INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA

Diversificação do complexo *Allobates femoralis* (Anura, Dendrobatidae) em florestas da Amazônia brasileira: desvendando padrões atuais e históricos.

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Manaus - AM
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PEDRO IVO SIMÕES

ORIENTADORA: Dra. ALBERTINA PIMENTEL LIMA
CO-ORIENTADORA: Dra. IZENI PIRES FARIAS

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BANCA EXAMINADORA DO TRABALHO ESCRITO:

Nome (instituição)	**Parecer**
Jeff Podos (University of Massachusetts)	Aprovado
Walter Hödl (Universität Wien)	Aprovado
José Manuel Padial (Uppsala University)	Aprovado
Robert Jehle (University of Salford)	Aprovado

BANCA EXAMINADORA DA DEFESA PÚBLICA DA TESE:

Nome	**Parecer**
José Antonio Alves Gomes (INPA)	Aprovado
Marcelo Menin (UFAM)	Aprovado
Mario Cohn-Haft (INPA)	Aprovado

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Sinopse:

São analisados aspectos da diversidade e evolução de um grupo de espécies de anuros filogeneticamente relacionados, amplamente distribuídos na Bacia Amazônica e historicamente reconhecidos como um único táxon: *Allobates femoralis*. Descreve-se uma nova espécie para o grupo e delimita-se a ocorrência de linhagens que representam potenciais novas espécies. É estudado o efeito do rio Madeira como barreira vicariante entre populações do grupo. Aponta-se que segmentos ao longo do rio não tiveram a mesma eficácia em prevenir a migração de indivíduos entre margens no passado. Por fim, é realizada a caracterização genética de uma zona de contato onde duas espécies do grupo hibridizam naturalmente.

Palavras-Chave: Herpetologia, Taxonomia, Biogeografia, Filogeografia, Evolução, Hibridização, Rio Madeira

À memória de Esther Mores.

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RESUMO

Neste estudo, analiso aspectos da diversidade e evolução de um grupo de espécies de anuros filogeneticamente relacionados, amplamente distribuídos na bacia Amazônica e historicamente reconhecidos como um único táxon: *Allobates femoralis*. O primeiro capítulo aborda as relações sistemáticas entre diversas populações alopátricas deste grupo e formaliza o reconhecimento e descrição de uma nova espécie: *Allobates hodli*. Também é apontada a ocorrência de outras linhagens monofiléticas, possuidoras de fenótipos acústicos e morfológicos característicos, indicadas como potenciais espécies crípticas. No segundo capítulo, avalio o efeito do rio Madeira sobre a diferenciação genética, morfológica e acústica de populações de *A. femoralis* distribuídas ao longo de seus interflúvios, além de aplicar análises filogeográficas para verificar a congruência entre os padrões de diferenciação observados e os padrões esperados caso o rio Madeira tenha funcionado como uma barreira vicariante desde sua formação. Análises filogenéticas e populacionais baseadas em marcadores moleculares mitocondriais apontam padrões condizentes com a hipótese de que o leito do rio Madeira represente uma barreira histórica proporcionando o isolamento entre populações de margens opostas. Porém, a eficácia do rio como barreira vicariante é variável ao longo de seu curso, sendo reportados prováveis eventos de dispersão entre margens em pontos entre o médio e o alto curso do rio. Populações amostradas em um mesmo interflúvio não possuem morfologia ou vocalizações mais similares entre si do que quando comparadas a populações amostradas na margem oposta, indicando que a diferenciação de caracteres fenotípicos é influenciada por outros mecanismos evolutivos. No terceiro capítulo, apresento a caracterização genética de uma zona de contato entre *A. femoralis* e *A. hodli*, localizada no alto rio Madeira. A análise de marcadores moleculares mitocondriais e microssatélites sugerem que hibridização natural entre as duas espécies é mais freqüente na linha central geográfica da zona de contato, decaindo abruptamente em um raio inferior a dois quilômetros à jusante e à montante desta área. Estimativas de diversidade genética obtidos em áreas adjacentes à zona de contato suportam a existência de seleção contra híbridos oriundos do cruzamento direto entre indivíduos parentais pertencentes às duas espécies.

ABSTRACT

In this study, I analyze aspects of the diversity and evolution of a group of phylogenetically related anuran species, which are widely distributed along the Amazon basin, and which have been historically recognized as belonging to a single taxon: *Allobates femoralis*. The first chapter addresses the systematic relationships between several allopatric populations of this group and formalizes the recognition and description of a new species: *Allobates hodli*. It also highlights the occurrence of additional monophyletic lineages, which present particular acoustic and morphological phenotypes, and are indicated as potential cryptic species. In the second chapter, I evaluate the effect of the Madeira River on the genetic, morphological, and acoustic differentiation between *A. femoralis* populations distributed along both interfluves, applying phylogeographic analyses in order to verify the congruence between observed differentiation patterns and those expected if the Madeira River has functioned as a vicariant barrier since its origin. Phylogenetic and population analyses based on mitochondrial molecular markers suggests patterns that are coincident with the hypothesis that the Madeira River channel represents a historical barrier causing the isolation between populations from opposite riverbanks. However, the effectiveness of the river as a vicariant barrier is variable along its course, and possible events of dispersal between riverbanks are reported for localities between its middle and upper course. Morphology and calls of populations sampled on the same interfluve are not more similar to each other in when compared to populations sampled on the opposite riverbank, suggesting that differentiation of phenotypic characters is influenced by additional evolutionary mechanisms. In the third chapter, I present the genetic characterization of a contact zone between *A. femoralis* and *A. hodli*, located on the upper Madeira River. Analyses of mitochondrial and microsatellite molecular markers suggest that natural hybridization between the two species is more frequent along the geographic central line of the contact zone, decaying abruptly less than two kilometers downstream and upstream of this area. Genetic diversity estimates measured at sites adjacent to the contact zone support the existence of selection against hybrids originating from direct crosses between parental individuals belonging to the two species.

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INTRODUÇÃO GERAL

Ocupando uma área estimada em mais de seis milhões de km^2, a bacia Amazônica concentra grande parte da biodiversidade do planeta, não raramente sendo relatadas estimativas de números de espécies por unidade de área superiores às esperadas por comparações com outros ecossistemas tropicais (Gentry, 1988; Betts *et al.*, 2008). Apesar do amplo reconhecimento da região amazônica como um dos últimos biomas terrestres tropicais a apresentar grandes extensões contínuas sob baixo impacto de ações antrópicas, o conhecimento científico sobre a biodiversidade contida nesta área avança à velocidade geralmente mais lenta do que a pressão exercida por processos de expansão das fronteiras agrícolas e das frentes colonização que a sucedem (Laurance *et al.*, 2004; Betts *et al.*, 2008).

Uma melhor compreensão a respeito da diversidade contida em um determinado grupo taxonômico, e a respeito de sua distribuição, tem sido dificultada não apenas pela falta de acesso por pesquisadores a áreas distantes dos centros regionais de pesquisa, mas também pela dependência histórica entre a prática taxonômica e a caracterização de espécies baseada exclusivamente em morfologia. Recentemente, a integração de dados filogeográficos e comportamentais a caracterizações morfológicas tradicionais é proposta como uma estratégia mais adequada à identificação de linhagens evolutivas distintas, especialmente entre espécies cujos sistemas reprodutivos relacionam-se fortemente a caracteres comportamentais e cuja diferenciação morfológica recíproca é sutil (Bickford *et al*., 2006). Este tipo de abordagem tem se mostrado útil principalmente à detecção de

espécies de anuros até então desconhecidas, ou mascaradas pela taxonomia vigente (Padial *et al*., 2009; 2010; Fouquet *et al*., 2007a, b; Vieites *et al*., 2009).

Além da elucidação de questões taxonômicas e sistemáticas, a análise conjunta de informações sobre a distribuição da variabilidade genética obtida a partir de análises filogeográficas e de dados sobre a distribuição de caracteres fenotípicos permite inferências mais precisas sobre a história evolutiva da linhagem de interesse (Avise, 2000; Knowles, 2009), fornecendo pistas de como a história geológica e demográfica influenciaram sua diversificação fisiológica, morfológica e comportamental. A despeito de grande controvérsia entre estudiosos do tema (Endler, 1982; Bush, 1994; Haffer 1997; Colinvaux *et al*., 2000), grande parte dos modelos evolutivos propostos para a bacia Amazônica relacionam a diversificação entre grupos de organismos amazônicos a eventos históricos envolvendo barreiras vicariantes determinadas por ciclos climáticos (*e.g.* Haffer, 1969) ou pela dinâmica geológica e hidrológica da bacia (Hoorn, 1994; Gascon *et al*., 1998; 2000; Lougheed *et al*., 1999; Hoorn & Wesselingh, 2010).

Dentre estes modelos, a hipótese de rios como barreiras se destaca como o mais antigo (Wallace, 1852). Segundo interpretações mais recentes (Caparella, 1987; Colwell, 2000; Gascon *et al*., 2000), grandes rios amazônicos deveriam representar obstáculos intransponíveis para alguns organismos, dificultando a dispersão de indivíduos e, consequentemente, reduzindo o fluxo gênico entre suas populações. Uma vez isoladas em margens opostas, estas populações passariam a sofrer processos evolutivos independentes, podendo se tornar diferentes linhagens evolutivas ao longo do tempo. Desacreditada por estudos anteriores (Gascon *et al.* 1998; 2000; Lougheed *et al*., 1999) realizados ao longo do rio Juruá, um tributário meridional do rio Amazonas, a influência

de outros grandes rios amazônicos sobre a diferenciação genética e fenotípica entre populações de anfíbios anuros foi pouco estudada. Se o mesmo padrão observado para o Rio Juruá não for verdadeiro para outros rios da bacia, o paradigma de homogeneidade entre grupos habitantes de margens distintas pode trazer conseqüências graves para o planejamento de estratégias de conservação da biodiversidade da região (Azevedo-Ramos & Galatti, 2002).

Enquanto hipóteses a respeito da origem da biodiversidade amazônica têm recebido alguma atenção em estudos biogeográficos, os processos evolutivos que a mantém raramente são mencionados. Em especial, conseqüências evolutivas derivadas do contato secundário entre espécies que divergiram em alopatria causada por uma barreira histórica são desconhecidas entre anuros da Amazônia brasileira. A hibridização natural entre espécies a partir de contato secundário geralmente envolve apenas um pequeno número de indivíduos, mas trata-se de um fenômeno comum em relação ao número de espécies, subespécies ou morfotipos entre os quais é reportada (Mallet, 2005; Genovart, 2009). Um dos paradigmas iniciais a respeito da hibridização natural propunha que barreiras reprodutivas existentes entre espécies são eficientes a ponto de manter cruzamentos interespecíficos em freqüências muito baixas e, quando ocorrendo, originando híbridos inviáveis, estéreis ou com baixo potencial de sobrevivência ou reprodução. Tal paradigma relacionava-se diretamente à adoção irrestrita do conceito biológico de espécies, tendo o isolamento reprodutivo como pré-requisito para o reconhecimento de dois grupos de organismos como pertencentes a espécies distintas (Mayr, 1996, sintetiza este ideário, principalmente à luz dos padrões observados entre espécies animais). Assim, a hibridização era vista como um processo inócuo, quando

considerados seus efeitos potenciais sobre a evolução das espécies ou subespécies envolvidas. De fato, estudos mais recentes, envolvendo tanto espécies de plantas quanto de animais, constatam que a hibridização entre linhagens divergentes ao longo de zonas de contato secundário não apenas têm efeitos significativos sobre a diversidade genética e a dinâmica populacional destas linhagens, como também que tais efeitos são extremamente variáveis entre táxons e entre suas populações, opondo-se ao antigo paradigma de inocuidade (Mallet, 1995; Arnold *et al*., 1999; Coyne e Orr, 2004).

Allobates femoralis

Allobates femoralis (Boulenger, 1883) é um anuro diurno e terrestre, pertencente à família Dendrobatidae, amplamente distribuído em florestas de terra-firme (não inundáveis) na bacia Amazônica. É geralmente encontrado em atividade sobre o folhiço ou entre troncos caídos, em ambientes florestais (Roithmair, 1994; Lescure & Marty, 2000). A reprodução da espécie ocorre durante a estação chuvosa e a postura dos ovos é realizada em folhas sobre o chão, sendo os girinos transportados posteriormente pelos machos até corpos d´água próximos. Machos de *A. femoralis* são extremamente territoriais durante a época reprodutiva e vocalizam a partir de sítios elevados em relação ao chão dentro de seus territórios, os quais mantêm por períodos que variam de alguns dias a até mais de um mês (Roithmair, 1992, 1994; Rodríguez & Duellman, 1994). As vocalizações de anúncio, utilizadas para a atração de fêmeas e demarcação dos territórios, são constituídas pela repetição regular de um grupo de notas curtas e moduladas em freqüência (Hödl, 1987).

O comportamento estacionário de *A. femoralis*, com territórios de diferentes machos distribuídos pelo menos alguns metros entre si, permite que um indivíduo seja gravado e capturado sem que o procedimento interfira gravemente na atividade de indivíduos vizinhos (Hödl, 1987; Roithmair, 1992). Estas características, aliadas a um suposto potencial para respostas genéticas e fenotípicas a eventos históricos ou ecológicos (devido a sua restrição a florestas de terra-firme), tornam *A. femoralis* uma espécie ideal para estudos de variação interpopulacional em caracteres acústicos, genéticos e morfológicos.

Estudos anteriores constataram a ocorrência de dois morfotipos de *Allobates femoralis* na margem esquerda do alto rio Madeira e descreveram variações morfológicas e acústicas coincidentes com a separação de populações pelo leito do rio (Simões *et al*., 2008). Os dois morfotipos têm distribuição exclusiva, um morfotipo nunca ocorrendo na área onde ocorre o segundo. Entretanto, indivíduos de ambos os morfotipos são encontrados em uma zona de contato perpendicular ao rio e coincidente com o limite entre duas unidades de relevo. Até então, a ausência de uma base de dados moleculares e de amostragens em outros pontos da distribuição da espécie haviam impossibilitado inferências mais expressivas sobre as relações evolutivas e a biogeografia das linhagens que compõem este sistema. Ao longo dos três capítulos a seguir, analiso e discuto estes temas contando com resultados obtidos a partir de uma amostragem mais abrangente sobre a variabilidade genética, morfológica e comportamental do grupo *Allobates femoralis*.

OBJETIVOS

Os objetivos gerais de cada capítulo foram os seguintes:

Capítulo I – Elucidar as relações sistemáticas entre um dos morfotipos de *Allobates femoralis* encontrados no alto rio Madeira e as demais populações reconhecidas sob o mesmo táxon, propondo os rearranjos taxonômicos necessários;

Capítulo II - Avaliar a influência do rio Madeira sobre a variabilidade genética, acústica e morfológica entre populações do complexo *Allobates femoralis*, testando seu efeito como uma barreira à dispersão de indivíduos da espécie e elucidando as relações filogeográficas entre as populações estudadas;

Capítulo III – Caracterizar geneticamente a zona híbrida entre os dois morfotipos de *Allobates femoralis* descrita para o alto rio Madeira, avaliando a ocorrência, freqüência e extensão geográfica de eventos de hibridização e introgressão genética entre as duas linhagens.

Capítulo I[1]

[1] Manuscrito formatado de acordo com as normas da revista Zootaxa. Publicado no volume 2406, páginas 1 a 28, em abril de 2010. O posicionamento das espécies aqui estudadas na Família Aromobatidae reflete a taxonomia proposta por Grant *et al.* 2006, mais tarde revisada por Santos *et al*. 2009, que propõem a ressureição da família Dendrobatidae, sendo esta última classificação utilizada nos Capítulos II e III.

Title: The description of a cryptic species related to the pan-Amazonian frog *Allobates femoralis* (Boulenger 1883) (Anura: Aromobatidae).

Corresponding Author: Pedro Ivo Simões

E-mail: pedroivo@inpa.gov.br

Authors: Simões, Lima & Farias

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The description of a cryptic species related to the pan-Amazonian frog *Allobates femoralis* (Boulenger 1883) (Anura: Aromobatidae).

PEDRO IVO SIMÕES[1,3], ALBERTINA P. LIMA[1] & IZENI PIRES FARIAS[2]
[1]*Coordenação de Pesquisas em Ecologia, Instituto Nacional de Pesquisas da Amazônia, Manaus, AM, Brazil.*
[2]*Laboratório de Evolução e Genética Animal, Departamento de Biologia, Universidade Federal do Amazonas, Manaus, AM, Brazil.*
[3]*Corresponding author: pedroivo@inpa.gov.br*

Abstract

We describe a new species of litter frog from western Brazilian Amazon previously referred to as *Allobates femoralis* (Boulenger 1883). The new species is allopatric to *A. femoralis* and its known occurrence is restricted to terra-firme forests on the left bank of the upper Madeira River and southeastern State of Acre. This species is distinguished from *A. femoralis* and from other species in the *A. femoralis* group by presenting two-note advertisement calls and conspicuous reddish-orange color on ventral surfaces of hind limbs and posterior abdomen. Phylogenetic analyses based on a fragment of the 16S rRNA mitochondrial gene suggest the new species is the sister group to a clade referred to as *A. femoralis* occurring in southern State of Acre, from which it is distinguished by six unambiguous nucleotide substitutions, in addition to exclusive advertisement calls and color patterns. The new species is more distantly related to *A. femoralis* sensu stricto occurring near the *A. femoralis* type locality in the Peruvian Amazon. Summarizing evidence from molecular phylogenetic analysis, genetic distances and available data on advertisement calls, we identify one possible case of genetic introgression between lineages in this group and highlight the potential for the description of more species within the *A. femoralis* complex.

Key words: Amazonia, *Allobates hodli* sp. nov. , Brazil, Dendrobatoidea, *femoralis*, new species, species complex, taxonomy.

Resumo

Nós descrevemos uma nova espécie de rã de folhiço para a Amazônia Brasileira ocidental, a qual foi previamente tratada como *Allobates femoralis* (Boulenger 1883). A nova espécie é alopátrica em relação a *A. femoralis* e sua ocorrência conhecida é restita a florestas de terra-firme na margem esquerda do alto rio Madeira e sudeste do Estado do Acre. Esta espécie se distingue de *A. femoralis* e de outras espécies do grupo *A. femoralis* por possuir cantos de anúncio constituídos por duas notas e coloração laranja-avermelhada na superfície ventral dos membros posteriores e abdôme posterior. Análises filogenéticas baseadas em um fragmento do gene mitocondrial 16S rRNA sugerem que a nova espécie é o grupo-irmão de um clado reconhecido como *A. femoralis* que ocorre no sul do Estado do Acre, do qual se distingue por seis substituições nucleotídicas não-ambíguas, além de padrões exclusivos de vocalizações de anúncio e de coloração. A nova espécie é evolutivamente mais distante de *A. femoralis* sensu stricto, que ocorrem

próximos à localidade-tipo de *A. femoralis* na Amazônia peruana. Sumarizando evidências obtidas através de análise filogenética molecular, distâncias genéticas e dados disponíveis sobre vocalizações de anúncio, nós identificamos um possível caso de introgressão genética entre linhagens deste grupo e enfatizamos o potencial para a descrição de mais espécies dentro do complexo *A. femoralis*.

Palavras-chave: Amazônia, *Allobates hodli* sp. nov., Brasil, Dendrobatoidea, *femoralis*, nova espécie, complexo de espécies, taxonomia.

Introduction. For some time researchers have pointed out that the existence of cryptic species within widespread anuran taxa could be frequent in the Amazon basin (Wynn and Heyer 2001; Azevedo-Ramos & Galatti 2002). Such suggestions now receive great support from recent work providing evidence for the existence of cryptic lineages within different families of frogs (Fouquet *et al.* 2007; Twomey & Brown 2008; Brown & Twomey 2009; Lötters *et al.* 2009; Padial & De la Riva 2009). Adding to the conservative nature of some morphological characters frequently used in taxonomic studies, the lack of extensive behavioral databases and very long distances between sampling sites compromise the diagnosis of cryptic lineages and the accurate determination of their distributions.

Allobates Zimmermann & Zimmermann (1988) is the most species-rich and widespread genus within the family Aromobatidae (Grant *et al.* 2006). Forty-four *Allobates* species are currently recognized, distributed in lowland forests from the eastern slope of the Andes, across the Amazonian lowlands of Bolivia, Colombia, Ecuador, Peru and Brazil, and reaching the Guyana Shield and Atlantic forests of Brazil (Lötters *et al.* 2007; Frost 2009). New species of *Allobates* are regularly found in the Amazonian lowlands (Lima & Caldwell 2001; Caldwell & Lima 2003; Lima *et al*. 2007) and recent species redescriptions that include behavioral, reproductive mode and larval morphology data from type locality populations (Caldwell *et al.* 2002; Lima *et al*. 2009) will likely increase the rate of species discoveries in this region. Although sampling efforts are still deficient, many of the recently described species apparently have limited distributions, and revisionary studies of currently widely distributed taxa will probably result in the discovery of many new species.

Recently, comprehensive studies of the phylogenetic relationships and evolution of the Amazonian poison-frogs (Grant *et al*. 2006; Santos *et al*. 2009) have indicated the existence of elevated genetic divergence between lineages of a ground-dwelling frog, *Allobates femoralis*, and proposed that this taxon consists in a complex of cryptic species. *Allobates femoralis* is widely distributed throughout primary, non-flooded forest areas in the Amazon Basin. During the last 30 years, several populations belonging to this taxon have been the subject of numerous studies, ranging from acoustic and visual communication (Hödl 1987; Narins *et al*. 2003; Hödl *et al.* 2004; Amézquita *et al.* 2005; 2006; Göd *et al.* 2007) to territorial and reproductive behavior (Roithmair 1992; 1994; Ringler *et al.* 2009), with numerous authors pointing out the existence of conspicuous population variation in morphology, acoustic signal detection, advertisement call characteristics, color and genetic traits (Lutz & Kloss 1952; Hödl 1987; Lougheed *et al.* 1999; Amézquita *et al*. 2006; 2009; Simões *et al*. 2008).

In this study, we aim to add to the findings reported in Simões *et al*. (2008) and Amézquita *et al*. (2009) on the acoustic, morphological and genetic differentiation of a geographically restricted group found in southwestern Brazilian Amazon that presents a two-note advertisement call, previously referred to as *Allobates femoralis*. This group is allopatric (and in two instances, parapatric) to populations of *Allobates femoralis* that resemble that from type locality in call characteristics, color pattern and morphology. The locations of two contact zones between these lineages are provided herein. We describe this group as a new species, presenting detailed information on morphology, behavioral traits, geographic distribution, as well as phylogenetic and genetic differentiation data based on mitochondrial DNA. Additionally, we use available mtDNA sequences and records of advertisement calls to explore the relationships between the new species and other populations referred to as *A. femoralis*, identifying cryptic lineages that might be potential subjects for future taxonomic investigation.

Material and Methods. Specimens described here were deposited in the herpetology section of the zoological collection of Instituto Nacional de Pesquisas da Amazônia (INPA-H), in Manaus, Brazil, coming from field work carried out in four localities in the extreme southeast of the State of Acre (in January 2003) and along the left bank of the upper Madeira River (from November 2004 to February 2005) in northern state of Rondônia (Fig. 1). Specimens were collected as part of studies addressing the geographic variation in populations of the group *Allobates femoralis*. Complementary information on field procedures and more comprehensive data on the study area can be found in Simões *et al*. (2008) and Amézquita *et al*. (2009).

We examined and measured all specimens in the laboratory using a digital caliper or a micrometer on a dissecting microscope to the nearest 0.01 mm. Measurements and terminology, as well as diagnostic characters, followed Lima *et al*. (2007). Some diagnostic characters were included following Grant *et al*. (2006) and Lötters *et al*. (2007). Measurements were: snout to vent length (SVL), head length from tip of snout to posterior edge of maxilla articulation (HL), head width at the level of maxilla articulation (HW), snout length (SL), eye-to-nostril distance from anterior corner of the eye to the center of nostril (EN), internarial distance (IN), eye length from anterior to posterior corner (EL), interorbital distance (IO), maximum diameter of tympanum (TYM), forearm length from proximal edge of palmar tubercle to outer edge of flexed elbow (FAL), lengths from proximal edge of palmar tubercle to tips of fingers I, II and III (HAND I, HAND II, HAND III), width of disk on Finger III (WFD), thigh length from the posterior extremity of the coccyx to the outer edge of flexed knee (THL), tibia length from outer edge of flexed knee to heel (TIL), foot length from proximal edge of outer metatarsal tubercle to tip of Toe IV (FL), width of disk on Toe IV (WTD). Additionally, we measured arm length from anterior corner of arm insertion to the outer edge of flexed elbow (AL), the length from proximal edge of palmar tubercle to tip of Finger IV (HAND IV) and tarsus length from heel to the distal edge of inner metatarsal tubercle (TAR).

Descriptions of color in life were based in direct observation of specimens during field work and photographs by A. P. Lima and Walter Hödl.

Four tadpoles were used for description. These tadpoles were obtained from a clutch collected in the locality of Abunã on 15 January, 2005. Tadpoles were raised in laboratory until stage 36 of Gosner (1960), anesthetized in a solution of lidocaine and

preserved in 10% formalin on 28 January, 2005. Measurements and terminology for description of tadpoles follow McDiarmid and Altig (1999). Measurements were: total length from tip of snout to tip of tail (TL), body length from tip of snout to body-tail insertion (BL), tail length from body–tail insertion to tip of tail (TAL), body width at spiracle level (BW), body height at spiracle level (BH), tail muscle maximum width (TMW), tail muscle maximum height (TMH), tail maximum height (TH), head width at the level of the eyes (HWLE), interorbital distance (IOD) and internostril distance (IND).

We recorded advertisement (n = 60) and courtship (n = 4) calls of males collected in the localities along the upper Madeira River (Cachoeira do Jirau, Mutum-Paraná and Abunã) using a Sony WM-D6C tape recorder (2004, Sony Corr., Japan) and AKG 568 EB directional microphone (2003, AKG acoustics GMBH, Austria), positioned approximately 1 m away from the calling individual. All recordings were made at 06:30-18:00 h and air temperature at the moment of recording was registered. Recordings were digitized from tapes using Raven 1.2 software (Charif *et al*. 2004) at a sample rate of 22050 Hz and 16 bits sample format.

From the recording of each individual, we sampled three advertisement calls from which we measured spectral and temporal parameters, according to procedures described in Simões *et al.* (2008). Measurements were: silent interval between calls (SIC), silent interval between first and second note (SIN), duration of call (DC), duration of first note (D1), duration of second note (D2), maximum frequency of call (MFC), highest frequency of call (HFC), lowest frequency of call (LFC), maximum frequency of first note (MFN1), highest frequency of first note (HFN1), lowest frequency of first note (LFN1), maximum frequency of second note (MFN2), highest frequency of second note (HFN2), lowest frequency of second note (LFN2). Courtship calls were recorded opportunistically during the recording of advertisement calls, and the number of calls obtained from a total four individuals varied. Therefore, measurements (DC, MFC, and number of pulses) were obtained from a single call or from all available calls. In the latter case, values presented are the averages among all available calls.

Samples of muscle and liver tissue preserved in 95% ethanol were obtained from individuals collected in the three localities along the upper Madeira River (Cachoeira do Jirau, Mutum-Paraná, Abunã, Fig. 1) and were housed at Coleção de Tecidos de Genética Animal at Universidade Federal do Amazonas (CTGA – ICB/UFAM), Manaus, Brazil. Additional tissue samples were obtained from populations referred to as *A. femoralis* in 10 other localities in Brazilian Amazonia (Fig. 1). Two of these populations (Monte Alegre, Lower Jirau, Fig. 1) are located immediately outside contact zones with the species described herein.

Total genomic DNA extraction was carried out from samples using cetyl trimethyl ammonium bromide (CTAB) protocol (modified from Doyle & Doyle 1987). We used primers 16Sar and 16Sbr (Palumbi 1996) to amplify a 518 b.p. partial sequence of the 16S rRNA mitochondrial gene via polymerase chain reaction (PCR) from total genomic DNA. PCR reactions used a final volume of 16 µL and contained 6.7 µL ddH_2O, 2.0 µL of 25 mM $MgCl_2$, 1.5 µL of 10 mM dNTPs (2.5mM each dNTP), 1.5 µL of 10X amplification buffer (75 mM Tris HCl, 50 mM KCl, 20 mM $(NH_4)2SO_4$), 1.5 µL of a 2 µM solution of each primer, 0.3 µL of Taq DNA Polymerase 5 U/µL (Biotools, Spain) and 1 µL of DNA (about 30 ng/µL). PCR conditions had a pre-heating step of 92°C for 60 s, followed by 35 cycles of denaturation at 92° for 60 s, primer annealing at

50°C for 50 s and primer extension at 72°C for 90 s. A final extension step occurred at 72°C for 5 min. Sequencing reactions were performed according to manufacturer's recommended ABI BigDye Terminator Cycle Sequencing protocol, using primer 16Sbr and an annealing temperature of 50°C. Sequencing was performed in an automatic ABI 3130xl Sequencer.

Sequences were aligned using the ClustalW algorithm (Thompson *et al.* 1994) implemented in BioEdit (Hall 1999) and checked by eye. Final data set included 72 sequences of the new species (27 of topotypic individuals from Cachoeira do Jirau), plus 96 additional sequences of *A. femoralis* from the additional 10 sampling sites, as well as 28 sequences from reference *A. femoralis* populations (Fig. 1, Table 5). Reference sequences included one sequence from a locality close to Yurimaguas, Loreto (collected at Shucshuyacu, 20 km from *A. femoralis* type-locality), one sequence from Tarapoto, San Martin, (130 km from *A. femoralis* type-locality) and one sequence from Panguana (400 km from *A. femoralis* type-locality), all in Peru. To date, Sucshuyacu (site 12 in Fig. 1B), near Yurimaguas, is considered the site closest to the *A. femoralis* type-locality from where DNA sequences were made available. Other reference sequences include individuals sampled in Ecuador, Colombia, Suriname and other sites in Peruvian and Brazilian Amazon. *Aromobates nocturnus*, *Anomaloglossus stepheni*, *Allobates talamancae*, *Allobates nidicola* and *Allobates zaparo* were used as outgroups. The first four taxa are considered basal to *A. femoralis*, and *A. zaparo* is considered its sister species (Grant *et al.* 2006; Santos *et al.* 2009). Reference and outgroup sequences were all obtained from GenBank (Table 5). Uncorrected pairwise genetic distances between groups were calculated in MEGA (Tamura *et al.* 2007). Data set was reduced to unique haplotypes (including outgroups) for phylogenetic analysis. Phylogenetic analysis was performed in Treefinder (Jobb 2008) under the Maximum Likelihood criterion with GTR+I+G model of substitution, selected via Akaike information criterion as implemented in Modeltest 3.7 (Posada & Crandall 1998).

Natural history observations were made opportunistically during field work by P. I. Simões and A. P. Lima. Additionally, stomachs of 81 preserved individuals from the three localities along the upper Madeira River were dissected under stereoscopic microscope for a brief analysis of diet. Prey items were identified to order and quantified as simple frequencies (number of stomachs containing item / total non-empty stomachs examined).

***Allobates hodli* sp. nov**

Figures 2–5.

Epipedobates femoralis Hödl *et al.* 2004 p. 823, Catuaba, Acre population (partim).
Allobates femoralis Amézquita *et al.* 2006 p. 1877, Catuaba, Acre population (partim); Lötters *et al.* 2007 p. 307, Fig. 379; Simões *et al.* 2008 p. 610, Fig. 2B. (partim); Amézquita *et al*. 2009, Fig. 1, Catuaba pattern (partim).

Holotype. INPA–H 16555 (original field number APL 2014). Adult male, collected by P. I. Simões and A. P. Lima after recording of advertisement calls at 07:55 h, 25th of

November 2004, at Cachoeira do Jirau, on the left bank of the upper Madeira River (09.3347° S, 64.7375° W), approximately 125 km upstream from the city of Porto Velho, Estado de Rondônia, Brazil.

Paratopotypes. INPA-H 16541–16554, INPA-H 16556–16569 (original field numbers APL 2000–2013, 2015–2018, 2022–2030, 2032), 6 females, 22 males. Collected in the same locality as holotype, 23-25 November 2004 by P. I. Simões and A. P. Lima.

Paratypes. All from Brazil. **Acre:** INPA-H 11621–11640, , 4 females, 17 males, Fazenda Catuaba, Municipality of Rio Branco, 10.0742° S, 67.6249° W, collected in February 2004 by A. P. Lima. **Rondônia:** INPA-H 16578, 16584–16587, 16589, 16591–16592, 16597, 16602–16603, 16605–16607, 16611–16614, 16620–16624, 16626, 16628, 16631, 16633, 16636–16637, 16639–16641, 16643, 16645–16646, 16648, 13 females, 26 males, collected on the left bank of the upper Madeira River, across the river from the village of Fortaleza do Abunã, 160 km upstream from the city of Porto Velho, 72 km upstream from Cachoeira do Jirau, 9.5160° S, 65.3249° W, collected 05-08 January 2005 by P.I. Simões and A.P. Lima. INPA-H 16596, 16730, 16739, 16756, 16758, 16767, 16771, 16777–16778, 16788, 16805, 16818–16819, 2 females, 11 males, collected on the left bank of the upper Madeira River, across the river from the village of Mutum-Paraná, 121 km upstream from the city of Porto Velho, 34 km upstream from Cachoeira do Jirau, 9.5732° S, 64.9211° W, collected 10–13 January 2005 by P. I. Simões and A. P. Lima.

Etymology. The specific epithet is a patronym for Dr. Walter Hödl, an Austrian biologist and professor who pioneered research on behavior and acoustic communication in anurans. For the past two decades, Walter and his students have dedicated special attention to the *Allobates femoralis* complex.

Diagnosis. The new species is assigned to the genus *Allobates* by the combination of the following characters: presence of a pale dorsolateral stripe, dorsal skin texture granular posteriorly, basal webbing present only between Toes III and IV, Finger I longer than Finger II, finger discs generally weakly expanded (moderately expanded on Finger I), median lingual process absent, testes not pigmented, dark collar absent on throat, oral disk of tadpoles emarginate, not umbelliform. *Allobates hodli* is distinguished in life from all other species of *Allobates* (except *Allobates femoralis*, *Allobates myersi* and *Allobates zaparo*) for presenting relatively large body-size (average SVL = 24.76 ± 1.08 mm, males and females pooled), by the lack of brown or light-brown colors or patterning on dorsum and lateral surface of body, and by presenting dark and white marbling on anterior ventral surface of body, replaced by solid reddish-orange color on the ventral surface of hind limbs .

Allobates hodli is distinguished from other taxa and morphotypes that form the *A. femoralis* complex by presenting advertisement calls consisting of groups of two notes repeated in series or bouts (instead of groups of one, three or four notes), and by presenting a conspicuous reddish-orange coloration on the ventral surface of legs, instead of an exclusively black and white reticulated pattern, observed in *A. femoralis*. *Allobates hodli* also has diffuse reddish-orange and black patches on dorsal surface of thighs, as opposed to regular, pale (yellowish to red) longitudinal flash marks extending onto the

entire dorsal surface of thighs, generally margined by dark patches, observed in typical *A. femoralis*.

A. hodli is distinguished from *A. zaparo* and *A. myersi* by the color of dorsum, which is uniformly black/dark-brown in *A. hodli* (Fig.2), but reddish in *A. zaparo* and brown to light-brown in *A. myersi*. *Allobates myersi* also lacks a pale dorsolateral stripe.

A. hodli is largely sympatric to *Ameerega picta*, a dendrobatid frog that presents similar body size and color pattern. However, *A. hodli* can be distinguished from *Ameerega picta* by lacking a bright (orange to red) flash mark on calf region.

Description of holotype. Morphological measurements of holotype are presented in Table 1. Body robust, head slightly wider than long (HL/HW = 0.94) (Fig. 3A). Eye diameter slightly larger than distance from nostril to anterior corner of the eye. Nares located posterolaterally to tip of snout, directed posterolaterally, visible in ventral and anterior view. Center of nostril not visible dorsally. Canthus rostralis convex from tip of snout to nostril, straight from nostril to anterior corner of the eye. Loreal region vertical. Tympanum well visible, with maximum diameter horizontal, corresponding to 44% the maximum diameter of the eye. Maxillary teeth present. Tongue length twice as large as wide, attached anteriorly on first third. Median lingual process absent. Choanae round. A single vocal sac is present, corresponding to most of the area of the medial and posterior subgular region. Vocal sac round when expanded. When retracted, vocal sac forms two lateral slits at the level of maxilla articulation (Fig. 3, B).

Skin granular on dorsum and dorsal surface of legs. Granules round, more developed on dorsal surface of urostyle region and shanks. Skin smooth ventrally and laterally. Dermal flap above cloaca absent.

Palmar tubercle slightly triangular. Thenar tubercle well-developed, oval to elliptic, maximum diameter 1.28 times smaller than maximum diameter of palmar tubercle. Subarticular tubercles of Fingers II, III and IV are round, small, never exceeding the width of phalanges. Subarticular tubercle of Finger I elliptic, 1.21 times larger than thenar tubercle in maximum diameter. Supernumerary tubercles absent. Carpal pad and metacarpal ridges absent on hands. No fringes or webbing on fingers. A distal tubercle on finger IV is weakly developed (Fig. 3C). Finger I is slightly (1.08 times) longer than Finger II. Length of finger IV does not reach distal subarticular tubercle of finger III when fingers are pressed against each other. Relative lengths of fingers: IV < II < I < III. Finger III not swollen. Disc of Finger I moderately expanded, edges of disk corresponding approximately to width of digital shaft, disc width 1.37 times the width of adjacent phalange. Discs of Fingers II, III and IV weakly expanded, edges of discs corresponding approximately to half or less than half width of digital shafts, 1.26, 1.32 and 1.39 times the width of adjacent phalanges, respectively.

Length of shank corresponding to 48% of snout-to-vent length (Table 1). Tarsal keel is tubercle-like, strongly curved at its proximal end, flattening towards the metatarsal tubercle. Metatarsal fold evident (but not folding over itself) running from the base of Toe V towards metatarsal tubercle, but not reaching it. Preaxial edge of tarsus smooth, with no fringe. Basal webbing present only between Toes III and IV, and II and III. Relative lengths of toes: I < II < V < III < IV (Fig. 3D). Disc of Toe I weakly expanded, edges of disc corresponding to less than half the width of digital shaft, disk

width 1.25 times the width of adjacent phalange. Discs of toes II, III, IV and V moderately expanded, edges of disks corresponding approximately to width of their respective digital shafts, width of discs 1.54, 1.44, 1.52 and 1.46 times the width of adjacent phalanges, respectively.

Variation in type series. Morphological measurements of individuals constituting type series are presented in Table 1. Morphological characters described for the holotype apply to all individuals in type series, except for the following: Males slightly smaller (4.42%, in average) than females. Head slightly longer than wide in males (HL/HW = 1.04) and females (HL/HW = 1.05) in average. Maximum diameter of tympanum corresponding to approximately half the maximum diameter of the eye in males and females (Table 1). Vocal sac and slits absent in females.

Palmar tubercle round to slightly triangular. A distal tubercle on finger IV is present in 28 of a total 83 (34.1%) inspected specimens, but is absent or weakly developed in the remaining 54 specimens (65.9%).

Color in life. Males and females do not present dimorphism in relation to color and color pattern. Dorsal surface of body solid black to solid dark-brown (Fig. 2B). Lateral surface of body solid black. Dorsolateral line white, thinner than lateral line (Fig. 2A, 2E). When continuous with flash marks on thighs, dorsolateral line becomes reddish-orange on groin. Lateral line white. Gular region solid black to dark bluish-gray in males and females (Fig. 2D). In males, vocal sacs usually with a paler bluish-gray color when inflated. Mid abdomen white with irregular black to dark-gray blotches or speckling, merging with solid dark color of gular region. Abdomen bright reddish-orange posteriorly, with dark irregular spots appearing marginally from lateral edges. Ventral surfaces of hind limbs also bright reddish-orange, sometimes with small marginal dark spots (Fig. 2C, 2D). Plantar surface of feet brown. Ventral surfaces of arms bright reddish-orange, with bright yellow flash marks extending from dorsal surface of upper arms. Black to dark-gray spot ventrally on upper arm, at the point of body insertion, continuous with gular region pattern. Dorsal surfaces of posterior and anterior limbs reddish to brick-brown (Fig. 2B, 2E). Dorsal and rear surfaces of thighs with irregular bright reddish-orange flash marks or patterning, same color as ventral surfaces of legs, with irregular black or dark-brown blotches or spots. Granules on dorsal surface of shanks usually darker than overall color of shanks. A yellow flash mark is present dorsally on upper arms, at the point of body insertion (Fig. 2B, 2E). The iris is evident, with metallic yellowish-brown pigmentation.

Color in life of juveniles. Color of juveniles after metamorphosis is the same of adults. Dorsum and flanks are solid black to dark-brown, with dorsolateral and lateral lines white and conspicuous. Limbs generally reddish-brown. Bright yellow flash marks are present dorsally on the upper arms, and may reach the elbow. Dorsal surface of thighs with conspicuous longitudinal bright reddish-orange flash marks, lacking black or dark-brown blotches or spots (Fig. 2F).

Color in alcohol of holotype. Dorsum is solid black to dark-brown. A thin, pale white dorsolateral line is present, continuous from groin at hind limb insertion, over the orbit and nostril, to the tip of snout (Fig. 3A). A pale white lateral line is present, broader than dorsolateral stripe, running from groin, over the insertion of arm, below nostril, to tip of upper lip. Lateral stripe is continuous on both sides of the body. Lateral surface between dorsolateral and lateral stripe solid black. Color of gular region and throat is solid black to dark-brown. Abdomen color is white with irregular black blotches or speckling. Abdomen color becomes solid black/dark-brown from chest towards the gular region. The black/dark-brown speckling over white background pattern is replaced posteriorly by a solid pale-tan pattern, continuous with the ventral color pattern of hind limbs (Fig. 3B).

Arms uniformly very pale-brown in dorsal view, paler on the axilla and carpal/metacarpal regions. Irregular dark blotches appear on dorsal surfaces of tarsus and fingers. Arms uniformly pale white to pale tan in ventral view, with a black patch (continuous with color pattern of gular region) on anterior surface of the arm. Surface of outer lateral edge of forearm and metacarpal region same color as dorsal surface of arms, extending laterally from elbow and reaching the palm of hands and ventral surface of fingers (Fig. 3C). Legs are pale brown in dorsal view. Irregular pale, unpigmented patches, as well as irregular black blotches and spots are present on dorsal and rear surfaces of thighs (Fig. 3A). The area immediately around vent is darker than the overall surface of thighs. Dorsal surfaces of shanks with darker granules. Inner dorsal surface of tarsal region is lighter than overall pattern of legs. Toes are generally darker than tarsal region in dorsal view. Ventral surface of legs is uniformly pale-tan with small brown spots appearing marginally from outer edges. Ventral surfaces of tarsal region and toes darker, same color as dorsal surface of legs (Fig. 3B, 3D). Tongue is cream-colored; large intestine (removed for the analysis of diet) is unpigmented. Testes are unpigmented.

Color variation in type series. Color in alcohol described for the holotype apply to all individuals in type series, except for the following: Lateral stripe is usually continuous, but can be interrupted in some individuals, on one or on both sides of body. Gular region and throat solid black in females, solid black to dark-brown in males. Mature oocytes are pigmented, with black pigment concentrated on animal pole.

Description of tadpoles. Tadpole measurements were obtained from four tadpoles in developmental stage 36 (Table 2). Tadpoles correspond to a lot under the same collection number, INPA-H 23693. The largest tadpole (TL = 24.7 mm, Fig. 4) was used for detailed description.

Body is depressed, body width (6.0 mm) larger than body depth (4.8 mm), body length 16.1 mm. Snout nearly round, flattened anterodorsally in lateral view (Fig. 4C). Tip of snout flattened anteriorly in dorsal view (Fig. 4A). Nares small, directed anterolaterally, located 0.8 mm anterior to the eye, and 1.0 mm posterior to tip of snout. Nostrils narrowly spaced, distance between nostrils 0.9 mm. Eyes dorsal, directed dorsolaterally, 0.9 mm in maximum length, located 1.8 mm posterior to tip of snout.

Distance between medial margins of the eyes is 1.4 mm. Spiracle single, sinistral, forming a free tube opening posterodorsally below body axis in lateral view, 5.0 mm posterior from tip of snout (Fig. 4C). Vent tube medial, free, 0.9 mm in length, opening dextrally.

Tail musculature reaches maximum depth (2.4 mm) approximately at the end of first third of tail length, and maximum width at body-tail insertion (2.6 mm). Ventral tail fin originates at body-tail insertion. Dorsal tail fin originates slightly posterior (0.8 mm) to body-tail insertion, and reaches maximum high 14.5 mm from tip of snout, corresponding to the region of maximum tail depth. At maximum depth of tail, depth of musculature is 1.4 mm, dorsal fin 1.5 mm and ventral fin 1.1 mm.

Oral disc is positioned anteroventrally, emarginate laterally, transversely elliptical, 2.6 mm in transverse width. Anterior labium continuous with snout, 2.6 mm in length. Marginal papillae absent dorsally on anterior labium (gap 1.7 mm, 74% of total anterior labium length), present only laterally, on its outer margins. Posterior labium free from body wall, 2.4 mm in length, with a single row of marginal papillae. All papillae with rounded tips (Fig. 5).

Labial tooth row formula is 2(2)/3(1). Rows A-1 and A-2 with same length (2.1 mm), A-2 with a large medial gap (0.6 mm). Rows P-1, P-2 and P-3 with same length (2.0 mm), P-1 presenting a very narrow medial gap (< 0.1 mm), best evidenced by a break between subjacent tooth ridges. Upper jaw sheath arch-shaped, 1.1 mm in length (42 % of oral disk width), 0.1 mm in width. Cutting edge serrate, with serrations not extending to lateral process of the upper jaw. Lower jaw sheath deeper than upper jaw, V-shaped, 0.8 mm in length, with serrate cutting edge (Fig. 5).

Color in preservative is dark to light tan. Body is darker than tail, with scattered brown melanophores in higher densities on anterior dorsum. High concentrations of melanophores also appear posteriorly on dorsum at the top and on flanking regions of tail muscle insertion (Fig. 4A). Melanophores are evenly distributed on anterior ventral surface of body. Posterior ventral surface of body is transparent, not pigmented, and intestines are clearly visible through skin (Fig. 4B). Tail musculature is light tan, tail fins transparent, with scattered brown melanophores forming irregular blotches on tail surface (Fig. 4C).

Advertisement and courtship calls description and variation. Advertisement calls of *Allobates hodli* consist in trills of calls formed by two whistle-like notes with ascending frequency modulation (Fig. 6A, 6C). Measurements of advertisement call characteristics of holotype and average values for 60 males from type series are presented on Table 3. The average maximum frequency of calls within type series is 3425.0 ± 184.7 Hz, and average duration of calls (summed durations of first and second notes, and inter-note silent interval) 0.164 ± 0.011 s. First note is less modulated (average difference between lower and higher frequency = 470.7 ± 94.8 Hz) than the second note (average difference between lower and higher frequency = 740.7 ± 115.0 Hz) and shorter in duration (0.033 ± 0.004 s, in comparison to second note, 0.056 ± 0.007 s).

Courtship calls of *A. hodli* are quite distinct from their advertisement calls and are constituted by a continuous pulsed tone (Fig. 6D), emitted only in the presence of females near the male's calling perch. Average maximum frequency and duration of courtship

calls obtained from calls of four individuals were 3190.2 Hz and 0.571 s, respectively (Table 4). The average pulse emission rate between calls was 125.2 pulses per second.

Molecular phylogeny and genetic distances. From an initial sequence database containing 203 16S rDNA sequences (including outgroups), a total of 93 unique haplotypes were used in the phylogenetic analysis (Table 5). Phylogenetic reconstructions support the existence of two basal clades within the *Allobates femoralis* group, both forming the sister clade to *Allobates zaparo* (Fig. 7A). One of the basal clades contains *Allobates hodli* and the second contains samples from areas nearby *Allobates femoralis* type-locality, which we refer to as *Allobates femoralis* sensu stricto (clade femo 04, Fig. 7B). *A. hodli* is marginally paraphyletic to populations that occur in the southern reaches of the Brazilian State of Acre (clade Acre 01, Fig. 7B), which present advertisement calls constituted by four notes and color pattern more similar to that of *A. femoralis* than to that of *A. hodli*. Samples from the locality Monte Alegre were not clearly positioned within *A. hodli* or Acre 01 clade. This locality probably corresponds to a relictual contact zone between these clades. *Allobates hodli* and clade Acre 01 form the sister group to a third clade occurring in the northern and western forests of the State of Acre, in Brazil, and along the Madre de Dios River, in Peru (clade Acre 2, Fig. 7B). Despite the clear differentiation in advertisement calls and color pattern, average uncorrected pairwise genetic distance between *A. hodli* and clade Acre 01 does not exceed 1.5%, while distance between *A. hodli* and clade Acre 02 exceeds 2.0% (Table 6). Despite the low levels of genetic divergence from clade Acre 01, *A. hodli* is differentiated from this clade by six unambiguous character state changes in the 16S rDNA fragment analyzed. The observed high levels of genetic similarity between *A. hodli* and clade Acre 01 and the relatively restricted distribution of the latter clade brings up the possibility of that clade Acre 01 originated from past genetic introgression from the widely distributed clade Acre 02 into *A. hodli* along the western portion of its geographic distribution (see Discussion below).

The basal clade containing *A. hodli* and the Acre 01 and Acre 02 clades is the sister group to the clade including *A. femoralis* sensu stricto (clade femo 04, Fig. 7B) and other populations of *A. femoralis* from Peru, Ecuador, Colombia, Suriname, and Brazil. Within this clade, samples from Ecuador form a highly supported clade (clade femo 01, Fig. 7B), which is weakly supported as the sister group to all the remaining *A. femoralis* populations included in this basal clade. Samples from the upper Madeira River basin (clade femo 02, Fig. 7B) and from the Brazilian state of Pará (clade femo 03, Fig. 7B) formed well supported clades, which together are the sister group to a weakly supported clade including *A. femoralis* sensu stricto and additional samples from Iquitos, Panguana (both in Peru), Reserva Ducke (Brazil), and Leticia (Colombia). Average pairwise genetic distances between samples in this basal clade and *A. hodli* ranged from approximately 3.9% (between *A. hodli* and *A. femoralis* from Suriname) to 4.9% (between *A. hodli* and clade femo 04, which contains *A. femoralis* sensu stricto). *Allobates hodli* is distinguished from clades femo 01–04 by at least 23 unambiguous character state changes in the 16S rDNA fragment analyzed. The lack of support and the existence of highly divergent sequences found within clade femo 04 suggest elevated levels of genetic variability

between populations across the Peruvian Amazon, and additional sampling is necessary in order to clarify their phylogenetic relationships.

Natural history notes. *Reproduction and behavior*. Observations were made during the rainy season, when males were found calling during the day from sunrise (time of earlier recording 07:15, INPA-H 16602) to sunset (time of later recording 18:15 h, INPA-H 16592). The number of individuals calling generally decreased around mid-day. Males called from sites slightly elevated from the forest floor, such as logs or perches among fallen branches. Individuals were also frequently observed on the bases of small palm trees and on rocks. Males are territorial, approaching portable amplifiers when we executed playback recordings of their own calls, calls of other males from the same population or calls of *A. femoralis* males from the upper Madeira River near the calling site of the focal male. Courtship calls were emitted only in the presence of females in male's territory, but further courtship, oviposition or larvae relocation behaviors were not observed. One tadpole clutch was collected at Abunã on 15 January 2005. The clutch was found on the ground, over a dead leaf, less than 1 m from a male's calling site. The clutch was transported to Manaus, and tadpoles were raised until developmental stage 36 for tadpole description or until complete metamorphosis for observations of color pattern ontogeny (see above).

In the localities of Abunã and Cachoeira do Jirau, juveniles were frequently found close to small streams inside the forest. Although tadpoles were not found in those streams, there is a possibility that this species uses such water bodies or temporary ponds created by their sporadic overflow as sites for tadpole deposition.

Diet. From 81 dissected stomachs, 24 (29.6%) were empty. Considering only stomachs that contained prey, ants (Formicidae) and adult coleopterans were the most frequent items found, each found in 25 (43.8%) stomachs. Spiders were found in 12 (21.0%) and dipterans in 9 (15.8%) stomachs. Other less frequent items found were isopterans (5 stomachs, 8.8%), miriapods (3 stomachs, 5.26%), coleopteran larvae (3 stomachs, 5.26%), hemipterans (2 stomachs, 3.5%), other hymenopterans (2 stomachs, 3.5%) and terrestrial dipteran larvae (2 stomachs, 3.5%). Collembolans, orthopterans, blattarians and acari were found each in a single stomach.

Distribution. Known distribution of *Allobates hodli* is restricted to southwestern Brazilian Amazonia (coordinates are given in Table 5), from the locality of Cachoeira do Jirau (09.3347° S, 64.7375° W), in the Municipality of Porto Velho, to the eastern reaches of the Municipality of Rio Branco, in the state of Acre (10.0742° S, 67.6249° W). The eastern boundary of the species' distribution is well known, as it reaches a contact zone with a population of *Allobates femoralis* (clade femo 02, Fig. 7B) on the left bank of the upper Madeira River, about 1 km downstream of the Jirau rapids (9.3206° S, 64.7225° W). The westernmost site of occurrence of *A. hodli* is located in the vicinity of the city of Rio Branco, in Fazenda Catuaba (site 4, Fig. 1A). South of Rio Branco, in a district known as Monte Alegre (site 5, Fig. 1A), *A. hodli* is replaced by another

552 population (clade Acre 01, Fig. 7B) which presents typical *A. femoralis* coloration and 4-
553 note advertisement calls.
554 The species is not known to occur on the right bank of the Madeira and Mamoré
555 Rivers (it is possible that these rivers represent barriers to the distribution of this species),
556 thus its southernmost record is also Fazenda Catuaba, probably reaching forest remnants
557 south of the city of Rio Branco. The northern distribution limit for the species is
558 unknown.
559
560
561 **Discussion.** The taxon *Allobates femoralis* has already been considered a complex of
562 closely related species by many authors (e.g. Grant *et al*. 2006; Lötters *et al.* 2007; Santos
563 *et al.* 2009). Recent studies considered the phylogenetic relationships of this group in a
564 higher taxonomic context (Grant *et al.* 2006; Santos *et al.* 2009), agreed in relation to the
565 existence of cryptic species under this taxon. Grant *et al.* (2006) argue that, in spite of
566 forming a monophyletic group, pronounced genetic distances between sampling sites
567 (3.9–14.6%, cytocrome *b*) are indicative of multiple (at least eight) species. In a more
568 recent approach, Santos *et al.* (2009) estimated *A. femoralis* comprised nine distinct
569 species that diversified within the Amazon Basin 5.4–8.7 million years ago. However,
570 these studies only circumstantially addressed phylogenetic relationships within the *A.*
571 *femoralis* clade, using samples from localities separated by hundreds of kilometers. A
572 detailed description of phenotypes, as well as the distribution of each group/species was
573 beyond the scope of these works. No sequences from the known distribution range of *A.*
574 *hodli* were included in these studies, and *Allobates hodli* represents a new taxon, additive
575 to the number of cryptic species presumed by the studies of Grant *et al.* (2006) and
576 Santos *et al.* (2009).
577 The existence of conspicuous genetic differences between the individuals from
578 Catuaba and other *A. femoralis* populations (including reference populations from
579 Reserva Ducke, Treviso, Leticia and Panguana) was observed by Amézquita *et al.*
580 (2009), based on a 306 b.p. fragment of the cytochrome *b* mitochondrial gene. Despite
581 the pronounced geographic distances between most populations sampled, authors argue
582 that genetic distances observed between Fazenda Catuaba and other populations were
583 larger than expected to be explained by geographic distance alone, and are largely
584 correlated to phenotypic distances, considering combined data on morphometrics,
585 acoustic properties of calls, and color pattern.
586 *Allobates hodli* is the first species of this complex to be described since the
587 description of *A. myersi* by Pyburn (1981). It has a relatively well-known distribution and
588 is characterized by unambiguous molecular, morphological and behavioral characters that
589 make this taxon distinguishable from all other clades included in the *A. femoralis*
590 complex and their close relatives. To our knowledge *A. hodli* is the only species in the
591 *Allobates femoralis* complex (sensu Lötters *et al.* 2007) on Brazilian territory to present
592 advertisement calls constituted by the repetition of groups of two frequency-modulated
593 notes. Similar 2-note advertisement calls have been noted for *A. myersi* in Colombia
594 (Pyburn 1981) and *A. zaparo* in Ecuador (Read 2000).
595 Detailed morphological and acoustic comparisons between 2-note call populations
596 from the left bank of the upper Madeira River (herein described as *A. hodli*) and 4-note
597 call populations distributed in other localities in this area were presented in Simões *et al.*

(2008). The study also highlighted the coincidence between the distribution of both groups and the boundaries between distinct geomorphological domains. Although relationships between habitat variation and underlying geomorphology is largely unknown in this area, summary of evidence of phenotypic differentiation and restricted distribution point to the rejection of the hypothesis of current ecological exchangeability (sensu Crandall *et al.* 2000) between individuals of these two groups, but this issue deserves further testing using niche-modeling approaches. The reciprocal monophyly between basal clades containing *A. hodli* and *A. femoralis* from the upper Madeira River (clade femo 02, Fig. 7B) points to past genetic isolation that remains in recent time, despite of their occurrence in sympatry across a narrow contact zone downstream of Cachoeira do Jirau (Simões *et al.* 2008).

Advertisement calls are considered the most conspicuous sexual signals in frogs and the first pre-mating signals perceived by a distant female, playing a crucial role in female attraction and sexual selection by females, besides mediating territorial male-to-male interactions. All other signals, including courtship calls, are usually emitted once the female is already within a male's territory (Gerhardt & Huber 2002; Wells 2007). Our results highlight the existence of clear differentiation in the number of notes in advertisement calls of *A. hodli* and other populations referred to as *A. femoralis*. Despite this difference, playback trials using calls of *A. femoralis* and *A. hodli* (performed in populations of the upper Madeira River) triggered aggressive phonotactic behavior in males of both species. However, the existence and strength of call differentiation effects on sexual selection by females belonging to the *A. femoralis-A. hodli* complex are yet to be tested.

Genetic introgression is not uncommon among amphibians (Hofman, S. & Szymura 2007; Vogel & Johnson 2008; Brown & Twomey 2009). In cases of relaxed selection on signal recognition, hybridization would likely take place at suture zones, allowing for genetic introgression, and thus rendering polyphyletic or paraphyletic molecular phylogenies (Funk & Omland 2003). In addition to a paraphyletic mtDNA phylogeny, the restricted geographic distribution of clade Acre 01 (which present 4-note advertisement calls and color patterns similar to those of clade Acre 02) suggests that it could have arisen from past genetic introgression from clade Acre 02 into *A. hodli* along the western distribution of the latter (McGuire et al. 2007; Brown & Twomey 2009). This hypothesis remains to be tested with nuclear DNA markers and experiments on female sexual selection. The current range of extant primary forest in this region (reduced to very small patches) makes it difficult to sample more individuals in the area between Fazenda Catuaba and Monte Alegre. Ongoing deforestation across this area will likely increase the geographic isolation between *A. hodli* and populations of clades femo 01 and femo 02 occurring in southern and western State of Acre.

Although apparently allowing for some hybridization along contact zones (at least in the past), differences in advertisement calls between *A. hodli* and other clades referred to as *A. femoralis* are geographically fixed, and are maintained along the remaining areas of sympatry. The 2-note advertisement calls of *A. hodli* are also clearly distinguished from the 4-note calls of individuals sampled in areas south of *A. femoralis* type locality in Yurimaguas (individuals were recorded in Chazuta, 70 km, and Pongo de Cainarachi, 45 km, Fig. 8), which are highly allopatric to *A. hodli*. In addition to fixed differences in such bioacoustic characters, we describe differentiation in morphological traits that are

not variable among reference populations of *A. femoralis* and *A. hodli*, such as the reddish-orange color on ventral surface of posterior abdomen and hind limbs, and diffuse flash marks on thighs in *A. hodli*. These characters are also clearly distinguished from those observed in populations inhabiting localities close to *A. femoralis* type-locality (Barranquita, 36 km south of Yurimaguas, Fig. 9), and as such can be treated as diagnostic characters. When combined with generally high levels of genetic differentiation in 16S rDNA relative to reference *A. femoralis* populations, our results match the criteria proposed by Vieites et al. (2009) for validation of a candidate taxon, according to which *A. hodli* should be regarded as a distinct species.

Summarizing information from the mtDNA phylogeny and available records of advertisement calls, we suggest that there is potential for taxonomic reappraisal of other geographically restricted populations which are currently recognized under the name *Allobates femoralis*. Namely, populations from Ecuador (clade femo 01, Fig. 7B) and southwestern Amazon Basin (clade Acre 02, Fig. 7B) represent putative new taxa, with characteristic phenotypes and relatively well known geographic distribution. Although presenting lower between-clade genetic distances, populations from the Madeira and Tapajós River basins (clades femo 02 and 03) represent geographically structured monophyletic lineages, and further population genetics studies should address the existence of current gene flow between them.

Samples from Colombian and northern Peruvian Amazon that constitute clade femo 04 (Fig. 7B) probably represent populations of nominal *A. femoralis*. Silvertone (1976) designated a male individual collected in Yurimaguas, in the Huallaga River, Peru, as the *A. femoralis* lectoype, as the same individual was used in the original description by Boulenger in 1883. Although samples from the immediate vicinity of Yurimaguas were not available for this study, calls (Fig. 8, also see Amézquita 2009), photographs (Fig. 8, also see Pyburn 1981), and DNA sequences (Table 5) obtained in localities near the type locality suggest that populations of *A. femoralis* distributed across Departamento Loreto, in Peru, and Departamento Amazonas, in Colombia, present similar advertisement calls and color pattern, and thus we propose represent *A. femoralis* sensu stricto. In the future, increased sampling across northwestern Colombian Amazon, southern Peruvian Amazon, and Bolivia will possibly reveal a wider geographic distribution for this clade. However, at least two advertisement call phenotypes are known to exist across this region, and our DNA sequence analyses pointed to the existence of high levels of genetic divergence between samples collected in this area. Mapping the boundaries between these distinct acoustic morphotypes, and including more samples from each population in new phylogenies will allow us testing the hypothesis of reciprocal monophyly and current gene-flow between them, in addition to elucidating their evolutionary relationships in relation to the remaining species that form the *Allobates femoralis* complex.

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References

Amézquita, A., Castellanos, L. & Hödl, W. (2005) Auditory matching of male *Epipedobates femoralis* (Anura: Dendrobatidae) under field conditions: the role of spectral and termporal call features. *Animal Behavior,* 70, 1377–1386.

Amézquita, A., Hödl, W., Lima, A.P., Castellanos, L., Erdtmann, L. & Araújo, M.C. (2006) Masking interference and the evolution of the acoustic communication system in the Amazonian dendrobatid frog *Allobates femoralis*. *Evolution,* 60(9), 1874–1887.

Amézquita, A., Lima, A.P., Jehle, L., Castellanos, L., Ramos, O., Crawford, A.J., Gasser, H. & Hödl, W. (2009) Calls, colours, shapes, and genes: A multi-trait approach to the study of geographic variation in the Amazonian frog *Allobates femoralis*. *Biological Journal of the Linnean Society*, 98, 826–838.

Azevedo-Ramos, C. & Galatti, U. (2002) Patterns of amphibian diversity in Brazilian Amazonia: Conservation implications. *Biological Conservation,* 103, 103–111.

Brown, J.L. & Twomey, E. (2009) Complicated histories: three new species of poison frogs of the genus Ameerega (Anura: Dendrobatidae) from north-central Peru. *Zootaxa,* 2049, 1–38.

Caldwell, J.P., Lima, A.P., & Keller, C. (2002) Redescription of *Colostethus marchesianus* (Melin, 1941) from its type locality. *Copeia*, 2002, 157–165.

Caldwell, J.P. & Lima, A.P. (2003) A new species of *Colostethus* with a nidicolous tadpole. *Herpetologica*, 59, 219–234.

Charif, R.A., Clark, C.W. & Fristrup, K.M. (2004) *Raven 1.2 User's Manual*. Cornell Laboratory of Ornithology, Ithaca, NY.

Clough, M. & Summers, K. (2000) Phylogenetic systematics and the biogeography of the poison frogs: Evidence from mitochondrial DNA sequences. *Biological Journal of The Linnean Society*, 70, 515–540.

Crandall, K.A., Bininda-Emonds, O.R.P., Mace, G.M. & Wayne, R.K. (2000) Considering evolutionary processes in conservation biology. *Trends in Ecology and Evolution*, 15(7), 290–295.

Doyle, J.J. & Doyle, J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19, 11–15.

Fouquet, A., Vences, M., Salducci, M.-D., Meyer, A., Marty, C., Blanc, M. & Gilles, A. (2007) Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the *Scinax ruber* and *Rhinella margaritifera* species groups. *Molecular Phylogenetics and Evolution*, 43, 567–582.

Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., de Sá, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.E., Blotto, B.L., Moller, P., Drewes, R.C., Nussbaun, R.A., Lynch, J.D., Green, D.M. & Wheeler, W.C. (2006) The amphibian tree of life. *Bulletin of the American Museum of Natural History*, 279, 1–370.

Frost, D.R. (2009) *Amphibian Species of the World: an Online Reference. Version 5.3* (12 February, 2009). American Museum of Natural History, New York, USA. Available from: http://research.amnh.org/herpetology/amphibia/ (05 June 2009).

Funk, D.J. & Omland, K.E. (2003) Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics*, 34, 397–423.

Gerhardt, H.C. & Huber, F. (2002) Acoustic communication in insects and anurans: Common problems and diverse solutions. University of Chicago Press, Chicago, pp. 327–367.

Göd, M., Franz, A., & Hödl, W. (2007) The influence of internote-interval variation of the advertisement call on the phonotactic behaviour in male *Allobates femoralis* (Dendrobatidae). *Amphibia-Reptilia*, 28, 227–234.

Gosner, K.L. (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, 16, 183–190.

Grant, T., Frost, D.R., Caldwell, J.P., Gagliardo, R., Haddad, C.F.B., Kok, P.J.R., Means, B.D., Noonan, B.P., Schargel, W. & Wheeler, W.C. (2006) Phylogenetic systematics of dart-poison frogs and their relatives (Anura: Athesphatanura: Dendrobatidae). *Bulletin of the American Museum of Natural History*, 299, 1–262.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.

Hödl, W. (1987) *Dendrobates femoralis* (Dendrobatidae): a handy fellow for frog bioacoustics. *Proceedings of the 4th Ordinary General Meeting of the Societas Europaea Herpetologica*. Nijmegen, 1987, 201–204.

Hödl, W., Amézquita, A. & Narins, P.M. (2004) The rôle of call frequency and the auditory papillae in phonotactic behavior in male dart-poison frogs *Epipedobates femoralis* (Dendrobatidae). *Journal of Comparative Physiology A*, 190, 823–829.

Hofman, S. & Szymura, J.M. (2007) Limited mitochondrial DNA introgression in a *Bombina* hybrid zone. *Biological Journal of the Linnean Society*, 91, 295–306.

Jobb, G. (2008) *TREEFINDER version of October 2008*, distributed by the author, Munich, Germany. Available from: http://www.treefinder.de/ (27/05/2009).

Lima, A.P. & Caldwell, J.P. (2001) A new species of *Colostethus* with sky blue digits. *Herpetologica*, 57, 180–189.

Lima, A.P., Sanchez, D.E.A. & Souza, J.R.D. (2007) A new Amazonian species of frog genus *Colostethus* (Dendrobatidae) that lays its eggs on undersides of leaves. *Copeia 2007*, 1, 114–122.

Lima, A.P., Caldwell, J.P. & Strussmann, C. (2009) Redescription of *Allobates brunneus* (Cope) 1887 (Anura: Aromobatidae: Allobatinae), with a description of the tadpole, call, and reproductive behavior. *Zootaxa*, 1988, 1–16.

Lötters, S., Jungfer, K.-H., Henkel, F.W. & Schmitz, W. (2007) *Poison Frogs. Biology, Species & Captive Husbandry*. Chimaira, Frankfurt/Main, 668 pp.

Lötters, S., Schmitz, A., Reichle, S., Rödder, D. & Quennet, V. (2009) Another case of cryptic diversity in poison frogs (*Dendrobatidae: Ameerega*) - description of a new species from Bolivia. *Zootaxa*, 2028, 20–30.

Lutz, B. & Kloss, G.R. (1952) Anfíbios anuros do alto Solimões e Rio Negro: apontamentos sobre algumas formas e suas vicariantes. *Memórias do Instituto Oswaldo Cruz*, 50, 621–677.

McDiarmid, R.W. & Altig, R. (1999) Body plan: development and morphology. In: *Tadpoles: the biology of anuran larvae*. R.W. McDiarmid & R. Altig (eds). The University of Chicago Press, Chicago, pp. 24–51.

McGuire, J.A., Linkem, C.W., Koo, M.S., Hutchison, D.W., Lappin, A.K, Orange, D.I., Lemos-Espinal, J., Riddle, B. & Jaeger, J.R. (2007) Mitochondrial introgression and incomplete lineage sorting through space and time: phylogenetics of Crothaphytid lizards. *Evolution*, 61(12), 2879–2897.

Narins, P.M., Hödl, W. & Grabul, D.S. (2003) Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *PNAS*, 100 (2), 577–580.

Padial, J.M. & De la Riva, I. (2009) Integrative taxonomy reveals cryptic Amazonian species of Pristimantis (Anura, Strabomantidae). *Zoological Journal of the Linnean Society*, 155, 97–122.

Palumbi, S.R. (1996) Nucleic acids II: the polymerase chain reaction. *In*: Hillis, D.M., Moritz, C. & Mable, B.K. (Eds) *Molecular Systematics*. Sinauer & Associates Inc., Sunderland, Massachusetts, pp. 205–247.

Posada, D. & Crandall K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, 14(9), 817–818.

Pyburn, W.F. (1981) A new poison-dart frog (Anura: Dendrobatidae) from the forest of southeastern Colombia. *Proceedings of the Biological Society Washington*, 94, 67–75.

Read, M. (2000) *Frogs of the Ecuatorian Amazon*: a guide to their calls. Morley Read Productions, Cornwall, England. Digital media (CD).

Ringler, M., Ursprung, E. & Hödl, W. (2009) Site fidelity and patterns of short- and long-term movement in the brilliant-thighed poison frog *Allobates femoralis*. *Behavioral Ecology and Sociobiology*, 63(9), 1281–1293.

Roberts, J.L., Brown, J.L., von May, R., Arizabal, W., Schulte, R. & Summers, K. (2006) Genetic divergence and speciation in lowland and montane Peruvian poison frogs. *Molecular Phylogenetics and Evolution*, 49, 149–164.

Roithmair, M.E. (1992) Territoriality and male mating success in the Dart-Poison Frog, *Epipedobates femoralis* (Dendrobatidae, Anura). *Ethology*, 92, 331–343.

Roithmair, M.E. (1994) Field studies on reproductive behaviour in two dart-poison frog species (*Epipedobates femoralis*, *Epipedobates trivittatus*) in Amazonian Peru. *Herpetological Journal*, 4, 77–85.

Santos J.C., Coloma, L.A., Cannatella, D.C. (2003) Multiple, recurring origins of aposematism and diet specialization in poison frogs. *Proceedings of the National Academy of Science USA*, 100 (22), 12792–12797.

Santos, J.C., Coloma, L.A., Summers, K., Caldwell, J.P., Ree, R. & Cannatella, D.C. (2009) Amazonian amphibian diversity is primarily derived from Late Miocene Andean lineages. *PLOS Biology*, 7(3), 1–14.

Simões, P.I., Lima, A.P., Magnusson, W.E., Hödl, W. & Amézquita, A. (2008) Acoustic and morphological differentiation in the frog *Allobates femoralis*: Relationships with the upper Madeira River and other potential geological barriers. *Biotropica*, 40(5), 607–614.

Tamura K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4*:* Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596–1599.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994) Improved sensitivity of profile searches through the use of sequence weights and gap excision. *Computer Applications in the Biosciences*, 10(1), 19–29.

Twomey, E. & Brown, J.L. (2008) A partial revision of the *Ameerega hahneli* complex (Anura: Dendrobatidae) and a new cryptic species from the East-Andean versant of Central Peru. *Zootaxa*, 1757, 49–65.

Vieites, D.R., Wollenberg, K.C., Andreone, F., Köhler, J., Glaw, F. & Vences, M. (2009) Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *PNAS*, 106(20), 8267–8272.

Vogel, L.S. & Johnson, S.G. (2008) Estimation of hybridization and introgression frequency in toads (Genus: *Bufo*) using DNA sequence variation at mitochondrial and nuclear loci. *Journal of Herpetology*, 42(1), 61–75.

Wells, K.D. (2007) *The ecology and behavior of amphibians*. The University of Chicago Press, Chicago, pp. 278–282.

Wynn, A. & Heyer, W.R. (2001) Do geographically widespread species of tropical amphibians exist? An estimate of genetic relatedness within the Neotropical frog *Leptodactylus fuscus* (Schneider, 1799) (Anura: Leptodactylidae). *Tropical Zoology*, 14, 255–285.

FIGURE 1. Relative location and denomination of (A) sampling sites in the Brazilian States of Acre and Rondônia, and (B) sampling sites and locations from where *Allobates femoralis* 16S rRNA mtDNA reference sequences were available in the Amazon Basin (shaded in paler gray). Yellow dots represent the distribution of *Allobates hodli* sp. nov. Light-green, dark-green, and black dots represent localities of samples referred to as *A. femoralis*, including two sites (5 and 9) where *A. hodli* reaches contact zones with these populations. Dot colors stand for major lineages recovered by phylogenetic analysis of a partial sequence of the 16S rRNA mitochondrial gene (see text and Fig. 7). Site 20, Yurimaguas, is considered the closest to *A. femoralis* type locality.

FIGURE 2. Color in life of *Allobates hodli* n. sp. (A) Lateral view of and adult male from Abunã, in Rondônia. (B) Dorsal view of a male from Cachoeira do Jirau, Rondônia. (C) Ventral view of an adult male from Fazenda Catuaba, in Acre, photographed through a transparent plastic bag. Note bright reddish–orange color of posterior abdomen and ventral surface of legs. (D) Ventral view of a male (left) and a female (right) from Cachoeira do Jirau. (E) Dorsolateral view of a male from Cachoeira do Jirau. Note irregular reddish-orange and black blotches and spots on dorsal surface of thighs and bright yellow flash marks on upper arms. (E) Juvenile from Abunã, photographed in laboratory after completion of metamorphosis. Photos A–C taken under natural light conditions. Photos A, B, D and E taken in July 2004; C in January 2003; A–E by Walter Hödl. F taken in February 2005 by A.P. Lima.

FIGURE 3. (A) Dorsal and (B) ventral views of *Allobates hodli* holotype (INPA-H 16555), a male collected at Cachoeira do Jirau in November, 2004. This individual lacks the disgestive tract and liver, removed for diet and genetic analyses respectively. (C) and (D) Hand and foot of *A. hodli* holotype.

FIGURE 4. (A) Dorsal view of preserved *Allobates hodli* tadpole in developmental stage 36 collected at Abunã, on the left bank of the upper Madeira River, in Rondônia, Brazil, on January 2005 (INPA-H 23693). (B) and (C) Ventral and lateral views of the same tadpole, respectively.

FIGURE 5. Oral disc of *Allobates hodli* tadpole from Abunã (INPA-H 23693).

FIGURE 6. The advertisement calls of *Allobates hodli* are constituted by trills of two notes repeated in series. (A) Waveform and sonogram of advertisement calls of *A. hodli* holotype (INPA–H 16555) recorded at Cachoeira do Jirau, Rondônia, at 07:55 h., in November 2004, air temperature 25.3°C, scaled to evidence ascending frequency modulation of notes. (B) Advertisement call of holotype in a larger scale, evidencing continuous repetition of two-note calls. (C) Advertisement call of an *A. hodli* male paratype (SVL = 24.62 mm) from Abunã, Rondônia, recorded at 24.7°C. (D) Courtship call of one *A. hodli* male (INPA-H 16553, SVL = 23.84 mm) recorded at type locality at 09:00 h, in November 2004, air temperature 26.3°C.

FIGURE 7. Maximum Likelihood phylogenetic tree reconstructed from unique haplotypes of a 518 b.p. fragment of the mitochondrial gene 16S rRNA of *Allobates*

femoralis and *Allobates hodli* sp. nov. sampled in 13 localities in Brazilian Amazon. Data set included reference sequences from Peru, Colombia, Ecuador, Suriname and other localities in Brazil obtained from GenBank. (A) Phylogenetic position of the clade including *A. hodli* sp. nov. and *A. femoralis* in relation to outgroups supports *Allobates zaparo* as their sister group. Clade labels represent support values from 5000 bootstrap replicates (only values above 50 are shown). (B) Relative phylogenetic placement of clades within the ingroup. Sample oscillograms and sonograms of advertisement calls from populations within clades display natural variation in number of notes. *A. hodli* is closely related to populations of *A. femoralis* from the southern Brazilian state of Acre (clade Acre 01), which present a four-note advertisement call and color pattern characteristic of *A. femoralis*. Both form the sister group to samples from northwestern Acre and from the Madre de Dios River basin (clade Acre 02). The basal clade containing *A. hodli* and Acre 01 and Acre 02 clades is the sister group to a basal clade containing *A. femoralis* sensu stricto (placed in the weakly supported clade femo 04) and the other reference sequences from populations referred to as *A. femoralis* (clades femo 01, femo 02, femo 03, femo 04). Samples from Ecuador form a divergent and well supported clade, with advertisement calls formed by a single note. Individuals from Panguana (placed in clade femo 04) present a distinctive 3-note advertisement call (not shown). All remaining populations have advertisement calls constituted by four notes. Calls from Yasuní, Ecuador, published by Read (2000). Calls from Pongo de Cainarachi, Peru (about 45 km south from *A. femoralis* type-locality in Yurimaguas) provided by Jason L. Brown. (C) Corresponding values on axes of oscillograms and sonograms of advertisement call samples.

FIGURE 8. Advertisement calls of *Allobates femoralis* recorded in (A) Chazuta (6.5419°S, 76.1083°W) and (B) Pongo de Cainarachi (6.2974°S, 76.2343°W), both localities in San Martin, south of *A. femoralis* type-locality in Yurimaguas. In both sites, calls are constituted by groups of four frequency-modulated notes. Sounds appearing with peak frequency at approximately 4.0 kHz in B are background noise. Recordings are courtesy of Jason L. Brown.

FIGURE 9. (A), (B) and (C) Dorsolateral color pattern of three specimens of *Allobates femoralis* photographed near Barranquita (6.2653°S, 76.0434°W), 36 km from *Allobates femoralis* type locality in Yurimaguas, Loreto, Peru. (D) Ventral view of same individual B, showing exclusively black and white color patterning on belly and ventral surface of thighs. Photos are courtesy of Jason L. Brown.

TABLE 1. Measurements (in mm) and proportions of *Allobates hodli* holotype (INPA-H 16555) and type series. Males and females present size dimorphism, females generally larger than males. Values in type series columns represent mean ± standard deviation (minimum value observed in the series – maximum value observed in the series).

		Type series	
Measurements	Holotype	Males (*n* = 76)	Females (*n* = 25)
SVL	23.99	24.41 ± 1.13 (22.2-27.3)	25.54 ± 1.05 (23.6-28.1)
HL	7.52	8.19 ± 0.49 (7.3 – 9.7)	8.54 ± 0.34 (7.9-9.4)
HW	8.01	7.84 ± 0.56 (4.5-9.1)	8.11 ± 0.31 (7.5-8.6)
SL	4.00	4.16 ± 0.50 (2.0-5.0)	4.49 ± 0.50 (3.2-5.5)
EN	2.30	2.36 ± 0.35 (1.9-3.1)	2.51 ± 0.28 (1.9-3.1)
IN	3.90	3.74 ± 0.22 (3.1-4.2)	3.89 ± 0.19 (3.5-4.2)
EL	2.70	2.94 ± 0.24 (2.0-3.4)	3.07 ± 0.23 (2.4-3.6)
IO	7.30	7.70 ± 0.38 (7.0-8.6)	7.96 ± 0.37 (7.2-8.8)
TYM	1.20	1.49 ± 0.15 (1.1-1.9)	1.60 ± 0.18 (1.2-2.0)
AL	5.23	5.26 ± 0.61 (4.11-6.83)	5.25 ± 0.93 (4.4-9.0)
FAL	6.46	6.23 ± 0.48 (5.0-7.1)	6.30 ± 0.46 (4.9-7.1)
H1	5.33	5.16 ± 0.32 (4.4-6.0)	5.24 ± 0.36 (4.5-6.0)
H2	4.91	4.61 ± 0.31 (3.7-5.7)	4.60 ± 0.28 (4.0-5.3)
H3	6.12	6.08 ± 0.31 (5.3-6.8)	6.10 ± 0.30 (5.6-6.9)
H4	4.54	4.21 ± 0.29 (3.6-4.9)	4.14 ± 0.35 (3.5-4.9)
WFD	0.80	0.79 ± 0.08 (0.6-0.9)	0.77 ± 0.07 (0.6-0.9)
THL	11.02	10.78 ± 0.67 (7.5-12.6)	10.47 ± 1.29 (5.5-12.1)
TIL	11.46	11.22 ± 0.52 (8.2-12.0)	11.29 ± 0.62 (9.2-12.3)
TAR	7.51	6.73 ± 0.63 (4.3-10.0)	6.74 ± 0.40 (5.9-7.6)
FL	9.95	10.33 ± 0.79 (7.3-11.5)	10.42 ± 0.65 (8.4-11.4)
WTD	1.10	1.05 ± 0.10 (0.8-1.2)	1.05 ± 0.09 (0.8-1.3)
HL/SVL	0.31	0.34 ± 0.02 (0.29–0.39)	0.33 ± 0.01 (0.30–0.36)
HW/SVL	0.33	0.32 ± 0.02 (0.18–0.36)	0.32 ± 0.01 (0.30–0.34)
TL/SVL	0.48	0.46 ± 0.02 (0.34–0.50)	0.44 ± 0.02 (0.37-0.48)
TYM/EL	0.44	0.51 ± 0.06 (0.39–0.65)	0.52 ± 0.06 (0.41-0.64)
ENA/EL	0.85	0.81 ± 0.12 (0.63–1.35)	0.82 ± 0.12 (0.72-1.17)

TABLE 2. Measurements (in mm) of four *A. hodli* tadpoles (INPA-H 23693) in developmental stage 36 of Gosner (1960) raised in laboratory from an egg clutch collected in Abunã, on the left bank of the upper Madeira River, in Rondônia, Brazil.

	Individuals				
Measurements	I	II	III	IV	X ± *s.d.*
TL	24.7	23.3	21.4	21.4	*22.7 ± 1.6*
BL	16.1	14.6	13.2	13.1	*14.3 ± 1.4*
TAL	16.1	14.6	13.2	13.1	*14.3 ± 1.4*
BW	6.0	6.3	6.2	5.5	*6.0 ± 0.4*
BH	4.8	4.1	4.5	3.9	*4.3 ± 0.4*
TMW	2.6	2.5	2.4	2.1	*2.4 ± 0.2*
TMH	2.4	2.1	2.1	2.2	*2.2 ± 0.1*
TH	4.5	4.0	4.0	3.6	*4.0 ± 0.4*
HWLE	5.6	5.3	5.4	5.2	*5.4 ± 0.2*
IOD	1.4	1.5	1.5	1.4	*1.5 ± 0.1*
IND	0.9	0.9	0.8	0.8	*0.9 ± 0.1*

TABLE 3. Advertisement call measurements of *Allobates hodli* holotype (INPA-H 16555) and type series collected in three localities along the upper Madeira River, in Rondônia, Brazil. Values in type series column represent mean ± standard deviation (minimum value observed in the series – maximum value observed in the series). Holotype was recorded at 07:55 h, air temperature during recording was 25.3°C. Average snout to vent length among 60 recorded males was 24.39mm ± 1.11mm (22.26mm-27.31mm). and average air temperature at the time of recording 26.52°C ± 1.46°C (23.3°C -29.8°C).

Measurements	Holotype	Type series (*n* = 60)
SIC (s)	0.207	0.218 ± 0.044 (0.128-0.357)
SIN (s)	0.082	0.074 ± 0.007 (0.062-0.099)
DC (s)	0.170	0.164 ± 0.011 (0.140-0.198)
D1 (s)	0.035	0.033 ± 0.004 (0.020-0.047)
D2 (s)	0.053	0.056 ± 0.007 (0.039-0.079)
MFC (Hz)	3565.53	3425.0 ± 184.7 (2991.3-3897.5)
HFC (Hz)	4002.40	3831.3 ± 174.6 (3262.1-4223.5)
LFC (Hz)	3186.73	3029.6 ± 124.5 (2713.3-3240.8)
MFN1 (Hz)	3488.37	3319.6 ± 141.5 (2971.6-3610.4)
HFN1 (Hz)	3702.83	3552.2 ± 157.5 (3087.1-3964.5)
LFN1 (Hz)	3226.47	3082.3 ± 130.7 (2779.5-3287.8)
MFN2 (Hz)	3637.30	3482.7 ± 193.8 (2977.0-3895.7)
HFN2 (Hz)	4011.43	3838.2 ± 175.1 (3262.1-4254.2)
LFN2 (Hz)	3260.73	3099.8 ± 121.4 (2787.3-3333.4)

TABLE 4. Courtship call measurements of *Allobates hodli* from type locality at Cachoeira do Jirau (INPA-H 16553 and 16567) and from Abunã (INPA-H 16606 and 16621), on the left bank of the upper Madeira River, in Rondônia, Brazil. More than one courtship call was available for INPA-H 16567 and INPA-H 16621, and values represent the averages between all available calls.

	Individual sampled (INPA-H #)				
	16553	16567	16606	16621	X ± *s.d.*
Nº of calls analysed	*1*	*6*	*1*	*2*	
Temperature (°C)	26.3	26.3	28.2	26.0	*26.7 ± 1.0*
SVL (mm)	23.84	24.75	25.47	23.94	*24.5 ± 0.7*
DC (s)	0.624	0.800	0.402	0.457	*0.571 ± 179*
MFC (Hz)	2960.8	2865.7	3488.4	3446.0	*3190.2 ± 322.6*
Nº of pulses	73	90	61	55	*70 ± 15*
Nº of pulses/second	117.0	113.0	151.7	119.0	*125.2 ± 17.8*

TABLE 5. Sample names and available sample information for sequences of *Allobates hodli* sp nov., reference *Allobates femoralis* sequences and outgroup sequences included in the molecular phylogenetic analysis. Clades correspond to monophyletic groups presented in Figure 7B).

Sample name	Clade	Locality	Coordinates	16S	Reference
Ecuador 1	femo 01	Cuyabeno, Sucumbios, Ecuador	-	AF128572	Clough & Summers, 2000
Ecuador 2	femo 01	Cuyabeno, Sucumbios, Ecuador	0°0’ S, 76°10’W	DQ502093	Grant *et al.* 2006
Ecuador 3	femo 01	Ecuador	-	AY364543	Santos *et al*. 2003
Ecuador 4	femo 01	Parque Nac. Yasuni, Ecuador	-	EU342535	Santos *et al*. 2009
Ecuador 5	femo 01	Cuyabeno, Sucumbios, Ecuador	0°0’ S, 76°10’W	DQ502093	Grant *et al.* 2006
Ecuador 6	femo 01	Cuyabeno, Sucumbios, Ecuador	0°0’ S, 76°10’W	DQ502228	Grant *et al.* 2006
Ecuador 7	femo 01	Cuyabeno, Sucumbios, Ecuador	-	DQ342534	Santos *et al*. 2009
LowerJirau 1	femo 02	Lower Jirau, Rondônia, Brazil	9.311°S, 64.717°W	GU017446	this study
LowerJirau 2	femo 02	Lower Jirau, Rondônia, Brazil	9.311°S, 64.717°W	GU017447	this study
LowerJirau 3	femo 02	Lower Jirau, Rondônia, Brazil	9.311°S, 64.717°W	GU017448	this study
LowerJirau 4	femo 02	Lower Jirau, Rondônia, Brazil	9.311°S, 64.717°W	GU017449	this study
Jaci-Paraná 1	femo 02	Jaci-Paraná, Rondônia, Brazil	9.169°S, 64.429°W	GU017456	this study
Jaci-Paraná 2	femo 02	Jaci-Paraná, Rondônia, Brazil	9.169°S, 64.429°W	GU017452	this study
Jaci-Paraná 3	femo 02	Jaci-Paraná, Rondônia, Brazil	9.169°S, 64.429°W	GU017451	this study
Jaci-Paraná 4	femo 02	Jaci-Paraná, Rondônia, Brazil	9.169°S, 64.429°W	GU017454	this study

Jaci-Paraná 5	femo 02	Jaci-Paraná, Rondônia, Brazil	9.169°S, 64.429°W	GU017453	this study
Jaci-Paraná 6	*femo* 02	Jaci-Paraná, Rondônia, Brazil	9.169°S, 64.429°W	GU017455	this study
Jaci-Paraná 7	*femo* 02	Jaci-Paraná, Rondônia, Brazil	9.169°S, 64.429°W	GU017450	this study
Jaci-Paraná 8	*femo* 02	Jaci-Paraná, Rondônia, Brazil	9.169°S, 64.429°W	GU017457	this study
Guajará-Mirim 1	*femo* 02	Guajará-Mirim, Rondônia, Brazil	10°19’S, 64°33’W	DQ283045	Frost *et al*. 2006
Guajará-Mirim 2	*femo* 02	Guajará-Mirim, Rondônia, Brazil	10°19’S, 64°33’W	EU342537	Santos *et al*. 2009
Guajará-Mirim 3	*femo* 02	Guajará-Mirim, Rondônia, Brazil	10°19’S, 64°33’W	DQ502088	Grant *et al*. 2006
Mutum-Paraná(R) 1	*femo* 02	Mutum-Paraná (right bank), Rondônia, Brazil	9.641°S, 64.886°W	GU017458	this study
Mutum-Paraná(R) 2	*femo* 02	Mutum-Paraná (right bank), Rondônia, Brazil	9.641°S, 64.886°W	GU017462	this study
Mutum-Paraná(R) 3	*femo* 02	Mutum-Paraná (right bank), Rondônia, Brazil	9.641°S, 64.886°W	GU017459	this study
Mutum-Paraná(R) 4	*femo* 02	Mutum-Paraná (right bank), Rondônia, Brazil	9.641°S, 64.886°W	GU017461	this study
Mutum-Paraná(R) 5	*femo* 02	Mutum-Paraná (right bank), Rondônia, Brazil	9.641°S, 64.886°W	GU017460	this study
Suriname	-	Sipaliwini, Suriname	3°5.7’N, 56°28.3’W	DQ502246	Grant *et al*. 2006
Trairão 1	*femo* 03	Trairão, Pará, Brazil	4.683°S, 56.022°W	GU017477	this study
Trairão 2	*femo* 03	Trairão, Pará, Brazil	4.683°S, 56.022°W	GU017478	this study
Trairão 3	*femo* 03	Trairão, Pará, Brazil	4.683°S, 56.022°W	GU017479	this study
Trairão 4	*femo* 03	Trairão, Pará, Brazil	4.683°S, 56.022°W	GU017480	this study
Treviso 1	*femo* 03	Fazenda Treviso, Pará, Brazil	3.158°S, 54.859°W	GU017475	this study

Treviso 2	*femo* 03	Fazenda Treviso, Pará, Brazil	3.158°S, 54.859°W	GU017474	this study
Treviso 3	*femo* 03	Fazenda Treviso, Pará, Brazil	3.158°S, 54.859°W	GU017476	this study
Iquitos 1	*femo* 04	Iquitos, Loreto, Peru	-	DQ523023	Roberts *et al.* 2006
Iquitos 2	*femo* 04	Iquitos, Loreto, Peru	-	DQ523025	Roberts *et al.* 2006
Iquitos 3	*femo* 04	Iquitos, Loreto, Peru	-	DQ523040	Roberts *et al.* 2006
Leticia	*femo* 04	Cerca Viva, Leticia, Amazonas, Colombia	-	EU342536	Santos *et al.* 2009
Panguana	*femo* 04	Panguana, Peru	-	DQ502117	Grant *et al*. 2006
Yurimaguas	*femo* 04	Shucshuyacu, Yurimaguas, Loreto, Peru	6.032°S, 75.857°W	DQ523072	Roberts *et al.* 2006
ReservaDucke	*femo* 04	Reserva Ducke, Amazonas, Brazil	-	DQ502113	Grant *et al.* 2006
Tarapoto	*femo* 04	Saposoa, Tarapoto, San Martin, Peru	6.771°S, 76.941°W	DQ523082	Roberts *et al.* 2006
hodli – Abunã 1	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017423	this study
hodli – Abunã 2	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017429	this study
hodli – Abunã 3	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017431	this study
hodli – Abunã 4	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017432	this study
hodli – Abunã 5	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017424	this study
hodli – Abunã 6	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017430	this study
hodli – Abunã 7	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017434	this study
hodli – Abunã 8	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017435	this study

hodli – Abunã 9	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017426	this study
hodli – Abunã 10	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017436	this study
hodli – Abunã 11	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017428	this study
hodli – Abunã 12	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017427	this study
hodli – Abunã 13	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017433	this study
hodli – Abunã 14	*hodli*	Abunã, Rondônia, Brazil	9.516°S, 65.324°W	GU017425	this study
hodli – Jirau 1	*hodli*	Cachoira do Jirau, Rondônia, Brazil	9.335°S, 64.737°W	GU017444	this study
hodli – Jirau 2	*hodli*	Cachoira do Jirau, Rondônia, Brazil	9.335°S, 64.737°W	GU017445	this study
hodli – Jirau 3	*hodli*	Cachoira do Jirau, Rondônia, Brazil	9.335°S, 64.737°W	GU017443	this study
hodli – Mutum-Paraná(L) 1	*hodli*	Mutum-Paraná (left bank), Rondônia, Brazil	9.573°S, 64.921°W	GU017441	this study
hodli – Mutum-Paraná(L) 2	*hodli*	Mutum-Paraná (left bank), Rondônia, Brazil	9.573°S, 64.921°W	GU017437	this study
hodli – Mutum-Paraná(L) 3	*hodli*	Mutum-Paraná (left bank), Rondônia, Brazil	9.573°S, 64.921°W	GU017438	this study
hodli – Mutum-Paraná(L) 4	*hodli*	Mutum-Paraná (left bank), Rondônia, Brazil	9.573°S, 64.921°W	GU017440	this study
hodli – Mutum-Paraná(L) 5	*hodli*	Mutum-Paraná (left bank), Rondônia, Brazil	9.573°S, 64.921°W	GU017442	this study
hodli – Mutum-Paraná(L) 6	*hodli*	Mutum-Paraná (left bank), Rondônia, Brazil	9.573°S, 64.921°W	GU017439	this study
MonteAlegre 1	-	Monte Alegre, Acre, Brazil	10.346°S, 67.518°W	GU017469	this study
MonteAlegre 2	-	Monte Alegre, Acre, Brazil	10.346°S, 67.518°W	GU017468	this study
Brasiléia 1	Acre 01	Brasiléia, Acre, Brazil	10.965°S, 68.733°W	GU017463	this study

Brasiléia 2	Acre 01	Brasiléia, Acre, Brazil	10.965°S, 68.733°W	GU017464	this study
Seringal	Acre 01	Seringal da Cachoeira, Acre, Brazil	10.833°S, 69.381°W	GU017467	this study
BR-317 1	Acre 01	BR-317, Acre, Brazil	10.820°S, 69.192°W	GU017465	this study
BR-317 2	Acre 01	BR-317, Acre, Brazil	10.820°S, 69.192°W	GU017466	this study
CruzeiroDoSul 1	Acre 02	Cruzeiro do Sul, Acre, Brazil	7.956°S, 72.077°W	GU017470	this study
CruzeiroDoSul 2	Acre 02	Cruzeiro do Sul, Acre, Brazil	7.956°S, 72.077°W	GU017473	this study
CruzeiroDoSul 3	Acre 02	Cruzeiro do Sul, Acre, Brazil	7.956°S, 72.077°W	GU017472	this study
CruzeiroDoSul 4	Acre 02	Cruzeiro do Sul, Acre, Brazil	7.956°S, 72.077°W	GU017471	this study
PortoWalter 1	Acre 02	Porto Walter, Acre, Brazil	8°15’S, 72°46’W	DQ502091	Grant *et al.* 2006
PortoWalter 2	Acre 02	Porto Walter, Acre, Brazil	-	EU342533	Santos *et al.* 2009
PortoWalter 3	Acre 02	Porto Walter, Acre, Brazil	8°15’S, 72°46’W	DQ502092	Grant *et al.* 2006
PortoWalter 4	Acre 02	Porto Walter, Acre, Brazil	-	EU342532	Santos *et al.* 2009
PortoWalter 5	Acre 02	Porto Walter, Acre, Brazil	9°34’S, 72°46’W	DQ502231	Grant *et al.* 2006
MadreDeDios 1	Acre 02	Puerto Maldonado, Cusco Amazônico, Peru	-	DQ501990	Grant *et al.* 2006
MadreDeDios 2	Acre 02	Puerto Maldonado, Cusco Amazônico, Peru	-	DQ502014	Grant *et al.* 2006
MadreDeDios 3	Acre 02	Puerto Maldonado, Cusco Amazônico, Peru	-	DQ502015	Grant *et al.* 2006
CuzcoAmazonico	Acre 02	Boca Manu, Cuzco, Peru	-	DQ523069	Roberts *et al*. 2006
nidicola	-	Castanho, Amazonas, Brasil	-	EU342518	Santos *et al*. 2009

nocturnus	-	Trujillo, Venezuela	-	DQ502154	Grant *et al.* 2006
stepheni	-	Reserva Ducke, Amazonas, Brazil	-	DQ502107	Grant *et al.* 2006
talamancae	-	Quibdo, Choco, Colombia	-	EU342510	Santos *et al*. 2009
zaparo 1	-	Ecuador	-	AY364578	Santos *et al*. 2003
zaparo 2	-	Ecuador	-	AY364579	Santos *et al*. 2003
zaparo 3	-	Pastaza, Ecuador	-	EU342530	Santos *et al*. 2009

TABLE 6: Mean uncorrected pairwise genetic distances between major clades of the Maximum Likelihood phylogenetic tree obtained from 518 b.p. fragment of the 16S rRNA gene of *Allobates hodli* sp. nov. and reference populations of *Allobates femoralis*. Denominations in first column correspond to those in Fig. 7. Samples from Suriname and Monte Alegre were not placed within any major clade and their relative genetic distances are calculated separately.

	n	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Allobates hodli* sp. nov.	*72*													
2 Acre 01	*13*	0.014												
3 Acre 02	*17*	0.022	0.021											
4 Monte Alegre	*2*	0.010	0.012	0.015										
5 femo 01	*7*	0.040	0.040	0.036	0.035									
6 femo 02	*41*	0.044	0.051	0.045	0.043	0.029								
7 femo 03	*19*	0.040	0.046	0.041	0.039	0.024	0.016							
8 femo 04	*8*	0.049	0.055	0.051	0.047	0.028	0.021	0.023						
9 Suriname	*1*	0.039	0.046	0.040	0.038	0.024	0.007	0.012	0.016					
10 *Allobates zaparo*	*3*	0.062	0.064	0.056	0.055	0.049	0.052	0.049	0.050	0.048				
11 *Allobates nidicola*	*1*	0.090	0.098	0.091	0.089	0.098	0.098	0.098	0.095	0.095	0.093			
12 *Allobates talamancae*	*1*	0.101	0.111	0.108	0.102	0.103	0.108	0.107	0.101	0.103	0.108	0.107		
13 *Anomaloglossus stepheni*	*1*	0.135	0.140	0.132	0.129	0.140	0.148	0.141	0.146	0.145	0.137	0.131	0.149	
14 *Aromobates nocturnus*	*1*	0.130	0.137	0.128	0.129	0.135	0.130	0.13	0.137	0.131	0.133	0.141	0.149	0.119

Fig. 1

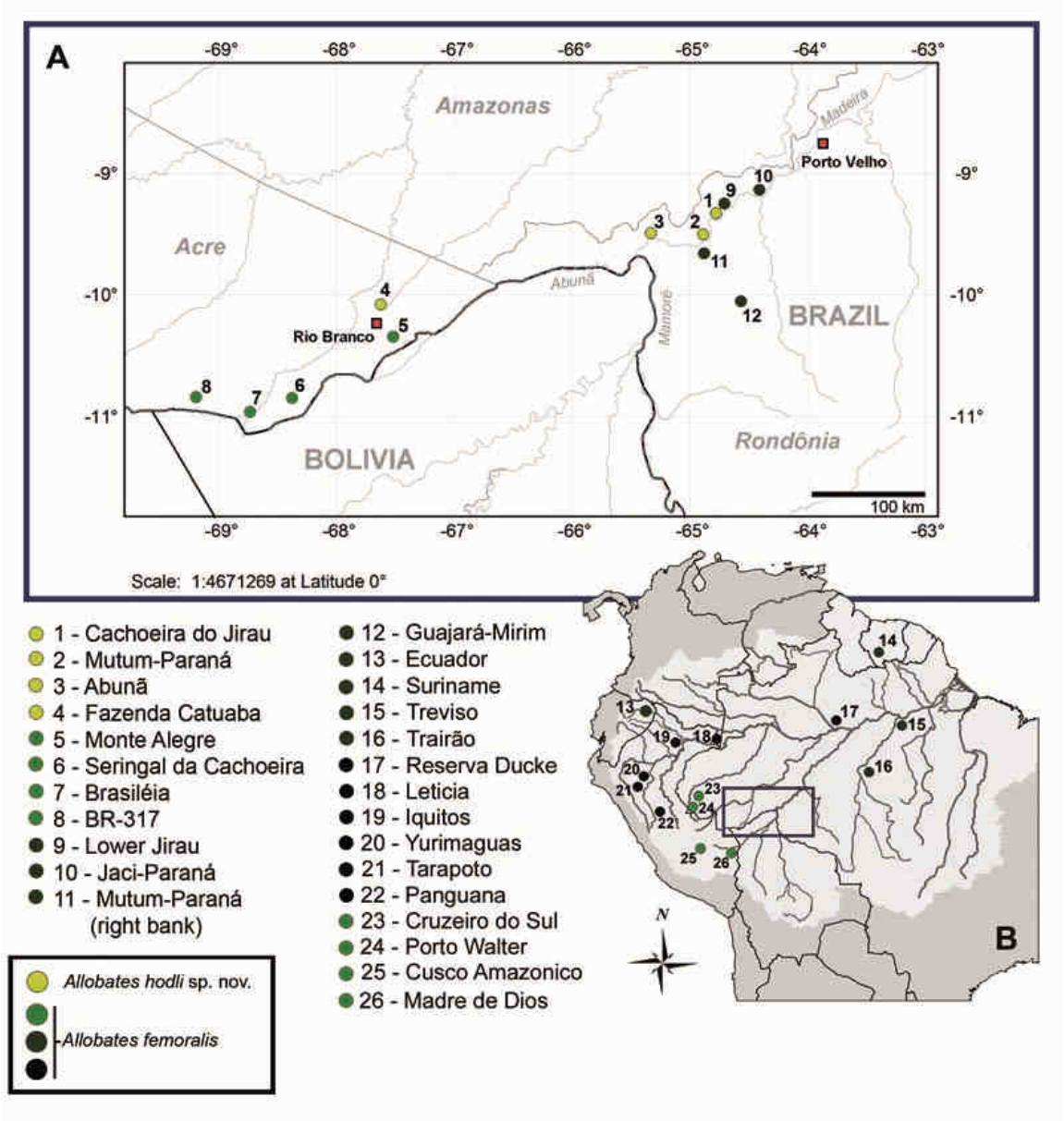

A
-69°
-68°
-67°
-66°
-65°
-64°
-63°
-9°
-10°
-11°
Amazonas
Acre
Madeira
Porto Velho
Abunã
Mamoré
Rio Branco
BRAZIL
BOLIVIA
Rondônia
100 km
Scale: 1:4671269 at Latitude 0°
1 - Cachoeira do Jirau
2 - Mutum-Paraná
3 - Abunã
4 - Fazenda Catuaba
5 - Monte Alegre
6 - Seringal da Cachoeira
7 - Brasiléia
8 - BR-317
9 - Lower Jirau
10 - Jaci-Paraná
11 - Mutum-Paraná (right bank)
12 - Guajará-Mirim
13 - Ecuador
14 - Suriname
15 - Treviso
16 - Trairão
17 - Reserva Ducke
18 - Leticia
19 - Iquitos
20 - Yurimaguas
21 - Tarapoto
22 - Panguana
23 - Cruzeiro do Sul
24 - Porto Walter
25 - Cusco Amazonico
26 - Madre de Dios
Allobates hodli sp. nov.
Allobates femoralis
N
B

Fig.2

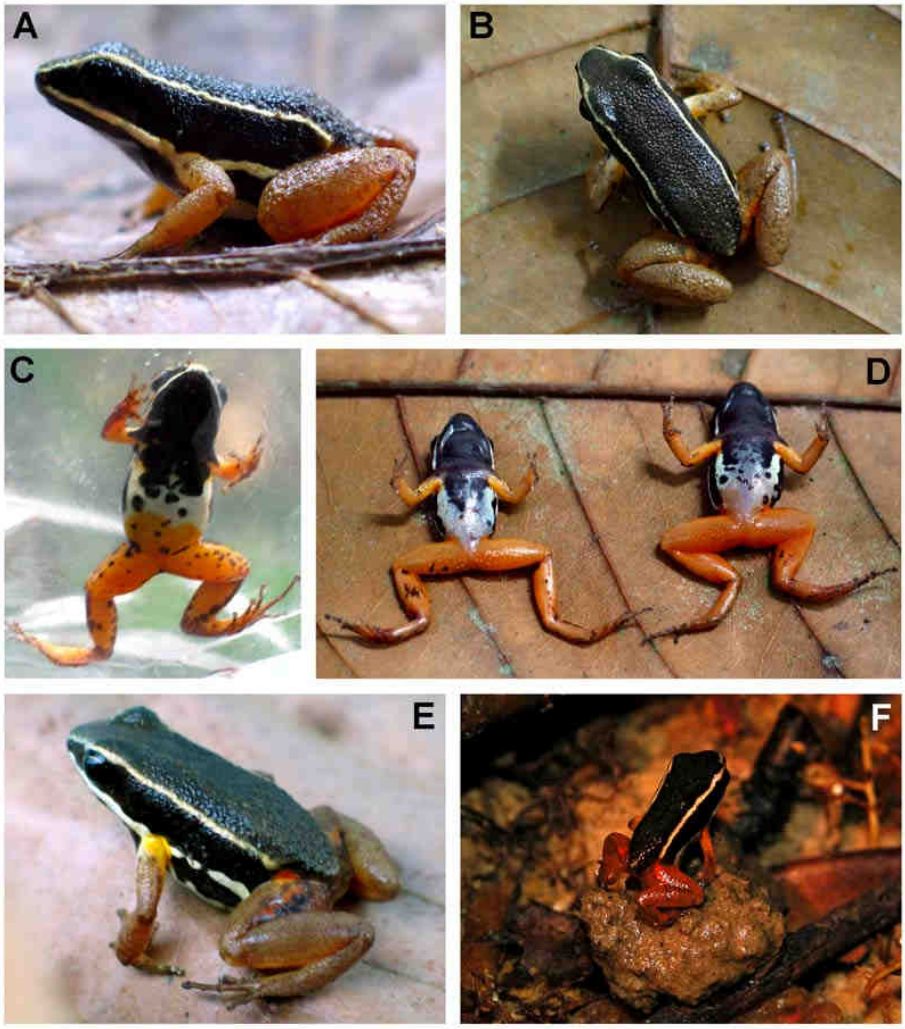


Fig. 3

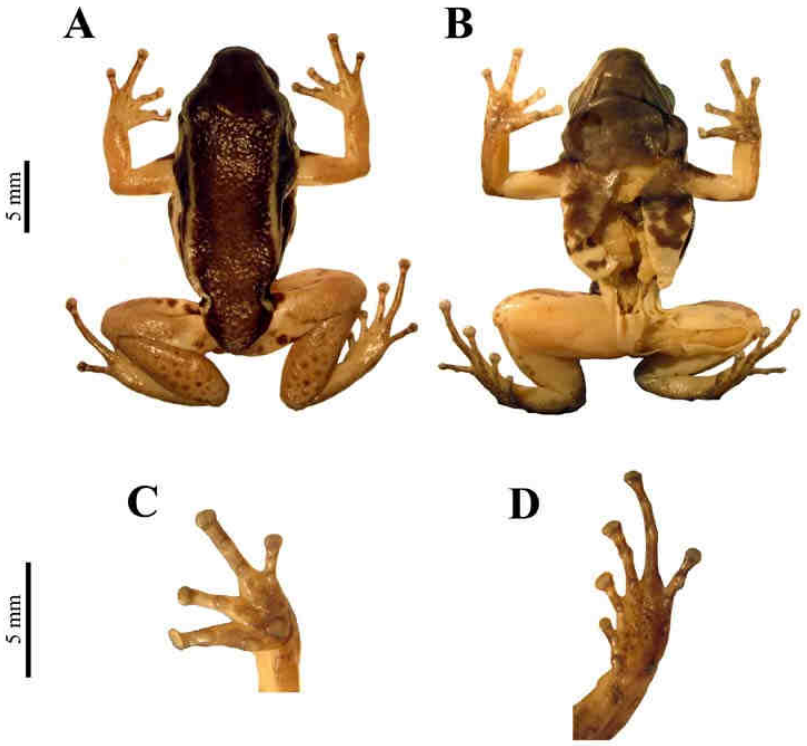


Fig. 4

Fig. 5

Fig. 6

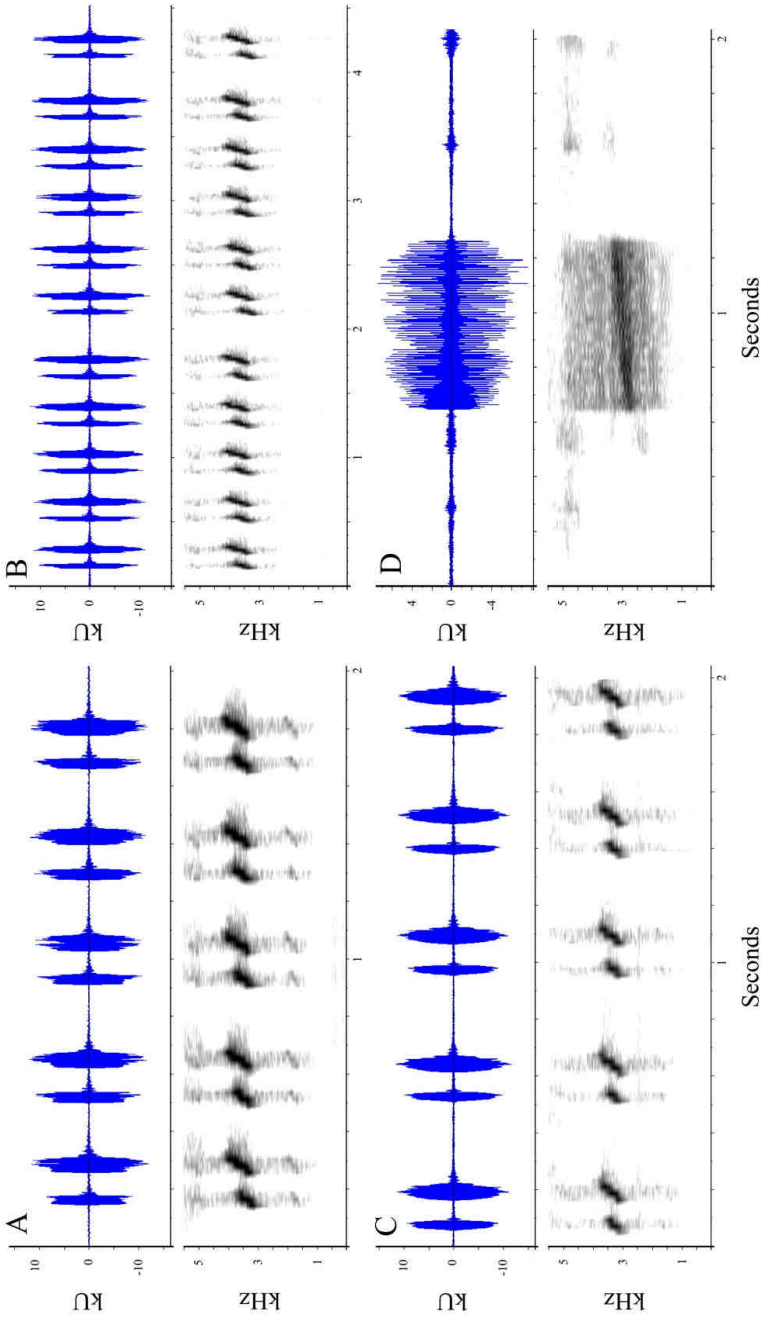


Fig. 7

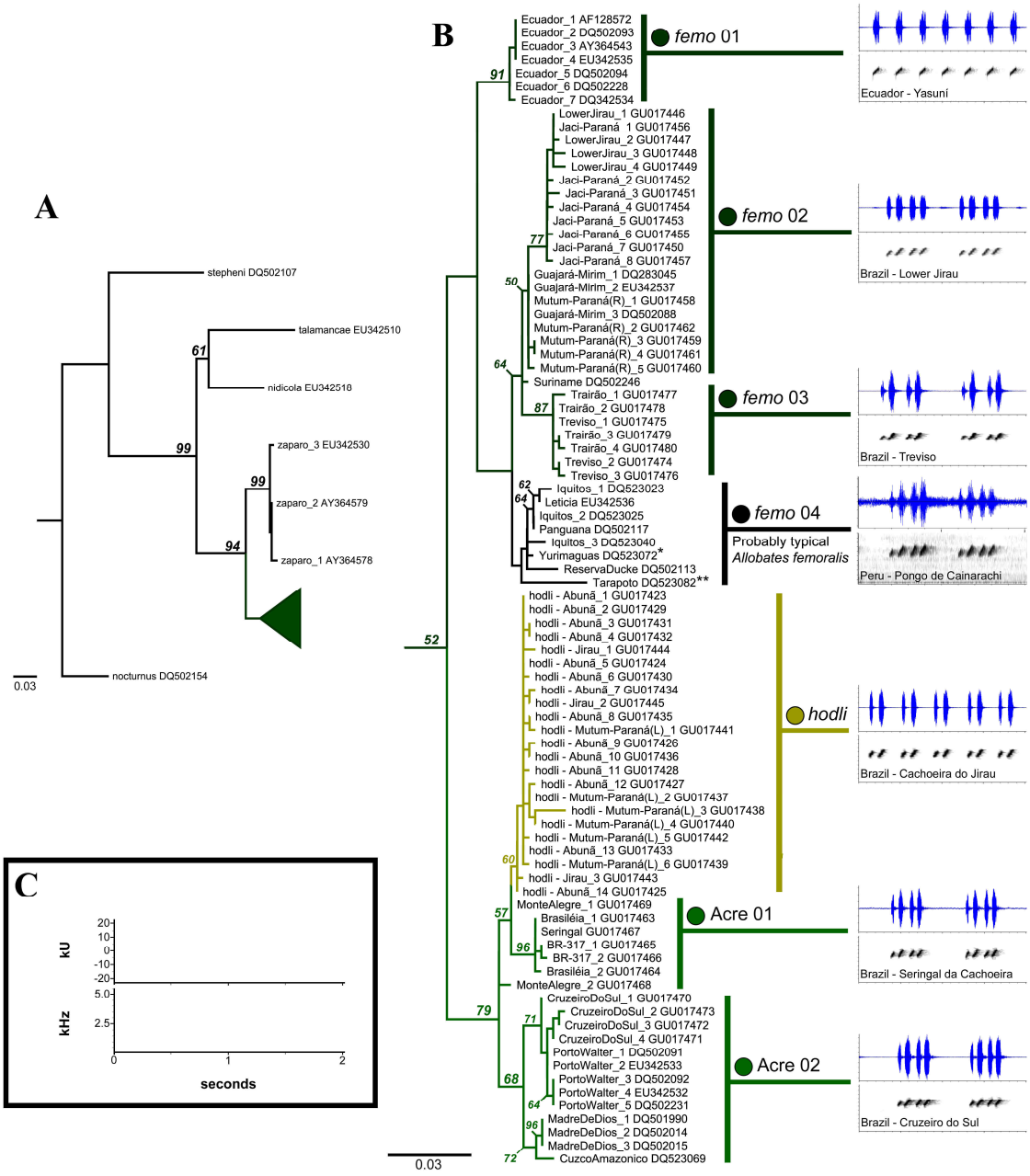

A
stepheni DQ502107
talamancae EU342510
nidicola EU342510
zaparo_3 EU342530
zaparo_2 AY364579
zaparo_1 AY364578
nocturnus DQ502154
0.03
B
Ecuador_1 AF128572
Ecuador_2 DQ502093
Ecuador_3 AY364543
Ecuador_4 EU342535
Ecuador_5 DQ502094
Ecuador_6 DQ502228
Ecuador_7 DQ342534
femo 01
Ecuador - Yasuní
LowerJirau_1 GU017446
Jaci-Paraná_1 GU017456
LowerJirau_2 GU017447
LowerJirau_3 GU017448
LowerJirau_4 GU017449
Jaci-Paraná_2 GU017452
Jaci-Paraná_3 GU017451
Jaci-Paraná_4 GU017454
Jaci-Paraná_5 GU017453
Jaci-Paraná_6 GU017455
Jaci-Paraná_7 GU017450
Jaci-Paraná_8 GU017457
Guajará-Mirim_1 DQ283045
Guajará-MIrIm_2 EU342537
Mutum-Paraná(R)_1 GU017458
Guajará-Mirim_3 DQ502088
Mutum-Paraná(R)_2 GU017462
Mutum-Paraná(R)_3 GU017459
Mutum-Paraná(R)_4 GU017461
Mutum-Paraná(R)_5 GU017460
femo 02
Brazil - Lower Jirau
Suriname DQ502246
Trairão_1 GU017477
Trairão_2 GU017478
Treviso_1 GU017475
Trairão_3 GU017479
Trairão_4 GU017480
Treviso_2 GU017474
Treviso_3 GU017476
femo 03
Brazil - Treviso
Iquitos_1 DQ523023
Leticia EU342536
Iquitos_2 DQ523025
Panguana DQ502117
Iquitos_3 DQ523040
Yurimaguas DQ523072*
ReservaDucke DQ502113
Tarapoto DQ523082**
femo 04
Probably typical
Allobates femoralis
Peru - Pongo de Cainarachi
hodli - Abunã_1 GU017423
hodli - Abunã_2 GU017429
hodli - Abunã_3 GU017431
hodli - Abunã_4 GU017432
hodli - Jirau_1 GU017444
hodli - Abunã_5 GU017424
hodli - Abunã_6 GU017430
hodli - Abunã_7 GU017434
hodli - Jirau_2 GU017445
hodli - Abunã_8 GU017435
hodli - Mutum-Paraná(L)_1 GU017441
hodli - Abunã_9 GU017426
hodli - Abunã_10 GU017436
hodli - Abunã_11 GU017428
hodli - Abunã_12 GU017427
hodli - Mutum-Paraná(L)_2 GU017437
hodli - Mutum-Paraná(L)_3 GU017438
hodli - Mutum-Paraná(L)_4 GU017440
hodli - Mutum-Paraná(L)_5 GU017442
hodli - Abunã_13 GU017433
hodli - Mutum-Paraná(L)_6 GU017439
hodli - Jirau_3 GU017443
hodli - Abunã_14 GU017425
hodli
Brazil - Cachoeira do Jirau
MonteAlegre_1 GU017469
Brasiléia_1 GU017463
Seringal GU017467
BR-317_1 GU017465
BR-317_2 GU017466
Brasiléia_2 GU017464
MonteAlegre_2 GU017468
Acre 01
Brazil - Seringal da Cachoeira
CruzeiroDoSul_1 GU017470
CruzeiroDoSul_2 GU017473
CruzeiroDoSul_3 GU017472
CruzeiroDoSul_4 GU017471
PortoWalter_1 DQ502091
PortoWalter_2 EU342533
PortoWalter_3 DQ502092
PortoWalter_4 EU342532
PortoWalter_5 DQ502231
MadreDeDios_1 DQ501990
MadreDeDios_2 DQ502014
MadreDeDios_3 DQ502015
CuzcoAmazonico DQ523069
Acre 02
Brazil - Cruzeiro do Sul
0.03
C
kU
kHz
seconds

Fig. 8

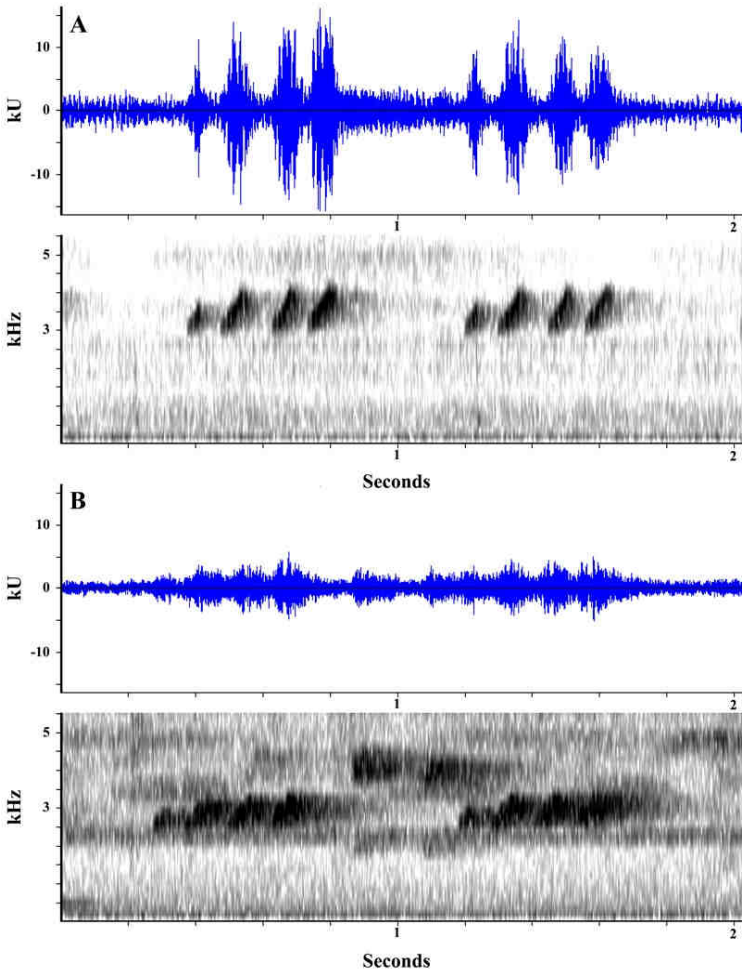
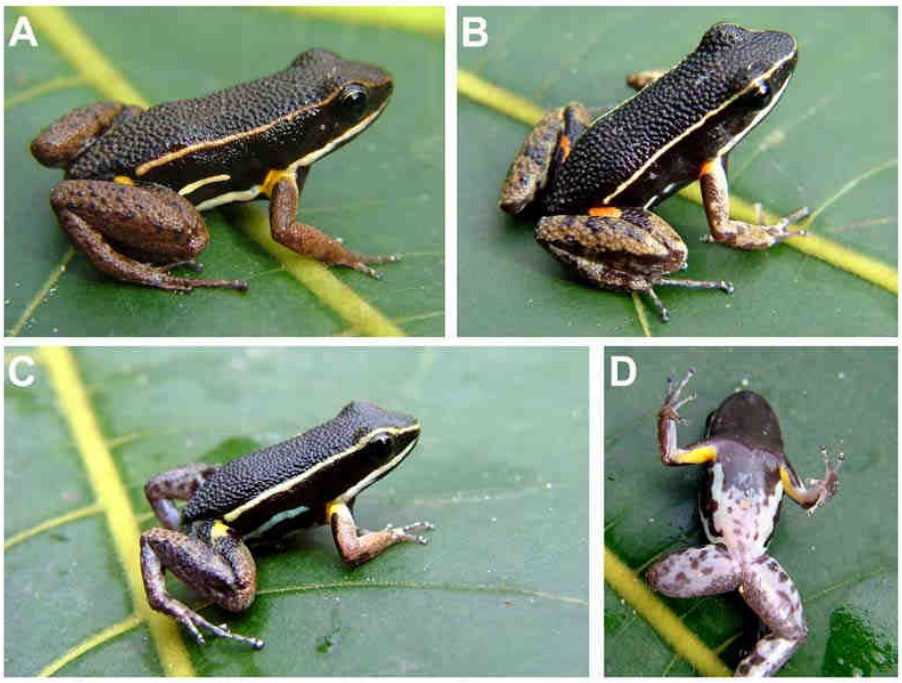


Fig. 8



Capítulo II[2]

[2] Manuscrito formatado de acordo com as normas da revista Journal of Biogeography. Não submetido.

Article type: Original article

Article title: Revisiting the river-barrier hypothesis of diversification in the Amazonian lowlands: effects of the Madeira River on differentiation patterns of the brilliant-thighed frog, *Allobates femoralis*.

Authors names: Pedro Ivo Simões[1*], Albertina Pimentel Lima[1] & Izeni Pires Farias[2].

Authors addresses:

1 - Coordenação de Pesquisas em Ecologia, Instituto Nacional de Pesquisas da Amazônia, Caixa Postal 478, CEP 69011-970, Manaus, AM, Brazil (pedroivo@inpa.gov.br, lima@inpa.gov.br)

2 - Universidade Federal do Amazonas (UFAM), Departamento de Biologia, Laboratório de Evolução e Genética Animal (LEGAL), Av. Rodrigo Octávio Jordão Ramos, 3000, 69077-000 Manaus, AM, Brazil (izeni_farias@ufam.edu.br).

**Corresponding author*

Running head: River barrier effects on anuran population differentiation

ABSTRACT

Aim: Our goal was to evaluate possible effects of one of the largest lowland Amazonian rivers on the phylogeography and genetic and phenotypic differentiation of the dendrobatid frog *Allobates femoralis*, and to compare the observed patterns with predictions posed by the classic river-barrier hypothesis of vicariance.

Location: Madeira River basin, Southwestern Brazilian Amazon.

Methods: We established 17 sampling localities along the Madeira River, on both riverbanks, from the upper course to the river's mouth, where we recorded and collected individuals of *A. femoralis*. We applied phylogenetic analysis on a mtDNA dataset to test for reciprocal monophyly between clades occupying opposite riverbanks. A larger dataset containing only 16S rDNA sequences was used in genetic population analysis and tests of genetic differentiation between riverbanks. Published data on the ages of *A. femoralis* lineages were used to estimate divergence times. Phenotypic differentiation and correlation with geographic distances were evaluated by means of multivariate analysis of variance and partial Mantel tests on principal components representing bioacustic and morphometric variables.

Results: *Allobates femoralis* populations on opposite sides of the Madeira River do not constitute monophyletic clades. Haplotype sharing was detected between localities on opposite riverbanks in the upper Madeira River. Signals of population expansion support the rapid colonization of the upper left riverbank following dispersal events from the right

riverbank. Genetic and morphological variation was generally larger between populations on the same riverbanks than between populations on opposite riverbanks. While the river channel accounts for part of the population divergence in morphology, other mechanisms such as isolation by distance and environmental selection might account for the remaining phenotypic variation between populations.

Main conclusions: According to strict interpretations of the classic river-barrier hypothesis, the Madeira River has not been an effective vicariant barrier since its establishment. Our data support that climatically or tectonically induced channel course dynamics allowed dispersal events across the upper to middle course of the river occurred during the Pleistocene, possibly masking a vicariant event triggered by the river's origin.

Keywords: Amazon Basin, Brazil, river-barrier hypothesis, Madeira River, Dendrobatidae, *Allobates femoralis*, phylogeography, phenotypic differentiation, genetic differentiation.

INTRODUCTION

Some of the most interesting questions regarding the Amazonian lowlands are related to the overwhelming number of species found throughout its whole range, and to the geographic distribution of each one of them. Several evolutionary mechanisms have been suggested as the driving forces behind the outstanding levels of species richness and current distribution patterns observed in this region (reviewed in Haffer, 1997; Moritz *et al*., 2000; Antonelli *et al*., 2010), but lack of congruence between these patterns and available geologic data frequently led to episodes of controversy and debate (Bush, 1994; Bush & Oliveira, 2006; Colinvaux *et al*., 2000). The hypothesis that large Amazonian rivers act as effective vicariant barriers since their establishment is perhaps the oldest evolutionary mechanism proposed to this region (Colwell, 2000), and its foundations rely upon early accounts of Amazonian species distributions (Wallace, 1852). More contemporary interpretations of the hypothesis predict rivers to work as a potential vicariant mechanism capable of splitting populations of a formerly widespread species and preventing subsequent gene flow. Once isolated in opposite sides of a large river, populations would go through distinct evolutionary pathways, becoming independently evolving lineages. From this simple vicariant model, follow the expectations that the barrier effect would be stronger according to river width (*i.e.* towards the river's mouth) and highly pronounced among *terra-firme* (not seasonally flooded) forest specialists (Caparella, 1987; Gascon *et al*., 1998, 2000; Colwell, 2000).

Criticism to the river hypothesis of vicariance is widespread in literature addressing the phylogeography of Amazonian vertebrates. Most of it was unleashed by supporters of Pleistocene refuge hypothesis (Haffer, 1974; 1997; Haffer & Prance, 2002) or derived from

studies carried out along the Juruá River, a large (albeit highly meandering) southern tributary of the Amazon River. These studies rejected to a great extent the role of this river as a possible barrier to dispersal of individuals based on community and population analyses using many sources of data (*e.g.* patterns of species composition, population variation in morphology and allozymes, mitochondrial DNA phylogenies), obtained from a varied array of small-mammal and amphibian taxa (Silva & Patton, 1998; Gascon *et al*., 1998, 2000; Lougheed *et al.*, 1999). In that system, similarity among communities in relation to species composition of was more frequently related to geographic distances between sampling sites or to habitat type (seasonally-flooded *várzea* forests *versus* non-flooded *terra-firme* forests) than to their split by the river channel (Gascon *et al*., 2000). Population and phylogenetic analyses largely supported the existence of high levels of differentiation between populations sampled in forests along the headwaters and those inhabiting areas along the lower course of the Juruá (Patton *et al*., 1994; Gascon *et al*., 1998; Silva & Patton, 1998; Lougheed *et al.*, 1999). Based on correlations between estimated ages and locations of divergent clades with Miocene orogenic events in the western Amazon, these patterns lead to increased support to the hypothesis that tectonic ridges played a key role as past vicariant barriers that shaped the current distribution of genetic diversity of focal taxa.

The effectiveness of tectonic arches as potential physical barriers has been also questioned, as most of these geological structures date from the Paleozoic and Mesozoic and are largely covered by deep layers of sedimentary deposits dating from the Cretaceous and Cenozoic, and as reports on their location and characterization on literature are often conflicting (Rossetti *et al*., 2005; Wesselingh & Salo, 2006). Tectonic processes such as the activation of structural arches most likely affected the historical distribution of the lowland

97 Amazonian biota by influencing the evolution and settlement of major drainage systems
98 throughout the Neogene, especially from late Miocene (Hoorn, 1994; Wesselingh, 2006;
99 Nogueira, 2008; Figueiredo *et al.*, 2009). Following Miocene, the gradual establishment of
100 continental and regional drainages under tectonic and climatic control led the Amazonian
101 lowlands to frequent shifts in channel course orientation, water discharge, and depositional
102 regimes, which are imprinted in the interfluves of most large Amazonian rivers as a mosaic
103 of distinct sedimentary units (Latrubesse & Franzinelli, 2002; Latrubesse, 2003; Rossetti *et*
104 *al*., 2005). The diversity of geomorphological compartments subject to unique levels of
105 tectonic activity should itself cause abrupt environmental shifts along the interfluves, which
106 might be an important factor triggering biological differentiation, despite (or in addition to)
107 current river location (Rossetti *et al*., 2005; Wesselingh & Salo, 2006).
108 The existence of environmentally induced effects on population differentiation
109 (several examples are reviewed in Coyne & Orr, 2004) and species distributions (Tuomisto
110 *et al*., 2003), and the often cited possibility of dispersal events across headwater regions or
111 following channel re-orientation or river drought (Haffer, 1974; Noonan & Wray, 2006),
112 should not completely rule out the importance of large Amazonian rivers as putative
113 vicariant barriers. Rather, these events could be potentially traced back by the analysis of
114 patterns that are incongruent with a primarily vicariance-based model represented by large
115 rivers (Cracraft & Prum, 1988).
116 Not many studies evaluating the role of rivers as mechanisms potentially capable of
117 triggering and maintaining biological differentiation were carried out along the Madeira
118 River. The Madeira is the largest southern tributary of the Amazon River, both in drainage
119 area and water discharge, the latter being four times the average volume discharged by the
120 Juruá (Latrubesse, 2003). It is a well known suture zone between many *terra-firme* taxa,

121 limiting major areas of endemism and biogeographic provinces in the Amazonian lowlands
122 (Haffer, 1974; Ron, 2000; Roosmalen *et al*., 2002; Silva *et al*., 2005, Morrone, 2006). Two
123 studies directly addressed population differentiation and phylogeography of *terra-firme*
124 vertebrate taxa along the Madeira River, focusing primarily on birds. Two species of
125 flycatchers, *Hemitriccus zosterops* and *Hemitriccus minor* (family Tyranidae), present
126 pronounced genetic differentiation between riverbanks, despite no corresponding variation
127 in vocal or morphological traits (Cohn-Haft, 2000). A recent study addressing the
128 phylogeography of three phylogenetically distinct species of birds, *Glyphorynchus*
129 *spirurus* (family Furnariidae), *Willisornis poecilinotus* (family Thamnophilidae) and
130 *Schiffornis turdina* (family Tityridae), revealed high levels of genetic (mtDNA)
131 differentiation between populations occupying opposite riverbanks (Fernandes *et al.*, in
132 press). In the three species analyzed, populations grouped according to riverbank constitute
133 reciprocally monophyletic sister lineages. More than one group of genetically distinct
134 populations of *Glyphorynchus spirurus* occur along the right bank, and population
135 structuring was attributed to direct geographic isolation effects caused by two large
136 tributaries that cross the Madeira-Tapajós interfluve (the Aripuanã and Ji-Paraná rivers),
137 which are recognized as important dispersal boundaries to primates (Roosmalen *et al*.,
138 2002).
139 Such coincidences between distribution limits of vertebrate species and an
140 increasing body of evidence supporting genetic population structuring on opposite sides of
141 the Madeira River and some of its tributaries suggest the Madeira River basin is an
142 interesting system for the investigation of geographically-influenced evolutionary
143 mechanisms. In this context, anuran populations inhabiting non-flooded forests along the
144 Madeira riverbanks have been largely overlooked. Works counting on intensive sampling

145 throughout the entire river course, aiming at a more precise evaluation of river effects on
146 phenotypic or genetic differentiation, are inexistent.
147 *Allobates femoralis* is probably the anuran taxon that received most attention
148 considering population differentiation studies in the Madeira River basin. A detailed study
149 on population variation in advertisement calls and morphometric traits along the upper
150 course of the river revealed pronounced quantitative differences between populations
151 inhabiting opposite riverbanks (Simões *et al*., 2008). The same study reported the existence
152 of a distinct morphotype distributed exclusively on the upper left bank of the river, reaching
153 a contact zone with a more widespread form of *Allobates femoralis* at the locality of
154 Cachoeira do Jirau, coinciding with the limits between two geomorphological units with
155 particular origins and topography (DNPM, 1978a; Souza Filho *et al*., 1999). The systematic
156 relationships between this morphotype and other populations of *Allobates femoralis*
157 occurring on the upper Madeira River were recently addressed, and the group received
158 species status based on mtDNA molecular phylogeny, overall acoustic and morphological
159 differentiation, and its maintenance along the contact zone at Cachoeira do Jirau (Simões *et*
160 *al*., 2010). The distribution of this new species, restricted to the left bank, was interpreted as
161 additional evidence to the role of the Madeira River as an effective barrier to the dispersal
162 of individuals. Stronger phylogeographic inferences were hindered by the lack of sampling
163 sites downstream of Porto Velho, along the middle and lower stretches of the river.
164 In this study, we evaluate the influence of the Madeira River on the differentiation
165 of genetic, acoustic, and morphological traits between *Allobates femoralis* populations in
166 terra-firme forests of opposite riverbanks along its entire course, while testing for the null
167 hypothesis that these characters are correlated with geographic distance between samples. If
168 the Madeira River has effectively prevented the dispersal of individuals between riverbanks

since its entrenchment, we expect populations inhabiting opposite riverbanks to be reciprocally monophyletic in a mtDNA molecular phylogeny. Additionally, we expect populations inhabiting the same riverbank to be more similar to each other in terms of genetic and phenotypic traits, when compared to populations inhabiting opposite riverbanks. When considering a neutrally evolving mitochondrial gene, we expect to observe a linear increase of the genetic distance between populations on immediately opposite riverbanks towards the mouth of the river.

METHODS

Study area

The study area consists of *terra-firme* forests along both sides of the Madeira River, from the locality of Mutum-Paraná, in the upper Madeira River, to localities near the river's mouth (Fig. 1). Along the upper segment of the study area (from Mutum-Paraná to the city of Porto Velho), the river is channeled through a continuous plateau formed predominantly by Pleistocene sediments, which is replaced at some locations by exposed fragments of a highly eroded plateau associated with Pre-Cambrian sediments and by outcrops of the granitic cratonic basement (DNPM, 1978a; Souza Filho *et al.*, 1999; Bettencourt *et al.*, 2010). Along this segment, the river has an average width of 1 km, running through a system of successive rapids. Only narrow areas of the margins are seasonally flooded and colonized by open pioneer vegetation in months of low water levels (DNPM 1978a).

Approximately 40 km downstream of Porto Velho, plateaus are replaced by the Amazon Plain, characterized by recent alluvial sediments deposited on a sedimentary basin

dating from Pliocene to Pleistocene (DNPM, 1978a,b). The Madeira River runs through the Amazon Plain along its middle and lower course, which are characterized by strong sedimentary dynamics allowing for the formation of marginal lakes, channels, and islands, and by wide floodplains covered by *várzea* (seasonally flooded) forests on riverbanks. At some sites, the plateaus (which remain continuous along the Madeira-Tapajós and Madeira-Purus interfluves) extend from the most central areas of the interfluves, reaching the riverbanks (DNPM, 1978b). Much of the area adjacent to the river's mouth was remodeled by the activation of normal and strike-slip tectonic faults of recent geological ages (Quaternary) that induced channel orientation of the Madeira and Amazon Rivers, as evidenced by palaeochannels and meander relicts (Costa *et al*., 2001). These events originated sedimentary compartments on each riverbank that are distinct from those observed along the middle course of the Madeira River (Costa *et al*., 2001; Rossetti *et al*., 2005).

Sampling design

In order to evaluate patterns of genetic and phenotypic differentiation along the Madeira River, we reanalyzed acoustic and morphometric data from samples obtained in eight localities along the upper course of the river (localities 1–8 - Table 1, Fig. 1), presented in a previous study (Simões *et al.*, 2008), and included genetic data obtained from the same individuals. We sampled two extra sites on opposite banks along the transition between the upper and middle courses of the river (localities 9 and 10), and two localities on its middle course (localities 11 and 12). Five localities were sampled on the river's lower course, from its confluence with the Aripuanã River to the river's mouth

(localities 13–17). The lack of a paired sampling site on the left bank opposite to locality 13 (Novo Aripuanã) was due to the existence of massive extensions of flooded forests on that riverbank, which prevent access to *terra-firme* forests within the central region of the interfluve. We conducted acoustic searches in two *terra-firme* forest sites immediately opposite to localities 14 and 15 (coordinates 4.3317° S / 59.6736° W, and 3.5424° S / 59.7789° W, respectively), but no *A. femoralis* populations were found after two days of searches in each locality, despite the presence of other dendrobatid species typical of *terra-firme* environments (*e.g. Ameerega trivittata, Allobates caeruleodactylus, Allobates nidicola*).

Data collection

Field excursions for data collection were carried out in several opportunities from November 2004 to February 2009, always during the rainy season (November–March). *Allobates femoralis* is usually found in habitats of primary forest, although tolerant to forested environments subject to some degree of disturbance (*e.g.* selective logging and forest fragmentation - Caldwell & Araújo, 2005; Tocher *et al.*, 2001). The species is vocally active during the day and can be easily detected in these habitats by their advertisement calls. We conducted acoustic searches in each sampling locality and wherever a population was found, we tried to record and capture as many individuals as possible, to a maximum of 16.

Recordings were done using whether a Sony WM-D6C (2004, Sony Corr., Japan) or a Marantz PMD660 (2005, D&M Professional, U.S.A.) recorder, and Senheiser directional microphones (2006, Sennheiser Electronic Corporation, U.S.A.) positioned about 1 m from

241 focal male. At least three calling bouts (a series of >10 calls) were registered for each
242 individual. Air temperature at the time of recording was registered with a digital
243 thermometer. We manually captured all recorded individuals, which were later anesthetized
244 in a solution of lidocaine, fixed in 10% formalin, tagged, and preserved in 70 percent
245 ethanol solution. Muscle samples from each individual were extracted and preserved in 95-
246 98 percent ethanol before fixation procedures. Additional tissue samples were obtained
247 from females collected opportunistically during field work. In one locality (Manaquiri,
248 Table 1) we did not find males in calling activity, but seven individuals were sampled for
249 genetic analyses. Voucher specimens were deposited in the herpetology section of the
250 zoological collection of Instituto Nacional de Pesquisas da Amazônia (INPA-H), in
251 Manaus, Brazil. Tissue samples were housed at Coleção de Tecidos de Genética Animal at
252 Universidade Federal do Amazonas (CTGA – ICB/UFAM), Manaus, Brazil.
253
254 *Acoustic data collection*
255
256 Advertisement call recordings were analyzed in Raven 1.2 software (Charif *et al.*,
257 2004). From each individual recorded, we chose the calling bout with less background
258 noise and selected three calls for detailed analyses. We discarded warm-up calls at the
259 beginning of each calling bout, as well as the last two calls of each bout, to avoid sampling
260 frequency and timing variations originating from vocal interactions with neighbors, or due
261 to fatigue (Gerhardt & Huber, 2002). We conducted spectral analyses on the set of selected
262 calls following a fast Furier transform with frequency resolution of 82 Hz and 2048 points.
263 Temporal variables (durations of calls, notes, and silent intervals) were measured directly
264 from sample oscillograms. A total 24 acoustic variables (nine temporal, 15 spectral – Table

6) were obtained from each call sample, and their final values for each individual represent the arithmetic mean value between the three call samples. We did not include in the analyses variables relating to calling bouts (call/note emission rates, duration of calling bouts, and silent intervals between calling bouts) because they are normally influenced by behavioral responses to the presence of observers. Mean values and standard deviations of call traits in each locality are available in Table S1 (Supplementary Material).

Morphometric data collection

Voucher specimens were measured under a dissecting microscope using a digital caliper (precision 0.01 mm) and graduated ocular lens (precision 0.10 mm). We recorded 19 direct external morphometric measures of head, body and limbs (Table 7). All measurements were done on the left side of the body. Snout-to-vent length (SVL) was measured separately to the nearest 0.01mm, and used as a covariate in analyses of phenotypic differentiation, as described below. Arithmetic mean values and standard deviations of morphometric traits in each sampling locality are provided in Table S2.

Molecular data acquisition

Total genomic DNA was extracted from preserved muscle tissues using a cetyl trimethyl ammonium bromide (CTAB) protocol, modified from Doyle & Doyle (1987). We used primers 16Sar e 16Sbr (Palumbi, 1996) and Cytb AF.f and Cytb AF.r (this study - 5' GACACCTCAATAGCYTTCTC 3' and 5' CGAAATGTTAGGSTRCGTTGAT 3', respectively) to amplify a 507 bp fragment of the 16S rRNA and a 610 bp fragment of the

289 citocrome *b* mitochondrial genes. These fragments correspond to positions 3972 to 4503,
290 and 16497 to 17106 of the complete mitochondrial genome of *Xenopus leavis* (Roe *et al*.,
291 1985), respectively. The mitochondrial 16S rRNA gene has been regarded as one of the
292 best markers for the study of systematic relationships among anurans because priming sites
293 are largely conserved (Vences *et al*., 2005; Fouquet *et al*., 2007). Small fragments of the
294 cytocrome *b* gene have been successfully used in previous phylogenetic works addressing
295 the systematic relationships in the Dendrobatoidea superfamily (Grant *et al*., 2006), and in
296 case-studies that dealt specifically with the evolution and biogeography of *Allobates*
297 *femoralis* (Lougheed *et al*., 1999; Amézquita *et al*., 2009).
298 DNA amplification via polymerase chain reaction (PCR) used mixes with a final
299 volume of 16 µL, containing 6.7 µL ddH$_2$O, 2.0 µL of 25mM MgCl$_2$, 1.5 µL of 10 mM
300 dNTPs (2.5mM each dNTP), 1.5 µL of 10X amplification buffer (75mM Tris HCl, 50 mM
301 KCl, 20 mM (NH4)2SO4), 1.5 µL of a 2 µM solution of each primer, 0.3 µL of Taq DNA
302 polimerase 5 U/µL (Biotools, Spain) and 1 µL of DNA (about 30 ng/ µL). Reaction
303 conditions had a pre-heating step at 92°C for 60 s, 35 cycles of denaturation at 92°C for 60
304 s, primer annealing at 50°C for 50 s, and primer extension at 72°C for 90 s, followed by
305 final extension step of five minutes at 72°C. Sequencing reactions were carried out after
306 PCR product purification with exonuclease and alkaline phosfatase (Fermentas Life
307 Sciences, Canada) and followed ABI BigDye Terminator Cycle Sequencing Kit protocols,
308 as indicated by the manufacturer. Forward primers were used in the sequencing reactions
309 and an annealing temperature of 50°C was applied. The resulting single-stranded products
310 were resolved in an ABI 3130xl automatic sequencer.
311 Sequence alignment was carried out separately for each locus in Bioedit (Hall,
312 1999). We used ClustalW algorithm (Thompson *et al.*, 1994) to generate preliminary

alignments, which were subsequently checked by eye and corrected by comparison with the original chromatographs. Amino acid translations were checked in MEGA 4.1 (Tamura *et al*., 2007) for the existence of premature stop codons for the cytochrome *b* segment.

Analysis

Phylogenetic analyses

Phylogenetic analyses used a concatenated data set of individuals sequenced for both 16S rRNA and cytocrome *b* genes. The incongruence length difference test (ILD test – Farris *et al*., 1995) implemented in *PAUP** 4.0b10 (Swofford, 1998) was used to verify the existence of heterogeneity in the phylogenetic signal between partitions represented by each locus. Phylogenetic reconstructions were carried out under maximum likelihood (ML) criterion in TREEFINDER (Jobb, 2008), and via Bayesian inference (BI) as implemented in MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). *Allobates hodli* was used as outgroup in tree reconstructions by both methods.

Selection of the best model of sequence evolution for the concatenated data set was done via Akaike information criterion using jModeltest (Posada, 2008). The selected model (transversional model with a gamma distribution, TVM+G) was applied to ML phylogenetic reconstruction. Branch support of the resulting ML tree topology was computed by bootstrap analysis with 5.000 replicates. Bayesian inference analysis was run for two million generations, with sampling frequency of chains set to every 100th generation. We applied four simultaneous independent runs, and Metropolis coupling with four heated chains was used to improve distribution sampling. We used TRACER v.1.4

(Rambaut & Drummond, 2007) to generate plots of the log-likelihood scores, tree lengths and values of model parameters against generation numbers to evaluate at which step chains became stationary, and to decide on the burn-in. The first 20.000 trees were consequently discarded as burn-in steps. Convergence was later confirmed by evaluation of the Potential Scale Reduction Factor (PSRF) after summarizing sampled model and tree parameter values in MRBAYES 3.1.2 (Ronquist *et al*., 2009).

The best tree topology obtained in the ML analysis was compared to an alternative tree, constraining clades originating from the same riverbank as reciprocally monophyletic. We used the SH test of tree topology (Shimodaira & Hasegawa, 1999) to test whether the likelihood of the constrained tree topology was significantly different from that of the best ML tree. The SH test is considered to be conservative and not prone to Type 1 errors or misleading results (Buckley, 2002). The SH test was performed in TREEFINDER (Jobb, 2008), applying the TVM+G model of evolution and 100.000 replicates.

Population analyses

As a larger number of successful amplifications were available for the 16S rDNA gene in comparison to cytocrome *b* (153 sequences, against 227 for 16S rDNA), we restricted our genetic population analysis to the 16S rDNA dataset. We used the complete set of 16S rDNA sequences to construct an haplotype network using statistical parsimony (Templeton *et al*., 1992), as implemented in TCS 1.21 (Clement *et al*., 2000), applying a 95% connection limit and considering gaps as a 5^{th} character state, to graphically assess the genealogical relationships among samples, as well as the distribution of haplotypes between riverbanks. As evidences of river effects on genetic structuring of populations, we tested

361 whether greater genetic diversity is observed between than within riverbanks by means of a
362 molecular analysis of variance (AMOVA – Excoffier *et al*., 1992), as implemented in
363 Arlequin (Excoffier *et al*., 2005). Additionally, fixation indexes (*Fst* - Wright, 1951) and
364 average genetic distances (Kimura 2-parameters – Kimura, 1980) were measured between
365 sampling localities as rough estimates of relative genetic structuring that could be
366 confronted with further expectations under the river-barrier hypothesis. Measures were
367 obtained in DnaSP v.5.10 (Librado & Rozas, 2009) and MEGA 4.1, respectively.
368 We estimated the most probable number of genetic clusters formed by the sampled
369 16S rDNA sequences by applying a Bayesian analysis of population structure on nucleotide
370 frequencies, as implemented in BAPS 5 (Corander & Tang, 2007; Corander *et al*., 2008).
371 The number of genetic clusters was treated as a random parameter (option "fixed-*K*" was
372 disabled), and the upper bound to the number of clusters was set from one to 17, the latter
373 value corresponding to the number of sampling localities. Three independent runs were
374 performed for each upper bound value, and selection of the most probable cluster
375 configuration was made by comparing the log-likelihood values of the best models.
376 The same 16S rDNA data set was used to compute haplotype and nucleotide
377 diversity in each of the genetic clusters indicated by BAPS. We used Tajima's D (Tajima,
378 1989), Fu's FS (Fu, 1997), and Ramos-Onsins & Rozas's R_2 (Ramos-Onsins & Rozas,
379 2002) tests to evaluate whether mutations in our data set are selectively neutral, checking
380 for the possibility of past population expansion events or selective sweeps. Statistical
381 significance for these tests was estimated via coalescent simulations (Hudson, 1990) with
382 10.000 replicates in DnaSP v.5.10. Additional evidence for population expansion were
383 inferred from mismatch distributions in each cluster, and tested by a similar coalescent
384 simulation procedure using the sum of square deviations (SSD - Schneider & Excoffier,

1999) between observed and expected mismatch values, as well as the Harpending's raggedness index (Hri – Harpending, 1994) , as implemented in Arlequin (Excoffier *et al*., 2005), using 5.000 parametric bootstrap replicates.

Divergence time estimation

A reduced 16S rDNA alignment consisting of unique haplotypes recovered from population analyses described above was used for estimation of divergence times. As a calibration point we used the average time of divergence between clades that include *Allobates femoralis* and *Allobates hodli*, estimated as approximately 4.5 mya (Santos *et al*., 2009). As the time of divergence estimates present a large variation under the 95% confidence interval, we repeated the analysis described below applying approximate minimum and maximum ages (2.5 and 7.0 mya, respectively), as estimated by Santos *et al.* (2009) for the same clade. We used available 16S rDNA sequences of *Anomaloglossus stepheni* and *Allobates zaparo* (Genbank accession numbers DQ502108 and AY364578, respectively) as outgroups. New model parameters were estimated for the reduced 16S rDNA dataset in jModeltest. Maximum likelihood analysis was used to reconstruct a new tree in PAUP*, and to obtain the likelihood of the same tree with a molecular clock model enforced. A likelihood ratio test (Huelsenbeck & Crandall, 1997) rejected the hypothesis of homogenous rate of evolution among branches, suggesting the molecular clock model as inappropriate ($LR=88.76$, $P<0.001$, $df=44$). Thus, we adopted a Local Rate Minimum Deformation (LRMD) model as the calibration method and applied it to Jukes-Cantor distance tree generated from the same alignment, as implemented in TREEFINDER. The LRMD method assumes rates along branches to be more similar to those of neighboring

409 edges, and as similar as possible to estimated local rates. Detailed information on the
410 method can be found in TREEFINDER's manual (Jobb, 2008).
411
412 *Phenotypic differentiation*
413
414 To test whether the phenotypic differentiation of *Allobates femoralis* was greater
415 between than within riverbanks, we applied multivariate analysis of variance (MANOVA)
416 statistics. First, we calculated the arithmetic means of the acoustic and morphometric
417 variables among all sampled individuals for each of the 16 sampling localities. Using the
418 mean values for each locality reduces the power of subsequent tests, but avoids the pseudo-
419 replication caused by the repetition of the same value for decimal geographic coordinates,
420 which are important covariates used to account for geographic distance between
421 populations. The number of independent acoustic and morphometric variables was reduced
422 by a principal component analysis (PCA). The distribution of localities along the two first
423 components (which accounted for more than 80% of total phenotypic variation – see
424 Results) was evaluated graphically for the detection of clusters. The first and second
425 principal components where subsequently used as dependent variables on a multivariate
426 analysis of variance (MANOVA) model, to test whether variances between populations on
427 opposite riverbanks where significantly larger than those observed between populations
428 inhabiting the same riverbank. Geographic coordinates (in decimal degrees), and mean
429 snout-to-vent length (SVL) of individuals in each sampling locality, were included in the
430 model as covariates to account for effects of body-size and geographic distances on
431 phenotypic variation (Reis *et al*., 1990; Legendre *et al*., 2002). Although air temperature is
432 known to affect call traits (Ryan, 1988; Gerhardt & Huber, 2002), mean values for each

433 locality are potentially not informative because of temperature variation along the day.
434 Thus, air temperature was not used as a covariate in the MANOVA model. All analyses
435 were carried out in SYSTAT v.10 (Wilkinson, 1990).
436
437 *Geographic correlates of phenotypic and genetic variation*
438
439 We evaluated correlations between the geographic distance between sampling sites
440 and genetic and phenotypic distances between their respective *A. femoralis* populations by
441 applying a series of Mantel tests on distance matrices derived from genetic, acoustic and
442 morphological data sets (Mantel, 1967). Partial Mantel tests were applied to check for
443 correlations between genetic/phenotypic distances among populations and their split by the
444 river channel while controlling for effects of geographic distance between sampling sites
445 (Smouse *et al*., 1986; Legendre, 2000). Phenotypic distance matrices between localities
446 were generated by calculating pairwise Euclidean distances between their scores on first
447 and second acoustic and morphometric principal components produced by a PCA analysis
448 as described above. As acoustic and morphometric's first principal components were
449 strongly correlated with body size (linear regression r^2=0.89, $F_{1,14}$=121.2. P<0.001 for
450 morphometric PC1; r^2=0.53, $F_{1,14}$=15.7, P=0.001 for acoustic PC1) , we regressed them
451 against the corresponding mean SVL values for each sampling locality, and used the
452 residuals as new size-independent phenotypic variables, from which we calculated new
453 Euclidean distance matrices. Distance matrices were calculated independently for each
454 phenotypic variable, and for each pair of corresponding variables (acoustic PC1 regression
455 residual + PC2, morphometric PC1 regression residual + PC2). Euclidean distances
456 between mean SVL measures for each sampling locality were calculated, and applied as a

457 body-size distance matrix, which was analyzed separately. Average genetic distances
458 (Kimura 2-parameters) between sampling localities calculated from the 16S rDNA dataset
459 used in the genetic population analysis were used to build the genetic distance matrix.
460 To test for river influences on the differentiation of phenotypic traits, we applied a
461 binary correspondence matrix designating a value "0" for localities within the same
462 riverbank, and value "1" for localities on opposite riverbanks. This binary matrix was then
463 tested for correlations with body size, acoustic, morphometric, and genetic distance
464 matrices. As the effects of historical barriers and isolation by distance on differentiation are
465 often overlapped (Telles & Diniz-Filho, 2005), Mantel tests between binary and phenotypic
466 matrices were performed while controlling for the effect of a third matrix, containing the
467 linear geographic distance between localities, measured in kilometres. Additionally, we
468 tested for the existence of correlations between genetic and phenotypic distances with the
469 geographic distances between sampling localities using simple Mantel tests. All tests were
470 done in ZT (Bonnet & Van de Peer, 2002) using permutation of the residuals of the null
471 models (Anderson & Legendre, 1999), and applying 10.000 randomizations.
472
473
474 **RESULTS**
475
476 **Phylogenetic analysis**
477
478 The concatenated data set (16S rDNA + cytobrome *b*) contained 94 unique
479 haplotypes of *Allobates femoralis* (GenBank accession numbers provided in Table S3). No
480 incompatibility between data matrices constituted by fragments of the two mtDNA loci was

481 detected (ILD test *P* value = 1 - (72/100) = 0.28), hence justifying the concatenation of data
482 sets.

483 Phylogenetic reconstructions using ML and BI rendered best trees with similar
484 topologies (Fig. 2). Both analyses point samples from Democracia (locality 11, Fig. 1) as
485 the sister group to the clade containing samples from Careiro and Manaquiri (localities 16
486 and 17, Fig. 1). These clades constitute a well supported left riverbank basal clade, which is
487 the sister group to a clade containing all samples from the right riverbank and samples from
488 the upper left riverbank. The latter form a clade nested within the right riverbank clade, and
489 includes samples from Humaitá, on the right riverbank (locality 9 - Fig. 1). This clade is the
490 sister-group to a clade formed by samples from the remaining sites along the right bank of
491 the upper Madeira River (localities 1, 4, 6, 8 - Fig. 1). Both upper Madeira clades form the
492 sister clade to samples from localities on the right bank along the river's middle to lower
493 course. These are split in two well supported clades which are their reciprocal sister groups:
494 one formed by samples from Manicoré (locality 12 - Fig. 1), and other formed by samples
495 from Novo Aripuanã, Borba, and Nova Olinda do Norte (localities 13, 14, 15 - Fig. 1).

496 The SH test of tree topology rejected an alternative tree assuming clades on the
497 same riverbank as monophiletic lineages as a better phylogenetic hypothesis than that
498 represented by the best tree obtained by the previous ML analysis (Likelihood Ratio=
499 88.76, df=44, SH's $P<0.001$).

500

501 **Population analyses**

502

503 Population analyses were based exclusively on the 16S rDNA fragment samples,
504 rendering a total 227 sequences that corresponded to 44 unique 16S rDNA haplotypes

505 (Table 2). Overall haplotype distribution between sampling sites indicate haplotype sharing
506 among sites on the right bank of the middle to lower course of the river (except from one
507 individual, all samples from Novo Aripuanã, Borba and Nova Olinda do Norte share the
508 same haplotype), and between sites on the same riverbank along the upper course. A single
509 haplotype (H25 - Table 2, Fig. 3) is shared between populations occurring in opposite
510 riverbanks, on the region comprising the transition between the upper and middle course of
511 the Madeira River, in the localities of Santo Antônio and Humaitá. A greater number of
512 intermediate (not sampled) mutational steps separate haplotypes from the left riverbank on
513 the middle to lower course of the river from the remaining haplotypes that constitute the
514 genealogy. Haplotypes found on the left bank of the upper course of the river are
515 comparatively separated by fewer mutational steps from haplotypes occurring on the right
516 bank (Fig. 3).
517 The AMOVA indicated that 20.28 percent of the overall genetic variation is
518 explained by the division of samples in groups according to riverbank (F_{CT} = 20.27,
519 P=0.005), while 66.66 percent of the remaining variation was observed among sampling
520 localities, and 13.07 percent among samples within sampling sites. Elevated values for *Fst*
521 estimates were generally observed between sampling sites (Table 3), reflecting overall high
522 levels of population structuring, except between localities in the upper Madeira River. A
523 pattern of increasing genetic distances between paired sites on immediate opposite
524 riverbanks towards the river's mouth is evident, but do not follow a linear trend.
525 Comparisons between sites on either the middle (Manicoré and Democracia) or the lower
526 (Nova Olida and Careiro) course of the river rendered greater genetic distances than those
527 observed between sampling sites on the upper course (Table 3, Fig. 4). Genetic distances

dropped to zero between sites on opposite riverbanks in Humaitá and between the tree localities on the right bank of the lower course (Novo Aripuanã, Borba and Nova Olinda).

Bayesian analysis of genetic structure based in 16S rDNA samples supported the existence of seven distinct genetic clusters (log ML value = -1265.8319; probability = 0.99), roughly corresponding to the major well-supported clades recovered by the phylogenetic analyses, and indicating the existence of population structuring within riverbanks. The most probable configuration of genetic clusters generated by BAPS suggests that genotypes belonging to clusters widespread in the upper right riverbank are also present on the left bank, in Santo Antônio and Humaitá (Fig. 5). Neutrality tests indicated signals of population size changes in clusters constituted by samples of Careiro+Manaquiri, samples from Jaci-Paraná+Morrinho+Santo Antônio on the right bank, and in the cluster constituted by samples from Lower Jirau+Jaci-Paraná+Morrinho+Santo Antônio on the left bank (Table 4). Only the latter cluster received statistical support for signs of past demographic changes considering simulations over the three tests applied, including Ramos-Onsins & Rozas's R_2, which is more appropriate to small sample sizes (Ramos-Onsins & Rozas, 2002). By the analysis of mismatch distributions (Fig. 6) only for the cluster formed by samples from Manicoré+Novo Aripuanã+Borba+ Nova Olinda the null hypothesis of population expansion was rejected by tests of both parameters (SSD and Hri), indicating stable population size over time (Table 5). Analysis based on SSD rejected the hypothesis of population expansion for the clade formed by samples from Jaci-Paraná+Morrinho+Santo Antônio on the right bank. However, the same clade presented signs of population growth according to Fu's *F*s, which is considered as having superior statistical power (Ramos-Onsins & Rozas, 2002).

552 **Estimated divergence times**

553

554 Divergence times as estimated by the LRMD method on a reduced 16SrDNA
555 dataset indicate the time of first divergence between left and right riverbank clades to be
556 Late Pliocene (around 2.8 mya). Applying the same method using the mean minimum and
557 maximum ages estimated by Santos *et al.* (2009) to calibrate the same tree, divergence time
558 is supposed to have happened between 4.3 and 1.4 mya. All subsequent cladogenetic events
559 are indicated as no older than Early Pleistocene. Time for the most recent common ancestor
560 between populations on the left and right bank of the upper Madeira River as inferred from
561 the mean calibration age was estimated as younger than 1 mya. (Fig. 7), probably having
562 occurred some time between 1.5 and 0.6 mya.

563

564 **Acoustic and morphological differentiation**

565

566 Along the study area, all sampled populations of *Allobates femoralis* presented a
567 uniform pattern of advertisement calls, constituted by four notes with ascending frequency
568 modulation (example sonograms are shown in Fig. 8). Thus, attribution of homology
569 between variables measured from calls recorded in different sampling sites was
570 straightforward.

571 Principal component analysis on mean values of 24 acoustic variables of *Allobates*
572 *femoralis* recorded in 16 localities along the Madeira River recovered two first principal
573 components accounting for approximately 85 percent of the total variation in call traits.
574 Spectral variables (relating to call and note frequencies) had high loadings on the first
575 component (PC 1), while the second component (PC 2) accounted for most of the variation

relative to durations of notes and call, and silent intervals (Table 6). Populations inhabiting the right riverbank presented higher levels of acoustic variation in relation to populations of the left riverbank. The latter presented a more aggregated distribution along both principal components, although no clear clustering of populations belonging to the same riverbank could be observed in this analysis (Fig. 9a).

The lack of clearly delimited acoustic groups defined by principal component analysis was supported by the multivariate analysis of variance, which suggested that populations inhabiting opposite riverbanks are not distinct, in average, in relation to characterisitcs of their advertisement calls (*Pillai trace*=0.176, *P*=0.345, df =11), despite effects of body size (*Pillai trace*=0.425, *P*= 0.048, df =11), and geographic distance between sampling localities (*Pillai trace*=0.500, *P*< 0.022, df =11) on call traits.

Principal component analysis on mean values of 19 morphometric variables generated two first principal components accounting for over 86 percent of the total variation in external continuous morphological traits. As it is typical of PCA on morphometric traits, all variables had heavy and positive loadings on the first component (PC 1), which accounts for most of the size-dependent variation on morphological characters (Green, 2001). The second principal component (PC 2) accounted mostly for head and limb measurements (Table 7). Graphical analyses of the distribution of sample means along these principal components (Fig. 9b) suggest that populations on the left bank present greater variability on size-dependent characters in relation to populations inhabiting the right bank.

No evident clustering relating to riverbanks was observed along principal components, and differentiation between populations on opposite riverbanks only approached significance (*Pillai trace*=0.383, *P*=0.070, df =11), when accounting for effects

of the covariates body-size (*Pillai trace*=0.895, *P*<0.001, df =11) and geographic distance between localities (*Pillai trace*=0.579, *P*<0.009, df =11).

Geographic correlates of acoustic, morphological and genetic differentiation

Partial Mantel tests indicate significant correlation between genetic, body-size, and morhometric distances (as represented by residual variation of morphometric PC1) and the origin of samples according to riverbank, despite correlations between geographic distance between sampling sites and genetic and body-size differences (Table 8). Acoustic distances (as represented by residual variation of acoustic PC1), as well as morphometric distances measured from combined first and second PCs, were correlated to geographic distance between sampling sites, but not to riverbanks. Variation on the remaining phenotypic traits or combinations of traits was not correlated to linear geographic distance or to sampling site distribution on different riverbanks, suggesting that other evolutionary mechanisms might be involved.

DISCUSSION

Although scientists have long recognized the large Amazonian rivers as the most evident boundaries between species belonging to various groups of vertebrates (Wallace, 1952; Haffer, 1974; Roosmalen *et al*., 2002; Hayes & Sewlal, 2004), several attempts have failed to prove their role as effective vicariant barriers, mostly based on resulting patterns that conflicted with rigorous *a priori* expectations about reciprocal monophyly between

625 riverbanks, and about the extent of genetic and phenotypic differentiation observed between
626 samples collected on opposite riverbanks, in comparison with samples originating from the
627 same riverbank. As exemplified by studies focusing on anuran populations, the river-barrier
628 hypothesis was largely discarded with basis on results obtained along the Juruá River,
629 which did not evidence clear differences in species composition between riverbanks, and
630 uncovered instances of more pronounced genetic divergence between samples collected in
631 the headwaters when compared to samples collected downstream (Gascon *et al*., 1998,
632 2000; Lougheed *et al*., 1999). These led to increasing support to the alternative hypothesis
633 that tectonic ridges were the most important historical factor to have influenced current
634 differentiation patterns among focal species. This model was readily adopted, for example,
635 in studies addressing the phylogenetic relationships among dendrobatid frogs (Symula *et*
636 *al*., 2003), although actual position of arches were, at best, imprecise (Wesselingh & Salo,
637 2006). Later, at least one study carried out in the basins of some of the largest Amazonas
638 western tributaries (Marañon and Napo) reported that individuals belonging to the
639 *Ranitomeya* (*Dendrobates*) *ventrimaculata* complex sampled in nearby locations on
640 opposite riverbanks are generally more distantly related, in comparison to individuals
641 collected at greater geographic distances in the same interfluves (Noonan & Wray, 2006),
642 stimulating further research on the influence of rivers as differentiation mechanisms.
643 Despite early reports on the coincidence between population differentiation of
644 *Allobates femoralis* and riverbed position on the upper Madeira River (Simões *et al*., 2008),
645 increased sampling along a much larger scale led to results that are discordant with the
646 hypothesis that the river channel has permanently prevented dispersal of *Allobates*
647 *femoralis* individuals across riverbanks. Considering the large number of characters, and
648 analytical methods employed, only a few revealed patterns in agreement with predictions of

649 the river-barrier hypothesis in its more restrictive definition. For instance, samples in
650 opposite riverbanks do not constitute reciprocally monophyletic groups (and this hypothesis
651 does not receive a better support in likelihood based comparisons), genetic differentiation
652 does not increase linearly towards the river's mouth when samples from immediately
653 opposite sites are compared, although the highest values of genetic distances are observed
654 between populations on opposite sides of the middle and lower courses of the river. Most of
655 the population genetic and phenotypic variation occurs along the riverbanks, not across
656 them. However, we argue that deviations from the pattern expected by the river-barrier
657 hypothesis likely resulted from point dispersal events following preceding cladogenetic
658 events which are highly coincident with the river's origin and dynamics.
659
660 **Phylogenetic patterns and time of divergence**
661
662 Controversy exists in relation to the time of establishment of the Amazon fluvial
663 system as a prevailing eastward drainage connecting rivers originating on the slopes of the
664 Andes chain to the Atlantic. Recent estimates based on sedimentological data from the
665 Amazon fan suggest that from 6.8 mya the Amazon was a large, entrenched river, carrying
666 large quantities of sediments with Andean and cratonic origins, although the
667 transcontinental river system might be as old as 11.8 M.a. (Figueiredo *et al*., 2009). The
668 adoption of this rather early time for the river system onset contributed, for example, to the
669 ruling out of rivers as important mechanisms of genetic differentiation in leaf-cutter ants
670 (*Atta* spp.) in favor of isolation in Pleistocene refugia (Solomon et al., 2008). An alternative
671 interpretation based on palinological and sedimentological data along the Solimões and
672 Amazon Rivers, as well as zircon dating of sediments along these transects, suggests a

673 more complex scenario, with sub-basins limited by structural arches (such as the Purus
674 Arch) that prevailed until post Late Miocene times (Nogueira, 2008). Our results support a
675 first split between basal *Allobates femoralis* clades on opposite riverbanks dating at least
676 from Early Pliocene, and most probably during Late Pliocene, thus in concordance with the
677 establishment of the Madeira River as a event subsequent to the onset of the Amazon
678 within this time spam (Roddaz *et al*., 2010).

679 Divergence events within subclades of the Madeira River *A. femoralis* phylogeny
680 also track back the Pliocene–Pleistocene dynamics of large tributaries on the right bank of
681 the Madeira River. Variation in water discharge and repositioning of main channels of the
682 Aripuanã and Ji-Paraná rivers are fairly well documented and both sub-basins seem to
683 constitute extensive megafan regions with headwaters fixed on the cratonic basement and
684 highly variable main channel orientation (Latrubesse, 2003; Wilkinson *et al*., 2010). Such
685 dynamic history of river courses could be partially related to genetic divergence between
686 the clade containing samples from Nova Olinda do Norte+Borba+Novo Aripunã (which are
687 located outside the megafan influenced environment and harbour very similar genetic
688 populations) and the clade constituted by Manicoré samples. The same rationale is
689 applicable to explain the divergence between those clades and the clade formed by samples
690 from the upper Madeira River.

691 Recent interpretations of the river-barrier hypothesis propose that river width is the
692 main factor determining the strength of the channel as a vicariant barrier. In fact, earlier
693 studies point that the lower courses of large Amazonian rivers restrict more avian and
694 primate taxa than their upper courses (Roosmallen, 2002; Hayes & Sewlal, 2004; Borges,
695 2007), and gene flow between populations of the saddle-back tamarin (*Saguinus fuscicollis*)
696 is restricted to the headwater region of the Juruá River (Peres *et al*., 1994). Our results

697 support the inexistence of recent gene flow between populations of *Allobates femoralis*
698 across riverbanks on the middle to lower to course of the Madeira River. These might have
699 happened in recent geological times along restricted sites along the upper to middle course,
700 as described below.
701
702 **Tracking dispersal events**
703
704 A possible case of dispersal of *A. femoralis* from the right to the left bank in the area
705 of the Santo Antônio rapids was raised in a previous study, based in increased
706 morphological and acoustic similarity between the population of the left bank at Santo
707 Antônio and the remaining populations inhabiting the right bank of the upper Madeira
708 River (Simões *et al*., 2008). Our results revealed that not only individuals on both margins
709 share mtDNA haplotypes along this area, but that most individuals sampled approximately
710 200 km downstream, in the vicinities of Humaitá, carry the same haplotype (H25 - Table 2,
711 Fig. 3). Haplotype sharing along a restricted section of the riverbanks (H25 is not observed
712 in any other locality sampled) supports the hypothesis of recent gene flow mediated by
713 dispersal across the river channel better than an alternative scenario of incomplete lineage
714 sorting following splitting of populations by the river or any other vicariant barrier
715 (McGuire *et al*., 2007). Neutrality tests also reject the hypothesis of constant population
716 size for populations inhabiting the upper left riverbank from Lower Jirau to Santo Antônio,
717 hinting at rapid population expansion following dispersal to this area. Increasing genetic
718 distances from Humaitá-Santo Antônio towards localities upstream (Morrinho, Jaci-Paraná
719 and Lower Jirau) suggest that more than a single dispersal event from the right bank might
720 have occurred.

721 Curiously, haplotype sharing or genetic signs of dispersal across riverbanks were
722 not observed among the three avian taxa studied by Fernandes *et al*. (in press) along any
723 segment of the Madeira River, despite the apparent absence of acoustic and morphological
724 variation between their populations. Sister clades on opposite riverbanks are discussed as
725 deeply divergent lineages, probably isolated for more than 2.0 million years. These results
726 are intriguing when considering the potential dispersion capacity of birds when compared
727 to that of a territorial dendrobatid frog, highly intolerant to open or seasonally flooded
728 areas. How exactly *A. femoralis* was able to disperse across riverbanks despite large
729 physical and physiological limitations can only be answered when a more detailed climatic
730 and geological history of the Madeira River basin is available. Considering the current
731 knowledge about the evolution of the Amazon basin and the biology of *A. femoralis*, we
732 suggest dispersion events might have been mediated by climatic oscillations and
733 subsequent river channel re-orientation.
734 Sediment analyses west of the Amazon fan detected considerable fluctuations in
735 water discharge in recent geological times (< 14.000 years ago), and pointed periods of
736 extreme drought in the Amazon Basin during the Late Pleistocene (Maslin & Burns, 2000).
737 Reduced water discharge was probably caused by effects of global lower temperatures on
738 reducing regional rainfall and on decreasing rates of melting of the Andean ice caps. As a
739 tropical river of primarily Andean origin, the Madeira water level is extremely dependent
740 on Andean meltwater discharge and precipitation seasonality. The analysis of sedimentary
741 deposits on its extreme upper course reveal drastic changes in coarseness of material
742 carried by the river from Pliocene to current time, highly supporting variation in water
743 discharge regimes (Westaway, 2006). Although the upper course of the Madeira River has
744 been strongly entrenched and stable for a long period of time (spamming hundreds of

745 thousands to millions of years - Westaway, 2006), it reaches the Amazon Plains
746 downstream of Santo Antônio, where strong discharge variation and sedimentary dynamics
747 could generate frequent shifts on the orientation of the main channel, and the migration of
748 sedimentary islands from one margin to another. If these large blocks of terrain contained
749 expressive areas of not seasonally flooded forest, individuals of *A. femoralis* could have
750 been passively transported between riverbanks.

751 At least two other dendrobatid species distributed throughout the right bank of the
752 upper Madeira River, and across the State of Rondônia present restricted distributions on
753 the upper left bank: *Adelphobates* (*Dendrobates*) *quinquevittatus* and *Ameerega picta*
754 (Caldwell & Myers, 1990; personal observations by the authors). Their distribution on that
755 riverbank is probably interrupted downstream of the Santo Antônio rapids, as these two
756 species are not known to occur in forests close to Humaitá. Both taxa represent potential
757 candidates for comparative studies involving dispersal-vicariance analysis (*e.g.* Zink *et al*.,
758 2000) or other statistical phylogeographic approaches (*e.g.* Carnaval *et al*., 2009), in order
759 to corroborate the existence of dispersion events across margins, as well a clearer estimate
760 of their ages.

761 The hypothesis of channel shifts might also apply to the lower course of the river,
762 where a great fraction of the plains covering the right riverbank was exposed during the
763 Quaternary, following the migration of the Amazon River channel to the north, as it
764 occupied its current location (Costa *et al*., 2001). Signs of population expansion of *A.*
765 *femoralis* clade formed by samples from Careiro and Manaquiri possibly reflect
766 colonization following the establishment of not seasonally flooded environments across this
767 area.

768

Comparisons with patterns described from the Juruá River

Allobates femoralis figured as an important model species in the phylogeographic assessments along the Juruá River (Gascon *et al*., 1998; Lougheed *et al*., 1999). Lougheed *et al*. (1999) discussed the tectonic ridge hypothesis on the basis of the existence of a highly divergent headwater clade, but did not discard the possibility of that clade constituting an independent evolutionary lineage whose divergence from downstream *A. femoralis* populations predated the establishment of the Juruá River. In that case, authors suggested that samples placed in major clades within the phylogeny obtained in their study would be more fitted as subjects for the evaluation of a river-barrier effect.

The possibility that *A. femoralis* constitute a species complex was confirmed by systematic studies (Grant *et al*., 2006; Santos *et al*., 2009), and detailed systematic evaluation of *A. femoralis* was made recently available for the upper Madeira River system and other localities in the Amazon (Simões *et al*., 2010). Some western populations referred to as *A. femoralis* were indicated as deeply divergent evolutionary lineages, immediately receiving species status (*Allobates hodli*) or being regarded as candidate cryptic species (*e.g*. populations inhabiting the Brazilian state of Acre and the Madre de Dios River basin). Although no samples from Porangaba, in Acre (the headwater clade of Lougheed *et al*., 1999), were included in the study by Simões *et al.* (2010), we hypothesize that they do consist of a distinct evolutionary lineage, with recent evolution being independent from eastern (*i.e.* Juruá's middle and lower course) populations.

By observing the phylogenies obtained by Lougheed *et al*. (1999) and those presented herein, a similar pattern emerges when basal western clades (*A. hodli* and the "Porangaba" clade) are treated as outgroups. A more basal split is observed between

populations of opposite riverbanks, followed by subsequent “state reversion” from a clade occupying primarily the right riverbank to a more derived clade with representative individuals occurring on both riverbanks. Despite the highly dynamic course of the Juruá River, with fast changing channel course and movement of sedimentary islands, widespread dispersal events across margins are probably prevented by *Allobates femoralis* habitat requirements, which restrict the species to *terra-firme* forests on more stable plateaus of distinct higher altitude.

Differentiation of phenotypic traits

Phenotypic differentiation between riverbanks was more evident for body-size than for other traits considered. Population variation in individual body-size can be affected by both environmental and demographic processes (Thorpe et al., 2005). As proposed earlier, distinct erosive and depositional regimes occurred along large fractions of the Madeira riverbanks, originating areas with unique edaphic properties (Costa *et al*., 2001; Rossetti *et al*., 2005; Bettencourt *et al*., 2010). Whether local edaphic variation contributes to the establishment of environmental mosaics or clines along *terra-firme* forests close to the Madeira River channel is unknown. However, soil and terrain characteristics are known to deeply affect forest structure (Castilho et al., 2006), and influence the abundance of some terrestrial breeding anuran species in central areas of the Brazilian Amazon (Menin *et al*., 2007). The existence of body-size differentiation across riverbanks, and the existence of greater size-free morphological variation within than between riverbanks supports that selective pressures mediated by environmental variation play a complementary row in overall morphological differentiation in the studied *A. femoralis* populations.

As with the size-free morphological variables, and opposed to the patterns observed in the upper Madeira River (Simões *et al*., 2008), we were unable to detect river effects on call trait differentiation when considering populations along the entire river length. Alternatively, geographic distances between sampling sites seem to correlate, at least partially, with call divergence. This result is in accordance with a previous study supporting that genetic drift (and hence isolation by distance) plays an important role in causing a clinal acoustic divergence pattern in *A. femoralis* populations sampled primarily along the curse of the Amazon River (Amézquita *et al*., 2009). The evolution of advertisement call traits in anurans can be further influenced by many sources of local selective pressures, including sexual selection by females (Boul *et. al*., 2007) and natural selection mediated by predators (Bernal *et al*., 2007) or co-active species competing in acoustic space, although the latter was considered to be unrelated to *A. femoralis* call divergence in an earlier study (Amézquita *et al*., 2006). These mechanisms are generally not independent from one another, and their approximate weights on shaping the current variation pattern in advertisement calls of *A. femoralis* deserve further testing through experimental approaches.

Future research and concerns

Our results highlight the existence of greater population sub-structuring than expected considering the traditional biogeographic delimitation of the Madeira River basin, as comprehending two major areas of endemism (the Madeira-Tapajós interfluve and the Inambari area of endemism, extending from the left bank to the Andean slopes to the west) divided by the river channel. The high levels of genetic differentiation between some of the

841 monophyletic groups found along our study area suggest that at least three major regions
842 harbour distinct evolutionary significant units (ESU's – Moritz, 1994), those corresponding
843 to the upper Madeira River (including both riverbanks from Mutum-Paraná to Humaitá),
844 and both the right and left riverbanks of the middle to lower river course. This can be a key
845 information for the planning of long term conservation strategies, especially if the same
846 distribution pattern is observed among species-level phylogenies of co-occurring anurans.
847 However, our data also show population structuring at a finer scale, suggesting that
848 management procedures targeted on conservation of current genetic diversity within ESU's
849 in the face of contemporary threats should consider these more geographically restricted
850 units and related demographic data into account (Moritz, 1995).
851 A especial case relates to the upper section of the Madeira River channel and
852 adjacent *terra-firme* environments, as they experience drastic environmental changes
853 related to the settlement of a complex of hydroelectric power plants, whose dams will be
854 located immediately downstream of sampling sites at Lower Jirau and Santo Antônio
855 (Laurance *et al*., 2004; Clemons, 2007; Switkes, 2008). Although population level
856 evolutionary patterns are unlikely to be changed due to channel obstruction and damming
857 (because their effect on population isolation and reconnection events are probably
858 innocuous considering evolutionary time), habitat loss following increased colonization and
859 development of human communities along this transect (Perz *et al*., 2008) can eventually
860 produce a profound effect on the observed levels of genetic diversity and traceable
861 evolutionary relationships between the *A. femoralis* populations studied herein. In that
862 sense, our results are largely applicable to a broader conservation biology context, as the
863 patterns described above can be compared to future assessments on *A. femoralis* genetic

diversity, potentially revealing contemporary impacts of human-induced environmental changes on wildlife evolution.

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REFERENCES

Amézquita, A., Hödl, W., Lima, A.P., Castellanos, L., Erdtmann, L. & Araújo, M.C. (2006) Masking interference and the evolution of the acoustic communication system in the Amazonian dendrobatid frog *Allobates femoralis*. *Evolution*, 60(9), 1874–1887.

Amézquita, A., Lima, A.P., Jehle, R., Castellanos, L., Ramos, O., Crawford, A.J., Gasser, H. & Hödl, W. (2009) Calls, colours, shapes, and genes: A multi-trait approach to the study of geographic variation in the Amazonian frog *Allobates femoralis*. *Biological Journal of the Linnean Society*, 98, 826–838.

Anderson, M.J. & Legendre, P. (1999) An empirical comparison of permutation methods

for tests of partial regression coefficients in a linear model. *Journal of Statististical Computation and Simulation*, 62, 271–303.

Antonelli, A., Quijada-Mascareñas, A., Crawford, A.J., Bates, J.M., Velazco, P.M. & Wüster, W. (2010) Molecular studies and phylogeography of Amazonian tetrapods and their relation to geological and climatic models. *Amazonia, landscape and species evolution* (ed. by C. Hoorn, C. and F.P. Wesselingh), pp. 386–403. Blackwell Publishing, Oxford.

Bernal, X., Page, R.A., Rand, A.S., & Ryan, M.J. (2007) Cues for eavesdroppers: Do frog calls indicate prey density and quality? *The American Naturalist*, 169(3), 409–415.

Bettencourt, J.S., Leite Jr., W.B, Ruiz, A.S., Matos, R., Payolla, B.L. & Tosdal, R.M. (2010) The Rondonian-San Ignacio Province in the SW Amazonian Craton: An overview. *Journal of South American Earth Sciences*, 29, 28–46.

Bonnet, E. & Van de Peer, Y. (2002) ZT: a software tool for simple and partial Mantel tests. *Journal of Statistical Software*, 7, 1–12.

Borges, S. (2007) Análise biogeográfica da avifauna da região oeste do baixo Rio Negro, amazônia brasileira. *Revista Brasileira de Zoologia*, 24(4), 919–940.

Boul, K.E., Funk, W.C., Darst, C.R., Cannatella, D.C. & Ryan, M.J. (2007) Sexual selection drives speciation in an Amazonian frog. *Proceedings of the Royal Society B*, 274, 399–406.

Buckley, T.R. (2002) Model misspecification and probabilistic tests of topology: Evidence from empirical data sets. *Systematic Biology*, 51(3), 509–523.

Bush, M. B. (1994) Amazonian speciation: a necessarily complex model. *Journal of Biogeography*, 21, 5–17.

Bush, M.B. & Oliveira, P.E. (2006) The rise and fall of the refugial hypothesis of Amazonian speciation: a paleoecological perpective. *Biota Neotropica*, 6 (1), 1–17.

Caldwell, J.P. & Myers, C.W. (1990) A new poison frog from Amazonian Brazil, with further revision of the *quinquevittatus* group of *Dendrobates*. *American Museum Novitates*, 2988, 1–21.

Caldwell, J.P. & Araújo, M.C. (2005) Amphibian faunas of two eastern Amazonian rainforest sites in Pará, Brazil. *Occasional Papers of the Sam Noble Oklahoma Museum of Natural History*, 16, 1–41.

Capparella, A.P. (1987) *Effects of riverine barriers on genetic differentiation of Amazonian forest undergrowth birds*. PhD Thesis, Luisiana State University, Baton Rouge.

Carnaval, A.C., Hickerson, M.J., Haddad, C.F.B., Rodrigues, M.T. & Moritz, C. (2009) Stability predicts genetic diversity in the Brazilian Atlantic Forest hotspot. *Science*, 323, 785–789.

Castilho, C.V., Magnusson, W.E., Araújo, R.N.O., Luizão, R.C.C., Luizão, F.J., Lima, A.P. & Higuchi, N. (2006) Variation in aboveground tree live biomass in a central Amazonian forest: Effects of soil and topography. *Forest Ecology and Management*, 234, 85–96.

Charif, R.A., Clark C.W. & Fristrup, K.M. (2004) *Raven 1.2 User's Manual*. Cornell Laboratory of Ornithology, Ithaca, NY.

Clement, M., Posada, D. & Crandall, K.A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657–1659.

Clemons, K. S. (2007) Hydroeletric dams: transboundary environmental effects and international law. *Florida State University Law Review*, 36, 487–536.

Cohn-Haft, M. (2000) *A case study in amazonian biogeography: Vocal and DNA sequence variation in* Hemitriccus *flycatchers*. PhD Thesis, Luisiana State University, Baton Rouge.

Colinvaux, P.A., De Oliveira, P.J. & Bush, M.B. (2000) Amazonian and neotropical plant communities on glacial time-scales: The failure of the aridity and refuge hypotheses. *Quaternary Science Reviews*, 19, 141–169.

Colwell, R.K. (2000) A barrier runs through it . . . or maybe just a river. *Proceedings of the National Academy of Science*, 97(25), 13470–13472.

Corander J. & Tang , J. (2007) Bayesian analysis of population structure based on linked molecular information. *Mathematical Biosciences*, 205, 19–31.

Corander, J., Marttinen, P., Sirén J. & Tang, J. (2008) Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics*, 9, 539.

Costa, J.B.S., Bemerguy, R.L., Hasui, Y. & Borges, M.S. (2001) Tectonics and paleogeography along the Amazon River. *Journal of South American Earth Sciences*, 14, 335–347.

Coyne, J.A. & Orr, H.A. (2004) *Speciation*. Sinauer Associates Inc., Sunderland.

Cracraft, J. & Prum, R.O. (1988) Patterns and processes of diversification: speciation and historical congruence in some neotropical birds. *Evolution*, 42(3), 603–620.

DNPM a - Departamento Nacional da Produção Mineral – Brasil (1978) *Projeto RADAMBRASIL – Levantamento dos recursos naturais, Vol.18, Folha SC 20, Porto*

Velho: Geologia, geomorfologia, pedologia, vegetação e uso potencial da terra. Departamento Nacional da Produção Mineral, Rio de Janeiro.

DNPM b - Departamento Nacional da Produção Mineral – Brasil (1978) *Projeto RADAMBRASIL – Levantamento dos recursos naturais, Vol.19, Folha SB 20, Purus: Geologia, geomorfologia, pedologia, vegetação e uso potencial da terra*. Departamento Nacional da Produção Mineral , Rio de Janeiro.

Doyle, J. J. & Doyle, J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19, 11–15.

Excoffier, L., Smouse, P. & Quattro, J. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitocondrial DNA restriction data. *Genetics*, 131, 479–491.

Excoffier, L. G., Laval, G. & S. Schneider (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, 1, 47–50.

Farris, J.S., Källersjö, M., Kluge, A.G. & Bult, C. (1995) Constructing a significance test for incongruence. *Systematic Biology*, 44(4), 570–572.

Fernandes, A.M., Cohn-Haft, M., Hrbek, T. & Farias, I.P. (2010) Comparative phylogeography of three species of passerine birds in the Madeira River basin: cryptic endemism in a threatened landscape. *Journal of Biogeography* (in press).

Figueiredo, J., Hoorn, C., van der Ven, P. & Soares, E. (2009) Late Miocene onset of the Amazon River and the Amazon deep-sea fan: Evidence from the Foz do Amazonas Basin. *Geology*, 37(7), 619–622.

Fouquet, A., Gilles, A., Vences, M., Marty, C., Blanc, M., Gemmell, N.J. (2007) Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *Plos One*, 10, 1–10.

Fu, Y.X. (1997) Statistical tests of neutrality of mutations againstpopulation growth, hitchhiking and background selection. *Genetics*, 147, 915–925.

Gascon, C., Lougheed, S.C. & Bogart, J. P. (1998) Patterns of genetic population differentiation in four species of Amazonian frogs: A test of the Riverine Barrier Hypothesis. *Biotropica*, 30(1), 104–119.

Gascon, C., Malcolm, J.R., Patton, J.L., Silva, M.N.F., Bogarti, J.P., Lougheed, S.P., Peres, C.A., Neckel, S. & Boag, P.T. (2000) Riverine barriers and the geographic distribution of Amazonian species. *PNAS*, 97(25), 13672–13677.

Gerhardt, H.C. & Huber, F. (2002) *Acoustic communication in insects and anurans*: *common problems and diverse solutions*. University of Chicago Press, Chicago.

Grant, T., Frost, D.R., Caldwell, J.P., Gagliardo, R., Haddad, C.F.B., Kok, P.J.R., Means, B.D., Noonan, B.P., Schargel, W. & Wheeler, W.C. (2006) Phylogenetic systematics of dart-poison frogs and their relatives (Anura: Athesphatanura: Dendrobatidae). *Bulletin of the American Museum of Natural History*, 299, 1–262.

Green, A.J. (2001) Mass/Length residuals: Measures of body condition or generators of spurious results? *Ecology*, 82(5), 1473–1483.

Haffer, J. (1974) Avian speciation in Tropical South America: With a systematic survey of the toucans (Ramphastidae) and jacamars (Galbulidae). *Publications of the Nuttall Ornithological Club*, 14, 1–390.

Haffer, J. (1997) Alternative models of vertebrate speciation in Amazonia: an overview. *Biodiversity and Conservation*, 6, 451–476.

Haffer, J. & Prance, G.T. (2002) Impulsos climáticos da evolução na Amazônia durante o Cenozóico: sobre a teoria dos Refúgios da diferenciação biótica. *Estudos Avançados*, 16, 175–206.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.

Harpending, R.C. (1994) Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology*, 66, 591–600.

Hayes, F.E. & Sewlal, J.N. (2004) The Amazon River as a dispersal barrier to passerine birds: effects of river width, habitat and taxonomy. *Journal of Biogeography*, 31, 1809–1818.

Hoorn, C. (1994) An environmental reconstruction of the palaeo-Amazon River system (Middle–Late Miocene, NW Amazonia). *Palaeogeography, Palaeoclimatology, Palaeoecology*, 112, 187–238.

Hudson, R.R. (1990) Gene genealogies and the coalescent process. *Oxford Surveys in Evolutionary Biology*, 7, 1–44.

Huelsenbeck, J.P. & Crandall, K.A. (1997) Phylogeny estimation and hypothesis testing using Maximum Likelihood. *Annual Review of Ecology and Systematics*, 28, 437–466.

Jobb, G. (2008) *TREEFINDER*, version of October 2008. Distributed by the author at www.treefinder.de, Munich, Germany.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16, 111–120.

Latubresse, E.M. (2003) The Late-Quaternary Palaeohydrology of large South American fluvial systems. *Palaeohydrology: Understanding Global Change* (ed. by K. J. Gregory and G. Benito), pp. 193–212. John Wiley & Sons Ltd, Hoboken, USA.

Latubresse, E.M. & Franzinelli, E. (2002) The Holocene alluvial plain of the middle Amazon River, Brazil. *Geomorphology*, 44, 241–257.

Laurance, W.F., Albernaz, A.K.M., Fearnside, P.M., Vasconcelos, H.L. & Ferreira, L.V. (2004) Deforestation in Amazonia. *Science*, 304, 1109.

Legendre, P. (2000) Comparison of permutation methods for the partial correlation and partial Mantel tests. *Journal of Statistical Computation and Simulation*, 67, 37–73.

Legendre, P., Dale, M.R.T., Fortin, M.J., Gurevitch, J., Hohn, M. & Myers, D. (2002) The consequences of spatial structure for the design and analysis of ecological field surveys. *Ecography*, 25, 601–615.

Librado, P. & Rozas, J. (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451–1452.

Lougheed, S.C., Gascon, C., Jones, D.A., Bogart, J.P. & Boag, P.T. (1999) Ridges and rivers: a test of competing hypothesis of Amazonian diversification using a dart-poison frog (*Epipedobates femoralis*). *Proceedings of the Royal Society London*, 266, 1829–1835.

Mantel, N. (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27,209–220.

Maslin, M.A. & Burns, S.J. (2000) Reconstruction of the Amazon Basin effective moisture availability over the past 14,000 years. *Science*, 290, 2285–2287.

McGuire, J.A., Linkem, C.W., Koo, M.S., Hutchison, D.W., Lappin, A.K., Orange, D.I., Lemos-Espinal, J., Riddle, B.R. & Jaeger, J.R. (2007) Mitochondrial introgression and incomplete lineage sorting through space and time: phylogenetics of the Crotaphytid lizards. *Evolution*, 61(12), 2879–2897.

Menin, M., Lima, A.P., Magnusson, W.E. & Waldez, F. (2007) Topographic and edaphic effects on the distribution of terrestrially reproducing anurans in Central Amazonia: mesoscale spatial patterns. *Journal of Tropical Ecology*, 23, 539–547.

Moritz, C. (1994) Defining evolutionarily significant units for conservation. *Trends in Ecology and Evolution*, 9, 373–375.

Moritz, C. (1995) Uses of Molecular Phylogenies for Conservation. *Philosophical Transactions: Biological Sciences*, 349(1327), 113–118.

Moritz, C., Patton, J.L., Schneider, C.J. & Smith, T.B. (2000) Diversification of rainforest faunas: An integrated molecular approach. *Annual Review of Ecology and Systematics*, 31, 533–563.

Morrone, J.J. (2006) Biogeographic areas and transition zones of Latin America and the Caribbean Islands based on panbiogeographic and cladistic analyses of the entomofauna. *Annual Review of Entomology*, 51, 467–94.

Nogueira, A.C.R. (2008) Guinada para o Atlântico. *Scientific American Brasil – Amazônia*, 1, 22–27.

Noonan, B.P. & Wray, K.P. (2006) Neotropical diversification: the effects of complex history on diversity within the poison frog genus *Dendrobates*. *Journal of Biogeography*, 33, 1007–1020.

Palumbi, S.R. (1996) Nucleic acids II: the polymerase chain reaction. *Molecular Systematics* (ed. by D.M. Hillis, C. Moritz, and B.K. Mable), pp. 205–247. Sinauer & Associates Inc., Sunderland, Massachusetts.

Patton, J.L., Silva, M.N.F. & Malcolm, J.R. (1994) Gene genealogy and differentiation among arboreal spiny rats (Rodentia: Echymidae) of the Amazon Basin: a test of the riverine barrier hypothesis. *Evolution*, 48, 1314–1323.

Peres, C.A., Patton, J.L. & Silva, M.N.F. (1996) Riverine Barriers and gene flow in Amazonian saddle-back tamarins. *Folia Primatologica*, 67(3), 113–124.

Perz, S., Brilhante, S., Brown, F., Caldas, M., Ikeda, S, Mendoza, E., Overdevest, C., Reis, V., Reyes, J.F., Rojas, D., Schmink, M., Souza, C., Walker, R. (2008) Road building, land use and climate change: prospects for environmental governance in the Amazon. *Philosophical Transactions Royal Society B*, 363, 1889–1895.

Posada, D. (2008) jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution*, 25, 1253–1256.

Rambaut, A. & Drummond, A.J. (2007) *Tracer v1.4*. Distributed by the authors at http://beast.bio.ed.ac.uk/Tracer.

Ramos-Onsins, S.E. & Rozas, J. (2002) Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, 19, 2092–2100.

Reis,S.F., Pessoa, L.M. & Strauss, R.E. (1990) Application of size-free canonical discriminant analysis to studies of geographic differentiation. *Revista Brasileira de Genética*, 13, 509–520.

Roddaz, M., Hermoza, W., Mora, A., Baby, P., Parra, M., Christophoul, F., Brusset, S. & Espurt, N. (2010). Cenozoic sedimentary evolution of the Amazonian foreland basin

system. *Amazonia, landscape and species evolution* (ed. by C. Hoorn, C. and F.P. Wesselingh), pp. 61–88. Blackwell Publishing, Oxford.

Roe, B.A., Ma, D.P., Wilson, R.K. & Wong, J.F. (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. *Journal of Biological Chemistry*, 260(17), 9759–9774.

Ron, S. (2000) Biogeographic area relationships of lowland Neotropical rainforest based on raw distributions of vertebrate groups. *Biological Journal of the Linnean Society*, 71, 379–402.

Ronquist, F. & Huelsenbeck, J. P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572–1574.

Ronquist, F., van der Mark, P. & Huelsenbeck, J.P. (2009) Bayesian phylogenetic analysis using MrBayes. *The Phylogenetic Handbook* (ed. by P. Lemey, M. Salemi and A. Vandamme), pp. 210–266. Cambridge University Press, New York.

Roosmalen, M.G.M. van, Roosmalen, T. van & Mittermeier, R.A. (2002) A taxonomic review of the Titi Monkeys, genus *Callicebus*, Tomas, 1903, with the description of two new species, *Callicebus bernhardi* and *Callicebus stephennashi*, from Brazilian Amazonia. *Neotropical Primates*, 10 (Suppl.), 1–52.

Rossetti, D.F., de Toledo, P.M. & Góes, A.M. (2005) New geological framework for Western Amazonia (Brazil) and implications for biogeography and evolution. *Quaternary Research*, 63, 78–89.

Ryan, M.J. (1988) Energy, calling, and selection. *American Zoologist*, 28, 885–898.

Santos, J.C., Coloma, L.A., Summers, K., Caldwell, J.P., Ree, R. & Cannatella, D.C. (2009) Amazonian amphibian diversity is primarily derived from Late Miocene Andean lineages. *PLOS Biology*, 7(3), 1–14.

Schneider, S. & Excoffier, L. (1999) Estimation of demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: Application to human mitochondrial DNA. *Genetics*, 152, 1079–1089.

Shimodaira, H. & Hasegawa, M. (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* 16(8), 1114–1116.

Silva, J.M.C., Rylands, A.B. & Fonseca, G.A.B. (2005) The fate of the Amazonian areas of endemism. *Conservation Biology*, 19(3), 689–694.

Silva, M.N.F. & Patton, J. L. (1998) Molecular phylogeography and the evolution and conservation of mammals. *Molecular Ecology*, 7, 475–486.

Simões, P.I., Lima, A.P., Magnusson, W.E., Hödl, W. & Amézquita, A. (2008) Acoustic and morphological differentiation in the frog *Allobates femoralis*: Relationships with the upper Madeira River and other potential geological barriers. *Biotropica*, 40(5), 607–614.

Simões, P.I., Lima, A.P. & Farias, I.P. (2010) The description of a cryptic species related to the pan-Amazonian frog *Allobates femoralis* (Boulenger 1883) (Anura: Aromobatidae). *Zootaxa*, 2406, 1–28.

Smouse, P.E., Long, J.C. & Sokal, R.R. (1986) Multiple regression and correlation extensions of the Mantel test of matrix correspondence. *Systematic Zoology*, 35, 727–32.

Solomon, S.E., Bacci, M., Martins, J., Vinha, G.G., & Mueller, U.G. (2008) Paleodistributions and comparative molecular phylogeography of leafcutter ants (*Atta* spp.) provide new insight into the origins of Amazonian diversity. *Plos ONE*, 3(7), 1–15.

Souza Filho, P.W.M., Quadros, M.L.E.S., Scandolara, J.E., Silva Filho, E.P. & Reis, M.R. (1999) Compartimentação morfoestrutural e neotectônica do sistema fluvial Guaporé-Mamoré-Alto Madeira, Rondônia – Brasil. *Revista Brasileira de Geociências*, 29(4), 469–476.

Switkes, G. (2008) *Águas Turvas: Alertas sobre as conseqüencias de barrar o maior afluente do Amazonas*. International Rivers, São Paulo.

Swofford, D.L. (1998) *PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods)*, version 4. Sinauer Associates, Sunderland, Massachusetts.

Symula, R., Schulte, R. & Summers, K. (2003) Molecular systematics and phylogeography of Amazonian poison frogs of the genus *Dendrobates*. *Molecular Phylogenetics and Evolution*, 26, 452–475.

Tajima, F. (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123, 585–595.

Tamura K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4*:* Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596–1599.

Telles, M.P.C. & Diniz-Filho, J.A.F. (2005) Multiple Mantel tests and isolation-by-distance, taking into account long-term historical divergence. *Genetics and Molecular Research*, 4(4), 742–748.

Templeton, A.R., Crandall, K.A. & Sing, C.F. (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetic*s, 132, 619–633.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994) Improved sensitivity of profile searches through the use of sequence weights and gap excision. *Computer Applications in the Biosciences*, 10(1), 19–29.

Thorpe, R.S., Reardon, J.T., & Malhotra, A. (2005) Common garden and natural selection experiments support ecotypic differentiation in the Dominican anole (*Anolis oculatus*). *The American Naturalist*, 165(4), 495–504.

Tocher, M.D., Gascon, C. & Meyer, J. (2001) Community composition and breeding success of Amazonian frogs in continuous forest and matrix habitat aquatic sites. *Lessons from Amazonia*: *The ecology and conservation of a fragmented forest* (ed. by R.O Bierregaard, C .Gascon, T. Lovejoy and R. Mesquita), pp. 235–247. Yale University Press, New Haven & London.

Tuomisto, H., Ruokolainen, K. & Yli-Halla, M. (2003) Dispersal, environment, and floristic variation of Western Amazonian forests. *Science*, 299, 241–244.

Vences, M., Thomas, M., Meijden, A. van der, Chiari, Y. & Vieites, D.R. (2005) Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Frontiers in Zoology*, 2(5), 1–12.

Wallace, A. R. (1852) On the monkeys of the Amazon. *Proceedings of the Zoological Society of London*, 20, 107–110.

Wesselingh, F.P. (2006) Miocene long-lived lake Pebas as a stage of mollusk radiations, with implications for landscape evolution in western Amazonia. *Scripta Geologica*, 133, 1–17.

Wesselingh, F.P. & Salo, J.A. (2006) A Miocene perspective on the evolution of the Amazonian biota. *Scripta Geologica*, 133, 439–458.

Westaway, R. (2006) Late Cenozoic sedimentary sequences in Acre state, southwestern Amazonia: Fluvial or tidal? Deductions from the IGCP 449 fieldtrip. *Journal of South American Earth Sciences*, 21, 120–134.

Wilkinson, L. (1990) *SYSTAT: the system for statistics*. SYSTAT inc., Evanston.

Wilkinson, M.J., Marshall, L.G., Lundberg, J.G. & Kreslavsky, M.H. (2010). Megafan environments in northern South America and their impact on Amazon Neogene aquatic ecosystems. *Amazonia, landscape and species evolution* (ed. by C. Hoorn, C. and F.P. Wesselingh), pp. 162–184. Blackwell Publishing, Oxford.

Wright, S. (1951) The genetical structure of populations. *Ann. Eugenics*, 15, 323–354.

Zink, R.M., Blackwell-Rago, R.C., & Ronquist, F. (2000) The shifting roles of vicariance in biogeography. *Proceedings of the Royal Society London*, 267, 497–503.

Table 1: Names, positions according to riverbank, and coordinates (in decimal degrees) of the 17 sampling localities along the Madeira River, in the sates of Rondônia and Amazonas, Brazil.

Site	Locality name	Riverbank	Latitude	Longitude
1	Mutum-Paraná	Right	9.6414° S	64.8859° W
2	Lower Jirau	Left	9.3114° S	64.7172° W
3	Jaci-Paraná	Left	9.1694° S	64.4289° W
4	Jaci-Paraná	Right	9.2045° S	64.3620° W
5	Morrinho	Left	9.0199° S	64.2172° W
6	Morrinho	Right	9.0158° S	64.0914° W
7	Santo Antônio	Left	8.8309° S	64.0206° W
8	Santo Antônio	Right	8.6550° S	64.0195° W
9	Humaitá	Right	7.5488° S	62.8772° W
10	Humaitá	Left	7.0228° S	63.1028° W
11	Democracia	Left	5.8058° S	61.4453° W
12	Manicoré	Right	5.8231° S	61.2986° W
13	Novo Aripuanã	Right	5.1503° S	60.3467° W
14	Borba	Right	4.4342° S	59.6236° W
15	Nova Olinda do Norte	Right	3.8744° S	59.0461° W
16	Careiro	Left	3.3708° S	59.8683° W
17	Manaquiri	Left	3.4272° S	60.6150° W

Table 2: Distribution of 16SrDNA haplotypes of *Allobates femoralis* among 17 sampling localities* along the Madeira River, Brazil. Haplotype H25 is the only haplotype shared between population inhabiting opposite riverbanks, corresponding to localities between the municipality of Humaitá (Amazonas) and Cachoeira do Santo Antônio (Rondônia).

	Locality																
Haplotype	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
H01	13	-	-	4	-	4	-	4	-	-	-	-	-	-	-	-	-
H02	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H03	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H04	-	19	3	-	-	-	1	-	-	-	-	-	-	-	-	-	-
H05	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H06	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H07	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H08	-	-	7	-	1	-	-	-	-	-	-	-	-	-	-	-	-
H09	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H10	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H11	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H12	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H13	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H14	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
H15	-	-	-	5	-		-	6	-	-	-	-	-	-	-	-	-
H16	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-
H17	-	-	-	6	-	7	-	8	-	-	-	-	-	-	-	-	-
H18	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
H19	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
H20	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
H21	-	-	-	-	11	-	-	-	-	-	-	-	-	-	-	-	-
H22	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-
H23	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
H24	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
H25	-	-	-	-	-	-	3	-	6	11	-	-	-	-	-	-	-
H26	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
H27	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
H28	-	-	-	-	-	-	-	-	-	-	8	-	-	-	-	-	-
H29	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-
H30	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
H31	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
H32	-	-	-	-	-	-	-	-	-	-	-	15	-	-	-	-	-
H33	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
H34	-	-	-	-	-	-	-	-	-	-	-	-	15	11	5	-	-
H35	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
H36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
H37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
H38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
H39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
H40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	2
H41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1

*1=Mutum-Paraná (right bank), 2=Lower Jirau (left bank), 3= Jaci-Paraná (left bank), 4=Jaci-Paraná (right bank), 5=Morrinho (left bank), 6=Morrinho (right bank), 7=Santo Antônio (left bank), 8=Santo Antônio (right bank), 9=Humaitá (right bank), 10=Humaitá (left bank), 11=Democracia (left bank), 12=Manicoré (right bank), 13=Novo Aripuanã (right bank), 14=Borba (right bank), 15=Nova Olinda do Norte (right bank), 16=Careiro (left bank), 17=Manaquiri (left bank).

Table 3: Relative *Fst* fixation index (lower left matrix) and average Kimura 2-parameter genetic distances (upper right matrix) between *Allobates femoralis* collected in 17 sampling localities along the Madeira River. Measures were obtained from a 507 b.p. of the mitochondrial 16S rDNA fragment.

Locality		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Mutum	**1**		*0.009*	*0.008*	*0.004*	*0.009*	*0.005*	*0.006*	*0.004*	*0.004*	*0.005*	*0.028*	*0.011*	*0.004*	*0.005*	*0.004*	*0.023*	*0.031*
L. Jirau	**2**	0.907		*0.003*	*0.011*	*0.004*	*0.012*	*0.005*	*0.011*	*0.007*	*0.007*	*0.039*	*0.018*	*0.013*	*0.013*	*0.013*	*0.026*	*0.032*
Jaci (L)	**3**	0.801	0.436		*0.009*	*0.004*	*0.010*	*0.005*	*0.009*	*0.005*	*0.006*	*0.037*	*0.016*	*0.012*	*0.012*	*0.012*	*0.025*	*0.032*
Jaci (R)	**4**	0.484	0.804	0.700		*0.011*	*0.004*	*0.006*	*0.003*	*0.004*	*0.005*	*0.032*	*0.011*	*0.008*	*0.008*	*0.008*	*0.023*	*0.031*
Morri.(L)	**5**	0.810	0.554	0.291	0.720		*0.012*	*0.006*	*0.011*	*0.007*	*0.007*	*0.039*	*0.018*	*0.013*	*0.013*	*0.013*	*0.026*	*0.033*
Morri.(R)	**6**	0.490	0.788	0.693	0.000	0.711		*0.008*	*0.003*	*0.006*	*0.006*	*0.033*	*0.012*	*0.008*	*0.009*	*0.008*	*0.025*	*0.033*
S.Ant.(L)	***7***	0.649	0.539	0.388	0.486	0.486	0.513		*0.006*	*0.002*	*0.002*	*0.034*	*0.013*	*0.010*	*0.010*	*0.010*	*0.021*	*0.028*
S.Ant.(R)	**8**	0.517	0.813	0.710	0.000	0.728	0.002	0.493		*0.004*	*0.005*	*0.032*	*0.011*	*0.008*	*0.008*	*0.008*	*0.023*	*0.031*
Huma.(R)	**9**	0.918	0.930	0.780	0.640	0.795	0.651	0.200	0.651		*0.000*	*0.033*	*0.011*	*0.008*	*0.009*	*0.008*	*0.018*	*0.027*
Huma.(L)	**10**	0.858	0.887	0.736	0.598	0.759	0.617	0.173	0.607	0.000		*0.033*	*0.011*	*0.009*	*0.009*	*0.009*	*0.019*	*0.027*
Democr.	**11**	0.965	0.971	0.947	0.952	0.944	0.914	0.928	0.929	0.982	0.971		*0.031*	*0.023*	*0.023*	*0.023*	*0.019*	*0.027*
Manicoré	**12**	0.954	0.965	0.917	0.838	0.912	0.823	0.858	0.846	0.987	0.958	0.976		*0.006*	*0.007*	*0.006*	*0.021*	*0.029*
N. Aripu.	**13**	0.918	0.964	0.898	0.786	0.894	0.761	0.826	0.802	1.000	0.963	0.975	0.979		*0.000*	*0.000*	*0.018*	*0.027*
Borba	**14**	0.853	0.938	0.870	0.752	0.870	0.731	0.797	0.768	0.960	0.926	0.959	0.929	0.000		*0.000*	*0.019*	*0.027*
N. Olinda	**15**	0.918	0.964	0.898	0.786	0.893	0.761	0.826	0.802	1.000	0.963	0.975	0.979	0.000	0.000		*0.018*	*0.027*
Careiro	**16**	0.947	0.948	0.914	0.890	0.910	0.879	0.875	0.894	0.955	0.938	0.927	0.953	0.955	0.936	0.955		*0.011*
Manaqui.	**17**	0.800	0.805	0.780	0.762	0.783	0.760	0.737	0.764	0.782	0.773	0.768	0.794	0.782	0.773	0.782	0.414	

Table 4: Summary statistics of genetic polymorphism parameters and results of neutrality tests performed on seven genetic clusters of *Allobates femoralis* collected in 17 sampling localities. Clustering was estimated via Bayesian analysis of population structure in BAPS. Localities numbers from 1 to 17 correspond to sites presented in Table 1 and Fig. 1. *n* = sample size; *h* = number of haplotypes; *S* = number of segregating sites; π = average pairwise distance between samples in the same cluster, plus or minus one standard deviation; D_T = Tajima's *D*; *Fs* = Fu's *Fs*; R_2 = Ramos-Onsins & Roza's R2. Tests signs followed by 95% stand for probability of result via coalescent simulations adopting a 95% confidence interval.

Cluster	**Localities**	***n***	***h***	***S***	**π ± 1 S.D.**	***DT***	***Dt* 95%**	***Fs***	***Fs* 95%**	R_2	R_2 **95%**
1	16+17	19	6	7	0.00146±0.00146	-2.1100	0.007*	-3.335	0.032*	0.1058	0.180
2	2+3+5+7	56	14	14	0.00287±0.00029	-1.5682	0.035*	-8.002	0.005*	0.0479	0.016*
3	1+4+6+7+8+9+10	63	9	9	0.00265±0.00023	-0.8109	0.234	-2.407	0.174	0.0723	0.223
4	4+6+8	25	4	3	0.00062±0.00026	-1.5041	0.086	-2.442	0.051*	0.0940	0.102
5	12+13+14+15	48	4	6	0.00296±0.00033	0.2558	0.663	2.050	0.875	0.1251	0.677
6	11	12	3	3	0.00099±0.00058	-1.6293	0.097	-0.614	0.351	0.1984	0.661
7	7	4	3	3	0.00298±0.00109	-0.7544	0.535	-0.288	0.361	0.2764	0.327

Table 5: Tests of demographic expansion based on the sum of squared deviation (SSD) between observed and expected mismatch distributions, and Harpending's raggedness index (Hri) preformed on seven genetic clusters of *Allobates femoralis* along the Madeira River. Localities numbers from 1 to 17 correspond to sites presented in Table 1 and Fig. 1. *n* = number of samples. Values of $P < 0.05$ reject the null hypothesis population expansion through time.

Clade	Localities	*n*	Mismatch obs. mean	Mismatch obs. variance	SSD	*P* (SSD)	Hri	*P* (Hri)
1	16+17	19	0.737	1.042	0.008612	0.38	0.133545	0.66
2	2+3+5+7	56	1.443	1.121	0.008333	0.10	0.062074	0.37
3	1+4+6+7+8+9+10	63	1.333	1.069	0.007707	0.27	0.048525	0.59
4	4+6+8	25	1.027	2.133	0.315577	0.00*	0.104577	1.00
5	12+13+14+15	48	1.487	2.429	0.182474	0.07*	0.538710	0.06*
6	11	12	0.985	0.907	0.001122	0.95	0.051882	0.93
7	7	4	1.500	1.100	0.005536	0.92	0.083333	0.99

Table 6: Loadings of the first five principal components generated by a principal component analysis on 24 acoustic variables means measured from advertisement calls of *Allobates femoralis* males recorded in 16 sites along the Madeira River, Brazil. Spectral variables had higher scores on PC 1, while PC 2 summarized variation related to duration of notes and calls.

		Loadings				
Variable	**Variable type**	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC5**
Silent intervals between calls	Temporal	-0.395	0.750	0.027	-0.355	-0.116
Call duration	Temporal	-0.235	0.953	0.146	0.107	-0.029
1st note duration	Temporal	0.409	0.696	-0.456	-0.102	-0.340
2nd note duration	Temporal	-0.161	0.838	-0.36	0.370	0.006
3rd note duration	Temporal	0.116	0.822	-0.424	-0.22	0.173
4th note duration	Temporal	-0.283	0.812	-0.242	0.304	0.257
Silent interval between 1st and 2nd notes	Temporal	-0.446	0.619	0.516	0.061	0.343
Silent interval between 2nd and 3rd notes	Temporal	-0.303	0.595	0.541	0.300	-0.394
Silent interval between 3rd and 4th notes	Temporal	-0.234	0.698	0.509	-0.370	0.007
1st note maximum frequency	Spectral	0.981	0.070	0.017	0.061	-0.092
1st note lowest frequency	Spectral	0.983	0.013	0.106	0.113	-0.028
1st note highest frequency	Spectral	0.954	0.238	-0.037	-0.047	-0.152
2nd note maximum frequency	Spectral	0.977	0.088	0.055	0.076	0.095
2nd note lowest frequency	Spectral	0.981	-0.027	0.086	0.105	-0.055
2nd note highest frequency	Spectral	0.958	0.192	-0.003	-0.103	-0.041
3rd note maximum frequency	Spectral	0.981	0.074	0.059	-0.032	0.107
3rd note lowest frequency	Spectral	0.982	-0.053	0.079	0.083	-0.004
3rd note highest frequency	Spectral	0.953	0.157	-0.005	-0.207	0.068
4th note maximum frequency	Spectral	0.967	0.120	0.067	0.135	0.121
4th note lowest frequency	Spectral	0.973	-0.082	0.057	0.090	0.018
4th note highest frequency	Spectral	0.959	0.160	-0.001	-0.135	0.052
Maximum frequency of call	Spectral	0.975	0.060	0.106	0.013	0.071
Lowest frequency of call	Spectral	0.977	-0.005	0.074	0.126	-0.036
Highest frequency of call	Spectral	0.956	0.165	0.021	-0.158	0.055
Eigenvalues		*14.974*	*5.435*	*1.474*	*0.839*	*0.588*
% of total variance explained		**62.392**	**22.644**	**6.140**	**3.495**	**2.449**

Table 7: Loadings of the first five principal components generated by a principal component analysis on 19 morphometric variables means of *Allobates femoralis* males recorded in 16 localities along the Madeira River, Brazil. PC1 accounts for most of the size-dependent variation in morphology.

	Loadings				
Variable	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC5**
Head length	0.943	-0.22	0.196	-0.009	0.045
Head width	0.837	-0.336	0.257	0.175	0.221
Snout length	0.904	-0.092	0.267	-0.18	-0.23
Eye to nostril distance	0.716	-0.661	0.102	-0.003	-0.092
Distance between nostrils	0.817	0.198	0.456	-0.175	0.192
Maximum diameter of eye	0.834	-0.021	-0.352	-0.319	0.117
Distance between orbits	0.921	-0.115	0.258	-0.166	0.071
Maximum diameter of tympanum	0.819	-0.482	0.06	0.212	-0.05
Forearm length	0.951	-0.063	0.048	0.031	-0.209
Length of Finger I	0.961	0.135	-0.113	0.084	-0.05
Length of Finger II	0.98	0.027	-0.104	-0.007	0.017
Length of Finger III	0.957	0.208	-0.141	0.064	-0.054
Width of Finger III disc	0.648	0.606	0.359	0.152	-0.158
Tibia length	0.977	-0.035	-0.154	0.046	0.05
Foot length	0.916	0.115	-0.274	0.104	0.011
Width of Toe IV disc	0.805	0.457	0.184	0.153	0.15
Leg length	0.916	0.223	-0.062	-0.219	-0.086
Arm length	0.905	0.042	-0.381	0.001	-0.007
Tarsus length	0.884	0.035	-0.407	0.102	0.064
Eigenvalues	*14.807*	*1.601*	*1.210*	*0.398*	*0.281*
% of total variance explained	**77.932**	**8.427**	**6.368**	**2.095**	**1.479**

Table 8: Statistical predictions of simple and partial Mantel tests evaluating correlations between phenotypic, genetic and geographic distances of *Allobates femoralis* sampled in 16 localities along the Madeira River. Partial Mantel tests model notation corresponds to "MATRIX 1" X "MATRIX 2"."COVARIATE MATRIX".

Model	***r***	***P***
DGeo X DGen	0.302	**0.025***
DGeo X DSVL	0.199	**0.047***
DGeo X DMPC1	-0.042	0.384
DGeo X DMPC2	0.390	**0.004***
DGeo X DMT	0.373	**0.004***
DGeo X DAPC1	0.353	**0.009***
DGeo X DAPC2	-0.003	0.554
DGeo X DAT	0.132	0.153
River X Dgen	0.196	**0.004***
River X DSVL	0.402	**0.004***
River X DMPC1	0.413	**0.006***
River X DMPC2	-0.070	0.167
River X DMT	-0.009	0.502
River X DAPC1	-0.057	0.023
River X DAPC2	0.054	0.176
River X DAT	0.025	0.317
DGen X River.Dgeo	0.229	**0.000***
DSVL X River.DGeo	0.427	**0.002***
DMPC1 X River.DGeo	0.412	**0.007***
DMPC2 X River.DGeo	-0.045	0.301
DMT X River.DGeo	0.02	0.346
DAPC1 X River.DGeo	-0.033	0.353
DAPC2 X River.DGeo	0.054	0.180
DAT X River.DGeo	0.035	0.264

DGeo=Geographic distance; River=riverbank (binary); DGen=Genetic distance (mean uncorrected pairwise); DSVL= Body size distance; DMPC1/DMPC2/DMT=Morphometric distances based on the first, second, and first and second combined components of a principal component analysis (PCA) on morphometric variables, respectively; DAPC1/DAPC2/DAT=Acoustic distances based on the first, second, and first and second combined components of a PCA on acoustic variables, respectively. First morphometric and acoustic components were regressed against SVL and residuals were used for Euclidean distance calculations.

Figure 1: (a) Location of study area in lowlands within the Brazilian Amazon basin in South America; (b) Distribution of 17 *Allobates femoralis* sampling localities along both riverbanks of the Madeira River, in the states of Rondônia and Amazonas, Brazil. White dots assigned as "N1" and "N2" correspond to two localities where adequate habitat for *A. femoralis* is present, but no populations where found; (c) Denomination of sampling localities.

Figure 2: Phylogenetic trees recovered from (a) Maximum Likelihood, and (b) Bayesian phylogenetic analysis on a concatenated dataset containing fragments of the 16S rRNA and cytocrome *b* mitochondrial genes of *Allobates femoralis* specimens collected in 17 localities along the Madeira River. Branch labels correspond to branch support estimated by bootstrap analysis in (a), and to clade posterior probabilities in (b). Only values above 75 are displayed in (a).

Figure 3: Haplotype network built from 227 16S rDNA sequences of *Allobates femoralis* collected in 17 localities along the Madeira River. Areas of ellipses are proportional to frequency of individuals bearing that haplotype. Numbers refer to haplotype designations provided on Table 2, which also contains geographic locations of haplotypes. Small dots and transverse bars represent not sampled (missing) intermediate haplotypes. The bar between the two trees represents the precedence of samples within clades according to riverbank.

Figure 4: Mean genetic distances (Kimura 2-parameters) between paired sampling localities on immediate opposite riverbanks of the Madeira River according to their distribution from the extreme upper course to the river's mouth. Genetic distances are more pronounced in comparisons between populations on the middle to lower course of the river, but a linear pattern of increasing genetic differentiation towards the river's mouth could not be evidenced.

Figure 5: Barplot resulting from a Bayesian analysis of genetic differentiation on 227 individual 16S rDNA sequences of *Allobates femoralis* collected in 17 sampling localities along the Madeira River. Distinct patterns or grayscale shades represent each of seven genetic clusters estimated in BAPS. Individuals are sorted according to sampling localities.

Figure 6. Mismatch distributions constructed using pairwise differences among mtDNA 16S rDNA samples from seven clusters resulting from a Bayesian analysis of genetic differentiation on 227 *Allobates femoralis* individuals collected along the Madeira River.

Figure 7: Chronogram of divergence times estimated by a Local Rate Minimum Deformation model on a 16S rDNA genetic distance tree of *Allobates femoralis* populations along the Madeira River. Values on left column correspond to estimated divergence times of each clade. Mean approximate time of divergence between A. *femoralis* and *A. hodli* (a) was used to calibrate tree. First split between the Madeira River clades were estimated as Late Pliocene (b) and might reflect the onset of the main river channel. Most subsequent divergence events occurred during Pleistocene. A possible case of dispersal from right to left riverbank was estimated as 0.9 M.a. (c). Darker bars departing from (b) and (c)

represent variation on possible time of divergence based on maximum and minimum ages estimated for divergence between *A. femoralis* and *A. hodli*.

Figure 8: Samples of representative advertisement call and morphological patterns of *Allobates femoralis* males along the Madeira River, in Brazil. Graphics correspond to oscillograms (upper, blue) and sonograms (lower, grayscale) of advertisement calls, denoting absence of variation in number of notes between populations in distinct sampling sites. Arrows (a) and (b) correspond to geographic location of the mouths of the Aripuanã and Ji-Paraná Rivers, respectively.

Figure 9: Distribution of mean values for each *Allobates femoralis* sampling locality along the first two principal components generated by a principal component analysis on (a) 24 acoustic variables of advertisement calls; and (b) 19 external morphometric variables obtained from male individuals. Symbols L and R stand for sampling sites located on left and right riverbanks of the Madeira River, respectively. Symbol labels correspond to the 16 sampling localities (see Table 1, Figure 1).

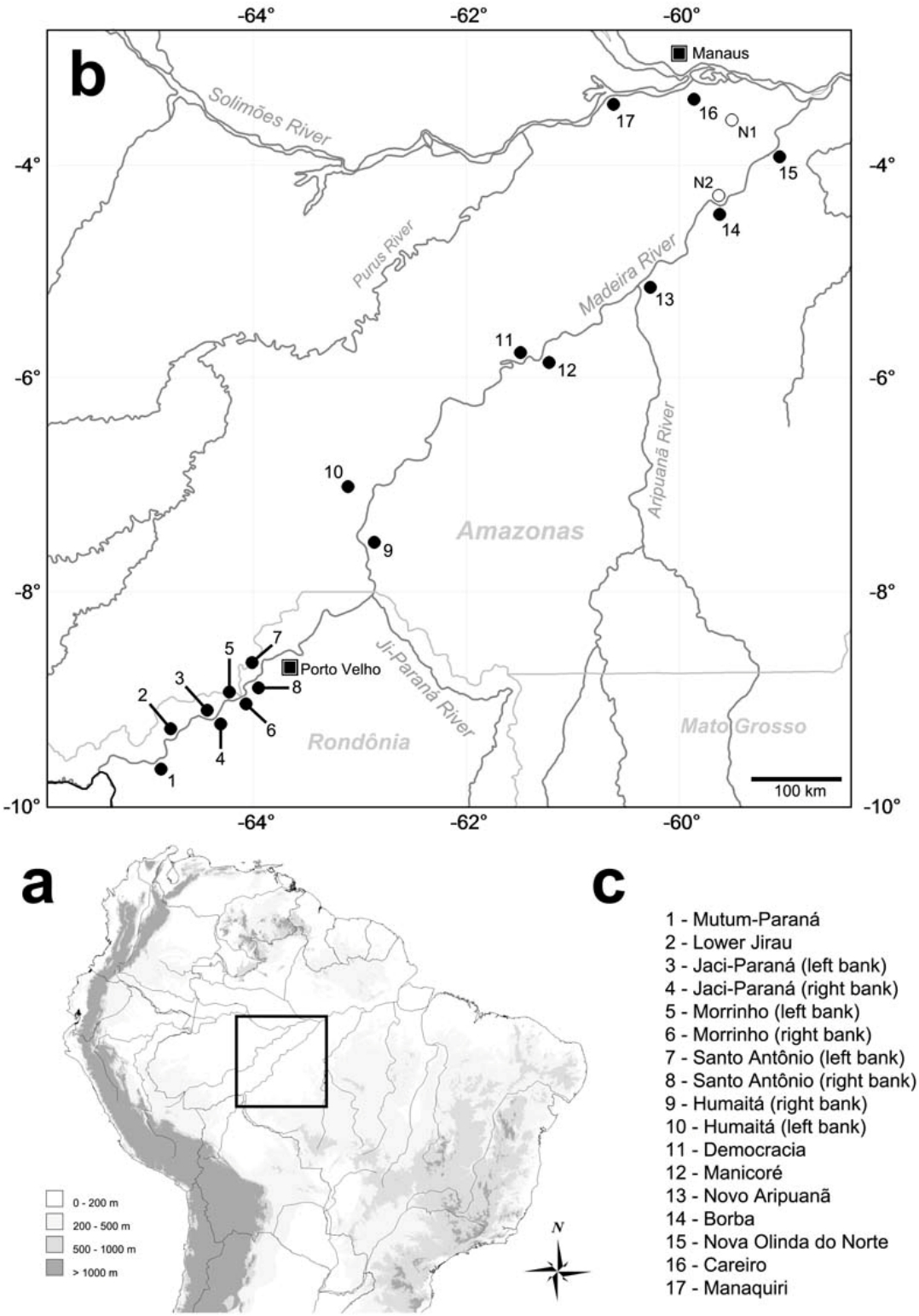
b
-64°
-62°
-60°
-4°
-6°
-8°
-10°
Manaus
Solimões River
Purus River
Madeira River
Aripuanã River
Ji-Paraná River
Amazonas
Rondônia
Mato Grosso
Porto Velho
N1
N2
100 km
a
0 - 200 m
200 - 500 m
500 - 1000 m
> 1000 m
N
c
1 - Mutum-Paraná
2 - Lower Jirau
3 - Jaci-Paraná (left bank)
4 - Jaci-Paraná (right bank)
5 - Morrinho (left bank)
6 - Morrinho (right bank)
7 - Santo Antônio (left bank)
8 - Santo Antônio (right bank)
9 - Humaitá (right bank)
10 - Humaitá (left bank)
11 - Democracia
12 - Manicoré
13 - Novo Aripuanã
14 - Borba
15 - Nova Olinda do Norte
16 - Careiro
17 - Manaquiri

Fig. 1

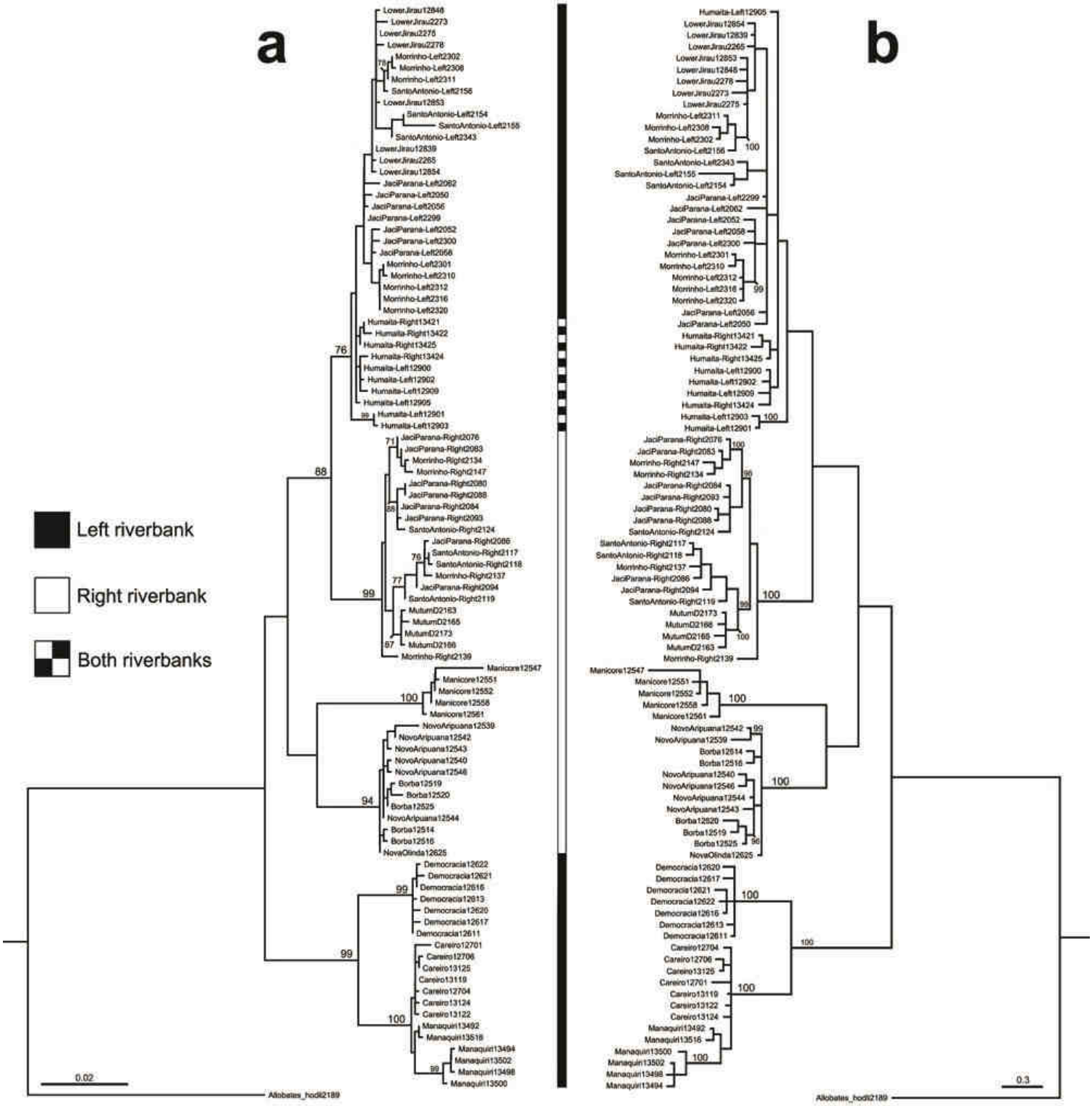
a
b
Left riverbank
Right riverbank
Both riverbanks
0.02
0.3
Allobates_hodl2189

Fig. 2

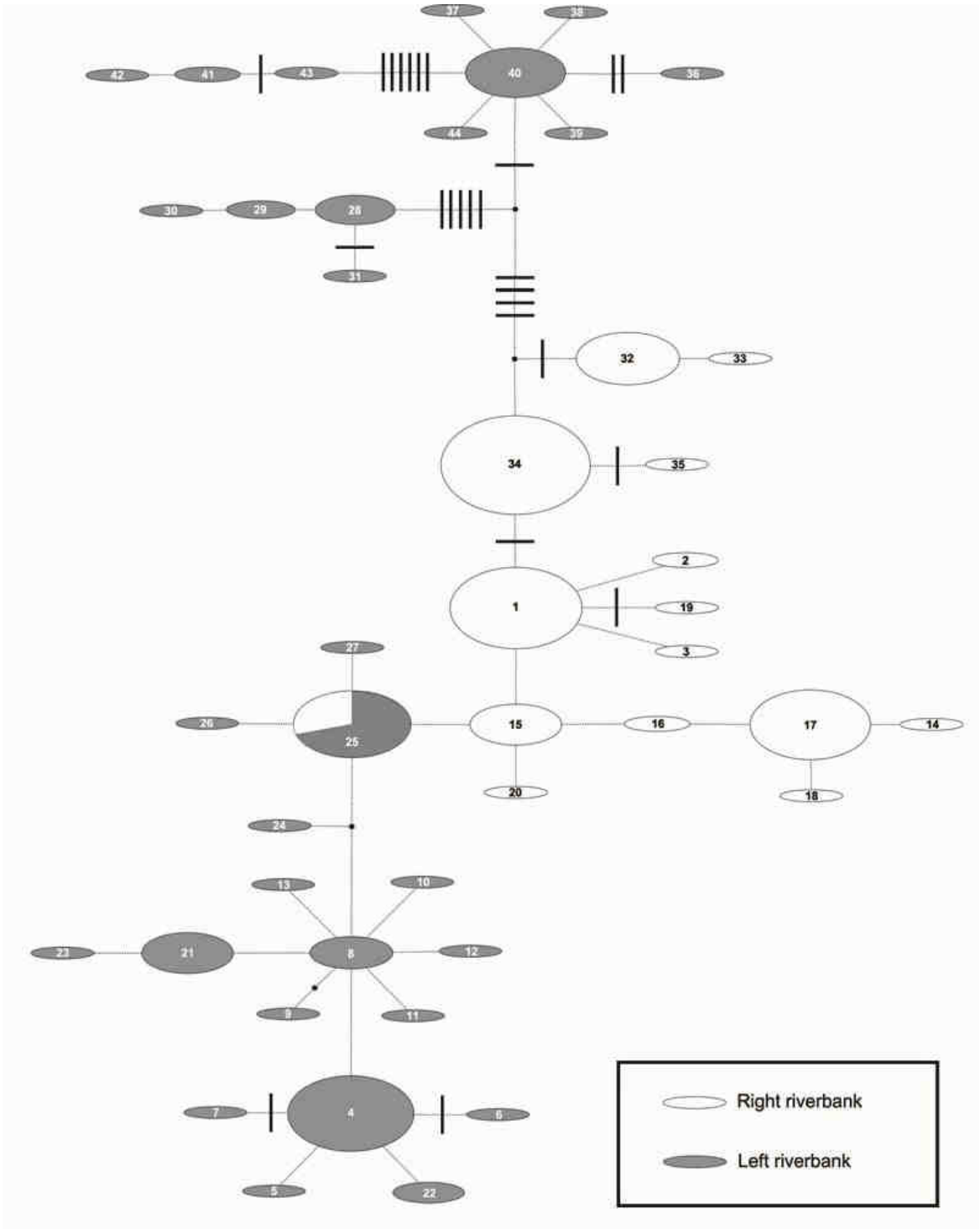
37
38
42
41
43
40
36
44
39
30
29
28
31
32
33
34
35
2
1
19
3
27
26
25
15
16
17
14
20
18
24
13
10
23
21
8
12
9
11
7
4
6
5
22
Right riverbank
Left riverbank

Fig. 3

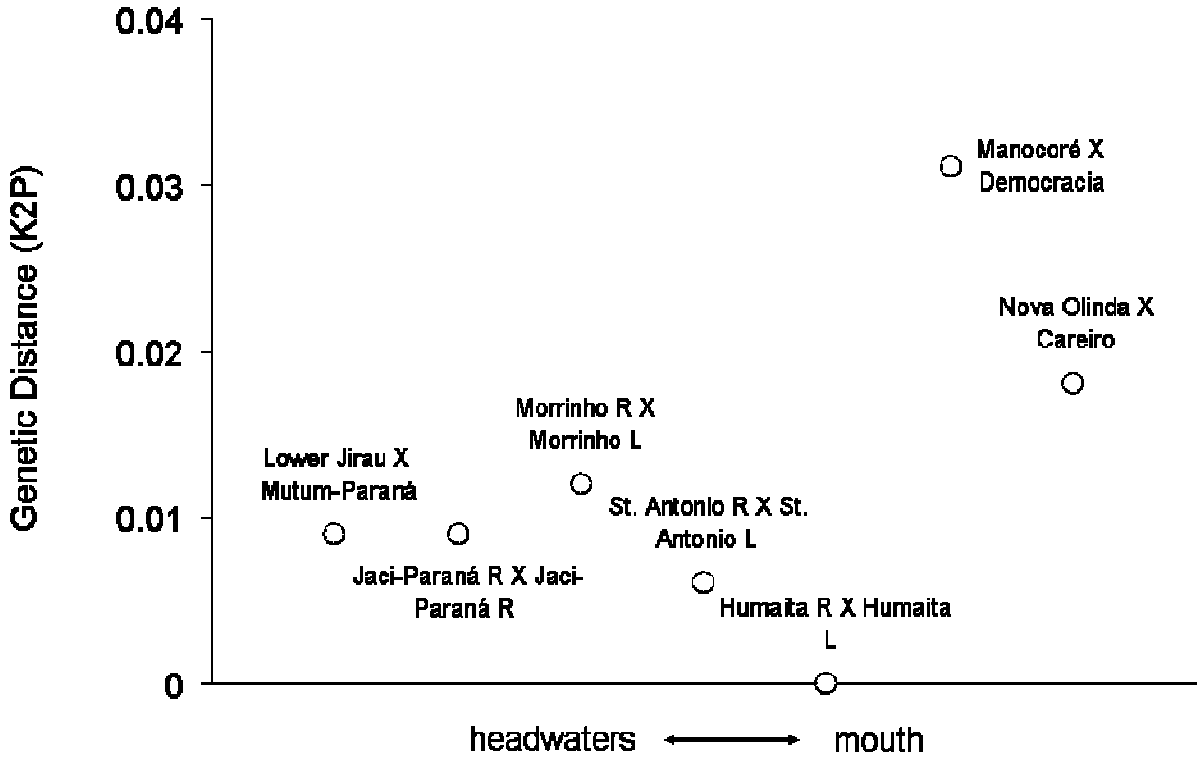
0.04
0.03
0.02
0.01
0
Genetic Distance (K2P)
Manocoré X Democracia
Nova Olinda X Careiro
Morrinho R X Morrinho L
Lower Jirau X Mutum-Paraná
St. Antonio R X St. Antonio L
Jaci-Paraná R X Jaci-Paraná R
Humaita R X Humaita L
headwaters
mouth

Fig.4

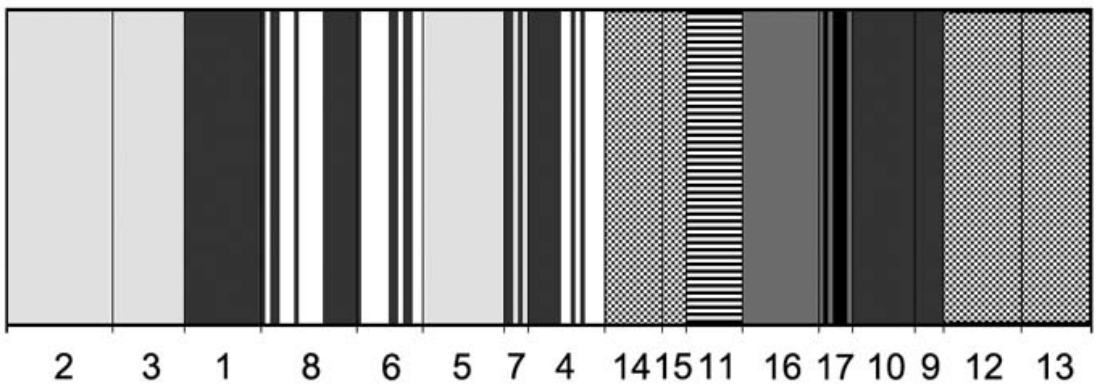
2 3 1 8 6 5 7 4 14 15 11 16 17 10 9 12 13

Fig.5

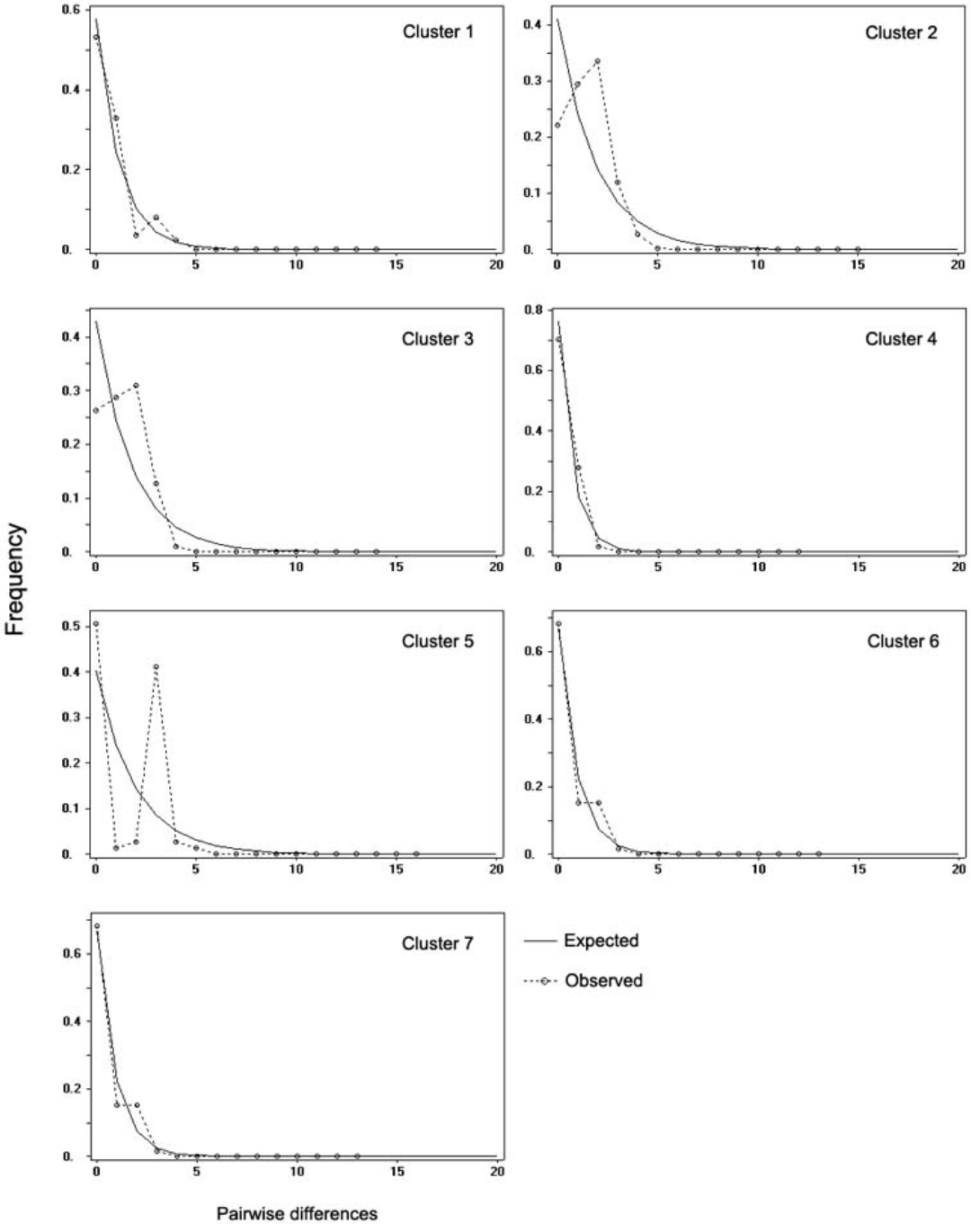
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Expected
Observed
Frequency
Pairwise differences

Fig. 6

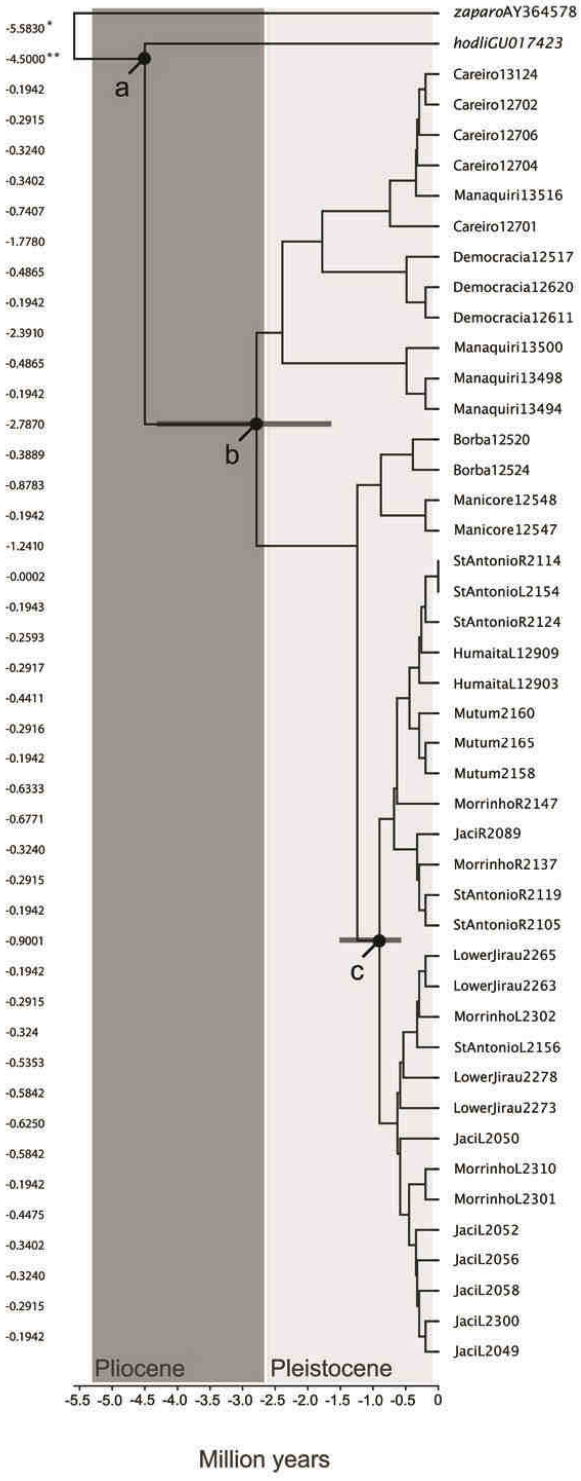
-5.5830*
-4.5000**
-0.1942
-0.2915
-0.3240
-0.3402
-0.7407
-1.7780
-0.4865
-0.1942
-2.3910
-0.4865
-0.1942
-2.7870
-0.3889
-0.8783
-0.1942
-1.2410
-0.0002
-0.1943
-0.2593
-0.2917
-0.4411
-0.2916
-0.1942
-0.6333
-0.6771
-0.3240
-0.2915
-0.1942
-0.9001
-0.1942
-0.2915
-0.324
-0.5353
-0.5842
-0.6250
-0.5842
-0.1942
-0.4475
-0.3402
-0.3240
-0.2915
-0.1942
a
b
c
zaparoAY364578
hodliGU017423
Careiro13124
Careiro12702
Careiro12706
Careiro12704
Manaquiri13516
Careiro12701
Democracia12517
Democracia12620
Democracia12611
Manaquiri13500
Manaquiri13498
Manaquiri13494
Borba12520
Borba12524
Manicore12548
Manicore12547
StAntonioR2114
StAntonioL2154
StAntonioR2124
HumaitaL12909
HumaitaL12903
Mutum2160
Mutum2165
Mutum2158
MorrinhoR2147
JaciR2089
MorrinhoR2137
StAntonioR2119
StAntonioR2105
LowerJirau2265
LowerJirau2263
MorrinhoL2302
StAntonioL2156
LowerJirau2278
LowerJirau2273
JaciL2050
MorrinhoL2310
MorrinhoL2301
JaciL2052
JaciL2056
JaciL2058
JaciL2300
JaciL2049
Pliocene
Pleistocene
-5.5 -5.0 -4.5 -4.0 -3.5 -3.0 -2.5 -2.0 -1.5 -1.0 -0.5 0
Million years

Fig.7

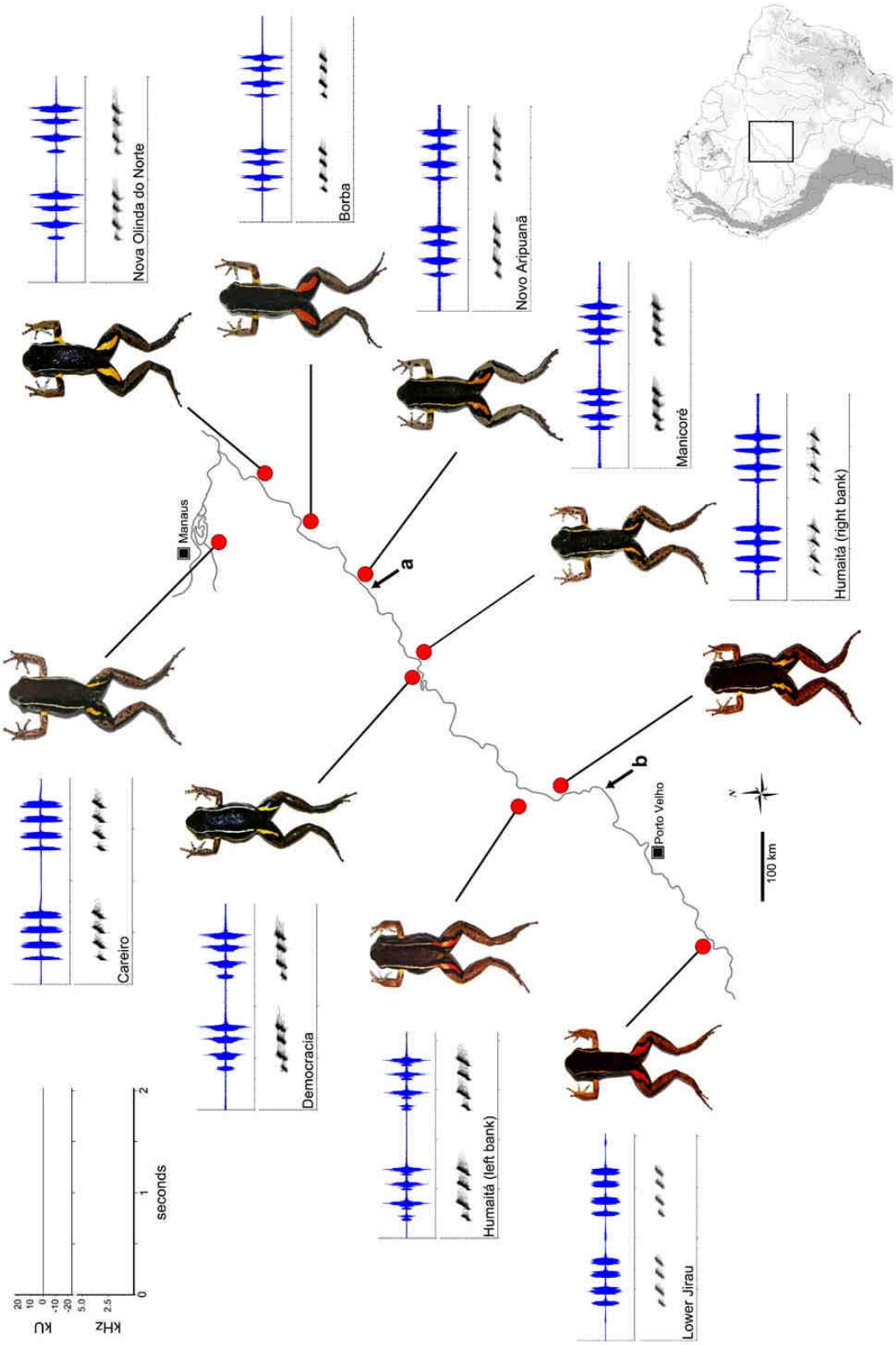
Nova Olinda do Norte
Borba
Novo Aripuanã
Manicoré
Humaitá (right bank)
Careiro
Democracia
Humaitá (left bank)
Lower Jirau
Manaus
Porto Velho
a
b
100 km
N
kU
kHz
seconds

Fig. 8

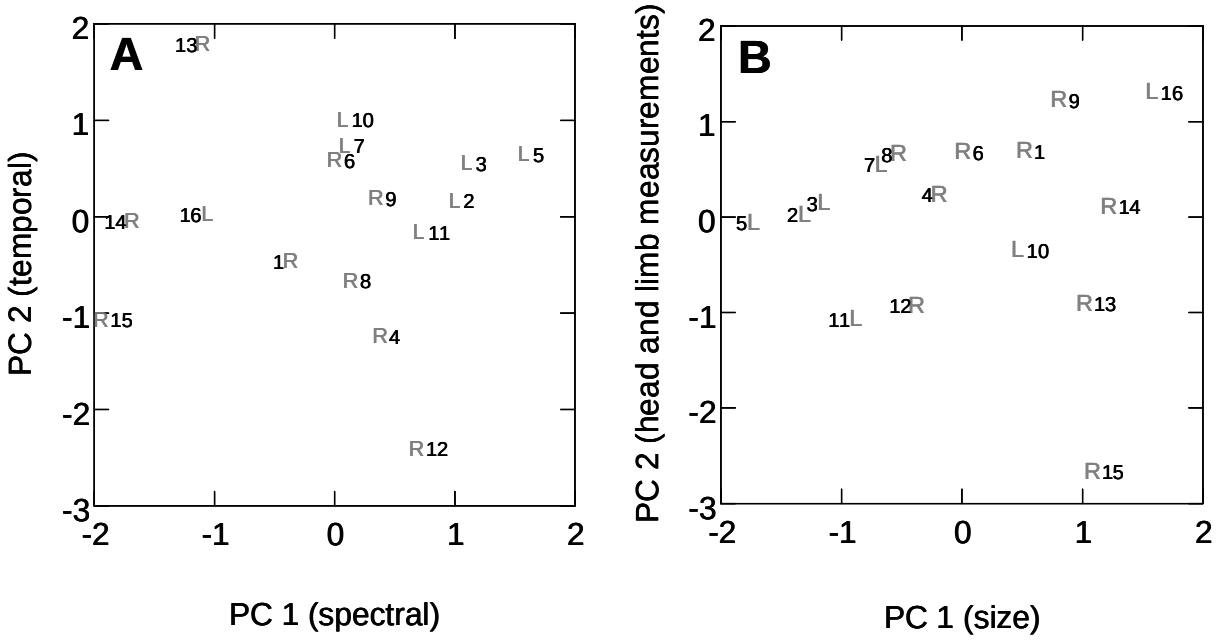
A
B
PC 2 (temporal)
PC 1 (spectral)
PC 2 (head and limb measurements)
PC 1 (size)
13R
L10
L7
R6
L3
L5
R9
L2
14R
16L
L11
1R
R8
R15
R4
R12
R9
L16
7L
8R
R6
R1
4R
2L
3L
5L
R14
L10
11L
12R
R13
R15

Fig. 9

APPENDIX I

List of voucher specimens examined.

Humaitá (right riverbank): INPA-H 26462–26468; **Humaitá (left riverbank):** 26355–26367; **Democracia:** INPA-H 26324–26335; **Manicoré:** INPA-H 26469–26470, INPA-H 26472, INPA-H 26474–26487; **Novo Aripuanã:** INPA-H 26392–26394, INPA-H 26396, INPA-H 26398–26399, INPA-H 26403, INPA-H 26405–26406, INPA-H 26408, INPA-H 26413–26414, INPA-H 26416–26418; **Borba:** APL 12511, APL 12514–12522, APL 12524–12526, APL 12529; **Nova Olinda do Norte:** INPA-H 26336–26341; **Careiro:** INPA-H 26439, INPA-H 26445–26448, INPA-H 26450–26461; **Manaquiri:** INPA-H 26492–26498. **Localities along the upper Madeira river:** INPA-H 16570-16577, INPA-H 16579-16583, INPA-H 16588, INPA-H 16590, INPA-H 16593–16595, INPA-H 16598-16601, INPA-H 16604, INPA-H 16608–16610, INPA-H 16615–16619, INPA-H 16629-16630, INPA-H16642, INPA-H 16644, INPA-H 16649–16817, INPA-H 16820–16826.

SUPPLEMENTARY MATERIAL

Table S1: Arithmetic means and standard deviations of 24 acoustic variables* obtained from *Allobates femoralis* males in 16 localities along the Madeira River, in Brazil. *N* corresponds to total number of individuals sampled. Values in remaining columns correspond to mean±one standard deviation.

Locality	*N*	ICS	D4	MF4	LOF4	HIF4	SIL3	D3	MF3	LOF3
Lower Jirau-L	15	0.436± 0.039	0.074 ±0.007	3587.6 ±139.4	3102.8 ±81.0	3859.4 ±115.4	0.063 ±0.007	0.065 ±0.007	3481.6 ±140.3	3071.6 ±76.2
Jaci-Paraná-R	12	0.444± 0.025	0.070 ±0.008	3384.1 ±144.9	2978.6 ±77.8	3777.2 ±144.7	0.058 ±0.006	0.060 ±0.006	3368.7 ±137.1	2959.2 ±84.9
Jaci-Paraná_L	16	0.453± 0.080	0.077 ±0.009	3580.4 ±121.8	3101.3 ±78.3	3888.5 ±118.4	0.061 ±0.005	0.068 ±0.005	3469.2 ±144.8	3068.3 ±85.3
Morrinho-R	13	0.520± 0.076	0.076 ±0.008	3376.1 ±137.8	2920.1 ±100.5	3696.1 ±137.8	0.058 ±0.008	0.071 ±0.010	3296.3 ±137.2	2903.0 ±63.2
Morrinho-L	13	0.491± 0.052	0.078 ±0.006	3669.5 ±136.7	3151.2 ±74.4	3933.6 ±120.0	0.055 ±0.007	0.072 ±0.006	3590.2 ±150.5	3121.1 ±53.7
Mutum-Paraná-R	14	0.456± 0.050	0.074 ±0.009	3270.0 ±209.2	2846.1 ±117.2	3691.9 ±143.1	0.055 ±0.007	0.067 ±0.008	3203.8 ±190.9	2823.2 ±106.5
St. Antônio-R	14	0.450± 0.042	0.071 ±0.005	3349.0 ±188.1	2936.4 ±107.0	3709.1 ±158.4	0.055 ±0.006	0.066 ±0.006	3277.9 ±159.4	2912.2 ±100.5
St. Antônio-L	5	0.590± 0.083	0.074 ±0.005	3348.4 ±272.4	2928.1 ±72.6	3790.8 ±101.6	0.064 ±0.006	0.065 ±0.005	3282.3 ±187.4	2922.6 ±26.1
Nova Olinda-R	4	0.486 ±0.063	0.075 ±0.007	3085.0 ±160.6	2658.0 ±56.5	3342.6 ±89.9	0.057 ±0.008	0.061 ±0.006	3020.4 ±170.7	2656.9 ±72.3
Democracia-L	12	0.463 ±0.045	0.076 ±0.011	3430.5 ±205.1	3016.7 ±145.5	3869.5 ±171.4	0.053 ±0.007	0.071 ±0.010	3398.6 ±194.7	2988.4 ±141.5
Humaitá-L	3	0.566 ±0.082	0.075 ±0.010	3356.7 ±145.0	2856.9 ±87.6	3738.1 ±78.5	0.065 ±0.003	0.074 ±0.004	3349.6 ±156.7	2859.3 ±94.4
Borba-R	12	0.488 ±0.046	0.078 ±0.011	3054.8 ±119.8	2698.8 ±112.3	3421.4 ±145.6	0.059 ±0.007	0.066 ±0.013	2993.5 ±112.0	2701.1 ±111.2
N. Aripuanã-R	3	0.596 ±0.145	0.088 ±0.005	3225.1 ±106.0	2797.2 ±50.4	3571.8 ±32.5	0.063 ±0.003	0.072 ±0.008	3119.9 ±57.6	2771.0 ±74.4
Manicoré-R	12	0.400 ±0.052	0.067 ±0.005	3402.3 ±155.6	3095.4 ±299.8	3711.4 ±235.6	0.043 ±0.008	0.059 ±0.005	3344.3 ±137.0	3041.2 ±281.8
Careiro-L	8	0.569 ±0.080	0.076 ±0.008	3158.9 ±73.3	2800.4 ±70.1	3589.4 ±65.5	0.068 ±0.006	0.070 ±0.009	3158.9 ±79.6	2771.5 ±62.2
Humaitá-R	5	0.509 ±0.130	0.073 ±0.012	3346.9 ±80.3	3027.8 ±104.4	3712.4 ±94.7	0.066 ±0.011	0.067 ±0.009	3338.3 ±90.0	2996.2 ±85.0

*ICS= Inter-call silent interval (s); D4: duration of fourth note (s); MF4, LOF4, HIF4= maximum, lowest and highest frequencies of fourth note (Hz); SIL3= silent interval between fourth and third notes (s); D3= duration of third note (s); MF3, LOF3= maximum and lowest frequencies of third note (Hz).

Table S1: continued.

Locality	*N*	HIF3	SIL2	D2	MF2	LOF2	HIF2	SIL1	D1	MF1
Lower Jirau-L	15	3782.2 ±109.6	0.102 ±0.012	0.073 ±0.007	3531.6 ±158.1	3059.2 ±73.1	3804.9 ±102.7	0.073 ±0.010	0.044 ±0.004	3276.6 ±81.5
Jaci-Paraná-R	12	3707.2 ±149.2	0.091 ±0.011	0.067 ±0.007	3348.1 ±160.1	2945.9 ±92.8	3728.9 ±146.2	0.065 ±0.007	0.041 ±0.007	3178.6 ±132.2
Jaci-Paraná_L	16	3827.0 ±115.3	0.099 ±0.009	0.076 ±0.008	3481.1 ±175.7	3049.6 ±84.1	3837.7 ±122.1	0.067 ±0.006	0.050 ±0.007	3320.3 ±153.4
Morrinho-R	13	3634.2 ±133.2	0.098 ±0.012	0.078 ±0.008	3340.1 ±143.8	2890.4 ±61.4	3670.1 ±130.6	0.067 ±0.006	0.051 ±0.008	3145.2 ±102.3
Morrinho-L	13	3886.0 ±121.6	0.087 ±0.007	0.077 ±0.005	3612.9 ±167.3	3095.0 ±59.7	3890.1 ±130.2	0.066 ±0.007	0.051 ±0.005	3407.1 ±99.4
Mutum-Paraná-R	14	3635.7 ±135.1	0.089 ±0.005	0.075 ±0.009	3237.2 ±216.2	2799.0 ±103.6	3655.6 ±129.5	0.062 ±0.008	0.048 ±0.008	3032.9 ±120.3
St. Antônio-R	14	3669.1 ±161.5	0.087 ±0.008	0.072 ±0.005	3311.7 ±179.0	2894.5 ±100.2	3680.4 ±154.2	0.063 ±0.008	0.049 ±0.005	3158.7 ±122.9
St. Antônio-L	5	3722.7 ±75.3	0.107 ±0.008	0.073 ±0.004	3299.5 ±227.9	2895.8 ±41.9	3755.0 ±121.6	0.071 ±0.005	0.051 ±0.003	3160.3 ±80.8
Nova Olinda-R	4	3284.1 ±96.0	0.098 ±0.004	0.073 ±0.010	3066.2 ±150.7	2625.0 ±93.8	3291.5 ±92.5	0.071 ±0.008	0.038 ±0.006	2833.4 ±138.0
Democracia-L	12	3804.8 ±169.0	0.080 ±0.010	0.075 ±0.010	3396.4 ±197.4	2944.2 ±143.8	3786.8 ±166.6	0.060 ±0.008	0.052 ±0.009	3203.9 ±182.8
Humaitá-L	3	3691.4 ±84.9	0.099 ±0.006	0.077 ±0.008	3344.8 ±164.5	2864.4 ±88.0	3728.0 ±92.9	0.064 ±0.009	0.054 ±0.006	3182.1 ±190.8
Borba-R	12	3350.9 ±144.9	0.103 ±0.009	0.077 ±0.013	3033.1 ±127.4	2666.8 ±113.4	3385.0 ±143.9	0.071 ±0.007	0.045 ±0.009	2940.6 ±119.9
Novo Aripuanã-R	3	3473.5 ±63.8	0.104 ±0.007	0.085 ±0.003	3170.7 ±151.2	2766.0 ±68.4	3524.3 ±60.9	0.074 ±0.004	0.051 ±0.003	2994.9 ±122.0
Manicoré-R	12	3627.9 ±222.7	0.081 ±0.010	0.068 ±0.005	3374.7 ±151.8	3025.6 ±318.8	3652.8 ±224.0	0.050 ±0.008	0.043 ±0.004	3247.4 ±154.7
Careiro-L	8	3559.9 ±78.7	0.086 ±0.006	0.071 ±0.009	3146.2 ±71.5	2721.7 ±47.7	3484.4 ±66.0	0.073 ±0.006	0.043 ±0.005	2952.4 ±88.0
Humaitá-R	5	3670.7 ±102.4	0.098 ±0.013	0.073 ±0.008	3354.1 ±82.0	2985.3 ±84.2	3679.8 ±101.6	0.063 ±0.009	0.050 ±0.011	3234.9 ±59.6

*HIF3= highest frequency of third note (Hz); SIL2= silent interval between third and second notes (s); D2= duration of second note (s); MF2, LOF2, HIF2= maximum, lowest and highest frequencies of second note (Hz); SIL1= silent interval between second and first notes (s); D1= duration of first note (s); MF1= maximum frequency of first note (Hz).

Table S1: continued.

Locality	*N*	LOF1	HIF1	DC	MFC	LOFC	HIFC
Lower Jirau-L	15	3026.9 ±71.6	3505.9 ±82.9	0.497 ±0.031	3513.5 ±170.0	3011.9 ±74.6	3860.3 ±112.7
Jaci-Paraná-R	12	2902.4 ±106.9	3418.8 ±169.1	0.456 ±0.017	3377.4 ±151.6	2880.7 ±91.1	3796.3 ±152.1
Jaci-Paraná_L	16	3015.1 ±90.9	3581.6 ±141.0	0.502 ±0.029	3484.9 ±162.6	2999.4 ±73.5	3898.2 ±120.8
Morrinho-R	13	2865.3 ±63.2	3407.0 ±110.0	0.504 ±0.044	3341.9 ±137.4	2843.2 ±64.2	3699.1 ±134.0
Morrinho-L	13	3074.1 ±61.5	3638.9 ±126.4	0.489 ±0.021	3593.2 ±169.2	3056.4 ±64.6	3937.4 ±123.3
Mutum-Paraná-R	14	2780.8 ±91.9	3325.8 ±102.2	0.474 ±0.034	3228.9 ±208.7	2738.7 ±105.0	3695.5 ±157.0
St. Antônio-R	14	2850.1 ±91.7	3427.5 ±163.7	0.466 ±0.016	3320.5 ±185.2	2840.4 ±96.3	3720.6 ±154.2
St. Antônio-L	5	2888.2 ±36.5	3481.2 ±85.5	0.510 ±0.012	3311.0 ±221.6	2844.8 ±38.1	3794.9 ±117.7
Nova Olinda-R	4	2600.9 ±93.6	3039.0 ±107.6	0.475 ±0.012	3065.7 ±157.0	2590.4 ±83.5	3340.2 ±78.5
Democracia-L	12	2907.4 ±152.7	3501.6 ±171.2	0.472 ±0.036	3371.4 ±203.7	2900.1 ±139.8	3863.0 ±171.7
Humaitá-L	3	2831.5 ±118.6	3498.8 ±126.8	0.508 ±0.019	3347.2 ±160.6	2829.0 ±122.4	3738.5 ±85.7
Borba-R	12	2676.8 ±118.9	3152.7 ±140.9	0.504 ±0.040	2995.9 ±114.0	2648.5 ±111.9	3413.6 ±143.6
Novo Aripuanã-R	3	2716.9 ±85.2	3294.2 56.7	0.543 ±0.024	3124.1 ±104.1	2729.2 ±77.3	3575.1 ±47.5
Manicoré-R	12	2955.8 ±215.8	3420.9 ±190.5	0.416 ±0.027	3351.2 ±140.7	2978.1 ±344.3	3702.4 ±252.9
Careiro-L	8	2697.7 ±69.8	3190.9 ±101.5	0.488 ±0.033	3140.6 ±81.9	2690.5 ±45.0	3603.5 ±57.7
Humaitá-R	5	2909.1 ±117.3	3491.2 ±80.3	0.495 ±0.020	3352.0 ±91.6	2925.9 ±88.3	3733.6 ±75.9

*LOF1, HIF1= lowest and highest frequencies of first note (Hz); DC= duration of call (s); MFC, LOFC, HIFC= maximum, lowest and highest frequencies of call (Hz).

Table S2: Arithmetic means and standard deviations of snout-to-vent length (SVL) and 19 external morphometric variables* obtained from *Allobates femoralis* males in 16 localities along the Madeira River, in Brazil. *N* corresponds to total number of individuals sampled. Values in remaining columns correspond to mean±one standard deviation.

Locality	***N***	**SVL (mm)**	**HL (mm)**	**HW (mm)**	**SL (mm)**	**ENO (mm)**	**IN (mm)**	**EL (mm)**	**IO (mm)**	**TYM (mm)**	**FAL (mm)**
Lower Jirau-L	17	24.74± 0.85	8.28 ± 0.43	7.98 ± 0.39	4.35 ± 0.39	2.55 ± 0.21	3.65 ± 0.27	2.99 ± 0.27	7.68 ± 0.41	1.58 ± 0.13	6.50 ± 0.27
Jaci-Paraná-R	12	26.50 ± 0.82	8.67 ± 0.42	8.19 ± 0.33	4.86 ± 0.42	2.71 ± 0.34	4.07 ± 0.21	3.13 ± 0.18	8.24 ± 0.35	1.68 ± 0.13	7.17 ± 0.28
Jaci-Paraná_L	17	25.21 ± 1.2	8.34 ± 0.32	7.87 ± 0.35	4.44 ± 0.33	2.49 ± 0.25	3.88 ± 0.16	3.06 ± 0.19	7.89 ± 0.17	1.56 ± 0.18	6.63 ± 0.19
Morrinho-R	13	26.65 ± 1.07	8.74 ± 0.31	8.29 ± 0.33	4.75 ± 0.46	2.76 ± 0.29	4.25 ± 0.24	3.19 ± 0.12	8.17 ± 0.26	1.66 ± 0.15	7.00 ± 0.26
Morrinho-L	13	24.76 ± 1.22	8.18 ± 0.44	7.97 ± 0.43	4.12 ± 0.50	2.46 ± 0.29	3.74 ± 0.19	2.95 ± 0.29	7.56 ± 0.24	1.58 ± 0.13	6.49 ± 0.28
Mutum-Paraná-R	14	26.87 ± 1.37	8.88 ± 0.46	8.45 ± 0.36	4.95 ± 0.35	2.66 ± 0.21	4.34 ± 0.20	3.14 ± 0.17	8.41 ± 0.30	1.71 ± 0.17	7.09 ± 0.34
St. Antônio-R	15	26.26 ± 0.98	8.68 ± 0.33	8.25 ± 0.36	4.63 ± 0.46	2.50 ± 0.24	4.17 ± 0.15	3.21 ± 0.18	8.15 ± 0.30	1.66 ± 0.14	6.89 ± 0.35
St. Antônio-L	5	25.04 ± 1.22	8.34 ± 0.22	8.15 ± 0.34	4.48 ± 0.34	2.50 ± 0.38	3.96 ± 0.19	3.18 ± 0.15	7.90 ± 0.34	1.56 ± 0.15	6.82 ± 0.38
Nova Olinda-R	4	27.67 ± 1.87	9.62 ± 0.24	9.02 ± 0.43	4.98 ± 0.36	3.43 ± 0.10	4.28 ± 0.10	3.33 ± 0.34	8.70 ± 0.39	2.00 ± 0.22	7.46 ± 0.42
Democracia-L	12	24.89 ± 0.75	8.27 ± 0.35	7.96 ± 0.42	4.51 ± 0.29	2.72 ± 0.30	3.71 ± 0.19	3.10 ± 0.21	7.94 ± 0.31	1.63 ± 0.25	6.96 ± 0.33
Humaitá-L	8	26.37 ± 0.60	8.90 ± 0.36	8.49 ± 0.24	4.68 ± 0.29	2.81 ± 0.25	3.96 ± 0.16	3.36 ± 0.21	8.15 ± 0.21	1.85 ± 0.19	7.10 ± 0.29
Borba-R	12	27.34 ± 1.43	9.45 ± 0.55	8.38 ± 0.41	5.11 ± 0.45	3.08 ± 0.41	4.29 ± 0.24	3.45 ± 0.16	8.54 ± 0.37	1.89 ± 0.16	7.61 ± 0.37
Novo Aripuanã-R	11	27.18 ± 1.20	9.33 ± 0.64	8.64 ± 0.55	5.05 ± 0.52	3.13 ± 0.53	4.12 ± 0.18	3.34 ± 0.22	8.35 ± 0.42	1.86 ± 0.21	7.45 ± 0.35
Manicoré-R	12	26.19 ± 0.90	8.50 ± 0.34	7.97 ± 0.17	4.63 ± 0.37	2.86 ± 0.29	3.78 ± 0.13	3.42 ± 0.17	7.94 ± 0.25	1.73 ± 0.17	6.80 ± 0.21
Careiro-L	14	27.29 ± 0.78	9.18 ± 0.50	8.55 ± 0.39	4.48 ± 0.45	2.78 ± 0.44	4.23 ± 0.18	3.39 ± 0.18	8.39 ± 0.26	1.85 ± 0.21	7.67 ± 0.28
Humaitá-R	5	26.92 ± 0.70	8.89 ± 0.36	8.15 ± 0.48	4.80 ± 0.37	2.64 ± 0.34	4.16 ± 0.09	3.50 ± 0.12	8.34 ± 0.34	1.62 ± 0.13	7.19 ± 0.40

*HL=head length from jaw articulation to tip of snout; HW=head width measured at jaw articulation level; SL=snout length from anterior corner of the eye to tip of snout; ENO: distance from anterior corner of the eye to nostril; IN=distance between nostrils; EL=maximum diameter of the eye; IO= inter-orbital distance; TYM=maximum diameter of tympanum; FAL=forearm length.

Table S2: Continued.

	N	H1 (mm)	H2 (mm)	H3 (mm)	WFD (mm)	TL (mm)	FL (mm)	WTD (mm)	LL (mm)	AL (mm)	TAR (mm)
Lower Jirau-L	17	5.21 ± 0.27	4.65 ± 0.26	6.08 ± 0.24	0.82 ± 0.07	11.30 ± 0.28	10.69 ± 0.35	1.07 ± 0.08	10.61 ± 0.71	4.95 ± 0.60	6.75 ± 0.32
Jaci-Paraná-R	12	5.51 ± 0.24	5.10 ± 0.22	6.62 ± 0.27	0.85 ± 0.08	12.16 ± 0.29	11.30 ± 0.45	1.18 ± 0.10	11.53 ± 0.41	5.23 ± 0.27	6.99 ± 0.35
Jaci-Paraná-L	17	5.18 ± 0.21	4.66 ± 0.22	6.21 ± 0.19	0.76 ± 0.06	11.31 ± 0.35	10.73 ± 0.31	1.11 ± 0.09	10.92 ± 0.66	4.99 ± 0.37	7.20 ± 0.36
Morrinho-R	13	5.42 ± 0.26	5.07 ± 0.21	6.51 ± 0.31	0.88 ± 0.08	12.09 ± 0.42	11.01 ± 0.46	1.24 ± 0.12	11.65 ± 0.53	5.33 ± 0.36	6.66 ± 0.63
Morrinho-L	13	5.09 ± 0.36	4.55 ± 0.23	5.98 ± 0.28	0.75 ± 0.06	11.36 ± 0.47	10.82 ± 0.56	1.45 ± 1.95	10.84 ± 0.53	4.88 ± 0.40	7.23 ± 0.23
Mutum-Paraná-R	14	5.68 ± 0.31	5.17 ± 0.33	6.76 ± 0.23	0.88 ± 0.09	12.30 ± 0.39	11.48 ± 0.48	1.18 ± 0.11	11.87 ± 0.55	5.41 ± 0.36	6.94 ± 0.38
St. Antônio-R	15	5.38 ± 0.25	4.96 ± 0.19	6.33 ± 0.21	0.81 ± 0.07	11.93 ± 0.36	10.81 ± 0.39	1.12 ± 0.10	11.54 ± 0.53	4.70 ± 0.42	7.41 ± 0.27
St. Antônio-L	5	5.38 ± 0.28	4.83 ± 0.24	6.43 ± 0.30	0.80 ± 0.00	11.88 ± 0.22	11.17 ± 0.76	1.12 ± 0.13	11.54 ± 0.16	5.24 ± 0.45	6.56 ± 0.64
Nova Olinda-R	4	5.68 ± 0.38	5.46 ± 0.35	6.71 ± 0.28	0.78 ± 0.17	13.06 ± 0.74	11.80 ± 0.36	1.15 ± 0.10	11.56 ± 1.27	5.04 ± 0.50	7.00 ± 0.34
Democracia-L	12	5.24 ± 0.32	4.87 ± 0.33	6.16 ± 0.26	0.73 ± 0.08	11.80 ± 0.40	10.96 ± 0.68	0.98 ± 0.10	11.00 ± 0.45	5.57 ± 0.43	6.93 ± 0.31
Humaitá-L	8	5.82 ± 0.17	5.31 ± 0.39	6.79 ± 0.18	0.81 ± 0.08	12.64 ± 0.25	11.55 ± 0.42	1.21 ± 0.10	11.58 ± 0.52	5.95 ± 0.43	7.66 ± 0.44
Borba-R	12	5.89 ± 0.24	5.39 ± 0.25	7.01 ± 0.42	0.93 ± 0.13	12.74 ± 0.52	11.85 ± 0.48	1.28 ± 0.14	12.14 ± 0.68	5.38 ± 0.38	7.22 ± 0.31
Novo Aripuanã-R	11	6.01 ± 0.43	5.41 ± 0.32	7.01 ± 0.42	0.83 ± 0.08	12.88 ± 0.79	11.59 ± 1.10	1.16 ± 0.15	12.35 ± 0.69	5.66 ± 0.58	6.68 ± 0.45
Manicoré-R	12	5.35 ± 0.19	4.94 ± 0.24	6.45 ± 0.28	0.73 ± 0.07	12.05 ± 0.39	11.13 ± 0.61	1.08 ± 0.09	11.51 ± 0.40	5.74 ± 0.49	7.07 ± 0.46
Careiro-L	14	6.20 ± 0.20	5.66 ± 0.32	7.43 ± 0.30	0.93 ± 0.08	13.49 ± 0.49	12.52 ± 0.37	1.34 ± 0.11	12.37 ± 0.69	5.79 ± 0.47	7.73 ± 0.44
Humaitá-R	5	5.86 ± 0.27	5.52 ± 0.27	7.00 ± 0.22	0.86 ± 0.11	12.91 ± 0.37	12.21 ± 0.44	1.20 ± 0.12	12.16 ± 0.46	5.46 ± 0.38	8.08 ± 0.24

*H1=length of Finger I; H2=length of Finger II; H3=legth of Finger III; WFD=width of Finger III disc; TL=tibia length; FL=foot length; WTD=width of Toe IV disc; LL=leg length; AL=arm length; TAR=tarsus length.

Table S3: Sample names, locality of origin, and accession numbers for sequences used in the mtDNA phylogenetic analyses of *Allobates femoralis* along the Madeira River, Brazilian Amazon.

Sample designation	Locality	Coordinates	16S rDNA	Cyt b
Allobates_hodli2189	Abunã		GU017423	Submitted
LowerJirau2275	Lower Jirau	9.3114° S, 64.7172° W	Submitted	Submitted
LowerJirau2265	Lower Jirau	9.3114° S, 64.7172° W	GU017447	Submitted
LowerJirau2273	Lower Jirau	9.3114° S, 64.7172° W	GU017448	Submitted
LowerJirau2278	Lower Jirau	9.3114° S, 64.7172° W	GU017449	Submitted
LowerJirau12848	Lower Jirau	9.3114° S, 64.7172° W	Submitted	Submitted
LowerJirau12853	Lower Jirau	9.3114° S, 64.7172° W	Submitted	Submitted
LowerJirau12839	Lower Jirau	9.3114° S, 64.7172° W	Submitted	Submitted
LowerJirau12854	Lower Jirau	9.3114° S, 64.7172° W	Submitted	Submitted
JaciParana-Left2050	Jaci-Paraná – left bank	9.1694° S, 64.4289° W	GU017451	Submitted
JaciParana-Left2056	Jaci-Paraná – left bank	9.1694° S, 64.4289° W	GU017454	Submitted
JaciParana-Left2052	Jaci-Paraná – left bank	9.1694° S, 64.4289° W	GU017452	Submitted
JaciParana-Left2062	Jaci-Paraná – left bank	9.1694° S, 64.4289° W	Submitted	Submitted
JaciParana-Left2058	Jaci-Paraná – left bank	9.1694° S, 64.4289° W	GU017455	Submitted
JaciParana-Left2300	Jaci-Paraná – left bank	9.1694° S, 64.4289° W	Submitted	Submitted
JaciParana-Left2299	Jaci-Paraná – left bank	9.1694° S, 64.4289° W	Submitted	Submitted
Morrinho-Left2301	Morrinho – left bank	9.0199° S, 64.2172° W	Submitted	Submitted
Morrinho-Left2302	Morrinho – left bank	9.0199° S, 64.2172° W	Submitted	Submitted
Morrinho-Left2310	Morrinho – left bank	9.0199° S, 64.2172° W	Submitted	Submitted
Morrinho-Left2311	Morrinho – left bank	9.0199° S, 64.2172° W	Submitted	Submitted
Morrinho-Left2312	Morrinho – left bank	9.0199° S, 64.2172° W	Submitted	Submitted
Morrinho-Left2320	Morrinho – left bank	9.0199° S, 64.2172° W	Submitted	Submitted
Morrinho-Left2308	Morrinho – left bank	9.0199° S, 64.2172° W	Submitted	Submitted
Morrinho-Left2316	Morrinho – left bank	9.0199° S, 64.2172° W	Submitted	Submitted
SantoAntonio-Left2154	Santo Antônio – left bank	8.8309° S, 64.0206° W	Submitted	Submitted
SantoAntonio-Left2343	Santo Antônio – left bank	8.8309° S, 64.0206° W	Submitted	Submitted
SantoAntonio-Left2156	Santo Antônio – left bank	8.8309° S, 64.0206° W	Submitted	Submitted
SantoAntonio-Left2155	Santo Antônio – left bank	8.8309° S, 64.0206° W	Submitted	Submitted
Humaita-Right13421	Humaitá – right bank	7.5488° S, 62.8772° W	Submitted	Submitted
Humaita-Right13422	Humaitá – right bank	7.5488° S, 62.8772° W	Submitted	Submitted
Humaita-Right13424	Humaitá – right bank	7.5488° S, 62.8772° W	Submitted	Submitted
Humaita-Right13425	Humaitá – right bank	7.5488° S, 62.8772° W	Submitted	Submitted
Humaita-Left12900	Humaitá – left bank	7.0228° S, 63.1028° W	Submitted	Submitted
Humaita-Left12901	Humaitá – left bank	7.0228° S, 63.1028° W	Submitted	Submitted
Humaita-Left12902	Humaitá – left bank	7.0228° S, 63.1028° W	Submitted	Submitted
Humaita-Left12905	Humaitá – left bank	7.0228° S, 63.1028° W	Submitted	Submitted
Humaita-Left12903	Humaitá – left bank	7.0228° S, 63.1028° W	Submitted	Submitted
Humaita-Left12909	Humaitá – left bank	7.0228° S, 63.1028° W	Submitted	Submitted
SantoAntonio-Right2117	Santo Antônio – right bank	8.6550° S, 64.0195° W	Submitted	Submitted
SantoAntonio-Right2118	Santo Antônio – right bank	8.6550° S, 64.0195° W	Submitted	Submitted
SantoAntonio-Right2119	Santo Antônio – right bank	8.6550° S, 64.0195° W	Submitted	Submitted
SantoAntonio-Right2124	Santo Antônio – right bank	8.6550° S, 64.0195° W	Submitted	Submitted
Morrinho-Right2134	Morrinho – right bank	9.0158° S, 64.0914° W	Submitted	Submitted
Morrinho-Right2137	Morrinho – right bank	9.0158° S, 64.0914° W	Submitted	Submitted
Morrinho-Right2139	Morrinho – right bank	9.0158° S, 64.0914° W	Submitted	Submitted
Morrinho-Right2147	Morrinho – right bank	9.0158° S, 64.0914° W	Submitted	Submitted
JaciParana-Right2086	Jaci-Paraná – right bank	9.2045° S, 64.3620° W	Submitted	Submitted
JaciParana-Right2088	Jaci-Paraná – right bank	9.2045° S, 64.3620° W	Submitted	Submitted
JaciParana-Right2093	Jaci-Paraná – right bank	9.2045° S, 64.3620° W	Submitted	Submitted

Table S3: Continued.

Sample designation	Locality	Coordinates	16S rDNA	Cyt b
JaciParana-Right2093	Jaci-Paraná – right bank	9.2045° S, 64.3620° W	Submitted	Submitted
JaciParana-Right2094	Jaci-Paraná – right bank	9.2045° S, 64.3620° W	Submitted	Submitted
JaciParana-Right2084	Jaci-Paraná – right bank	9.2045° S, 64.3620° W	Submitted	Submitted
JaciParana-Right2076	Jaci-Paraná – right bank	9.2045° S, 64.3620° W	Submitted	Submitted
JaciParana-Right2080	Jaci-Paraná – right bank	9.2045° S, 64.3620° W	Submitted	Submitted
JaciParana-Right2083	Jaci-Paraná – right bank	9.2045° S, 64.3620° W	Submitted	Submitted
MutumD2173	Mutum-Paraná – right bank	9.6414° S, 64.8859° W	Submitted	Submitted
MutumD2166	Mutum-Paraná – right bank	9.6414° S, 64.8859° W	GU017461	Submitted
MutumD2165	Mutum-Paraná – right bank	9.6414° S, 64.8859° W	GU017460	Submitted
MutumD2163	Mutum-Paraná – right bank	9.6414° S, 64.8859° W	Submitted	Submitted
Manicore12552	Manicoré	5.8231° S, 61.2986° W	Submitted	Submitted
Manicore12558	Manicoré	5.8231° S, 61.2986° W	Submitted	Submitted
Manicore12561	Manicoré	5.8231° S, 61.2986° W	Submitted	Submitted
Manicore12551	Manicoré	5.8231° S, 61.2986° W	Submitted	Submitted
Manicore12547	Manicoré	5.8231° S, 61.2986° W	Submitted	Submitted
Borba12525	Borba	4.4342° S, 59.6236° W	Submitted	Submitted
Borba12514	Borba	4.4342° S, 59.6236° W	Submitted	Submitted
Borba12516	Borba	4.4342° S, 59.6236° W	Submitted	Submitted
Borba12519	Borba	4.4342° S, 59.6236° W	Submitted	Submitted
Borba12520	Borba	4.4342° S, 59.6236° W	Submitted	Submitted
NovoAripuana12539	Novo Aripuanã	5.1503° S, 60.3467° W	Submitted	Submitted
NovoAripuana12542	Novo Aripuanã	5.1503° S, 60.3467° W	Submitted	Submitted
NovoAripuana12543	Novo Aripuanã	5.1503° S, 60.3467° W	Submitted	Submitted
NovoAripuana12544	Novo Aripuanã	5.1503° S, 60.3467° W	Submitted	Submitted
NovoAripuana12546	Novo Aripuanã	5.1503° S, 60.3467° W	Submitted	Submitted
NovoAripuana12540	Novo Aripuanã	5.1503° S, 60.3467° W	Submitted	Submitted
NovaOlinda12625	Nova Olinda do Norte	3.8744° S, 59.0461° W	Submitted	Submitted
Democracia12611	Democracia	5.8058° S, 61.4453° W	Submitted	Submitted
Democracia12613	Democracia	5.8058° S, 61.4453° W	Submitted	Submitted
Democracia12616	Democracia	5.8058° S, 61.4453° W	Submitted	Submitted
Democracia12617	Democracia	5.8058° S, 61.4453° W	Submitted	Submitted
Democracia12620	Democracia	5.8058° S, 61.4453° W	Submitted	Submitted
Democracia12622	Democracia	5.8058° S, 61.4453° W	Submitted	Submitted
Democracia12621	Democracia	5.8058° S, 61.4453° W	Submitted	Submitted
Careiro12704	Careiro	3.3708° S, 59.8683° W	Submitted	Submitted
Careiro12706	Careiro	3.3708° S, 59.8683° W	Submitted	Submitted
Careiro12701	Careiro	3.3708° S, 59.8683° W	Submitted	Submitted
Careiro13125	Careiro	3.3708° S, 59.8683° W	Submitted	Submitted
Careiro13119	Careiro	3.3708° S, 59.8683° W	Submitted	Submitted
Careiro13122	Careiro	3.3708° S, 59.8683° W	Submitted	Submitted
Careiro13124	Careiro	3.3708° S, 59.8683° W	Submitted	Submitted
Manaquiri13494	Manaquiri	3.4272° S, 60.6150° W	Submitted	Submitted
Manaquiri13498	Manaquiri	3.4272° S, 60.6150° W	Submitted	Submitted
Manaquiri13500	Manaquiri	3.4272° S, 60.6150° W	Submitted	Submitted
Manaquiri13502	Manaquiri	3.4272° S, 60.6150° W	Submitted	Submitted
Manaquiri13516	Manaquiri	3.4272° S, 60.6150° W	Submitted	Submitted
Manaquiri13492	Manaquiri	3.4272° S, 60.6150° W	Submitted	Submitted

Capítulo III[3]

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Authors: Pedro Ivo Simões*[1], Albertina P. Lima[1] & Izeni P. Farias[2].

Title: Restricted natural hybridization between two species of litter frogs on a threatened landscape in southwestern Brazilian Amazonia.

1 - Coordenação de Pesquisas em Ecologia, Instituto Nacional de Pesquisas da Amazônia, Caixa Postal 478, CEP 69011-970, Manaus, AM, Brazil (pedroivo@yahoo.com.br, lima@inpa.gov.br)

2 - Universidade Federal do Amazonas (UFAM), Departamento de Biologia, Laboratório de Evolução e Genética Animal (LEGAL), Av. Rodrigo Octávio Jordão Ramos, 3000, 69077-000 Manaus, AM, Brazil (izeni_farias@ufam.edu.br).

***Corresponding author:**

E-mail: pedroivosimoes@yahoo.com.br

Phone: 55 92 3643-1832 / 55 92 8165-6549

Fax: 55 92 3643-1909

Abstract

Natural hybridization between allopatric species following secondary contact has been poorly documented for Neotropical anurans inhabiting the Amazonian lowlands. We conducted a genetic survey across a contact zone between two species of litter frogs, *Allobates hodli* and *Allobates femoralis* (family Dendrobatidae), located on the left riverbank of the upper Madeira River, State of Rondônia, Brazil. We obtained tissue samples from 11 sampling sites on both riverbanks, covering approximately a 400 km long transect. We evaluated the genetic relationships between samples using haplotype networks and a distance-based phylogenetic tree obtained from a dataset of 16S rRNA mtDNA sequences. Estimates of genetic diversity, population structure, and identification of sites where genetic admixture occurred were carried out by means of frequency-based methods and Bayesian inference on mtDNA and a set of four microsatellite loci, including samples collected throughout the study area. A reduced dataset including only microsatellite loci genotyped from samples on the left riverbank was applied in assignment tests for detecting levels of admixture at the contact zone and adjacent sampling sites, and for detecting and quantifying hybrid individuals. Our results suggest that genetic introgression between *A. hodli* and *A. femoralis* is greatly restricted to the core area of the contact zone, where potential hybrids are less frequent than parental genotypes. Effects on the genetic variability of adjacent populations are only detected at sites located 1.5 km downstream and upstream of the core area, suggesting the existence of negative selection against hybrids, possibly mediated by postzygotic isolation mechanisms.

Keywords

Amazon, Madeira River, hybrid zone, genetic introgression, Dendrobatidae, *Allobates femoralis*

Introduction

The succession of geological and climatic events occurring from Late Miocene to the present influenced current geographic distribution of animal species in the Amazonian lowlands (see Hoorn and Wesselingh 2010 for a recent review). Most speciation models considered for this region rely strongly on vicariance, and the retraction of past geological or ecological barriers is thought to have triggered the range expansion of many lineages that diverged in isolation, many of which reached secondary contact zones with other, closely related lineages (Haffer 1997; Moritz et al. 2000). Considering species that are distributed in primary rainforests not subject to seasonal flooding (i.e. intolerant to open habitats and floodplains), Amazonian rivers represent obvious boundaries to the geographic range expansion of lineages that evolved in allopatry. However, secondary contact zones are not always coincident with the current location of river channels and suture lines are sometimes found on the same riverbank, often perpendicularly to river channels (Haffer 1997).

Several evolutionary outcomes can be expected from natural secondary contact between two lineages that diverged in allopatry, depending on the extent of neutral or adaptive differentiation accumulated between those lineages while isolated (Barton and

Hewitt 1985; Coyne and Orr 2004; Allendorf and Luikart 2007). One is the formation of hybrid swarms, or populations predominantly constituted by hybrid individuals, originating from several generations of crosses between hybrid individuals or backcrosses between hybrids and parental populations (Seehausen 2004). A second outcome is expected when parental lineages diverged phenotypically and became adapted to distinct extremes of an environmental gradient, rendering a clinal or patchy contact zone, with frequency and direction of hybridization largely related to resource or habitat distribution, rendering a smooth gradient or a mosaic of parental and hybrid genotypes (e.g. Vorndran et al. 2002; Keller et al. 2008). The third and most common outcome of secondary contact is the establishment of very narrow hybrid zones, dependent on the balance between selection against hybrid individuals and migration of parental genes from adjacent populations. These are frequently referred to as “tension zones”, and can be characterized by the presence of parental genotypes within samples and geographically limited introgression of parental lineages or hybrid genotypes from the core area of the contact zone into the distribution of the second parental lineage (Barton and Hewitt 1985; Arnold et al. 1999; Jiggins and Mallet 2000). The local evolution of lineages following secondary contact is generally unpredictable as these models are density dependent, and selection regimes can change in time according, for example, to environmental conditions (Levin et al. 1996; Grant and Grant 1997). However, a few evolutionary trends can be presumed from the characterization of hybrid zones (such as the geographic replacement of parental populations by hybrid swarms, or the establishment of hybrid sinks reducing local genotypic variability), often with potential use for conservation planning (Seehausen et al. 2007; Dawe et al. 2009; Hird and Sullivan 2009).

Hybrid zones or suture lines between closely related anuran species are frequently found along limited transects of their peripheral geographic distribution (Barton and Hewitt 1985; Jiggins and Mallet 2000; Wells 2007; Lemmon et al. 2007; Vogel and Johnson 2008; Moritz et al. 2009). However, the occurrence of contact zones and the description of areas of possible genetic introgression between divergent lineages of Amazonian lowland anurans have been poorly documented in the literature (e.g. point records are briefly mentioned in Brown and Twomey 2009; Simões et al. 2010).

In early 2005, a narrow and well-delimited contact zone between two species of Amazonian frogs of the genus *Allobates* (Family Dendrobatidae) was discovered on the left riverbank of the upper Madeira River (Simões et al. 2008). The contact zone coincides with the boundary between two geomorphological units, evidenced on the channel of the river by a group of large rapids, locally known as Cachoeira do Jirau. At the time, the two species were thought to represent distinct morphotypes of the widespread brilliant-thighed poison frog, *Allobates femoralis* (Simões et al. 2008; Amézquita et al. 2009). Recently, summing information on the geographic distribution, mtDNA molecular phylogeny, and available evidence on morphological and acoustic differentiation, one of the former morphotypes was described as a new species, which has a restricted geographic distribution, being parapatric to, and highly divergent from the *A. femoralis* populations inhabiting the upper Madeira River basin (Simões et al. 2010).

Despite its recognition for at least five years, the contact zone between *A. hodli* and *A. femoralis* has not been subject to detailed studies aiming at its characterization and current evolutionary dynamics. As the two species are not each other's sister clades (Simões et al. 2010), and have been distinct lineages for at least 2.5 million years (and most probably for around 4.5 million years - Santos et al. 2009), the presence of mtDNA markers

typical of one of the lineages within the genome of the other can be unambiguously attributed to genetic introgression rather than to incomplete lineage sorting from a recent polymorphic common ancestor.

In this study, we conduct a genetic characterization of the contact zone between *A. femoralis* and the recently described *Allobates hodli*, evaluating the occurrence of hybridization between these two species. Additionally, we evaluate how secondary contact affects the local distribution of genetic variability in comparison to nearby populations of both species using mtDNA and microsatellite markers. As current development policies are ubiquitous along this segment of the Madeira River (Clemons 2007), our main goal is to provide a first insight into the natural patterns of genetic structure among these model species. This information can be used as a valuable guideline for monitoring programs aiming at accessing the impacts of contemporary environmental changes resulting from such policies.

Methods

Study area

The study area comprises *terra-firme* (not seasonally flooded) forests along a ≈400 km segment of the upper Madeira River, in southwestern Brazilian Amazon, from the village of Fortaleza do Abunã, in Rondônia, to the vicinities of the city of Humaitá, State of Amazonas (Fig. 1). Along this segment, the Madeira River is generally entrenched, 1 km wide in average, flowing fast through a system of successive rafts and rapids. Expressive

areas of floodplains occur adjacent to the river channel only downstream of Porto Velho, corresponding to areas included in the municipality of Humaitá (DNPM 1978).

Within the study area, *Allobates hodli* is distributed exclusively on the left riverbank, occurring from localities across the river from Fortaleza do Abunã to the level of the Cachoeira do Jirau rapids. Downstream of the Cachoeira do Jirau rapids, and across the right riverbank, *A. hodli* is replaced by *A. femoralis* (Fig. 1b). The two species are easily distinguished by their advertisement calls (Fig. 1c), in addition to characteristic color patterns (Simões et al. 2010).

Although the contact zone between *A. hodli* and *A. femoralis* is restricted to the left riverbank, mtDNA haplotype sharing is known to occur between *A. femoralis* populations on opposite banks in regions near Humaitá (Simões et al. unpublished data). Possible cases of DNA introgression between *A. femoralis* inhabiting the right bank of the upper Madeira River and *A. hodli* have not been verified in previous studies. Therefore, samples from three sites on the right riverbank (9, 10, and 11, Fig. 1b) were used to evaluate potential genetic admixture between these two groups of populations prior to hybridization analysis, which were restricted to samples collected along the left riverbank.

Molecular data acquisition

Allobates hodli and *Allobates femoralis* muscle tissue samples were housed at Coleção de Tecidos de Genética Animal at Universidade Federal do Amazonas (CTGA – ICB/UFAM – Appendix I), Manaus, Brazil, originating from field work carried out at 11 sampling sites along the study area (Table 1, Fig. 1b), which were visited in different occasions between 2004–2009 by P.I.Simões and A.P. Lima. Fragments of the 16S rRNA

mitochondrial gene for some of these locations were already available on GenBank (Appendix II). We complemented the available 16S dataset by including additional sequences obtained from samples from the same localities and sequences from the remaining sampling sites. Sequences and microsatellite markers used in population and hybridization analyses were amplified according to the following laboratory protocols.

Total genomic DNA was extracted from preserved muscle or liver tissue samples using a cetyl trimethyl ammonium bromide (CTAB) protocol (modified from Doyle and Doyle 1987). We used primers 16Sar and 16Sbr (Palumbi, 1996) to amplify a 507 b.p. fragment of the 16S rRNA mitochondrial gene via polymerase chain reaction (PCR). PCR reactions used a final volume of 16 µL and contained 6.7 µL ddH_2O, 2.0 µL of 25 mM $MgCl_2$, 1.5 µL of 10 mM dNTPs (2.5mM each dNTP), 1.5 µL of 10X amplification buffer (75 mM Tris HCl, 50 mM KCl, 20 mM $(NH_4)2SO_4$), 1.5 µL of a 2 µM solution of each primer, 0.3 µL of Taq DNA Polymerase 5 U/µL (Biotools, Spain) and 1 µL of DNA (about 30 ng/µL). PCR conditions had a pre-heating step of 92°C for 60 s, followed by 35 cycles of denaturation at 92° for 60 s, primer annealing at 50°C for 50 s and primer extension at 72°C for 90 s. A final extension step occurred at 72°C for 5 min. Sequencing reactions were performed according to manufacturer's recommended ABI BigDye Terminator Cycle Sequencing protocol, using primer 16Sbr and an annealing temperature of 50°C. Sequencing was performed in an automatic ABI 3130xl sequencer (Applied Biosystems).

In addition to 16S rRNA sequences, we used four pairs of primers described by Jehle et al. (2008) in order to amplify four microsatellite loci from samples from both species (Epifem 03, Epifem 05, Epifem 12 and Epifem 13). PCR reactions used a final volume of 10.5 µL, and cointained 2.6 µL ddH_2O, 1.3 µL 25mM $MgCl_2$, 1.3 µL 10 mM dNTPs, 2.0 µL of 10X amplification buffer, 1.0 µL of a 2 µM solution of reverse primer,

0.5 µL of a 2 µM solution of forward primer, 0.5 µLof a 2 µM solution of the M13 primer, 0.3 µL µL of Taq DNA Polymerase 5 U/µL and 1.0 µL of DNA (30 ng/µL). PCR conditions used pre-heating step of 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s and extension at 72°C for 45 seconds. The annealing of M13 primers occurred subsequently, applying 15 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 45 s, and extension at 72°C for 45 seconds. Final extension occurred at 72°C for 30 min. PCR products were genotyped in an automatic ABI 3130xl sequencer. Resulting genotypes were inspected in GeneMapper 4.0 (Applied Biosystems), and allele sizes were inferred by comparisons with peaks of known size produced by ROX-labeled size standards (DeWoody et al. 2004).

The six remaining pairs of primers described by Jehle et al. (2008) either rendered monomorphic alleles across the study populations (Epifem 06), or failed to successfully amplify the respective microsatellite markers in all (Epifem16, Epifem17) or in a set of particular populations (Epifem 09, Epifem 14, Epifem15). These markers were characterized from a single population from the vicinities of Santarém, State of Pará, Brazil, located at least 1050 km from our study area, and might not be applicable to all populations referred to as *A. femoralis*, which comprise a group genetically divergent cryptic species (Grant et al. 2006; Santos et al. 2009), possibly due to substitutions on primer annealing sites.

Mitochondrial DNA analyses

The 16S rDNA sequences were initially aligned using the ClustalW algorithm (Thompson et al. 1994) implemented in BioEdit (Hall 1999), verified by eye, and corrected

manually, when necessary. Gaps and substitutions were checked by comparisons with the original cromatographs. In order to evaluate the genealogical relationships among haplotypes and overall haplotype distributions, haplotype networks were built from the resulting alignment by methods of statistical parsimony (Templeton et al. 1992) using TCS 1.21 (Clement et al. 2000), and applying a 95% connection limit, considering gaps as a 5^{th} character state. Analysis of DNA polymorphism and estimates of genetic diversity were carried out in DnaSP v.5.10 (Librado and Rozas 2009) for samples of each species and from each sampling site, separately.

We applied a Bayesian analysis of population structure on nucleotide frequencies (Corander and Tang 2007; Corander et al. 2008) over the 16S rDNA database in order to estimate the most probable number of genetic clusters formed by samples along the study area, and to evaluate the existence of sites where mtDNA introgression between clusters occurred. Analysis were run in BAPS 5 (Corander and Tang 2007; Corander et al. 2008), taking the number of clusters as a random parameter and setting the upper bound to one or up to eleven clusters (the latter corresponding to the total number of sampling sites). Five independent runs were performed for each upper bound value, and selection of the most probable cluster configuration was made by comparing the log-likelihood values of the best models. The evolutionary relationships between samples were further verified by reducing the 16S rDNA database to unique haplotypes, from which we obtained a Neighbor-Joining tree (Saitou and Nei 1987) based on Tamura-Nei genetic distances (Tamura and Nei 1993) in MEGA 4.1 (Tamura et al. 2007).

Population structure and hybridization analysis using microsatellites

Description of microsatellite loci variability and evaluation of genetic diversity parameters for each sampling site were carried out in GENALEX 6 (Peakall and Smouse 2006). Measures of *F*st between sampling sites based on Weir and Cockerham estimates and heterozygote deficit within populations (*F*is) were calculated in FSTAT 2.9.3.2 (Goudet 2001).

We investigated the existence of large scale population structuring and admixture within the study area based on microsatellite markers using Bayesian inference, as implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000). This model-based approach uses information on allele frequencies and assumes Hardy-Weinberg and linkage equilibrium between loci within each inferred genetic cluster to approximate the posterior probability of the actual number of clusters. Once reliable information on the number and distribution of clusters is obtained, it is possible to assign the proportion of each individual's genome that originated from a particular cluster. In a preliminary analysis, we used samples from all sites, and assumed the number of possible genetic clusters formed by these samples (K) to vary from one to ten. This analysis was run for 15 iterations with one million MCMC replicates after 100.000 initial replicates which were discarded as burn-in, applying an admixture model and considering allele frequencies to be independent from each other. The most probable number of clusters was selected graphically according to the mean increase in posterior probabilities observed from each value of K to K+1 between all iterations. The results allowed us to confirm the number of clusters suggested by the mtDNA population structure analysis described above and to identify sampling sites where genetic admixture between riverbanks occurred. Results were also used to select populations experiencing no (or very reduced) genetic admixture regimes on the left riverbank, which could be assigned as pure parental populations in the hybridization analyses described below.

239 Analyses of hybridization focused on samples from sites 3, 4 and 5, corresponding
240 to the core contact zone between the two species (site 4), and to sites located ≈1.5 km
241 upstream (site 3) and downstream (site 5). Assignment tests were carried out in STRUCTURE
242 2.3.3 and in NEWHYBRIDS (Anderson and Thompson 2002). Like STRUCTURE, the
243 NEWHYBRIDS method is capable of estimating the probability of assignment of each
244 individual to a particular genotype class by accessing allele frequency variation between
245 species (i.e. the two methods do not depend on diagnostic loci, with exclusive alleles fixed
246 in each species). In addition to estimating the probability of assignment of one individual to
247 one of the species that are potentially hybridizing (as in STRUCTURE), the NEWHYBRIDS
248 method estimates the posterior probability of that individual belonging to a particular
249 hybrid generation or category based on expected genotypic frequencies (i.e. F_1, F_2, parental
250 backcrosses). Both methods provide the posterior probability of membership of each
251 individual to an alternative genotypic category, allowing for posterior inferences about
252 evolutionary mechanisms regulating the hybrid zone dynamics (Jiggins and Mallet 2000).
253 Recent tests show that these methods do not outperform each other when using
254 microsatellite data, producing complementary results (Sanz et al. 2009).
255 Species assignment probabilities were accessed in STRUCTURE by setting the
256 number of possible genetic clusters (K) to two (*A. hodli* / *A. femoralis*). As very strong data
257 are necessary to overcome misclassification when priors on pure parental populations are
258 provided, we did not include any prior information about parental populations in the
259 STRUCTURE analysis. Analysis parameters were similar to the previous analysis considering
260 all samples, applying 100.000 burn-in replicates followed by one million MCMC replicates
261 after 100.000, considering an admixture model and independent allele frequencies. Average

values between 20 iterations are presented for all individuals (q_i) and for within-sampling site (Q) membership coefficients.

For the NEWHYBRIDS analysis, we employed four distinct frequency categories: pure *A. femoralis*, pure *A. hodli*, F_1, and F_2 hybrids. As the small number of loci would probably prevent the correct distinction between pure parental lineages and hybrids originating from backcrosses (Boecklen and Howard 1997), parental backcross categories were not considered. Information on species origin was provided for putative parental individuals of pure *A. hodli* or *A. femoralis* origin collected in sampling sites not close to the contact zone, which were selected from the previous population structure analyses (see Results). This was done by applying the "z" option to the input file, as recommended by the software's programmers. NEWHYBRIDS analysis was run for five million sweeps after 500.000 burn-in steps, applying Jeffreys-type prior distributions to allele frequency and mixing proportion parameters.

Results

Mitochondrial 16S rDNA sequence analysis

We obtained 16S rDNA sequences from 222 individuals distributed throughout the 11 sampling sites in the study area. These corresponded to 47 unique haplotypes, which were generally species-exclusive and constituted independent haplotype clusters in the TCS parsimony network analysis (Fig. 2, Fig. 3). Both *Allobates femoralis* and *Allobates hodli* haplotypes were found in the core area of the contact zone, but haplotypes associated with the *A. hodli* lineage are more diverse among Contact Zone samples (six haplotypes, against

286 a single *A. femoralis* haplotype). A single case of mitochondrial DNA introgression
287 between species was detected approximately 1.5 km downstream of the contact zone, at
288 Lower Jirau (site 5, Fig. 1b, Fig. 3), where one *A. femoralis* male (original field number /
289 tissue collection number APL-2276) with typical four-note advertisement call carried an *A.*
290 *hodli* haplotype. No *A. femoralis* haplotypes occurring on the right riverbank were found
291 among *A. hodli* samples or among *A. femoralis* samples upstream of Jaci-Paraná (site 6 –
292 Fig. 3).
293 Genetic diversity estimates were generally lower for pooled samples of *A. hodli*, in
294 comparison to pooled samples of *A. femoralis* (Table 2). Within *A. femoralis*, samples from
295 the right riverbank had lower values for genetic diversity estimates than samples from the
296 left riverbank. Estimates measured for each sampling site separately (Table 2) revealed a
297 sudden increase in nucleotide diversity (π) and genetic diversity estimates (Θ_π, Θ_S) from
298 Jirau (site 3) towards the Contact Zone (site 4), reflecting the mixed occurrence of *A. hodli*
299 and *A. femoralis* haplotypes at this site. Estimated values drop dramatically from the
300 Contact Zone towards Lower Jirau (site 5), except for the genetic diversity based on the
301 number of segregating sites (Θ_S), which increases discretely at this site. Conversely,
302 haplotype diversity drops from the Contact Zone towards Lower Jirau, where only six
303 haplotypes are observed among 33 samples. Additional cases of lowered haplotype
304 diversity are found in Humaitá, at sampling sites on both riverbanks (sites 8 and 9).
305 Bayesian analysis of population structure on the complete mtDNA dataset indicated
306 the existence of three genetic clusters (log ML = -915.3804; posterior probability = 0.99927
307 – Fig. 4). Two clusters correspond to *A. femoralis* samples, and are roughly structured
308 according to riverbanks (Fig. 4), with some degree of admixture on the left bank, at
309 sampling sites 6, 7 and 8, reflecting haplotype sharing between localities across the river, as

seen above. The third cluster corresponds to *A. hodli* samples from sites 1, 2 and 3, and admixture with the *A. femoralis* cluster exclusive of the left bank occurs in the contact zone at site 4. The same *A. femoralis* individual (APL-2276) reported above as possessing an *A. hodli* haplotype at site 5 was placed in the *A. hodli* cluster.

The Neighbor-Joining tree based on genetic distances between unique 16S rDNA haplotypes revealed two highly supported (bootstrap value = 99%) monophyletic clades (Fig. 5), corresponding to *A. hodli* and *A. femoralis* samples. No subclades are supported according to the bootstrap analysis (all bootstrap values < 40%). Haplotypes representative of both clades are found in the contact zone.

Population structure analysis inferred from microsatellites

The four microsatellite loci were successfully genotyped from samples of a total 195 individuals from the 11 sampling sites. Among these, Epifem 05 had the lowest number of alleles and heterozygote genotypes (Table 3). Estimates of microsatellite diversity per locus at each sampling site (Table 4) indicate a slight increase in number of alleles at the Contact Zone and Lower Jirau, while observed heterozygosity was generally lower at both sampling sites immediately adjacent to the Contact Zone. Values for all diversity estimates decrease abruptly at the level of Santo Antônio, on the left bank (site 7). Estimates averaged between all loci maintain a similar pattern (Table 5). Heterozygote deficit within sampling sites (estimated as *F*is) was relatively pronounced at Jirau (Table 5), approximately 1.5 km upstream the core area of the contact zone, and corresponding to a predominantly *A. hodli* population (see below). At this site, a large number of private

alleles are also found, in comparison to adjacent sampling sites. *F*st values between sampling sites were generally low, rarely exceeding 0.2 (Table 6).

Based on the average between 15 iterations run in STRUCTURE, the posterior probability among alternative numbers of clusters plateaus at K=3 (Fig. 6), with an abrupt decrease in the magnitude of likelihood change from K=3 to K=4. Selecting three as the actual number of genetic clusters rendered geographic distribution of clusters based on microsatellite markers similar to that obtained with mtDNA data (as suggested by BAPS analysis described above) (Fig. 7). One of the clusters is constituted by *A. hodli* samples, and is distributed from Abunã (site 1) to Jirau (site 3), experiencing admixture with one of the two *A. femoralis* clusters at the Contact Zone. Considering the two *A. femoralis* clusters, one is restricted to the left riverbank, from the Contact Zone to Jaci-Paraná, and the second occurs downstream on the same riverbank, as well as in all sampling sites on the right riverbank. As the two clusters meet at Jaci-Paraná, we removed three samples that had proportions of membership (q_i) to the downstream/right bank cluster superior to 90% from the Jaci-Paraná pool. The remaining 12 individuals from Jaci-Paraná were used in the subsequent analysis of hybridization as a sample of pure parental *A. femoralis* genotypes. Importantly, no evidence of recent introgression or admixture from the right to the left riverbank upstream of Jaci-Paraná was evident from mtDNA or microsatellite markers.

Hybridization analysis

Hybridization analyses were performed on 145 individuals from six sampling sites on the left riverbank. Individuals from Abunã and Mutum-Paraná (sites 1 and 2) were considered pure parental *Allobates hodli* populations (n = 34), while individuals from Jaci-

357 Paraná (n = 12) were considered pure *Allobates femoralis*. The analyses focused on the
358 remaining individuals (n = 99), sampled across the contact zone from Jirau to Lower Jirau
359 (sites 3, 4 and 5).

360 Bayesian admixture analysis conducted in STRUCTURE revealed a steep trend in the
361 average proportion of species membership associated to each population from Jirau to
362 Lower Jirau, largely concentrated in the core area of the contact zone (site 4). Proportions
363 of membership to one of the two species estimated from overall samples at this core area
364 were almost equivalent, indicating a high level of genetic admixture (Fig. 8). Estimated
365 average proportions of membership to *A. hodli* (Q_h) increases abruptly upstream, while
366 membership to *A. femoralis* (Q_f) increases on the opposite direction. Average individual
367 membership coefficients highlight the presence of extensive admixture in the core area of
368 the contact zone, with introgression of only a few individuals bearing genotypes attributed
369 to the alternate species at adjacent sites downstream and upstream (Fig. 9).

370 The NEWHYBRIDS analysis confirmed the presence of possible hybrid individuals in
371 the core area of the contact zone (5 of 30 individuals with >50% posterior probability of
372 assignment to hybrid categories at Contact Zone site), but the frequency of individuals
373 bearing hybrid genomes decreases abruptly at immediately adjacent sampling sites (Fig. 9).
374 Among 10 females genotyped from the core area of the contact zone, only one had a
375 posterior probability superior to 40% of belonging to a hybrid class. Among the 20 males
376 collected at the same site, six (30% of total males) surpassed this threshold.

377 All hybrids were strongly attributed to F_2 genotypic class, and are consequently
378 considered to be more closely related to parental genotypes than expected for F_1
379 generations. The contact zone has a clear bimodal pattern, with a few individuals presenting
380 high probabilities of bearing intermediate genotypes, and parental genotypes being frequent

even in the contact zone's core (Fig. 9). The analyses assigned a 60% probability of a pure *A. femoralis* origin and 40% probability of a F_2 hybrid origin to the *A. femoralis* male found to bear an *A. hodli* haplotype collected at Lower Jirau (APL-2276).

Discussion

Results from genetic analyses across the contact zone between *Allobates hodli* and *Allobates femoralis* on the left riverbank of the upper Madeira River suggest that it conforms better to a tension zone model than to a case of insipient hybrid swarm or to a clinal model with gradual replacement of genetic characteristics from one species towards the alternate species range (Barton and Hewitt1985). As typical of such tension zones, genetic admixture and hybridization between the two species is greatly restricted to the core area of the contact zone (namely, to sampling site 4). This is reflected in local genetic diversity estimates, as indexes based on nucleotide and allele diversity increase at the core zone as a result of admixture between genomes of both species. On the other hand, estimates based on haplotype diversity and heterozigosity indicate reduced diversity immediately downstream and severe heterozygote deficit upstream of this area, supporting the existence of selective pressures preventing gene flow past the areas adjacent to the core zone.

A single case of mtDNA introgression was observed from *A. hodli* towards the distribution of *A. femoralis* at Lower Jirau (site 5), where one *A. femoralis* male carried an *A. hodli* haplotype. This represents a frequency of less than 4 % of introgressed *A. hodli* haplotypes into *A. femoralis* distribution, only 1.5 km away from the core hybrid zone. This individual was subsequently assigned to a *A. femoralis* / F_2 hybrid origin by analysis of

405 microsatellite markers, and probably results from a considerable number of backcrosses
406 involving hybrid individuals and *A. femoralis*. Haplotypes characteristic of *A. hodli* prevail
407 in frequency and richness at the core area, while no *A. femoralis* haplotypes are found
408 upstream (and consequently within *A. hodli* geographic distribution).

409 Narrow contact zones with a bimodal pattern of genotypic distribution are usually
410 related to prezygotic barriers to gene flow, mediated by assortative mating or fertilization
411 (Jiggins and Mallet 2000). Strong prezygotic selection by females is a phenomenon
412 common to anuran hybrid zones, which are often characterized by marked character
413 displacement and reinforcement driven by female selection on acoustic traits (Höbel and
414 Gerhardt 2003; Pfennig 2003; Hoskin et al. 2005). Although the artificial manipulation of
415 advertisement calls are known to have effects on male to male aggressive behavior in the
416 *Allobates femoralis* group (Hödl et al. 2004; Göd et al. 2007), playback experiments
417 broadcasting natural calls within territories of *A. femoralis* and *A. hodli* males along the
418 Maderia River contact zone detected no differences in aggressive (phonotatic) behavior
419 towards conspecific or heterospecific calls (L.K. Erdtmann and P.I. Simões, unpublished
420 data). Tests addressing female mate choice are still needed in order to corroborate the
421 existence and the strength of a behavioral reproductive barrier. However, the available
422 evidence obtained so far from male response to playback experiments and the presence of
423 hybrids along the contact zone suggest that any behavioral prezygotic barriers between the
424 parental species are, at least, leaky.

425 Thus, current data offer better support the hypothesis that the maintenance of the
426 current contact zone is related to postzygotic isolation mechanisms. Among these, the
427 existence of genetic incompatibilities over multiple loci, or reduced fitness of F_1 hybrids
428 (which are apparently rare across the contact zone area) are examples of possible intrinsic

429 and extrinsic factors regulating contact zone position and width. While a reduced number of
430 polymorphic genetic markers are generally sufficient to point out the existence of hybrid
431 individuals along a contact zone, discrimination between alternate hybrid classes will often
432 demand many more markers. Particularly, the distinction between parental populations and
433 backcrosses might require several dozens (Boecklen and Howard 1997).
434 Although fitness can vary between hybrid generations, and reduced F_1 survival does
435 not necessarily imply in absence of F_2 hybrids, hybrid classification according to
436 NEWHYBRIDS should be viewed with caution due to the small number of microsatellites
437 employed. This analysis is conservative in the sense that hybrids are classified according to
438 genotype frequencies among all loci expected under Mendelian laws of inheritance
439 (Anderson and Thompson 2002). Thus, although useful for revealing the frequency and
440 extent of hybridization between both study species along the left bank of the upper Madeira
441 River, these results allow us to make no strong assumptions about genetic bottlenecks
442 affecting particular hybrid generations, as some F_1 hybrids might have been misclassified
443 as F_2.
444 It is important to stress that all individuals collected in the core area of the contact
445 zone were adults, and most of the individuals to which considerable probability of
446 belonging to an hybrid class were male. These observations suggest possible sexually-
447 related trends on hybridization dynamics, such as increased viability or survival of hybrid
448 males. Future analysis including sex-linked genetic markers will be useful for clarifying
449 these trends. A broader array of neutral markers should also be applied to precisely confirm
450 the assignment of individuals to distinct hybrid classes, ruling out more elaborate
451 hypothesis such as hybrid breakdown by unviable admixture of genetic backgrounds among
452 F_2 or backcross progeny (Burton et al. 2006).

Natural or human induced environmental changes can rapidly shift the prevailing balance between gene flow from parental populations and localized selection against hybrids along narrow hybrid zones by their effects on available resources and/or population density (Grant and Grant 1993, 2002; Haig et al. 2004; Keller et al. 2008; Genovart 2009). Currently, the *A. hodli*/*A. femoralis* hybrid zone on the left bank of the upper Madeira River seems to be stabilized by selective pressures against hybrids or genetic incompatibility mechanisms, being restricted in width to less than three kilometers, largely coincident with the transition zone between distinct geomorphological compartments. Contemporary developmental projects have been increasing along the upper course of the Madeira River, and include the construction of two hydroelectric power plants at the level of Santo Antônio and Jirau rapids (Clemmons 2007). Apart from the direct effects of dam building on populations inhabiting the vicinities of the current Contac Zone, power-line and road systems associated with these power plants will much probably induce fast human colonization along this entire section of the Madeira River basin (Laurance et al. 2004; Perz et al. 2008). Habitat loss or micro-climatic alterations following changes in land use could break the ongoing balance described for the *A. hodli*/*A. femoralis* hybrid zone. Thus, our results represent a valuable record with direct application in monitoring short-term effects of the recently established power plant systems and human-induced environmental changes on a well-delimited evolutionary system.

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References

Allendorf FW, Luikart G (2007) Conservation and the genetics of populations. Blackwell, Oxford

Amézquita A, Lima AP, Jehle R, Castellanos L, Ramos O, Crawford AJ, Gasser H, Hödl W (2009) Calls, colours, shapes, and genes: A multi-trait approach to the study of geographic variation in the Amazonian frog *Allobates femoralis*. Biol J Linn Soc 98: 826–838

Anderson EC, Thompson, EA (2002) A model-based method for identifying species hybrids using multilocus genetic data. Genetics 160: 1217–1229

Arnold ML, Bulger MR, Burke JM, Hempel AL, Williams JH (1999) Natural hybridization: how low can you go and still be important? Ecology 80(2): 371–381

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. Ann Rev Ecol Syst 16: 133–148

Boecklen WJ, Daniel JH (1997) Genetic analysis of hybrid zones: numbers of markers and power resolution. Ecology 78(8): 2611–2616

Brown J, Twomey, E (2009) Complicated histories: three new species of poison frogs of the genus *Ameerega* (Anura: Dendrobatidae) from north-central Peru. Zootaxa 2049: 1–38

Burton RS, Ellison CK, Harrison JS (2006) The sorry state of F2 hybrids: Consequences of rapid mitochondrial DNA evolution in allopatric populations. Am Nat 168 (Supplement): 14–24

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9: 1657–1659

Clemons KS (2007) Hydroeletric dams: transboundary environmental effects and international law. Fla State University Law Rev 36: 487–536

Corander J, Tang J (2007) Bayesian analysis of population structure based on linked molecular information. Math Biosci 205: 19–31

Corander J, Marttinen P, Sirén J, Tang J (2008) Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. BMC Bioinforma 9: 539

Coyne JA, Orr HA (2004) Speciation. Sinauer, Sunderland

Dawe KL, Shafer ABA, Herman TB, Stewart DT (2009) Diffusion of nuclear and mitochondrial genes across a zone of secondary contact in the maritime shrew, *Sorex maritimensis*: implications for the conservation of a Canadian endemic mammal. Conserv Genet 10: 851–857

DeWoody JA, Shupp J, Kenefic L, Busch J, Murfitt L, Keim P (2004) Universal method for producing ROX-labeled size standards suitable for automated genotyping. Biotechniques 37: 348–352

DNPM - Departamento Nacional da Produção Mineral – Brasil (1978) *Projeto RADAMBRASIL* – Levantamento dos recursos naturais, Vol.18, Folha SC 20, Porto Velho: Geologia, geomorfologia, pedologia, vegetação e uso potencial da terra. Departamento Nacional da Produção Mineral, Rio de Janeiro

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19: 11–15

Göd M, Franz A, Hödl W (2007) The influence of internote-interval variation of the advertisement call on the phonotactic behaviour in male *Allobates femoralis* (Dendrobatidae). Amphibia-Reptilia 28: 227–234

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www.unil.ch/izea/softwares/fstat.html

Grant PR, Grant BR (1992) Hybridization of bird species. Science 256(5054): 193–197

Grant PR, Grant BR (2002) Unpredictable evolution in a 30-year study of Darwin's finches. Science 296: 707–711

Grant T, Frost DR, Caldwell JP, Gagliardo R, Haddad CFB, Kok PJR, Means BD, Noonan BP, Schargel W, Wheeler WC (2006) Phylogenetic systematics of dart-poison frogs and their relatives (Anura: Athesphatanura: Dendrobatidae). Bull Am Mus Nat Hist 299: 1–262

Haffer J (1997) Alternative models of vertebrate speciation in Amazonia: An overview. Biodiv Conserv 6: 451–476

Haig SM, Mullins TD, Forsman ED, Trail PW, Wennerberg L (2004) Genetic identification of Spotted Owls, Barred Owls, and their hybrids: legal implication of hybrid identity. Conserv Biol 18: 1347–1357

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98

Hird S, Sullivan J (2009) Assessment of gene flow across a hybrid zone in red-tailed chipmunks (*Tamias ruficaudus*). Mol Ecol 18: 3097–3109

Höbel G, Gerhardt HC (2003) Reproductive character displacement in the acoustic communication system of gree tree frogs (*Hyla cinerea*). Evolution 57(4): 894–904

Hödl W, Amézquita A, Narins PM (2004) The rôle of call frequency and the auditory papillae in phonotacticbehavior in male dart-poison frogs *Epipedobates femoralis* (Dendrobatidae). J Comparative Physiol A 190: 823–829

Hofman S, Szymura JM (2007) Limited mitochondrial DNA introgression in a *Bombina* hybrid zone. Biol J Linn Soc 91: 295–306

Hoorn C, Wesselingh FP (2010) Amazonia, landscape and species evolution. Blackwell, Oxford

Hoskin CJ, Higgie H, McDonald KR, Moritz C (2005) Reinforcement drives rapid allopatric speciation. Nature 437: 1353–1356

Jehle R, Gasser H, Pfunder M, Amézquita A, Lima AP, Hödl W (2008) Ten polymorphic microsatellite loci for *Allobates femoralis*, an Amazonian dendrobatoid frog. Mol Ecol Resour 8(6): 1326–1328

Jiggins CD, Mallet J (2000) Bimodal hybrid zones and speciation. Trends Ecol Evol 15(6): 250–255

Keller B, Wolinska J, Manca M, Spaak P (2008) Spatial, environmental and anthropogenic effects on the taxon composition of hybridizing *Daphnia*. Phil Trans R Soc B 363: 2943–2952

Laurance WF, Albernaz AKM, Fearnside PM, Vasconcelos HL, Ferreira LV (2004) Deforestation in Amazonia. Science 304: 1109.

Lemmon EM, Lemmon AR, Collins JT, Lee-Yaw JA, Cannatella DC (2007) Phylogeny-based delimitation of species boundaries and contact zones in the trilling chorus frogs (*Pseudacris*). Mol Phylogen Evol 44: 1068–1082

Levin DA, Francisco-Ortega J, Jansen RK (1996) Hybridization and the extinction of rare plant species. Conserv Biol 10(1): 10–16.

Librado P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452

Moritz C, Patton JL, Schneider CJ, Smith TB (2000) Diversification of rainforest faunas: an integrated molecular approach. Ann Rev Ecol Syst 31: 533–563

Moritz C, Hoskin CJ, MacKenzie JB, Phillips BL, Tonione M, Silva N, VanDerWal J, Williams LE, Graham CH. (2009) Identification and dynamics of a cryptic suture zone in tropical rainforest. Proc R Soc B 276: 1235–1244

Palumbi SR (1996) Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Moritz C, Mable BK (Eds) Molecular Systematics. Sinauer, Sunderland, pp 205–247

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6: 288–295

Perz S, Brilhante S, Brown F, Caldas M, Ikeda S, Mendoza E, Overdevest C, Reis V, Reyes JF, Rojas D, Schmink M, Souza C, Walker R. (2008) Road building, land use and climate change: prospects for environmental governance in the Amazon. Phil Trans R Soc B 363: 1889–1895

Pfennig KS (2003) A test of alternative hypothesis for the evolution of reproductive isolation between spadefoot toads: support for the reinforcement hypothesis. Evolution 57(12): 2842–2851

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945–959

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425

Santos JC, Coloma LA, Summers K, Caldwell JP, Ree R, Cannatella DC (2009) Amazonian amphibian diversity is primarily derived from Late Miocene Andean lineages. PLOS Biol 7(3): 1–14

Sanz N, Araguas RM, Fernández R, Vera M, García-Marín JL (2009) Efficiency of markers and methods for detecting hybrids and introgression in stocked populations. Conserv Genet 10: 225–236

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19(4): 198–206

Seehausen O, Takimoto G, Roy D, Jokela J (2007) Speciation reversal and biodiversity dynamics with hybridization in changing environments. Mol Ecol 17(1): 30–44

Simões, PI, Lima AP, Magnusson WE, Hödl W, Amézquita A (2008) Acoustic and morphological differentiation in the frog *Allobates femoralis*: Relationships with the upper Madeira River and other potential geological barriers. Biotropica 40(5): 607–614

Simões PI, Lima AP, Farias IP (2010) The description of a cryptic species related to the pan-Amazonian frog *Allobates femoralis* (Boulenger 1883) (Anura: Aromobatidae). Zootaxa 2406: 1–28

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10: 512–526

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4*:* Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132: 619–633

Thompson JD, Higgins DG, Gibson TJ (1994) Improved sensitivity of profile searches through the use of sequence weights and gap excision. Comput Applications Biosci 10(1): 19–29

Vogel LS, Johnson SG (2008) Estimation of hybridization and introgression frequency in toads (Genus: *Bufo*) using DNA sequence variation at mitochondrial and nuclear loci. J Herpetology 42(1): 61–75

Vorndran IC, Reichwaldt E, Nürnberger B (2002) Does differential susceptibility to predation in tadpoles stabilize the *Bombina* hybrid zone? Ecology 83(6): 1648–1659

Wells KD (2007) The ecology and behavior of amphibians. The University of Chicago Press, Chicago

Table 1 Designations, position according to riverbank, clade/species attribution and coordinates of 11 sampling sites along the upper Madeira River, in southwestern Brazilian Amazon.

Site	Locality name	Riverbank	Clade	Latitude	Longitude
1	Abunã	Left	*A. hodli*	9.5160°S	65.3249°W
2	Mutum-Paraná	Left	*A. hodli*	9.5732°S	64.9211°W
3	Jirau	Left	*A. hodli*	9.3347°S	64.7375°W
4	Contact Zone	Left	*A. hodli / A femoralis*	9.3206°S	64.7225°W
5	Lower Jirau	Left	*A. femoralis*	9.3114° S	64.7172° W
6	Jaci-Paraná	Left	*A. femoralis*	9.1694° S	64.4289° W
7	Santo Antônio	Left	*A. femoralis*	8.8309° S	64.0206° W
8	Humaitá	Left	*A. femoralis*	7.0228° S	63.1028° W
9	Humaitá	Right	*A. femoralis*	7.5488° S	62.8772° W
10	Santo Antônio	Right	*A. femoralis*	8.6550° S	64.0195° W
11	Mutum-Paraná	Right	*A. femoralis*	9.6414° S	64.8859° W

Table 2 Mitochondrial 16S rDNA genetic diversity estimates along a transition zone between *Allobates hodli* and *Allobates femoralis* sampled along the upper Madeira River. Estimates are presented for all samples pooled, for each species (excluding samples from the core area of the Contact Zone, as both species are present at that site, and species attirbution is not straightforward), and separately for each one of 11 sampling sites.

Group / Sampling site	***n***	***nH***	***Hd***	π	***S***	Θ_π	Θ_S
Study area (all samples)	222	47	0.888±0.011	0.024±0.0004	46	0.0248	0.0157
Allobates hodli	72	19	0.735±0.041	0.002±0.0003	18	0.0025	0.0075
Allobates femoralis (samples pooled)	107	23	0.850±0.020	0.006±0.0007	36	0.0061	0.0142
Allobates femoralis (right bank)	41	8	0.768±0.047	0.003±0.0032	7	0.0032	0.0032
Allobates femoralis (left bank)	61	15	0.720±0.053	0.005±0.0013	32	0.0047	0.0141
Abunã	34	10	0.717±0.072	0.002±0.0003	8	0.002	0.0039
Mutum-Paraná (left)	11	7	0.818±0.119	0.004±0.0013	9	0.0041	0.0061
Jirau	27	6	0.638±0.068	0.002±0.0003	6	0.0018	0.0031
// Contact Zone //	43	7	0.678±0.052	0.025±0.0009	29	0.0257	0.0138
Lower Jirau	33	6	0.333±0.105	0.003±0.0025	28	0.0036	0.0142
Jaci-Paraná	15	7	0.771±0.100	0.002±0.0005	7	0.0023	0.0043
St. Antônio (left)	5	3	0.700±0.218	0.003±0.0010	3	0.0032	0.0029
Humaitá (left)	13	3	0.295±0.156	0.006±0.0003	2	0.0006	0.0013
Humaitá (right)	6	1	0.000±0.000	0.000±0.0000	0	0	0
St. Antônio (right)	19	5	0.743±0.004	0.003±0.0003	4	0.003	0.0023

N = Sample size; *nH* = number of haplotypes; *Hd* = haplotype diversity; π = nucleotide diversity; *S* = number of segregating sites; Θ_π = genetic diversity according to nucleotide diversity; Θ_S = Genetic diversity according to the number of segregating sites.

Table 3 Characteristics of the four microsatellite loci described by Jehle et al. (2008) for *Allobates femoralis* used in this study, sampled from total 195 individuals of *A. femoralis* and *A. hodli* collected in the study area along the upper Madeira River.

Locus	Repeat motif	No. of alleles	Allele size range	% of rare alleles (freq. < 0.05)	Observed heterozigosity	% missing data*
Epifem 03	$(GATA)_{11}$	29	188–294	0.65	0.653	0.01
Epifem 05	$(CATA)_3(AT)_3(AC)_{18}$	11	102–122	0.54	0.241	0.02
Epifem 12	$(TATC)_{15}$	40	134–210	0.92	0.575	0.01
Epifem 13	$(CTAT)_{20}$	49	206–334	0.91	0.774	0.00

*Measured as the number of individuals lacking information for the referred locus from a total 195 individuals genotyped.

Table 4 *Allobates hodli* and *Allobates femoralis* microsatellite diversity estimated per locus at 11 sampling sites along the upper Madeira River.

Sampling site	Locus	*n*	Na	Ne	Ho	He
1	Epifem 03	21	14	8.647	0.476	0.884
Abunã	Epifem 05	21	4	1.947	0.429	0.486
	Epifem 12	20	12	8.333	0.700	0.880
	Epifem 13	21	15	10.889	0.762	0.908
2	Epifem 03	13	12	8.667	0.692	0.885
Mutum-Paraná (left)	Epifem 05	12	4	2.165	0.500	0.538
	Epifem 12	13	10	6.500	0.769	0.846
	Epifem 13	13	12	7.860	0.923	0.873
3	Epifem 03	26	15	7.553	0.346	0.868
Jirau	Epifem 05	27	5	2.881	0.370	0.653
	Epifem 12	27	13	3.488	0.407	0.713
	Epifem 13	27	14	9.113	0.704	0.890
4	Epifem 03	30	12	6.716	0.567	0.851
Contact Zone	Epifem 05	30	4	2.270	0.333	0.559
	Epifem 12	30	14	9.730	0.633	0.897
	Epifem 13	30	22	13.740	0.900	0.927
5	Epifem 03	41	13	8.301	0.707	0.880
Lower Jirau	Epifem 05	40	5	1.264	0.100	0.209
	Epifem 12	42	16	9.484	0.452	0.895
	Epifem 13	42	20	12.466	0.881	0.920
6	Epifem 03	15	12	8.333	0.867	0.880
Jaci-Paraná	Epifem 05	14	4	1.562	0.357	0.360
	Epifem 12	14	9	4.506	0.357	0.778
	Epifem 13	15	17	13.235	0.600	0.924
7	Epifem 03	7	6	4.455	0.857	0.776
Santo Antônio (left)	Epifem 05	7	3	2.333	0.000	0.571
	Epifem 12	7	4	2.800	0.429	0.643
	Epifem 13	7	5	4.261	0.714	0.765
8	Epifem 03	13	9	6.377	0.846	0.843
Humaitá (left)	Epifem 05	13	3	2.268	0.077	0.559
	Epifem 12	13	16	8.667	0.846	0.885
	Epifem 13	13	13	9.941	0.615	0.899
	Epifem 03	7	8	5.765	0.857	0.827
Humaitá (right)	Epifem 05	7	2	1.690	0.000	0.408
	Epifem 12	7	7	5.765	0.571	0.827
	Epifem 13	7	11	8.909	0.714	0.888
10	Epifem 03	10	9	6.667	0.800	0.850
Santo Antônio (right)	Epifem 05	10	5	3.846	0.000	0.740
	Epifem 12	10	6	4.762	0.700	0.790
	Epifem 13	10	7	5.000	0.700	0.800
11	Epifem 03	10	10	6.667	0.800	0.850
Mutum-Paraná (right)	Epifem 05	10	4	1.709	0.100	0.415
	Epifem 12	10	9	6.897	0.800	0.855
	Epifem 13	10	10	5.556	0.600	0.820

n = number of samples; Na = number of alleles; Ne = number of effective alleles; Ho = observed heterozygosity; He = expected heterozigosity.

Table 5 Average microsatellite diversity (± standard errors) and estimate of heterozygote deficit (*F*is) obtained from four microsatellite loci of *Allobates hodli* and *Allobates femoralis* from 11 sampling sites along the upper Madeira River.

Site	Na	Ne	Np	He	*F*is
1	11.250 ± 2.496	7.454 ± 1.922	0.250 ± 0.250	0.790 ± 0.101	0.274
2	9.500 ± 1.893	6.298 ± 1.448	0.000 ± 0.000	0.785 ± 0.083	0.122
3	11.750 ± 2.287	5.759 ± 1.525	1.750 ± 1.109	0.781 ± 0.058	0.431
4	13.000 ± 3.697	8.114 ± 2.422	0.250 ± 0.250	0.809 ± 0.085	0.264
5	13.500 ± 3.697	7.879 ± 2.373	1.250 ± 0.479	0.726 ± 0.173	0.274
6	10.500 ± 2.723	6.909 ± 2.524	1.000 ± 0.408	0.736 ± 0.129	0.292
7	4.500 ± 0.645	3.462 ± 0.527	0.000 ± 0.000	0.689 ± 0.049	0.344
8	10.250 ± 2.810	6.813 ± 1.685	0.750 ± 0.479	0.797 ± 0.080	0.289
9	7.000 ± 1.871	5.532 ± 1.480	1.750 ± 1.109	0.737 ± 0.111	0.343
10	6.750 ± 0.854	5.069 ± 0.588	0.500 ± 0.289	0.795 ± 0.023	0.355
11	8.250 ± 1.436	5.207 ± 1.202	1.250 ± 0.479	0.735 ± 0.107	0.267

Na = number of alleles; Ne = number of effective alleles;Np = number of private alleles; He = expected heterozigosity.

Table 6 *F*st values between 11 sampling sites along the upper Madeira River based on Weir and Cockerham estimators, obtained from four microsatellite loci of *Allobates hodli* and *Allobates femoralis*. Sampling sites 1, 2, and 3 correspond to *A. hodli* samples, while sites 5 to 11 are considered to be exclusively *A. femoralis*.

		1	2	3	4	5	6	7	8	9	10	11
1	**Abunã**	0.0000										
2	**Mutum (L)**	0.0280	0.0000									
3	**Jirau**	0.0835	0.0800	0.0000								
4	**Contact Zone**	0.0965	0.0846	0.0898	0.0000							
5	**Lower Jirau**	0.1885	0.1823	0.1848	0.0402	0.0000						
6	**Jaci-Paraná**	0.1765	0.1728	0.1801	0.0639	0.0471	0.0000					
7	**St. Antônio (L)**	0.1913	0.1871	0.2012	0.1561	0.1839	0.1449	0.0000				
8	**Humaitá (L)**	0.1397	0.1307	0.1551	0.0742	0.0788	0.0676	0.1138	0.0000			
9	**Humaitá (R)**	0.1559	0.1624	0.1668	0.0702	0.0597	0.0375	0.1013	0.0424	0.0000		
10	**St. Antônio (R)**	0.1281	0.1309	0.1161	0.1080	0.1574	0.1268	0.1451	0.0908	0.0893	0.0000	
11	**Mutum (R)**	0.1624	0.1353	0.1802	0.1673	0.2084	0.1832	0.1994	0.0970	0.1672	0.1094	0.0000

(R) and (L) correspond to right and left riverbanks, respectively, for sites with the same denomination. Numbers correspond to site locations and coordinates as presented in Fig. 1 and Table 1.

Fig. 1 (a) Relative location of study area in northern South America; **(b)** Distribution and denomination of 11 sampling sites along the study area in the upper Madeira River, southwestern Brazilian Amazonia. White filled dots correspond to distribution of *Allobates hodli*, and black filled dots to the distribution of *Allobates femoralis*. The two species meet at a contact zone on the left riverbank (sampling site 4) adjacent to the Cachoeira do Jirau rapids; **(c)** *A. hodli* and *A. femoralis* can be distinguished by characteristic advertisement calls, constituted by two notes in *A. hodli* and four notes in *A. femoralis*. Sonograms represent calls of one individual from Abunã (site 1) and one individual from Lower Jirau (site 5).

Fig. 2 (a) Haplotype network built from 16S rDNA sequences of *Allobates hodli* using statistical parsimony. Areas of ellipses proportional to haplotype frequency. Small dots on lines represent to missing intermediate haplotypes. Colors stand for haplotype origin according to sampling site; **(b)** Relative position of sampling sites along the upper Madeira River.

Fig. 3 (a) Haplotype network built from 16S rDNA sequences of *Allobates femoralis*, and **(b)** origin according to location of sampling sites along the upper Madeira River. Areas of ellipses are proportional to haplotype frequency. Dots along lines stand for missing intermediate haplotypes.

Fig. 4 Bayesian analysis of population structure on a 16S rRNA mitochondrial gene dataset obtained from 222 individuals indicates the existence of three genetic clusters along the upper Madeira River, corresponding to one *Allobates hodli* cluster and two *Allobates femoralis* clusters. Genetic admixture between clusters occurs on the *hodli*/*femoralis* contact zone on the left riverbank, and between the two *femoralis* clades downstream on the same riverbank.

Fig. 5 Neighbor-Joining tree constructed from 48 unique *Allobates hodli/Allobates femoralis* 16S rDNA haplotypes found along the study area. The two species for monophyletic groups, with samples collected in a contact zone distributed among both clades. Tip labels correspond to sampling site followed by tissue collection number. Basal clades are highly supported (bootstrap value = 99%).

Fig. 6 Posterior probability of data according to the possible number of genetic clusters (K=1-10, *x* axis) formed by samples of *Allobates hodli* and *Allobates femoralis* from 11 sampling sites along the upper Madeira River. Signs for each value of K represent the arithmetic mean and standard deviations between 15 iterations run in STRUCTURE 2.3.3.

Fig. 7 Barplot of membership coefficients (q_i) obtained in STRUCTURE assigning samples of *Allobates hodli* and *Allobates femoralis* to three genetic clusters inferred from data on four microsatellite loci. The two darker clusters represent *A. femoralis* clusters (see text). Numbers on *x* axis refer to sampling localities presented in Table 1 and Fig. 1. Arrows indicate individuals removed from the Jaci-Paraná sample in posterior analyses of hybridization.

Fig. 8 Average proportions of membership to *Allobates hodli* (Q_h) and *Allobates femoralis* (Q_f) in each of six sampling sites along the upper Madeira River estimated in STRUCTURE 2.3.3. Proportions for each sampling site correspond to the mean value between 20 independent iterations. Site 4 corresponds to the core area of the contact zone between the two species.

Fig. 9 Barplots of hybrid category assignments generated by NEWHYBRIDS (upper graph) and membership coefficients generated by STRUCTURE (lower graph). Each vertical column on both graphs represents one of 145 individuals of *Allobates hodli* and *Allobates femoralis* originating from six sampling sites on the left bank of the upper Madeira River (center). For the NEWHYBRIDS analysis, Abunã and Mutum were assigned as containing pure *A. hodli* individuals, while Jaci was regarded as a pure *A. femoralis* sample. Both analysis support the hybrid zone as strongly bimodal, with few putative hybrids greatly restricted to the core of the contact zone. Frequency of F_1 hybrids is almost negligible in comparison to F_2 hybrids (but see text).

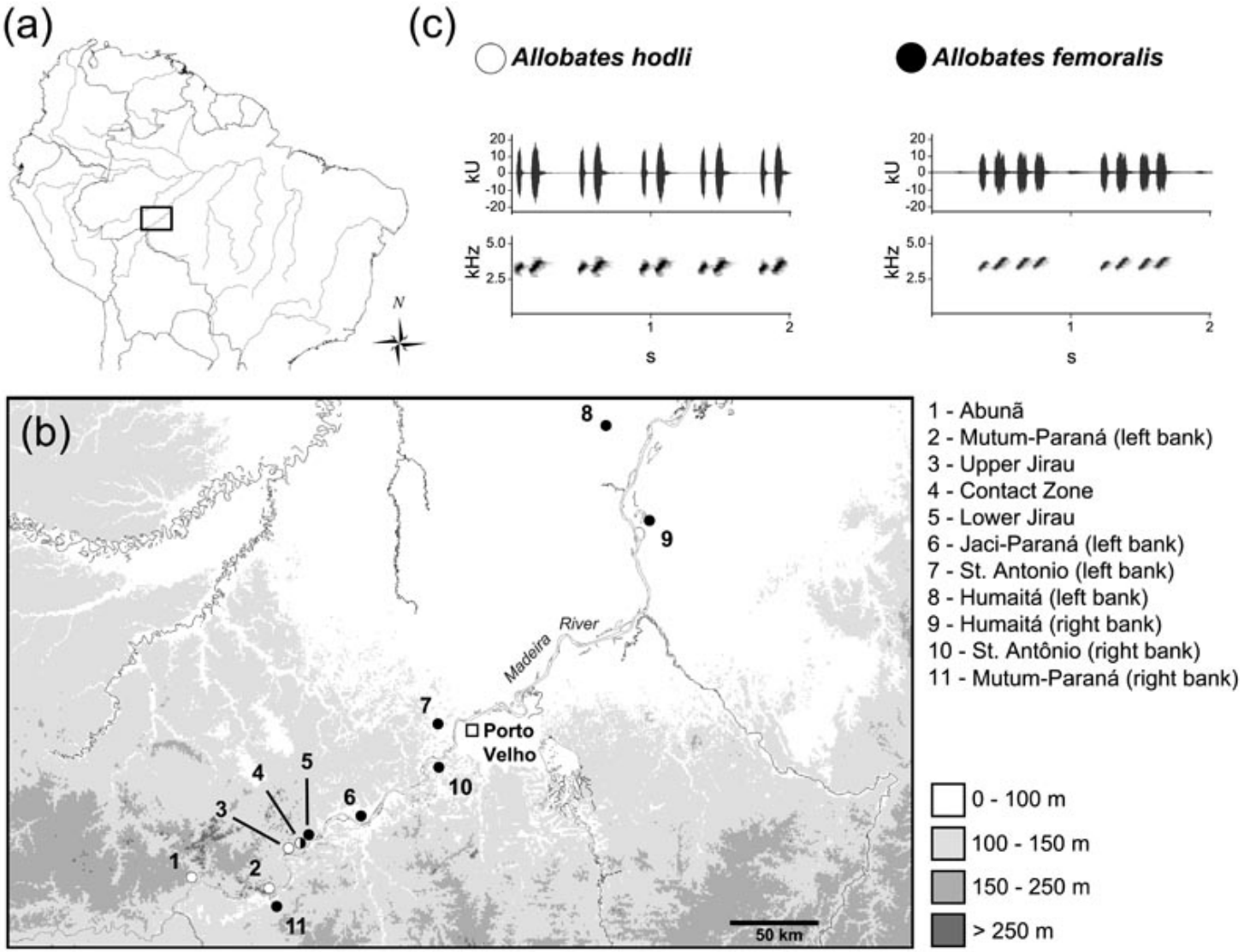
(a)
N
(c)
Allobates hodli
Allobates femoralis
kU
kHz
s
(b)
Madeira River
Porto Velho
50 km
1 - Abunã
2 - Mutum-Paraná (left bank)
3 - Upper Jirau
4 - Contact Zone
5 - Lower Jirau
6 - Jaci-Paraná (left bank)
7 - St. Antonio (left bank)
8 - Humaitá (left bank)
9 - Humaitá (right bank)
10 - St. Antônio (right bank)
11 - Mutum-Paraná (right bank)
0 - 100 m
100 - 150 m
150 - 250 m
> 250 m

Fig. 1

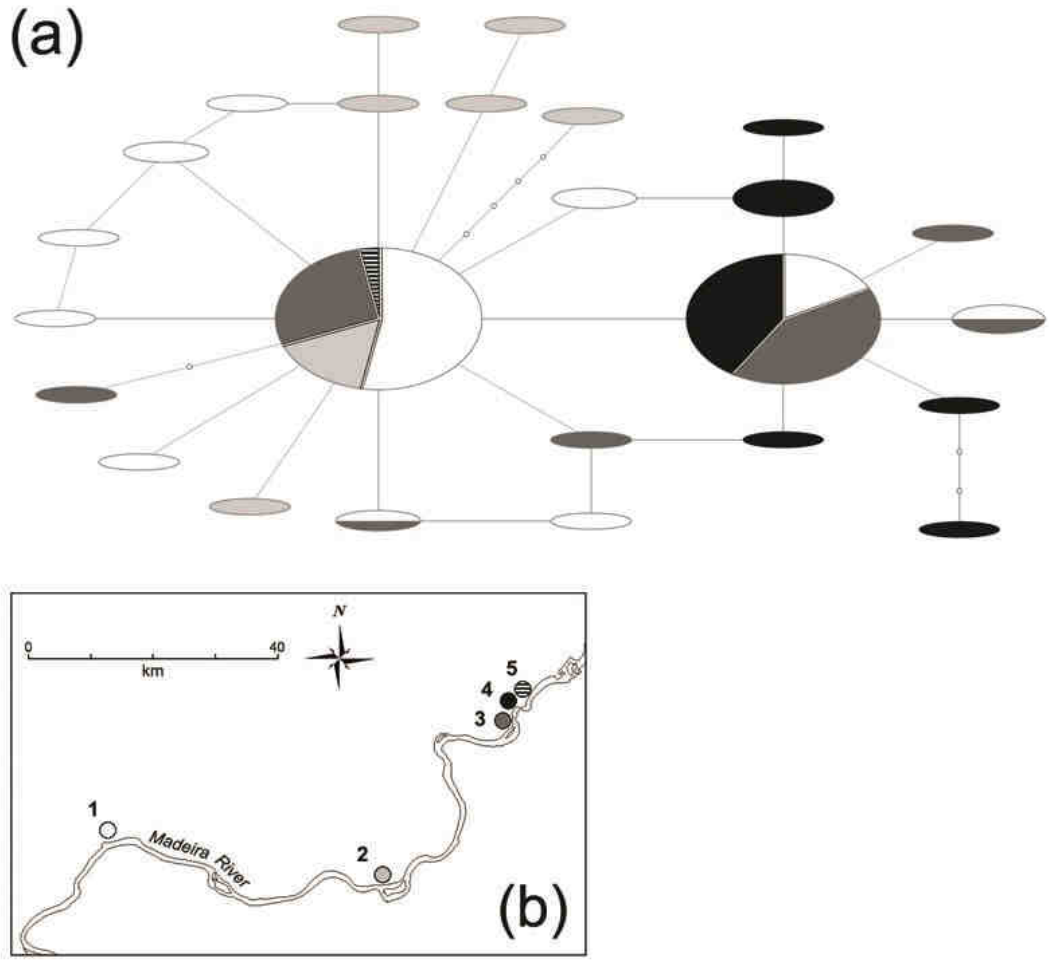

Fig. 2

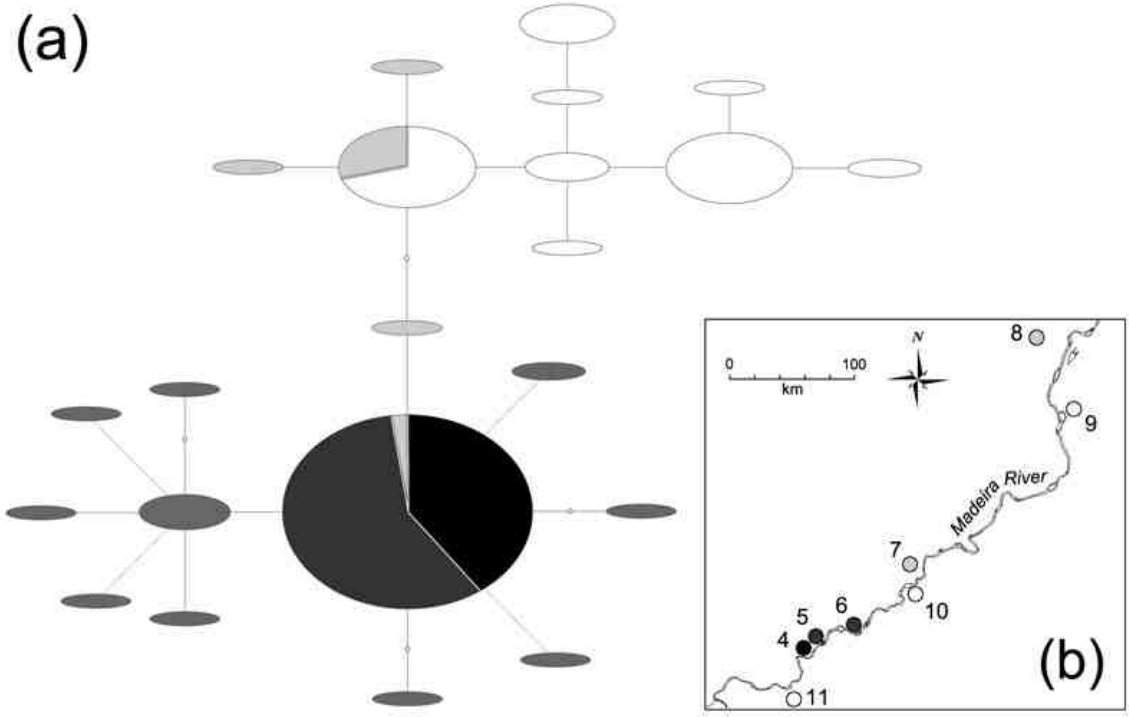

Fig. 3

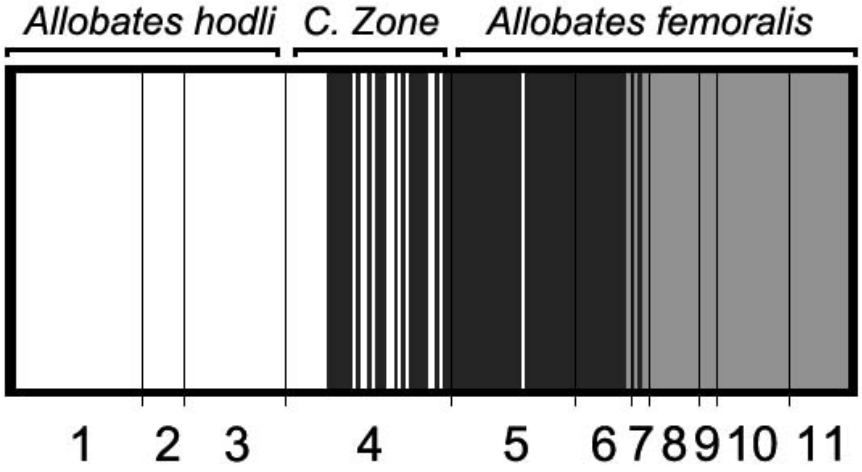
Allobates hodli
C. Zone
Allobates femoralis
1
2
3
4
5
6
7
8
9
10
11

Fig. 4

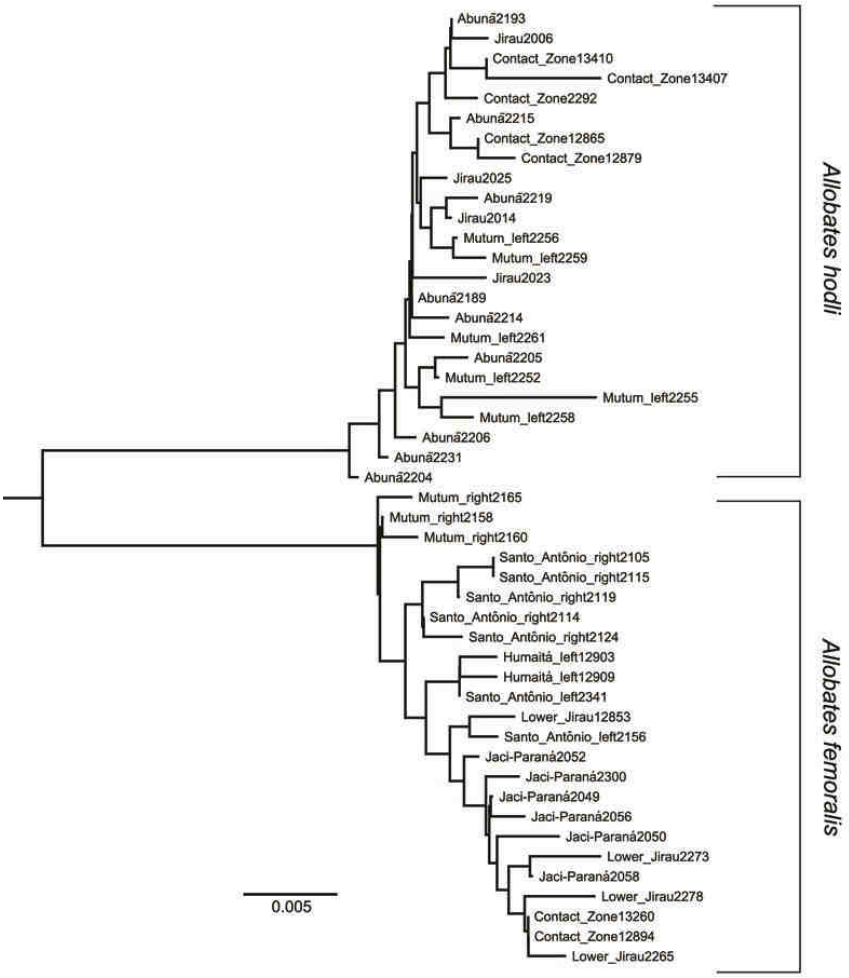
Abunã2193
Jirau2006
Contact_Zone13410
Contact_Zone13407
Contact_Zone2292
Abunã2215
Contact_Zone12865
Contact_Zone12879
Jirau2025
Abunã2219
Jirau2014
Mutum_left2256
Mutum_left2259
Jirau2023
Abunã2189
Abunã2214
Mutum_left2261
Abunã2205
Mutum_left2252
Mutum_left2255
Mutum_left2258
Abunã2206
Abunã2231
Abunã2204
Mutum_right2165
Mutum_right2158
Mutum_right2160
Santo_Antônio_right2105
Santo_Antônio_right2115
Santo_Antônio_right2119
Santo_Antônio_right2114
Santo_Antônio_right2124
Humaitá_left12903
Humaitá_left12909
Santo_Antônio_left2341
Lower_Jirau12853
Santo_Antônio_left2156
Jaci-Paraná2052
Jaci-Paraná2300
Jaci-Paraná2049
Jaci-Paraná2056
Jaci-Paraná2050
Lower_Jirau2273
Jaci-Paraná2058
Lower_Jirau2278
Contact_Zone13260
Contact_Zone12894
Lower_Jirau2265
0.005
Allobates hodli
Allobates femoralis

Fig. 5

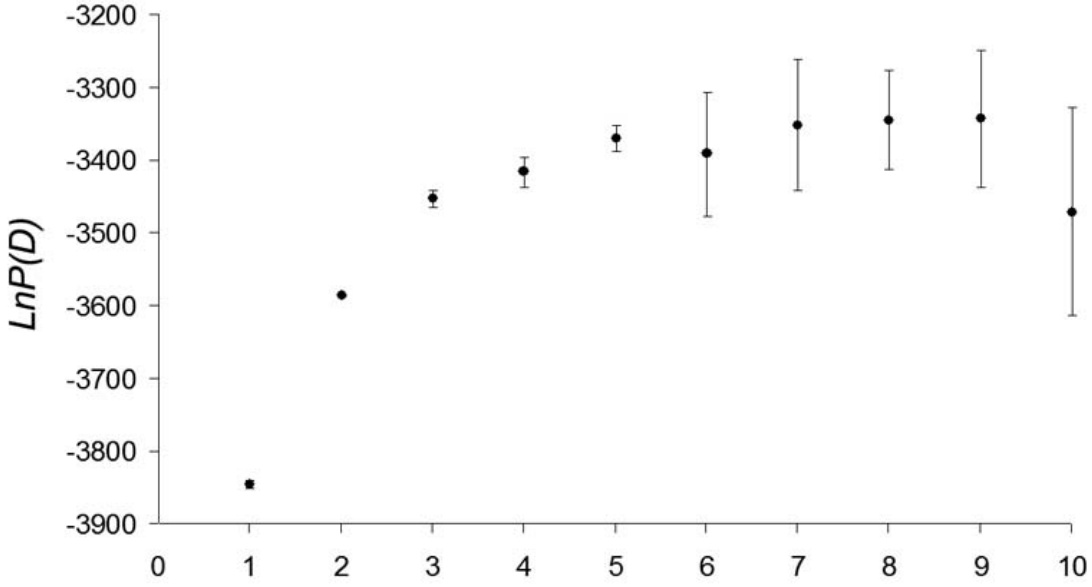
-3200
-3300
-3400
-3500
-3600
-3700
-3800
-3900
LnP(D)
0
1
2
3
4
5
6
7
8
9
10

Fig. 6

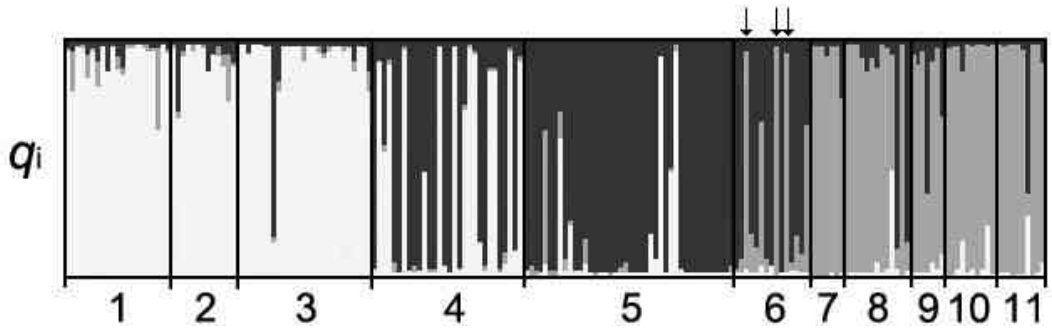
qi
1
2
3
4
5
6
7
8
9
10
11

Fig. 7

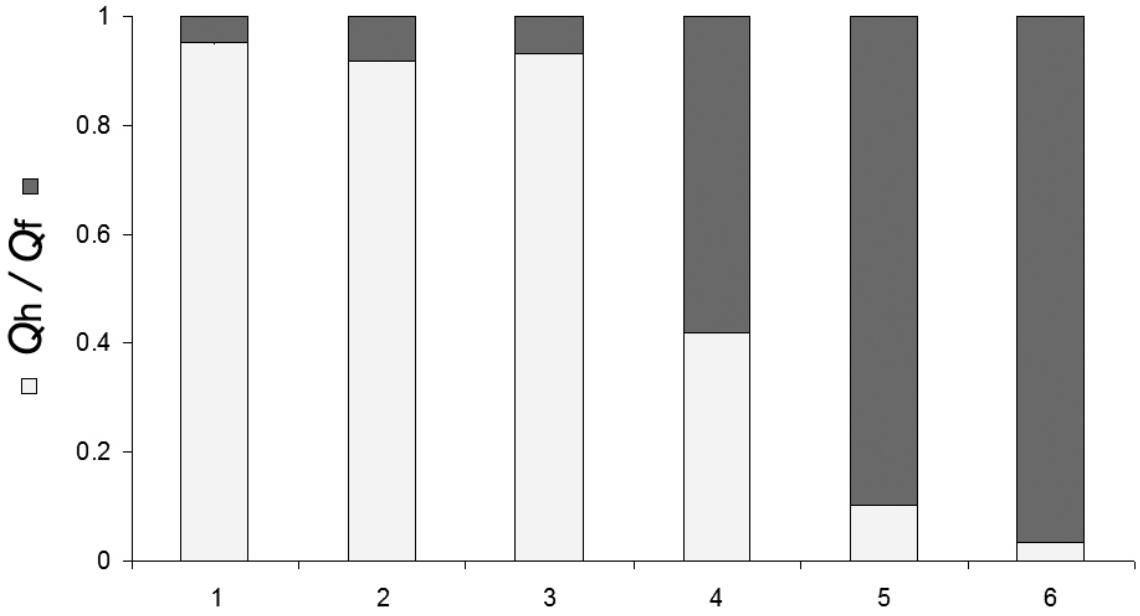

Fig. 8

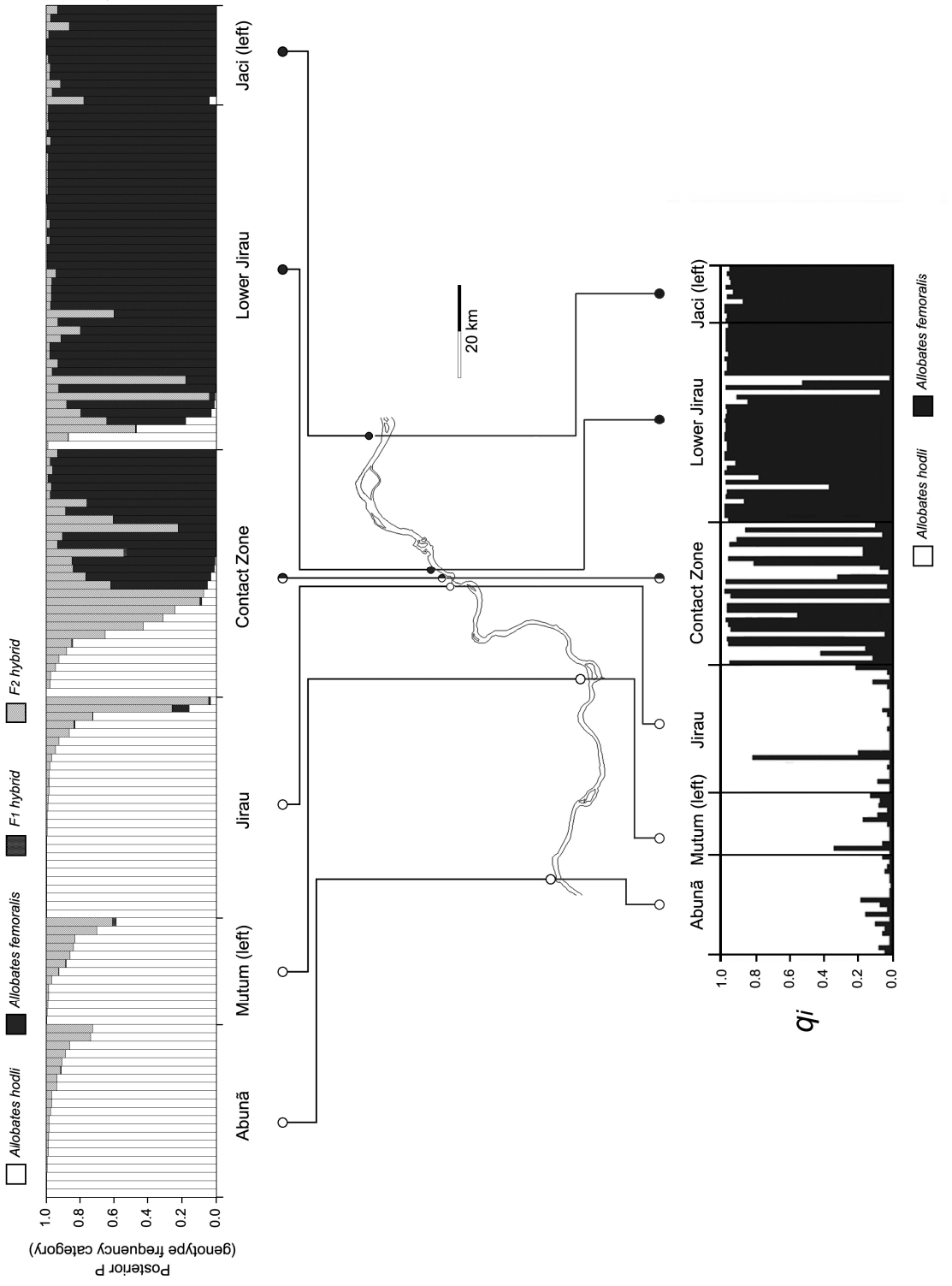
Allobates hodli
Allobates femoralis
F1 hybrid
F2 hybrid
Posterior P
(genotype frequency category)
1.0
0.8
0.6
0.4
0.2
0.0
Abunã
Mutum (left)
Jirau
Contact Zone
Lower Jirau
Jaci (left)
20 km
qi
Allobates hodli
Allobates femoralis

Fig. 9

Appendix I

List of *Allobates hodli* and *Allobates femoralis* tissue samples used in this study. Tissues are housed at Coleção de Tecidos de Genética Animal at Universidade Federal do Amazonas (CTGA – ICB/UFAM), Manaus, Brazil, and numbered according to original field numbers, all prefixed by "APL".

16S rRNA analyses:

Abunã: 2189 2190 2191 2192 2193 2194 2195 2196 2199 2202 2203 2204 2205 2206 2207 2208 2212 2213 2214 2215 2217 2218 2219 2220 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232; **Mutum-Paraná (left riverbank):** 2250 2252 2253 2254 2255 2256 2258 2259 2260 2261 2262; **Jirau:** 2000 2001 2002 2003 2004 2005 2006 2008 2009 2028 2029 2030 2032 2027 2010 2011 2012 2013 2014 2015 2016 2017 2018 2023 2024 2025 2026; **Contact Zone:** 2270 2269 2291 2292 2293 2294 2295 2296 12864 12865 12866 12867 12877 12878 12879 12880 12881 12883 12884 12891 12892 12893 12894 12895 12896 13419 13261 13259 13399 13400 13404 13410 13407 13418 13419 13415 13260 13405 13405 13406 13414; **Lower Jirau:** 2263 2264 2265 2266 2267 2268 2272 2273 2274 2275 2276 2277 2278 2280 2281 2283 2284 2285 2286 2287 2288 2289 2290 12838 12839 12840 12848 12849 12850 12851 12852 12853 12854; **Jaci-Paraná**: 2049 2050 2051 2052 2054 2055 2056 2057 2058 2060 2061 2062 2063 2299 2300; **St. Antônio (left riverbank):** 2155 2156 2157 2343 2341; **Humaitá (left riverbank**): 12900 12901 12902 12903 12904 12905 12906 12907 12908 12909 12910 12911 12912; **Humaitá (right riverbank):** 13421 13422 13423 13424 13425 13426; **St. Antônio (right riverbank):** 2104 2105 2106 2109 2107 2111 2112 2114 2115 2116 2117 2118 2119 2120 2123 2124 2125 2127 2131; **Mutum-Paraná (right riverbank):** 2158 2160 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2176 2180 2182.

Microssatellite analyses:

Abunã: 2189 2191 2193 2194 2195 2196 2199 2201 2202 2203 2205 2206 2224 2225 2226 2227 2228 2229 2230 2231 2232; **Mutum-Paraná (left riverbank):** 2249 2250 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262; **Jirau:** 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2011 2012 2013 2014 2016 2017 2018 2022 2023 2024 2025 2026 2027 2028 2029 2030 2032; **Contact Zone:** 12864 12865 12866 12867 12877 12878 12879 12880 12881 12882 12883 12884 12891 12892 12893 12894 12895 12896 13259 13260 13261 13262 13400 13401 13404 13405 13406 13407 13408; **Lower Jirau:** 2263 2264 2265 2266 2267 2268 2269 2270 2272 2273 2274 2275 2276 2277 2278 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 12838 12839 12840 12847 12848 12849 12850 12851 12852 12853 12854; **Jaci-Paraná:** 2051 2052 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2299 2300; **St. Antônio (left riverbank):** 2154 2155 2156 2157 2341 2343 2344; **Humaitá (left riverbank):** 12900 12901 12902 12903 12904 12905 12906 12907 12908 12909 12910 12911 12912; **Humaitá (right riverbank):** 13420 13421 13422 13423 13424 13425 13426; **St. Antônio (right riverbank):** 2111 1212 2113 2114 2115 2116 2117 2118 2119 2124; **Mutum-Paraná (right riverbank):** 2160 2165 2167 2168 2169 2170 2171 2172 2173 2176.

Appendix II

Table S1 GenBank accession numbers for 16S rRNA sequences of *Allobates hodli* and *Allobates femoralis* used in this study. Sample number refer to tissue identification according to original collection number (APL series), which are also applied at Coleção de Tecidos de Genética Animal at Universidade Federal do Amazonas (CTGA – ICB/UFAM), Manaus, Brazil, where samples are deposited.

Sampling site	Sample number	GenBank accession number
Abunã	2189	GU017423
Abunã	2193	GU017425
Abunã	2204	GU017426
Abunã	2205	GU017427
Abunã	2206	GU017428
Abunã	2214	GU017430
Abunã	2215	GU017431
Abunã	2219	GU017434
Abunã	2231	GU017436
Mutum-Paraná (left riverbank)	2252	GU017437
Mutum-Paraná (left riverbank)	2255	GU017438
Mutum-Paraná (left riverbank)	2256	GU017439
Mutum-Paraná (left riverbank)	2258	GU017440
Mutum-Paraná (left riverbank)	2259	GU017441
Mutum-Paraná (left riverbank)	2261	GU017442
Jirau	2006	GU017443
Jirau	2014	Submitted
Jirau	2023	GU017444
Jirau	2025	GU017445
Contact Zone	2292	Submitted
Contact Zone	12865	Submitted
Contact Zone	12879	Submitted
Contact Zone	12894	Submitted
Contact Zone	13260	Submitted
Contact Zone	13407	Submitted
Contact Zone	13410	Submitted
Lower Jirau	12853	GU017446
Lower Jirau	2265	GU017447
Lower Jirau	2273	GU017448
Lower Jirau	2278	GU017449
Jaci-Paraná	2049	GU017450
Jaci-Paraná	2050	GU017451
Jaci-Paraná	2052	GU017452
Jaci-Paraná	2056	GU017454
Jaci-Paraná	2058	GU017455
Jaci-Paraná	2300	GU017457
St. Antônio (left riverbank)	2156	Submitted

Table S1 Continued.

Sampling site	Sample number	GenBank accession number
St. Antônio (left riverbank)	2341	Submitted
Humaitá (left riverbank)	12903	Submitted
Humaitá (left riverbank)	12909	Submitted
St. Antônio (right riverbank)	2105	Submitted
St. Antônio (right riverbank)	2114	Submitted
St. Antônio (right riverbank)	2115	Submitted
St. Antônio (right riverbank)	2119	Submitted
St. Antônio (right riverbank)	2124	Submitted
Mutum-Paraná (right)	2158	GU017458
Mutum-Paraná (right)	2160	GU017459
Mutum-Paraná (right)	2165	GU017460

CONCLUSÕES GERAIS

Através da análise de um banco de dados mais amplo em relação a estudos anteriores, foi possível reavaliar o arranjo sistemático corrente para o grupo *Allobates femoralis*, confirmando que as populações reconhecidas sob este nome constituem, na verdade, um complexo de espécies alopátricas. A integração entre informações provindas de análises filogenéticas moleculares, e o mapeamento da distribuição geográfica dos clados e dos fenótipos acústicos e morfológicos observados entre estas populações, permitiu a descrição de uma espécie altamente divergente das populações típicas de *Allobates femoralis*. Esta espécie, *Allobates hodli*, tem distribuição restrita ao alto rio Madeira, em Rondônia, e ao sul do Acre. Os mesmos resultados apontaram a existência de outras espécies crípticas, de distribuição relativamente restrita, que aguardam descrição formal.

A avaliação do efeito do rio Madeira sobre a diferenciação genética e fenotípica entre populações de *Allobates femoralis* mostra um padrão concordante com a hipótese de que o rio tenha representado uma barreira vicariante efetiva entre estas populações no passado, provavelmente durante o Plioceno tardio. A coincidência entre o padrão filogeográfico obtido através da análise de dados moleculares e a separação das populações pelo leito do rio é quebrada apenas na região de transição entre o médio e o alto curso do rio, onde a similaridade genética entre populações de margens opostas sugerem a ocorrência de eventos de dispersão entre margens, provavelmente durante o Pleistoceno. Estes eventos poderiam ser resultado do reposicionamento da calha do rio, ocasionado pela ação conjunta entre a dinâmica de sedimentação do leito do rio e oscilações climáticas extremas, típicas daquele período.

A diferenciação morfológica e acústica entre as populações do rio Madeira parecem não estar correlacionadas com a diferenciação observada a partir de marcadores moleculares supostamente neutros. Este resultado pode ser tomado como uma evidência de que os caracteres fenotípicos respondem mais rapidamente a forças seletivas locais, havendo pouca relação entre sua variação e a estrutura genética entre as populações deste sistema. É importante ressaltar que os resultados obtidos ao longo do rio Madeira sugerem que estudos que contam com poucas amostras pontuais oriundas de cada interflúvio podem não revelar padrões filogeográficos verdadeiros, não detectando, por exemplo, eventos de dispersão recentes entre interflúvios. Alternativamente, coletas pontuais podem amostrar apenas populações originadas por estes eventos, ignorando a forte estruturação genética observada entre populações distribuídas ao longo dos interflúvios, e produzindo interpretações evolutivas errôneas. Estas poderiam trazer conseqüências severas para o planejamento de estratégias de conservação baseadas na distribuição da variabilidade genética e fenotípica.

Por fim, a caracterização genética da zona de contato entre *Allobates hodli* e *Allobates femoralis* na margem esquerda do alto rio Madeira confirmou a ocorrência de hibridização entre as duas espécies. Porém, a ocorrência de híbridos e a introgressão genética entre as populações é restrita geograficamente, uma vez que a freqüência de indivíduos potencialmente híbridos e ou de introgressão genética entre as espécies decai drasticamente pouco mais de um quilômetro acima e abaixo da linha central da zona de contato. Os padrões observados sugerem a ocorrência de seleção contra híbridos, provavelmente mediada por mecanismos de isolamento reprodutivo pós-zigótico. Estudos citogenéticos e experimentos laboratoriais envolvendo cruzamentos entre as espécies seriam formas adequadas de se confirmar esta hipótese.

Uma vez que os interflúvios do rio Madeira são hoje o cenário para grandes projetos de desenvolvimento, incluindo a restauração de grandes rodovias e o estabelecimento de um sistema de hidrelétricas ao longo do alto curso do rio, os resultados apresentados aqui podem servir como base de referência para o monitoramento dos efeitos em longo prazo de tais projetos sobre a estrutura populacional, variabilidade e dinâmica evolutiva de anfíbios anuros ecologicamente restritos a ambientes florestais.

REFERÊNCIAS BIBLIOGRÁFICAS

Allendorf, F.W.; Luikart, G. 2007. *Conservation and the genetics of populations*. Blackwell Publishing, Oxford.

Amézquita, A.; Castellanos, L.; Hödl, W. 2005. Auditory matching of male *Epipedobates femoralis* (Anura: Dendrobatidae) under field conditions: the role of spectral and termporal call features. *Animal Behavior* 70: 1377–1386.

Amézquita, A.; Hödl, W.; Lima, A.P.; Castellanos, L.; Erdtmann, L.; Araújo, M.C. 2006. Masking interference and the evolution of the acoustic communication system in the Amazonian dendrobatid frog *Allobates femoralis*. *Evolution* 60(9): 1874–1887.

Amézquita, A.; Lima, A.P.; Jehle, R.; Castellanos, L.; Ramos, O.; Crawford, A.J.; Gasser, H.; Hödl, W. 2009. Calls, colours, shapes, and genes: A multi-trait approach to the study of geographic variation in the Amazonian frog *Allobates femoralis*. *Biological Journal of the Linnean Society* 98: 826–838.

Anderson, M.J.; Legendre, P. 1999. An empirical comparison of permutation methods for tests of partial regression coefficients in a linear model. *Journal of Statististical Computation and Simulation* 62: 271–303.

Arnold, M.L.; Bulger, M.R.; Burke, J.M.; Hempel, A.L.; Williams, J.H. 1999. Natural hybridization: how low can you go and still be important? *Ecology* 80(2): 371–381

Avise, J.C. 2000. *Phylogeography: the history and formation of species*. Harvard University Press, Cambridge.

Azevedo-Ramos, C.; Galatti, U. 2002. Patterns of amphibian diversity in Brazilian Amazonia: Conservation implications. *Biological Conservation* 103: 103–111.

Barton, N.H.; Hewitt, G.M. 1985. Analysis of hybrid zones. *Annual Review of Ecology and Systematics* 16: 133–148.

Bernal, X.; Page, R.A.; Rand, A.S.; Ryan, M.J. 2007. Cues for eavesdroppers: Do frog calls indicate prey density and quality? *The American Naturalist* 169(3): 409–415.

Bettencourt, J.S.; Leite Jr., W.B.; Ruiz, A.S.; Matos, R.; Payolla, B.L.; Tosdal, R.M. 2010. The Rondonian-San Ignacio Province in the SW Amazonian Craton: An overview. *Journal of South American Earth Sciences* 29: 28–46.

Betts, R.A.; Malhi, Y.; Roberts, J.T. 2008. The future of the Amazon: new perspectives from climate, ecosystem and social sciences. *Phylosophical Transactions of the Royal Society B* 363: 1729–1735.

Bickford, D.; Lohman, D.J.; Navjot, S.S.; Ng P.K.L.; Meier, R.; Winker, K.; Ingram, K.K.; Das, I. 2006. Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution 22: 148–155.

Bonnet, E.; Van de Peer, Y. 2002. ZT: a software tool for simple and partial Mantel tests. *Journal of Statistical Software* 7: 1–12.

Boecklen, W.J.; Daniel, J.H. 1997. Genetic analysis of hybrid zones: numbers of markers and power resolution. Ecology 78(8): 2611–2616.

Borges, S. 2007. Análise biogeográfica da avifauna da região oeste do baixo Rio Negro, amazônia brasileira. *Revista Brasileira de Zoologia* 24(4): 919–940.

Boul, K.E.; Funk, W.C.; Darst, C.R.; Cannatella, D.C.; Ryan, M.J. 2007. Sexual selection drives speciation in an Amazonian frog. *Proceedings of the Royal Society B* 274: 399–406.

Brown, J.L.; Twomey, E. 2009. Complicated histories: three new species of poison frogs of the genus Ameerega (Anura: Dendrobatidae) from north-central Peru. *Zootaxa* 2049: 1–38.

Buckley, T.R. 2002. Model misspecification and probabilistic tests of topology: Evidence from empirical data sets. *Systematic Biology* 51(3): 509–523.

Bush, M. B. 1994. Amazonian speciation: a necessarily complex model. *Journal of Biogeography* 21: 5–17.

Burton, R.S.; Ellison, C.K.; Harrison, J.S. 2006. The sorry state of F2 hybrids: Consequences of rapid mitochondrial DNA evolution in allopatric populations. *The American Naturalist* 168 (Supplement): 14–24.

Bush, M.B.; Oliveira, P.E. 2006. The rise and fall of the refugial hypothesis of Amazonian speciation: a paleoecological perpective. *Biota Neotropica* 6 (1): 1–17.

Caldwell, J.P.; Myers, C.W. 1990. A new poison frog from Amazonian Brazil, with further revision of the *quinquevittatus* group of *Dendrobates*. *American Museum Novitates* 2988: 1–21.

Caldwell, J.P.; Lima, A.P.; Keller, C. 2002. Redescription of *Colostethus marchesianus* (Melin, 1941) from its type locality. *Copeia* 2002: 157–165.

Caldwell, J.P.; Lima, A.P. (2003) A new species of *Colostethus* with a nidicolous tadpole. *Herpetologica* 59: 219–234.

Caldwell, J.P.; Araújo, M.C. 2005. Amphibian faunas of two eastern Amazonian rainforest sites in Pará, Brazil. *Occasional Papers of the Sam Noble Oklahoma Museum of Natural History* 16: 1–41.

Capparella, A.P. 1987. *Effects of riverine barriers on genetic differentiation of Amazonian forest undergrowth birds*. Tese de Doutorado, Luisiana State University, Baton Rouge.

Carnaval, A.C.; Hickerson, M.J.; Haddad, C.F.B.; Rodrigues, M.T.; Moritz, C. 2009. Stability predicts genetic diversity in the Brazilian Atlantic Forest hotspot. *Science* 323: 785–789.

Castilho, C.V.; Magnusson, W.E.; Araújo, R.N.O.; Luizão, R.C.C.; Luizão, F.J.; Lima, A.P.; Higuchi, N. 2006. Variation in aboveground tree live biomass in a central Amazonian forest: Effects of soil and topography. *Forest Ecology and Management* 234: 85–96.

Charif, R.A.; Clark C.W.; Fristrup, K.M. 2004. *Raven 1.2 User's Manual*. Cornell Laboratory of Ornithology, Ithaca, NY.

Clement, M.; Posada, D.; Crandall, K.A. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657–1659.

Clemons, K. S. 2007. Hydroeletric dams: transboundary environmental effects and international law. *Florida State University Law Review* 36: 487–536.

Clough, M.; Summers, K. 2000. Phylogenetic systematics and the biogeography of the poison frogs: Evidence from mitochondrial DNA sequences. *Biological Journal of The Linnean Society* 70: 515–540.

Cohn-Haft, M. 2000. *A case study in amazonian biogeography: Vocal and DNA sequence variation in* Hemitriccus *flycatchers*. Tese de Doutorado, Luisiana State University, Baton Rouge.

Colinvaux, P.A.; De Oliveira, P.J.; Bush, M.B. 2000. Amazonian and neotropical plant communities on glacial time-scales: The failure of the aridity and refuge hypotheses. *Quaternary Science Reviews* 19: 141–169.

Colwell, R.K. 2000. A barrier runs through it . . . or maybe just a river. *Proceedings of the National Academy of Science* 97(25): 13470–13472.

Corander J.; Tang , J. 2007. Bayesian analysis of population structure based on linked molecular information. *Mathematical Biosciences* 205: 19–31.

Corander, J.; Marttinen, P.; Sirén J.; Tang, J. 2008. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics* 9: 539.

Costa, J.B.S.; Bemerguy, R.L.; Hasui, Y.; Borges, M.S. 2001. Tectonics and paleogeography along the Amazon River. *Journal of South American Earth Sciences* 14: 335–347.

Coyne, J.A.; Orr, H.A. 2004. *Speciation*. Sinauer Associates Inc., Sunderland.

Cracraft, J.; Prum, R.O. 1988. Patterns and processes of diversification: speciation and historical congruence in some neotropical birds. *Evolution* 42(3): 603–620.

Crandall, K.A.; Bininda-Emonds, O.R.P.; Mace, G.M.; Wayne, R.K. 2000. Considering evolutionary processes in conservation biology. *Trends in Ecology and Evolution* 15(7): 290–295.

Dawe, K.L.; Shafer, A.B.A.; Herman, T.B.; Stewart, D.T. 2009. Diffusion of nuclear and mitochondrial genes across a zone of secondary contact in the maritime shrew, *Sorex maritimensis*: implications for the conservation of a Canadian endemic mammal. *Conservation Genetics* 10: 851–857.

DeWoody, J.A.; Shupp, J.; Kenefic, L.; Busch, J.; Murfitt, L.; Keim, P. 2004. Universal method for producing ROX-labeled size standards suitable for automated genotyping. *Biotechniques* 37: 348–352.

DNPM - Departamento Nacional da Produção Mineral – Brasil. 1978. *Projeto RADAMBRASIL – Levantamento dos recursos naturais, Vol.18, Folha SC 20, Porto Velho: Geologia, geomorfologia, pedologia, vegetação e uso potencial da terra*. Departamento Nacional da Produção Mineral, Rio de Janeiro.

DNPM - Departamento Nacional da Produção Mineral – Brasil. 1978. *Projeto RADAMBRASIL – Levantamento dos recursos naturais, Vol.19, Folha SB 20, Purus: Geologia, geomorfologia, pedologia, vegetação e uso potencial da terra*. Departamento Nacional da Produção Mineral , Rio de Janeiro.

Doyle, J.J.; Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Excoffier, L.; Smouse, P.; Quattro, J. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitocondrial DNA restriction data. *Genetics* 131: 479–491.

Excoffier, L. G.; Laval, G.; Schneider, S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47–50.

Farris, J.S.; Källersjö, M.; Kluge, A.G.; Bult, C. 1995. Constructing a significance test for incongruence. *Systematic Biology* 44(4): 570–572.

Fernandes, A.M.; Cohn-Haft, M.; Hrbek, T.; Farias, I.P. 2010. Comparative phylogeography of three species of passerine birds in the Madeira River basin: cryptic endemism in a threatened landscape. *Journal of Biogeography* (in press).

Figueiredo, J.; Hoorn, C.; van der Ven, P.; Soares, E. 2009. Late Miocene onset of the Amazon River and the Amazon deep-sea fan: Evidence from the Foz do Amazonas Basin. *Geology* 37(7): 619–622.

Fouquet, A.; Gilles, A.; Vences, M.; Marty, C.; Blanc, M.; Gemmell, N.J. 2007a. Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *Plos One* 10: 1–10.

Fouquet, A.; Vences, M.; Salducci, M.-D.; Meyer, A.; Marty, C.; Blanc, M.; Gilles, A. 2007b. Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the *Scinax ruber* and *Rhinella margaritifera* species groups. *Molecular Phylogenetics and Evolution* 43: 567–582.

Frost, D.R.; Grant, T.; Faivovich, J.; Bain, R.H.; Haas, A.; Haddad, C.F.B.; de Sá, R.O.; Channing, A.; Wilkinson, M.; Donnellan, S.C.; Raxworthy, C.J.; Campbell, J.E.; Blotto, B.L.; Moller, P.; Drewes, R.C.; Nussbaun, R.A.; Lynch, J.D.; Green, D.M.; Wheeler, W.C. 2006. The amphibian tree of life. *Bulletin of the American Museum of Natural History* 279: 1–370.

Frost, D.R. 2009. *Amphibian Species of the World: an Online Reference. Version 5.3* (http://research.amnh.org/herpetology/amphibia/). Acesso: 05/06/2009.

Fu, Y.X. 1997. Statistical tests of neutrality of mutations againstpopulation growth, hitchhiking and background selection. *Genetics* 147: 915–925.

Funk, D.J.; Omland, K.E. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics* 34: 397–423.

Gascon, C.; Lougheed, S.C.; Bogart, J. P. 1998. Patterns of genetic population differentiation in four species of Amazonian frogs: A test of the Riverine Barrier Hypothesis. *Biotropica* 30(1): 104–119.

Gascon, C.; Malcolm, J.R.; Patton, J.L.; Silva, M.N.F.; Bogarti, J.P.; Lougheed, S.P.; Peres, C.A.; Neckel, S.; Boag, P.T. 2000. Riverine barriers and the geographic distribution of Amazonian species. *PNAS* 97(25): 13672–13677.

Gentry, A.H. 1988. Tree species richness of upper Amazonian forests. *Proceedings of the National Academy of Science* USA 85: 156–159.

Gerhardt, H.C.; Huber, F. 2002. *Acoustic communication in insects and anurans*: *common problems and diverse solutions*. University of Chicago Press, Chicago.

Göd, M.; Franz, A.; Hödl, W. 2007. The influence of internote-interval variation of the advertisement call on the phonotactic behaviour in male *Allobates femoralis* (Dendrobatidae). *Amphibia-Reptilia* 28: 227–234.

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183–190.

Goudet, J. 2001. *FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3)* (http://www.unil.ch/izea/softwares/fstat.html). Acesso: 20/11/2009.

Grant, P.R.; Grant, B.R. 1992. Hybridization of bird species. *Science* 256(5054): 193–197.

Grant, P.R.; Grant, B.R. 2002. Unpredictable evolution in a 30-year study of Darwin's finches. *Science* 296: 707–711.

Grant, T.; Frost, D.R.; Caldwell, J.P.; Gagliardo, R.; Haddad, C.F.B.; Kok, P.J.R.; Means, B.D.; Noonan, B.P.; Schargel, W.; Wheeler, W.C. 2006. Phylogenetic systematics of dart-poison frogs and their relatives (Anura: Athesphatanura: Dendrobatidae). *Bulletin of the American Museum of Natural History* 299: 1–262.

Green, A.J. 2001. Mass/Length residuals: Measures of body condition or generators of spurious results? *Ecology* 82(5): 1473–1483.

Haffer, J. 1969. Speciation in Amazonian forest birds. *Science* 165: 131-137.

Haffer, J. 1974. Avian speciation in Tropical South America: With a systematic survey of the toucans (Ramphastidae) and jacamars (Galbulidae). *Publications of the Nuttall Ornithological Club* 14: 1–390.

Haffer, J. 1997. Alternative models of vertebrate speciation in Amazonia: an overview. *Biodiversity and Conservation* 6: 451–476.

Haffer, J.; Prance, G.T. 2002. Impulsos climáticos da evolução na Amazônia durante o Cenozóico: sobre a teoria dos Refúgios da diferenciação biótica. *Estudos Avançados* 16: 175–206.

Haig, S.M.; Mullins, T.D.; Forsman, E.D.; Trail, P.W.; Wennerberg, L. 2004. Genetic identification of Spotted Owls, Barred Owls, and their hybrids: legal implication of hybrid identity. Conservation Biology 18: 1347–1357.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.

Harpending, R.C. 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology* 66: 591–600.

Hayes, F.E.; Sewlal, J.N. 2004. The Amazon River as a dispersal barrier to passerine birds: effects of river width, habitat and taxonomy. *Journal of Biogeography* 31: 1809–1818.

Hird, S.; Sullivan, J. 2009. Assessment of gene flow across a hybrid zone in red-tailed chipmunks (*Tamias ruficaudus*). *Molecular Ecology* 18: 3097–3109.

Höbel, G.; Gerhardt, H.C. 2003. Reproductive character displacement in the acoustic communication system of gree tree frogs (*Hyla cinerea*). *Evolution* 57(4): 894–904.

Hödl, W. 1987. *Dendrobates femoralis* (Dendrobatidae): a handy fellow for frog bioacoustics. *Proceedings of the 4th Ordinary General Meeting of the Societas Europaea Herpetologica*. Nijmegen, 1987, 201–204.

Hödl, W.; Amézquita, A.; Narins, P.M. 2004. The rôle of call frequency and the auditory papillae in phonotactic behavior in male dart-poison frogs *Epipedobates femoralis* (Dendrobatidae). *Journal of Comparative Physiology A* 190: 823–829.

Hofman, S.; Szymura, J.M. 2007. Limited mitochondrial DNA introgression in a *Bombina* hybrid zone. *Biological Journal of the Linnean Society* 91: 295–306.

Hoorn, C. 1994. An environmental reconstruction of the palaeo-Amazon River system (Middle–Late Miocene, NW Amazonia). *Palaeogeography, Palaeoclimatology, Palaeoecology* 112: 187–238.

Hoorn, C.; Wesselingh, F.P. 2010. Amazonia, landscape and species evolution. Blackwell Publishing, Oxford.

Hoskin, C.J.; Higgie, H.; McDonald, K.R.; Moritz, C. 2005. Reinforcement drives rapid allopatric speciation. *Nature* 437: 1353–1356.

Hudson, R.R. 1990. Gene genealogies and the coalescent process. *Oxford Surveys in Evolutionary Biology* 7: 1–44.

Huelsenbeck, J.P.; Crandall, K.A. 1997. Phylogeny estimation and hypothesis testing using Maximum Likelihood. *Annual Review of Ecology and Systematics* 28: 437–466.

Jehle, R.; Gasser, H.; Pfunder, M.; Amézquita, A.; Lima, A.P.; Hödl, W. 2008. Ten polymorphic microsatellite loci for *Allobates femoralis*, an Amazonian dendrobatoid frog. Molecular Ecology Resources 8(6): 1326–1328.

Jiggins, C.D.; Mallet, J. 2000. Bimodal hybrid zones and speciation. *Trends in Ecology and Evolution* 15(6): 250–255.

Jobb, G. 2008. *TREEFINDER* (www.treefinder.de). Acesso: 20/04/2009.

Keller, B.; Wolinska, J.; Manca, M.; Spaak, P. 2008. Spatial, environmental and anthropogenic effects on the taxon composition of hybridizing *Daphnia*. *Philosophical Transactions of the Royal Society B* 363: 2943–2952.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.

Knowles L.L. 2009. Statistical phylogeography. *Annual Review of Ecology, Evolution, and Systematics* 40: 593–612.

Latubresse, E.M. 2003. The Late-Quaternary Palaeohydrology of large South American fluvial systems. *In:* Gregory, K.J.; Benito, G. (Eds). *Palaeohydrology: Understanding Global Change*. John Wiley & Sons Ltd, Hoboken. p. 193–212.

Latubresse, E.M.; Franzinelli, E. 2002. The Holocene alluvial plain of the middle Amazon River, Brazil. *Geomorphology* 44: 241–257.

Laurance, W.F.; Albernaz, A.K.M.; Fearnside, P.M.; Vasconcelos, H.L.; Ferreira, L.V. 2004. Deforestation in Amazonia. *Science* 304: 1109.

Legendre, P. 2000. Comparison of permutation methods for the partial correlation and partial Mantel tests. *Journal of Statistical Computation and Simulation* 67: 37–73.

Legendre, P.; Dale, M.R.T.; Fortin, M.J.; Gurevitch, J.; Hohn, M.; Myers, D. 2002. The consequences of spatial structure for the design and analysis of ecological field surveys. *Ecography* 25: 601–615.

Lescure, J.; Marty, C. 2000. Atlas des amphibiens de Guyane. *Patrimoines Naturels* 45: 1–288.

Lemmon, E.M.; Lemmon, A.R.; Collins, J.T.; Lee-Yaw, J.A.; Cannatella, D.C. 2007. Phylogeny-based delimitation of species boundaries and contact zones in the trilling chorus frogs (*Pseudacris*). *Molecular Phylogenetics and Evolution* 44: 1068–1082.

Levin, D.A.; Francisco-Ortega, J.; Jansen, R.K. 1996. Hybridization and the extinction of rare plant species. *Conservation Biology* 10(1): 10–16.

Librado, P.; Rozas, J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25: 1451–1452.

Lima, A.P.; Caldwell, J.P. 2001. A new species of *Colostethus* with sky blue digits. *Herpetologica* 57: 180–189.

Lima, A.P.; Sanchez, D.E.A.; Souza, J.R.D. 2007. A new Amazonian species of frog genus *Colostethus* (Dendrobatidae) that lays its eggs on undersides of leaves. *Copeia 2007*(1): 114–122.

Lima, A.P.; Caldwell, J.P.; Strussmann, C. 2009. Redescription of *Allobates brunneus* (Cope) 1887 (Anura: Aromobatidae: Allobatinae), with a description of the tadpole, call, and reproductive behavior. *Zootaxa* 1988: 1–16.

Lötters, S.; Jungfer, K.-H.; Henkel, F.W.; Schmitz, W. 2007. *Poison Frogs. Biology, Species & Captive Husbandry.* Chimaira, Frankfurt.

Lötters, S.; Schmitz, A.; Reichle, S.; Rödder, D.; Quennet, V. 2009. Another case of cryptic diversity in poison frogs (*Dendrobatidae: Ameerega*) - description of a new species from Bolivia. *Zootaxa* 2028: 20–30.

Lougheed, S.C.; Gascon, C.; Jones, D.A.; Bogart, J.P.; Boag, P.T. 1999. Ridges and rivers: a test of competing hypothesis of Amazonian diversification using a dart-poison frog (*Epipedobates femoralis*). *Proceedings of the Royal Society London* 266: 1829–1835.

Lutz, B.; Kloss, G.R. 1952. Anfíbios anuros do alto Solimões e Rio Negro: apontamentos sobre algumas formas e suas vicariantes. *Memórias do Instituto Oswaldo Cruz* 50: 621–677.

Mallet, J. 2005. A species definition for the modern systhesis. *Trends in Ecology and Evolution* 10(7): 294–299.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* 27: 209–220.

Maslin, M.A.; Burns, S.J. 2000. Reconstruction of the Amazon Basin effective moisture availability over the past 14,000 years. *Science* 290: 2285–2287.

Mayr, E. 1996. What is a species and what is not? *Philosophy of Science* 63(2): 262–277.

McDiarmid, R.W.; Altig, R. 1999. Body plan: development and morphology. *In:* McDiarmid, R.W.; Altig, R. (Eds). *Tadpoles: the biology of anuran larvae*. The University of Chicago Press, Chicago p. 24–51.

McGuire, J.A.; Linkem, C.W.; Koo, M.S.; Hutchison, D.W.; Lappin, A.K.; Orange, D.I.; Lemos-Espinal, J.; Riddle, B.R.; Jaeger, J.R. 2007. Mitochondrial introgression and incomplete lineage sorting through space and time: phylogenetics of the Crotaphytid lizards. *Evolution* 61(12): 2879–2897.

Menin, M.; Lima, A.P.; Magnusson, W.E.; Waldez, F. 2007. Topographic and edaphic effects on the distribution of terrestrially reproducing anurans in Central Amazonia: mesoscale spatial patterns. *Journal of Tropical Ecology* 23: 539–547.

Moritz, C. 1994. Defining evolutionarily significant units for conservation. *Trends in Ecology and Evolution* 9: 373–375.

Moritz, C. 1995. Uses of Molecular Phylogenies for Conservation. *Philosophical Transactions: Biological Sciences* 349(1327): 113–118.

Moritz, C.; Patton, J.L.; Schneider, C.J.; Smith, T.B. 2000. Diversification of rainforest faunas: An integrated molecular approach. *Annual Review of Ecology and Systematics* 31: 533–563.

Moritz, C.; Hoskin, C.J.; MacKenzie, J.B.; Phillips, B.L.; Tonione, M.; Silva, N.; VanDerWal, J.; Williams S.E., Graham, C.H. 2009. Identification and dynamics of a cryptic suture zone in tropical rainforest. *Proceedings of the Royal Society* B 276: 1235–1244.

Morrone, J.J. 2006. Biogeographic areas and transition zones of Latin America and the Caribbean Islands based on panbiogeographic and cladistic analyses of the entomofauna. *Annual Review of Entomology* 51: 467–94.

Narins, P.M.; Hödl, W.; Grabul, D.S. 2003. Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *PNAS* 100(2): 577–580.

Nogueira, A.C.R. 2008. Guinada para o Atlântico. *Scientific American Brasil – Amazônia* 1: 22–27.

Noonan, B.P.; Wray, K.P. 2006. Neotropical diversification: the effects of complex history on diversity within the poison frog genus *Dendrobates*. *Journal of Biogeography* 33: 1007–1020.

Padial, J.M.; De la Riva, I. 2009. Integrative taxonomy reveals cryptic Amazonian species of Pristimantis (Anura, Strabomantidae). *Zoological Journal of the Linnean Society* 155: 97–122.

Padial, J.M.; Miralles, A.; De La Riva, I.; Vences, M. 2010. The integrative future of taxonomy. *Frontiers in Zoology* 7: 1–16.

Palumbi, S.R. 1996. Nucleic acids II: the polymerase chain reaction. *In:* Hillis, D.M.; Moritz, C.; Mable, B.K. (Eds). *Molecular Systematics*. Sinauer & Associates Inc., Sunderland, Massachusetts. p. 205–247.

Patton, J.L.; Silva, M.N.F.; Malcolm, J.R. 1994. Gene genealogy and differentiation among arboreal spiny rats (Rodentia: Echymidae) of the Amazon Basin: a test of the riverine barrier hypothesis. *Evolution* 48: 1314–1323.

Peakall, R.; Smouse, P.E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

Peres, C.A.; Patton, J.L.; Silva, M.N.F. 1996. Riverine Barriers and gene flow in Amazonian saddle-back tamarins. *Folia Primatologica* 67(3): 113–124.

Perz, S.; Brilhante, S.; Brown, F.; Caldas, M.; Ikeda, S; Mendoza, E.; Overdevest, C.; Reis, V.; Reyes, J.F.; Rojas, D.; Schmink, M.; Souza, C.; Walker, R. (2008) Road building, land use and climate change: prospects for environmental governance in the Amazon. *Philosophical Transactions Royal Society B* 363: 1889–1895.

Pfennig, K.S. 2003. A test of alternative hypothesis for the evolution of reproductive isolation between spadefoot toads: support for the reinforcement hypothesis. *Evolution* 57(12): 2842–2851.

Posada, D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* 25: 1253–1256.

Posada, D.; Crandall K.A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14(9): 817–818.

Pritchard, J.K.; Stephens, M.; Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.

Pyburn, W.F. 1981. A new poison-dart frog (Anura: Dendrobatidae) from the forest of southeastern Colombia. *Proceedings of the Biological Society Washington* 94: 67–75.

Rambaut, A.; Drummond, A.J. 2007. *Tracer v1.4*. (http://beast.bio.ed.ac.uk/Tracer). Acesso: 10/07/2009.

Ramos-Onsins, S.E.; Rozas, J. 2002. Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution* 19: 2092–2100.

Read, M. 2000. *Frogs of the Ecuatorian Amazon*: a guide to their calls. Morley Read Productions, Cornwall.

Reis,S.F.; Pessoa, L.M.; Strauss, R.E. 1990. Application of size-free canonical discriminant analysis to studies of geographic differentiation. *Revista Brasileira de Genética* 13: 509–520.

Ringler, M.; Ursprung, E.; Hödl, W. 2009. Site fidelity and patterns of short- and long-term movement in the brilliant-thighed poison frog *Allobates femoralis*. *Behavioral Ecology and Sociobiology* 63(9): 1281–1293.

Roberts, J.L.; Brown, J.L.; von May, R.; Arizabal, W.; Schulte, R.; Summers, K. 2006. Genetic divergence and speciation in lowland and montane Peruvian poison frogs. *Molecular Phylogenetics and Evolution* 49: 149–164.

Rodríguez, L.O.; Duellman, W.E. 1994. *Guide of the frogs of the Iquitos Region, Amazonian Peru*. University of Kansas Publications [Natural History Museum - Special Publications, 22], Lawrence, Kansas.

Roe, B.A.; Ma, D.P.; Wilson, R.K.; Wong, J.F. 1985. The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. *Journal of Biological Chemistry* 260(17): 9759–9774.

Roithmair, M.E. 1992. Territoriality and male mating success in the Dart-Poison Frog, *Epipedobates femoralis* (Dendrobatidae, Anura). *Ethology* 92: 331–343.

Roithmair, M.E. 1994. Field studies on reproductive behaviour in two dart-poison frog species (*Epipedobates femoralis*, *Epipedobates trivittatus*) in Amazonian Peru. *Herpetological Journal* 4: 77–85.

Ron, S. 2000. Biogeographic area relationships of lowland Neotropical rainforest based on raw distributions of vertebrate groups. *Biological Journal of the Linnean Society* 71: 379–402.

Ronquist, F.; Huelsenbeck, J. P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Ronquist, F.; van der Mark, P.; Huelsenbeck, J.P. 2009. Bayesian phylogenetic analysis using MrBayes. *In*: Lemey, P.; Salemi, M.; Vandamme, A. (Eds). *The Phylogenetic Handbook*. Cambridge University Press, New York. p. 210–266.

Roosmalen, M.G.M.; Roosmalen, T.; Mittermeier, R.A. 2002. A taxonomic review of the Titi Monkeys, genus *Callicebus*, Tomas, 1903, with the description of two new species, *Callicebus bernhardi* and *Callicebus stephennashi*, from Brazilian Amazonia. *Neotropical Primates* 10 (Suppl.): 1–52.

Rossetti, D.F.; de Toledo, P.M.; Góes, A.M. 2005. New geological framework for Western Amazonia (Brazil) and implications for biogeography and evolution. *Quaternary Research* 63: 78–89.

Ryan, M.J. 1988. Energy, calling, and selection. *American Zoologist* 28: 885–898.

Saitou, N.; Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406–425.

Santos J.C.; Coloma, L.A.; Cannatella, D.C. 2003. Multiple, recurring origins of aposematism and diet specialization in poison frogs. *Proceedings of the National Academy of Science USA* 100 (22): 12792–12797.

Santos, J.C.; Coloma, L.A.; Summers, K.; Caldwell, J.P.; Ree, R.; Cannatella, D.C. 2009. Amazonian amphibian diversity is primarily derived from Late Miocene Andean lineages. *PLOS Biology* 7(3): 1–14.

Sanz, N.; Araguas, R.M.; Fernández, R.; Vera, M.; García-Marín, J.L. 2009. Efficiency of markers and methods for detecting hybrids and introgression in stocked populations. *Conservation Genetics* 10: 225–236.

Schneider, S.; Excoffier, L. 1999. Estimation of demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: Application to human mitochondrial DNA. *Genetics* 152: 1079–1089.

Seehausen, O. 2004. Hybridization and adaptive radiation. *Trends in Ecology and Evolution* 19(4): 198–206.

Seehausen, O.; Takimoto, G.; Roy, D.; Jokela, J. 2007. Speciation reversal and biodiversity dynamics with hybridization in changing environments. Molecular Ecology 17(1): 30–44.

Shimodaira, H.; Hasegawa, M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* 16(8): 1114–1116.

Silva, J.M.C.; Rylands, A.B.; Fonseca, G.A.B. 2005. The fate of the Amazonian areas of endemism. *Conservation Biology* 19(3): 689–694.

Silva, M.N.F.; Patton, J. L. 1998. Molecular phylogeography and the evolution and conservation of mammals. *Molecular Ecology* 7: 475–486.

Simões, P.I. 2006. Divergência acústica e morfológica em populações de *Allobates femoralis* (Anura, Dendrobatidae) do alto Rio Madeira. Dissertação [Mestrado]. Instituto Nacional de Pesquisas da Amazônia. Manaus.

Simões, P.I.; Lima, A.P.; Magnusson, W.E.; Hödl, W.; Amézquita, A. 2008. Acoustic and morphological differentiation in the frog *Allobates femoralis*: Relationships with the upper Madeira River and other potential geological barriers. *Biotropica* 40(5): 607–614.

Simões, P.I.; Lima, A.P.; Farias, I.P. 2010. The description of a cryptic species related to the pan-Amazonian frog *Allobates femoralis* (Boulenger 1883) (Anura: Aromobatidae). *Zootaxa* 2406: 1–28.

Smouse, P.E.; Long, J.C.; Sokal, R.R. 1986. Multiple regression and correlation extensions of the Mantel test of matrix correspondence. *Systematic Zoology* 35: 727–32.

Solomon, S.E.; Bacci, M.; Martins, J.; Vinha, G.G.; Mueller, U.G. 2008. Paleodistributions and comparative molecular phylogeography of leafcutter ants (*Atta* spp.) provide new insight into the origins of Amazonian diversity. *Plos ONE* 3(7): 1–15.

Souza Filho, P.W.M.; Quadros, M.L.E.S.; Scandolara, J.E.; Silva Filho, E.P.; Reis, M.R. 1999. Compartimentação morfoestrutural e neotectônica do sistema fluvial Guaporé-Mamoré-Alto Madeira, Rondônia – Brasil. *Revista Brasileira de Geociências* 29(4): 469–476.

Switkes, G. 2008. *Águas Turvas: Alertas sobre as conseqüencias de barrar o maior afluente do Amazonas*. International Rivers, São Paulo.

Swofford, D.L. 1998. *PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods)*, version 4. Sinauer Associates, Sunderland, Massachusetts.

Symula, R.; Schulte, R.; Summers, K. 2003. Molecular systematics and phylogeography of Amazonian poison frogs of the genus *Dendrobates*. *Molecular Phylogenetics and Evolution* 26: 452–475.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123: 585–595.

Tamura, K.; Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10: 512–526.

Tamura K.; Dudley, J.; Nei, M.; Kumar, S. 2007. MEGA4*:* Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596–1599.

Telles, M.P.C.; Diniz-Filho, J.A.F. 2005. Multiple Mantel tests and isolation-by-distance, taking into account long-term historical divergence. *Genetics and Molecular Research* 4(4): 742–748.

Templeton, A.R.; Crandall, K.A.; Sing, C.F. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetic*s 132: 619–633.

Thompson, J.D.; Higgins, D.G.; Gibson, T.J. 1994. Improved sensitivity of profile searches through the use of sequence weights and gap excision. *Computer Applications in the Biosciences* 10(1): 19–29.

Thorpe, R.S.; Reardon, J.T.; Malhotra, A. 2005. Common garden and natural selection experiments support ecotypic differentiation in the Dominican anole (Anolis oculatus). *The American Naturalist* 165(4): 495–504.

Tocher, M.D.; Gascon, C.; Meyer, J. 2001. Community composition and breeding success of Amazonian frogs in continuous forest and matrix habitat aquatic sites. *In:* Bierregaard, R.O.; Gascon, C.; Lovejoy, T.; Mesquita, R. (Eds). *Lessons from Amazonia*: *The ecology and conservation of a fragmented forest*. Yale University Press, New Haven. p. 235–247.

Tuomisto, H.; Ruokolainen, K.; Yli-Halla, M. 2003. Dispersal, environment, and floristic variation of Western Amazonian forests. *Science* 299: 241–244.

Twomey, E.; Brown, J.L. 2008. A partial revision of the *Ameerega hahneli* complex (Anura: Dendrobatidae) and a new cryptic species from the East-Andean versant of Central Peru. *Zootaxa* 1757: 49–65.

Vences, M.; Thomas, M.; Meijden, A. van der; Chiari, Y.; Vieites, D.R. 2005. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Frontiers in Zoology* 2(5): 1–12.

Vieites, D.R.; Wollenberg, K.C.; Andreone, F.; Köhler, J.; Glaw, F.; Vences, M. 2009. Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy os Science USA* 106(20): 8267–8272.

Vogel, L.S.; Johnson, S.G. 2008. Estimation of hybridization and introgression frequency in toads (Genus: *Bufo*) using DNA sequence variation at mitochondrial and nuclear loci. *Journal of Herpetology* 42(1): 61–75.

Vorndran, I.C.; Reichwaldt, E.; Nürnberger, B. 2002. Does differential susceptibility to predation in tadpoles stabilize the *Bombina* hybrid zone? *Ecology* 83(6): 1648–1659.

Wallace, A. R. 1852. On the monkeys of the Amazon. *Proceedings of the Zoological Society of London* 20: 107–110.

Wells, K.D. 2007. *The ecology and behavior of amphibians*. The University of Chicago Press, Chicago. p. 278–282.

Wesselingh, F.P. 2006. Miocene long-lived lake Pebas as a stage of mollusk radiations, with implications for landscape evolution in western Amazonia. *Scripta Geologica* 133: 1–17.

Wesselingh, F.P.; Salo, J.A. 2006. A Miocene perspective on the evolution of the Amazonian biota. *Scripta Geologica* 133: 439–458.

Westaway, R. 2006. Late Cenozoic sedimentary sequences in Acre state, southwestern Amazonia: Fluvial or tidal? Deductions from the IGCP 449 fieldtrip. *Journal of South American Earth Sciences* 21: 120–134.

Wilkinson, L. 1990. *SYSTAT: the system for statistics*. SYSTAT inc., Evanston.

Wright, S. 1951. The genetical structure of populations. *Annual Eugenics* 15: 323–354.

Wynn, A.; Heyer, W.R. 2001. Do geographically widespread species of tropical amphibians exist? An estimate of genetic relatedness within the Neotropical frog *Leptodactylus fuscus* (Schneider, 1799) (Anura: Leptodactylidae). *Tropical Zoology* 14: 255–285.

Zink, R.M.; Blackwell-Rago, R.C.; Ronquist, F. 2000. The shifting roles of vicariance in biogeography. *Proceedings of the Royal Society London* 267: 497–503.

ANEXOS[4]

[3] Pareceres emitidos pelas bancas examinadoras da aula de qualificação, da versão escrita da tese e da defesa pública da tese, respectivamente.

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PIPG BTRN
PROGRAMA INTEGRADO DE PÓS-GRADUAÇÃO EM BIOLOGIA TROPICAL E RECURSOS NATURAIS

Ministério da Ciência e Tecnologia — BRASIL UM PAÍS DE TODOS — GOVERNO FEDERAL

AULA DE QUALIFICAÇÃO

PARECER

Aluno(a): **PEDRO IVO SIMÕES**
Curso: ECOLOGIA
Nível: Doutorado
Orientador(a): ALBERTINA P. LIMA (INPA)

Título:

"DIVERSIFICAÇÃO DO COMPLEXO *Allobates femoralis* (ANURA, DENDROBATIDAE) EM FLORESTAS DA AMAZÔNIA BRASILEIRA: INTEGRANDO FATORES CAUSAIS GENÉTICOS, HISTÓRICOS E ECOLÓGICOS".

BANCA JULGADORA:

TITULARES:

MARISTERRA RODRIGUES LEMES (INPA)
MÁRCIO LUIZ DE OLIVEIRA (INPA)
CELSO MORATO DE CARVALHO (INPA)
RENATO CINTRA SOARES (INPA)
JEFF PODOS (University of Massachusetts)

SUPLENTES:

VERA MARIA F. DE ALMEIDA E VAL (INPA)
JORGE IVAN REBELO PORTO (INPA)

EXAMINADORES	PARECER		ASSINATURA
MARISTERRA RODRIGUES LEMES	(X) Aprovado	( ) Reprovado	
MÁRCIO LUIZ DE OLIVEIRA	(X) Aprovado	( ) Reprovado	
CELSO MORATO DE CARVALHO	(X) Aprovado	( ) Reprovado	
RENATO CINTRA SOARES	(X) Aprovado	( ) Reprovado	
JEFF PODOS	(X) Aprovado	( ) Reprovado	
VERA MARIA F. DE ALMEIDA E VAL	( ) Aprovado	( ) Reprovado	
JORGE IVAN REBELO PORTO	( ) Aprovado	( ) Reprovado	

Manaus(AM), 20 de novembro de 2006

OBS: O candidato respondeu com segurança às questões formuladas demonstrando conhecimento do assunto sendo aprovado por unanimidade.

INPA - DCPG
RECEBIDO
Em: 21/11/06
Hora: 9:20
Visto:

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Instituto Nacional de Pesquisas da Amazônia - INPA
Programa de Pós-graduação em Ecologia

Avaliação de tese de doutorado

Título: **Diversificação do complexo Allobates femoralis (Anura, Dendrobatidae) em florestas da Amazônia brasileira: desvendando padrões atuais e históricos**

Aluno: **PEDRO IVO SIMÕES**

Orientador: **Albertina P. Lima** Co-orientador: **Izeni Pires Farias**

Avaliador: **Walter Hödl**

Por favor, marque a alternativa que considerar mais apropriada para cada ítem abaixo, e marque seu parecer final no quadro abaixo

	Muito bom	Bom	Necessita revisão	Reprovado
Relevância do estudo	(x)	( )	( )	( )
Revisão bibliográfica	(x)	( )	( )	( )
Desenho amostral/experimental	(x)	( )	( )	( )
Metodologia	(x)	( )	( )	( )
Resultados	(x)	( )	( )	( )
Discussão e conclusões	(x)	( )	( )	( )
Formatação e estilo texto	(x)	( )	( )	( )
Potencial para publicação em periódico(s) indexado(s)	(x)	( )	( )	( )

PARECER FINAL

(x) **Aprovada**

( ) **Aprovada com correções** (indica que as modificações mesmo extensas podem ser incluídas a juízo do orientador)

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( ) **Reprovada** (indica que o trabalho não tem o nível de qualidade adequado para uma tese)

Vienna, Austria **4.9.2010**

Local **Data** **Assinatura**

Comentários e sugestões podem ser enviados como uma continuação desta ficha, como arquivo separado ou como anotações no texto impresso ou digital da tese. Por favor, envie a ficha assinada, bem como a cópia anotada da tese e/ou arquivo de comentários por e-mail para pgecologia@gmail.com e claudiakeller23@gmail.com ou por correio ao endereço abaixo. O envio por e-mail é preferível ao envio por correio. Uma cópia digital de sua assinatura será válida.

Endereço para envio de correspondência:

Claudia Keller
DCEC/CPEC/INPA
CP 478
69011-970 Manaus AM
Brazil

Instituto Nacional de Pesquisas da Amazônia - INPA
Graduate Program in Ecology

Referee evaluation sheet for PhD thesis

Title: **Diversificação do complexo Allobates femoralis (Anura, Dendrobatidae) em florestas da Amazônia brasileira: desvendando padrões atuais e históricos**

Candidate: **PEDRO IVO SIMÕES**

Supervisor: **Albertina P. Lima** Co-supervisor: **Izeni Pires Farias**

Examiner: **Robert Jehle**

Please check one alternative for each of the following evaluation items, and check one alternative in the box below as your final evaluation decision.

	Excellent	Satisfactory	Needs improvement	Not acceptable
Relevance of the study	( )	(x)	( )	( )
Literature review	(x)	( )	( )	( )
Sampling design	(x)	( )	( )	( )
Methods/procedures	(x)	( )	( )	( )
Results	( )	(x)	( )	( )
Discussion/conclusions	( )	(x)	( )	( )
Writing style and composition	(x)	( )	( )	( )
Potential for publication in peer reviewed journal(s)	(x)	( )	( )	( )

FINAL EVALUATION

(x) **Approved without changes**

( ) **Approved with changes** (no need for re-evaluation by this reviewer)

( ) **Potentially acceptable, conditional upon review of a corrected version** (The candidate must submit a new version of the thesis, taking into account the corrections asked for by the reviewer. This new version will be sent to the reviewer for a new evaluation only as acceptable or not acceptable)

( ) **Not acceptable** (This product is incompatible with the minimum requirements for this academic level)

Salford **17/08/2010**

Place **Date** **Signature**

Additional comments and suggestions can be sent as an appendix to this sheet, as a separate file, and/or as comments added to the text of the thesis. Please, send the signed evaluation sheet, as well as the annotated thesis and/or separate comments by e-mail to pgecologia@gmail.com and claudiakeller23@gmail.com or by mail to the address below. E-mail is preferred. A scanned copy of your signature is acceptable.

Mailing address:

Claudia Keller
DCEC/CPEC/INPA
CP 478
69011-970 Manaus AM
Brazil

Instituto Nacional de Pesquisas da Amazônia - INPA
Programa de Pós-graduação em Ecologia

Avaliação de tese de doutorado

Título: **Diversificação do complexo Allobates femoralis (Anura, Dendrobatidae) em florestas da Amazônia brasileira: desvendando padrões atuais e históricos**

Aluno: **PEDRO IVO SIMÕES**

Orientador: **Albertina P. Lima** Co-orientador: **Izeni Pires Farias**

Avaliador: **José Manuel Padial Fregenal**

Por favor, marque a alternativa que considerar mais apropriada para cada ítem abaixo, e marque seu parecer final no quadro abaixo

	Muito bom	Bom	Necessita revisão	Reprovado
Relevância do estudo	(X)	( )	( )	( )
Revisão bibliográfica	(X)	( )	( )	( )
Desenho amostral/experimental	(X)	( )	( )	( )
Metodologia	(X)	( )	( )	( )
Resultados	(X)	( )	( )	( )
Discussão e conclusões	(X)	( )	( )	( )
Formatação e estilo texto	(X)	( )	( )	( )
Potencial para publicação em periódico(s) indexado(s)	(X)	( )	( )	( )

PARECER FINAL

(X) **Aprovada**

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Granada, Spain, 23 August 2010, 

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Claudia Keller
DCEC/CPEC/INPA
CP 478
69011-970 Manaus AM
Brazil

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Avaliação de tese de doutorado

Título: **Diversificação do complexo Allobates femoralis (Anura, Dendrobatidae) em florestas da Amazônia brasileira: desvendando padrões atuais e históricos**

Aluno: **PEDRO IVO SIMÕES**

Orientador: **Albertina P. Lima** Co-orientador: **Izeni Pires Farias**

Avaliador: **Jeff Podos**

Por favor, marque a alternativa que considerar mais apropriada para cada ítem abaixo, e marque seu parecer final no quadro abaixo

	Muito bom	Bom	Necessita revisão	Reprovado
Relevância do estudo	(x)	( )	( )	( )
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PARECER FINAL

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____Amherst, MA, EUA____, _____12 August 2010___, _____
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Claudia Keller
DCEC/CPEC/INPA
CP 478
69011-970 Manaus AM
Brazil

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ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA DO INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA.

Ao 1º dia do mês de outubro do ano de 2010, às 14:30 horas, na sala de aula do Programa de Pós- Graduação em Ecologia do Instituto Nacional de Pesquisas da Amazônia PPG-ECO/INPA, reuniu-se a Comissão Examinadora de Defesa Pública, composta pelos seguintes membros: o(a) Prof(a). Dr(a). **José Antonio Alves Gomes**, do Instituto Nacional de Pesquisas da Amazônia, o(a) Prof(a). Dr(a). **Mário Cohn-Haft**, do Instituto Nacional de Pesquisas da Amazônia e o (a) Prof(a). Dr(a). **Marcelo Menin**, da Universidade Federal do Amazonas, tendo como suplentes o(a) Prof(a). Dr(a). Lúcia Helena Rapp Py-Daniel, do Instituto Nacional de Pesquisas da Amazônia e o(a) Prof(a). Dr(a). Marina Anciães, do Instituto Nacional de Pesquisas da Amazônia, sob a presidência do(a) primeiro(a), a fim de proceder a argüição pública da **TESE DE DOUTORADO** de **PEDRO IVO SIMÕES**, intitulada "Diversificação do complexo *Allobates femoralis* (Anura, Dendrobatidae) em florestas da Amazônia brasileira: desvendando padrões atuais e históricos", orientado(a) pelo(a) Prof(a). Dr(a). Albertina Pimentel Lima , do Instituto Nacional de Pesquisas da Amazônia e co-orientado(a) pelo(a) Prof(a). Dr(a). Izeni Pires Farias, da Universidade Federal do Amazonas.

Após a exposição, o(a) discente foi argüido(a) oralmente pelos membros da Comissão Examinadora, tendo recebido o conceito final:

☒ APROVADO(A) ☐ REPROVADO(A)

☒ POR UNANIMIDADE ☐ POR MAIORIA

Obs. Com distinção e louvor

Nada mais havendo, foi lavrada a presente ata, que, após lida e aprovada, foi assinada pelos membros da Comissão Examinadora.

Prof(a).Dr(a). José Antonio Alves Gomes

Prof(a).Dr(a). Mário Cohn-Haft

Prof(a).Dr(a). Marcelo Menin

Coordenação PPG-ECO/INPA