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Explorando processos que geram variação de cor *Adelphobates galactonotus*, uma espécie de sapo colorido e venenosoendémico da Amazônia Oriental

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Manaus, Amazonas
Setembro, 2016

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Sinopse:

Estudou-se possíveis processos que direcionam a variação de cor em *Adelphobates* nio *galactonotus*, sapo dendrobatideo e aposemático, endêmico do leste de Amazônia Brasileira. Foi avaliada a coloração como mecanismo de defesa contra predadores e as relações filogeneticas com a distribuição das cores ao longo da sua distribuição utilizando sequências de dois genes mitocondriais (mtDNA) e milhares de marcadores de representação reduzida do genoma de polimorfismos únicos de nucleotídeos (*single nucleotide polimorphism* - SNPs).

Palavras-chave: Aposematismo, predação, polimorfismo de cor, Dendrobatidae, genômica, SNP, sapo venenoso, adaptação local

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RESUMO

Coloração aposemática pode servir como defesa contra predadores visualmente orientados, porque sinais conspícuos são fácies de detectar e podem ser prontamente associados a impalatabilidade. Contudo, os processos evolutivos que direcionam a coloração aposemática são enigmáticos, porque para ser eficaz um sinal aposemático precisa ser consistente e comum e, ainda, é necessário que seja selecionado nas baixas frequências iniciais. Nessa tese nós amostramos *Adelphobates galactonotus*, anuro dendrobatídeo que ocorre na Amazônia Oriental brasileira e com coloração dorsal que varia geograficamente, como modelo de estudo sobre processos que geram variação de cor em dendrobatídeos. No capítulo I nós avaliamos se há seleção local mediada por predadores sobre a coloração de *A. galactonotus* em duas localidades próximas que contém exclusivamente morfotipos azul ou laranja. Nós mostramos que não houve diferença na freqüência de ataques realizados por predadores visualmente orientados (aves) entre modelos que possuíam a coloração nativa, a coloração introduzida, não suportando a hipótese de que a seleção local mediada por predadores visualmente orientados é a causa da variação geográfica e da origem evolutiva independente de diferentes cores aposemáticas em *A. galactonotus*. No capitulo II nós investigamos se os morfotipos de cor teriam evoluído independentemente múltiplas vezes e se os padrões de seleção se associam com estes, utilizando sequências de dois genes mitocondriais (mtDNA) e milhares de marcadores de representação reduzida do genoma de polimorfismos únicos de nucleotídeos (*single nucleotide polimorphism* - SNPs). Encontramos partição genética associada ao rio Xingu. Usando mtDNA, o tempo de divergência genética estimado, entre sapos de lados opostos desse rio, foi 4.8 milhões de anos atrás e linhagens genéticas levaram a diferentes divergências de cor ao longo do Pleistoceno, sugerindo que uma mesma cor evoluiu independentemente múltiplas vezes, ao leste e oeste do rio Xingu. 16 SNPs mostraram-se altamente associados à cor, sugerindo um papel na determinação da cor. Nós propomos que a rápida evolução da diversidade de cores iniciou-se provavelmente em populações geograficamente isoladas durante períodos de fragmentação de hábitat associados ao Pleistoceno.

Exploring processes that generate color variation in *Adelphobates galactonotus*, a dendrobatid frog species, colored and poisonous, endemic of the eastern Amazon

ABSTRACT

An aposematic coloration could serve like a defense against visually oriented predators because conspicuous signals are easy to detect, memorize and associate with unpalatabilty. However, the evolutionary processes driving aposematic coloration are enigmatic, because to be effective, an aposematic signal needs to be consistent and common, and also, should be selected at initial low frequencies. In this thesis we sampled *Adelphobates galactonotus*, a dendrobatid anuran distributed at the east of the Brazilian Amazon, south Amazonas River, and with a dorsal coloration varying geographically, as a model to explain the processes generating color variation in dendrobatids. In Chapter I we assess if there is local upon coloration of *A. galactonotus* selection mediated by predators in two close localities containing exclusively blue or orange morfotypes. We show that there was no difference in the attack frequency by visually oriented predators (birds) among models with native coloration, an introduced coloration or a brown control coloration, not supporting the hypothesis that local selection mediated by visually oriented predators is the cause of geographic variation and independent evolutionary origin of different aposematic colors in *A. galactonotus*. In Chapter II we investigate whether color morphs have evolved independently several times and if selection patterns are associated with these, using sequences of two mitochondrial genes (mtDNA) and thousands of single nucleotide polimorphisms (SNPs). We found a strong genetic partitioning associated with Xingu River. Using mtDNA, the estimated divergence time between frogs from opposite riverbanks, was 4.8 million years ago (m.y.a.) and genetic linages conduced to different color divergences along the Pleistocene, suggesting that the same color evolved independently several times, east and west of the Xingu River. 16 SNPs were highly associated to color, suggesting a role in color determination. We propose that rapid evolution of color diversity probably began on populations geographically isolated during habitat fragmentation periods associated to Pleistocene.

Sumário

Lista de tabelas

Lista de figuras

Capítulo I. No predatory bias with respect to color familiarity for the aposematic *Adelphobates galactonotus* (Anura: Dendrobatidae)

Capítulo II. A colors burst: the evolution of polymorphism in the warning coloration of the Amazonian poison frog *Adelphobates galactonotus*

Introdução geral

A natureza apresenta uma grande variedade de cores, o que tem instigado pesquisadores de diferentes grupos taxonômicos (e.g., plantas, borboletas, besouros, peixes, cobras, aves, anuros) a investigar como essas cores evoluíram e os processos que direcionaram os padrões de coloração que observamos hoje. De modo geral, a literatura tem mostrado que diferentes processos, independentemente ou em conjunto, podem ter direcionado a diversificação das cores por meio de fatores como seleção natural, seleção sexual, deriva genética e isolamento por distancia.

A coloração cumpre uma variedade de funções, dependendo do contexto da história natural das espécies. Essas funções não são necessariamente excludentes e incluem proteção solar, aposematismo, mimetismo ou uso em interações comportamentais, como reconhecimento intraespecífico, seleção sexual e competição intrasexual (Hödl & Amézquita 2001; Reynolds & Fitzpatrick 2007; Patrick & Sasa 2009; Mills & Patterson 2009). Desse modo, padrões de cor em animais são importantes para seu desempenho reprodutivo e sobrevivência, estando assim frequentemente relacionados a mecanismos ecológicos e evolutivos (Mills & Patterson 2009).

A função ecológica da cor divide-se em dois tipos principais: coloração de advertência e mimetismo. A coloração de advertência, ou aposematismo, refere-se às colorações exóticas ou conspícuas, geralmente associadas às caraterísticas nocivas. Uma coloração aposemática frequentemente consiste de tons brilhantes de vermelho, amarelo, azul ou branco, algumas vezes em contraste com a cor preta (Gamberale & Tullberg 1998; Joron & Mallet 1998; Lindström 1999; Toledo & Haddad 2009). Esse tipo de coloração serve como um sinal de advertência para predadores visualmente orientados, que aprendem a associar impalatabilidade ou toxicidade com coloração contrastante e brilhante (Endler & Mappes 2004). O mimetismo refere-se à semelhança adaptativa em sinal entre espécies em uma localidade, sendo que o sinal imitado geralmente é aposemático (Joron *et al.* 1999).

Toledo e Haddad (2009) sugeriram uma divisão do mimetismo em três grandes grupos: camuflagem, onde um organismo se assemelha com parte do ambiente; homotipia, que consiste na imitação mimética de outro objeto; e homotipia defensiva, o qual inclui o mimetismo müllerianо, que por sua vez descreve espécies não palatáveis as quais exibem padrões convergentes (Guilford & Dawkins 1993), e o mimetismo aritmético, onde espécies

palatáveis, semelhantes e simpátricas, apresentam taxas de predação proporcionais às suas frequências relativas: quanto mais abundantes, menor a chance de um individuo ser predado.

A coloração exibida pelos organismos pode variar inter ou intraespecificamente. Diferenças na cor são usualmente explicadas como resposta à seleção natural (Joron & Mallet 1998) ou seleção sexual (Summers *et al.* 1999; Galeotti *et al.* 2003; Reynolds & Fitzpatrick 2007; Maan & Cummings 2008; Brown *et al.* 2010), podendo levar a especiação (Jiggins *et al.* 2001). A ocorrência de dois ou mais fenótipos de cores diferentes e geneticamente determinados, em organismos de uma mesma espécie, é chamado polimorfismo de coloração (McKinnon & Pierotti 2010). O polimorfismo de coloração pode ser de dois tipos: indivíduos de diferentes cores em uma mesma localidade ou indivíduos com cor diferente em cada localidade, esse ultimo conhecido como politipismo. Esses tipos de polimorfismo de coloração podem estar relacionados ao dimorfismo sexual ou variações ontogenéticas, ou ainda ocorrer independentemente desses fatores (Toledo & Haddad 2009). Assim, O polimorfismo de coloração tem sido investigado, com a intenção de entender e explicar os mecanismos que o geraram, em diferentes espécies tais como: répteis e anfíbios (Forsman & Shine 1995; Rudh *et al.* 2007) - frequentemente encontrado em anuros (Heyer 1997; Hoffman & Blouin 1999), aves (Fowlie & Krüger 2003; Galeotti *et al.* 2003; Roulin & Wink 2004; Roulin 2004; Pryke & Griffith 2007), insetos (Smith *et al.* 1988; Forsman & Appelqvist 1999; Nielsen & Watt 2000; Unsicker *et al.* 2008), mamíferos (Klinka & Reimchen 2009), aracnídeos (Oxford 2005), moluscos (Goodhart 1987; Whiteley *et al.* 1997; Hayashi & Chiba 2004; Rodrigues & Silva Absalão 2005), entre outros.

Os anuros da família Dendrobatidae (sapos venenosos ou *"poison-arrow frogs*") ocorrem apenas na região Neotropical, desde o sul da Nicarágua até o Peru, a Bolívia e o Brasil. Estes sapos possuem ampla variação de coloração corpórea, inter e intraespecíficamente, e têm sido considerados aposemáticos, apresentando uma coloração conspícua de advertência para suas toxinas cutâneas (Silverstone 1975; Vences *et al.* 2000; Grant *et al.* 2006). De fato, as cores conspícuas de dendrobatídeos têm sido frequentemente associadas a defesa contra predadores por meio de impalatabilidade, toxicidade, ou capacidade de resistir ou escapar de predadores (Summers & Clough 2001; Toledo & Haddad 2009; Maan & Cummings 2009). Saporito *et al* (2007) usaram modelos de argila para demostrar que a coloração de *Oophaga pumilio* (Dendrobatidae) serve como defesa contra predadores visualmente orientados. Outros estudos com dendrobatídeos usaram modelos de argila ou parafina para testar o papel da cor na seleção por predadores (seleção natural), e,

apesar de mostrarem resultados contrastantes quanto a predação associada a padrões de coloração locais ou novos, todos indicam que cores vibrantes previnem a predação. Novas formas coloridas podem ser mais propensas a sofrer ataques em comparação às formas locais (Noonan & Comeault 2009; Hegna *et al.* 2012), ambas podem ser igualmente atacadas (Amézquita *et al.* 2013), ou ainda cores menos conspícuas podem ser menos atacadas, independente de serem formas novas ou locais.

No capitulo I desta tese investigou-se se a seleção natural mediada por predação é um mecanismo que explicaria a variação de cor em *Adelphobates galactonotus*, um sapo dendrobatídeo, colorido e venenoso, endêmico do leste da Amazônia brasileira, utilizando modelos de parafina, semelhantes em cor e formas a indivíduos vivos.

Evidência experimental indica que as cores brilhantes dos sapos venenosos do Neotrópico não servem apenas para deter predadores, mas também como sinais utilizados pelas fêmeas para escolha de parceiros reprodutivos (Summers *et al.* 1999; Reynolds & Fitzpatrick 2007; Noonan & Comeault 2009). A seleção sexual afeta a evolução da coloração nas espécies, porque características de cores e padrões de coloração também podem funcionar para atrair parceiro e até no reconhecimento do mesmo (Summers *et al.* 1999; Jiggins *et al.* 2001).

Alguns estudos envolvendo polimorfismo de coloração em Dendrobatídeos têm sido desenvolvidos com *Oophaga pumilio*, espécie com ampla variação na coloração ao longo de sua distribuição; esses estudos têm encontrado relação entre a variação de cor e a seleção sexual. Summers *et al*. (1999), usando dois morfotipos de *Oophaga pumilio*, encontraram que as fêmeas de cada população preferem sapos da mesma cor, sugerindo que as diferenças de cor são sinais visuais utilizados por esses sapos, sendo que Reynolds & Patrick (2007) nas mesmas populações além da preferencia pela mesma cor destacam a dupla funcionalidade da cor como defesa contra predadores e na escolha de par. Os resultados de Brown *et al*. (2010) sugerem que não só a seleção sexual pode ser um sinal filogenético para explicar o polimorfismo, mas também outros fatores como diferenças nas comunidades de predadores, luminosidade do ambiente e/ou grau de toxicidade contribuem na evolução e desenvolvimento de diferentes sinais eficientes.

Ferramentas genéticas e os possíveis mecanismos que explicam a variação de cor

O conhecimento dos processos que determinam padrões de diversidade biológica é fundamental para a conservação da biodiversidade como entidade evolutiva. Relações

filogenéticas frequentemente refletem a relação entre a distribuição dos indivíduos, fazendo dos estudos filogeográficos uma ferramenta importante na investigação das influências históricas na distribuição das espécies naturais (Avise *et al.* 1987, Beebee 2005, Zeisset & Beebee 2008).

O processo de diversificação das espécies está fortemente ligado ao tectonismo e ao clima, mas o momento da origem e as causas evolutivas dessa diversidade são ainda tema de debate (Moritz *et al.* 2000; Hoorn *et al.* 2010). Diversas hipóteses têm sido propostas para explicar os padrões biogeográficos observados na região Neotropical e na Amazônia, em específico. Por exemplo, barreiras paleogeográficas têm sido propostas como barreiras importantes ao fluxo gênico (soerguimento dos Andes no Cenozoico, os arcos resultantes e as incursões marinhas do Mioceno). Hoorn *et al*. (2010) evidenciaram que a elevação dos Andes foi fundamental para a evolução das paisagens e dos ecossistemas amazônicos, e que os padrões de biodiversidade atuais estão profundamente enraizados no pré-Quaternário. Haffer (1969, 1992) propôs que as florestas tropicais se expandiram e contraíram durante os ciclos glaciais (hipótese dos refúgios), e que essas contrações separaram a floresta em pequenos refúgios, que promoveram a divergência e especiação. A teoria fluvial propõe que populações animais e vegetais na Amazônia separaram-se pelo desenvolvimento do sistema fluvial, onde os leitos dos rios e suas várzeas atuam como barreiras à dispersão. Originalmente proposta por Wallace (1852 *apud* Moritz *et al.* 2000), a hipótese dos rios como barreiras diz que os principais rios da Amazônia, propiciam a especiação alopátrica.

Análises filogenéticas são portanto componentes chave para nosso entendimento do polimorfismo fenotípico em espécies aposemáticas. Em particular, o entendimento da história evolutiva das populações que variam amplamente nas características aposemáticas é crítico para a formulação de predições e suposições em relação às forças que podem atuar na evolução dessas características (Wang & Shaffer 2008a). Reconstruções filogenéticas, permitem, por exemplo, realizar inferências em relação as taxas relativas de evolução dos fenótipos (Drummond *et al.* 2012).

A evolução da variação da cor em sapos aposemáticos, tem sido associada a vários processos evolutivos, tais como: evolução da cor múltiplas vezes, i.e., de forma independente (Wang & Shaffer 2008a), seleção sexual (Summers *et al.* 1999; Reynolds & Fitzpatrick 2007; Richards-Zawacki *et al.* 2012), seleção natural (pressão por predação) (Saporito *et al.* 2007a; Noonan & Comeault 2009; Hegna *et al.* 2011, 2012; Pröhl & Ostrowski 2011) e história demográfica (Gehara *et al.* 2013) atuando nos processos de diversificação da cor. Variação de

cor também tem sido associada a processos de hibridização, seguidos de contato secundário (Medina *et al.* 2013) e variação da cor como resultado de processos evolutivos ecológicos e históricos mediados por vicariancia direcionada por clima e seleção por predadores (Comeault & Noonan 2011), além de diversificação de sinais de advertência com o estabelecimento de anéis de mimetismo mülleriano (Symula *et al.* 2001; Darst *et al.* 2006; Twomey *et al.* 2013, 2014) também foram evidenciados.

Relações de isolamento por distancia (IBD) são geralmente esperadas ao longo de paisagens contiguas, e a variação de cor pode assim estar relacionada com a distância genética, geográfica ou ambiental. Estudos testando o efeito do IBD na variação da cor, em diferentes escalas espaciais, têm encontrado fortes associações em alguns casos (e.g., *O. pumilio,* Wang & Summers 2010) ou nenhuma associação (e.g., *Parasemia plantaginis,* Hegna *et al.* 2015). Uma seleção continua de genes específicos pode atuar na manutenção do polimorfismo intraespecífico da cor, o que pode ser testado com reconstruções filogenéticas e técnicas contemporâneas (sequenciamento de alto rendimento) que permitam a identificação de potenciais sinais de seleção em relação à cor. No capitulo II, utilizamos marcadores de DNA mitocondrial e milhares de marcadores genéticos do tipo SNPs amostrados ao longo da distribuição de *A. galactonotus*, para obter o padrão filogenético da diversificação da cor da espécie. Com esses dados verificamos se o tempo de diversificação da cor em *A. galactonotus* é consistente com o tempo de diversificação de outros sapos dendrobatideos; se os agrupamentos de cores semelhantes, mas geograficamente isolados, representam eventos de diversificação independentes e se a coloração de *A. galactonotus* se encontra sob seleção.

Objetivo geral

Investigar possíveis processos que geram variação de cor em *Adelphobates galactonotus*, sapo dendrobatídeo, colorido e venenoso.

Objetivos específicos

- Avaliar se o polimorfismo de coloração de *A. galactonotus* é uma resposta à pressão por predação.

- Investigar se os morfotipos de cor em *A. galactonotus* evoluíram independentemente múltiplas vezes e testar se os sinais de seleção identificados em nível genômico associam-se com estes morfotipos.

CAPÍTULO I

Rojas, D., Stow, A., Amézquita, A., Simões P.I. & Lima A.P. No predatory bias with respect to colour familiarity for the aposematic *Adelphobates galactonotus* (Anura: Dendrobatidae).2015. *Behaviour* 152:1637-1657

No predatory bias with respect to color familiarity for the aposematic *Adelphobates galactonotus* (Anura: Dendrobatidae)

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Summary

Aposematic coloration deters visually oriented predators because conspicuous signals are easier to detect and associate with unpalatability. Consequently, brightly colored prey that are novel are predicted to be preyed on more than those with bright but typical colors. Here we evaluated whether predatory bias is associated with the color differences observed at two different localities for a large conspicuously colored and poisonous Amazonian frog, *Adelphobates galactonotus*. At each locality, predation experiments were carried out using frog models of two naturally occurring colors of the study species (blue and orange) and a control (brown). We found no evidence that novel colors were more vulnerable to predation than local colors. These results do not therefore support our hypothesis that predatory bias explains the geographic variation of color in *A. galactonotus*.

Keywords: Aposematism, poison frogs, *Adelphobates galactonotus*, Evolution, predation, color variation

Introduction

That conspicuous colors warn visually oriented predators of prey unpalatability has been demonstrated in a wide range of taxa (Oxford & Gillespie, 1998; Mallet & Joron, 1999; Saporito et al., 2007b; Mochida, 2011). Although color and pattern variation is often observed in aposematic species (Joron et al., 1999; Darst & Cummings, 2006; Wang & Shaffer, 2008), it is unexpected and poorly understood. Selection should favor monomorphic within-species coloration so that the signal is easily learnt by predators (Harvey et al., 1982; Endler, 1988; Mallet & Joron, 1999). This paradox has been stated recurrently in the literature (Stevens & Ruxton, 2014; Rojas et al., 2014a) and several hypotheses have been tested, including

environmental differences and predator variation, Müllerian mimicry, dietary differences, mate choice and differences in behavior.

Müllerian mimicry, where aposematic species in sympatry adopt the same warning signals, appears widespread and has been demonstrated in *Heliconius* butterflies (Kapan, 2001), Appalachian millipedes (Marek & Bond, 2009) and dendrobatid frogs (Symula et al., 2001). That temporal variation in predation can generate color variation in aposematic species is shown by the larvae of the wood tiger moth (*Parasemia plantaginis*), where seasonality results in a higher proportion of naïve predators at the start of the season. This has driven the evolution of a more cryptic, quick developing form predominating early on in the season, when a less effective signal is not as costly (Lindstedt et al., 2008). Slower developing morphs have a brighter, more effective signal aimed at predators that have learnt to be wary from earlier experiences (Lindstedt et al., 2009). Moreover, spatial variation in predator community composition can facilitate the divergence of warning signals (Nokelainen et al. 2014). In addition, color variation in tiger moths and other aposematic species, such as desert locusts (*Schistocera gregaria*) and ladybird beetles (*Harmonia axyridis, Coccinella septempunctata*) can also result from dietary differences (Grill & Moore, 1998; Despland & Simpson, 2005; Lindstedt et al., 2010; Blount et al., 2012; Stevens & Ruxton, 2014). Similarly, skin toxins in dendrobatid frogs are sequestered from arthropods (Saporito et al., 2004) and, as a consequence, toxicity, and the cost of predation to the predator can vary depending on geographic location and habitat use (Saporito et al., 2007a). Also, intraspecific color variation has been associated with variation in chemical defenses in some species: seaslugs (Cortesi & Cheney, 2010), ladybirds (Bezzerides et al., 2007; Blount et al., 2012), paper wasps (Vidal-Cordero et al., 2012) and poison frogs (Maan & Cummings, 2012). An additional process that can maintain color variation is mate choice. Color acts as a cue for mate choice in numerous animals, and for aposematic species, assortative mating on the basis

of color maintains color variation (Jiggins et al., 2001; Summers et al., 1999; Reynolds & Fitzpatrick, 2007). This need not be independent of predation pressure, where lower fitness of hybrids reinforces assortative mating with respect to color, asmay be the case for the strawberry poison arrow frogs (*Oophaga pumilio*) (Maan & Cummings, 2008; Richards-Zawacki & Cummings, 2011).

The role of predation in selecting different colors has been investigated in a variety of invertebrates (Forsman & Appelqvist, 1999; Svádová et al., 2009) and vertebrate groups (Blanco & Bertellotti, 2002; Husak et al., 2006; Farallo & Forstner, 2012) and there has been much work on aposematic dendrobatid frogs (Darst & Cummings, 2006; Noonan & Comeault, 2009; Chouteau & Angers, 2011; Comeault & Noonan, 2011; Hegna et al., 2011). Field experiments carried out to test the relationships between predation and aposematism have predicted that model animals with conspicuous colors will be less frequently attacked than those with a less conspicuous color, since the former may not be edible. Because aposematism probably involves some learning, where predators easily learn to associate unpalatability, toxicity, or resistance with bright coloration (Cott, 1940; Endler, 1991; Gamberale & Tullberg, 1998; Lindström et al., 1999; Endler & Mappes, 2004), predators should also attack the aposematic forms they know less frequently than aposematic forms that are new to them.

Field experiments can yield results different from laboratory-based trials. For example in the laboratory, red forms of the wood tiger moth were less frequently preyed by avian predators when compared to orange forms. However, this difference was not apparent when both forms were exposed to the native multi-predator environment (Lindstedt et al., 2011). In field-based studies, consistent support for predatory bias with respect to color has not been achieved for several taxonomic groups. For example, in an island population of *O. pumilio*, the local color was attacked more frequently by birds than the non-local colors (Hegna et al.,

2012). Explanations included neophobia, predators possessing a pre-existing search image, and different habitat use of the local morph, which are more arboreal than elsewhere (Hegna et al., 2012).

Intra-specific variation of conspicuous coloration is often found in members of the Dendrobatidae family, where highly contrasting colors advertise the presence of skin toxins (Silverstone, 1975; Myers & Daly, 1983; Summers & Clough, 2001). The relationship between bright coloration and predation has been tested in five species of aposematic dendrobatid frogs, with contrasting outcomes. For *Dendrobates tinctorius* (Noonan & Comeault, 2009), *O. pumilio* (Hegna et al., 2012), and *Ranitomeya imitator* (Chouteau & Angers, 2011) individuals with novel colors are more often preyed upon than those with local colors. However, predators avoided equally both the novel and local color morphotypes of *O. histrionica* (Amézquita et al., 2013, see summary table 1 supplementary material) and, less conspicuous morphs of *O. granulifera* were less attacked irrespective of whether they were of local or novel coloration (Willink et al., 2014).

Here we evaluate whether levels of predation are associated with color in the poisonous diurnal frog *Adelphobates galactonotus* (Family Dendrobatidae), which is endemic to the eastern Amazon Basin, south of the Amazon River (Fig. 1). Throughout its distribution, *A. galactonotus* can have yellow, orange to red, white, light-blue or black dorsal coloration (Hoogmoed & Avila-Pires, 2012). Different morphotypes are patchily distributed across its geographic range and are not known to occur in sympatry. Given the relatively large size of *A. galactonotus* it is an ideal species to further investigate the relationship between predation frequency and local versus novel color types in poison frogs. This is because for some taxa, differences in the efficiency of aposematic signals can vary in accordance to the total size of the signal and color pattern. For example, *Oophaga pumilio* varies in color and size along its distribution and aposematism is less efficient than being cryptic when body size is small

(Rudh, 2013). Thus, a species like *A. galactonotus,* for which the signal is two or three times larger than most species of dendrobatid frogs, potentially possess a stronger and more conspicuous aposematic signal. Here we use colored paraffin wax models in two populations of *A. galactonotus* with contrasting color morphs (blue and orange). We tested at each locality, 1) whether aposematic models are attacked less frequently than the brown ones, and 2) whether local forms are less attacked than novel ones.

Materials and methods

Predation experiments were conducted from the 19th of January to the 15th of February 2013, in two localities in the Caxiuanã Bay region in the municipality of Portel, Pará State, Brazil (Fig. 1). One locality was on the western bank of the bay (ICMBio station– FLONA Caxiuanã, Instituto Chico Mendes de Conservação da Biodiversidade, 51°26'02.8"W 1°47'37.6"S) and the other on the eastern bank (Village of Brabo, 51°25'14.1"W 1°57'45.3"S). We used paraffin wax models equivalent in size and colors to live *Adelphobates galactonotus* (SVL in cm, average ± SD: animals = 3.62 ± 0.25, models = 4.06 ± 0.12). Models were coated with non-toxic, odorless paint (Natural Colors® Acrilex) in three colors (Fig. 2): (1) orange, representing *A. galactonotus* coloration found at ICMBio station but not at the village of Brabo, (2) blue, representing coloration at the village of Brabo but not occurring at ICMBio station, and (3) brown, as controls simulating non-aposematic frogs. Most dendrobatids lack UV reflectance, which allowed us to adjust the color of the models by visual comparison (Noonan & Comeault, 2009; Summers et al., 2003). We compared the color reflectance of ten models of each color with living animals (five orange from ICMBio and three blue from Village of Brabo) using an Ocean Optics spectrometer and the software Spectra Suite®.

Reflectance was analyzed in the R program (package pavo; Maia et al., 2013) and these data confirmed the absence of UV reflectance on models (Fig. 2).

Experimental design

In each study area, we set up 24 transects where the models were exposed to predators for 6 days. Each transect was 20 m wide and 100 m long with a central trail and separated by at least 200m from one another. Twenty-one models (seven of each color) were distributed along each transect at 5 m intervals. Each model was placed at a different orthogonal distance in relation to the central trail. Model colors and distance to the main trail were chosen randomly using a computer pseudorandom number generator. When necessary, models were replaced by a different color in order to avoid positioning three consecutive models with the same color. Model location alternated between left and right sides of the central line, starting on the left at each transect and were exposed for a total of 144 h. In order to minimize the possibility that predators develop a familiarity with model placement, after every 72 h of each trial, all the models were collected, models that were attacked were replaced by models of the same color, and a new session was initiated (as described above). Each attacked model was photographed to assist with evaluating the characteristics of the bite marks in order to identify the type of animal (bird, mammal, lizard or insect) responsible for the attack. In addition, all models were inspected using a stereoscopic microscope and, combined with visual appraisal of the photographs, we could confidently assign the bites as avian, mammalian, lizard or invertebrate. We analyzed avian predators separately because they are presumably the most visually oriented predators, they are the most diverse vertebrate taxon in the Amazon rainforest (Nores, 2000) and consequently might represent the main source of selective pressure on color type.

To confirm our expectation of V shaped bite marks from avian attacks we conducted two experimental trials near a forest remnant in the campus of the Instituto Nacional de Pesquisas da Amazônia (INPA) in Manaus, central Brazilian Amazonia. Models were positioned on ripe papaya fruits distributed a few meters from fruit trees, frequently visited by birds, and collected after 24h in order to record attack marks. We also collected fruits with evidence of bird attacks and photographed both fruits and models (Fig. 3a, b). These records were used to identify characteristically V shaped avian bites on models used in the field experiments (Fig. 3c).

Statistical analysis

To assess for spatial autocorrelation, the association between attack frequency and geographic distances (Euclidean) between transects was examined in three Mantel tests: one including both localities and one for each locality.

We used a Generalized Linear Model (GLM) with a binomial distribution to test whether model color (two aposematic: blue and orange, and one non-aposematic: brown) and the experimental locality (ICMBio and Village of Brabo) influenced the probability of attack (binary; attacked or not attacked). Two GLM's were used, one where the dependent variable included all attacks by vertebrates and another where the dependent variable only included avian attacks. As predictor variables, we included the native color (1: model of the local color, 0: model of a different color from local). Transect identity was also included in the GLMs to further test for spatial effects. A post-hoc test (Tukey's HSD) was conducted to compare the proportion of bitten models for each pair of colors. Mantel tests were performed with the program R (R Development Core Team, 2014), and the regressions with SYSTAT 12.0 (SYSTAT©, 2007).

Results

Summary of attack frequencies

The effective number of models used in the experiment was 1008 at each locality. At ICMBio station 1005 models were recovered and 998 models were recovered at the Village of Brabo and the 13 missing models were excluded from further analysis. Of the 1005 models recovered at the ICMBio station locality, 174 models (17.3%) had evidence of attacks, and of the 998 models recovered at the Village of Brabo locality, 143 (14.3%) had been attacked (Fig. 4).

Of the 174 models that were attacked at ICMBio station locality, where the local color of *A. galactonotus* is orange, 18 models had bite marks from invertebrates and these were scored as 'not attacked' for further analysis. Vertebrates had attacked the remaining 156 models with the number of attacks on blue, orange and brown models being 63, 48 and 45 respectively. Of these, avian predators attacked 110 models (10.9% of all the recovered models; 42 blue, 33 orange and 35 brown).

Of the 143 attacked models at the Village of Brabo location, where the color of *A. galactonotus* is blue, 73 were identified with invertebrate bite marks and not considered in further analysis. The remaining 70 models had vertebrate bite marks (blue, 40; orange, 16; brown, 14). Of these 70 models, avian predators attacked 39 models (3.8% of all the recovered models; blue, 17; orange, 14; brown, 8).

Mantel tests demonstrated that there was no correlation between the geographical distances among transects and attack frequencies at both localities combined (P=0.257) or for each locality separately (ICMBio, P = 0.512; Brabo, P = 0.162).

Are there differences in bite frequencies among colors?

The frequency of attacks from all vertebrates (data pooled across both locations) did not support the prediction that novel colors would suffer a higher frequency of attacks

compared to local colors (GLM, R^2 = 0.039, N = 2003, p = 0.542, F = 0.373, df = 1). The distribution of attack frequencies for each color type did not differ between transects (p = 0.533, F = 0.948, df = 23). However, there was a significant association between color and attack frequency ($p < 0.001$, F = 8.050, df = 2). Pairwise comparisons between colors showed that blue colored models carried a significantly higher proportion of attacks (Tukey's HSD; blue – orange, p = 0.001; blue–brown p = 0.004). All other pairwise comparisons were non-significant. We found that models were significantly ($p < 0.0001$, F = 38.992, df = 1) less likely to be attacked by vertebrates at the village of Brabo locality than at the ICMBio station locality (Fig. 4).

When only attacks by birds were considered there was no evidence of predatory bias with respect to color (individually for each locality and data pooled across localities, GLM, R^2 = 0.033, N = 2003; p = 0.292, F = 1.231, df = 2). Similarly, avian attacks were not significantly associated with transect location (p = 0.372, F = 1.071, df =23). Pairwise comparisons between different colors suggest that each color had a similar risk of being attacked by birds (Tukey's HSD; $p > 0.05$ for all comparisons). Finally, we found that models were significantly ($p < 0.0001$, F = 40.623, df = 1) less likely to be attacked by avian predators at the village of Brabo location than at the ICMBio station location (Fig. 4).

Discussion

Our data do not support the prediction that, for a given locality, predator recognition of native color results in a higher frequency of attacks on novel colors. Furthermore, there is no evidence that seemingly more conspicuous colors ward off attacks. We put forward several explanations for our observation, including the methodology we used, the environment in which the tests were carried out, the small spatial scale over which color variation in *A. galactonotus* occurs, and the possibility that predatory selection is no longer occurring.

Given that we could only be confident in assigning bite marks as avian or mammalian, it is possible that bite marks represent investigation by animals that don't prey on *A. galactonotus*. It is also possible that the color matching of our models wasn't accurate enough, and all models were seen as novel and equally intriguing, or frightening, to the panel of potential predators. Although we did not have a control in place to test for this, comparison with other model-based tests of predation, carried out in a similar fashion, suggest that inquisitive pecking, or biting, are unlikely to completely mask differences in the frequency of predatory attacks between colors. In similar model-based tests of predation carried out on dendobatid frogs in Central and South America, significantly less predation on local aposematic colors were observed (Noonan & Comeault, 2009; Hegna et al., 2011; Comeault & Noonan, 2011). In these studies color matching was carried out by eye, and so unlikely to be any more accurate than the color matching carried out here. Furthermore, if inquisitive bites on our models were more commonplace, and masking predatory attempts, then the proportion of models attacked are likely to be higher than in those similar studies where predatory bias was shown. Yet, the proportion of attacked models was more or less equivalent in our study with the percentage of bitten models in similar studies varying from 0.2% to 27% (0.8% to 6.2% models bitten per day), while in our study the percentage of bitten models varied from 3.8% to 17.3% (0.65% to 2.6% models bitten per day; Supplementary material: Table 1).

Other methodological explanations for a lack of predatory bias among colors are that brown models were partially cryptic, being difficult to detect due to their similarity with the color of leaf litter on the substrate. In contrast, predatory trials on other poison frogs (none of which occur in sympatry with *A. galactonotus* at our study localities) suggest that brown models are more frequently attacked than bright-colored models (Rojas et al. 2014b). Although, in one trial, uniformly brown models were placed on green leaves (Chouteau &

265 Angers, 2011), where the model color is in contrast with the background. Another possible
266 explanation for the equivalent level of predation on each of the model colors used here, is the
267 presence of cryptic and toxic species in the study area (*Ameerega hanheli* and *Allobates*
268 *femoralis*), so that predators are recognizing all frogs as unpalatable.

269 With the exception of a higher frequency of attacks on blue colors, we found little
270 evidence of predator bias with respect to color. *In situ* experiments like ours, and those using
271 other poison frogs, exposed models to a variety of predators, many of which have multiple
272 prey targets. It seems plausible, if not likely, that predators are trained to avoid a wide array of
273 conspicuous colors. In addition, given that different color types can be located in very close
274 proximity along contact zones, predators may be exposed to two or more color types of *A.*
275 *galactonotus*. Furthermore, in an environment, such as the Amazon rainforest, where toxic
276 defense is relatively frequent, predators may be generally wary. Finally, a methodological
277 flaw common to all static-model experiments is that the aposematic signals are most
278 conspicuous during movement, and might be cryptic when stationary (Ruxton et al., 2004).
279 The inherent problem with static models may also explain why the brown models were
280 attacked in an equal proportion to colors assumed to be conspicuous in the field (Paluh et al.,
281 2014).

282 An exception to the lack of predatory bias was that the blue models were more
283 frequently attacked by non-avian vertebrates, and we suspect that mammalian predation might
284 be responsible. In many mammals, prey detection occurs principally by olfactory cues, but
285 color might still influence the identification of prey (Melin et al., 2007). It is plausible that the
286 blue color, being lighter (Fig. 2), is more contrasting with the leaf litter at night (Endler, 1993)
287 and exposes this color to nocturnal carnivorous mammals that generally forage when *A.*
288 *galactonotus* is not active. Because the models were exposed during both day and night, the

289 predation events recorded may not reflect selection specifically against blue coloration in *A.*
290 *galactonotus*.

291 While we cannot completely rule out the presence of predatory bias with respect to
292 color, our results contrast to similarly carried out tests, and this at least suggests that there
293 may not be strong color based selection presently occurring. It is possible that aposematic
294 coloration in *A. galactonotus* evolved under different conditions and the maintenance of color
295 variation could potentially be through mate choice and assortative mating with respect to
296 color. Indeed, assortative mating based on color cues, and directional selection through
297 increased predation on hybrids has been used to explain the maintenance of color variation
298 (Summers et al., 1999; Jiggins et al., 2001; Reynolds & Fitzpatrick, 2007; Servedio et al.,
299 2011; Cummings & Crothers, 2013). It seems conceivable that assortative mating with
300 respect to color could be maintained at points of contact, even if directional predatory
301 selection is relaxed. Particularly if color morphs have been historically separated for long
302 enough that genetic differentiation results in selection against color hybrids for other reasons.
303 These could be purely genetic, such as the accumulation of novel mutations, chromosomal
304 rearrangements and the effects of genetic drift resulting in negative epistatic interactions or
305 even hybrid sterility (Orr & Turelli, 2001; Coyne & Orr, 2004; Presgraves, 2010; Wolf et al.,
306 2010). It is plausible that historical processes, such as retraction to separate refugia during
307 glacial maxima allowed for the required level of genetic divergence. These sorts of genetic
308 processes, rather than predation, might reinforce hybrid boundaries and thus maintain color
309 polymorphisms via reproductive character displacement (Richards-Zawacki & Cummings,
310 2011). Characterizing the level of genetic divergence among different color types of *A.*
311 *galactonotus* will help evaluate the role genetic incompatibility in maintaining color variation.

312 We also showed that experimental locality influenced the overall frequency of attacks
313 upon models, with a significantly lower number of attacks on models distributed in village of

Brabo compared to models distributed in ICMBio station. This difference could be attributed to environmental differences. ICMBio station is located within a protected conservation unit, the National Forest of Caxiuanã (FLONA Caxiuanã), where human population density and hunting pressure are low. Village of Brabo is located in a populated area, where many inhabitants depend on hunting for subsistence and impacts of the environment may be greater. It is possible that the abundance of birds and mammals is higher in areas of lower human density. Differences in the abundance of avian predators have been shown to be advantageous or disadvantageous for conspicuous or inconspicuous species, suggesting that spatial variation in predator communities play a role in the maintenance of warning signaling variation (Valkonen et al., 2012). Similarly, the variation in predator community composition, which generates a geographical mosaic of selection, has been suggested as a factor promoting color variation in aposematic species (Nokelainen et al., 2014).

To further our knowledge of color variation in *A. galactonotus* there are several fundamental questions that need to be answered, such as whether there is assortative mating with respect to color, selection against hybrids, or variation in the toxicity of frogs between colors. In addition, a phylogeographic analysis across the distribution of this species will allow the environmental context, and time over which color variations evolved to be gauged. Collectively, these sorts of data can help resolve the extent to which color variation in *A. galactonotus* is maintained by predatory pressure, past or present, or other mechanisms derived from periods of isolation, such as reinforcement. If reproductive character displacement is the process by which color variation is maintained then we predict that the color differences between morphs should be maximal at the locations at which their distributions come in contact (Lambert et al., 2013).

Using models to explore predatory bias in respect to the color of aposematic poison frogs has not revealed a consistent pattern across species. In part, the differences in the

outcomes of these trials could be attributed to methodological limitations. However, it could also be that, in complex environments, these *in-situ* model-based experiments expose the models to predators that are wary of any conspicuous color or new object. It might also be that color variation was initially driven by predatory selection that is no longer occurring, and color variation is maintained by other processes. The maintenance of intraspecific variation of conspicuous colors appears to involve a more complex milieu of selective pressures, both past and present, than can be explained by predator bias alone.

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541

542 **Figures**

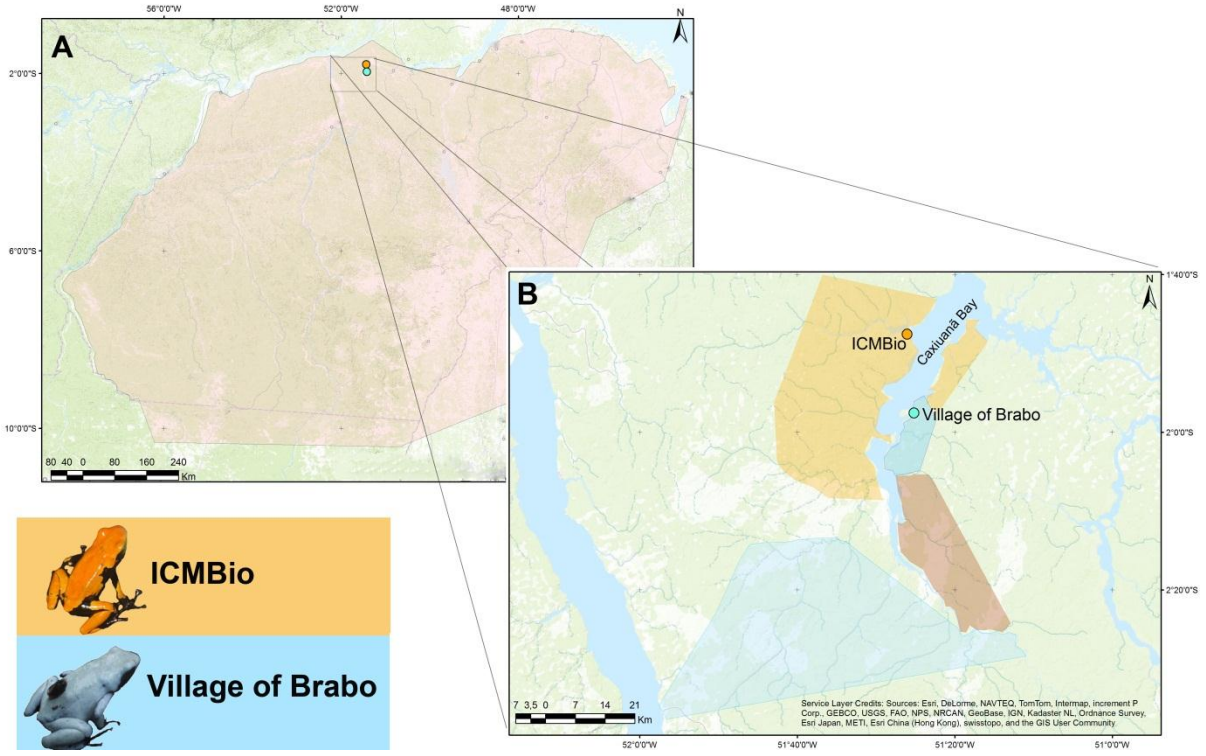

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Fig. 1 (A) Geographic location of the two study sites in Pará State, Brazil. (B) At ICMBio station, on the west bank of the Anapu River, the native color of *A. galactonotus* is bright orange. At Village of Brabo, at the east bank of the Anapu river, specimens are light-blue. The area highlighted in (A) represents the geographic distribution of *A. galactonotus* according to Hoogmoed & Avila-Pires (2012) and our data.

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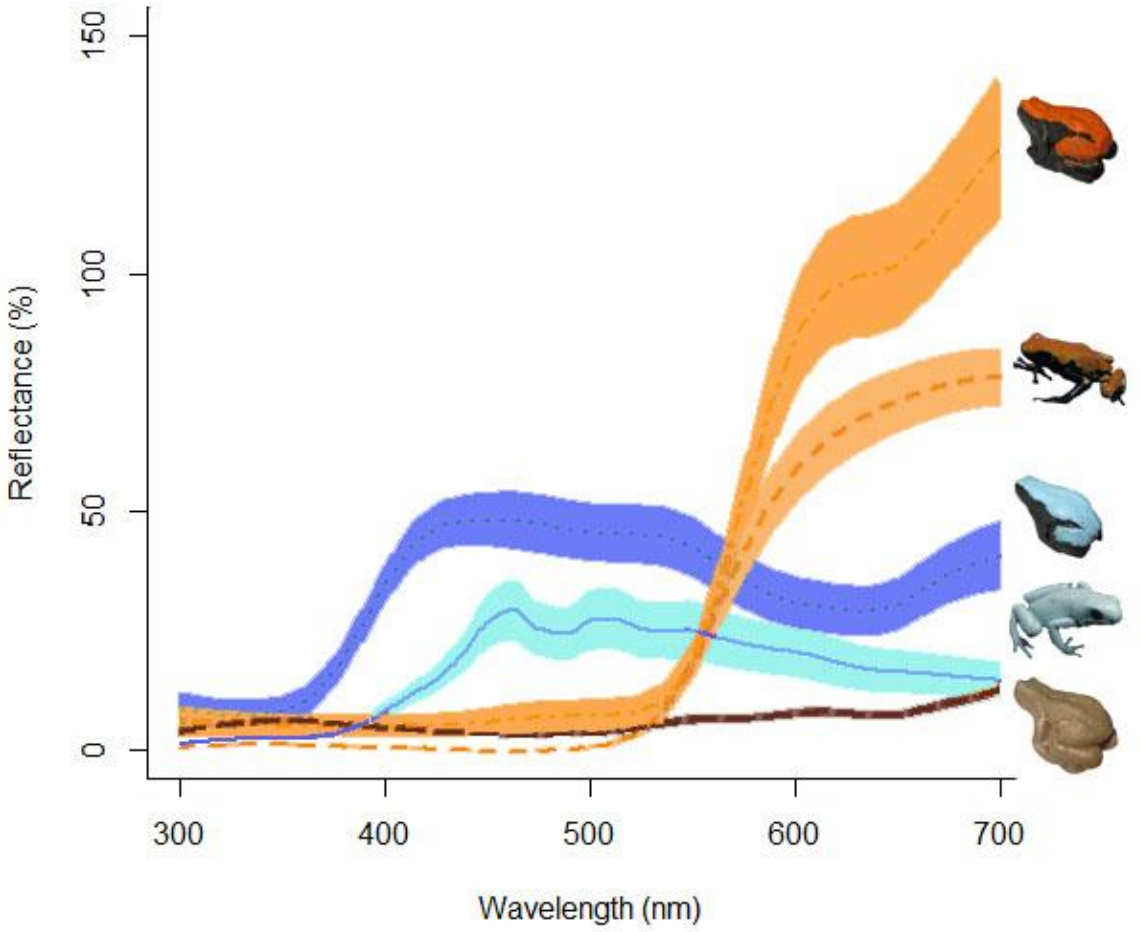

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Fig. 2 Reflectance spectra demonstrating that the wax models do not reflect UV, and in the respect are similar to living *A. galactonotus*. Shadows represent standard error.

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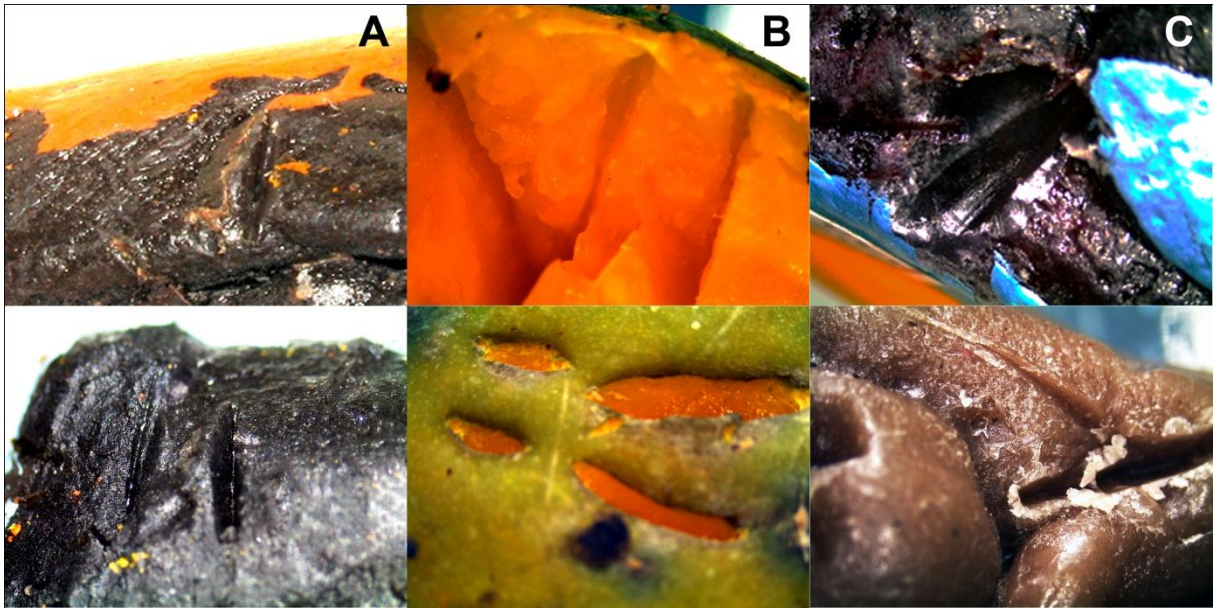

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Fig. 3 Bite marks by birds upon paraffin wax models. (A) Paraffin wax models used to characterize bird bite marks (B) ripe papaya fruits collected with evidence of avian bites, and (C) bird marks registered on models from the field experiments.

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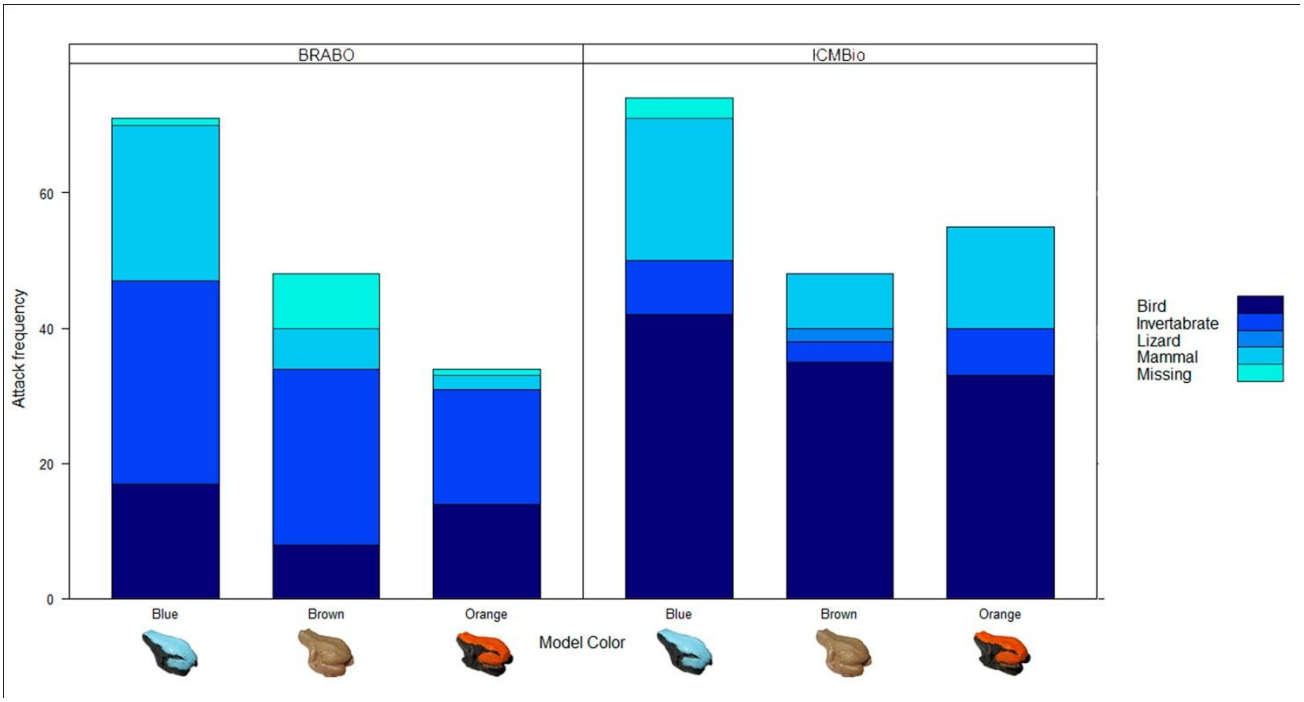

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Fig. 4 Bite frequency from different categories of predator on paraffin models that representthe brown phenotype and the two aposematic phenotypes (blue and orange) of *Adelphobates galactonotus* at the two study localities.

550

551 ## Appendix

Table A1. Summary table of studies addressing relationships between selection by predators and body coloration, using models (of clay or paraffin wax) representing local and novel morphs in aposematic poison frogs of the Family Dendrobatidae. The studies where trials were carried out in more than one locality, the data of each locality are shown separately. For the present study, the data are separated by vertebrate and avian predators for each locality.

Study	Species	Size (mm)	Occurrence Area (km²)	Dorsal colour	Attack proportion				Model material	Days	Background	Translocation	Check intervals (h)	Number of aposematic morphs	Species Presence/Absence	Minimum distance between models (m)	Effective N	Stats	p-value	Predators	Attack identification criteria	Main conclusion
					Brown	Other	Local1	Local2														
Amézquita et al. 2013	*Oophaga histriônica*	38	22 589.978	No uniform	0.95	0.75	0.70	-	Paraffin wax	22	Leaflitter	Yes	72	5	Absent	1	1200	GLM	0.0001	Birds, crabs, rodents	Beaks, claws, and rodent teeth	Predators generalize relevant cues from aposematic signals, so novel forms would be protected in the field.
					0.75	0.40	0.42	-				Yes			Present	1	1200		0.0001			
Chouteau & Angers 2011	*Ranitomeya imitator*	17-22	63 184.231	No uniform	0.13	0.24	0.06	-	Clay	3	Leaflitter	Yes	24	2	Present	5	900	X²	0.04	Birds, unknown predators	Birds: U-shaped	Different predator communities performing localized homogenizing selection on distinct aposematic signals.
					0.13	0.18	0.07	-		3		Yes		2	Present	5	900		0.04			
Present study	*Adelphobates galactonotus*	33-42	1 086 241.357	Uniform	0.11	0.12	0.10	-	Paraffin wax	12	Leaflitter	Yes	72	2	Present	5	1008	GLM	>0.05	Birds	Evidence of avian bites on ripe papaya fruits and models near of fruit trees; Mammals: bite marks leave by teeth; Lizards: teeth marks V-shaped	Predatory selection may not be a universal explanation for the maintenance of homogeneous and conspicuous colours.
					0.03	0.04	0.05	-		12		Yes			Present	5	1008		>0.05			
Present study	*Adelphobates galactonotus*			Uniform	0.14	0.19	0.14	-	Paraffin wax	12	Leaflitter	Yes	72	2	Present	5	1008	GLM	0.0001	Birds, mammals, lizards		
					0.05	0.05	0.12	-		12		Yes			Present	5	1008		0.0001			
Noonan & Comeault 2009	*Dendrobates tinctorius*	37-60	529 600.584	Uniform or no uniform	0.03	0.20	0.06	-	Clay	3	Leaflitter	No	72	2	Present	5	1260	G-test	0.001	Birds, rodents and unknown predators	N/A	Purifying role for predator selection, as brightly coloured novel forms are more lkely to suffer an attack than local aposematic and cryptic forms.

Hegna et al. 2012	*Oophaga pumilio*			No uniform	0.01	0.04	0.16	-	Clay	2	White paper or Leaflitter	No	48	2	Present	5	840	GLM	0.245	Birds	Birds: U- or V-shaped	Predation can be a factor shaping warning signal diversity through a variety of mechanisms.
Richards-Zawacki 2013	*Oophaga pumilio*			No uniform	-	-	0.31	0.35	Clay	2	Leaflitter	Yes	48	2	Present	2	800	X^2	0.223	Birds, mammals, crabs, and unknown	Birds: beaks; mammals: large incisors; crabs: claws	General avoidance of typical warning colours may contributed to the apparent stability of polymorphism.
					-	-	0.19	0.14		2		Yes	48	2	Present	2	800		0.662			
		17-24	64 704.682																			
Hegna et al. 2011	*Oophaga pumilio*			No uniform	-	-	0.20	0.27	Clay	2	White paper or Leaflitter	0	48	2	Present	5	1218	GLM	0.025	Birds, potential birds, mammals, arthropods and unknown	Birds: U- or V-shaped; mammals: spaces between teeth leave distinct ridges; arthropods (ants): small, paired linear marks of the mandibules	Contrasting colours did not affect attack rates by predators. The background coloration can potentially influence on wheter a predator chooses to attack.

CAPÍTULO II

Rojas, D., Lima, A.P., Simões, P.I., Avila-Pires, T.C.S., Hoogmoed, M.S., Bitar, Y.O.C., Kaefer, I.L., Amézquita, A. & Stow, A. A colors burst: the evolution of polymorphism in the warning coloration of the Amazonian poison frog *Adelphobates galactonotus*. Submetido à *Molecular Ecology*

A colors burst: the evolution of polymorphism in the warning coloration of the Amazonian poison frog *Adelphobates galactonotus*

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Keywords: Amazonia, aposematism, Dendrobatidae, population genomics, SNP, poison frog, local adaptation

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Running title: Evolution of color polymorphism in poison frogs

Abstract

To communicate distastefulness or toxicity, consistency of aposematic coloration might be assumed more effective, yet the dorsal coloration of the poison frog *Adelphobates galactonotus* varies throughout its distribution in eastern Brazilian Amazonia. Four dorsal colors (orange, yellow, brown and blue) occur patchily and repeatedly throughout the species range. To describe the geographic and evolutionary relationships among color morphotypes we measured dorsal coloration and reconstructed the phylogeny using sequences from two mitochondrial genes and Single Nucleotide Polymorphism (SNP) data. We applied ancestral character state analysis to test the hypothesis that similar colors found in different locations evolved independently. To identify loci potentially under selection with respect to color, we applied both an Fst outlier approach and SNP x color association tests using latent factor mixed effects modeling. SNP x color association tests identified 16 highly significant SNPs suggesting a role of selection in determining color. We found strong genetic partitioning associated with the Xingu River, in both mtDNA and SNP data sets. Using mtDNA, the time of genetic divergence between frogs on opposite riverbanks was estimated at 4.8 m.y.a. and genetic lineages leading to different colors split approximately during the Pleistocene. Ancestral character state analysis suggested that the same color types evolved independently several times, east and west of the Xingu River. Mitochondrial DNA haplotypes shared by frogs with different coloration support the conclusion of a recent color divergence. We suggest that the rapid evolution of color diversity probably arose in geographically isolated populations during periods of habitat fragmentation associated with the Pleistocene.

INTRODUCTION

A warning coloration, where contrasting and bright colors are related to the presence of toxins or other defenses in an organism, is often referred to as an aposematic coloration (Cott 1940; Guilford 1986), and is a widespread, yet poorly understood phenomenon (Endler & Greenwood 1988). The evolutionary processes that lead to aposematic coloration are still enigmatic, due to the need for aposematic signals be consistent and common in order to be effective as a defense mechanism (Cott 1940; Endler & Greenwood 1988), and yet, positively selected from an initial low frequency in the population where it originated. The outset of aposematic signaling in different animal groups has been hypothesized as the evolutionary consequence of either mimicry with co-occurring aposematic species (Joron & Mallet 1998), predatory neophobia (Lindström *et al.* 1999; Marples & Kelly 1999; Rowe & Guilford 1999; Ham *et al.* 2006) or gregariousness, because if a naïve predator attacks an aposematic individual and survives, it can then recognize nearby individuals bearing the same color as unpalatable (Guilford 1990; Gamberale & Tullberg 1998).

Intraspecific variation in aposematic coloration occurs in many taxa, such as plants (Lev-Yadun 2001), moths (Brakefield & Liebert 1985), butterflies (Joron & Mallet 1998; Joron *et al.* 1999; Speed & Ruxton 2005; Smith *et al.* 2013), beetles (Sagegami-Oba *et al.* 2007) and anurans (Summers *et al.* 1999; Siddiqi *et al.* 2004; Grant *et al.* 2006; Brusa *et al.* 2013). There is evidence of several, potentially interacting evolutionary processes leading to aposematic color variation. These include natural selection on variation in movement behavior, geographic differences in the local assemblage of predator species, and the presence of sympatric aposematic species (Endler & Mappes 2004; Valkonen *et al.* 2012; Rojas *et al.* 2014a; b). Additionally, color variation may be a consequence of geographic isolation and genetic drift (Hoffman *et al.* 2006) or sexual selection on color traits (Endler 1984).

Phylogenetic reconstructions provide the means to explore the evolutionary processes by which color variation arose (Wang & Shaffer 2008), such as whether particular color types evolved once or several times independently. They also allow inferences about the relative rate at which phenotypes evolved (Drummond *et al.* 2012). Further, isolation by distance (IBD) is generally expected across contigous landscapes, and color variation may be related to genetic or geographic distance predictors or both . The effect of IBD on color has been tested for different aposematic species across contrasting spatial scales, showing no association in some cases (e.g. wood tiger moth *Parasemia plantaginis;* Hegna *et al.* 2015) but a strong association in others, suggesting a role of selection on aposematic signals in order to trigger or reinforce reproductive isolation (e.g. strawberry poison frog *Oophaga pumilio*; Wang & Summers 2010). Therefore, ongoing selection for particular genes may act to maintain aposematic color polymorphisms within species, and this can be tested by combining phylogenetic reconstructions with a contemporary approach that identifies potential signatures of selection in relation to color. High-throughput sequencing techniques that produce thousands of genetic markers and the potential to detect selective sweeps across the genome are especially valuable in this respect (e.g. Hohenlohe *et al.* 2010).

Neotropical poison arrow frogs (family Dendrobatidae) possess aposematic coloration in combination with skin toxins, which likely evolved in tandem (Summers & Clough 2001) and both inter and intra-population variation in coloration exist (Amézquita *et al.* 2013; Richards-Zawacki *et al.* 2013; Rojas & Endler 2013). Interestingly, warning color differentiation seems to be a recent evolutionary process in most poison arrow frogs investigated so far, often with divergence in colors traced back to no earlier than the Pleistocene (Noonan & Gaucher 2006; Gehara *et al.* 2013).

Several evolutionary processes have been implicated in color divergence in aposematic frogs. In the Central American *O. pumilo*, different warning color morphotypes

evolved independently at multiple times (Wang & Shaffer 2008), with sexual selection (Summers *et al.* 1999; Reynolds & Fitzpatrick 2007; Richards-Zawacki *et al.* 2012), natural selection mediated by predation pressure (Saporito *et al.* 2007b; Noonan & Comeault 2009; Hegna *et al.* 2011, 2012; Pröhl & Ostrowski 2011), and demographic history (Gehara *et al.* 2013) being implicated in the process of color diversification. In the Andean harlequin poison frog *Oophaga histrionica*, some color phenotypes probably resulted from crosses between evolutionary lineages that diverged as a result of isolation by distance, suggesting that color variation can also arise from hybridization following secondary contact (Medina *et al.* 2013). In the northern Amazonian dyeing poison frog *Dendrobates tinctorius*, color variation is suggested to result from the joint effects of historical and ecological evolutionary processes, mediated by climate-driven vicariance and selection by predators (Comeault & Noonan 2011). Co-occurrence with different poison frog assemblages within the range of some species has led to the establishment of Müllerian mimicry rings and this has explained the rapid diversification of warning colors in some lowland Amazonian species, such as the poison frog *Ranitomeya imitator* and the nurse frog *Allobates femoralis* (Symula *et al.* 2001; Darst *et al.* 2006; Twomey *et al.* 2013, 2014).

The non-mimetic Amazonian poison frog, *Adelphobates galactonotus* (Steindachner, 1864), displays color variation throughout its distribution in eastern Amazonia, which comprises forest areas east of the Tapajós River to the Atlantic coast and south of the Amazon river down to the Amazon forest southern boundary with dry ecosystems in central and northeastern Brazil (Fig. 1) (Hoogmoed & Avila-Pires 2012). Different color morphotypes do not occur sympatrically, but replace each other repeatedly across the species' geographic range, in a mosaic-like fashion over a continuous rainforest landscape. The diversity and geographic distribution of *A. galactonotus* color types is unparalleled among other poison frog species not engaged in mimetic rings. A stunning diversity of color types have been reported

in the non-mimetic *O. histrionica* and *O. pumilio*, but their geographic distribution is several thousand km^2 narrower than that of *A. galactonotus*, spanning environmentally complex terrain in Central and Andean South America (montane, submontane, isthmic, insular), and the same color type is rarely observed in more than one region (Heinicke *et al.* 2007; Wang & Summers 2010; Medina *et al.* 2013). For these reasons, evolutionary mechanisms alternative to the ones raised to explain warning color variation in other poison frogs could be implicated in the origin of warning color diversity in *A. galactonotus*.

In a recent work, we demonstrated that the frequency of attacks by visually-oriented predators does not differ among wax dummies representing local and alternative color types of *A. galactonotus*, suggesting that local predation is not currently selecting for particular colors (Rojas *et al.* 2015). In the present study, we use field-collected dorsal color measurements, mtDNA sequence data, and a data set of several thousand single nucleotide polymorphisms (SNPs) sampled across the species geographic range in order to provide a phylogenetic pattern of color diversification in *A. galactonotus*. We then use these data sets to specifically test the following hypotheses: 1) that the timing of warning color diversification in *A. galactonotus* is consistent with the timing of color diversification in other Neotropical poison arrow frogs 2) That geographically isolated instances of similar color types represent independent evolutionary events 3) That warning color types in *A. galactonotus* are under selection.

METHODS

Study area

We sampled *Adelphobates galactonotus* across of its known geographic distribution in eastern Amazonia, south of the Amazon River, in the Brazilian state of Pará (Fig. 1). The area is crossed by two large southern tributaries of the Amazon, the Xingu and the Tocantins

rivers, both of which are implicated in sequential vicariant events relating to avian diversification (Aleixo 2004; Ribas *et al.* 2011; Avila-Pires *et al.* 2012). Our DNA sampling was concentrated to the west of the Tocantins River (Fig. 1), color sampling was more widely distributed, taking into account the literature (Hoogmoed & Avila-Pires, 2012).

Tissue sampling

A total of 220 individual samples were obtained from the tissue collection of Museu Paraense Emilio Goeldi, Belém, Brazil (MPEG; n = 84), and from fieldwork carried out from 2012 to 2014 (Table 1) in three regions from eastern Amazonia: Caxiuanã–Anapu river basin (Fig. 1A; n = 94), Tapajós-Jamanxim river basin (Fig. 1B; n = 40), and Carajás (Fig. 1C; n = 2). Tissue samples consisted of toe clips or muscle/liver tissue when voucher specimens were collected. Voucher specimens were deposited at the Osvaldo Rodrigues da Cunha Herpetological Collection of MPEG or at the Amphibian and Reptiles Collection of Instituto Nacional de Pesquisas da Amazônia (INPA-H) in Manaus, Brazil (Table S1). All tissue samples were stored in absolute ethanol prior to DNA extraction.

Color data collection

We measured the color of 99 live specimens at 17 localities distributed in the Caxiuanã-Anapu, Tapajós-Jamanxim and Carajás regions (2–13 specimens per locality; Table 1) with an OceanOptics spectrometer, and Spectra Suite® software. The spectrometer was calibrated for white and black before individual mesuarments. All mesuarments were obtained the same light conditions (on the laboratory with all lamps turned of and after 18:00 h). Individual reflectance spectra were obtained by averaging two dorsal measurements (head and lower dorsum). Spectra datasets were processed to average the spectra among individuals for each locality. The spectra analyses were conducted in the R statistical computing environment

(R Core Team 2014). The spectra were visualized using the *"aggplot"* function of the Pavo package (Maia *et al.* 2013). These analyses permit us to verify if the spectra are distinguishable, and validate the categories of color visually determined before measurements.

Mitochondrial sequence data

Total genomic DNA was extracted from preserved tissue samples using Promega® Wizard Extraction Kits, following the manufacturer´s guidelines. We amplified a 559 bp fragment of the Cytochrome Oxidase I (CO1) mitochondrial gene from 133 individuals using primers CHMF4 and CHMR4 (Che *et al.* 2012) and a 402 bp fragment of the 16S rDNA from 176 individuals using the primers 16Sar and 16Sbr (Palumbi 1996). PCR cycles were as follows: 30 s at 92 ˚C followed by 35 cycles of 92 ˚C for 10 s, 50 ˚C for 35 s, and 72 ˚C for 90 s, with a final extension of 10 min at 72 ˚C. PCR amplifications were purified for sequencing with ExoSAP-IT solution, then subjected to EtOH/EDTA precipitation and sequenced in the forward direction using an ABI sequencer 3031x (Applied Biosystems®).

Development of SNPs

Genomic DNA was extracted from 186 tissue samples (Table 1) using the GeneCatch™ Blood & Tissue Genomic Mini Prep Kit (Epoch Life Science, Inc). Approximately 0.5 µg of DNA was sent to Diversity Arrays Technology Pty. Ltd. (Canberra, Australia - http://www.diversityarrays.com) where SNPs discovery and genotyping was performed using the standard DartSeq™ protocol. DartSeq™ genotyping is a SNP-based genotyping-by-sequencing approach (Jaccoud *et al.* 2001; Kilian *et al.* 2012) where sequencing is carried out on an Illumina platform (Sansaloni *et al.* 2011) to genotype thousands of SNPs homogenously spaced across the genome (Petroli *et al.* 2012). We briefly outline the protocol below.

Library preparation

Template DNA was incubated in a 1X solution of Multi-Core™ restriction enzyme (RE) buffer (Promega) at 37°C for 2 hours to check genomic DNA quality. Approximately 100ng per µL of each sample was digested with a combination of *PstI and SphI* restriction enzymes. *PstI* and *Sphl* adapters and unique barcodes were ligated to each sample.

Each sample was amplified using PCR primers specific to barcode and adaptor sequences. PCR conditions were as follows: 1 min initial denaturation at 94 C°, followed by 30 cycles of 20 s denaturation (94 °C), 30 s annealing (58 °C) and 45 s extension (72 °C), and a final extension of 7 min at 72 °C. Using approximately 10 µL of each sample, all samples were pooled, diluted and denatured using NaOH in preparation for hybridization to the flow cell. The library was sequenced on an Illumina HiSeq®2500 platform (single read) using 77 cycles, resulting in 77bp long fragments. A proportion of the samples (>40%) were processed again through the whole library preparation protocol and downstream analysis to create the set of technical replicates that were used to assess the reproducibility of SNPs calls.

Quality control and initial SNP calling

Library sequences were converted to fastq format using the Illumina HiSeq®2500 software, and individuals were de-multiplexed based on the ligated barcode. Each read was assessed using Phred (Ewing & Green 1998) quality scores (Q-scores), and any reads containing Q-scores <25 were removed. All reads were checked against the DArT database and GenBank viral and bacterial sequences to identify potential contaminations. Following this primary workflow, SNPs were identified and called following the standard procedure in DArT proprietary pipeline DArTSoft14™ (Diversity Arrays Technology). The pipeline workflow is technically similar to the commonly used STACKS pipeline (Catchen *et al.*

2013), yet it differs from it as sequence clusters are first called from all samples pooled, prior to be called for each individual. All monomorphic sequence clusters were removed, and SNPs were called only if they were present in both homozygous and heterozygous forms. DArT pipeline also retains only SNPs with high balance between allele read depth and 'depth read depth' (average ratio of read depth between alleles = 0.75), reproducibility of >90% and minimum read depth of 5.

We further filtered the dataset using the following criteria:

1- Call rate of >98% i.e. less than 2% missing data

2- We only retained the first SNP in each fragment, to avoid creating a dataset containing closely linked loci.

3- 90% percent reproducibility

4- Average read depth for both alleles >5

5- Minor Allele Frequencies (MAF) >0.05

DNA sequences and statistics (call rate, polymorphic information content, heterozygosity, read depth and reproducibility) for all loci as well as genotypes for all individuals are deposited at Diversity Arrays Technology Pty. Ltd. (Canberra, Australia).

mtDNA analysis

Sequences were aligned using the ClustalW algorithm (Thompson *et al.* 1994) implemented in BioEdit 7.2.5 (version updated in November 2013) (Hall 1999). Substitutions were checked with the original chromatographs. In order to evaluate the genealogical relationships among haplotypes and overall haplotype distributions, a haplotype network for cytochrome oxidase I was 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. Analyses of genetic diversity at

16SrDNA and COI regions were carried out for each sampling location, and collectively for individuals on each side of the Xingu River using DnaSP v.5.10.01 (Librado & Rozas 2009).

Divergence time estimation

In order to estimate the divergence times for *Adelphobates galactonotus*, we conducted a Bayesian phylogenetic analysis in BEAST 1.8.0 (Drummond *et al.* 2012) using 59 unique mtDNA haplotypes recovered from a concatenated database of 16S rDNA and COI sequences (totaling 962 bp). Corresponding 16S rDNA and COI sequences from *A. castaneoticus*, *A. quinquevittatus* and *Phyllobates terribilis* were obtained from GenBank (accession numbers DQ502058, DQ502157, DQ502234, DQ502780, DQ502861 and DQ502906) and added to the database. Sequences were aligned with the MUSCLE algorithm (Edgar 2004) as implemented in MEGA 6.06 (Tamura *et al.* 2013). The database was partitioned according to the mtDNA regions. The most probable substitution model for each region was estimated independently in jModeltest 2.1.7 (Guindon & Gascuel 2003; Darriba *et al.* 2012). We defined the approximate average rate of evolution for each region based on results reported in Mueller (2006). As time calibration priors, we considered means and standard deviations previously estimated for the age of the most recent ancestor among *A. castaneoticus*, *A. galactonotus* and *A. qinquevittatus* (10.0 ± 2.5 m.y.a.) and between *A. galactonotus* and *A. castaneoticus* (8.0 ± 2.1 m.y.a.) (Santos *et al.* 2009). Remaining configurations, parameters and priors for the BEAST 1.8.0 input file followed recommendations provided by the software developers in accompanying documentation ("Relaxed phylogenetics and dating with confidence", by Drummond, Rambaut & Xie 2012). We applied a length of chain of 80 million generations with samples taken every eight thousand generations, and discarding 10% of the trees as burn-in, resulting in 9,000 sampled trees in the Markov Chain Monte Carlo (MCMC) run. The stationarity of the posterior

distributions for all model parameters, including medians and 95% Highest Posterior Density intervals (HPD) of the nodes was verified on Tracer 1.6.0 (Rambaut et al. 2014). The final consensus tree was obtained from the MCMC output using Tree Annotator 1.8.0 (Drummond *et al.* 2012).

Ancestral State Analysis

To test whether evolutionary transitions in dorsal coloration occurred a single time in *A. galactonotus* we conducted an ancestral character reconstruction. Briefly, dorsal coloration was collapsed into four character states according to the relative reflectance in short, medium and long-wavelenghts light (in decreasing order: blue, yellow, and orange), and absolute light reflectance (brown); these color categories are arguably distinguishable to a wide range of potential predators (Endler & Mappes 2004). A fifth state (patterned) was added for the outgroup species in the phylogenetic tree to indicate a general black dorsum crossed by white longitudinal stripes (*A. quinquevittatus*) or blotches (*A. castaneoticus*). Black dorsum with yellow/whitish dorsolateral stripes represents the ancestral color pattern in the genus *Phyllobates* as well, although the species we used here (*P. terribilis*) loses this pattern as adult and becomes uniformly yellow. As phylogenetic hypothesis, we used the ultrametric and binary tree obtained from the analysis of fragments of the 16S rDNA and COI mitochondrial genes. The reconstruction of ancestral color states was implemented with a continuous-time Markov model (Mk) on the R package *phytools* (Revell 2012), assuming equal rates of transition among them.

Outlier locus detection

To identify a subset of loci that are putatively under selection we used the Fst outlier approach implemented in *Bayescan* (Luikart *et al.* 2003), which uses a Bayesian method to

identify SNP loci with genetic differentiation (Fst) higher than expected by genetic drift alone. There are various methods that can be used to identify loci under selection, and each has different biases, including limited sensitivities (high rate of Type 2 errors) and susceptibilities to false discoveries (Type 1 errors). *Bayescan* has been found to exhibit the lowest Type 1 and Type 2 errors under a range of simulated scenarios (Narum & Hess 2011). In *Bayescan* posterior distributions are obtained using a Reversible-Jump Markov Chain Monte Carlo algorithm. We ran 20 pilot runs, followed by 100,000 iterations; that is, 5,000 samples with a thinning interval of 10 and a burn-in of 50,000 iterations. For the *Bayescan* analysis, using uninformative prior odds for the neutral model and the model including selection assumes that for each locus the models are equally likely. When sampling a large number of loci this can increase the probability of false discoveries. To minimize the chance of false discoveries we set the prior odds to 10 (corresponding to a prior belief that the selection model is 10 times less likely than the neutral model), which is the value suggested by the authors as appropriate for studies with a few thousands loci. Posterior odds are then calculated from Bayes Factors according to the formula PO=BF*P(M2)/P(M1), where P(M2) and P(M1) represent the prior probabilities for the model under selection and the neutral model respectively. We applied a False Discovery Rate (FDR, qval) of 5 % as a cut off value to identify loci under selection. Outlier analysis was performed using all SNP loci, filtered as described above, with individuals divided into 12 independent collection sites (‘populations’) from which five or more individuals were sampled (N = 173 total; 5-26 per site). The remaining individuals (N = 13) belonged to sites with less than five individuals collected and were excluded from both outlier and SNP x color association tests, however these individuals were included in analysis of neutral genetic structure and phylogenetic reconstruction based on SNP data.

Genetic structure using SNP data

We built a Neighbor Joining (NJ) tree of individuals using D_A distance (Nei *et al.* 1983) implemented in the software POPTREE2 (Takezaki *et al.* 2010). We performed a Principal Component Analysis (PCA) based on genetic distances (GD) using the R computing environment (packages: *adegenet* (Jombart & Ahmed 2011) and *ape* (Paradis *et al.* 2004). Because two distinct groups corresponding to samples from opposite sides of the Xingu River were observed in the PCA, separate PCAs using only data from a single side of the river were performed.

Bayesian assignment analysis, implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000), was used to assign individuals to genetic clusters (K) and estimate admixture proportions (Q) for each individual. The range of possible K was set from one to 21 (number of localities plus 3, as suggested by Evanno et al. (2005). The analysis was run for five iterations with a 10,000 burn-in period and MCMC of 10,000 replicates, setting an admixture model ($\alpha_{initial}$= 1.0; α_{max}= 10.0). The most probable number of genetic clusters (K) present in the data was defined following Evanno et al. (2005) using the Structure Harvester v. 6.0 program (Earl & vonHoldt 2012). To assess whether the removal of outlier loci (i.e. heterogeneity) was influencing patterns of genetic structure, we ran all of the above analyses a second time with the data set including outlier loci, and those without. In all cases our analyses produced very similar results and we present genetic structure analyses using the neutral SNP data only.

SNP x color association analysis

To identify SNPs that associate with different color morphotypes of *A. galactonotus*, we conducted correlation tests between individual color (four categories: blue, yellow, orange and brown) and SNP allele frequencies using the latent factor mixed models (LFMMs)

implemented within the software LFMM (Frichot *et al.* 2015). Only SNPs identified as Fst outliers were used in this analysis in order to target only those SNPs that are putatively under selection (see above). LFMMs consider genotypic matrix values as response variables in a linear regression model. LFMM simultaneously estimates the effects of hidden factors that represent population structuring due to background genetic variation or shared demographic history, defined by the number of latent factors that are usually determined by genetic clustering analysis (Frichot *et al.* 2015). After correcting for confounding effects, significant associations between SNP allele frequencies and observed ecological (or morphological) variables may be interpreted as evidence for selection at those loci (Frichot & François 2015). Compared to other outlier and environmental association analysis methods (see Rellstab *et al.* 2015), LFMM reduces the type I error (i.e. false positives) in datasets with complex underlying genetic structure (Frichot *et al.* 2013, 2015; de Villemereuil *et al.* 2014). We ran LFMM for the variable 'color' as implemented in the LEA package for R (Frichot & François 2015). The number of latent factors was chosen based on the results of STRUCTURE analysis. The program was run for 10000 iterations with a 5000 burn-in for five repetitions. Median z-scores were combined across runs, and p-values were adjusted for multiple tests using the Benjamini-Hochberg procedure (FDR = 5%) as outlined within the LEA package (Frichot & François 2015). The median genomic inflation factor (GIF or 'lambda') was calculated across runs according to Devlin and Roeder (1999) to evaluate the capacity to control the FDR, which is indicated by a GIF close to, or slightly below 1.0 (Frichot *et al.* 2015). DNA sequences containing SNPs associating with color were ran through the BLASTN online database to search for matches with functionally relevant genes that may relate to color synthesis.

Testing for Isolation-by-geographic distance

Spatial autocorrelation analyses were conducted to test for associations between geographic distance and genotypic similarity (r). The genetic distance matrix was constructed using only putatively neutral SNPs determined using Bayescan (above) from 12 localities (that contained more than five samples) in GenAlex 6.5 (Peakall & Smouse 2012). The same program was used to obtain a geographic distance matrix from the geographic coordinates of each locality and to run a spatial autocorrelation analysis between the two matrices. For all analyses we used the single population option with distance categories defined to maximise the number of pairwise comparisons, and where possible to allow for comparisons across landscapes at the same spatial scales. Spatial autocorrelation analyses were conducted for samples from each side of Xingu River separately. The geographic classes analysed for the east side of the Xingu River were 0-50, 51-200, 201-300 and 310-600 km and for the west side were 0-50 and 51-200 km. The 95% confidence intervals around r within each distance category were estimated by bootstrapping 9999 times, and the 95% confidence intervals around a random distribution (mean r = 0) was determined by 9999 permutations.

RESULTS

Geographic distribution of color morphotypes

Adelphobates galactonotus showed four coloration patterns occurring patchily and repeatedly throughout its geographic range: yellow: MV, BM, TJY; orange: ECFP, IC, MU, PB, CC, BA, TAR, BC, RE, TO, AT, AN, TJO; brown: SR, SA, PR, MA) and blue, which varies subtly from one locality to another. The blue color was found in ten localities (TAL, BR, MO, CA, BL, SS, SP, TJB, MP, JA) (Fig. 2 a to i, Table 1). At each sampling site, specimens were monomorphic in color pattern, exhibiting only slight variation in coverage of color patches. We did not observe any specimen with dorsal color pattern mixed or intermediate between the four color morphotypes.

399

400 *Haplotype network analysis*

401 We identified 37 variable sites and 35 haplotypes in 16S rDNA and 42 variable sites
402 and 34 haplotypes in the COI gene fragment (Fig. 3). Haplotype networks built using
403 statistical parsimony were similar for both mtDNA regions, hence only COI results are shown
404 (Fig. 3; see Table S2 for additional results). We found no association between color
405 morphotypes and the genetic structure inferred from mtDNA markers (i.e. specimens
406 belonging to different color categories shared haplotypes). Haplotype sharing does not occur
407 between *A. galactonotus* sampled in opposite sides of the Xingu River, suggesting that it
408 constitutes a barrier to gene flow.

409

410 *Phylogenetic analysis*

411 The final consensus tree inferred from a Bayesian phylogenetic analysis on unique
412 mtDNA haplotypes of *A. galactonotus* suggests that all color morphotypes evolved
413 independently and at multiple occasions along the species evolutionary history (Fig. 4).
414 Samples collected from regions west and east of the Xingu River form two well-supported
415 basal clades. The most recent common ancestor between specimens belonging to these clades
416 can be traced back to late Miocene, most probably around 4.8 million years ago (Fig. 4).
417 Differentiation of color morphotypes occurred more recently in evolutionary history, with
418 orange, yellow and blue morphotypes arising several times, most probably during the
419 Pleistocene.

420

421 *Ancestral state analysis*

422 The reconstruction of ancestral coloration in *A. galactonotus* strongly supports several
423 evolutionary origins for each color category within the two major clades (Fig. 5). Orange and

yellow morphs would have appeared at least four times from generally blue morphs in the Tapajós (West) clade. Although the ancestral state of the East clade is equivocal, the analysis reveals at least four evolutionary transitions between coloration dominated by generally long-wavelengths (orange, brown and yellow) and short-wavelengths(blue). Thus, although we are currently unable to postulate the coloration of an *A. galactonotus* common ancestor, we can confidently presume that colour transitions occurred several times within each of the major lineages.

Outlier loci detection and genetic structure

We identified a total of 7963 SNPs after bioinformatics filtering. A total of 1821 SNPs were identified as outlier loci using *Bayescan* and an FDR = 0.05 (23.7% of loci) (Fig. S1). Of these outlier loci, 1493 were found to be under positive (potentially diversifying or disruptive) selection (Fst = 0.73-0.85) and 328 loci under negative (or balancing) selection (Fst = 0.97-0.40), as indicated by Fst distributions (Fig. S1). All outlier loci were excluded from analysis of genetic structure, resulting in a total of 5872 SNPs. Principal Components (PC) Analysis using adegenet in R (Jombart & Ahmed 2011) with all neutral SNPs and all individuals (N = 186) resulted in the two first axes (PC1 and PC2) summarizing 64.71% of the total genetic variation. The distribution of specimens along these axes showed two well-defined genetic clusters corresponding to samples collected from the west and east of the Xingu River (Fig. 6a). Notably, genetic variability along PC2 is greater among samples collected from localities east of the Xingu River. SNP-based PCAs carried out separately for samples collected from each side of the Xingu River did not show grouping patterns associated with color nor locality (Fig 6b e c). The two groups, located on either side of the Xingu River were also recovered by the NJ tree topology (Fig. 7) with a high bootstrap

support. In addition, these two groups were supported by the Bayesian analyses in STRUCTURE (Fig 6).

Spatial autocorrelation analysis

Spatial autocorrelation analyses based on geographic distances and genotypic similarity showed that the genetic structure in *A. galactonotus* does not follow an isolation-by-distance pattern (Fig. S2). The first distance class shows that genotypic similarity among individuals sampled at the same locality is significantly greater than the sample wide average (r = 0). These data imply genotypic partitioning among localities. However, no pattern is evident when comparing the genetic similarity values for the distance categories larger than 50 km (r values: east: 50 km = -0.038, 200 km = -0.041, 300 km = -0.022, 600 km = -0.032; west: 50 km = -0.016, 200 km = -0.017). In other words, there is no evidence for localities further away being any more genetically dissimilar than locations in close proximity.

Detection of color-associated SNPs

SNP x color association analyses using LFMM (Frichot *et al.* 2015) were conducted with two latent factors defined, as informed by the STRUCTURE analysis where K = 2. Of the 1821 outlier SNPs tested, 16 SNPs significantly correlated with color morphotypes. Figure S3 shows the distribution of all SNP loci (N = 1821) in relation to their –log10 p-value for color, which after correction for multiple tests, resulted in a significance cut-off of approximately $P = 0.0004$. The genomic inflation factor (GIF), as specified by lambda, was equal to 1, indicating a strong capacity to detect false positives in the analysis. As determined via the *Bayescan* Fst outlier analysis (described above), 14 of these 16 SNPs were under negative (or balancing selection), and the remaining two exhibited high Fst values indicative

of positive (or divergent) selection (0.77 and 0.81, respectively; Table S3). BLASTN analysis of the DNA sequences of the 16 SNPs resulted in no significant matches to functional genes.

DISCUSSION

The current distribution of color morphotypes coupled with our genetic results show that *A. galactonotus* rapidly diversified into different colors most likely during the Pleistocene. Genetic partitioning between individuals sampled on each side of the Xingu River shows that the river channel constitutes a barrier to dispersal, which existed prior to warning color diversification that generated present day color morphotypes. Color variation is predominantly polyphyletic and the distribution of color morphotypes shows no obvious geographic pattern. The rapid diversification of color and the independent origin of the same colors multiple times suggest a simple genetic mechanism, whereby few mutational steps are required for color change. There are several possible explanations for how different colors became fixed in different localities. Broadly these could include strong divergent selection in sympatry, or the effects of geographic isolation, where both drift and selection might account for the rapid evolution of color variation in *A. galactonotus*. We argue that color divergence occurred in geographically isolated parts of the distribution, and have some genetic evidence that color has been under selection.

In anurans, as in other amphibians, fishes and reptiles, color is determined by three cell layers, the melanophores, the xantophores and the iridophores, each containing specific pigments (Hofreiter & Schöneberg 2010). Color variation in *A. galactonotus* could be due to one or a few point mutations related to genes involved in color determination via synthesis or destruction of pigments like pteridines, or in the metabolic pathways associated with the dietary acquisition, transport and integumentary accumulation of carotenoids, which could be responsible for the yellow/orange/brown coloration. If the carotenoids and pteridines are

497 removed, these colors disappear and the guanine crystals deeper in the epidermis are the main
498 determinants of the iridescent, bluish color (Bagnara & Matsumoto 2007; Bagnara *et al.*
499 2007). Following these sorts of mutations, we suggest that fixation of different colors
500 occurred in different localities. That is, alleles for different color morphs arose in isolation
501 (separately) and were then fixed. Isolation seems likely both with respect to the habitat use of
502 this species, and via historical processes. A naturally patchy distribution appears characteristic
503 of *A. galactonotus*, which might be associated with habitat heterogeneity including the
504 dependence on isolated and temporary water bodies, such as water accumulated in Brazil nut
505 pods or other phytotelmata (Hoogmoed & Avila-Pires 2012). This feature, coupled with the
506 typically low vagility of frogs, probably constrained the geographic extent of gene flow
507 between clades represented by different color types, as has also been reported for other
508 Neotropical ground-dwelling frogs (Fouquet *et al.* 2015). Additionally, given that much of the
509 genetic divergence occurred over the last 2.5 m.y.a., it seems reasonable to suppose that
510 environmental conditions, which have changed dramatically and repeatedly over that time
511 period (Cheng *et al.* 2013), probably acted to isolate parts of the *A. galactonotus* distribution.
512 Once geographically isolated, fixation of colors could be a faster result of divergent selection,
513 drift or some combination of both than in non-isolation scenarios.

514 In species with aposematic, conspicuous and polytypic coloration, the role of visually
515 oriented predators is frequently thought to drive diversification through natural selection (e.g.
516 Jiggins *et al.* 2001). However, recent field experiments using *A. galactonotus* wax models set
517 up in different localities failed to observe predatory bias toward particular color morphs
518 (Rojas *et al.* 2015). It is possible that this result reflected the experimental design, because
519 movement is relevant to prey detection by birds, which are the main predators of poison frogs
520 (Paluh *et al.* 2014). It is also possible that other forms of natural selection are driving color
521 variation in *A. galactonotus*. However, if natural selection is the process generating color

522 variation, then the polyphyletic nature of color and the lack of geographic pattern suggest that
523 this is happening at highly restricted scales.

524 Polyphyletic distributions of color morphotypes have been associated with sexual
525 selection, both theoretically and experimentally in several species (Iwasa & Pomiankowski
526 1995; Brunton 1998). It has been demonstrated that sexual selection can effectively and
527 rapidly drive a trait to fixation (Tazzyman & Iwasa 2010; Rudh *et al.* 2011) and thereby result
528 in polyphyletic evolution. *Adelphobates galactonotus* does not exhibit any obvious sexual
529 dichromatism, so if sexual selection is a driver of color divergence, it has not resulted in
530 visually conspicuous differences between the sexes (observations by the authors), as reported
531 in many frog species in which color plays a key role in mating choice (Bell & Zamudio 2012).
532 However, polymorphic species offer different phenotypes for females to choose, thus building
533 a scenario whereby this process could occur. Further work testing the presence of assortative
534 mating with respect to color is needed in order to evaluate whether different colors in *A.*
535 *galactonotus* are sexually selected by frogs.

536 We identified SNP loci putatively under positive (and to a lesser extent, balancing)
537 selection across the distribution of *A. galactonotus*, with some indication that there is
538 selection with respect to color based on a correlative SNP x color association approach.
539 Notably, the Fst outlier approach identified a high proportion of SNPs under putative
540 selection within our dataset, which we interpret cautiously due to potential Type 1 errors.
541 Despite this, our group of putative SNPs under selection acted to refine the set of loci from
542 which to perform SNP x color association tests, and we choose to not interpret signatures of
543 selection beyond these tests. Using a BLASTN search of the DNA sequences for all color-
544 associated loci, we were not able to identify any functionally relevant genes relating to color
545 or related metabolic processes that would further support a role of these particular loci being
546 associated with selection on color. Such annotations may be particularly difficult to obtain

where a trait is determined by few loci, or many loci spread throughout the genome, and thus the analysis would be greatly improved by mapping sequences to an annotated conspecific or closely related reference genome, which is currently unavailable. However, it is possible that some of the color-associated SNPs are physically linked to functionally relevant parts of the genome that may be under selection. Characterizing SNPs in genes directly relevant to color morphotypes may be unlikely with the current data set if a simple genetic mechanism requiring few loci underpin color differences, but the genetic mechanism governing color in *A. galactonotus*, as well as the type of pigments reflecting yellow and orange colors, remain unknown. Despite this, our results provide preliminary evidence that color has been under selection, and these data provide a step forward in characterizing the molecular basis of frog color polymorphism, pending the availability of additional genomic resources and chemical characterization of skin color pigments.

Isolation and divergence via genetic drift is another explanation for the polyphyletic nature of color in *A. galactonotus*. The most evident geographic pattern in our study system is that of a large river, the Xingu, implied as a geographic barrier between closely related evolutionary lineages, a classic diversification theory proposed by Alfred R. Wallace (Wallace 1854) and observed in different groups of Amazonian terrestrial vertebrates (Antonelli *et al.* 2010; Leite & Rogers 2013). Among Amazonian anurans, rivers have played important roles in intraspecific differentiation, influencing the distribution of genotypic and phenotypic diversity (Kaefer *et al.* 2013; Simões *et al.* 2014; Fouquet *et al.* 2015). Therefore, it is not surprising to find the Xingu River as a barrier to gene flow in *A. galactonotus*. Nonetheless, it is clear that color diversification occurred at a more recent temporal scale than the formation of the Xingu´s main river channel. Even allowing for the uncertainty around estimates of clade ages (Fig. 4), the timing of color diversification occurred well within the Pleistocene, a period of high and repetitive climatic instability especially in the eastern

572 portion of the Amazon basin (Maslin & Burns 2000; Vonhof & Kaandorp 2010; Cheng *et al.*
573 2013). Such cyclic disturbances might have generated intermittent isolation and small
574 population sizes through the contraction and expansion of adequate habitat. Although the
575 species is apparently tolerant to drier habitats (e.g. forest-Brazilian Cerrado, forest-
576 agricultural landscape ecotones), it was never reported at distances greater than a few
577 kilometers from forested areas (Hoogmoed & Ávila-Pires 2012). Under these conditions, it
578 seems plausible that drift might have contributed to the fixation of different color variants in
579 different localities following geographic isolation by climatically induced habitat shifts.

580 Irrespective of the mechanism that resulted in the fixation of different colors in
581 different localities (selection or drift), the observation that only a single color in any one
582 locality suggests evolutionary mechanisms that prevent recombination. Assuming that
583 divergence occurred at geographically isolated locations, then it is possible that color
584 differences might have been reinforced through assortative mating driven by lower hybrid
585 fitness. Narrow interspecific hybrid zones across unbroken rainforest landscapes have been
586 characterized in other Amazonian frogs (e.g. Brown & Twomey 2009; Simões *et al.* 2012)
587 suggesting that in these cases, hybridization lowers fitness. Therefore, further work
588 identifying contact zones between color types and the existence of assortative mating,
589 combined with genomic and pigment data collected both in the wild and in captivity will help
590 elucidate the processes maintaining the color variation in this species.

591

592

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Data Accessibility

The mtDNA sequences can be accessed in GenBank by the Accession numbers KU597806 - KU597873. Nuclear and mtDNA sequences and statistics (call rate, heterozygosity, read depth and reproducibility) for all loci as well as genotypes are accessible from the Dryad Digital Repository (doi: to be obtained). Nuclear DNA data is also held at Diversity Arrays Technology Pty. Ltd. (Canberra, Australia) and can be made available upon request.

Author Contributions

D.R., A.S. and A.P.L. designed the project. D.R., A.P.L., A.S. and P.I.S. collected samples. T.C.S.A-P. and M.S.H. contributed useful discussion. T.C.S.A-P., M.S.H. and Y.O.C.B. contributed with extra samples and discussion of results. D.R. and A.A. measured, analyzed and interpreted the color data. A.S., R.Y.D., P.I.S. and I.L.K analyzed and interpreted genetic data. D.R., I.L.K, R.Y.D. and A.S. wrote the manuscript with contributions from all co-authors.

1010 **Tables**

1011

Table 1. Locality names, their code and the regions from which *A. galactonotus* was sampled, number of specimens sequenced for cytochrome oxidase I and 16S rDNA regions and genotyped at SNPs; body color refers to the number of live specimens for which the reflectance spectrum of the dorsum was measured and color morph refers to the visually-based color morphotype of specimens from each locality.

Locality	Code	Region	COI	16S	SNP	Body color	Color morph
Estação Cientifica Ferreira Pena	EC	Caxiuanã Bay left bank		7	6		Orange
FLONA Caxiuanã – ICMBio Station	IC		8	14	11		Orange
FLONA Caxiuanã – Muju	MU			3	3		Orange
FLONA Caxiuanã – PPBio	PB		3	3	1		Orange
FLONA Caxiuanã – Cacoajo	CC		4	5	5	6	Orange
Bacuri Village	BA	Anapu River Right bank		6	6	9	Orange
Santa Maria	SM		3	4	5	6	Orange
Taperu Right	TAR		2	3	3	5	Orange
Taperu Left	TAL		4	4	4	6	Blue
Brabo Village	BR		8	7	8	3	Blue
Mojua River	MO			7	7	2	Blue
Cacoal, Atua River right bank	CA		2	4	3	6	Blue
São Raimundo	SR		2	4	3	3	Brown
Santo Amaro Village	SA			2	2	2	Brown
Prainha Village	PR		4	6	6	6	Brown
Angelim, Jacitara, Marapiranga	MA		9	11	11	13	Brown
Pracupi River and Anapu River south FLONA Caxiuanã	BL	Anapu River Left bank - Serra Sul – Carajás	10	10	16	13	Blue
FLONA Carajás	SS		2	2	6	2	Blue
Vila dos Cabanos	BC	Barcarena	2	2	2		Orange

Fazenda Riacho Monte Verde	MV	Monte Verde	5	10	9		Yellow
Porto de Moz	RE	Porto de Moz – Xingu River		2	2		Orange
Caracol, UHE Belo Monte	BM	Belo Monte – Xingu River	2	2	2		Yellow
Baião	TO	Tocantins River			2		Orange
Cachoeira do Espelho	AT	Altamira		3	5		Orange
Nazaré	SP	Senador Porfirio			2		Blue
Serra das Andorinhas	AN	Serra das Andorinhas	2	2	1		Orange
Tapajos River	TJ		9	6	4		Unknown
Tapajós MU	TJO	Tapajos River	10	12	8	4	Orange
Boca do Rato	TJY	Right bank	8	7	11	7	Yellow
Betel	TJB		22	17	21	6	Blue
Mina do Palito	MP	Jamanxim	7	6			Blue
Jamanxim river left bank	JA	River/Moraes de Almeida	5	3	13		Blue
Projeto ALPA	AL	Marabá	2	2			Brown

Figures

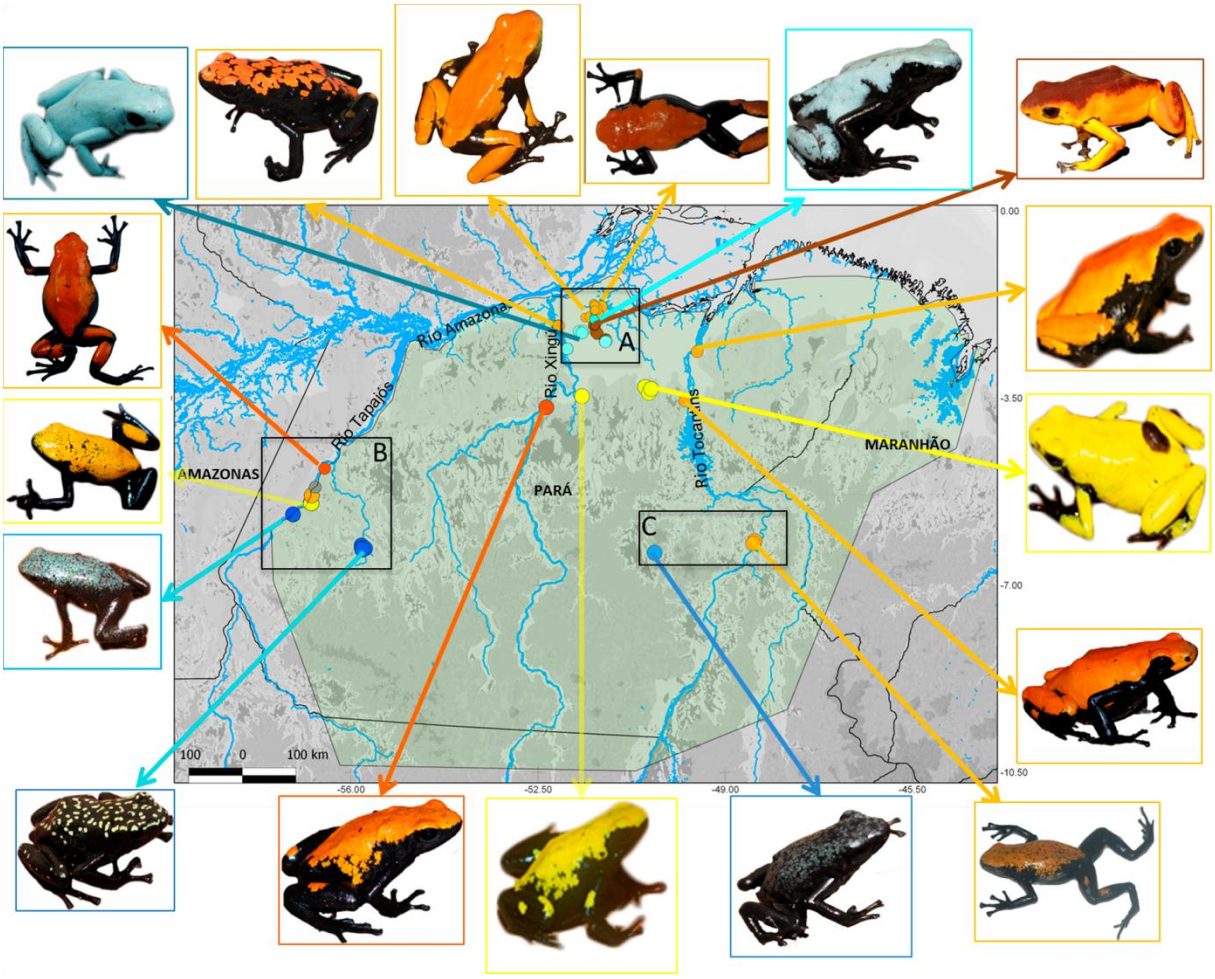

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Figure 1. Green shade: Geographic distribution of *Adelphobates galactonotus* in eastern Brazilian Amazonia, south of the Amazon and east of the Tapajós River. Dots: locations from where *A. galactonotus* tissue samples were collected. Colored dots are connected with pictures of representative local dorsal color morphotypes (yellow, orange, brown, blue), which may vary in the relative area covered by dark pigments. Grey dots indicate localitites where the local color morphotype is unknown (i.e. tissue samples obtained from preserved specimens with faint coloration). A, B and C indicate regions of fieldwork where color measurements were taken via light spectrophotometry, along with additional tissue samples: A) Caxiuanã-Anapu River basin; B) Tapajós-Jamanxim river basin. C) Carajás.

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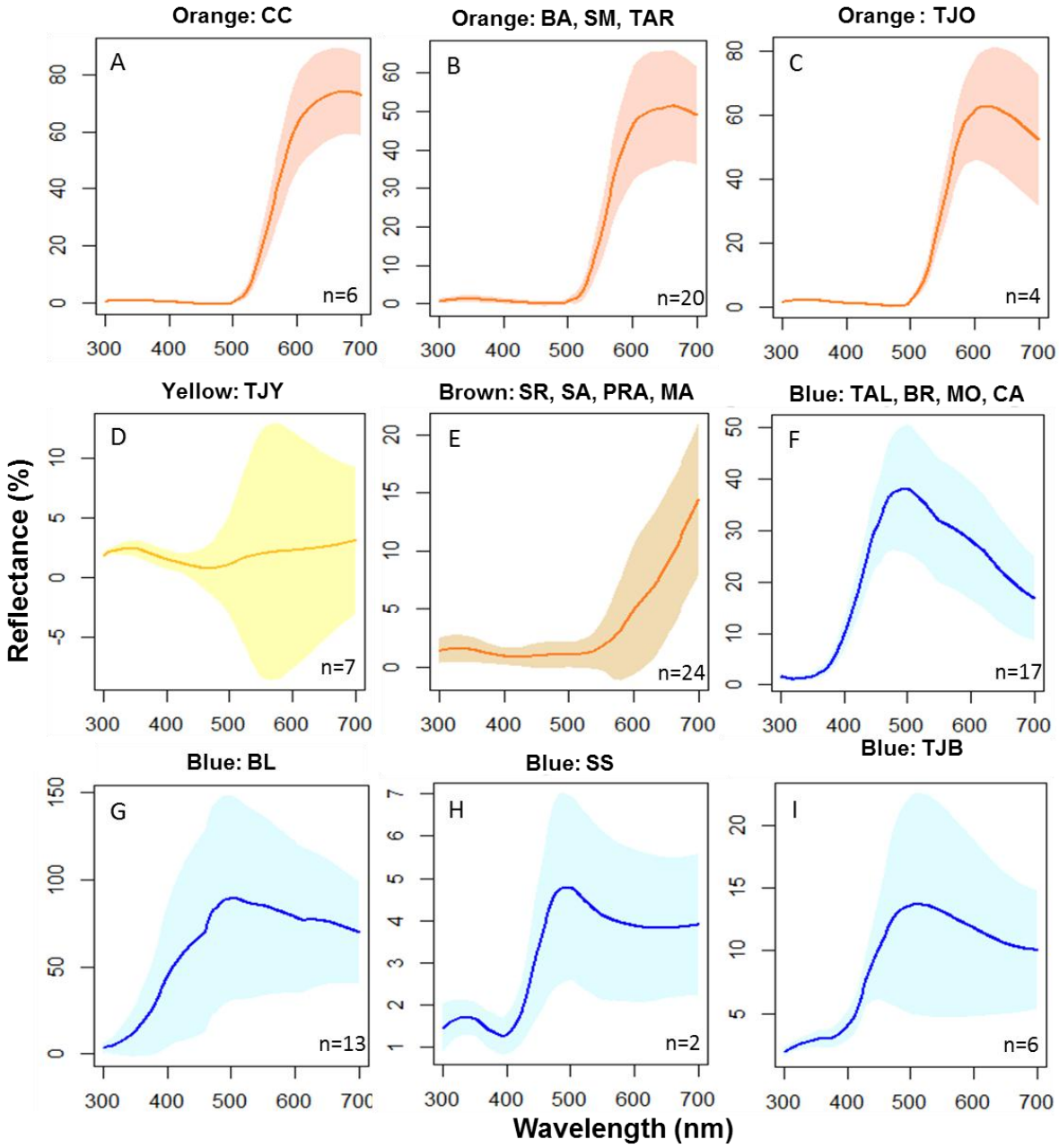

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Figure 2. Average reflectance spectra (lines) and standard deviation (shades) of dorsal coloration of *Adelphobates galactonotus* sampled in eastern Brazilian Amazonia. These reflectance patterns where used to categorize sampled specimens into four dorsal color categories: orange (A-C), yellow (D), orange (B–D), brown (E) and blue (F–I). Names and codes above graphs indicate the locality from where specimens where measured, as indicated in Table 1. n = number of specimens in each locality from which we obtained reflectance measurements.

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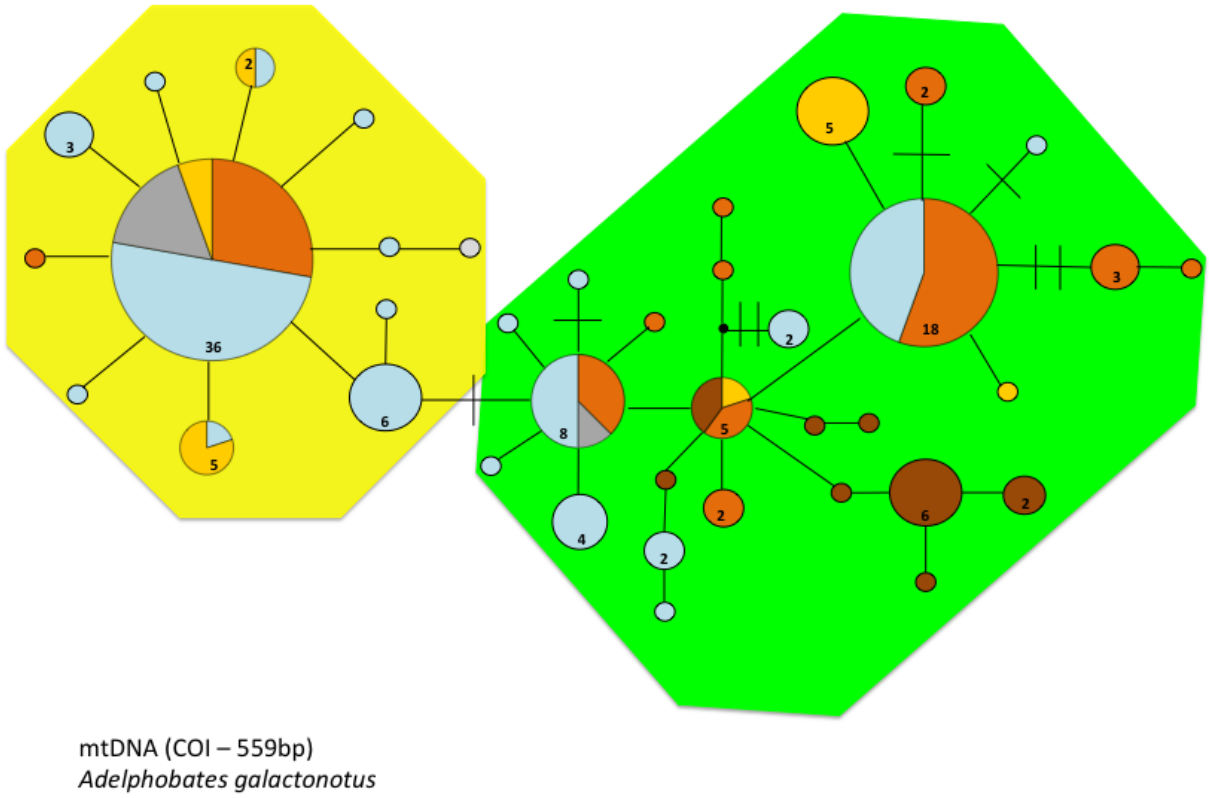

mtDNA (COI – 559bp)
Adelphobates galactonotus

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Figure 3. Haplotype network built from 133 sequences of a 559 bp fragment of the cytochrome oxidase I mitochondrial gene of *Adelphobates galactonotus*. Circle size is proportional to haplotype frequency. Circle colors represent relative frequency of visually-based color morphotypes (orange, yellow, brown and blue) carrying that particular mtDNA haplotype. Grey color represents sequences obtained from preserved specimens with unknown color morphotypes. Haplotypes in the yellow background portion correspond exclusively to samples collected west of the Xingu River. Haplotypes on green background correspond exclusively to samples collected east of that river.

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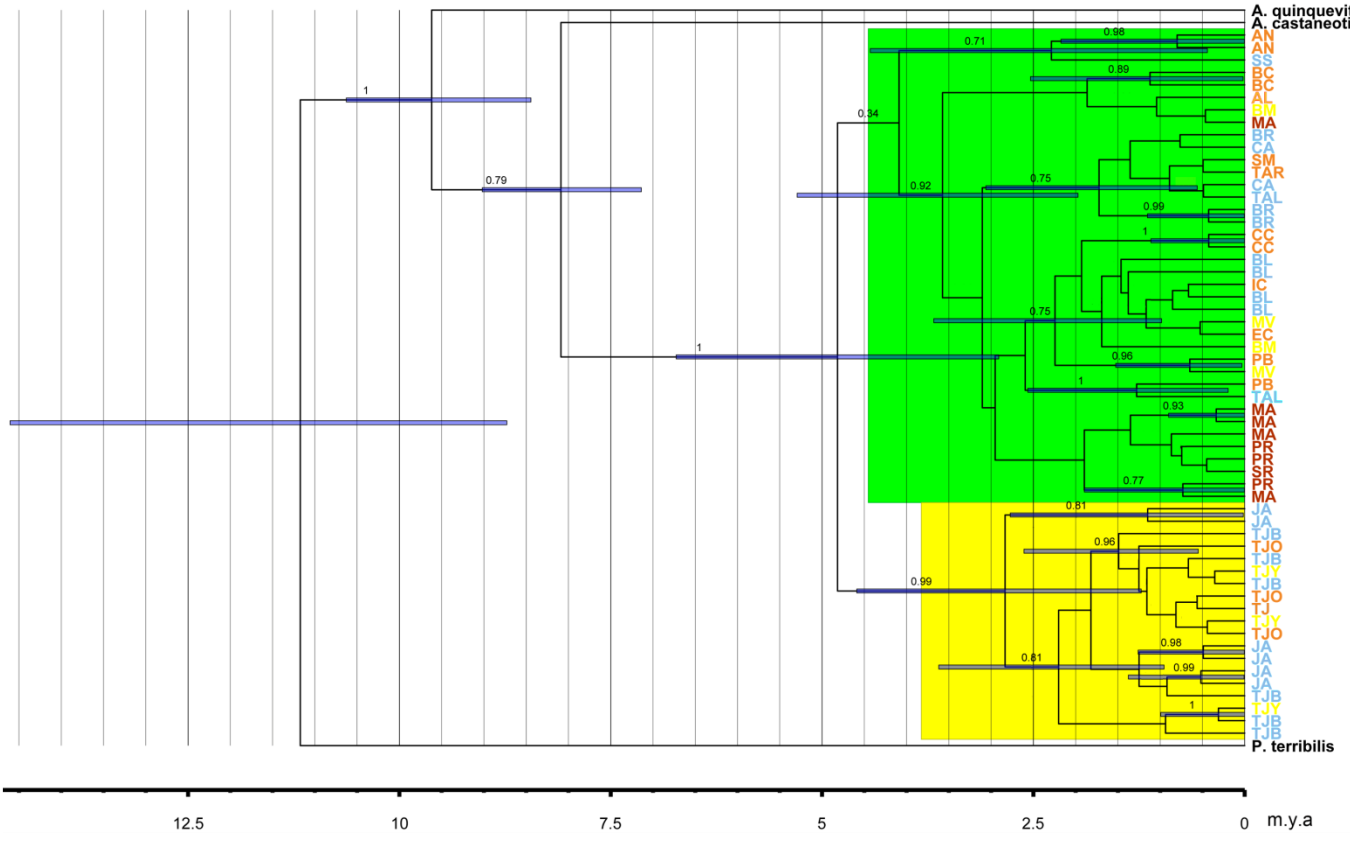

Figure 4. Time calibrated phylogenetic tree obtained via BEAST analysis based on fragments of the 16S rDNA and COI mitochondrial genes. *Adelphobates galactonotus* samples belong to two reciprocally monophyletic clades, corresponding to samples collected on opposite sides of the Xingu River (green and yellow shadows correspond to the east and west sites of Xingu River, respectively). Error bars on nodes represent the 95% highest posterior density interval of node ages. Values on branches stand for the posterior probability of nodes (only probabilities above 70% are shown). Tips are colored according to visually-based color in life of *A. galactonotus* specimens from which sequences derived (blue, orange, yellow, brown). Grey represents samples obtained from specimens of unknown color. Black tips correspond to outgroup sequences.

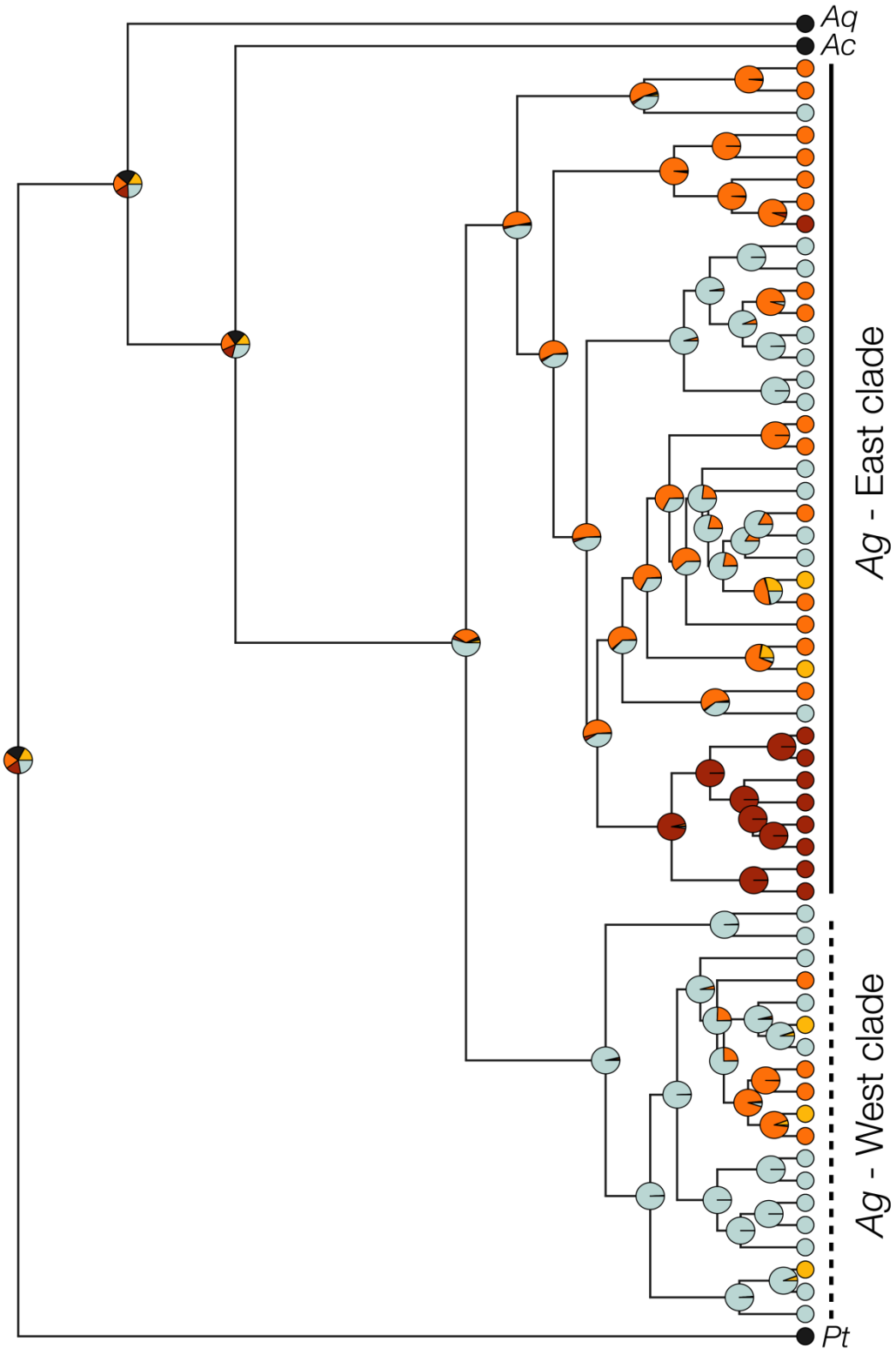

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Figure 5. Reconstruction of ancestral coloration in *Adelphobates galactonotus* as represented by four major functionality distinguishable colour categories: blue, yellow, orange, and brown. Black denotes the generally dark dorsum of ougroup species: *A. castaneoticus* (*Ac*), *A. quinquevittatus* (*Aq*) and the ancestor of the genus *Phyllobates* (*Pt*, see Methods). Pie charts at every node denote the Bayesian posterior probabilities for each colour state, empirically obtained from a continuous-time Markov model.

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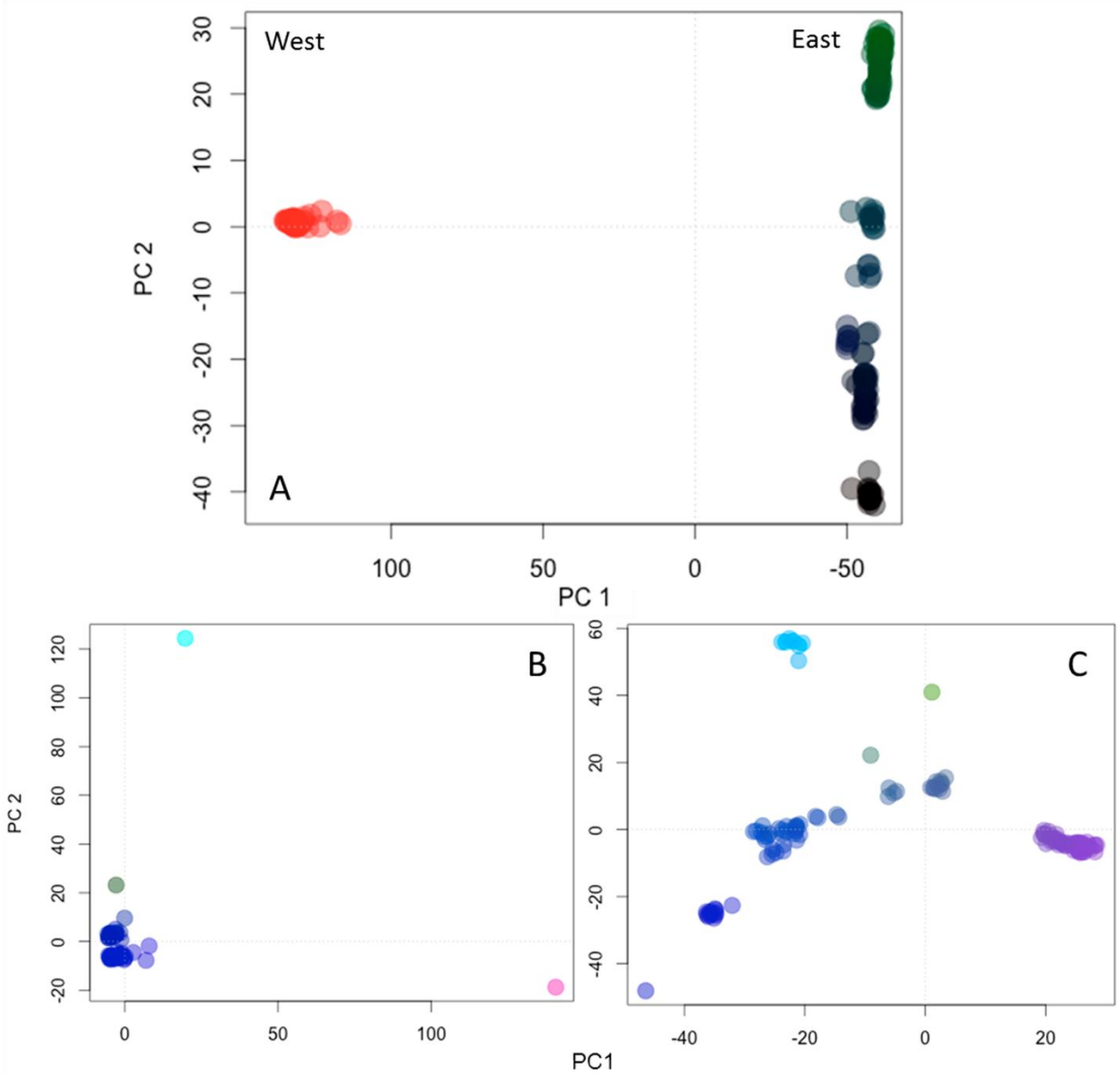

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Figure 6. Principal Component Analysis (PCA) using the genetic distances based on 5,872 SNPs. (A) PCA including all 186 samples showing two divergent genetic groups located on opposite sides of the Xingu River. (B) PCA including only samples collected on the west of the Xingu River. (C) PCA of the samples obtained from east of the Xingu River. Dot colors varies accordingly with genetic distances (similar colors for genetically closer specimens).

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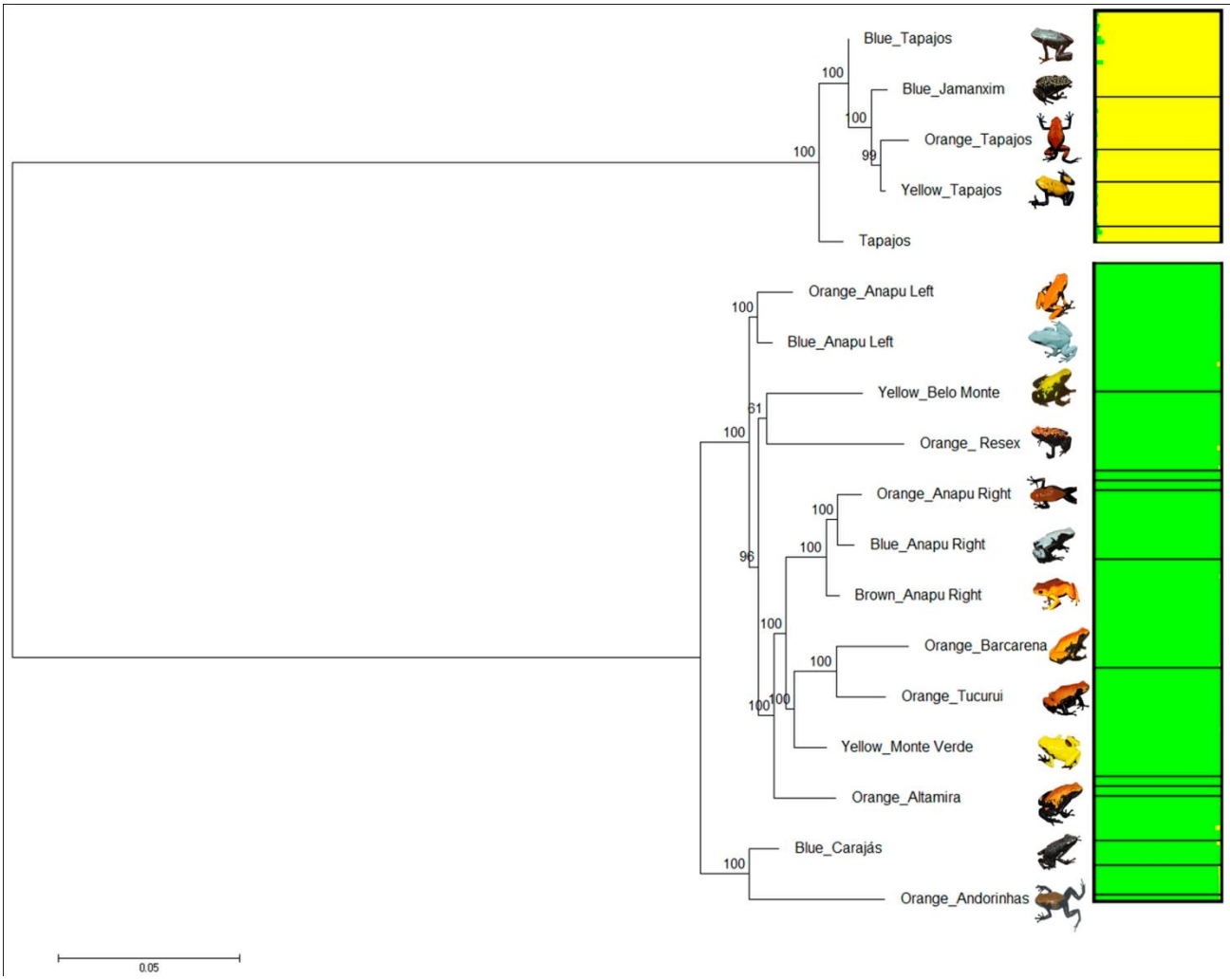

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Figure 7. Neighbor-Joining tree, using the genetic distances based on 186 samples and grouped by locality (tips). Bootstrap support is located at the nodes. Bar plot of membership coefficients obtained in STRUCTURE. Green and yellow colors of bar plot correspond to the west and east sites of Xingu River, respectively.

Supplementary Material:

A colors burst: the evolution of polymorphism in the warning coloration of the Amazonian poison frog *Adelphobates galactonotus*
Rojas et al.

Table S1. Accession number of haplotypes identified and samples sharing each haplotype

COI				16S			
Haplotype	Accession number	Genbank voucher	Sharing samples	Haplotype	Accession number	Genbank voucher	Sharing samples
Hap 1	KU597840	INPA-H035619	PJA258, PJA584, PJC264, PJC269, PJC275, PJC278, INPA-H035585, INPA-H035589, INPA-H035579, PJC086, PCJ114, PJC290, PJC421, PJC536, PJD174, PJD339,PJD367, PJD402, PJC550, APL20581, INPA-H035653, PJA302, PJC287, PJD135, PJD126, PJD125, PJD627, APL20575, APL20579, APL20582, APL20584, PJA261, PJA559, APL20524 MPEG24600, INPA-H035578, INPA-H035637, INPA-H035571, INPA-H035582, INPA-H035622, INPA-H035577, INPA-H035678, INPA-H035672, INPA-H035680, INPA-H035649, INPA-H035681, INPA-H035677, INPA-H035650, INPA-H03535660, INPA-H035683, INPA-H035584, INPA-H035676, INPA-H035629, INPA-H035616	Hap 1	KU597806	INPA-H035653	APL20538, APL20539, APL20577, MPEG34502, MPEG34503, PJC421, PJC422, INPA-H035589, PJD164, PJD174, PJD627, INPA-H035579
Hap 2	KU597841	INPA-H035590		Hap 2	KU597807	INPA-H035631	PJA302, PJA308, PJA559, INPA-H035600, INPA-035633, INPA-H035598, PJC203, PJC278, PJC114, PJC264, PJC269, PJC287, PJC289, INPA-H035585, PJD135, PJD126, PJD125, PJD339, PJD367, PJD402, PJD428, PJC550, PV3430, PJA258, PJA261, PJA289
Hap 3	KU597842	INPA-H035638	APL20543, MPEG34502, APL20514, APL20515, APL20540	Hap 3	KU597808	PJA579	PJC086
Hap 4	KU597843	INPA-H035598	PJC289, PJA289, PJA308, PJA570	Hap 4	KU597809	PJA584	PJC275
Hap 5	KU597844	PJD422	APL20577	Hap 5	KU597810	INPA-H035661	INPA-H035627, INPA-H035658, INPA-H035632, APL19250, INPA-H035625, INPA-H035580, INPA-H035626, INPA-H035681, INPA-H035677, INPA-H035586, INPA-H035592, INPA-H035650, INPA-H035679, INPA-H035656, INPA-H035660,

							INPA-H035630, INPA-H035609, INPA-H035584, INPA-H035570, INPA-H035655, INPA-H035676, INPA-H035647, INPA-H035629, APL19853, INPA-H035616, INPA-H035605, INPA-H035659, INPA-H035634, ICB33, MPEG22716, MPEG33757, MPEG34584, MPEG34589, MPEG34591, MPEG34592, MPEG34594, MPEG34595, , MPEG34601, INPA-H035574, INPA-H035573, INPA-H035615, INPA-H035654, INPA-H035673, INPA-H035608, INPA-H035606, INPA-H035665, MPEG22713, MPEG22714, MPEG34588, APL13487, APL13490, INPA-H035684, INPA-H035590, INPA-H035601, INPA-H035642, INPA-H035576, INPA-H035651, INPA-H035672, INPA-H035637, INPA-H035571, INPA-H035582, INPA-H035622, INPA-H035623, INPA-H035597, INPA-H035682, INPA-H035680, INPA-H035649, APL19245, APL19248, INPA-H035674, INPA-H035603, INPA-H035646, INPA-H035614, INPA-H035807, MPEG34500, MPEG25197, MPEG25198, INPA-H035610, MPEG22731, MPEG22732, MPEG22733, MPEG22735, MPEG22730, INPA-H035678, MPEG34609, MPEG34611, MPEG34619, MPEG34600, INPA-H035588, INPA-H035640, MPEG34613, MPEG34607
Hap 6	KU597845	PJD164		Hap 6	KU597811	INPA-H035602	MPPEG34608
Hap 7	KU597846	PJD428		Hap 7	KU597812	INPA-H035636	
Hap 8	KU597847	PV3430		Hap 8	KU597813	INPA-H035669	
Hap 9	KU597848	MPEG24588	MPEG24595	Hap 9	KU597814	INPA-H035683	
Hap 10	KU597849	INPA-H035652	INPA-H035624, INPA-H035643	Hap 10	KU597815	APL19849	MPEG31796
Hap 11	KU597850	INPA-H035662		Hap 11	KU597816	INPA-H035628	
Hap 12	KU597851	MPEG33757	MPEG33758	Hap 12	KU597817	INPA-H035671	
Hap 13	KU597852	MPEG25197	MPEG31796, MPEG34500, APL19250, INPA-H035630, APL1853	Hap 13	KU597818	INPA-H035675	
Hap 14	KU597853	MPEG25198		Hap 14	KU597819	APL20515	APL20525
Hap 15	KU597854	MPEG34623		Hap 15	KU597820	APL20524	
Hap 16	KU597855	MPEG34622		Hap 16	KU597821	APL20540	

Hap 17	KU597856	INPA-H035611	INPA-H035597, INPA-H035682, APL21111, INPA-H035625, INPA-H035675, INPA-H035684, INPA-H035601	Hap 17	KU597822	APL20542	
Hap 18	KU597857	INPA-H035807		Hap 18	KU597823	INPA-H035619	
Hap 19	KU597858	INPA-H035631	APL20523	Hap 19	KU597824	INPA-H035574	
Hap 20	KU597859	MPEG22730	MPEG22731, MPEG22732, MPEG22733, MPEG22735	Hap 20	KU597825	APL20611	
Hap 21	KU597860	INPA-H035587	APL20611	Hap 21	KU597826	INPA-H035587	MPEG34622, MPEG34623
Hap 22	KU597861	INPA-H035602	INPA-H035576, INPA-H035651, MPEG34584	Hap 22	KU597827	MPEG22715	
Hap 23	KU597862	INPA-H035642		Hap 23	KU597828	MPEG22717	
Hap 24	KU597863	INPA-H035674		Hap 24	KU597829	MPEG24595	
Hap 25	KU597864	INPA-H035603		Hap 25	KU597830	MPEG34602	
Hap 26	KU597865	INPA-H035610	INPA-H035626, INPA-H035628, INPA-H035586, INPA-H035570, INPA-H035655, APL19849, INPA-H035634	Hap 26	KU597831	MPEG34605	
Hap 27	KU597866	APL19248		Hap 27	KU597832	MPEG34615	
Hap 28	KU597867	APL19245		Hap 28	KU597833	INPA-H035613	MPEG34588
Hap 29	KU597868	INPA-H035679		Hap 29	KU597834	APL13455	
Hap 30	KU597869	INPA-H035647		Hap 30	KU597835	INPA-H035578	
Hap 31	KU597870	INPA-H035656		Hap 31	KU597836	INPA-H035577	
Hap 32	KU597871	MPEG34503		Hap 32	KU597837	MPEG33758	
Hap 33	KU597872	APL20525		Hap 33	KU597838	INPA-H035652	INPA-H035624, INPA-H035643, INPA-H035662
Hap 34	KU597873	APL20538	APL20541, APL20542	Hap 34	KU597839	MPEG34612	

Table S2. Mitochondrial COI and 16S rDNA genetic diversity estimates for Adelphobates galactonotus

Locality	COI							16S						
	n	nH	Hd	π	*S*	θ_π	θ_S	*n*	nH	Hd	π	*S*	θ_π	θ_S
Study area (all samples)	133	34	0.890±0.018	0.00646±0.00027	35	0.00652	0.01191	176	34	0.693±0.035	0.00639±0.00096	42	0.00644	0.01953
Estação Científica Ferreira Pena								7	3	0.524±0.209	0.00499±0.00290	7	0.00502	0.00721
FLONA Caxiuanã - ICMBio Station	8	1	0.000±0.000	0.00000±0.00000	0	0	0	13	6	0.641±0.150	0.01035±0.00540	21	0.01050	0.01750
FLONA Caxiuanã -Muju								3	2	0.667±0.314	0.00166±0.00078	1	0.00167	0.00167
FLONA Caxiuanã - PPBio	3	2	0.667±0.314	0.00239±0.00112	2	0.00239	0.00239	3	2	0.667±0.314	0.03980±0.01876	24	0.04203	0.04223
FLONA Caxiuanã - Cacoajo	4	2	0.500±0.265	0.00089±0.00047	1	0.00090	0.00098	5	2	0.400±0.237	0.00100±0.00059	1	0.00100	0.00120
Bacuri Village								6	3	0.600±0.215	0.00948±0.00332	9	0.00960	0.00999
Santa Maria	3	3	1.000±0.272	0.00358±0.00126	3	0.00359	0.00360	4	1	0.000±0.000	0.00000±0.00000	0	0	0
Taperu Right	2	1	0.000±0.000	0.00000±0.00000	0	0	0	3	1	0.000±0.000	0.00000±0.00000	0	0	0
Taperu Left	4	2	0.667±0.204	0.00239±0.00073	2	0.00239	0.00196	4	2	0.500±0.265	0.02494±0.01323	20	0.02580	0.02839
Brabo Village	8	3	0.679±0.122	0.00147±0.00037	2	0.00147	0.00138	7	2	0.286±0.196	0.00071±0.00049	1	0.00071	0.00102
Mojua River								7	2	0.286±0.196	0.00071±0.00049	1	0.00071	0.00102
Cacoal, Atua River right bank	2	2	1.000±0.500	0.00537±0.00268	3	0.00541	0.00541	4	2	0.500±0.265	0.00124±0.00066	1	0.00125	0.00136
São Raimundo	2	2	1.000±0.500	0.00358±0.00179	2	0.00359	0.00359	4	2	0.500±0.265	0.00873±0.00463	7	0.00883	0.00966
Santo Amaro Village								2	1	0.000±0.000	0.00000±0.00000	0	0	0
Prainha Village	4	3	0.833±0.222	0.00179±0.00061	2	0.00179	0.00196	6	2	0.333±0.215	0.00166±0.00107	2	0.00167	0.00219
Angelim, Jacitara, Marapiranga	9	4	0.694±0.147	0.00259±0.00051	3	0.00260	0.00199	11	2	0.182±0.144	0.00045±0.00036	1	0.00045	0.00085
Pracupi River and Anapu River south FLONA Caxiuanã	10	2	0.200±0.154	0.00072±0.00055	2	0.00072	0.00127	10	2	0.200±0.154	0.00050±0.00038	1	0.00050	0.00088
FLONA Carajás	2	1	0.000±0.000	0.00000±0.00000	0	0	0	2	2	1.000±0.500	0.00249±0.00125	1	0.00250	0.00250
Vila dos Cabanos	2	1	0.000±0.000	0.00000±0.00000	0	0	0	2	2	1.000±0.500	0.00498±0.00249	2	0.00501	0.00501
Fazenda Riacho Monte Verde	5	1	0.000±0.000	0.00000±0.00000	0	0	0	10	3	0.378±0.181	0.00813±0.00372	10	0.00822	0.00894
Porto de Moz								2	1	0.000±0.000	0.00000±0.00000	0	0	0
Caracol, UHE Belo Monte	2	2	1.000±0.500	0.00537±0.00268	3	0.00541	0.00541	2	1	0.000±0.000	0.00000±0.00000	0	0	0
Cachoeira do Espelho								3	2	0.667±0.314	0.00166±0.00078	1	0.00166	0.00166
Serra das Andorinhas	2	2	1.000±0.500	0.00179±0.00089	1	0.00179	0.00179	2	1	0.000±0.000	0.00000±0.00000	0	0	0
Tapajós River	6	1	0.000±0.000	0.00000±0.00000	0	0	0	6	2	0.333±0.215	0.00083±0.00054	1	0.00083	0.00109
Tapajós MU	11	2	0.182±0.144	0.00033±0.00026	1	0.00033	0.00061	12	3	0.439±0.158	0.00325±0.00200	7	0.00326	0.00585
Boca do Rato	8	4	0.750±0.139	0.00192±0.00047	3	0.00193	0.00208	7	4	0.714±0.181	0.00213±0.00071	3	0.00214	0.00306
Betel	22	6	0.411±0.131	0.00081±0.00029	5	0.00081	0.00247	17	3	0.581±0.068	0.00157±0.00027	2	0.00158	0.00148
Mina do Palito	7	3	0.714±0.127	0.00256±0.00052	3	0.00256	0.00220	6	3	0.600±0.215	0.00166±0.00069	2	0.00166	0.00219
Jamanxim river left bank	5	4	0.900±0161	0.00250±0.00057	3	0.00251	0.00259	3	2	0.667±0.314	0.00332±0.00156	2	0.00333	0.00333
Projeto ALPA	2	1	0.000±0.001	0.00000±0.00000	0	0	0	3	2	0.667±0.314	0.00166±0.00078	1	0.00166	0.00166

East side of Xingu river	74	23	0.888±0.025	0.00486±0.00041	26	0.00489	0.00981	126	24	0.443±0.057	0.00457±0.00129	37	0.00460	0.01815
West side of Xingu river	59	11	0.614±0.070	0.00138±0.00021	10	0.00139	0.00390	51	10	0.661± 0.058	0.00271±0.00061	14	0.00272	0.00792

Estimates are presented for all samples pooled, separately for each sampling site, and for each side of Xingu river

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 S3. Results of outlier analysis using Bayescan (Luikart et al. 2011) for 16 color associated SNPs lidentified using LFMM (Frichot et al. 2015). Shown are Locus ID, qval (indicator of false discovery rate, FDR), log10(qval), and Fst for each locus. The top two loci are under positive selection and the remainder are under balancing selection, as plotted in Table S1. The log10(qval) and Fst values here are equivalent to those presented in Figure S1 below, for reference.

Locus ID	qval	log10(qval)	Fst
2768	0.00058273	-3.2345	**0.81239**
4024	0.012246	-1.9136	**0.77087**
726	0.044564	-1.351	0.40448
2553	0.024675	-1.609	0.35494
1322	0.0035914	-2.4449	0.31325
3547	0.0065811	-2.1817	0.30626
66	0.00039447	-3.4045	0.28609
6796	0.00039447	-3.4089	0.28359
4469	0.0016972	-2.77211	0.27362
4955	0.00011688	-3.9586	0.25057
4119	0.00032507	-3.4881	0.2355
6046	0	0	0.22653
7157	0.00010117	-3.9956	0.22423
2436	0	0	0.22228
4194	0.00015487	-3.8239	0.21891
5842	0.00015487	-3.81	0.20437

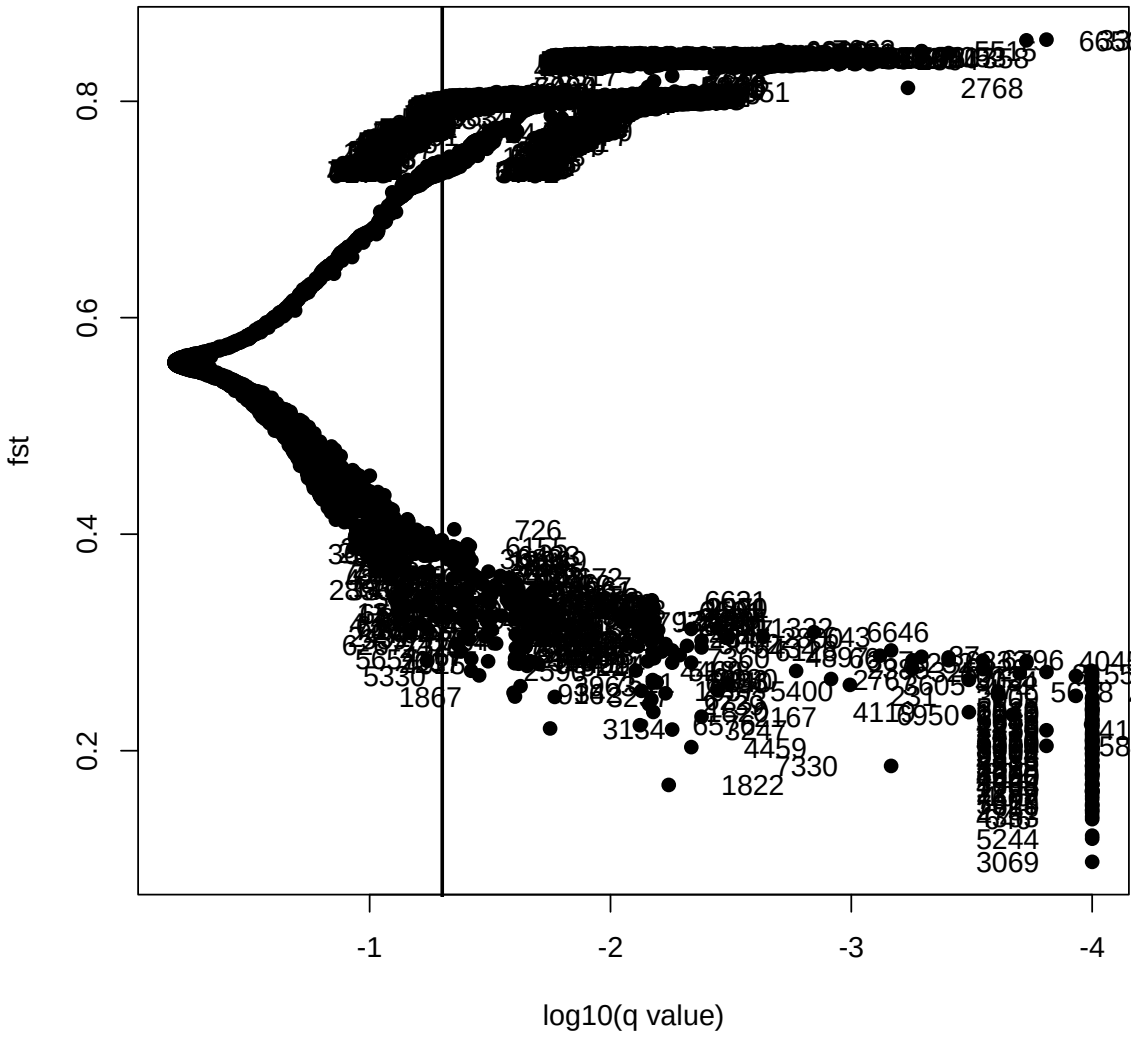

Figure S1. Plot produced by plot.bayescan function in the R statistical software environment showing outlier loci detected using the program Bayescan (Luikart *et al.* 2003) for *Adelphobates galactonotus*. Data were run using 7693 SNP loci divided into 12 populations. SNP loci are split at approximately Fst = 0.55 (x axis) in to upper (potentially postitive selection) and lower (potentially balancing selection) components. The Y-axis shows the log10(qvalue) which indicates the False Discovery Rate (FDR) associated with each SNP test. The black line indicates the implemented FDR cut-off of 0.05 applied within the analysis.

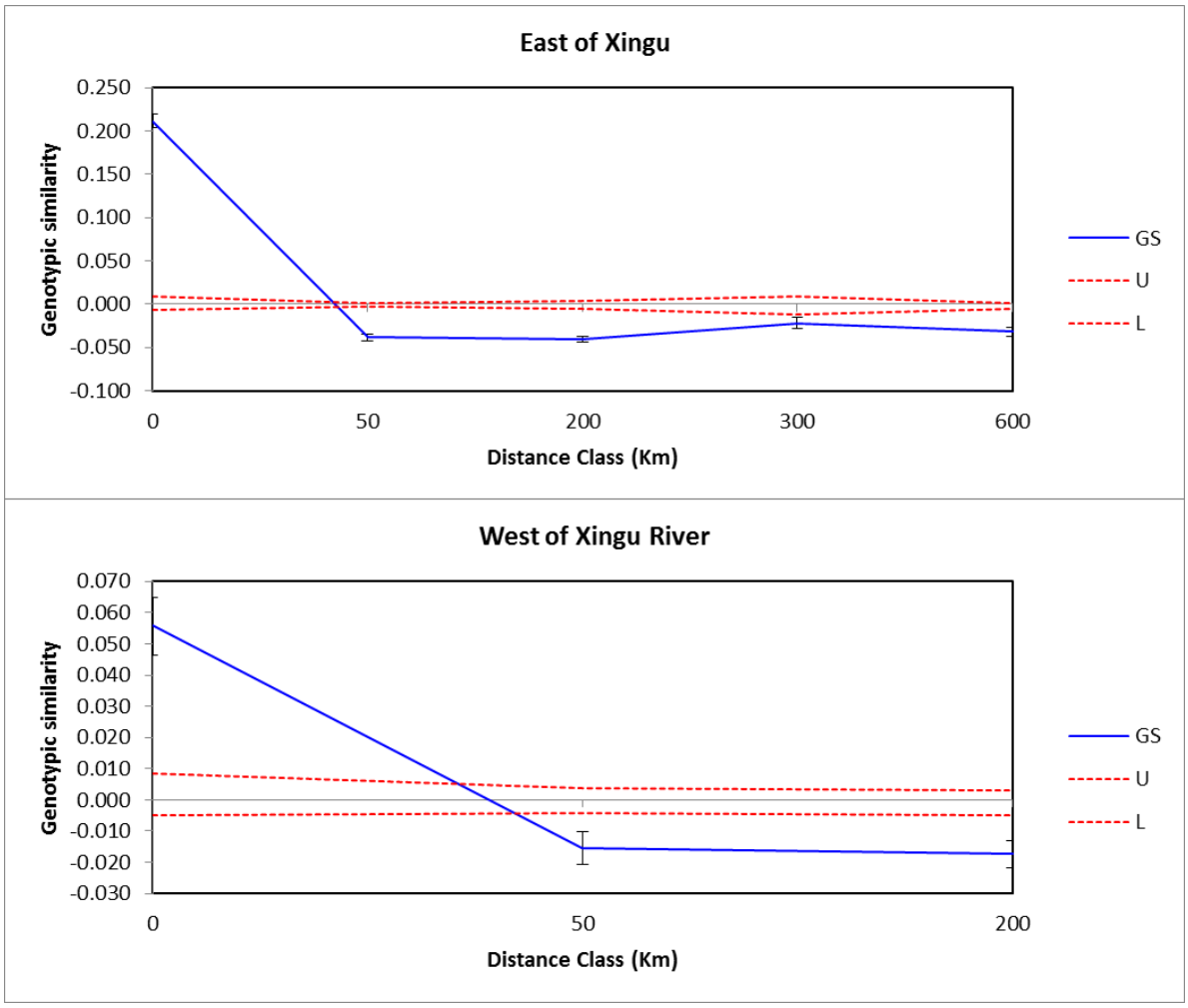

Figure S2. Spatial autocorrelation between genetic and geographic distances (A) among samples collected (A) east and (B) west of the Xingu River, showing no effect of geographic distance on the relatedness (genotypic similarity-GS) on both sides of the River. Red lines refer to upper (U) and lower (L) 95% confidence intervals around a random distribution.

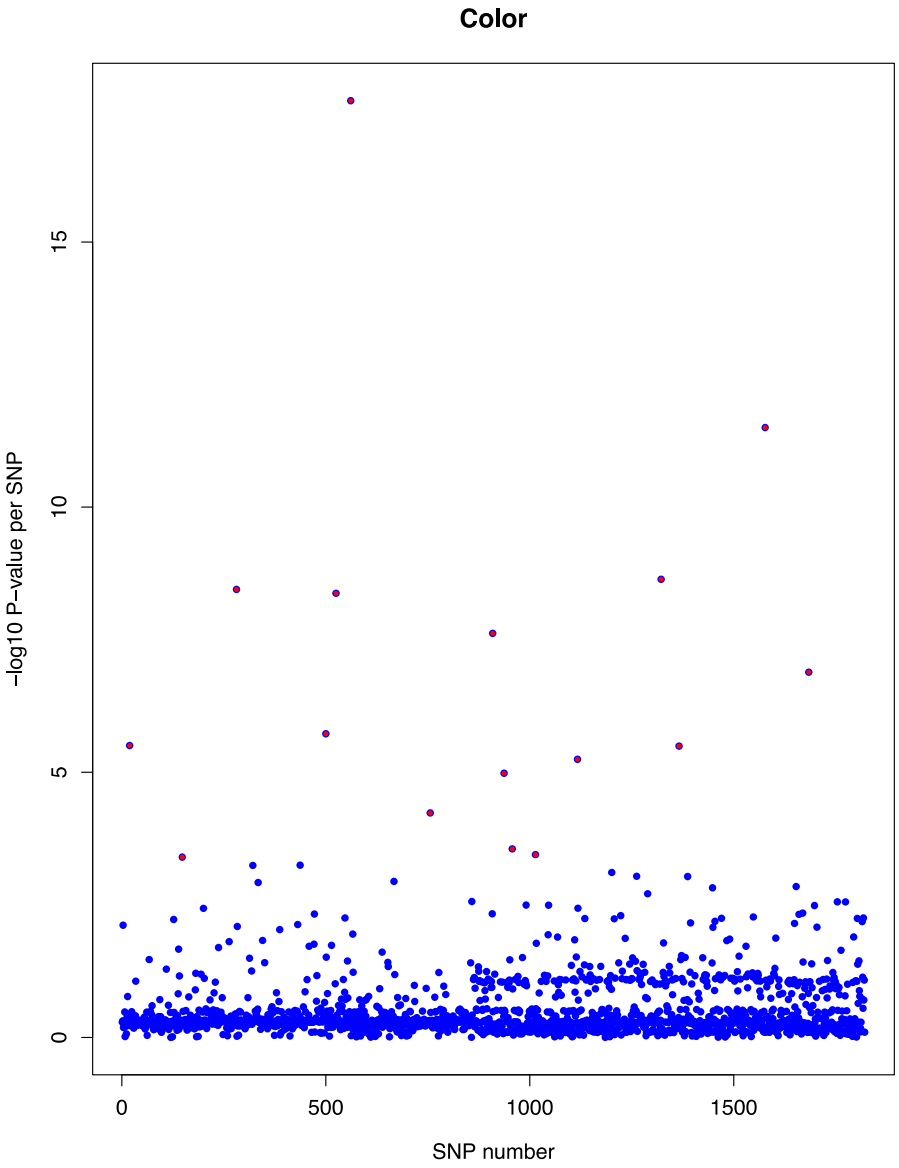

Figure S3. Manhatten plot of SNP x color morphotype analysis done using latent factor mixed modeling (implemented in LFMM software; Frichot et al. 2015) in *Adelphobates galactonotus*. SNP number refers to SNP ID (N= 1821) on the x-axis. The y-axis shows the log10 P-value for each SNP x color association test. Loci with high probability of being associated with color are shown as red dots (FDR < 0.5%). Blue dots show all other loci that were not significantly associating with color morphotype

Síntese

Apesar da variação de coloração intraespecífica ser frequente em diferentes taxa, os processos que a geram são pouco entendidos, pois não existe um mecanismo único que explique os padrões observados, e sim resultados variados e algumas vezes conflitantes para espécies relacionadas. Os resultados apresentados nos capítulos desta tese mostram que a analise individual de possíveis processos direcionando a variação de cor não necessariamente permitem chegar a uma resposta unânime da origem dos padrões de coloração atuais.

No primeiro capítulo, onde investigamos a predação como possível mecanismo direcionador da variação e distribuição das cores em *Adelphobates galactonotus*, nossos resultados foram diferentes ao esperado pela teoria, que preve novas formas coloridas em uma localidade são mais susceptíveis a sofrer ataques por predadores visualmente orientados. Assim, a variação de cor em *A. galactonotus* não é explicada por predação, pelo menos atualmente, nas localidades onde foram realizados os experimentos. Considerando a cor marrom como uma cor menos conspícua, era esperado que sofresse mais ataques do que as formas coloridas, como em outros estudos semelhantes. Essa falta de ataques sobre os modelos marrom pode ser associada a cripticismo parcial, dificultando sua a detecção visual pelos predadores; e também pela presença de outros anuros crípticos e venenosos fazendo que potenciais predadores reconheçam todas as cores como impalatáveis. Já que a predação, como força direcionadora da variação de cor, não pode ser totalmente descartada, é plausível que as cores em *A. galactonotus* tenham evoluido em condições diferentes às atuais, e estejam sendo mantidas por seleção sexual.

Entre outros possíveis fatores que podem afetar a distribuição das cores, assim como a efetividade do sinal aposemático, encontra-se a abundância de predadores (aves) e a composição da comunidade de predadores, que geram mosaicos geográficos de seleção. Processos históricos também podem contribuir na acumulação de mutações genéticas,

rearranjo de cromossomos e deriva genética. Assim, teorias como a dos refúgios são plausíveis para a divergência genética, que deve reforçar o isolamento por inviabilidade de híbridos em zonas de contato secundárias e inclusive, manter o polimorfismo de cor através do deslocamento de caracteres.

A abordagem experimental com modelos para explorar a predação como força direcionadora da variação da cor, não tem mostrado um padrão consistente entre as espécies. O que pode ser atribuído, em parte, a limitações metodológicas, e também porque em ambientes complexos, esse tipo de abordagem experimental, expõe os modelos a predadores cautelosos.

Ferramentas genéticas permitem explorar possíveis mecanismos relacionados a variação de cor nas espécies. A coloração de *A. galactonotus*, não exibe um padrão de variação geográfico e cores diferentes não ocorrem em uma mesma localidade. No segundo capitulo, usamos dois genes mitocondriais e marcadores moleculares do tipo SNPs, para explorar essa variação de cor em *A. galactonotus*. Nossos resultados mostraram uma rápida diversificação das cores, ocorrendo provavelmente durante o Pleistoceno. Os grandes rios tem se mostrado importantes na diferenciação intraespecífica para vários anuros amazônicos. Para *A. galactonotus* não foi diferente, pois o padrão geográfico mais evidente foi uma forte partição genética entre os indivíduos amostrados de cada lado do Rio Xingu, apontando o rio como uma barreira a dispersão, porem não relacionada com a variação de cor. A ausência de padrão geográfico na distribuição das cores em *A. galactonotus* e processos de divergência genética ocorrendo nos últimos 2.5 milhões de anos sugerem que a divergência de cores ocorreu isoladamente em diferentes partes da distribuição da espécie.

Mecanismos como mutações genéticas ocorrendo de forma isolada em diferentes localidades, isolamento, deriva, seleção sexual, podem estar relacionadas também ao processo de fixação das cores ao longo da distribuição de *A. galactonotus*. É possível que após a

divergência das cores de forma isolada, essa tenha sido reforçada por seleção sexual, através do baixo desempenho dos híbridos nas zonas de contacto.de contato secundário entre cores diferentes direcionado por híbridos com baixo desempenho. A seleção sexual que atua para reforçar o isolamento reprodutivo aparece como um potencial mecanismo na fixação das cores e geração de biodiversidade. Mas faz-se necessário conhecimento dos processos que resultam em variação espacial de uma característica para testar se o polimorfismo é um precursor da especiação. As características do nossa espécie modelo de estudo, a rápida diversificação e manutenção de diferentes cores em localidades próximas, proporcionam uma oportunidade para investigar mais a fundo estes mecanismos.

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ANEXOS

PG-ECO-INPA
PÓS GRADUAÇÃO EM ECOLOGIA

INPA
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GOVERNO FEDERAL
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AULA DE QUALIFICAÇÃO

PARECER

Aluno(a): DIANA PATRICIA ROJAS-AHUMADA
Curso: ECOLOGIA
Nível: DOUTORADO
Orientador(a): ALBERTINA PIMENTEL LIMA
Co-orientador(a): PEDRO IVO SIMÕES

Título:

"Usando uma espécie de sapo colorido e venenoso como modelo para explorar processos que geram variação em cor em dendrobatidae na Amazônia oriental"

BANCA JULGADORA:

TITULARES:
Marina Anciães (INPA)
Mario Cohn-Haft (INPA)
Jansen Alfredo Sampaio Zuanon (INPA)
Igor Luis Kaefer (INPA/CENBAM)
Carlos Gustavo Nunes da Silva (UFAM)

SUPLENTES:
Maria Claudia Gross (UFAM)
Paulo E. Dinelli Bobrowiec (INPA/CENBAM)

	PARECER		ASSINATURA
Marina Anciães (INPA)	(A) Aprovado	( ) Reprovado	
Mario Cohn-Haft (INPA)	( ) Aprovado	( ) Reprovado	
Jansen Alfredo Sampaio Zuanon (INPA)	( ) Aprovado	(X) Reprovado	
Igor Luis Kaefer (INPA/CENBAM)	(X) Aprovado	( ) Reprovado	
Carlos Gustavo Nunes da Silva (UFAM)	(X) Aprovado	( ) Reprovado	
Maria Claudia Gross (UFAM)	( ) Aprovado	( ) Reprovado	
Paulo E. Dinelli Bobrowiec (INPA/CENBAM)	(X) Aprovado	( ) Reprovado	

Manaus(AM), 22 de novembro de 2012

OBS: A banca recomenda que a aluna aprofunde o seu embasamento teórico sobre o tema da tese, e especialmente uma definição clara das premissas e hipóteses referentes aos capítulos da tese.

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BRASIL

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA DO INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA

Aos 30 dias do mês de setembro do ano de 2016, às 14:00 horas, no Auditório dos PPG`s ATU/CFT/ECO, Campus III, INPA/V8. Reuniu-se a Comissão Examinadora de Defesa Pública, composta pelos seguintes membros: o(a) Prof(a). Dr(a). **Marina Anciães**, do Instituto Nacional de Pesquisas da Amazônia - INPA, o(a) Prof(a). Dr(a). **Luiza Magalli Pinto Henriques,** do Instituto Nacional de Pesquisas da Amazônia - INPA, o(a) Prof(a). Dr(a). **Fernanda de Pinho Werneck**, do Instituto Nacional de Pesquisas da Amazônia - INPA, o(a) Prof(a). Dr(a) **Luis Felipe de Toledo Ramos Pereira**, da Universidade Estadual de Campinas - UNICAMP, o (a) Prof(a). Dr(a). **Sergio Henrique Borges**, da Universidade Federal do Amazonas – UFAM, sob a presidência do(a) primeiro(a), a fim de proceder a argüição pública do trabalho de **TESE DE DOUTORADO** de **DIANA PATRICIA ROJAS AHUMADA**, intitulado **"Usando Adelphobates galactonotus, uma espécie de sapo colorido e venenoso, como modelo para explorar processos que geram variação em cor em Dendrobatídeos na Amazônia Oriental"**, orientado pelo(a) Prof(a). Dr(a). Albertina Pimentel Lima, do Instituto Nacional de Pesquisas da Amazônia - INPA.

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

Nada mais havendo, foi lavrada a presente ata, que, após lida e aprovada, foi assinada pelos membros da Comissão Examinadora.

Prof(a).Dr(a). Marina Anciães

Prof(a).Dr(a). **Luiza Magalli Pinto Henriques**

Prof(a).Dr(a). Fernanda de Pinho Werneck

Prof(a).Dr(a). Luis Felipe de Toledo Ramos Pereira

Prof(a).Dr(a). Sérgio Henrique Borges

Coordenação PPG-ECO/INPA