs in relatively high densities on the forest floor (mean=0.024; SD=0.004 individuals per m<sup>2</sup>; Lima, A.P., unpubl. data). Currently, there is no evidence for a decline in the abundance or biomass of amphibians in the central portion of the biome (Deichmann et al., 2010). Individuals were deposited in the Herpetological Collection of the Instituto Nacional de Pesquisas da Amazônia (see Appendix), in Manaus, Brazil. To reduce possible measurement errors arising from variation between observers, a single researcher measured the phenotypic variables evaluated in this study.



**Fig. 1.** Sampling sites visited in this study: 1) Km 12 of Autazes Road, type locality of *Allobates nidicola*; 2) BR-319, km 260; 3) PPBio Manaquiri; 4) BR-319 Tupana; 5) Vila Gomes; 6) Borba; 7) Novo Aripuanã; 8) Road to Apuí; 9) Jacareacanga; 10) PARNA da Amazônia, type locality of *A. masniger*.



**Fig. 2.** Distribution of individual scores along the first and second principal components (PC) using external morphometric variables. In A, *Allobates nidicola* (triangles) and *A. masniger* (circles) from their respective type localities. In B, all sampled individuals from the left (triangles) and right (circles) sides of the Madeira River. In C, individuals from the localities Vila Gomes (left side of the Madeira River; triangles) and Borba (right side of the Madeira River; circles). The confidence ellipses are centred on the sample means of the two principal components.

### Morphological data

Twenty-three external morphometric measurements (in mm) were taken from the left side of the specimens, with the use of a stereoscopic microscope with a graduated ocular lens (precision 0.10 mm) and a digital caliper (to the nearest 0.01 mm). The following measurements were taken: snout-vent length (SVL); head length from angle of jaws to tip of snout (HL); head width at level of angle of jaws (HW); snout length from anterior corner of eye to tip of snout (SL); eye to nostril distance from anterior corner of eye to centre of nostril (EN); inter-nostril distance (IN); eye length from anterior to posterior corner (EL); inter-orbital distance (IO); diameter of tympanum (TYM); forearm length from proximal edge of palmar tubercle to outer edge of flexed elbow (FAL); upper arm length from trunk insertion to outer edge of flexed elbow (UAL); hand length from proximal edge of palmar tubercle to tip of fingers I, II, III and IV (HAND1, HAND2, HAND3 and HAND4); width of disc on finger III (WFD); tibia length from outer edge of flexed knee to heel (TL); foot length from proximal edge of outer metatarsal tubercle to tip of toe IV (FL); femur length (LL); diameter of palmar tubercle (DPT); width of tenar tubercle (WTT); width of disc on toe IV (WTD); width of finger III (WPF, Caldwell & Lima, 2003).

### Acoustic data

Each captured male had its advertisement call recorded for about three minutes with a Marantz PMD660 digital recorder (44.1 kHz sampling rate; 16-bit resolution) and an AKG 568 EB directional microphone positioned 1–1.5 m in front of the calling male. The advertisement call of both species is composed of a single note repeated rhythmically over time, thus forming sequences of notes lasting from two to five minutes. The spectral variables were obtained through the Selection Spectrum tool of the program Raven 1.2 (Window: Blackman; Discrete Fourier Transform: 1024 samples; 3dB filter bandwidth: 80.0 Hz, Charif et al., 2004). The measure used to represent each individual in the statistical analysis was the mean value of the parameters obtained from 20 notes and respective

intervals. The notes and intervals measured were arranged uniformly throughout the range of the central minute of the recording. We considered the following traits following Caldwell & Lima (2003): Note duration (s); Interval between notes (s); Lowest frequency (Hz); Highest frequency (Hz); Note modulation, calculated as the difference between the maximum and minimum frequencies (Hz); and Peak frequency (Hz).

### Statistical analyses

A principal components analysis (PCA) was used to reduce dimensionality and produce a smaller number of independent morphological and acoustic variables. After checking for normal distribution of data, we included the first two components (which were sufficient to explain ca. 50 percent of the total variation in our data sets) as dependent variables in a multivariate analysis of variance (MANOVA) model to test whether groups are phenotypically distinct. In order to highlight the biological meaning for the principal components (PC) generated, hereafter the variables with the highest loadings on a given component are provided in parentheses following its mention. In the acoustic analyses, the SVL and air temperature were included as covariables, given that they can affect spectral and temporal features of anuran calls (Ryan, 1988; Gerhardt and Huber, 2002).

We performed separate MANOVA analyses regarding morphological and acoustic differentiation between the type localities of the species and the pair Vila Gomes and Borba, which are located on opposite sides of the Madeira River, only 15 km distant from each other (Fig. 1). Whilst the first test sought to verify the taxonomic validity of both species, the second test aimed to evaluate the effect of the Madeira River as a barrier between individuals from the left and right sides of the river, practically independently of the effect of geographic distance.

Individuals from the left and right river sides were also tested regarding body shape and acoustic differentiation, through a discriminant function analysis (DFA). In order to express differences in shape between the groups, we used the following body ratios according to Verdade &

**Table 1.** Morphometric measurements of the individuals studied. Data are presented according to the side of the Madeira River (left, including individuals from the type locality of *A. nidicola*, and right, including individuals from the type locality of *A. masniger*). The columns for *A. nidicola* and *A. masniger* display measurements of individuals from the type locality of each of the species. Values are presented as mean  $\pm$  standard deviation (range of variation). Characters are given in mm and are coded according to the section Materials and Methods.

Morphometric traits	Left side (n=50)	Right side (n=50)	<i>A. nidicola</i> (n=10)	<i>A. masniger</i> (n=10)
SVL	19.62 $\pm$ 0.84 (18.2–21.4)	19.13 $\pm$ 1.00 (16.7–21.5)	19.74 $\pm$ 0.62 (18.8–21.0)	20.45 $\pm$ 0.70 (19.32–21.5)
HL	6.89 $\pm$ 0.44 (5.9–7.9)	6.57 $\pm$ 0.47 (5.8–7.7)	7.17 $\pm$ 0.40 (6.6–7.8)	7.02 $\pm$ 0.47 (5.9–7.6)
HW	6.89 $\pm$ 0.32 (6.3–7.6)	6.65 $\pm$ 0.44 (6.0–8.1)	7.04 $\pm$ 0.24 (6.7–7.5)	7.16 $\pm$ 0.44 (6.8–8.1)
SL	2.79 $\pm$ 0.19 (2.5–3.2)	2.56 $\pm$ 0.25 (2.0–3.0)	2.8 $\pm$ 0.27 (2.5–3.2)	2.25 $\pm$ 0.21 (2.0–2.7)
EN	2.00 $\pm$ 0.13 (1.6–2.3)	1.79 $\pm$ 0.18 (1.3–2.2)	2.06 $\pm$ 0.17 (1.8–2.3)	1.52 $\pm$ 0.12 (1.3–1.7)
IN	2.62 $\pm$ 0.17 (2.1–3.0)	2.58 $\pm$ 0.16 (2.3–3.0)	2.67 $\pm$ 0.06 (2.6–2.8)	2.72 $\pm$ 0.14 (2.5–3.0)
EL	2.67 $\pm$ 0.15 (2.3–3.1)	2.63 $\pm$ 0.14 (2.4–3.0)	2.73 $\pm$ 0.02 (2.6–3.0)	2.54 $\pm$ 0.13 (2.4–2.8)
IO	2.34 $\pm$ 0.23 (2.0–3.0)	2.28 $\pm$ 0.19 (1.9–2.8)	2.44 $\pm$ 0.27 (2.0–2.9)	2.34 $\pm$ 0.23 (2.0–2.8)
TYM	1.09 $\pm$ 0.13 (0.7–1.3)	1.02 $\pm$ 0.12 (0.8–1.3)	1.05 $\pm$ 0.13 (0.8–1.3)	1.01 $\pm$ 0.13 (0.9–1.3)
FAL	4.19 $\pm$ 0.22 (3.7–4.8)	4.29 $\pm$ 0.24 (3.6–4.9)	4.18 $\pm$ 0.23 (3.8–4.5)	4.35 $\pm$ 0.17 (4.1–4.6)
UAL	4.92 $\pm$ 0.28 (4.4–5.9)	4.90 $\pm$ 0.29 (4.3–5.5)	4.91 $\pm$ 0.21 (4.5–5.1)	5.12 $\pm$ 0.19 (4.7–5.5)
HAND1	3.37 $\pm$ 0.18 (3.0–3.8)	3.47 $\pm$ 0.22 (2.9–3.9)	3.31 $\pm$ 0.15 (3.0–3.5)	3.68 $\pm$ 0.15 (3.5–3.9)
HAND2	3.13 $\pm$ 0.15 (2.7–3.4)	3.08 $\pm$ 0.22 (2.7–3.7)	3.13 $\pm$ 0.12 (2.9–3.3)	3.38 $\pm$ 0.19 (3.1–3.7)
HAND3	4.39 $\pm$ 0.19 (3.9–4.7)	4.19 $\pm$ 0.28 (3.7–4.9)	4.41 $\pm$ 0.12 (4.2–4.6)	4.57 $\pm$ 0.25 (4.1–4.9)
HAND4	2.93 $\pm$ 0.17 (2.5–3.2)	2.79 $\pm$ 0.22 (2.3–3.2)	2.98 $\pm$ 0.18 (2.7–3.2)	3.05 $\pm$ 0.17 (2.7–3.2)
WFD	0.49 $\pm$ 0.08 (0.3–0.7)	0.48 $\pm$ 0.07 (0.3–0.6)	0.50 $\pm$ 0.05 (0.4–0.6)	0.59 $\pm$ 0.05 (0.5–0.6)
TL	8.77 $\pm$ 0.33 (8.1–9.8)	8.69 $\pm$ 0.44 (7.5–9.7)	8.88 $\pm$ 0.38 (8.4–9.8)	9.05 $\pm$ 0.33 (8.4–9.7)
FL	7.76 $\pm$ 0.30 (6.9–8.5)	7.69 $\pm$ 0.38 (6.9–8.5)	7.65 $\pm$ 0.34 (6.9–8.1)	7.82 $\pm$ 0.41 (7.2–8.5)
LL	8.88 $\pm$ 0.33 (8.1–10.0)	8.85 $\pm$ 0.42 (8.0–9.8)	8.77 $\pm$ 0.34 (8.1–9.2)	8.96 $\pm$ 0.48 (8.3–9.8)
DPT	0.64 $\pm$ 0.06 (0.5–0.8)	0.63 $\pm$ 0.07 (0.5–0.8)	0.62 $\pm$ 0.05 (0.5–0.7)	0.63 $\pm$ 0.03 (0.6–0.7)
WTT	0.43 $\pm$ 0.06 (0.3–0.5)	0.38 $\pm$ 0.06 (0.3–0.5)	0.47 $\pm$ 0.06 (0.4–0.5)	0.44 $\pm$ 0.06 (0.4–0.5)
WTD	0.68 $\pm$ 0.07 (0.5–0.8)	0.63 $\pm$ 0.09 (0.5–0.9)	0.65 $\pm$ 0.05 (0.6–0.7)	0.78 $\pm$ 0.05 (0.7–0.9)
WPF	0.38 $\pm$ 0.05 (0.3–0.5)	0.36 $\pm$ 0.06 (0.3–0.5)	0.40 $\pm$ 0.04 (0.3–0.5)	0.46 $\pm$ 0.03 (0.4–0.5)

Rodrigues (2007): LL/SVL, HAND3/SVL, FL/SVL, HW/HL, EN/HL, EL/HL, TYM/HL, TYM/EL, IN/HW, HAND1/HAND2, HAND2/HAND3, HAND4/HAND1. In this analysis the dependent variables were the body ratios, and the independent variables were the sides of the Madeira River. Because of the confounding effects of both temperature and body size on call parameters (Gerhardt & Huber, 2002), measurements were adjusted through linear regression on the DFA. Therefore, we conducted a DFA on temperature-adjusted data, thus preserving differences in SVL between the two species, and also a DFA on measurements adjusted for both temperature and SVL, in order to account for possible ontogenetic effects on call characteristics. The discriminant function was used to calculate the probability of classification of each individual collected in the putative range of the two species, by a Jackknifed classification matrix.

## RESULTS

### Morphology from type localities

A principal components analysis of the 23 morphometric measurements of males collected from both type localities ( $n=20$  individuals, Table 1) generated a first (SVL; 37.02%) and second (Head; 16.65%) component, which together explained 53.6% of the external morphological variation. There was a significant morphological difference between the samples from the type-localities of both species (MANOVA: *Pillai Trace*=0.74;  $F_{(2, 17)}=24.66$ ;  $P<0.005$ ; *SVL*=10.10;  $F_{(1, 18)}=20.43$ ;  $P<0.005$ ; *Head*=4.03;  $F_{(1, 18)}=4.83$ ;  $P=0.04$ ) (Fig. 2A).

### Morphology from river sides

After detecting a statistically significant morphological difference between individuals from their type localities, we included intermediate sampling points in order to test whether the segregation holds between the two sides of the Madeira River, an important geographic barrier in the area (see Fig. 1). A principal components analysis of the 23 morphometric measurements of males collected from both river sides ( $n=100$  individuals) generated a first (SVL; 35.77%) and a second (Head; 11.58%) component, which together explained 47.35% of the external morphological variation. We also observed a significant morphological difference between individuals from the right and left sides of the Madeira River (MANOVA: *Pillai Trace*=0.16;  $F_{(2, 97)}=9.75$ ;  $P<0.001$ ; *SVL*=5.95;  $F_{(1, 98)}=6.27$ ;  $P=0.014$ ; *Head*=10.62;  $F_{(1, 98)}=11.78$ ;  $P=0.001$ ) (Fig. 2B).

Given that our MANOVA results suggested the existence of distinct groups on each side of the Madeira River, we also tested the morphological difference between individuals from the localities Vila Gomes and Borba, which are located close together, but also separated by this barrier. A PCA of the 23 morphometric measurements of males collected from the two localities ( $n=20$  individuals) generated a first (SVL; 50.92%) and a second (Tubercles; 9.45%) component, which together explained 60.37% of the external morphological variation. We also observed a significant morphological difference between individuals from these two localities (MANOVA: *Pillai Trace*=0.82;  $F_{(2, 17)}=39.52$ ;  $P<0.001$ ; *SVL*=14.38;  $F_{(1, 18)}=56.07$ ;  $P<0.001$ ; *Tubercles*=1.25;  $F_{(1, 18)}=1.27$ ;  $P=0.274$ ) (Fig. 2C).

**Table 2.** Classification matrix based on the discriminant function analysis, using data for 100 males from both sides of the Madeira River. The left side includes individuals from the type locality of *Allobates nidicola* ( $n=50$ ), and the right side includes individuals from the type locality of *A. masniger* ( $n=50$ ). Body-shape traits were used to discriminate individuals based on morphology. Acoustic measurements were adjusted for temperature and for both temperature and body size through linear regressions. The number and percentage of individuals correctly assigned to each group are indicated.

	Right	Left	% correct
<i>Body shape traits</i>			
Right	43	7	86
Left	9	41	82
Jackknifed classification matrix			
Right	42	8	84
Left	12	38	76
<i>Temperature-adjusted acoustic traits</i>			
Right	41	9	82
Left	7	43	86
Jackknifed classification matrix			
Right	41	9	82
Left	8	42	84
<i>Temperature- and body size-adjusted acoustic traits</i>			
Right	36	14	72
Left	9	41	82
Jackknifed classification matrix			
Right	36	14	72
Left	11	39	78

Based on body proportions, the discriminant function analysis showed a significant difference between male individuals ( $n=100$ ) from the two sides of the Madeira River (*Pillai Trace*=0.52;  $F_{(12, 87)}=8.04$ ;  $P<0.001$ ). The classification matrix (Table 2) shows that the DFA was able to correctly identify 84% of individuals from the right side (including those from the type locality of *A. masniger*) and 76% of individuals from the left side (including those from the type locality of *A. nidicola*).

### The advertisement call of *Allobates masniger*

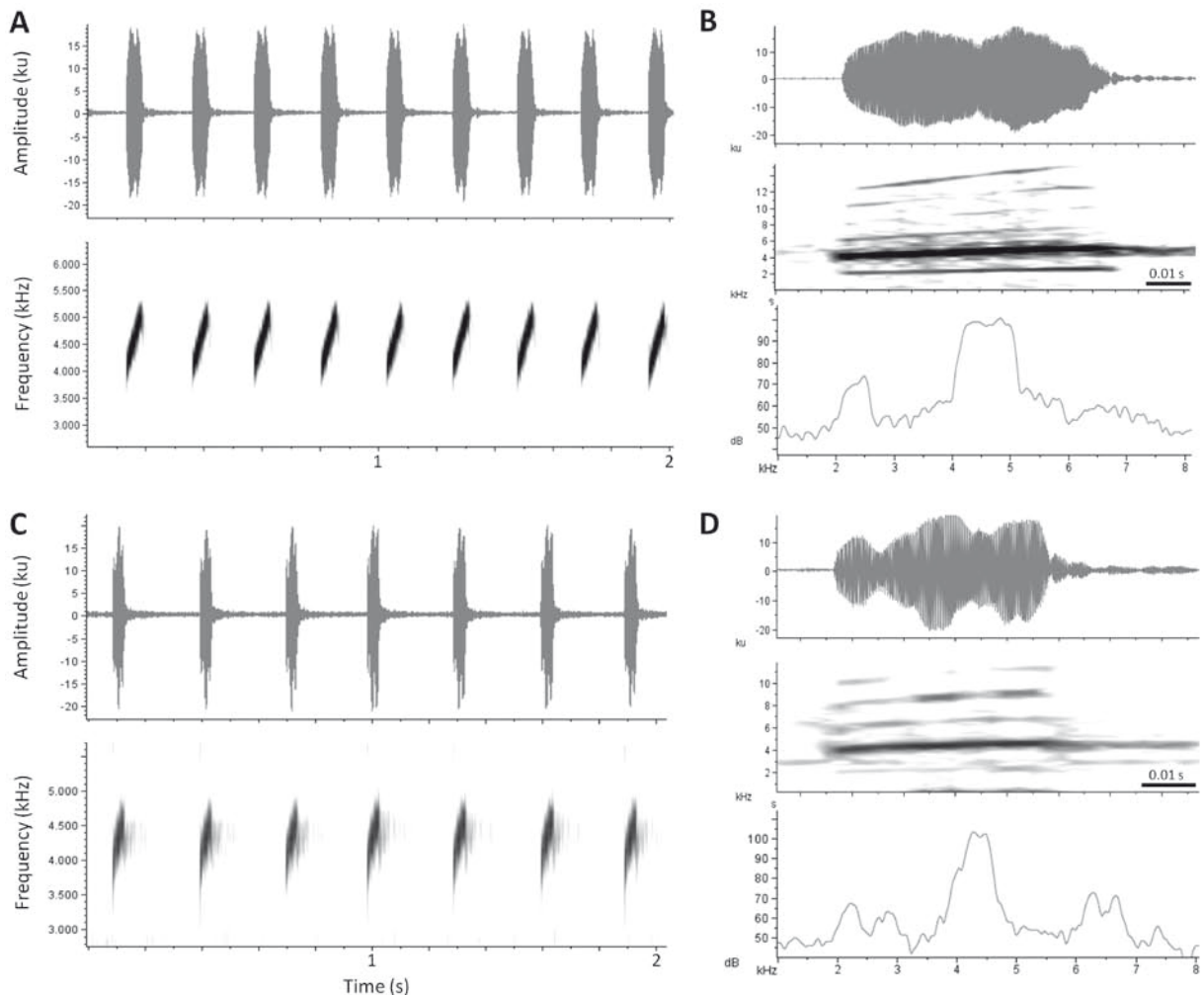
The call description is based on ten recordings from the Parque Nacional da Amazônia, Pará, Brazil, the type-locality of this species (Morales, 2002). It is composed of individual notes emitted in sequence (Fig. 3), with a mean frequency peak of 4566.5 Hz ( $\pm 92.9$ ). The note has an ascending frequency modulation, which ranges from 320.1 to 462.3 Hz. Mean note duration is 0.057 s ( $\pm 0.005$ ) and the mean interval between notes is 0.283 s ( $\pm 0.07$ ) (Table 3).

### Acoustics from type localities

A principal components analysis of the six acoustic measurements collected from the type localities of *A. masniger* and *A. nidicola* ( $n=20$  individuals, Table 3) generated a first (Spectral; 61.76%) and second (Temporal; 24.37%) component, which together explained 86.13% of the acoustic variation. There was a significant difference between the samples from the type localities of the two species (MANOVA: *Pillai Trace*=0.49;  $F_{(2, 13)}=7.46$ ;  $P=0.006$ ; *Spectral*=2.22;  $F_{(1, 16)}=9.97$ ;  $P=0.006$ ; *Temporal*=2.95;  $F_{(1, 16)}=3.84$ ;  $P=0.067$ ) (Fig. 4A).

### Acoustics from river sides

A PCA of the six acoustic measurements collected from both sides of the Madeira River ( $n=100$  individuals) generated a first (Spectral; 50.98%) and a second (Temporal; 20.40%) component, which together explained 71.38% of the total acoustic variation. We observed a significant difference in the advertisement calls between individuals from the right and left river sides



**Fig. 3.** Advertisement calls from the type localities of the species studied. (A) Oscillogram (above) and spectrogram (below) of a call sequence of an *A. masniger* male (SVL=18.5 mm; INPA-H 28196) recorded at Parque Nacional da Amazônia, municipality of Itaituba, Pará state, Brazil, at 0612 hours, air temperature 23.7 °C. (B) Oscillogram (above), spectrogram, and power spectrum (below) of a single call of this *A. masniger* male. (C) Oscillogram (above) and spectrogram (below) of a sequence of calls of an *A. nidicola* male (SVL=19.8 mm; INPA-H 28131) recorded at the locality Km 12 of Autazes Road, municipality of Autazes, Amazonas state, Brazil, at 0808 hours, air temperature 26.0 °C. (D) Oscillogram (above), spectrogram, and power spectrum (below) of a single call of this *A. nidicola* male.

**Table 3.** Advertisement call measurements of the individuals studied. Data are presented according to the side of the Madeira River (left, including individuals from the type locality of *A. nidicola*, and right, including individuals from the type locality of *A. masniger*). The columns for *A. nidicola* and *A. masniger* display measurements of individuals from the type locality of each of the species. Values are presented as mean±standard deviation (range of variation). Call traits are: note duration (ND, in s), interval between notes (IN, in s), lowest frequency (LF, in Hz), highest frequency (HF, in Hz), note modulation (NM, in Hz) and peak frequency (PF, in Hz).

Call traits	Left side (n=50)	Right side (n=50)	<i>A. nidicola</i> (n=10)	<i>A. masniger</i> (n=10)
ND	0.047±0.005 (0.034–0.060)	0.051±0.006 (0.038–0.064)	0.041±0.005 (0.034–0.050)	0.057±0.005 (0.047–0.064)
IN	0.282±0.050 (0.216–0.448)	0.346±0.084 (0.187–0.552)	0.274±0.038 (0.233–0.362)	0.283±0.07 (0.187–0.410)
LF	4023.1±246.6 (3555.7–4520.5)	4323.3±237.1 (3731.9–4918.7)	4069.0±129.5 (3821.1–4256.5)	4374.4±94.7 (4183.3–4520.8)
HF	4377.5±245.1 (3736.3–4836.0)	4723.97±206.2 (4194.6–5232.1)	4421.5±114.8 (4197.3–4576.5)	4752.8±99.5 (4545.3–4906.2)
NM	355.2±46.8 (286.6–486.2)	396.3±52.3 (313.4–542.6)	354.0±31.7 (319.6–409.9)	377.8±44.0 (320.1–462.3)
PF	4196.0±236.2 (3759.4–4689.9)	4522.1±211.0 (3998.4–5055.5)	4231.7±145.2 (3896.1–4413.4)	4566.5±92.9 (4362.6–4694.2)

(MANOVA: *Pillai Trace*=0.29;  $F_{(2, 95)}=19.61$ ;  $P<0.001$ ; *Spectral*=13.63;  $F_{(1, 96)}=28.33$ ;  $P<0.001$ ; *Temporal*=0.56;  $F_{(1, 96)}=0.76$ ;  $P=0.385$ ) (Fig. 4B).

A PCA of the six acoustic measurements collected from Vila Gomes and Borba ( $n=20$  individuals) generated a first (*Spectral*; 51.25%) and a second (*Temporal*; 19.06%) component, which together explained 70.31% of the advertisement call variation. We observed a significant acoustic difference between individuals from these two localities (*Pillai Trace*=0.54;  $F_{(2, 15)}=9.13$ ;  $P=0.003$ ; *Spectral*=1.80;  $F_{(1, 16)}=9.08$ ;  $P=0.008$ ; *Temporal*=0.76;  $F_{(1, 16)}=0.75$ ;  $P=0.398$ ) (Fig. 4C). The classification matrix (Table 2) showed that the DFA using temperature-adjusted data was able to correctly identify 82% of individuals from the right side (including those from the type locality of *A. masniger*), and 84% of individuals from the left side (including those from the type locality of *A. nidicola*).

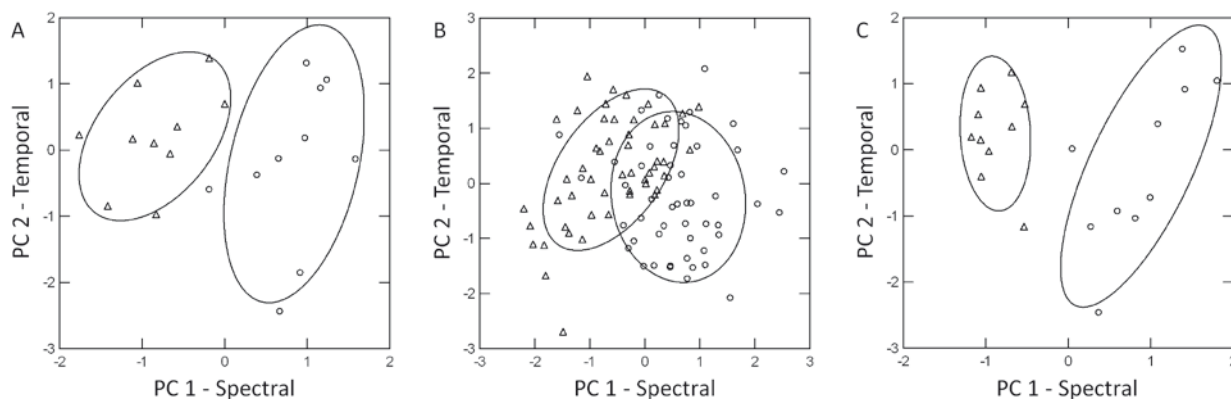
## DISCUSSION

The morphological and acoustic differences between individuals from the two river sides, as well as between the localities Vila Gomes and Borba, which are close together but separated by the Madeira River, indicates an allopatric distribution of divergent groups and the Madeira River as a geographic barrier. These results concord with earlier findings for *Allobates femoralis* (Simões et al., 2008; Simões et al., 2010) and provide further evidence of this river as a geographic barrier to dispersal in *Allobates*. Studies conducted at the Juruá River (Gascon et al., 1996; 1998; 2000; Loughheed et al., 1999) found no effect of a riverine barrier on frogs, probably because of the meandering nature of this river, which might allow passive movement of frogs along with the constant translation of blocks of land from one side to the other. These contrasting results may also be due to differences in river width, given that the effectiveness of a single river as a barrier was observed to vary according

to its width (Patton & da Silva, 1998). Studies have shown that Amazonian rivers do function as a geographic barrier for mammals (e.g., Ayres & Clutton-Brock, 1992; Peres et al., 1996) and birds (e.g., Hayes & Sewlal, 2004). However, for anurans, few studies have suggested that Amazonian rivers act as barriers (e.g., Noonan & Wray, 2006; Funk et al., 2007; Simões et al., 2008), and future studies, including other Amazonian tributaries, should enrich our knowledge about their role in promoting diversification at the species level.

We detected statistically significant phenotypic differences between *A. nidicola* and *A. masniger* in both morphological and bioacoustic characters, considering individuals from their type localities and also when assuming that they are allopatric when separated by the Madeira River. However, we did not find any diagnostic trait that could allow for their accurate differentiation. The ranges of variation of all analysed quantitative characters overlap, so there is no “phenotypic gap” that could provide us with elements to delimit the two species phenotypically and consequently determine their distributional ranges. Regardless of the species concept used to differentiate between taxonomic entities, diagnosability is required and should not be waived by any approach that is employed to discriminate between taxa (Goldstein & DeSalle, 2011). We support the use of integrative approaches that seek to find congruence between sets of characters that would be indicative of the evolutionary independence of a given lineage, and thus allow the lineage to be differentiated from other evolutionary entities (Padial et al., 2010), as is also highlighted by the general lineage concept of species (de Queiroz, 1998).

The spatial phenotypic differentiation detected in this study indicates that geography - through either isolation by distance or river effect - is involved in the mechanisms of differentiation, as observed in *A. femoralis* from Amazonia (Amézquita et al., 2009). In fact, visual inspection of our data in the multivariate space suggests



**Fig. 4.** Distribution of individual scores along the first and second principal components (PC) using acoustic variables. In A, *Allobates nidicola* (triangles) and *A. masniger* (circles) from their respective type localities. In B, all sampled individuals from the left (triangles) and right (circles) sides of the Madeira River. In C, individuals from the localities Vila Gomes (left side of the Madeira River; triangles) and Borba (right side of the Madeira River; circles). The confidence ellipses are centred on the sample means of the two principal components.

that both mechanisms are involved in the process, and future studies should be designed to disentangle these forces for a better comprehension of their role in phenotypic divergence and speciation. Differences in morphology and calls indicate genetic divergence in amphibians, but do not necessarily indicate interspecific status (Summers et al., 1997; Narins et al., 1998; Simões et al., 2008). Although morphology and calls are valid markers for biogeographic and evolutionary studies, in this study these attributes proved insufficient, as diagnostic features, to allow any decision regarding the taxonomic nature of the differences observed - i.e., whether they are intra- or interspecific. Hence, further lines of evidence such as behavioural, developmental and molecular traits would be needed. The male advertisement call plays a central role in female recognition and choice, being related to pre-zygotic isolation. Calls carry a phylogenetic signal and are considered important markers to help in assessing evolutionary differentiation in frogs (Erdtmann & Amézquita, 2009; Lehtinen et al., 2011). Calls have been used to resolve taxonomic issues without referring to molecular markers (Heyer, 2005; Angulo & Reichle, 2008; Barrio-Amorós & Santos, 2009; Angulo & Icochea, 2010). However, as observed in this study, genetic tools are needed to provide a better taxonomic resolution in many cases of cryptic species richness in *Allobates* (Simões et al., 2008; Amézquita et al., 2009; Simões et al., 2010).

Species descriptions in *Allobates* have employed morphological, bioacoustic, behavioural and molecular tools (e.g., Lima et al., 2007; Simões et al., 2010; Lima et al., 2010). Species redescrptions have also employed these classes of characters, which have proved essential for the resolution of many taxonomic problems in *Allobates*, as well as informing re-evaluations of the distributional boundaries of species (Caldwell et al., 2002; Lima & Keller, 2003; Lima et al., 2009). Although useful, in many cases additional traits are not available, and species of *Allobates* have also been described using classic taxonomic morphological approaches (Morales,

2002). Moreover, even the synonymisation of described species was proposed based solely on museum-preserved specimens (Verdade & Rodrigues, 2007). Based on our results presented here, we argue that further attention must be given to taxonomic decisions based on limited sets of data, especially because *Allobates* is presumed to comprise many cryptic and still-undescribed species (Caldwell et al., 2002; Grant et al., 2006; Montanarin et al., 2011).

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## APPENDIX: SPECIMENS STUDIED

INPA-H. Km 12 of Autazes Road: 28129, 28171, 28127, 28131, 28174, 28179, 28166, 28185, 28124, 28169; BR-319, km 260: 28153, 28178, 28181, 28156, 28168, 28183, 28173, 28186, 28189, 28175; PPBio Manaquiri: 28167, 28157, 28187, 28137, 28176, 28164, 28182, 28143, 28146, 28170; BR-319 Tupana: 28177, 28136, 28141, 28152, 28130, 28188, 28128, 28174, 28161, 28134; Vila Gomes: 28132, 28149, 28165, 28138, 28180, 28135, 28121, 28154, 28123, 28125; Borba: 28174, 28089, 28075, 28105, 28100, 28098, 28719, 28112, 28092, 28095; Novo Aripuanã: 28069, 28066, 28096, 28090, 28054, 28059, 28108, 28073, 28080, 28117; Road to Apuí: 28106, 28081, 28061, 28071, 28057, 28191, 28190, 28102, 28076, 28068; Jacareacanga: 28120, 28077, 28094, 28111, 28082, 28103, 28070, 28107, 28093, 28115; PARNA da Amazônia: 28193, 28196, 28206, 28213, 28207, 28200, 28208, 28207, 28214, 28109.

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