Calls, colours, shape, and genes: a multi-trait approach to the study of geographic variation in the Amazonian frog *Allobates femoralis*

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Evolutionary divergence in behavioural traits related to mating may represent the initial stage of speciation. Direct selective forces are usually invoked to explain divergence in mate-recognition traits, often neglecting a role for neutral processes or concomitant differentiation in ecological traits. We adopted a multi-trait approach to obtain a deeper understanding of the mechanisms behind allopatric divergence in the Amazonian frog, *Allobates femoralis*. We tested the null hypothesis that geographic distance between populations correlates with genetic and phenotypic divergence, and compared divergence between mate-recognition (acoustic) and ecological (coloration, body-shape) traits. We quantified geographic variation in 39 phenotypic traits and a mitochondrial DNA marker among 125 individuals representing eight populations. Geographic variation in acoustic traits was pronounced and tracked the spatial genetic variation, which appeared to be neutral. Thus, the evolution of acoustic traits tracked the shared history of the populations, which is unexpected for pan-Amazonian taxa or for mate-recognition traits. Divergence in coloration appeared uncorrelated with genetic distance, and might be partly attributed to local selective pressures, and perhaps to Batesian mimicry. Divergence in body-shape traits was low. The results obtained depict a complex evolutionary scenario and emphasize the importance of considering multiple traits when disentangling the forces behind allopatric divergence. ©2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, 98, 826–838.

ADDITIONAL KEYWORDS: allopatric divergence – Amazonas – anurans – bioacoustics – coloration – isolation by distance.

**INTRODUCTION**

Geographic variation is a necessary but not sufficient condition for species formation in allopatric models of speciation (Mayr, 1942). If behavioural traits that are related to mating begin to diverge in allopatry, the potential for speciation increases dramatically because among-population differences in mate-recognition traits may lead directly to pre-zygotic reproductive isolation (Lande, 1982; Panhuis et al., 2001). Although divergence in allopatry is accelerated by selective forces such as natural or sexual selection, nonselective forces such as genetic drift may also promote geographic variation (Wright, 1948; Lewontin & Krakauer, 1973; Avise, 2000; Nosil, 2007)
and even speciation through the stochastic divergence at extrinsic phenotypes (Irwin et al., 2005) or the accumulation of intrinsic Dobzhansky–Muller incompatibilities (Coyne & Orr, 1989). Although allopatric divergence does not always result in speciation, studying its origins will provide a better understanding of the initial stage of the speciation process under the most widely applicable speciation mode, the allopatric model (Coyne & Orr, 2004; Wiens, 2004).

Detailed studies of the role of genetic drift in geographic divergence of mate-recognition traits are surprisingly few in number (Ryan, Rand & Weigt, 1996; Wiens, 2004). The null hypothesis that divergence in mate-recognition traits is correlated with genetic divergence and geographic distance between populations should be regularly tested (Tilley, Verrell & Arnold, 1990; Panhuis et al., 2001; Boughman, 2002). Unexplained residual variation could be further tested for patterns predictable from the action of other evolutionary forces, such as ecological or sexual selection.

For two sister populations in allopatry, divergence at traits related to ecological performance (hereafter referred to as ecological traits) and reproduction (mate-recognition traits) may be differentially accelerated or constrained by selective forces relative to neutral traits (Spitze, 1993; Nosil, 2007). The rate of divergence might be similar if natural selection promotes geographic divergence in ecological traits, and reproductive incompatibility evolves secondarily via the concomitant divergence in mate-recognition traits (Rundle & Nosil, 2005). Alternatively, mate-recognition traits may diverge at faster rates compared to ecological traits if sexual selection promotes rapid divergence in both male traits and female preferences (Panhuis et al., 2001). The relative importance of different evolutionary forces for driving allopatric divergence can be better estimated with a multi-trait approach; for example, by comparing the degree of geographic differentiation between mate-recognition and ecological traits (Via, 2001).

In the present study, we adopt a multi-trait approach to study geographic variation in the frog, *Allobates femoralis* (Boulenger 1884; Anura: Aromobatidae). Previous studies indicated or documented geographic variation in calls, coloration pattern, and body size (Hödl, Amézquita & Narins, 2004; Amézquita et al., 2006). As in other anurans (Gerhardt & Huber, 2002), advertisement calls mediate mate recognition and antagonistic interactions in *A. femoralis*. The coloration pattern appears to play no role in intraspecific recognition (G. de Luna & A. Amézquita, pers. observ.), but may confer an ecological advantage if the coloration of *A. femoralis* mimics the coloration of toxic dendrobatid frogs in the eyes of potential predators (Darst, Cummings & Cannatella, 2006). Finally, body size and shape are generally considered to be evolutionarily conservative and of paramount importance in ecological and physiological performance (Barbault, 1988). By characterizing geographic variation in behavioural, ecological, and genetic traits among eight populations of *A. femoralis*, the present study (1) tested the null hypothesis that phenotypic divergence correlates with genetic divergence and geographic distance among populations; (2) compared the degree of geographic divergence between acoustic (i.e. mate recognition) traits and coloration and body-shape (i.e. ecological) traits; and (3) interpreted the resulting patterns using genetic distance at a presumably neutral locus as an estimation of time.

**MATERIAL AND METHODS**

We visited eight field sites distributed throughout the Amazon basin (Fig. 1) at the beginning of the rainy season. Most males were found at the edge of *terra firme* forest or associated with forest gaps, calling during daytime hours from elevated positions on the forest floor. Once we located a calling male, we (a) recorded the advertisement call (see below), (b) measured the air temperature at the place of calling, (c) captured the male, (d) photographed it, and (e) anesthetized it with commercial lidocaine and sacrificed it to obtain toe and liver samples for genetic analyses. Here, we present data obtained from 125 males (15–17 males per population). To reduce the potential measurement error as a result of among-observer variation, a single investigator measured each kind of phenotypic trait.

**ACOUSTIC ANALYSIS**

For each male, we recorded consecutive advertisement calls using a Sony WM D6C tape recorder and a microphone (AKG D-190-E, Shure BG4.1, or Sennheiser ME-62/K6) positioned at a distance of 0.5–1.5 m in front of the frog. Tape recordings were digitized at 22 kHz and spectral parameters of the calls were analysed calculating power spectra (Window: Blackman, DFT: 2048 samples, 3 dB filter bandwidth: 18.5 Hz) using the software CANARY, version 1.2.4 (Charif, Mitchell & Clark, 1995). Data from three calls per male were averaged to represent the smallest unit of statistical analysis. The within-male coefficient of variation ranged from 0.7% (call duration and spectral properties of the call) to 3% (note duration). Temporal and spectral parameters were measured using the terminology described in Cocroft & Ryan (1995). Low and high frequencies were measured at 20 dB (re 20 mPA) below the peak.
intensity, which is the value at which the signal energy could still be clearly distinguished from background noise.

COLORATION AND BODY SHAPE

We took lateral and dorsal photographs of each male with a Sony DSC-F717 digital camera. Images were taken between 07.00–11.00 h and 14.00–17.00 h, under cloudy conditions and under large trees close to or at the forest edge to ensure comparable patterns of high irradiance of most light wavelengths (Endler, 1993). Nonetheless, a Kodak colour card (Q-13, CAT 152 7654) was included in each picture to control for colour comparisons among study sites. All measurements on digital images were taken using the software SCION IMAGE (http://www.scioncorp.com).

To describe the colour pattern, we first measured the area covered by the dorsal line and the inguinal (femoral) patch and expressed it as percentage of the dorsal trunk area (Fig. 2). Second, we measured the relative brightness of seven body regions (Fig. 2) after applying red (r), green (g), and blue (b) filters to the digital picture. For each filter, a brightness score between 0 (dark) and 256 (light) was obtained as the average brightness value from five spots within each

Figure 1. Distribution of study populations of *Allobates femoralis* throughout the Amazon basin, and representative examples of advertisement calls and colour patterns. Photographs are not reproduced to scale. Oscillograms (blue) and sonagrams (grey) are shown of 2-s recordings of at least two advertisement calls. Calls consist of two notes in Catuaba, three notes in Panguana, and four notes in other populations. The geographic coordinates (degrees; latitude, longitude) are: Panguana (−9.6137, −74.9355), Leticia (−4.1233, −69.9491), Catuaba (−10.0742, −67.6249), Hiléia (−3.1977, −60.4425), Reserva Florestal Adolpho Ducke (−2.9333, −59.9744), Careiro (−3.3547, −59.8605), Treviso (−3.1491, −54.8403), and Arataí (3.9907, −52.5901).
body region. *Sensu* Endler (1990), each brightness score \((r, g, or b)\) was re-expressed as relative to the total brightness \([R'=r/(r+g+b), \ G'=g/(r+g+b), \ B'=b/(r+g+b)]\) and then used to derive the variables \((LM=R'-G'\) and \(B=B')\) that define a two-dimensional colour space for colour value of body parts. To summarize information on body proportions and absolute size, we measured eight morphological variables on the scaled digital images (Fig. 2).

**GENETIC ANALYSIS**

Genomic DNA was extracted using standard phenol-chloroform procedures (Sambrook & Russell, 2001). Polymerase chain reactions (PCRs) were conducted using shortened versions of Kocher’s universal primers L14841 (5′-CCATCCAACATCTCAGCATGAA-3′) and H15149 (5′-CCCTCAGAATGATATTGTCCTCA-3′) (Kocher et al., 1989), to amplify a 306-bp fragment of the cytochrome \(b\) (cyt \(b\)) mitochondrial gene. PCR followed standard protocols, with 1.5 mM MgCl\(_2\) in each reaction and an annealing temperature of 52 °C. Forward and reverse strands were sequenced independently on an ABI 3730 capillary sequencer. Sequences were aligned using CLUSTAL X, version 1.83 for Mac OS X (Thompson et al., 1997). No internal indels were observed, making alignment unambiguous. Amino acid translations were inferred using MacClade, version 4.06 for Mac OS X (Maddison & Maddison, 2003) and the lack of stop codons was confirmed using dnaSP, version 4.50 for Windows OS (Rozas et al., 2003).

To characterize the pattern of genetic variation, we calculated a median-joining (MJ) network (Bandelt, Forster & Röhl, 1999) using the Windows PC application NETWORK, version 4.112 (http://www.fluxus-engineering.com; for a review of techniques, see Posada & Crandall, 2001). Our MJ network was created using all 114 cyt \(b\) haplotypes, with all mutational differences weighted equally. The tolerance parameter, \(e\) (Bandelt et al., 1999), was left at its default value of zero, such that the resulting MJ network allowed a minimal amount of homoplasy and contained the smallest number of feasible links.

To accurately estimate genetic distances among populations, we applied a likelihood model of DNA sequence evolution. We evaluated the optimal evolutionary model of nucleotide substitution among the standard 56 models implemented in MODELTEST, version 3.6 for Unix (Posada & Crandall, 1998) and PAUP*, version 4.0b10 for Unix (Swofford, 1998) using the Akaike information criterion (Akaike,
The first model obtained from MODELTEST was used to calculate a Neighbour-joining tree (Saitou & Nei, 1987), which was then fed into a subsequent round of evaluation in MODELTEST. The resulting model and model-averaged parameter values (Posada & Buckley, 2004) were used to calculate genetic distances within and among populations. Because selective forces may influence within-population nucleotide polymorphism and among-population divergence, we tested the DNA sequence data for departure from the standard neutral model of molecular population genetics (Ballard & Kreitman, 1995). Animal mitochondrial DNA frequently shows an excess of amino acid polymorphism, indicative of slightly deleterious mutations (Rand & Kann, 1998). We tested for this and other potential selective forces using the McDonald–Kreitman test (McDonald & Kreitman, 1991). We also used Tajima’s D to test for an excess of low-frequency mutations, indicative of a recent selective sweep or population expansion (Tajima, 1989). All tests were conducted using dnaSP, version 4.50 for Windows OS (Rozas et al., 2003).

RESULTS

We found pronounced geographic variation in all phenotypic traits (Fig. 3; see also the Supporting information, Table S1). The phenotypic data were successfully reduced to a lower number of principal components (see the Supporting information, Table S2), as follows. Acoustic variables include call frequency (PC1), call duration (PC2), note duration (PC3), and inter-note interval (PC4); coloration variables include area of inguinal patch (PC1), back (PC2), axillary patch (PC3), and dorsal line area (PC4); and body shape variables include thigh width/body size (PC1), body width (PC2), and limb length (PC3). Temperature was significantly related to call frequency (linear regression, \( N = 112 \) frogs; \( F = 15.5, P < 0.001 \)) and inter-note interval (\( F = 20.5, P < 0.001 \)) and therefore we used the residuals (resPC1-Call frequency and resPC4-Inter-note interval) of the corresponding regressions for subsequent analyses.

The aligned cyt b fragments were comprised of 306 bp and 102 complete codons each, corresponding to positions 16348–16653 of the complete mitochondrial genome of Xenopus leavis, GenBank accession number NC_001573 (Roe et al., 1985). The chosen model was the Kimura (1981) three-parameter model + unequal base frequencies + gamma distributed rate heterogeneity among sites (Yang, 1994), or K81uf+G. The median-joining network based on 114 cyt b haplotypes closely resembled geography (Fig. 4). Because the genetic analysis revealed conspicuous differences between frogs from Catuaba compared to all other populations, we conducted further analyses both including and excluding individuals from this population.

The genetic data showed no evidence of departures from neutrality. We applied the McDonald–Kreitman test to Catuaba versus all other samples (\( P = 0.549 \), as well as to subsets of the data, such as Ducke-Carb-Hil versus Leticia-Panguana (\( P = 1.0 \)). We calculated Tajima’s D for the complete data set (\( D_T = 0.05303, P > 0.100 \)) and to all non-Catuaba sequences (\( D_T = 0.52999, P > 0.100 \)). To investigate the effect of missing data on these results, we removed the 20
shortest DNA sequences from the data set, repeated all analyses, and found identical results as above, except that for the McDonald–Kreitman test of Catuaba versus all other samples the \( P \)-value dropped slightly (\( P = 0.28227 \)).

Genetic distance was significantly correlated with geographic distance only when the Catuaba population was excluded (Mantel test; with Catuaba: \( r = 0.14, \ P = 0.260 \); without Catuaba: \( r = 0.60, \ P = 0.031 \)), suggesting that genetic differences between Catuaba and all other populations were much larger than predicted by geographic distance (Fig. 5A). Data visualization suggested some resemblance between phenotypic differences, genetic differences and the spatial distribution of populations (Fig. 4). Indeed, total phenotypic distance was correlated with genetic distance (with Catuaba: \( r = 0.66, \ P = 0.013 \); without Catuaba: \( r = 0.61, \ P = 0.005 \)) and, to a lower extent, with geographic distance between populations (with Catuaba: \( r = 0.36, \ P = 0.062 \); without Catuaba: \( r = 0.61, \ P = 0.014 \); Fig. 5B).

The first three functions of the discriminant function analysis (DF1 to DF3) explained 91% of the phenotypic variation. DF1 alone explained 67% of variation and revealed the largest differences between populations (i.e. the highest standardized coefficients of the discriminant function) in call duration, inguinal patch and inter-note interval (Fig. 6; see also the Supporting information, Table S3). After excluding the population of Catuaba (DF1 now explains 43% of variation), populations were more clearly differentiated in three out of four acoustic variables: call duration, note duration, and inter-note interval (Fig. 6; see also the Supporting information, Table S3). On average, the highest discriminant coefficients were for acoustic variables, followed by coloration and body shape variables (Fig. 6).

**Figure 3.** Geographic variation in acoustic, coloration, and body-shape traits among eight populations of the frog, *Allobates femoralis*. Values correspond to principal component scores (see the Supporting information, Table S2) that summarize covariation in original traits. For some variables, residual (‘res’) variation is shown, after statistically removing the effect of temperature.

DISCUSSION

Overall phenotypic divergence in *A. femoralis* appears to track the shared history of the populations as inferred from genetic similarity at a neutrally evolving mitochondrial DNA marker. However, when considering specific suites of phenotypic traits, contrasting patterns appear. (1) Acoustic traits are strongly differentiated among populations and this variation is significantly correlated with both genetic divergence and geographic distance, suggesting a role for genetic drift on allopatric divergence of acoustic traits. (2) At least one coloration trait, the inguinal patch, is strongly differentiated among populations but this variation is not correlated with genetic divergence or geographic distance, suggesting that geographic variation in this trait may be attributable at least in part to local selective forces. (3) Finally, body-shape traits show the lowest geographic structuring and no correlation with genetic divergence or geographic distance, suggesting possible constraints.

ACOUSTIC DIVERGENCE

Distant populations showed more genetic and acoustic divergence. Such correlations between acoustic, genetic and geographic distances fit the null hypothesis that neutral processes drive the evolution of the advertisement call in *A. femoralis*. A role for selective processes in allopatric divergence, however, cannot be precluded. Selective processes can generate correlations between genetic and phenotypic divergence at a deeper phylogenetic scale (Hansen & Martins, 1996), especially if the selective force is spatially variable. For example, similar patterns may be attributed to the selective effect of environmental clines (Lande, 1982; Wycherley, Doran & Beebee, 2002) or the inter-
action between the homogenizing effect of migration and the diversifying effect of local forces such as sexual selection (Thompson, 1999).

Regarding environmental clines, the study populations are mainly dispersed along an east–west axis (Fig. 1), coinciding with a cline in the amount and distribution of rainfall. Rainfall, to our knowledge, has been never invoked as a direct selective pressure on the evolution of anuran call traits such as call duration, note duration, and inter-note interval (Wilkczynski & Ryan, 1999; Gerhardt & Huber, 2002), nor do we do so in the present study. Nevertheless, rainfall may certainly affect anuran species richness and, thereby, the number of signalling species that co-occur with A. femoralis, which in turn might affect the evolution of their calls. However, this hypothesis has been tested and rejected: call divergence in A. femoralis was not related to the number and kind of syntopically and synchronically calling species (Amézquita et al., 2006). Alternatively, correlations with geographic distance may appear to be a result of migration promoting similarity among adjacent populations in the face of local selective pressures (Thompson, 1999). An homogenizing effect of migration among adjacent populations is very unlikely. The most proximal populations (Ducke, Hiléia, and Careiro) share no haplotypes, whereas the distant population pair, Arataï and Treviso, do share haplotypes (Fig. 4), suggesting recent migration. Significant spatial genetic structuring over short distances in mitochondrial and nuclear genes is not unusual in Neotropical amphibians (Crawford, 2003).

The evolution of the fauna and flora of the Amazonian basin is considered to reflect a variety of historical processes that may have variously interrupted or promoted gene flow among populations (Antonelli et al., 2009). Possible geographic barriers include unsuitable dry habitats (Haffer, 1969), geomorphic arches (Räsanen, Salo & Kalliola, 1987), and rivers (Gascon, Lougheed & Bogart, 1998; Lougheed et al., 1998), as well as marine incursions (Räsanen et al., 1995). None of these hypotheses predicts a relationship between genetic or phenotypic divergence and

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**Figure 5.** Pairwise relationships between (A) geographic, genetic, and (B) phenotypic distance among eight populations of the Amazonian frog, *Allobates femoralis*. A regression line is provided to illustrate statistically significant relationships, according to Mantel correlation analyses. Each data point represents the contrast between two populations, and white circles denote comparisons involving the population of Catuaba. Phenotypic distance is the Euclidean distance calculated from 11 parameters (total phenotypic distance) or from the corresponding subsets of four acoustic, four coloration, and three body-shape traits.
geographic distance. Instead, they predict a pattern of discontinuous variation across geographic barriers. A geographic pattern of isolation by distance in acoustic and genetic traits is somewhat surprising for a wide-ranging Amazonian species. A previous genetic approach (Fouquet et al., 2007) has, however, suggested that it might not be rare.

The aforementioned geographic hypotheses of Amazonian divergence are generally invoked to explain interspecific divergence, and may perhaps apply to the origin of the Catuaba population. As in Simões et al. (2008), we demonstrated the distinctiveness and discontinuous divergence of Catuaba frogs (Figs 4, 5), and suggest that the population of Catuaba may represent another taxonomic unit, whose divergence might be attributed to a geographic barrier different to the Madeira River, although this hypothesis demands further testing.

A geographic pattern of isolation by distance is also rather surprising given that mate-recognition signals, such as frog calls, are expected to be subject to strong constraining or diversifying selective pres-
Divergence of Coloration and Body-shape Traits

Geographic variation in body size and shape was on average very low, whereas colour variation was high. The functional role of size and shape in diverse aspects of anuran ecology and physiology likely imposes evolutionary constraints (Barbault, 1988; Lougheed et al., 2006). Among-population divergence in conspicuous coloration, particularly the inguinal patch, was high and uncorrelated with genetic or geographic distance. The role of body coloration in intraspecific communication in frogs is not yet clear (Hödl & Amézquita, 2001). In the poison frog Oophaga pumilio conspicuous coloration may play a role in mate choice (Summers et al., 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008), although previous studies on A. femoralis showed that inguinal and axillary colour patches are neither necessary (G. de Luna & A. Amézquita, pers. observ.) nor sufficient (Narins, Hödl & Grabul, 2003) to elicit male attacks on experimental dummies. Bright coloration in dendrobatid frogs is considered to serve mainly in interspecific communication, particularly the announcement of toxicity to potential predators (Santos, Coloma & Cannatella, 2003). Because no population of A. femoralis is known to be toxic, the conspicuously coloured inguinal and axillary patches may indicate Batesian mimicry on toxic syntopic species that bear similar coloration patterns, such as Amerega (Epipedobates) hahneli (Darst et al., 2006). Because bright axillary and inguinal patches are variable and widespread among toxic dendrobatid frogs, several of which co-occur with A. femoralis (A. P. Lima, W. Hödl, and A. Amézquita, pers. observ.), geographic variation in the inguinal patch of A. femoralis may result from the adaptive value of mimicking local toxic models.

The present study reveals that suites of phenotypic traits in A. femoralis may have diverged independently and that they differ both in the extent and mechanism of differentiation. Large geographic divergence occurred in acoustic traits that might affect mate recognition. A significant portion of this variation can be explained by shared history among populations, suggesting a substantial and unexpected role for neutral processes in allopatric divergence of mate-recognition signals. Coloration and body-shape traits revealed contrasting patterns, which are compatible with strong local selection pressures and evolutionary conservativeness, respectively. These findings depict an array of evolutionary processes underlying geographic divergence and emphasize the importance of considering multiple traits when disentangling the forces behind allopatric divergence and potential speciation.

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REFERENCES


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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary statistics of the acoustic, coloration, and body-shape traits measured on males of Allobates femoralis at eight populations: Leticia (LET), Hiléia (HIL), Careiro (CAR), Reserva Florestal Adolpho Ducke (DUC), Panguana (PAN), Catuaba (CAT), Treviso (TRE), and Aratai (ARA). For the geographic distribution of study populations, see Fig. 1. Temperature at which call recordings were made is provided below, following the acoustic parameters.

Table S2. Principal component analyses summarizing variation in acoustic, coloration, and body-shape traits measured on males of the frog Allobates femoralis from eight populations. Numbers represent loadings of original variables on principal components. The highest loadings are outlined in bold, when their value was higher than 0.6.

Table S3. Standardized coefficients (and percentage of explained variation) of the first four canonical discriminant functions that predict population membership of males of Allobates femoralis according to acoustic, coloration, and body-shape traits. Data are presented including (above) and excluding (below) the population of Catuaba. Phenotypic variables are principal components that summarize measured variables (see Table S2). The highest coefficients are outlined in bold, when their value was higher than 0.6.

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