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

ECOLOGIA, GENÔMICA DE PAISAGEM E STATUS TAXONÔMICO DO SAPO *ATELOPUS MANAUENSIS* (BUFONIDAE): UMA ABORDAGEM MULTIDISCIPLINAR APLICADA A CONSERVAÇÃO DE UMA ESPÉCIE EM RISCO DE EXTINÇÃO

RAFAEL FILGUEIRA JORGE

Manaus – Amazonas – Brasil
Agosto, 2020

RAFAEL FILGUEIRA JORGE

ECOLOGIA, GENÔMICA DE PAISAGEM E STATUS TAXONÔMICO DO SAPO *ATELOPUS MANAUENSIS* (BUFONIDAE): UMA ABORDAGEM MULTIDISCIPLINAR APLICADA A CONSERVAÇÃO DE UMA ESPÉCIE EM RISCO DE EXTINÇÃO

ORIENTADORA: Dra. ALBERTINA PIMENTEL LIMA

Tese apresentada ao Instituto Nacional de Pesquisas da Amazônia como requisito para obtenção do título de Doutor em Biologia (Ecologia).

Manaus – Amazonas – Brasil
Agosto, 2020

BANCA EXAMINADORA DO TEXTO E DA DEFESA ORAL PÚBLICA DA TESE

Dra. Cintia Cornelius Frisch
Universidade Federal do Amazonas (UFAM)

Dra. Fabiane Santana Annibale
Universidade Federal de Goiás (UFG)

Dra. Fernanda de Pinho Werneck
Instituto Nacional de Pesquisas da Amazônia (INPA)

Dra. Luciana Bolsoni Lourenço
Universidade Estadual de Campinas (UNICAMP)

Dr. Luís Felipe de Toledo Ramos Pereira
Universidade Estadual de Campinas (UNICAMP)

Aprovado por unanimidade

j82e Jorge, Rafael Filgueira
Ecologia, genômica de paisagem e status taxonômico do sapo Atelopus manauensis (Bufonidae): uma abordagem multidisciplinar aplicada a conservação de uma espécie em risco de extinção / Rafael Filgueira Jorge; orientador Albertina Pimentel Lima. -- Manaus:[s.l], 2020.
132 f.

Tese (Doutorado - Programa de Pós Graduação em Ecologia) -- Coordenação do Programa de Pós-Graduação, INPA, 2020.

1. Atelopus. 2. Ecologia. 3. Genômica de paisagem. 4. Taxonomia . 5. Conservação. I. Lima, Albertina Pimentel , orient. II. Título.

CDD: 598

Sinopse
Nesta tese foram utilizados dados moleculares, morfológicos e bioacústicos para definir o status taxonômico e nominar uma espécie candidata de *Atelopus* (Bufonidae) da região de Manaus. Foi investigada a influência da heterogeneidade ambiental nos limites geográficos, padrão de ocorrência e variação nas densidades de *Atelopus manauensis* para definir seu status de conservação. Por fim, foi utilizada uma abordagem de genômica de paisagem para investigar a influência da heterogeneidade ambiental na conectividade funcional e genética entre grupos genéticos de *Atelopus manauensis* para identificar possíveis ameaças que as mudanças ambientais antrópicas em andamento na área de ocorrência da espécie podem trazer para sua conservação, e assim propor medidas para evitar o isolamento dos grupos genéticos que compõe a espécie estudada.

Palavras-chave: 1. *Atelopus* 2. Taxonomia integrativa 3. Ecologia 4. Conservação 5. Adaptação local 6. Fluxo gênico 7. Amazônia central

Em homenagem ao meu pai, Marcos Jorge (in memoriam). Ele foi um grande exemplo de ética e dedicação à família, à sociedade e ao trabalho.

Dedico esta tese à minha mãe, Maria Elvira F. Jorge; ao meu irmão, Marcos Jorge Filho; e à minha amiga e companheira, Alessandra Cacela.

Agradecimentos

Eu agradeço a todos cidadãos e cidadãs brasileiros que financiam as atividades públicas de ensino, pesquisa e extensão do país, mesmo sem ver o retorno imediato. Espero que esta tese seja de alguma forma útil para melhorar a qualidade de vida da sociedade do Brasil.

Ao Instituto Nacional de Pesquisas da Amazônia (INPA) e ao Programa de Pós-Graduação em Ecologia (PPG-ECO) pela formação, estrutura e suporte institucional para o desenvolvimento dessa tese. Em especial, àqueles funcionários administrativos, técnicos e pesquisadores que lutam para manter a qualidade de ensino e pesquisa do instituto apesar das dificuldades.

Durante a coleta de dados e desenvolvimento desta tese recebi bolsa de estudos a nível de doutoramento nacional da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) e do Fundo de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) (edital nº 024/2014); taxa de bancada da CAPES via Programa de Excelência Acadêmica (PROEX – edital nº 0616/2018); e bolsa de estágio de doutoramento internacional da CAPES via Programa Doutorado Sanduíche no Exterior (PDSE – edital n° 41/2018). O projeto desta tese foi financiado pelo Conselho de Desenvolvimento Científico e Tecnológico (CNPq – edital nº: 401120/2016-3) concedido à Dra. Albertina P. Lima. Esses recursos foram fundamentais para desenvolvimento da presente tese.

À minha querida orientadora Albertina Pimentel Lima pela longa jornada ao meu lado desde o mestrado. Agradeço por ter acreditado em mim e no meu projeto, por ter sido tão dedicada e persistente no meu aprendizado e crescimento profissional e por todo conhecimento que vem compartilhando comigo ao longo desses anos.

Ao supervisor do estágio de doutorado no exterior Adam J. Stow pelo aceite em participar do meu projeto de tese e pela agradável recepção no Laboratório de Genética da Conservação na Macquarie University, em Sydney, Austrália. Sou grato pela contínua atenção e pelos grandiosos ensinamentos que vêm compartilhando comigo sobre genômica da conservação.

Aos avaliadores do projeto de pesquisa durante o processo seletivo do doutorado – Camila Cherem Ribas, Igor Luís Kaefer e Fernanda de Pinho Werneck – durante a aula de qualificação – Jansen Zuanon, Mario Cohn-Haft e Marina Anciães – e na versão escrita e defesa oral pública da tese – Cintia Cornelius, Fabiane Annibale, Felipe Toledo, Fernanda Werneck e Luciana Bolsoni – que contribuíram enormemente para o desenvolvimento desta tese e para o meu crescimento como pesquisador.

Ao Sr. Valzenir Albuquerque, que me ensinou muito sobre a fantástica floresta amazônica e seus povos durante minhas idas ao campo. Sou imensamente grato por ter vestido a camisa do meu projeto e se dedicado tanto em todas as nossas excursões.

Às dezenas de pessoas que me ajudaram durante as coletas de campo. Em especial, agradeço ao seu Zé (José da Silva Lopes), seu Sabá (Sebastião Sales), seu Edgar (“seu menino”), Letícia Melo (estudante de biologia da UFAM), Micaela e Raimundinho.

Devo um agradecimento especial ao Jabson Franco da Costa (*in memoriam*) que foi um grande amigo de Manaus e que me ajudou na excursão de campo mais árdua dessa jornada, na fazenda Rio Negro, Presidente Figueiredo. Você foi um exemplo de perseverança, alegria e superação.

Ao Marcelo Menin, Flávia R. C. Costa e Jansen Zuanon pelo empréstimo do pHmetro e esferodensiômetro. Foram enriquecedoras as breves trocas de ideias que tive com vocês.

Ao Niro Higuchi, José L. Camargo, Enrique Salazar, Gilmar Klein, Wagner V. de Sousa, Marcos Antônia S. Souza, Cristiano Gonçalves, à Tenente Sinandra dos S. Gomes e a todos os sargentos do Centro de Instruções de Guerra na Selva do Exército Brasileiro (CIGS/EB) pelas autorizações e apoio logístico na base LBA/ZF-2 e Estação Científica do ATTO, ARIE PDBFF, REBIO Uatumã, PARNA Anavilhanas, Mineração Taboca, Mil Madeireira, RDS Uatumã e área de treinamento do CIGS/EB, respectivamente.

À professora Dayse Aparecida da Silva da Universidade do Estado do Rio de Janeiro (UERJ) por ter aberto as portas do Laboratório de Diagnóstico por DNA (LDD) e fornecido toda a estrutura, materiais e conhecimentos necessários para a realização das minhas análises genéticas. Agradeço também os colegas do LDD que me guiaram pacientemente em todas etapas do processo de sequenciamento dos genes 16S e COI, em especial, Carolina Silva, Bruno Vieira e César Amaral.

À professora Dra. Cintia Cornelius e ao professor Dr. Tomas Hrbek por terem me supervisionado durante o estágio de docência nas disciplinas de Ecologia de Populações e Genética de Populações (UFAM), respectivamente, quando pude revisar e aprimorar meus conhecimentos nessas áreas do conhecimento que me fascinam.

Ao Bill Magnusson por ter aceitado em participar do segundo capítulo desta tese e com sua magnífica visão de cientista me ensinar a comunicar meus resultados. Obrigado por direta e indiretamente investir seu tempo no meu crescimento como pesquisador.

Ao Miquéias Ferrão por ter aceitado em participar do primeiro capítulo desta tese e contribuir para meu aprendizado em taxonomia.

Ao Anthony Ferreira e ao André Lima por me ajudarem nos experimentos de comunicação química de *Atelopus manauensis*. Em especial, sou enormemente grato ao Anthony Ferreira que me ajudou em diferentes etapas do meu doutorado.

À Marina Anciães e ao Fernando Teófilo pelo auxílio no uso do espectrofotômetro e pelos ensinamentos durante as análises e interpretações biológicas da variação de cores em animais. Agradeço especialmente ao Fernando, que me ajudou muito na medição de cores dos sapos.

À Andreza Melo e as meninas da secretaria adjunta dos PPGs-ECO/ATU/CFT do INPA, dona Valdecira, Jéssica, Raiza, Ana Serra e Marluce por me ajudarem sempre com a maior disposição a resolver as infinitas burocracias e pelos momentos de convivência no INPA.

Ao Dr. Erik Wild pela tradução e revisão do inglês do primeiro capítulo e pela revisão do inglês do abstract desta tese.

A todos pesquisadores/pesquisadoras, colaboradores/colaboradoras e alunos/alunas dos PPGs do INPA que me ensinaram muito durante as disciplinas que fiz ao longo do doutorado.

Aos participantes dos grupos de estudos Evolução e Biogeografia da Biota Amazônica (EBBA) e Biogeografia da Amazônia (BioGeoAm) pelas trocas de conhecimento durante as reuniões dos grupos que foram de grande importância para o desenvolvimento inicial desta tese.

Ao Laboratório de Análise Química Ambiental do INPA, em especial ao Sebastião Átila, Socorro, Aretusa e Luís por cordialmente analisar minhas amostras de água.

Ao Laboratório de Evolução e Genética Animal (LEGAL), em especial ao Érico Polo pelos ensinamentos em análises filogenéticas e biologia computacional.

Aos colegas do dia a dia no INPA V8: Paulinha, Rogério Hanada, Iza (Aleixo), Carol (Levis), Mari (Tolentino), Annelise, Elmo, Thiago (Bicudo), Lucas Bandeira, Elis, Sara, Alex, Kelly e Lucas Ferrante. Com certeza os dias foram mais descontraídos tendo vocês por perto.

Aos amigos da Macquarie University com que pude aprender bastante: Laura Fernandez e Bruno Buzatto. Obrigado pelos momentos de alegria.

Ao casal de amigos neozelandeses Louie e Telesia Tupoumalohi que foram verdadeiros companheiros em Sydney e fizeram os momentos longe da minha família passarem mais rápidos. Obrigado pelas risadas, alegria e cuidado.

Aos meus companheiros de república: Carlos, Diana, Otávio, Rodolpho, Ju, Julliane, Victor e Zina pelos inúmeros momentos de descontração, trocas de ideias sempre produtivas e animadas. Agradeço também aos meus amigos de longa data de Manaus: Gustavo, Yuri, Mariana e Marquinho. Tempo bom!

Ao meu querido irmão Marcos Jorge Filho, que sempre me ensinou muito sobre os caminhos da vida e a ter muita coragem para levantar a cabeça e seguir adiante mais forte após decepções. Valeu, irmãozinho!

Agradeço minha querida e protetora mãezinha, Marial Elvira Filgueira Jorge, por incentivar e financiar meus estudos e apoiar todas minhas decisões, e sempre me mostrar o lado positivo dos acontecimentos da vida. Você, sempre guerreira, me inspira a cada dia mais. Agradeço a fantástica educação que me deu. Obrigado, minha rainha!

Agradeço especialmente minha esposa, Alessandra Suzely Moda Cacela. Obrigado pela paciência, companheirismo e força para estar junto comigo nessa longa jornada fora de casa. Desculpe pela ausência, aflições e, muitas vezes, desânimo. Admiro, você é demais, é uma ótima chefa de família, além de me ensinar muito sobre geologia.

Science:

“If you don’t make mistakes, you’re doing it wrong.

If you don’t correct those mistakes, you’re doing it really wrong.

If you can’t accept that you’re mistaken, you’re not doing it at all.”

- Anon

RESUMO

O interflúvio entre os rios Negro e Uatumã (INU) concentra as áreas de pesquisa mais estudadas da floresta amazônica. Entretanto, ainda é desconhecida a distribuição geográfica e a diversidade genética de muitas espécies de anuros que habitam a região. Nessa paisagem está localizada a maior cidade da Amazônia, Manaus, que está expandindo sobre áreas de florestas contínuas de forma não planejada. Uma unidade evolutivamente significante e diferente de *Atelopus* foi revelada durante este estudo (Anura: Bufonidae: *Atelopus manauensis*). Esse gênero é considerado altamente ameaçado em toda a sua distribuição e esta espécie candidata, aqui descrita, tem a distribuição conhecida apenas nos arredores de Manaus. Portanto, o objetivo geral desta tese foi investigar a influência da heterogeneidade ambiental nos limites geográficos e na conectividade funcional e genética entre grupos genéticos de *Atelopus manauensis* e prover informações para definir e nominar essa unidade evolutivamente significante dentro do gênero *Atelopus*. No **primeiro capítulo**, foram utilizadas inferências filogenéticas para confirmar que *Atelopus manauensis* formava uma unidade evolutivamente significante. Assim confirmado, foram integrados dados moleculares, morfológicos e bioacústicos para nomear o *Atelopus* da região de Manaus e fornecer subsídios para sua conservação. Apesar de ter sido considerada por muito tempo parte de um complexo de espécies distribuído no oeste da Amazônia, *Atelopus manauensis* é uma espécie irmã de um clado distribuído no Escudo das Guianas. A espécie pode ser distinguida das espécies do clado irmão pelo canto, morfologia e padrão de coloração. Devido às semelhanças fenotípicas e ecológicas, os fatores que ameaçam outras espécies de *Atelopus* podem levar *Atelopus manauensis* a extinção. No **segundo capítulo**, foi investigada a influência da heterogeneidade ambiental do INU nos limites geográficos, padrão de ocorrência e variação das densidades de *Atelopus manauensis* e com isso avaliar seus status de conservação. Fatores relacionados ao clima, à estrutura da floresta e à extensão da área de inundação dos rios limitam a ocorrência de *Atelopus manauensis* a manchas de ambientes adequados em uma área reduzida e diretamente afetada pelo crescimento de Manaus. As densidades de *Atelopus manauensis* variam em resposta a mudanças sutis em características dos igarapés e das planícies aluviais adjacentes. Seguindo os critérios da IUCN, nós sugerimos que *Atelopus manauensis* seja classificada como "Em Perigo" e incluída em listas de espécies ameaçadas. No **terceiro capítulo,** foi utilizado um banco de dados genômicos para investigar a influência da heterogeneidade ambiental na distribuição geográfica da variação genética de *Atelopus manauensis*. Foram identificados seis grupos geneticamente estruturados, apesar da área geográfica reduzida da espécie. Existem diferenças adaptativas entre esses grupos, o que sugere que eles representam segmentos populacionais distintos e devem ser considerados individualmente como prioridades em esforços conservacionistas. A heterogeneidade ambiental (ex.: isolamento por ambiente e por resistência da paisagem), juntamente com o isolamento por distância geográfica, influenciam o fluxo gênico entre os grupos genéticos da espécie estudada. Existem evidências de endogamia em alguns dos grupos genéticos identificados e medidas para preservar a conectividade entre eles são urgentes para a conservação de *Atelopus manauensis*.

ABSTRACT

Ecology, landscape genomics and taxonomic status of the frog *Atelopus manauensis* (Bufonidae): a multidisciplinary approach applied to the conservation of a species in extinction risk

The Negro-Uatuma Rivers interfluve (NUI) concentrates the most studied research areas in the Amazon. Nonetheless, the geographic distribution and genetic diversity of many anuran species inhabiting the region are still unknown. The largest city in the Amazon, Manaus, is located within this landscape, and its unplanned growth is approaching continuous forests. The present study revealed a distinct evolutionary significant unit within *Atelopus* (Anura: Bufonidae: *Atelopus manauensis*). The genus is considered highly threatened throughout its distribution and this candidate species, described here, is known only for the surroundings of Manaus. Hence, the main goal of this thesis was to investigate the role of environmental heterogeneity in determining the geographic range and the functional and genetic connectivity among genetic lineages of *Atelopus manauensis* and provide information to define and nominate this evolutionary significant unit within the genus *Atelopus*. The **first chapter** uses phylogenetic inferences to confirm that *Atelopus* from the Manaus region comprises an evolutionary significant unit. After confirmation, molecular, morphological and bioacoustical data were integrated to assign a name to this *Atelopus* species and provide support for its conservation. Despite have long been assigned to a species complex distributed in the western Amazon, *Atelopus manauensis* is sister to a clade distributed in the Guiana Shield. The species can be distinguished from species of its sister clade by call, morphology and colour pattern. Due to phenotypic and ecological similarities, the same factors that threaten other *Atelopus* species may lead to the extinction *Atelopus manauensis*. The **second chapter** investigates the influence of the environmental heterogeneity of the NUI on the geographic range, occurrence pattern and variation in density of *Atelopus manauensis* to assess its conservation status. Factors related to climate, forest structure and extent of flooding-area of rivers limit the occurrence of *Atelopus manauensis* to patches of suitable habitats in a narrow geographic area directly affected by the expansion of Manaus. Variation in the density of the species is related to subtle changes in streams and stream bank characteristics. Following IUCN criteria, it is suggested that the conservation status of *Atelopus manauensis* should be “Endangered” and the species included in lists of threatened species. The **third chapter** employs a genomic data set to investigate the influence of environmental heterogeneity on functional and genetic connectivity among genetic lineages of *Atelopus manauensis*. Six genetically structured lineages were identified despite the limited geographic area of the species. There is evidence of adaptive differences among these six genetic lineages, suggesting the presence of six distinct population segments (DPSs) to be individually considered as priorities by conservation efforts. Environmental heterogeneity (isolation by environment and isolation by landscape resistance), along with isolation by geographic distance, influence gene flow among the genetic lineages of *Atelopus manauensis*. There is evidence of inbreeding for some of these lineages, making measures to preserve connectivity among them an urgent necessity for the conservation of *Atelopus manauensis*.

Key-words: 1. *Atelopus* 2. Integrative taxonomy 3. Ecology 4. Conservation 5. Local adaptation 6. Gene flow 7. central Amazonia

SUMÁRIO

LISTA DE TABELAS

CAPÍTULO I

165 m a.s.l. and forest biomass between 0-100 and 300-387 MG C ha^{-1}) and isolation by environment (IBE – altitude and forest biomass of sampled streams) on total genetic variation of *Atelopus manauensis*. The *P* values were obtained by ANOVA separately for IBD (*P* geo), IBR (*P* res 1-5) and IBE (*P* alt and *P* bio) variables. The inertia values are equivalent to variance, and the CP values show the constrained proportion of variance on genetic data captured by RDA. Bold *P* values show significant effects of IBD, IBR and/or IBE on total genetic variation. Abbreviations: example – IBR alt 0-20 – landscape resistance imposed by altitudes between 0 and 20 meters a.s.l.

Table 10. Coefficients from multiple linear regressions using PCoA scores representing genetic distance (3,121 SNPs data set) as response variable and PCoA scores representing geographic (IBD), landscape resistance (IBR – altitudes between 0-20, 20-60 and 125-165 m a.s.l. and forest biomass between 0-100 and 300-387 MG C ha^{-1}) and environmental (IBE – altitude and forest biomass of sampled streams) distances as predictor variables. Bold *P* values show significant effects of IBD, IBR and/or IBE on total genetic variation. When not specified, IBE includes altitude plus forest biomass of sampled streams as predictor variables in the models. **120**

Online Resource 1. Detailed information of 21 sampled streams from 14 drainage systems distributed in five catchments of the known distribution of the *Atelopus manauensis*. The geographic distribution of the sampled streams is showed in Figure 1 as numbers according to the numbers listed in the column "Stream number". **121**

Online Resource 2. Methods for DartSeqTM used by Diversity Arrays Technology (Canberra, Australia) to discover and genotype and for quality control and initial calling of SNPs of *Atelopus manauensis*. **122**

LISTA DE FIGURAS

INTRODUÇÃO GERAL

CAPÍTULO I

CAPÍTULO III

INTRODUÇÃO GERAL

A distribuição geográfica da biodiversidade amazônica vem sendo investigada a mais de um século (Wallace, 1852; Haffer, 1969; Leite e Rogers, 2013). Biogeógrafos têm demonstrado que processos históricos e ecológicos como, por exemplo, a dinâmica geológica, os ciclos paleoclimáticos extremos e a heterogeneidade ambiental da paisagem atual, atuando individualmente ou em combinação, determinaram os padrões de organização espacial da biodiversidade da região (Moraes *et al*., 2016; Godinho e Da Silva, 2018; Maximiano *et al*., 2020). O padrão biogeográfico geral da Amazônia tem sido atribuído a fatores históricos, como a formação e dinâmica dos grandes rios e seus afluentes (Fernandes *et al*., 2014; Oliveira *et al*., 2017; Moraes *et al*., 2020). Em contraste, um estudo recente demonstrou que fatores ecológicos relacionados ao clima, ao solo e a altitude são os principais determinantes do padrão biogeográfico geral de anuros amazônicos, em detrimento de barreiras vicariantes históricas (Vacher *et al*., 2020). Enquanto os processos e padrões macroevolutivos da biota amazônica têm sido amplamente estudados e ainda calorosamente debatidos (Ruokolinen *et al*., 2019; Santorelli *et al*., 2018), os processos microevolutivos relacionados com a distribuição da variação genética dentro dos limites geográficos das espécies têm sido pouco investigados.

Tais investigações são fundamentais para a conservação da diversidade biológica amazônica frente as drásticas modificações que a paisagem da região vem sofrendo, uma vez que a eficácia de ações conservacionistas a nível intraespecífico é alcançada ao maximizar a representatividade da diversidade genética acumulada historicamente entre diferentes populações de uma espécie e a variação genética adaptativa dessas populações (Moritz, 2002). A realização de estudos evolutivos em níveis populacionais depende de conhecimento ecológico detalhado sobre a abrangência geográfica e o padrão de distribuição das espécies (Pabijan *et al.*, 2020). Entretanto, estudos nesse sentido são limitados na Amazônia devido, principalmente, a lacuna de conhecimento e definição taxonômica refinada de parte considerável da biodiversidade da região, visto que a realização de estudos ecológicos depende da definição precisa de unidades taxonômicas operacionais (Gotelli, 2004).

Espécies são as unidades operacionais fundamentais para estudos ecológicos e a ausência de resolução taxonômica apropriada pode mascarar os padrões ecológicos associados a um organismo devido a adaptações ambientais espécie-específicas (Vogel Ely *et al*., 2017). Adicionalmente, a efetividade de listas de espécies ameaçadas depende da qualidade da delimitação taxonômica e sua ausência pode comprometer o direcionamento de investimentos e a própria efetividade de ações de conservação (Vogel Ely *et al*., 2017). Em áreas com elevada

riqueza de espécies, como a Amazônia, unidades evolutivamente significantes de diferentes organismos, por exemplo dos anuros, são incluídos em complexos de espécies nominais com ampla abrangência geográfica devido à falta de estudos taxonômicos detalhados (Vacher *et al*., 2020). A Amazônia concentra a maior diversidade de anuros do planeta e, ao mesmo tempo que este número é amplamente subestimado, a região também está sendo ameaçada pela implantação de obras de infraestrutura potencialmente impactantes (ex.: Fearnside, 2020). Essa lacuna de conhecimento taxonômico pode levar ao negligenciamento de tomadores de decisão dos níveis reais de perda da diversidade de anuros causados por estes grandes empreendimentos em planejamento (Kress *et al.*, 1998; Troudet *et al.*, 2017). Neste contexto, estudos taxonômicos envolvendo anuros amazônicos, principalmente de grupos mais vulneráveis às alterações antrópicas (ex.: espécies ambiente-específico) são urgentes para prevenir extinções em massas de espécies em decorrência de mudanças no uso da terra na Amazônia.

Os estudos taxonômicos com anuros eram baseados principalmente em dados morfológicos, o que está relacionado com a subestimação da riqueza real de espécies amazônicas. Atualmente, abordagens integrativas utilizando dados moleculares, morfológicos e bioacústicos têm beneficiado a avaliação do status taxonômico de grupos complexos com resolução taxonômica pobremente definida devido, por exemplo, a elevada sobreposição de caracteres externos entre linhagens (Ferrão *et al*., 2016, 2018, 2019; Kaefer *et al*., 2019). Uma vez que a devastação da floresta amazônica vem ocorrendo a uma velocidade sem precedentes, este avanço na avaliação do status taxonômico e na descrição formal de espécies pode beneficiar a realização de estudos ecológicos em escalas espaciais mais refinadas buscando investigar os fatores ambientais relacionados aos limites geográficos e variação das densidades das espécies.

Essas investigações são altamente pertinentes uma vez que a distribuição das composições de espécies em escalas locais na Amazônia é influenciada por variações sutis em características topográficas e da vegetação (Menin *et al*., 2007, 2011; Fraga *et al*., 2011; Bueno *et al*., 2012). Poucos estudos abordam a distribuição e as variações nas densidades de espécie em florestas de terra-firme da Amazônia, porém os estudos realizados com peixes (Espírito-Santo *et al*., 2013), anuros (Jorge *et al*., 2016) e camarões (Silva *et al*., 2019) na Reserva Florestal Adolpho Ducke (RFAD), Amazônia central, concluíram que variações nas características dos igarapés e das planícies aluviais adjacentes influenciam o padrão de ocorrência e a variação nas densidades destas espécies entre sistemas de drenagem. Tais informações são essenciais para a condução de estudos ecológicos mais detalhados aplicados a conservação de espécies ameaçadas. Entretanto, estudos investigando os padrões ecológicos

nas áreas geográficas as quais as espécies geralmente estão limitadas na Amazônia ainda são raros (ex.: Ferreira *et al*., 2018), mesmo que tais investigações sejam necessárias para identificar as ameaças e avaliar o status de conservação das espécies em escalas espaciais apropriadas para o delineamento de medidas conservacionistas na região (Fernandes, 2013).

O preenchimento desta lacuna taxonômica e ecológica em escalas refinadas permite a condução de estudos microevolutivos buscando identificar a contribuição de diferentes forças evolutivas na geração e manutenção da elevada diversidade biológica vista na Amazônia, favorecendo ainda o planejamento de estratégias efetivas de conservação (Moritz *et al.*, 2000). Estratégias conservacionistas buscam proteger porções da distribuição geográfica das espécies que abriguem tanto linhagens com algum nível de isolamento reprodutivo histórico, geradas por deriva e fluxo gênico, quanto linhagens adaptativas, mantidas por seleção natural. Assim, é garantida a preservação de todo potencial evolutivo das espécies, necessária para sua permanência na paisagem em condições ambientais atuais e futuras (Moritz, 2002). Portanto, a conservação da diversidade biológica na Amazônia depende do conhecimento da distribuição geográfica da variação genética intraespecífica e das forças evolutivas que a determinam.

A variação genética entre populações, distribuídas em porções distintas da área geográfica de uma espécie, surge devido a restrições geográficas na capacidade de dispersão dos indivíduos (Isolamento por distância geográfica – IDG; Wright, 1943), quando o acúmulo local de variantes genéticas por deriva genética é mais rápido do que o efeito homogeneizante do fluxo gênico (Slatkin, 1981, 1993). Adicionalmente ao IDG, a diferenciação entre populações geograficamente isoladas e ocupando partes distintas de gradientes ambientais é reforçada pelo acúmulo de variantes genéticas adaptativas, fixadas por seleção natural, quando a diferença genética aumenta devido a mudanças ambientais entre os locais ocupados por diferentes populações, independente da distância geográfica (Isolamento por Distância Ambiental - IDA; Wang e Bradburd, 2014). Em IDA, indivíduos dispersando entre ambientes distintos podem não sobreviver ou reproduzir, aumentando o isolamento reprodutivo e a diferenciação genética entre populações (Wang e Bradburd, 2014). Populações isoladas tendem a perder a variabilidade genética devido a endogamia, o que pode levar a extinção de populações locais por depressão endogâmica ou pela perda do potencial evolutivo. A preservação da variação genética pode ser garantida pela manutenção do fluxo gênico entre populações. Portanto, é necessário identificar as porções da paisagem que facilitam ou impedem o movimento de indivíduos e genes entre populações (Isolamento por Resistência da Paisagem – IRP; McRae, 2006), para prever a influência de alterações antrópicas no isolamento das

populações e guiar ações conservacionistas que visem a preservação ou o reestabelecimento da conectividade funcional e genética entre populações de espécies ameaçadas (Frankham, 2015; Balkenhol *et al.*, 2017).

As características naturais dos anuros os tornam um dos organismos mais ameaçados do planeta (Ruland e Jeschke, 2017). A modificação ou a contaminação de ambientes utilizados por adultos (ex.: mata ciliares) ou larvas (ex.: poças, riachos) de anuros, a fragmentação florestal e a contaminação por doenças infecciosas têm sido as principais ameaças para o grupo (Pabijan *et al*., 2020). Alguns anuros amazônicos, de gêneros conhecidamente sensíveis a alterações antrópica, estão ameaçados de extinção antes mesmo de serem descritos (ex.: *Atelopus*; Coloma *et al*., 2010; Jorge *et al*., 2020) e a descrição formal de tais espécies pode subsidiar estratégias para sua conservação. Um dos grupos de anuros mais ameaçados da Amazônia é o gênero *Atelopus* (96 espécies; Frost, 2020), do qual estimasse que 30 espécies foram extintas nas últimas décadas e outras 42 sofreram reduções populacionais drásticas, devido principalmente à perda de ambientes e a infecção pelo fungo patogênico *Batrachochytrium dendrobatidis* (La marca *et al*., 2005). No interflúvio entre o rio Negro e o rio Uatumã, Amazônia central, há a ocorrência de uma espécie de *Atelopus* incluída no complexo de espécies *Atelopus spumarius* sensu lato (Lötters *et al*., 2002). Porém, Lötters *et al.,* (2002) indicaram a possibilidade dessa população representar uma unidade evolutivamente significante esperando a descrição formal (adiante tratada como *Atelopus* sp.). *Atelopus* sp. é conhecida em apenas três localidades nos arredores da cidade Manaus, estado do Amazonas, Brasil. Espécies de *Atelopus* (Bufonidae) estão associadas a ambientes específicos (zonas ripárias) nas florestas tropicais onde ocorrem, sendo bons modelos para investigar a influência da heterogeneidade ambiental nos limites geográficos e na distribuição geográfica da variação genética. A expansão urbana de Manaus está se aproximando de áreas de floresta nativa onde provavelmente *Atelopus* sp. ocorre, tornando tais investigações urgentes para a conservação desta espécie candidata.

Nesse contexto, busquei na presente tese definir o status taxonômico para suceder com a descrição formal de *Atelopus* sp. (Capítulo 1); delimitar a abrangência geográfica para acessar o status de conservação desta espécie de *Atelopus* da região de Manaus, Amazonas, Brasil (Capítulo 2) e investigar a influência da heterogeneidade ambiental e da distância geográfica na distribuição espacial da variação genética de *Atelopus manauensis* (descrita no primeiro campítulo) para sugerir estratégias efetivas para conservação da espécie (Capítulo 3). Os resultados desses capítulos demonstraram como fatores históricos juntamente com a heterogeneidade ambiental contemporânea foram determinantes na diversificação

interespecífica entre *Atelopus manauensis* e as espécies do Escudo das Guianas, nos limites geográficos reduzidos da espécie e na marcante estruturação genética de *Atelopus manauensis* em sua pequena área geográfica. Mostro como a integração das diferentes linhas de pesquisas utilizadas nessa tese são fundamentais para guiar estratégias de conservação efetivas focadas em espécies ambiente-específico com distribuição geográfica restrita e fragmentada e ocupando paisagens tropicais ameaçadas por alterações antrópicas. O gênero *Atelopus* está ameaçado de extinção e estudos multidisciplinares buscando prever o impacto das ações humanas sobre espécies específicas podem subsidiar a conservação do gênero como um todo.

Espécie-Alvo

Atelopus manauensis é terrestre e diurna. Indivíduos machos do *A. manauensis* geralmente são encontrados sobre a serrapilheira ou sobre troncos caídos nas proximidades de igarapés em florestas de terra-firme da Amazônia central. Durante a estação chuvosa na região (novembro– abril), machos da espécie vocalizam a uma distância entre 0 até 10 metros das margens dos igarapés (Lima *et al*., 2006). Desovas da espécie são encontradas em poças conectadas aos igarapés e os girinos no próprio igarapé, geralmente fixados no substrato arenoso sob folhas mortas (Gascon, 1989). Foi demonstrado que a ocorrência e a variação nas densidades de *Atelopus manauensis* são descontínuas em resposta a variações sutis no pH e na vazão de igarapés em uma reserva nos arredores de Manaus (RFAD; Jorge *et al*., 2016).

Figura 1. Indivíduo macho (20,15 mm) de *Atelopus manauensis* fotografado na Reserva Florestal Adolpho Ducke, Manaus, Amazonas, Brasil.

Área de estudo

O interflúvio entre o rio Negro e o rio Uatumã é composto pela formação sedimentar Alter do Chão, pelo Grupo Trombetas e pelas rochas cristalinas do Escudo das Guianas, com altitudes variando entre 3 e 225 metros acima do nível do mar. Os solos da área são altamente heterogêneos, formados sob condições que vão desde muito (ex.: podzóis) até pouco úmidos (ex.: lateríticos) (Quesada *et al*., 2010). São predominantes as Florestas Ombrófilas Densas de terras baixas e submontanas, inserida em um mosaico de vegetações composto por Florestas Ombrófilas Abertas, Florestas Aluviais, Igapós, Campinaranas, Campinas, além de áreas desmatadas (Figura 2). O clima da região é tropical úmido, com temperatura anual média variando na área estudo entre 26,4ºC e 27,6ºC e pluviosidade média anual entre 2.028 e 3.006 mm (média entre os anos de 1970–2000; Hijmans *et al*., 2005).

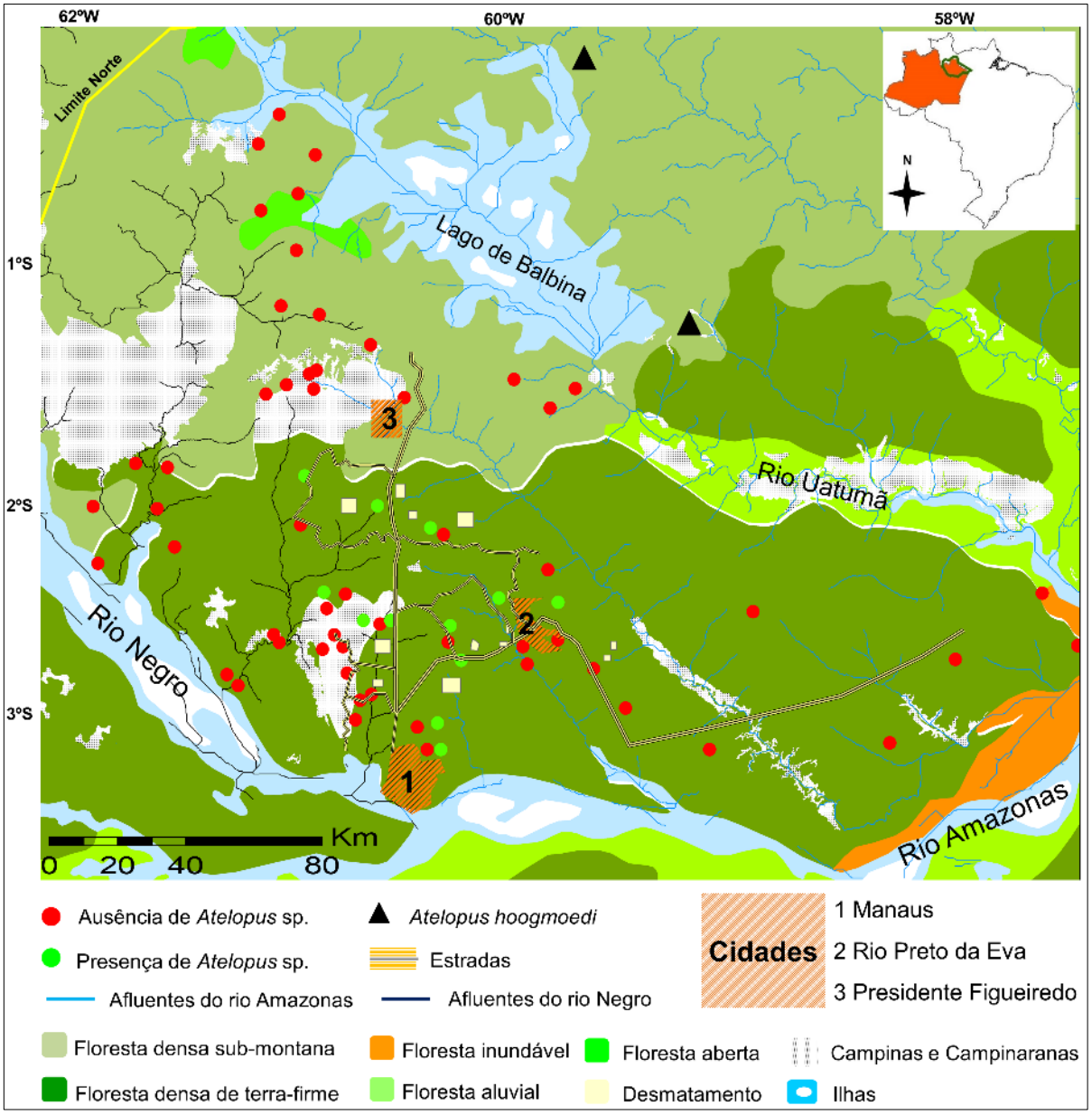


Figura 2. Área de estudo localizada entre os rios Negro e Uatumã (limites oeste e leste) e entre o rio Amazonas e áreas de florestas abertas ao norte da área de estudo (limites sul e norte). Essa paisagem é composta desde de áreas de campinas até áreas de florestas densas submontanas. As áreas de ocorrência da espécie (círculos verdes) são separadas por estradas principais e vicinais, além de áreas desmatadas e perímetros urbanos.

OBJETIVO GERAL

Investigar a influência da heterogeneidade ambiental nos limites geográficos na conectividade funcional e genética entre os grupos genéticos de *Atelopus manauensis* e prover informações para definir e nominar essa unidade evolutivamente significante dentro do gênero *Atelopus*.

OBJETIVOS ESPECÍFICOS

CAPÍTULO I

Integrar informações moleculares, morfológicas e bioacústicas para determinar o status taxonômico de *Atelopus manauensis* e proceder com a descrição formal da espécie (agora descrita), além de aprimorar o conhecimento das relações interespecíficas entre espécies de *Atelopus* distribuídas no Escudo das Guianas e nas terras baixas da Amazônia central.

CAPÍTULO II

Investigar a influência da heterogeneidade ambiental nos limites geográficos, no padrão de ocorrência e na variação das densidades de *Atelopus manauensis* para avaliar as ameaças para a conservação efetiva da espécie;

Utilizar os critérios IUCN como, por exemplo, a extensão de ocorrência, fragmentação das áreas de ocupação, qualidade dos ambientes ocupados e número de subpopulações em declínio, para acessar o status de conservação da espécie e avaliar qual categoria de risco que *Atelopus manauensis* se enquadra.

CAPÍTULO III

Caracterizar a variação genética e identificar partes da distribuição geográfica de *Atelopus manauensis* que abrigam grupos geneticamente distintos;

Investigar a existência de variações genéticas adaptativas entre grupos genéticos de *Atelopus manauensis* para identificar as características da paisagem que devem ser preservadas para manutenção do potencial evolutivo dos diferentes grupos genéticos que compõe a espécie;

Identificar quais características da paisagem facilitam ou impedem a conectividade funcional e genética entre grupos genéticos de *Atelopus manauensis* para projetar potenciais impactos antrópicos na perda da diversidade genética da espécie e sugerir porções da paisagem que devem ser protegidas para manter o fluxo gênico entre os grupos genéticos identificados.

Capítulo 1

Jorge, R.F., Ferrão, M. & Lima, A.P. Out of Bound: A New Threatened Harlequin Toad (Bufonidae, *Atelopus*) from the Outer Borders of the Guiana Shield in Central Amazonia Described Through Integrative Taxonomy. *Diversity, 12*, 310.

Article

Out of Bound: A New Threatened Harlequin Toad (Bufonidae, *Atelopus*) from the Outer Borders of the Guiana Shield in Central Amazonia Described through Integrative Taxonomy

Rafael F. Jorge [1],* , Miquéias Ferrão [2,3,*] and Albertina P. Lima [3]

[1] Programa de Pós-Graduação em Ecologia, Instituto Nacional de Pesquisas da Amazônia, Manaus 69067-375, Amazonas, Brazil

[2] Museum of Comparative Zoology, Harvard University, Cambridge, MA 02138, USA

[3] Coordenação de Biodiversidade, Instituto Nacional de Pesquisas da Amazônia, Manaus 69067-375, Amazonas, Brazil; lima@inpa.gov.br

* Correspondence: rafajorgebio@gmail.com (R.F.J.); uranoscodon@gmail.com (M.F.); Tel.: +55-21-97985-8790 (R.F.J.); +55-92-98230-8688 (M.F.)

http://zoobank.org/urn:lsid:zoobank.org:pub:D0C1ACD6-35B0-4C0E-B3AF-62C457465728

http://zoobank.org/urn:lsid:zoobank.org:act:5D9F4EF7-74D2-4A48-AE50-F42282439C86

Received: 22 June 2020; Accepted: 23 July 2020; Published: 10 August 2020

Abstract: We used integrative taxonomy to describe a new species of *Atelopus* from the lowlands of Central Amazonia in the region of Manaus, Amazonas, Brazil. The new species is geographically isolated from the southernmost species of *Atelopus* of the Guiana Shield. *Atelopus manauensis* species nova (sp. nov.) is characterized by the combination of the following characteristics: male snout-vent length range (SVL=19.1–26.4mm; n=11); dorsal and lateral skin smooth; ventral surface entirely white or white with cream-colored gular region; fingers and toes lacking subarticular tubercles and fringes.The advertisement call of the new species has a call duration of 689–840 ms, contains 15–26 pulses, is emitted at an average pulse rate of 25.5 pulses per second, and has a dominant frequency ranging 3088–3610 Hz. The genetic divergence between the new species and its morphologically most similar congeners (*A. spumarius* and *A. pulcher*) is greater than 4%. *Atelopus manauensis* sp. nov. is closely related to species of the *A. hoogmoedi* complex inhabiting the Guiana Shield. The new species has a small geographic distribution (approximately 4500 km2) in a landscape that is strongly threatened by the growth of Manaus, the largest city in Brazilian Amazonia. The new species is considered critically endangered and in need of urgent conservation measures.

Keywords: conservation; genetics; integrative taxonomy; morphology; vocalization**.**

Introduction

The genus *Atelopus* comprises 96 [1] diurnal (except for *A. nocturnus*) and small-sized (17–50 mm) species known as harlequin frogs, which are distributed from Central America to northern South America [2]. Although few species have been included in recent phylogenetic studies, recent phylogenies show *Atelopus* to contain two major clades: the Andean–Chocó–Central American clade, and the Amazonian–Guianan clade. The latter comprises the *tricolor* subclade (pre-Andean region of Peru and adjacent Bolivia) and the *flavescens-spumarius* subclade [3]. The *flavescens-spumarius* subclade includes species distributed in the upper Amazon Basin (Ecuador, Peru, and Colombia), the Guiana Shield (Guyana, Suriname, French Guiana, and Brazil) and the lowlands of Central Amazonia (Brazil). The alpha taxonomy of the species distributed in the Guiana Shield and surrounding lowlands is complex due to morphological, bioacoustics, and genetic similarities [3,4], and different recognized species may comprise a single polymorphic taxon [5]. Therefore, taxonomic studies integrating different traits (i.e., morphological, bioacoustics, and genetic) is needed to shed light on the taxonomy of *Atelopus* of the Guiana Shield and surrounding lowlands.

Atelopus spumarius was originally described from Iquitos, Peru [6], but due to the loss of the holotype, Lescure [7] assigned a neotype from Colonia, Peru. The forms that were initially treated as subspecies of *A. spumarius* distributed in the Andes (e.g., *A. s. andinus*; [8]) and in the Guiana Shield (*A. s. hoogmoedi* and *A. s. barbotini*; [7,9]) were latter considered different taxa by Lötters and De La Riva [10] and Lötters et al. (3,4). Additionally, *A. spumarius* sensu stricto (SS) exhibits considerable genetic differentiation (uncorrected p-distance > 3% from other species of the *A. spumarius* complex in the Guiana Shield, considering that *Atelopus* species within the Guiana Shield have less than 2% genetic differentiation [3]). Despite recent advances in understanding the phylogenetic relationships of the Amazonian–Guianan clade [4,11], there remain populations of *Atelopus* in areas of lowland forests adjacent to the Guiana Shield that require taxonomic review [3], such as the population in the region of Santarém (state of Pará) and the population in the region of Manaus (state of Amazonas), both of which are in Brazilian Amazonia.

Although occurring in the best studied portion of Brazilian Amazonia, the population of *Atelopus* in the region of Manaus and its surroundings has historically been assigned to different nominal species. The work of Zimmerman [12], Zimmerman and Bierregaard [13], and that of Zimmerman and Simberloff [14] assigned the name *Atelopus pulcher* to species collected from reserves in the region of Manaus. When describing the tadpole of an *Atelopus* collected as part

494 of the Projeto Dinâmica Biológica de Fragmentos Florestais (PDBFF—80 km northeast of
495 Manaus), Gascon [15] identified the population as *Atelopus pulcher* based mainly on the red
496 color of the hands and feet of adults at this locality being similar to those of individuals from
497 Peru and Ecuador, as described by Boulenger [16] and Peters [17]. However, the distribution
498 of *A. pulcher* is restricted to the eastern slope of the Andes (600–900 m above sea level, or m
499 a.s.l.) in these two countries [4]. In addition, both adults and tadpoles of *A. pulcher* in the eastern
500 portion of the Andes are distinct from those of other species in the *A. spumarius* complex [4].
501 Although they did not analyse adult *Atelopus* from the region of Manaus, Lötters et al. [4]
502 suggested that the species be treated as *Atelopus spumarius* sensu lato (SL) due to the red color
503 of the ventral surface of the hands and feet. This nomenclature has been followed by several
504 studies undertaken in this region (e.g., Lima et al. [18]; Rojas-Ahumada and Menin [19]; Menin
505 et al. [20]; Siqueira et al. [21]; Jorge et al. [22]; Rößler et al. [23]). However, *A. spumarius* SS
506 is restricted to the Upper Amazon Basin of Peru, Ecuador, and Colombia [4]. In addition, the
507 tadpole of *Atelopus* in the region of Manaus (PDBFF) differs from that of *A. spumarius*
508 described in Ecuador with regard to the position of the nostrils [24]. The present study employed
509 integrative taxonomy to describe the taxon of *Atelopus* from the region of Manaus as a new
510 species. This species is distinguished from others of the *A. spumarius* complex and of the
511 Guiana Shield by morphology, color pattern, advertisement call, and phylogenetic position.
512 Additionally, the new species is shown to be restricted to a small region of lowlands around
513 Manaus that suffers from strong anthropic pressure. Thus, the formal description of this species
514 will contribute to determining its conservation status and potentially help with the
515 implementation of conservationist measures.

516 **2. Materials and Methods**

517 *2.1. Sampling*

518 Individuals of the new species were collected manually in the morning (07:00–10:00) and
519 afternoon (15:00–17:00) at six locations in the municipality of Manaus (Amazonas, Brazil): (1)
520 Reserva Florestal Adolpho Ducke (RFAD; 2◦55J4.8” S, 59◦53J52.8” W; 109 m a.s.l.); (2)
521 training area of Centro de Instruções de Guerra na Selva do Exército Brasileiro (CIGS/EB;
522 2◦54J52.4” S, 59◦49J26.4” W; 61 m a.s.l.); (3) Cabo Frio camp (2◦3J20.4” S, 59◦55J51.6” W;
523 109 m a.s.l.); (4) Fazenda Dimona (2◦19J43.39” S, 60◦4J41.46” W; 102 m a.s.l.); (5) Fazenda
524 Experimental da Universidade Federal do Amazonas (FEUFAM; 2◦38J31.20” S, 60◦5J45.6”
525 W; 100 m a.s.l.); and (6) banks of a stream in a drainage system of middle Cuieiras River near
526 LBA/ZF2 scientific station (LBA/ZF2; 2◦33J32.40” S, 60◦13J48.36” W; 110 m a.s.l.). Fazenda

Dimona and Cabo Frio camp are located within the Área de Relevante Interesse Ecológico do Projeto Dinâmica Biológica de Fragmentos Florestais (ARIE PDBFF). Individuals collected at FEUFAM (2) and LBA/ZF2 (2) were used for genetic analyses.

For interspecific comparisons, eight adult males of *Atelopus hoogmoedi* were collected from two populations (four individuals of each population) with geographic distributions known to be the closest to the new species, both within the municipality of Presidente Figueiredo (state of Amazonas, Brazil): (1) Reserva Biológica do Uatumã (REBIO Uatumã; 1◦46J48" S, 59◦15J4.81" W; 80 m a.s.l.), 145 km straight-line distance from Manaus; and (2) Pitinga River drainage system (0◦42J52.74" S–60◦1J22.25" W; 177 m a.s.l.), 250 km straight-line distance from Manaus. Two individuals collected at REBIO Uatumã were used for genetic analyses.

The specimens were killed with a 5% lidocaine topical solution, fixed in 10% formaldehyde and preserved in 70% alcohol. Muscle tissue was extracted prior to fixation and kept in absolute alcohol. Specimens were deposited in the herpetology section of the zoological collection of the Instituto Nacional de Pesquisas da Amazônia (INPA-H), Manaus, Amazonas, Brazil. Specimens were collected under permit number 56,759 from Sistema de Autorização e Informação em Biodiversidade of Instituto Chico Mendes de Conservação da Biodiversidade (SISBIO/ICMBIO) and the study was approved by Comissão de Ética no Uso de Animais of INPA with registration number 002/2017 (CEUA/INPA).

Advertisement calls from two adult males of the new species were recorded in RFAD and one was recorded in Cabo Frio camp. The call of an uncollected male of the new species from RFAD presented by Lima et al. [18] was also used. Advertisement calls of seven males of *Atelopus hoogmoedi* were recorded: four males in REBIO Uatumã (INPA-H 041358, INPA-H 041359, INPA-H 041293, and INPA-H 041360) and three males on the banks of a stream of Pitinga River (INPA-H 041361, INPA-H 041362, and INPA-H 041363). Recordings were made with a Sennheiser K6/ME66 directional microphone coupled to a Marantz PMD660 digital recorder. The microphone was positioned approximately 150 cm from each individual, and recordings were made with a sampling rate of 44.1 kHz and sample size of 16 bits. Calls were recorded during the morning (08:00–11:00) and the average air temperature at the time of recording was approximately 26 ◦C.

In addition to the advertisement call (type 1 call), three other call types were recorded and analyzed: type 2, type 3, and type 4. Following Lötters et al. [25], the type 2 call represents the pure tone call; the type 3 call represents the short pure tone call; and the type 4 call represents

the pulsed short call. A type 2 call was recorded from a male in the drainage system on the east bank of the middle Puraquequara River located in CIGS/EB (SVL = 20.3 mm; 26.5 ◦C; 2◦51J50.4" S, 59◦48J54.0" W; 108 m a.s.l.; INPA-H 041372). Type 3 calls were recorded from the holotype in RFAD and from a male at the headwaters of the upper Cuieiras River located in Fazenda Dimona (SVL = 20.9 mm; 26.5 ◦C; 2◦19J43.3" S, 60◦4J41.46" W; 95 m a.s.l.; INPA-H 041373). The type 4 call was emitted by the holotype and recorded in laboratory. Type 2 and type 3 calls emitted by the holotype were recorded with a Sennheiser K6/ME66 directional microphone coupled to a Marantz PMD660 digital recorder. Type 2 and type 3 calls of other males and type 4 calls were recorded with a SONY PX333 digital recorder (format mp3, 192 kbps) with an internal microphone at 30 cm from the individuals. Files in mp3 format were converted to wav format (sampling rate 44.1 kHz, sample size 16 bits) to obtain acoustic parameters.

2.2. Phylogenetic analyses

To infer the phylogenetic relationships of the new species and its congeners, 28 16S ribosomal RNA gene (16S) sequences and seven cytochrome c oxidase subunit I gene (COI) sequences were selected from GenBank of the National Center for Biotechnology Information (NCBI). All sequences used in the current study were obtained from GenBank. The sequences correspond to 15 species of *Atelopus* [22 16S sequences of 13 described *Atelopus* species + one sequence of an undescribed species from the south bank of the Amazon River (municipality of Anapu, state of Pará, Brazil) + four sequences from individuals of the new species, and four COI sequences from individuals of the new species + two from *A. hoogmoedi* from REBIO Uatumã + one from *A. spurrelli*], including two 16S sequences of *A. hoogmoedi* from Guyana, two from French Guiana, two from REBIO Uatumã, and three from the municipality of Monte Alegre, state of Pará, Brazil. A 16S sequence and another COI sequence of *Rhinella marina* were selected for rooting the phylogenetic trees. See Table 1 for detailed information on the specimens used in the phylogenetic analyses and accession numbers for all sequences in GenBank. After concatenating and aligning the total of 28 sequences in Geneious 4.8.2 using default parameters [26], a final matrix was obtained containing 1274 base-pairs (bp) (614 bp 16S and 660 bp COI). PartitionFinder2 [27] was used to infer the best partition scheme and its respective models of nucleotide evolution through PhyML 3.0 [28] and Bayesian Information Criterion (BIC). Suggested partitions and models were as follows: 16S = GTR + G; COI\1 = SYM; COI\2 = F81; COI\3 = HKY. We used for phylogenetic inference the same 20 16S and all COI sequences used in Jorge et al. [29], including those sequences of the new species, only

593 adding eight new sequences, as follows: *A. hoogmoedi* (three sequences from the municipality
594 of Monte Alegre, state of Pará, Brazil—IDs 8–10 in Table 1), *A.* sp. Anapu, *A. barbotini* "B",
595 *A. oxapampae*, *A. tricolor*, and *R. marina* (IDs 7; 14; 26–28 in Table 1, respectively). Overall,
596 we obtained the same phylogenetic relationships and statistical support as obtained by Jorge et
597 al. [29], with a slight difference regarding the relationships of the French Guiana species, which
598 is likely because of the addition of new samples (see Results section, Section 3.1).

599 **Table 1.** Detailed information on the specimens used in the phylogenetic inferences: Species,
600 GenBank accession number, voucher, origin of specimens and source reference for sequences.

ID	Species	GenBank *16S*/*COI*	Voucher	Country	Source
1	***Atelopus manauensis* sp. nov.**	MT176236/MT184269	INPA-H 041289	Brazil	[29]
2	***Atelopus manauensis* sp. nov.**	MT176237/MT184270	INPA-H 041290	Brazil	[29]
3	***Atelopus manauensis* sp. nov.**	MT176238/MT184271	INPA-H 041291	Brazil	[29]
4	***Atelopus manauensis* sp. nov.**	MT176239/MT184272	INPA-H 041292	Brazil	[29]
5	*Atelopus hoogmoedi* REBIO Uatumã	MT176240/MT184273	INPA-H 041293	Brazil	[29]
6	*Atelopus hoogmoedi* REBIO Uatumã	MT176241/MT184274	INPA-H 041294	Brazil	[29]
7	*Atelopus* sp. Anapu	MK166205	KA14	Brazil	[30]
8	*Atelopus hoogmoedi* Monte Alegre	MK166206	LZA971	Brazil	[30]
9	*Atelopus hoogmoedi* Monte Alegre	MK166208	LZA990	Brazil	[30]
10	*Atelopus hoogmoedi* Monte Alegre	MK166211	LZA1046	Brazil	[30]
11	*Atelopus hoogmoedi*	JQ742148	IRSNB15781	Guyana	[31]
12	*Atelopus hoogmoedi*	JQ742149	IRSNB14477	Guyana	[31]
13	*Atelopus barbotini* "A"	EU672971	-	French Guiana	[32]
14	*Atelopus barbotini* "B"	GU183859	BPN1697	French Guiana	[33]
15	*Atelopus franciscus*	JQ742150	PK3306	French Guiana	[31]
16	*Atelopus* (*spumarius*) *hoogmoedi*	DQ283260	BPN754UTA	French Guiana	[34]
17	*Atelopus hoogmoedi*	EU672972	-	French Guiana	[32]
18	*Atelopus flavescens*	EU672970	-	French Guiana	[32]
19	*Atelopus seminiferus*	EU672976	-	Peru	[32]
20	*Atelopus spumarius*	EU672977	-	Peru	[32]
21	*Atelopus pulcher*	EU672973	KU211678	Peru	[32]
22	*Atelopus bomolochos*	GU252227	KU217468	Ecuador	[33]
23	*Atelopus peruensis*	GU252229	KU211631	Peru	[33]
24	*Atelopus spurrelli*	EU672975/DQ502895	MHNUC273	Colombia	[32,34]
25	*Atelopus loettersi*	EU672980	-	Peru	[32]
26	*Atelopus oxapampae*	EU672979	MTD1276	Peru	[32]
27	*Atelopus tricolor*	EU672978	MNCN5885	Bolivia	[32]
28	*Rhinella marina*	KR012644/ KR012546	QCAZ50702	Ecuador	[35]

601 Phylogenetic relationships were estimated using Maximum Likelihood (ML) and Bayesian
602 Inference (BI). The ML phylogenetic tree was estimated using IQ-TREE [36] with 10,000
603 ultrafast bootstraps; 10,000 iterations; a minimum correlation coefficient of 0.99; and 10,000
604 Shimodaira– Hasegawa approximate likelihood ratio replicates (SH-aLRT). The Bayesian
605 phylogenetic tree was inferred using MrBayes 3.2.6 [37] through four runs of 10 million
606 generations each using Monte Carlo via Markov Chains (MCMC) with four chains each.

607 Probabilities were sampled every 1000 generations. The tree was summarized after burning of
608 25% of the sampled trees. Uncorrected genetic distances (p-distance) and Kimura-2-parameters
609 (K2P; [38]) were calculated in MEGA 6.0 [39] using the 16S alignment.

610 *2.3. Morphological Analyses*

611 Specimens were measured using a digital magnifying glass with a millimeter lens
612 (measurements less than 10 mm) and a calliper with 0.01 mm precision (measurements greater
613 than 10 mm). The sex of specimens was determined by the presence/absence of nuptial pads
614 and vocal slits in males, or by the direct inspection of gonads. Thirteen morphometric
615 measurements were taken following Gray and Cannatella [40] and Coloma et al. [41]: snout-
616 vent length (SVL), sacrum width (SW), head width (HW), head length (HL), eye diameter
617 (EYDM), eye-to-nostril distance (EYNO), interorbital distance (IOD), internarial distance
618 (ITNA), length of flexed forearm (RDUL), hand length (HAND), thumb length (THBL), foot
619 length (FOOT), and tibia length (TL). Formulae for interdigital webbing followed Savage and
620 Heyer [42,43] and Myers and Duellman [44].

621 Populations of *Atelopus hoogmoedi* of REBIO Uatumã and Pitinga River are the
622 southernmost populations of the genus of the Guiana Shield and the geographically closest
623 populations to the new species described here. For this reason, we used morphometric
624 measurements of these populations to distinguish the new species from *A. hoogmoedi*. Three
625 cluster and classification analyses were used for this purpose: (1) Principal Component
626 Analysis (PCA); (2) Discriminant Analysis of Principal Components (DAPC; [45]); and (3)
627 Random Forest (RF; 46). The analyses were conducted in R [47] using SVL and 12
628 morphometric ratios (HW/SVL, HL/SVL, EYDM/SVL, EYNO/SVL, TL/SVL, IOD/SVL,
629 ITNA/SVL, RDUL/SVL, FOOT/SVL, HAND/SVL, THBL/SVL and SW/SVL) of 11 males of
630 the new species (Holotype, type series, paratypes and paratopotypes) and eight males of *A.*
631 *hoogmoedi* collected in REBIO Uatumã (4) and at Pitinga River (4). Paratype and paratopotype
632 specimens were included in the analyses so that the extreme minimum and maximum body
633 sizes observed among individuals of the new species could be included, and for the same reason
634 they were not included in the "type series". The PCA was performed using the "prcomp"
635 function of the *stats* package using the parameters "scale = T" and "center = T" to scale and
636 center the morphometric variables. A Multivariate Analysis of Variance (MANOVA) was
637 performed to test whether the multidimensional morphometric space occupied by the new
638 species differs significantly from that occupied by *A. hoogmoedi* in relation to the first two axes
639 of the PCA. The DAPC was performed using the "dapc" function of the *adegenete* 2.1.2

package [45]. As recommended by Jombart et al. [45], all principal components (PCs) and discriminant analysis eigenvalues (DAE) were used in the DAPC. Random Forest is a learning algorithm that builds classification trees from random subsamples of a database to then aggregate results and classify samples (specimens) into groups (species) [45]. Random Forest was conducted with 4,000 trees using the "randomForest" function of the *randomForest* 4.6-14 package [48]. Results are presented in the subsection "Morphological and Bioacoustic Analyses" of the section "Results". Raw morphologic measurements can be found in the Supplementary Material Table S1.

2.4. Bioacoustics

Calls were analysed in Raven 1.5 [49] and configured as follows: Blackman window; 3 dB Filter Bandwidth of 80 Hz; overlap of 80%; hop size of 4.1 ms; and discrete Fourier transform (DFT) size of 2048 samples. Temporal parameters were measured using oscillograms, and spectral parameters were measured using "power spectrum" graphs. Different sets of acoustic parameters were measured for each of the four call types.

Ten advertisement calls (type 1 call) from three males of the new species and 35 calls from four males of *Atelopus hoogmoedi* from REBIO Uatumã and three males from Pitinga River were analysed. Eleven parameters were measured in the advertisement calls, as follows: (1) call duration (Call_dur); (2) number of pulses (N_pulses); (3) pulse duration of first pulse (Puls_dur_1); (4) pulse duration of central pulse (Puls_dur_2), which usually precedes the last portion of the call when the pulses are emitted in a shorter time interval; (5) pulse duration of last pulse (Puls_dur_3); (6) pulse period of first three pulses, measured from the beginning of the first pulse to the end of the third pulse (Dur_3_first_puls); (7) pulse period of last three pulses, measured from the beginning of the antepenultimate pulse to the end of the last pulse (Dur_3_last_puls); (8) inter-pulse interval between first two pulses (Interpulse_1); (9) inter-pulse interval between central pulse and next pulse (Interpulse_2); (10) inter-pulse interval between last two pulses (Interpulse_3); (11) call dominant frequency measured using the "Peak Frequency" function (Domi_frequency); and (12) bandwidth. We also measured the advertisement calls of four males of *A. hoogmoedi* recorded by Costa-Campos and Carvalho [50] in Pedra Branca do Amapari (state of Amapá, Brazil), which is the closest population to the type locality of this species (Mont Atachi-Bacca, French Guiana) with data available to be re-analyzed in the same manner as the calls of the new species (recordings provided by the authors). As with the morphological data, three cluster and classification analyses (PCA+MANOVA, DAPC, and RF) were used to test whether the new species can be

673 distinguished from *Atelopus hoogmoedi* through the advertisement call. Analyses were
674 performed using the average of each acoustic parameter for each male, three of the new species
675 and 11 of *A. hoogmoedi*: four from REBIO Uatumã, three form Pitinga River, and four from
676 Amapá recorded by Costa-Campos and Carvalho [50]. Results are presented in the subsection
677 "Morphological and Bioacoustic Analyses" of the section "Results".

678 Six parameters were measured in type 2–4 calls, as follows: call duration, inter-call
679 interval, low frequency, high frequency, dominant frequency, and bandwidth. Low and high
680 frequencies, as well as the bandwidth, were measured 20 dB below the peak frequency in order
681 to avoid background noise. Dominant frequency and bandwidth were measured using the
682 functions "Peak frequency" and "Bandwidth 90%" in Raven, respectively. In addition, we
683 measured the inter-call interval within a series of type 2 calls, the number of pulses per call,
684 and the pulse duration of the first and last pulse of type 4 calls. The nomenclature of bioacoustic
685 traits followed Köhler [51].

686 *2.5. Natural history*

687 Information on the natural history of the new species was obtained through observations
688 made during the rainy seasons (December-March) of 2012–2013 and 2016–2019, in every
689 probable area of occurrence of the species, at different times of the day and under different
690 climatic conditions.

691 **3. Results**

692 *3.1. Phylogenetic analyses*

693 Both Bayesian Inference (BI) and Maximum Likelihood (ML) supported the *flavescens-*
694 *spumarius* clade as monophyletic (Figure 1), with *Atelopus pulcher* being the sister species of
695 all other species in the clade [ML support = 100, Posterior Probability (PP) = 1]. *Atelopus*
696 *spumarius* SS (lowlands of western Amazonia, upper Amazon Basin) and *A. seminiferus*
697 (eastern slope of the Andes) were inferred as sister species, forming a sister clade to the clade
698 composed of the species distributed in the Guiana Shield and surrounding lowlands of Central
699 Amazonia, and in the south bank of the Amazon River. Within this latter clade, the species from
700 Anapu was recovered with low support (ML = 68, PP = 0.64) as a sister to the clade composed
701 by the new species from Manaus and other species distributed in the Guiana Shield. In the latter
702 clade, the new species of Manaus is inferred as the sister to the Guiana Shield clade (ML = 69,
703 PP = 0.83). Samples of *A. hoogmoedi* from REBIO Uatumã formed a moderated-supported

subclade (ML = 78, PP = 0.94) within the Guiana Shield clade with five more samples of *A. hoogmoedi* from different locations in Brazil and Guyana (Figure 1). The internal relationships among the other species of the Guiana Shield (French Guiana) were not well supported in any of our reconstructions.

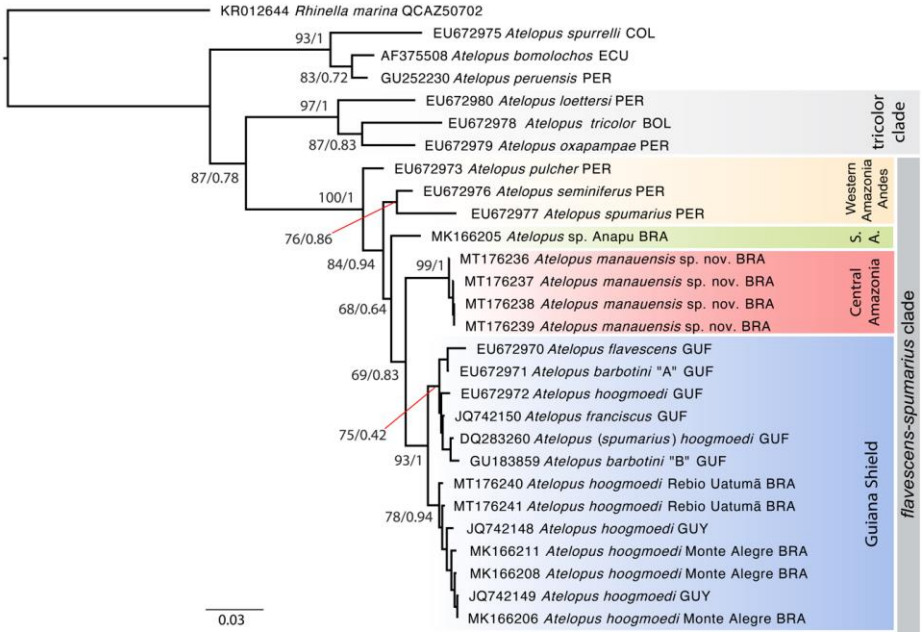


Figure 1. Phylogenetic reconstruction using Maximum Likelihood based on 614 base-pairs of 16S rRNA and 660 base-pairs of cytochrome c oxidase subunit I gene (COI). Node support is presented as bootstrap/posterior probability (PP). Horizontal yellow bar represents species distributed further west in Amazonia and on the eastern slope of the Andes; red bar represents the new species distributed around the city of Manaus (Brazil); blue bar denotes species distributed in the Guiana Shield. Abbreviations: (S.A.) south bank of the Amazon River—green bar.

Genetic distances between the new species and the other species of *Atelopus* included in the analyses (Table 2) ranged 2.3–11.6% (p-distance) and 2.3–12.7% (Kimura-2-paramenter, K2P). Genetic distances were relatively high between the new species and those with which it has been confused in the past: *A. spumarius* SS (p-distance = 4.7%; K2P = 4.9%) and *A. pulcher* (p-distance = 3.9%; K2P = 4.0%). Although *A. hoogmoedi* at REBIO Uatumã is the geographically closest population to the new species, the genetic distances between them varied between 2.5% (p-distance) and 2.6% (K2P). Similar values differentiate the new species and *A. hoogmoedi* of French Guiana (p-distance = 2.6%; K2P = 2.7%). The lowest values of genetic distance were between species of the *A. hoogmoedi* complex occurring in the Guiana Shield, which varied between 0.2–1.7% (p-distance) and 0.2–1.7% (K2P).

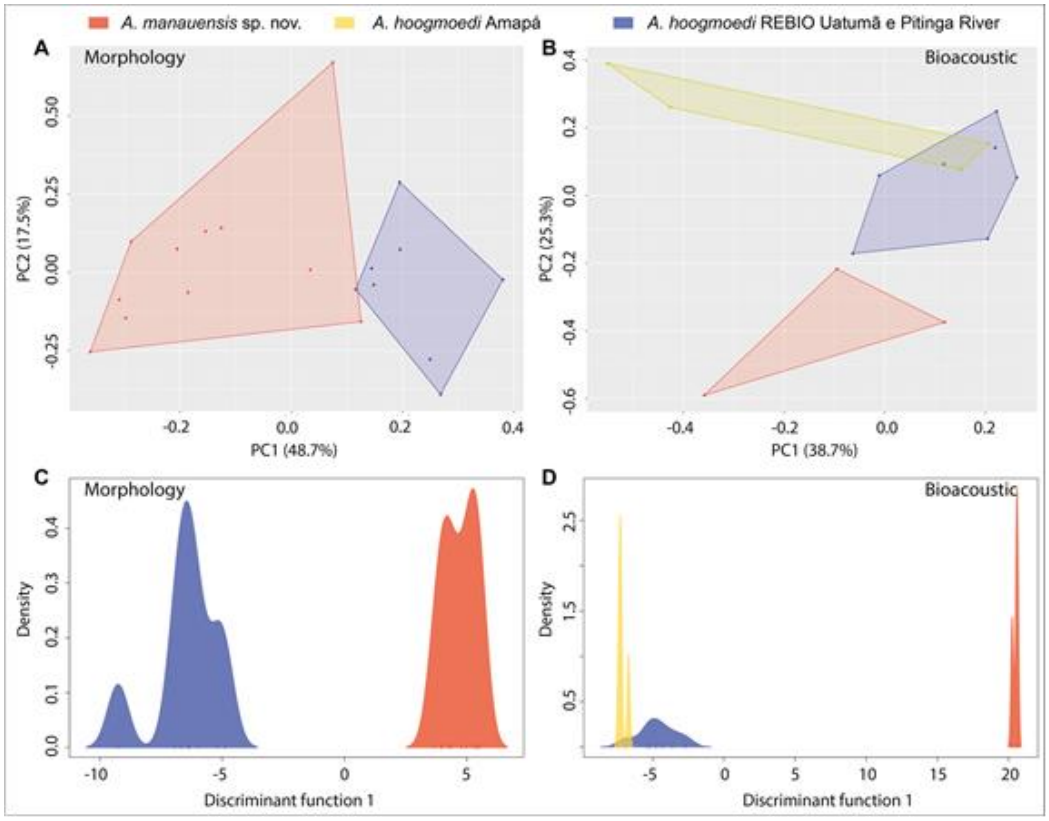
726 **Table 2.** Uncorrected p–distances (upper diagonal) and Kimura 2-Parameter (lower diagonal) between *Atelopus manauensis* sp. nov. and
727 species of the Amazonian-Guianan clade and of the Andean-Chocó-Central American clade. Genetic distances were calculated using 16S
728 rRNA sequences and are presented as percentages. Abbreviations: (COL) Colombia, (BOL) Bolivia, (PER) Peru, (ECU) Ecuador, (SS) sensu
729 stricto, (GUF) French Guiana, (BRA) Brazil, (GUY) Guyana, (RU) REBIO Uatumã, (MA) Monte Alegre, state of Pará, Brazil.

ID	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*A. spurrelli* COL		13.4	10.7	5.0	11.7	4.8	12.4	11.9	13.6	12.2	13.1	12.4	12.0	13.2	12.6	14.2	12.4	14.1	12.7
2	*A. tricolor* BOL	12.1		6.7	11.8	5.6	12.2	12.0	11.8	12.3	12.4	12.8	12.6	12.7	12.5	13.1	13.2	12.2	13.3	12.5
3	*A. loettersi* PER	9.9	6.3		10.7	6.1	11.1	13.7	10.7	12.3	11.6	13.1	12.0	11.2	12.0	12.3	12.7	11.7	13.1	11.9
4	*A. bomolochos* ECU	4.8	10.8	10.0		10.7	2.2	11.3	10.4	12.1	10.7	10.9	11.4	11.2	11.6	11.6	12.6	11.2	12.4	10.9
5	*A. oxapampae* PER	10.8	5.3	5.8	9.9		10.7	12.2	10.2	11.8	11.1	13.1	11.5	11.2	11.3	11.4	12.0	11.2	12.1	10.8
6	*A. peruensis* PER	4.6	11.1	10.3	2.2	9.9		11.1	10.3	11.6	10.4	10.8	10.9	10.7	11.3	11.1	12.1	10.7	12.3	10.5
7	*A. spumarius* SS PER	11.4	11.1	12.5	10.5	11.3	10.3		4.5	4.7	2.8	3.4	3.7	4.3	2.8	4.1	3.8	3.9	3.2	4.9
8	*A. pulcher* PER	11.0	10.9	9.9	9.6	9.5	9.6	4.3		3.4	2.6	3.3	2.7	3.4	2.7	3.1	2.8	2.9	3.1	4.0
9	*A. flavescens* GUF	12.4	11.3	11.3	11.2	10.9	10.7	4.6	3.3		3.2	2.6	1.1	1.7	0.4	1.5	1.1	1.3	0.8	3.0
10	*A. seminiferus* PER	11.2	11.4	10.7	9.9	10.3	9.7	2.7	2.6	3.1		2.4	2.2	2.8	2.2	2.5	2.7	2.4	2.6	3.2
11	*A.* sp. Anapu BRA	11.9	11.7	12.0	10.1	11.9	10.0	3.3	3.2	2.5	2.3		2.3	3.0	2.6	2.7	2.8	2.6	2.8	3.0
12	*A. franciscus* GUF	11.4	11.6	11.0	10.5	10.6	10.1	3.6	2.7	1.1	2.1	2.3		0.5	0.2	0.4	0.5	0.4	0.4	2.7
13	*A. barbotini* "B" GUF	11.0	11.7	10.4	10.4	10.3	9.9	4.2	3.3	1.7	2.7	2.9	0.5		0.8	0.7	1.0	1.3	1.1	3.0
14	*A. barbotini* "A" GUF	12.0	11.5	11.0	10.7	10.4	10.4	2.7	2.6	0.4	2.2	2.5	0.2	0.8		0.5	0.4	0.4	0.6	2.3
15	*A. hoogmoedi* GUF	11.6	11.9	11.2	10.7	10.5	10.2	4.0	3.0	1.4	2.5	2.7	0.4	0.7	0.5		0.4	0.6	0.3	2.9
16	*A. hoogmoedi* GUY	12.9	12.1	11.6	11.5	11.1	11.1	3.7	2.8	1.1	2.7	2.8	0.5	1.0	0.4	0.4		0.4	0.4	2.7
17	*A. hoogmoedi* RU BRA	11.4	11.3	10.7	10.4	10.3	9.9	3.8	2.9	1.3	2.3	2.5	0.4	1.2	0.4	0.6	0.4		0.2	2.7
18	*A. hoogmoedi* MA BRA	12.8	12.1	12.0	11.3	11.1	11.3	3.1	3.0	0.8	2.5	2.7	0.4	1.0	0.6	0.3	0.4	0.2		2.4
19	*A. manauensis* sp. nov. BRA	11.6	11.5	11.0	10.1	10.0	9.7	4.7	3.9	3.0	3.1	2.9	2.7	2.9	2.3	2.8	2.7	2.6	2.3	

730

731 *3.2. Morphological and Bioacoustic Analyses*

732 The multidimensional morphometric (Figure 2A) and bioacoustic (Figure 2B) spaces
733 occupied by *Atelopus manauensis* sp. nov. and the two geographically closest populations of *A.*
734 *hoogmoedi* (REBIO Uatumã and Pitinga River) were distinct for the first two axes of the PCAs
735 (MANOVA morphology: Pillai trace = 0.69, degrees of freedom (df) = 16, $p < 0.000001$;
736 MANOVA bioacoustic: Pillai trace = 1.00, df = 22, $p < 0.002$). The DAPCs were congruent
737 with the results of the PCAs and showed that the two species can be distinguished from each
738 other with maximum values of posterior probability of membership through morphology
739 (Figure 2C) and advertisement calls (Figure 2D).



740

741 **Figure 2.** Principal Component Analysis and Discriminant Analysis of Principal
742 Components: (A) PCA for morphological variables (males); (B) PCA for
743 advertisement call; (C) DAPC for morphological variables (males); and (D)
744 DAPC for advertisement call of *Atelopus manauensis* sp. nov. (orange – Manaus,
745 Amazonas); *A. hoogmoedi* (yellow – Amapá); and populations of *A. hoogmoedi*
746 of REBIO Uatumã and Pitinga River (blue – Presidente Figueiredo, Amazonas),
747 closest localities to *A. manauensis* sp. nov. with the occurrence of species of
748 *Atelopus*. All localities are in Brazil.

749 The variables that contributed most to the morphology DAPC were, in decreasing order,
750 ITNA, THBL and HW, while for the bioacoustic DAPC they were the pulse duration of the
751 central pulse, number of pulses, and pulse duration of the last pulse (Table 3). The
752 multidimensional acoustic space occupied by *Atelopus manauensis* sp. nov. and that occupied
753 by *A. hoogmoedi* of REBIO Uatumã, Pitinga River and Amapá in the first two axes of the PCA
754 (Figure 2B) were distinct (MANOVA bioacoustic: Pillai trace = 1.00; df = 22, P = 0.002). The
755 DAPC with advertisement call variables obtained 100% accuracy in attributing the calls of the
756 analysed individuals to their respective species (Figure 2D). The contribution of each acoustic
757 variable and the explanation value of the first two PCs are provided in Table 3.

758 **Table 3.** Contribution of each variable to the first two axes of the PCAs in
759 multidimensional morphological and acoustic spaces to explain the variation between
760 *Atelopus manauensis* sp. nov. and *A. hoogmoedi* of REBIO Uatumã, Pitinga River and
761 Amapá, Brazil. The total percentage of explanation of the variation between species and
762 populations for each axis are provided at the bottom. Abbreviations: (SVL) snout-vent
763 length, (SW) sacrum width, (HW) head width, (HL) head length, (EYDM) eye diameter,
764 (EYNO) eye-to-nostril distance, (IOD) interorbital distance, (ITNA) internarial distance,
765 (RDUL) length of flexed forearm, (HAND) hand length, (THBL) thumb length, (FOOT)
766 foot length, (TL) tibia length. Bold numbers highlight five variables with highest
767 contribution values for each Principal Component (PC).

Morphology	**PC1**	**PC2**	**Call**	**PC1**	**PC2**
SVL	**0.3731**	-0.0989	Call_dur	-0.2697	**0.3854**
HW	**-0.3729**	-0.0944	N_pulses	**0.3058**	**0.3564**
HL	**-0.3726**	-0.0171	Puls_dur_1	**-0.3007**	-0.0782
EYDM	**-0.3512**	-0.0744	Interpulse_1	**-0.4138**	-0.0042
ITNA	**-0.3482**	0.0172	Puls_dur_2	-0.2513	0.0300
IOD	-0.2834	-0.0745	Interpulse_2	**-0.3626**	-0.3038
EYNO	-0.0531	-0.0017	Puls_dur_3	0.0295	0.1371
SW	-0.3230	**-0.1655**	Interpulse_3	-0.2898	0.3132
FOOT	0.0910	**-0.5572**	Dur_3_first_puls	**-0.4367**	-0.0368
THBL	0.2361	**-0.2680**	Dur_3_last_puls	-0.2934	**0.3254**
HAND	0.0977	**-0.5271**	Domi_frequency	-0.1183	**-0.4472**
TL	-0.2663	-0.0910	bandwidth	0.0440	**-0.4483**
RDUL	-0.0798	**-0.5233**	–	–	–
Explanation (%)	48.7	17.5	Explanation (%)	38.7	25.3

768

769 *3.3. Taxonomic account*

770 *Atelopus manauensis* sp. nov.

771 LSID: urn:lsid:zoobank.org:act:5D9F4EF7-74D2-4A48-AE50-F42282439C86

772 *Atelopus pulcher*: Zimmerman [12]; Zimmerman & Bierregaard [13]; Zimmerman &
773 Simberloff [14]; Gascon [15].

774 *Atelopus spumarius* sensu lato: Lötters et al. [4]; Rößler et al. [23].

775 *Atelopus spumarius*: Jorge et al. [22].

776 *Atelopus* sp.: Jorge et al. [29].

777 **Examined material:** Holotype. INPA-H 041378 (labelled "HOLOTYPE" in the field),
778 adult male (Figure 3A,B) collected on 28th February 2019 by R.F. Jorge on the banks of a
779 stream in the Tinga drainage system, Reserva Florestal Adolpho Ducke (RFAD; 2°55'4.8" S,
780 59°53'52.8" W), middle course on the west bank of Puraquequara River, tributary of the north
781 bank of the Amazon River, municipality of Manaus, Amazonas, Brazil.

782 Paratopotypes. Eight adult specimens (six males and two females) collected at the same
783 stream of the Holotype. Five males collected on the same day of the Holotype INPA-H 041365,
784 INPA-H 041366, INPA-H 041367, INPA-H 041368 and INPA-H 041369 (field number ST1,
785 ST2, ST3, ST4 and ST5, respectively) by R.F. Jorge; one female and one male INPA-H 041377
786 and INPA-H 041376 (field number 29RD and 31RD) collected on 20th February 2017 by R.F.
787 Jorge; and a female INPA-H 011900 collected by D.J. Rodrigues on 29th April 2015. The
788 female INPA-H 041377 (29RD) is designated the Allotype (Figure 3C,D).

789 Paratypes. Four adult specimens (all males), all from the state of Amazonas. Two males
790 INPA-H 041370 and INPA-H 041371 (field number PTMP02-4 and PTMP02-5) collected by
791 R.F. Jorge on 17th January 2019 on the margins of a stream in the middle course of the east
792 bank of the Puraquequara River at CIGS/EB (2°54'52.4" S, 59°49'26.4" W) municipality of
793 Manaus; a male INPA-H 041374 (field number 56DI) collected by R.F. Jorge and V.S. Pimentel
794 on 1st December 2017 on the banks of a stream at the headwaters of the Cuieiras River, at
795 Fazenda Dimona of PDBFF (2°19'43.39" S, 60°4'41.46" W), municipality of Manaus; a male
796 INPA-H 041375 (field number 05ZF2) collected by R.F. Jorge and S.S. Sales on 19th January
797 2017 on the banks of a stream in the middle course of the Cuieiras River near LBA/ZF2
798 scientific station (a tributary of the east bank of the Negro River – 2°33'32.40" S, 60°13'48.36"
799 W), municipality of Manaus, Amazonas, Brazil.

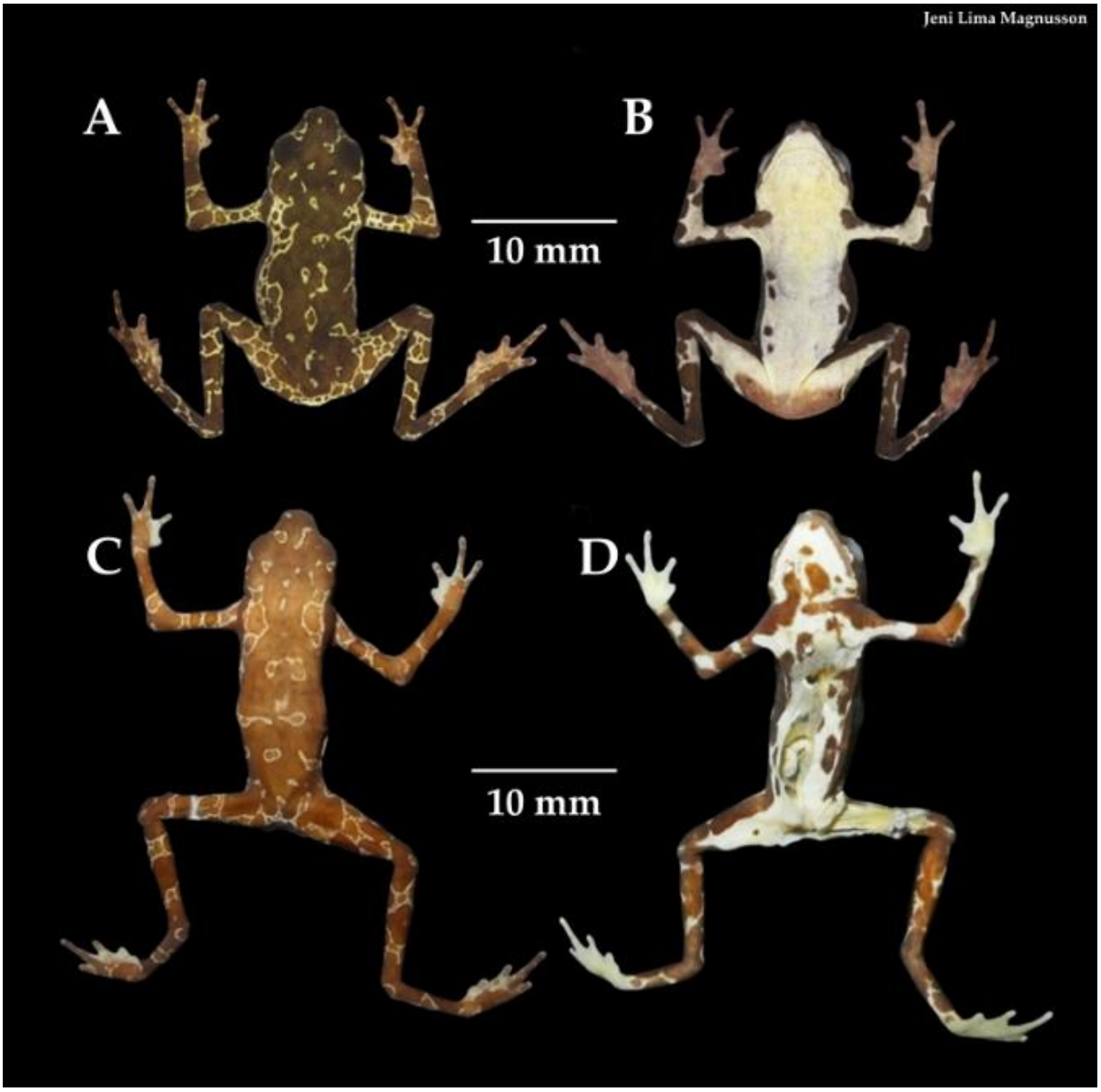


Figure 3. Specimens of *Atelopus manauensis* sp. nov. in preservative: (A) Dorsal and (B) Ventral view of the holotype (INPA-H 041378); (C) Dorsal and (D) Ventral view of the allotype (INPA-H 041377).

Referred specimens: Four adult specimens (all males), all from the state of Amazonas. Two males INPA-H 041289 and INPA-H 041290 (field number 02ZF2 and 03ZF2) collected by R.F. Jorge and S.S. Sales on 19th January 2017 on the banks of a stream in the middle course of the Cuieiras River near LBA/ZF2 scientific station (2°33'32.40" S, 60°13'48.36" W), municipality of Manaus; two males INPA-H 041291 and INPA-H 041292 (field number 16FU and 17FU) collected by R.F. Jorge and S.S. Sales on 26th January 2017 on the margins of a stream in the headwaters of the Taruma-Açu River at Fazenda Experimental da Universidade Federal do Amazonas (2°38'31.20" S, 60°5'45.6" W), municipality of Manaus. These specimens were used

for genetic analyses. Two males INPA-H 041372 and INPA-H 041373 (field number: PTAP03-1 and 55DI) were used to record the type 2 and type 3 calls.

Etymology: The specific epithet *manauensis* refers to the location of the occurrence of the new species, municipality of Manaus, state of Amazonas, Brazil.

Diagnosis: A small species of *Atelopus*; adult males SVL 19.1–26.4 mm (n = 11), adult females 27.9–28.8 mm (n = 2); interdigital webbing covering all of Finger I and a phalange of Finger II and rudimentary between other fingers; absence of serrated fringe on the sides of Finger III (Figure 4A,B); first phalange of Toe I atrophied, completely hidden in skin of the foot (similar to a callus), with no visible phalanges (Figure 5A,B); subarticular tubercles absent from hand and foot; palmar tubercles round and visible and plantar tubercles oval and poorly defined; the interdigital webbing reaches half of the third phalange of Toe IV and half of the first phalange of Toe V; in life, dorsum light brown to reddish brown with light yellow or light green reticulation network (Figure 6A–D); ventral surface of feet, hands, half of the posteroventral portion of thighs and half or all of the posteroventral portion of the cloacal region red (Figure 6E–H); throat, chest and central portion of belly white or whitish cream without spots in males, spotted in females; advertisement call consisting of a single multipulsed note with a call duration of 689–840 ms consisting of 15–26 pulses, with a dominant frequency of 3088–3610 Hz and bandwidth of 633–915 Hz (Figure 7A,B). Morphometric measurements of the holotype and type series are shown in Table 4.

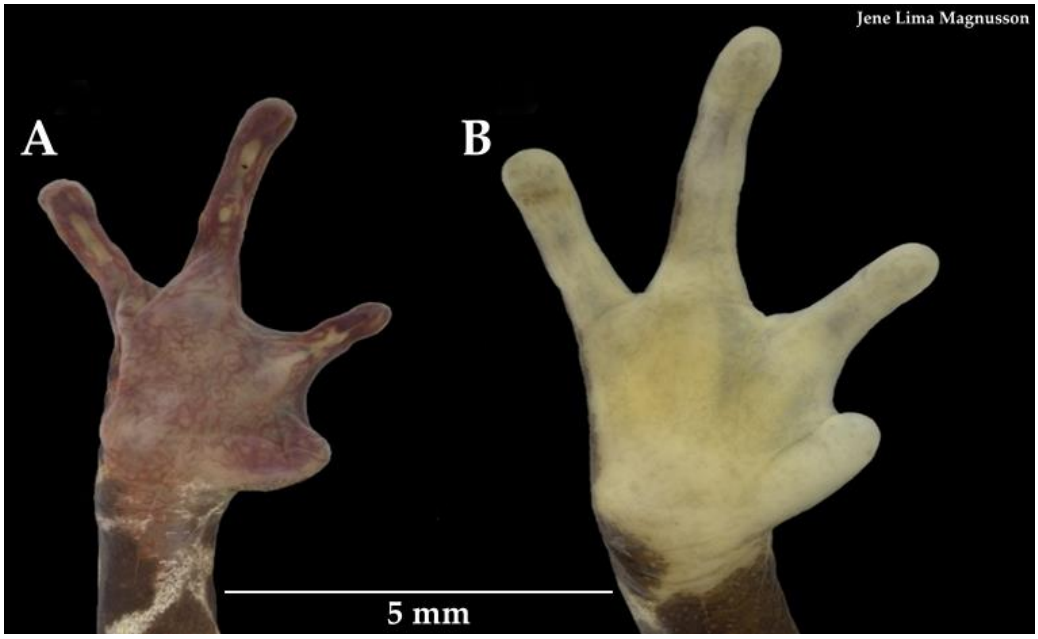
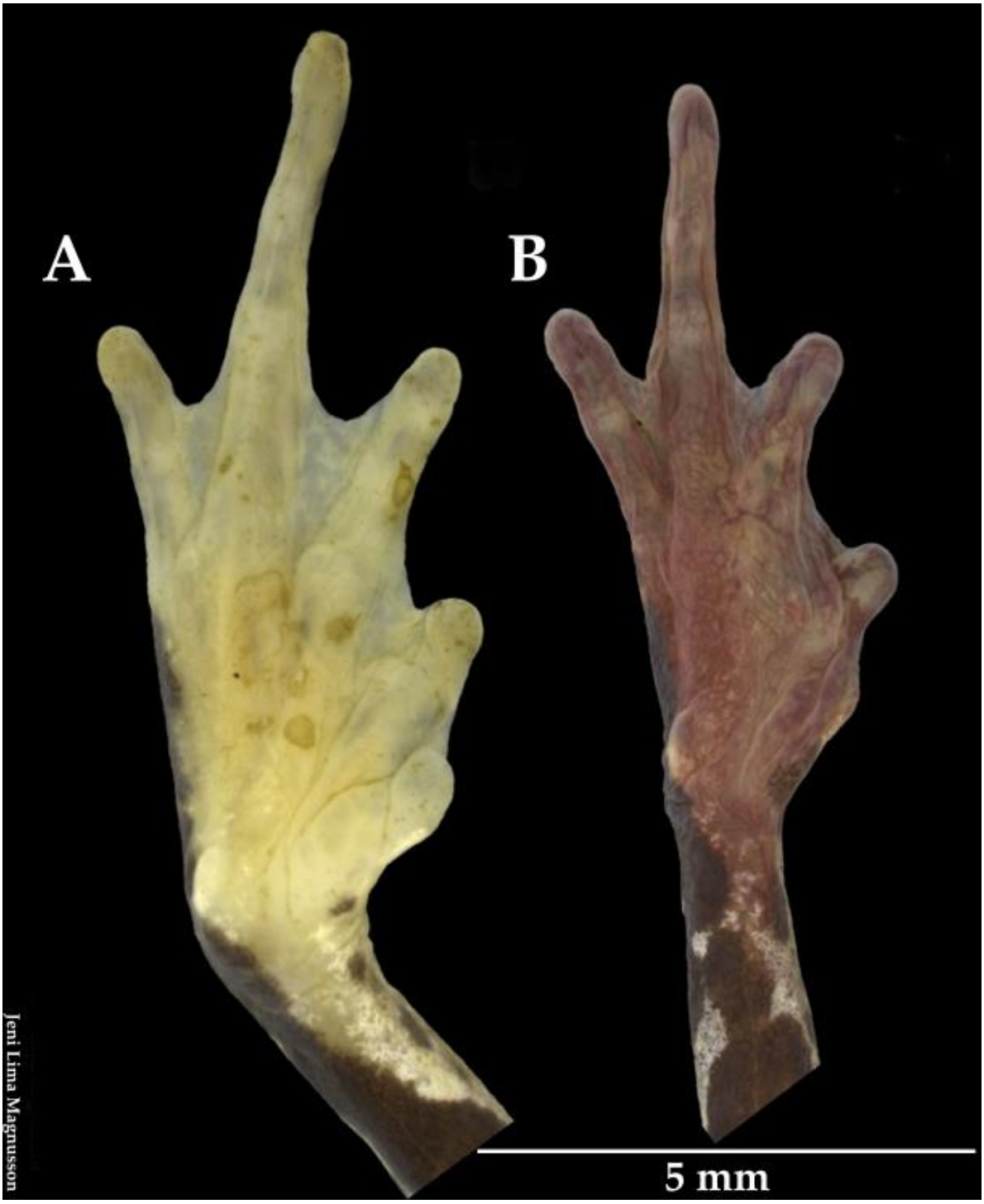


Figure 4. Ventral view of the hand of *Atelopus manauensis* sp. nov. in preservative: (A) holotype; (B) allotype.



834

835 **Figure 5.** Ventral view of the foot of *Atelopus manauensis* sp. nov. in preservative: (A)
836 allotype; (B) holotype.

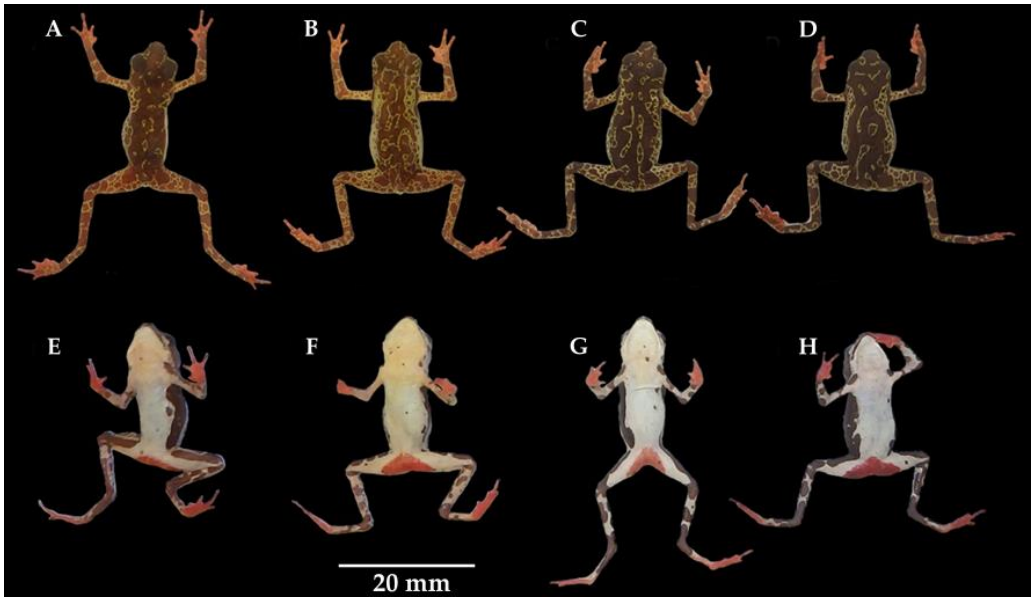


Figure 6. Coloration of the dorsal (upper images) and ventral (lower images) surfaces of the holotype and three males of type series of *Atelopus manauensis* sp. nov. recently euthanized: (A,E) INPA-H 041378, (B,F) INPA-H 041365, (C,G) INPA-H 041367, (D,H) INPA-H 041369. Note the reticulated network on the dorsum of each individual is unique; the variation in the distribution of the red spot on the thighs and posteroventral portion of the cloacal region; and the entirely white venter or white with a cream-colored gular region and head.

Table 4. Morphometric measurements of the holotype and type series of *Atelopus manauensis* sp. nov. Measurements of the holotype, followed by mean, standard deviation and maximum–minimum in parentheses for 11 adult males. Measurements of the two females are presented in the last column. Abbreviations for morphometric measurements are described in Material and Methods. Abbreviation: (*n*) sample size.

Measurement	**Holotype**	**Males (*n* = 11)**	**Females (*n* = 2)**
SVL	21.2	21.8 ± 2.4 (19.1–26.4)	27.9–28.8
SW	6.5	7.1 ± 1.1 (5.7–8.6)	8.4–8.5
HW	7.3	7.4 ± 0.5 (6.5–8.3)	8.3–8.4
HL	7.5	7.8 ± 0.6 (6.8–8.7)	8.9–9.2
EYDM	2.4	2.4 ± 0.2 (2.1–2.7)	2.7–3.0
EYNO	2.4	2.5 ± 0.3 (2.1–3.0)	2.8–3.0
IOD	3.0	2.9 ± 0.3 (2.5–3.6)	3.1–3.1
ITNA	2.6	2.7 ± 0.2 (2.4–3.1)	3.1–3.1
RDUL	6.3	6.7 ± 0.8 (6.0–8.2)	8.7–10.0
HAND	5.0	5.2 ± 0.6 (4.9–6.6)	6.4–7.5
THBL	2.2	2.2 ± 0.2 (1.9–2.5)	2.5–3.0
FOOT	7.3	7.7 ± 1.1 (6.4–10.4)	10.4–10.9
TL	9.8	10.3 ± 0.8 (9.8–11.9)	12.1–12.9

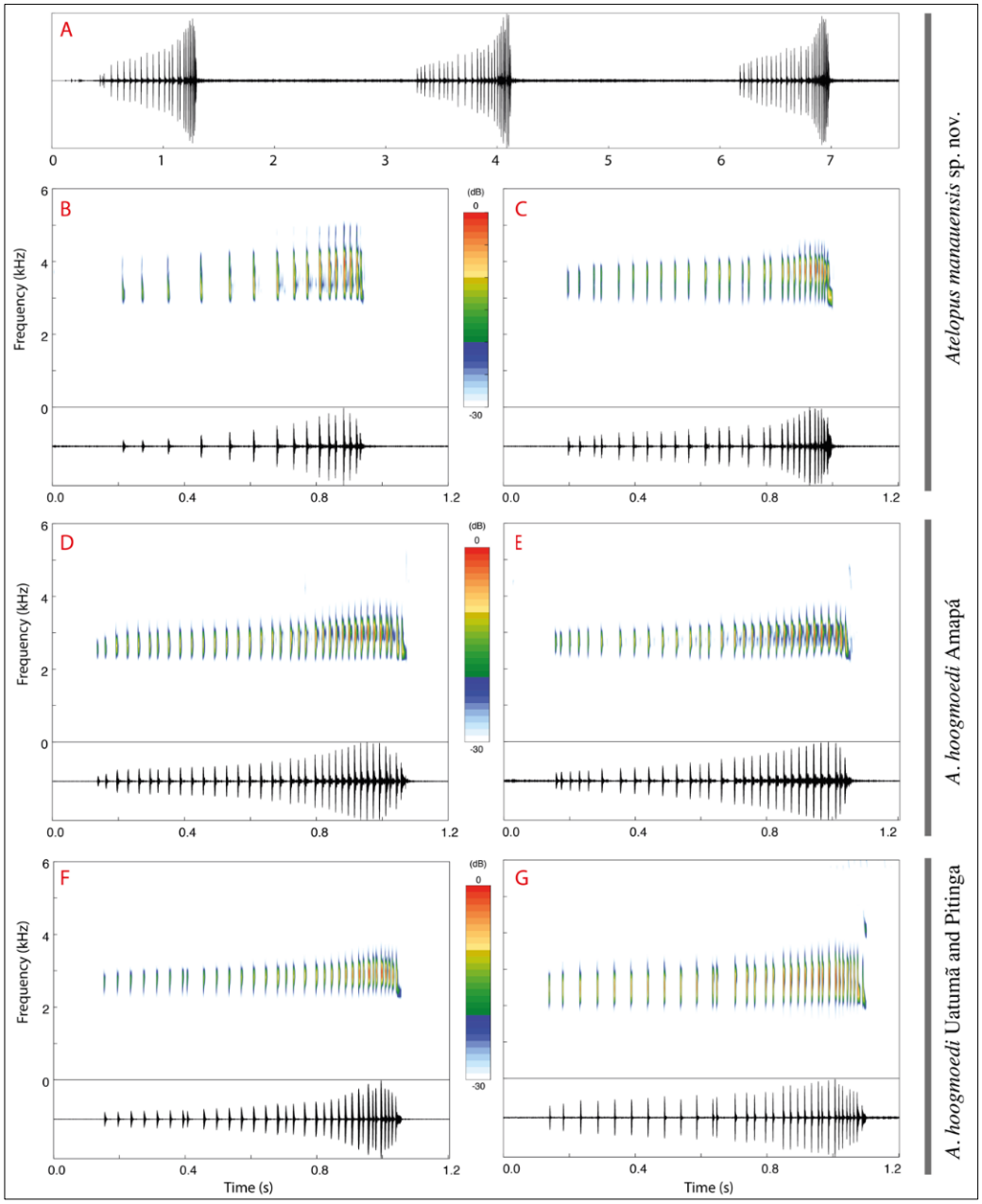


Figure 7. Advertisement call (type 1 call) of *Atelopus manauensis* sp. nov., and *A. hoogmoedi* of Amapá, of REBIO Uatumã, and of Pitinga River: (A) Oscillogram of three advertisement calls issued at regular intervals by an uncollected male of *A. manauensis* sp. nov. in Reserva Florestal Adolpho Ducke (RFAD), Manaus, Amazonas; (B) Spectrogram and oscillogram showing an advertisement call of the Holotype and (C) another uncollected specimen of *A. manauensis* sp. nov. in RFAD; (D,E) Spectrograms and oscillograms of the advertisement calls of two specimens of *A. hoogmoedi* of Amapá described by Campos & Carvalho [50]; (F,G) Spectrograms and oscillograms of males of *A. hoogmoedi* of REBIO Uatumã (F) and Pitinga River (G), Amazonas. All localities are in Brazil.

3.3.1. Taxonomic Comparisons

Interspecific morphological and bioacoustic comparisons were made between *Atelopus manauensis* sp. nov. and the geographically and phylogenetically closest species. Characteristics of the compared species are presented in parentheses, unless specified.

Atelopus manauensis sp. nov. differs from the population of *A. hoogmoedi* of REBIO Uatumã and Pitinga River by lacking basal and subarticular tubercles on the hands [*A. hoogmoedi* basal tubercles present on hands (Figure 8A) and feet and two subarticular tubercles on Toe IV (Figure 8B)]. *Atelopus manauensis* sp. nov. is smaller than any individual from the populations of *A. hoogmoedi* of REBIO Uatumã and Pitinga River and is differentiated by a maximum SVL of 26.4 mm for adult males (minimum SVL for *A. hoogmoedi* of REBIO Uatumã and Pitinga River 32.9 mm); dorsum light brown to reddish brown with a light yellow or light green reticulation network (dorsum dark brown to black with yellow reticulation network); ventral surface of hand and feet red in life, cream or brown in preservative, without spots (cream-colored with dark brown spots in life and in preservative); venter all white or white with a cream-colored gular region and head (bright yellow); red spot restricted to half of the posteroventral portion of thighs and all or half of the posteroventral portion of cloacal region red (black spot limited to the cloacal region); advertisement call with a maximum duration of 840 ms (1071 ms) consisting of up to 26 pulses (37 pulses) and a dominant frequency of 3088–3610 Hz (2498–3058 Hz) (Figure 7F,G).

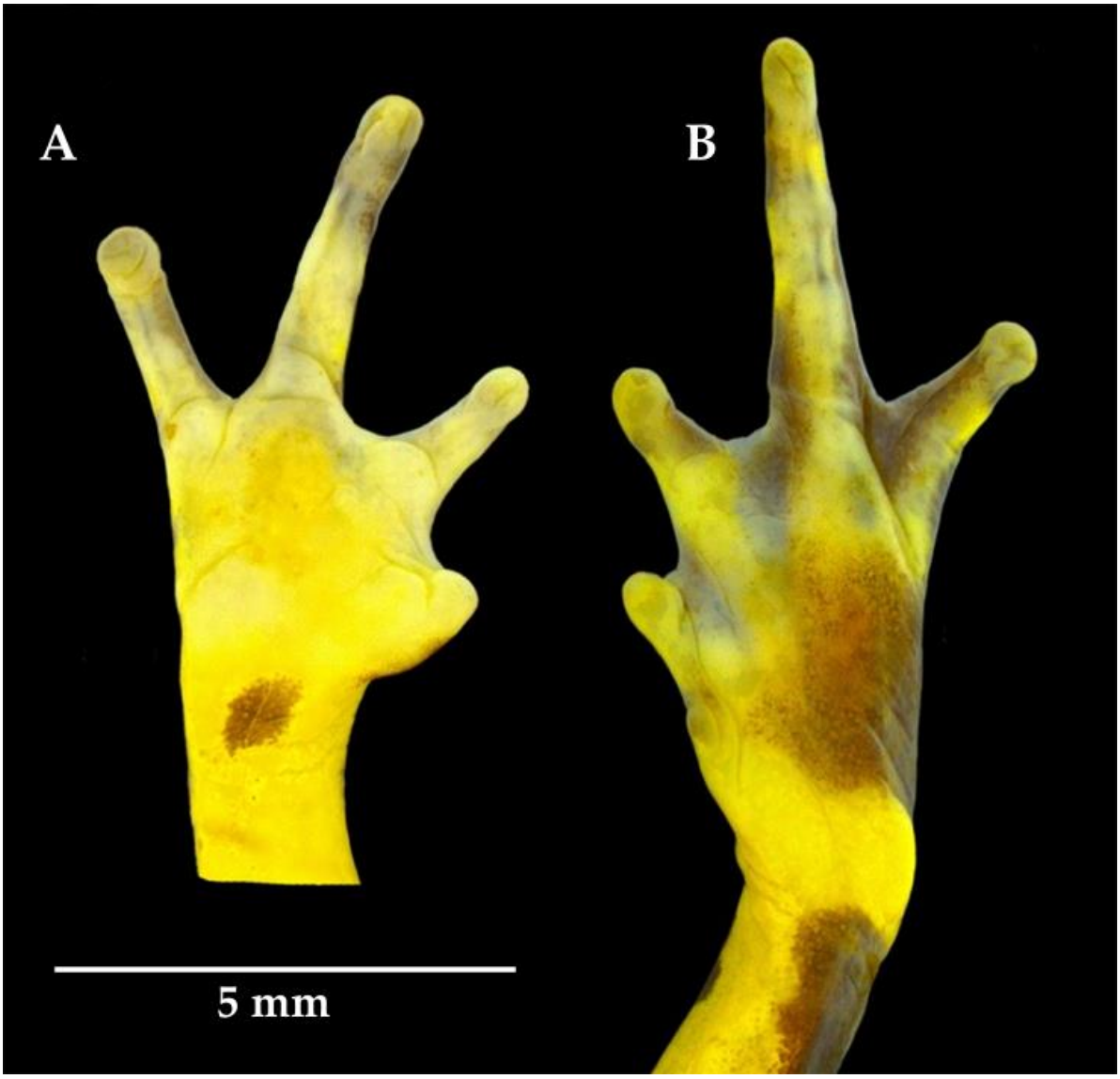


Figure 8. Ventral region of the hand (A) and foot (B) of *Atelopus hoogmoedi* from REBIO Uatumã (INPA-H 041293).

It differs from *Atelopus hoogmoedi* of Amapá (Brazil) by having a light brown to reddish brown dorsum with a light yellow or light green reticulation network (dark brown to black dorsum with yellow, orange or pink reticulation network); venter all white or white with cream-colored gular region and head (bright yellow); red spot restricted to half of the posteroventral region of thighs and half or all of the posteroventral portion of the cloacal region red (red spot covering the entire posteroventral portion of the cloacal region extending to near the knee and to the initial portion of the belly; photos C.E. Costa-Campos at AmphibiaWeb/*Atelopus hoogmoedi*); advertisement call duration of 689–840 ms (873–1710 ms; [50]) consisting of up to 26 pulses (35 pulses; [50]), dominant frequency of 3088–3610 Hz (2812–2838 Hz; [50]) and a bandwidth of 633–915 Hz (280–568 Hz; [50]) (Figure 7D,E).

Atelopus manauensis sp. nov. can be differentiated form *A. hoogmoedi* SS of French Guiana by a maximum SVL of 28.8 mm for females (minimum SVL 31.2 mm for females; [9]), tibia 47% of SVL (tibia varying from 43% to 45% of SVL among populations; [9]); dorsum light brown to reddish brown with light yellow or light green reticulation network (dorsum dark brown to black with yellow, orange or pink reticulation network; [52,53]); and advertisement call duration of 689–840 ms (1190–1200 ms; [7]) consisting of 15–26 pulses (40–42 pulses; [7]).

It differs from *Atelopus hoogmoedi* of Guyana by having a light brown to reddish brown dorsum with a light yellow or light green reticulation network (dark brown to black dorsum with yellow dorsolateral bands and marks with black spots; [54]); venter all white or white with a cream-colored gular region and head (venter, throat and head yellow, pink or orange with irregular black marks; [54]); interdigital webbing covering all of Finger I and a phalange of Finger II and rudimentary between other fingers (fingers unwebbed; [54]); longer interdigital webbing between toes IV and V (toes moderately webbed; [54]); and Toe I reduced (Toe I and Toe II much reduced; [54]).

The new species is distinguished from *Atelopus franciscus* by the larger size of females, a minimum SVL of 27.9 mm (maximum SVL of 26.5 mm; [9]); venter all white or white with a cream-colored gular region and head (venter and thighs red; [9]); and advertisement call duration of 689–840 ms (1340–1640 ms; [7]) consisting of 15–26 pulses (31–39 pulses; [7]).

Atelopus manauensis sp. nov. differs from *A. flavescens* by the smaller size of females, maximum SVL of 28.8 mm (minimum SVL of 31.5 mm; [9]), and of males, maximum SVL of 26.4 mm (minimum SVL of 27 mm; [9]); shorter tibia in females of 12.1–12.9 mm (minimum tibia length 13.0–16.0 mm; [9]); dorsum light brown to reddish brown with light yellow or light green reticulation network (dorsum varies in light yellow tones with small brown or light red vermiculation; [9]); venter all white or white with cream-colored gular region and head in males and white with rounded spots in females (venter pink-salmon in females and pink in males; [8]); and advertisement call duration of 689–840 ms (1340–1820 ms; [7]) consisting of 15–26 pulses (45–58 pulses; [7]).

The new species is distinguished from *A. barbotini* by having on average a longer tibia in males (TL/SVL = 0.47 in *A. manauensis* sp. nov.; TL/SVL = 0.44–0.45 in *A. barbotini*; [7]); dorsum light brown to reddish brown with light yellow or light green reticulation network (dorsum black with scattered sinuous lines of opaque red colors; Lescure [7]); and advertisement call duration of 689–840 ms (1300–1680 ms; [7]) consisting of 15–26 pulses (41–53 pulses; [7]).

Atelopus manauensis sp. nov. differs from *A. spumarius* SS by possessing a longer tibia, 47% of SVL (43% of SVL; [7]), although female sizes are similar; absence of small warts behind the eyes (warts present; [54]); and advertisement call consisting of up to 26 pulses (37 pulses; [7]).

The new species differs from *A. pulcher* by its smaller size, with a maximum SVL for females of 28.8 mm (minimum SVL of 32.0 mm; [4]); longer interdigital webbing between toes IV and V (shorter interdigital webbing; [4]); no subarticular tubercles on the hand or foot (ill-defined subarticular tubercles on fingers II, III and IV and toes II, III, IV and V; [4]); venter all white or white with a cream-colored gular region and head in males and white with dark brown rounded spots in females (light red venter; [4]); red spot restricted to half of the posteroventral region of thighs and all or half of the posteroventral portion of the cloacal region red in males and females (red spot covering the entire ventral region of the thighs and all of the posteroventral portion of the cloacal region extending to the belly in males, and all of the venter, thighs and head red with black spots in females; [4]); advertisement call duration 689–840 ms (1100–1300 ms; [4]) consisting of up to 26 pulses (47 pulses; [4]) and a dominant frequency of 3088–3610 Hz (2034–2824 Hz; [4]).

Finally, *Atelopus manauensis* sp. nov. differs from *A. seminiferus* by the smaller size of males and females: SVL 19.1–26.4 mm in males, 27.9–28.8 mm in females (SVL 33.8–35.2 mm in males, 40 mm in females; [56]); interdigital webbing covers two phalanges of Toe IV (interdigital webbing covers all of Toe IV; [57]); dorsum light brown to reddish brown with a light yellow or light green spots (dorsum uniformly dark brown or black with small yellow spots; [56,57]); venter all white or white with a cream-colored gular region and head (venter dark brown streaked with white and orange; [56]); and smooth sides (sides with tubercles; [57]).

Description of the Holotype: Body slender; neural spines not evident externally; head slightly longer than wide (HW/HL = 0.97); snout acuminate, with oval tip, dorsally concave; head length 30% of SVL; maxilla projected slightly over mandible; nostrils lateral and not visible from above; canthus rostralis concave between nostril and tip of snout and moderately straight between eye and nostril; loreal region concave; nostril closer to tip of snout than to eye; distance between nostrils and eye equal to eye diameter; distance between nostrils greater than eye diameter; tibia 51% of SVL; foot shorter than tibia (FOOT/TL = 0.74); relative sizes of fingers I < II < IV < III and toes I < II < III < V < IV, with first phalange of Toe I being atrophied and the toe hidden in the skin, similar to a callus; hand webbing rudimentary, present only between Finger I and Finger II; foot webbing, disregarding the Toe I in the form of a callus: II 0−2−, III 11/2–2 −1/2, IV 2 −1/3−1+ V; palmar tubercle well defined and rounded

961 (Figure 4A) and plantar tubercle poorly defined and oval (Figure 5A); subarticular tubercles
962 absent; thumb 44% of hand length (THBL/HAND = 0.44), covered by tiny brown keratinized
963 spikes (nuptial pads); dorsal skin smooth; belly with tiny black keratinized dots; gular region
964 smooth. In life, dorsal and lateral surfaces of the body with a lime-green reticulated network
965 spread irregularly on a chocolate brown background. Ventral region white with a cream-colored
966 gular region and head; lower part of limbs with brown bands on the sides. Red spot restricted
967 to half of the posteroventral region of thighs and half of the posteroventral portion of the cloacal
968 region (Figure 6E). In preservative, the dorsal surface was brown and the reticulated network
969 was pale yellow. Ventral region colored similar as in life, only the red spots on the hands, feet,
970 posteroventral region of thighs, and posteroventral portion of the cloacal region turn reddish
971 brown (Figure 3A–D).

972 **Vocalization:** The advertisement call of *Atelopus manauensis* sp. nov. consists of a single
973 multi-pulsed note issued at regular time intervals (Figure 7A). The mean call duration is 744 ±
974 84 ms (689–840 ms) and consists of 19 ± 6 pulses (15–26 pulses) (Figure 7B). Based on the
975 inter-pulse interval, the song can be temporally divided into two portions, with the pulses being
976 emitted more widely during the first two-thirds of the call than during the last third. The first
977 pulse and central pulse have similar average pulse durations of 7 ± 1 ms (6–8 ms) and 6 ± 1 ms
978 (5–7 ms), respectively. On the other hand, the last pulse is on average longer than all the other
979 pulses in the song with a pulse duration of 14 ± 6 ms (8–19 ms), with downward frequency
980 modulation (Figure 7C). The inter-pulse interval at the beginning and center of the song lasts
981 for 54 ± 16 ms (36–67 ms) and 50 ± 13 ms (40–65 ms), respectively, while the inter-pulse
982 interval between the last two pulses lasts for 7 ± 3 ms (4–9 ms). The total pulse period duration
983 of the first three pulses [132 ± 40 ms (92–172 ms)] is approximately three times longer than the
984 pulse period duration of the last three pulses of the call [43 ± 6 ms (38–49 ms)]. The call has a
985 dominant frequency of 3,334 ± 263 Hz (3,088–3,610 Hz) and a bandwidth of 743 ± 151 Hz
986 (633–915 Hz). The temporal and spectral parameters of the advertisement call of *Atelopus*
987 *manauensis* sp. nov., *A. hoogmoedi* of REBIO Uatumã, of Pitinga River, and of Amapá are
988 provided in Table 5.

989 Type 2 call (pure tone call) of *Atelopus manauensis* sp. nov. consists of an unpulsed short
990 note (Figure 9A–C) with a call duration of 137–217 ms (174 ± 24 ms, n = 15) and shows an
991 upward frequency modulation from the onset until the central portion of the call, and a
992 downward frequency modulation from the central until the final portion (Figure 9C). Calls are
993 commonly emitted singly (n = 12) and in a series of two (n = 5) or three (n = 2) calls. The
994 average inter-call interval within a series is 499 ± 39 ms (434–541 ms, n = 6) while the inter-

call interval between single calls is 2793 ± 1441 ms (963–4766 ms, n = 10). Type 2 calls have a dominant frequency of 2928–3143 Hz (3059 ± 75 Hz, n = 15), a low frequency of 2630–2692 Hz (2676 ± 19 Hz, n = 15), and a high frequency of 3052–3252 Hz (3171 ± 72 Hz, n = 15). The average bandwidth is 303 ± 55 Hz (215–388 Hz, n = 15).

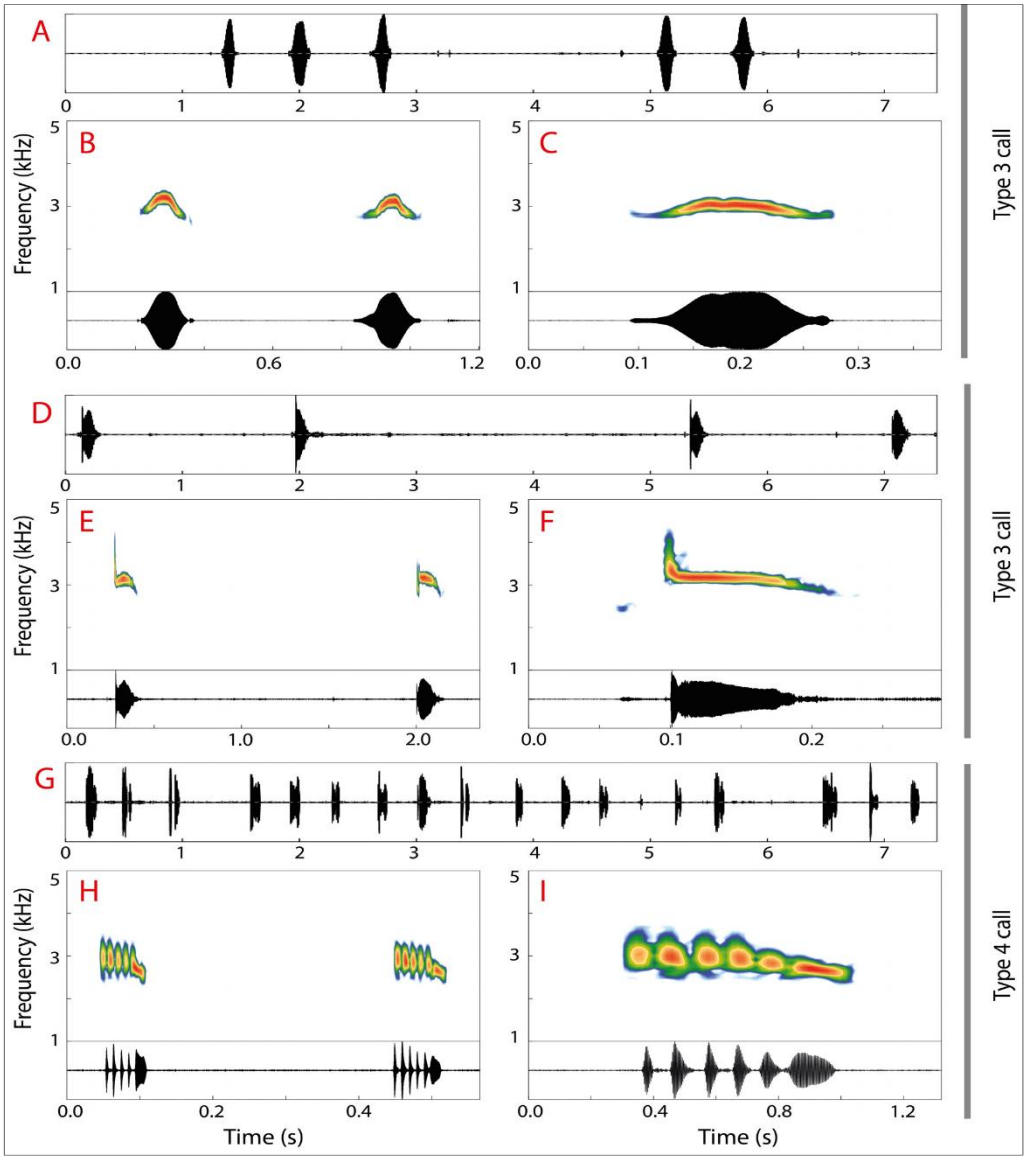


Figure 9. Type 2, type 3, and type 4 calls of *Atelopus manauensis* sp. nov.: (A) Oscillogram showing type 2 calls emitted within approximately 7.5 s; (B) Spectrogram and oscillogram showing two calls of the type 2 call; (C) A detailed view of one type 2 call; (D) Oscillogram showing type 3 calls emitted within approximately 7.5 s; (E) Spectrogram and oscillogram depicting two calls of the type 3 call; (F) A detailed view of one call of type 3 call; (G) Oscillogram showing type 4 calls emitted within approximately 7.5 s.; (H)

Spectrogram and oscillogram showing two calls of the type 4 call; (I) A detailed view of one call of type 4 call. Note the difference of frequency modulation among type 2–4 calls: (A–C) recorded at the training area of the Centro de Instruções de Guerra na Selva do Exército Brasileiro (CIGS/EB; SVL = 20.3 mm; air temperature = 26.5 ◦C; INPA-H 041372); (D–F) recorded at Fazenda Dimona (SVL = 20.9 mm; air temperature = 26.5 ◦C; INPA-H 041373); and (G–I) recorded from the holotype at Reserva Florestal Adolpho Ducke (SVL = 21.2 mm; INPA-H 041378).

The type 3 call (short pure tone call) of *Atelopus manauensis* sp. nov. is characterized by an unpulsed short note (Figure 9D–F) with a call duration of 57–165 ms (129 ± 31 ms, n = 15). The frequency modulation of type 3 calls rapidly descends during the first fifth portion, after which it declines slowly until the end of the call (Figure 9F). Type 3 calls are irregularly emitted and have an inter-call interval of 493–9264 ms (2295 ± 2812 ms, n = 10). Type 3 calls are characterized by having a dominant frequency of 2907–3100 Hz (3020 ± 66 Hz, n = 15), a low frequency of 2722–2907 Hz (2800 ± 69 Hz, n = 15), and a high frequency of 3031–3304 Hz (3174 ± 78 Hz, n = 15). Bandwidth ranges from 129 to 366 Hz (182 ± 75 Hz, n = 15).

Different from type 2 and type 3 calls, the type 4 call (short pulsed call) of *Atelopus manauensis* sp. nov. (Figure 9G–I) consists of a short-pulsed note with a downward frequency modulation. Type 4 calls have a call duration of 53–93 ms (77 ± 10 ms, n = 15), 4–8 pulses (6 ± 1 pulses, n = 15), and inter-call interval of 285–1394 ms (437 ± 285 ms, n = 15). The last pulse is always longer than the others (Figure 9I); the first pulse has a pulse duration of 4–10 ms (5 ± 2 ms, n = 15), while the last pulse has a pulse duration of 13–25 ms (17 ± 4 ms, n = 15). Type 4 calls are emitted with a dominant frequency of 2585–2842 Hz (2721 ± 80 Hz, n = 15), a low frequency of 2358–2482 Hz (2421 ± 46 Hz, n = 15), and a high frequency of 3181–3421 Hz (3319 ± 77 Hz, n = 15). The bandwidth of the type 4 call is 409–538 Hz (476 ± 42 Hz, n = 15).

Table 5. Advertisement call parameters for *Atelopus manauensis* sp. nov., two Brazilian populations of *A. hoogmoedi* of the Guiana Shield that are the geographically closest to the area of occurrence of *A. manauensis* sp. nov. (REBIO Uatumã and Pitinga River, Amazonas), and a Brazilian population of *A. hoogmoedi* (Pedra Branca do Amapari, Amapá) that is the geographically closest to the occurrence area of *A. hoogmoedi* SS in French Guiana. Abbreviations of the acoustic parameters are defined in Material and Methods. Abbreviation: (*n*) sample size.

Acoustic traits	***Atelopus manauensis* sp. nov. (*n* = 3)**	***Atelopus hoogmoedi* Amapá (*n* = 4)**	***Atelopus hoogmoedi* REBIO Uatumã e Pitinga River (*n* = 7)**
Call_dur (ms)	744 ± 84 (689–840)	1164 ± 387 (873–1710)	858 ± 89 (733–1071)
N_pulses	19 ± 6 (15–26)	29 ± 5 (23–35)	29 ± 5 (21–37)
Puls_dur_1 (ms)	7 ± 1 (6–8)	7 ± 1 (6–9)	6 ± 2 (3–11)
Puls_dur_2 (ms)	6 ± 1 (5–7)	8 ± 1 (7–9)	7 ± 2 (4–12)
Puls_dur_3 (ms)	14 ± 6 (8–19)	14 ± 5 (10–22)	15 ± 4 (7–27)
Interpulse_1 (ms)	54 ± 16 (36–67)	48 ± 33 (15–78)	35 ± 9 (19–50)
Interpulse_2 (ms)	50 ± 13 (40–65)	37 ± 11 (23–47)	32 ± 9 (15–54)
Interpulse_3 (ms)	7 ± 3 (4–9)	10 ± 3 (7–13)	7 ± 2 (4–13)
Dur_3_first_puls (ms)	132 ± 40 (92–172)	120 ± 66 (61–178)	88 ± 16 (56–116)
Dur_3_last_puls (ms)	43 ± 6 (38–49)	54 ± 5 (49–59)	42 ± 7 (30–59)
Domi_frequency (Hz)	3,334 ± 263 (3088–3610)	2825 ± 13 (2812–2838)	2,741 ± 161 (2498–3058)
Bandwidth (Hz)	743 ± 151 (633–915)	442 ± 127 (280–568)	555 ± 94 (388–754)

3.3.2. Variability

Male INPA-H 041376 is 4.6 mm larger than average in relation to total body size (SVL = 26.4 mm); its nuptial callus is a little evident; and the spot on the lower ventral portion was light red and changed to cream-color in preservative, similar to the two females. In general, the other males are similar to the holotype: nine individuals have an all-white venter and two have a white belly and cream-colored gular region and head; all males have the hands, feet, and half of the posteroventral portion of the thighs colored red, while seven individuals had this spot covering the entire posteroventral portion of the cloacal region and four had it covering only half of this region. There is a variation in the light yellow or light green reticulation network on a background of different shades of brown on the dorsum; each individual has its own unique pattern, as if it were an individual fingerprint (Figure 6A–D). In preservative, red spots on the hands, feet, posteroventral region of the thighs, and posteroventral portion of the cloacal region turn reddish brown in males and cream in females.

Some males (n = 3) have an eye diameter greater than the distance from the eye to the nostril, but in general it is the same or slightly smaller (ED/EYNO = 0.87–0.96). Females are considerably larger than males. Nuptial pads and vocal slits are present in males. Females have a wrinkled gular region, whereas this region is smooth and white or cream-colored in males; and females have a venter without spicules and with brown spots, unlike males who possess a venter with spicules and is rarely spotted.

3.4. Distribution and Natural History

The distribution of *Atelopus manauensis* sp. nov. is limited to the interfluve between the Negro and the Uatumã Rivers in the Alter do Chão formation (absent from the Guiana Shield and the Trombetas Group), within a narrow altitudinal range (61–125 m a.s.l.), and restricted to the micro-interfluve between the east bank of the Cuieiras River and the west bank of the Urubu River (Figure 10), which are tributaries of the Negro and the Amazon Rivers, respectively [29]. In this region, *Atelopus manauensis* sp. nov. inhabits the banks of small streams in dense ombrophilous forests of lowlands not flooded by large rivers [22]. Most of the individuals can be found within a four-meter strip along the banks of these streams, but some can be seen up to 10 meters form the bank.

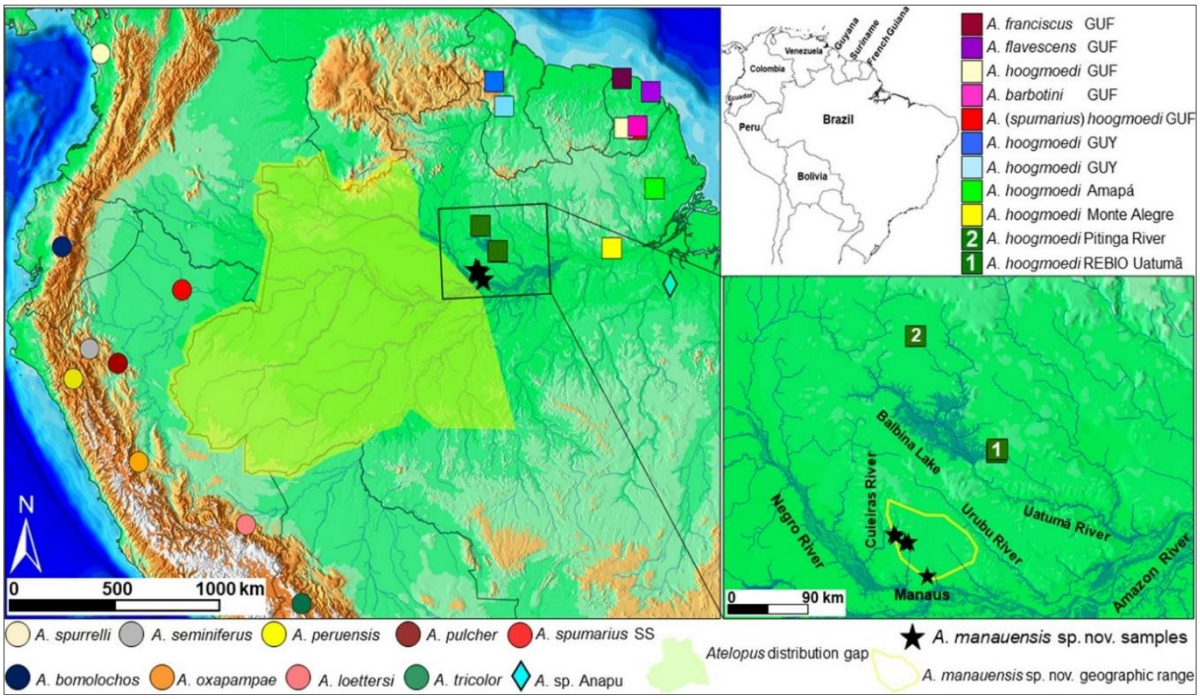


Figure 10. Distribution map for species of *Atelopus* used in the calculations of genetic distances and phylogenetic inferences, with colored circles representing species distributed to the west of Amazonia, on eastern and western versants of the Andes and Andean highlands; the squares representing species distributed in the Guiana Shield, and the green diamond a candidate species distributed on the south bank of the Amazon River, municipality of Anapu, state of Pará, Brazil [30]. The shaded polygon represents the portion of the Amazonian plain where there has been no record yet of a species of *Atelopus*, indicating a distribution gap for the genus (adapted from Lötters et al. [32]). Insert in the lower right shows the limits of the geographical distribution of *Atelopus manauensis* sp. nov. as represented by the yellow polygon between the Cuieiras and the Urubu Rivers; black stars refer to the type locality where the holotype, allotype and paratypes (RFAD) and individuals used for the genetics analyses (FEUFAM and LBA/ZF2) of *Atelopus manauensis* sp. nov. were collected; numbered dark green squares represent populations of *Atelopus hoogmoedi* of REBIO Uatumã (1) and of Pitinga River (2), on the east bank of the Uatumã River, the geographically closest *Atelopus* populations to the new species. Two specimens of *A. hoogmoedi* from REBIO Uatumã were used for genetic analyses.

Reproductive activity likely occurs from the beginning of the rainy season in the region (November) until March. On cloudy days males are less exposed, but when the sun's rays start to warm the forest floor, they look for sun spots and then start vocalizing at any time of the day. They vocalize and forage on roots, accumulated leaves, fallen tree trunks on the bank or over

streams, at the base of herbaceous plants or stemless palms and even climbing inclined trunks of small shrubs, usually during the day (0700–1000 h and 1500–1700 h) with milder temperatures. During the night it is common to see individuals resting more than 1 meter high on leaves of herbaceous plants on the banks of streams. The variety of calls emitted by *Atelopus* species is related to intra- and interspecific communication, but the functions of these calls have been poorly studied [25]. Type 1 calls (advertisement call) are used, as with other anurans, in the context of attracting reproductive mates, whereas type 2 calls (pure tone call) are likely used in an aggressive context such as in intraspecific interaction. Type 3 calls (pure tone short calls) are mostly of unknown function but may have a function between aggressive and release calls in the context of close-range interactions between conspecifics males with physical contact. Type 4 calls (pulsed short calls) clearly represent release calls [25]. Information on eggs and tadpoles of *Atelopus manauensis* sp. nov. can be found in Gascon [23]. There is no information on eggs or tadpoles of closely related species. Lötters et al. [4] recorded a female and a male of *A. pulcher* in auxiliary amplexus and, after 2–3 weeks, c.a. 600 unpigmented eggs were deposited in the water arranged in a single chain or string-like fashion [4].

4. Discussion

We used integrative taxonomy to describe a new species of *Atelopus* with a distribution mostly restricted to the municipality of Manaus, Brazil, including two of the most intensively studied forests in Amazonia (RFAD and PDBFF). *Atelopus manauensis* is the first species of the genus to be formally described for Brazil. Another *Atelopus* species known from Brazil is *A. hoogmoedi* that is distributed in the states of Amapá and Pará, but its type locality is in French Guiana. As is the case for several of its congeners, *A. manauensis* has a small area of occurrence (approximately 4500 km^2) and is critically threatened by urban expansion of the largest city in Brazilian Amazonia–Manaus [29].

Atelopus manauensis has ecological, morphological, and genetic similarities with species of *Atelopus* distributed c.a. 1500 km straight-line distance from Manaus, so the threats for extinctions identified for those species are likely to be relevant to *A. manauensis* as well. For example, the pathogenic fungus *Batrachochytrium dendrobatidis* (*BD*) has caused the extinction of numerous species of *Atelopus* [58], and the deforestation that is reaching Central Amazonia via highway BR-174 (Manaus-Boa Vista) could very likely facilitate its invasion into the area of occurrence of the new species. Although *BD* has not been found in populations of *A. manauensis* [29], an ongoing study indicates that *BD* infection would cause high mortality rates for the new species [59]. In addition to the threat of *BD*, the urban expansion of Manaus

and other nearby cities is fragmenting areas with aggregations of individuals of *A. manauensis*. The conservation status of *A. manauensis* was recently assessed as "Endangered" according to the criteria established by the International Union for Conservation of Nature, which are as follows: reduction of area of occurrence, area of occupation, and habitat quality; projected decline in the area of occurrence, area of occupation, and habitat quality; and area of occurrence less than 5000 km^2 (approximately 4500 km^2; [29]).

The interspecific phylogenetic relationships reconstructed in this study are in agreement with those of previous studies with regard to the monophyly of the clade distributed in the Guiana Shield [3,11,29]. Our phylogenetic analysis and that of Jorge et al. [29] were the first to show that *Atelopus manauensis* is sister to the clade containing the species of *Atelopus* distributed in the Guiana Shield. The genetic distances inferred between these species and *A. manauensis* vary between 2.3–2.8% (K2P) and 2.3–2.7% (p-distance) and are close to the average observed for anurans using 16S markers [60,61]. On the other hand, the genetic distances among species distributed in the Guiana Shield (*A. franciscus*, *A. flavescens*, *A. barbotini*, and *A. hoogmoedi*) were very low, ranging between 0.2–1.3% (K2P) and 0.2–1.5% (p-distance). This finding, together with the lack of bioacoustic divergences among the species of the Guiana Shield as reported by Costa-Campos & Carvalho [50], corroborate the suggestion of Kok [5] and Lötters et al. [3] that these species actually represent a single polymorphic species and that they should be synonymized.

In the past, *Atelopus manauensis* has also been mistaken for *A. pulcher* and *A. spumarius*. However, *A. spumarius* SS from Peru is the sister of *A. seminiferus*, which together form a clade positioned as the sister of the clade containing the species of the Guiana Shield and *A. manauensis*. *Atelopus pulcher* was finally allocated as a basal species to the aforementioned clade. The genetic distances between the new species and *A. pulcher* and *A. spumarius* SS were moderately high at 3.9% and 4.0% (p-distance) and 4.0% and 3.9% (K2P), respectively. In addition, species distributed in western Amazonia (i.e., *A. pulcher* and *A. spumarius* SS) and in the Guiana Shield (i.e., *A. hoogmoedi*) are geographically and ecologically isolated from each other and from *A. manauensis* due to historical biogeographical barriers, such as large rivers and mountains, and ecological barriers, such extensive areas of open, floodable forests that contain acidic water drainage systems to which the tadpole of *A. manauensis* is probably sensitive [22], preventing any possible contact of the new species with the genetically closest species of the *A. hoogmoedi* complex or with the morphologically most similar species of the *A. spumarius* complex.

Supplementary Material: The following are available online at www.mdpi.com/xxx/s1, **Table S1:** Morphologic measurements of *Atelopus manauensis* sp. nov. and *Atelopus hoogmoedi* of REBIO Uatumã (REUA) and Pitinga River. Abbreviations: (INPA-H) Herpetological section of the zoological collection of Instituto Nacional de Pesquisas da Amazônia, (SEX) sexes, (M) male, (F) female, (RFAD) Reserva Florestal Adolpho Ducke, (CIGS/EB) Centro de Instruções de Guerra na Selva do Exército Brasileiro, (FD) Fazenda Dimona, (REUA) Reserva Biológica do Uatumã, (Pitinga) Pitinga River, (SVL) snout-vent length, (SW) sacrum width, (HW) head width, (HL) head length, (EYDM) eye diameter, (EYNO) eye to nostril distance, (IOD) interorbital distance, (ITNA) internarial distance, (RDUL) length of flexed forearm, (HAND) hand length, (THBL) thumb length, (FOOT) foot length, (TL) tibia length.

Field Number	Voucher/INPA-H	SEX	Local	SVL	SW	HW	HL	EYDM	EYNO	ITNA	IOD	RDUL	HAND	THUL	FOOT	TIBL
HOLOTYPE	041378	M	RFAD	21.24	6.5	7.3	7.5	2.35	2.35	2.6	2.95	6.3	5	2.2	7.3	9.8
ST01	041365	M	RFAD	21.35	7.5	7.3	7.9	2.5	2.5	2.7	3.6	6.4	4.9	2.1	7.5	9.9
ST02	041366	M	RFAD	20.79	6.3	7.2	7.8	2.1	2.1	2.7	2.9	6.9	4.7	2.05	7.2	9.9
ST03	041367	M	RFAD	21.44	8.6	7.6	7.9	2.4	2.5	2.8	3	6.9	5.2	2.1	7.8	10.3
ST04	041368	M	RFAD	20.93	8.3	7.5	7.7	2.5	2.2	2.6	3.1	6.5	5.1	1.9	7.6	9.8
ST05	041369	M	RFAD	20.15	7.8	7.3	7.9	2.5	2.2	2.4	2.9	6.8	4.9	1.9	7.5	9.8
31RD	041376	M	RFAD	26.4	8.5	8.3	8.5	2.7	3	3.1	2.9	8.2	6.6	2.5	10.4	11.9
29RD	041377	F	RFAD	27.89	8.5	8.3	8.9	3	3	3.1	3	8.7	7.5	2.5	10.9	12.1
-	011900	F	RFAD	28.8	8.4	8.5	9.2	2.7	2.8	3.1	3.3	10	6.4	3	10.4	12.9
PTMP02-4	041370	M	CIGS/EB	24.96	6.7	7.8	8.7	2.7	2.9	2.9	31	7.9	6.2	2.4	8.8	11.7
PTMP02-5	041371	M	CIGS/EB	24.55	6.9	7.8	8.5	2.5	2.7	2.8	2.9	6.2	5.4	2.3	7.6	10.5
05ZF2	041375	M	LBA/ZF2	19.14	5.7	6.5	6.8	2.1	2.4	2.4	2.5	5.3	4.8	2.1	6.4	9.8
56DI	041374	M	FD	19.28	5.7	6.6	7	2.2	2.5	2.4	2.5	6	4.9	2.1	6.6	9.8
38UA	041358	M	REUA	29.37	8.2	9.3	9.5	2.7	3.4	3.2	3.7	8.4	6.5	3	10.4	13
39UA	041359	M	REUA	31.44	8.5	9.9	10.4	3	3.4	3.4	4.3	10.1	8	3.9	11.9	14
44UA	041293	M	REUA	32.9	8.6	10	10.6	3.1	3.5	3.6	3.8	9.6	8.1	4	12.3	13.1
46UA	041360	M	REUA	30	8.5	9.6	9.9	2.9	3.4	3.3	3.7	9.5	8	3.9	11.6	13
94UA	041361	M	Pitinga	29	8.2	9.4	9.7	2.7	3.5	3.1	3.4	9	7.2	2.9	10.5	14
95UA	041362	M	Pitinga	27.56	7.6	8.8	9.3	2.4	2.9	2.9	3.2	8.7	6.7	2.9	9.4	12.8
96UA	041363	M	Pitinga	28.28	7.7	9	9.5	2.7	3.2	3.1	3.7	8.9	6.9	3	9.9	13
97UA	041364	M	Pitinga	26.89	7.5	8.5	8.9	2.5	3.1	3	3.7	8.6	6.7	2.8	9.5	12.7

Author Contributions: Conceptualization, R.F.J. and A.P.L.; methodology, R.F.J., M.F. and A.P.L.; validation, R.F.J., M.F. and A.P.L.; formal analysis, R.F.J., M.F. and A.P.L.; investigation, R.F.J., M.F. and A.P.L.; resources, R.F.J., M.F. and A.P.L.; data curation, R.F.J.; writing—original draft preparation, R.F.J., M.F. and A.P.L.; writing—review and editing, R.F.J., M.F. and A.P.L.; visualization, R.F.J., M.F. and A.P.L.; supervision, M.F. and A.P.L.; project administration, R.F.J.; funding acquisition, A.P.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Universal), grant number 401120/2016-3 to A.P.L., and by Coordenação de Aperfeiçoamento do Pessoal de Nível Superior through Programa de Excelência Acadêmica (CAPES/PROEX), grant number 0616/2018. The R.F.J. received a scholarship from Coordenação de Aperfeiçoamento do Pessoal de Nível Superior in partnership with Fundação de Amparo à Pesquisa do Estado do Amazonas (CAPES/FAPEAM), grant number 24/2014. The Miquéias Ferrão received a postdoctoral fellowship from CNPq, grant number 154325/2018-0, and an Edward O. Wilson Biodiversity Postdoctoral Fellowship from the Museum of Comparative Zoology, Harvard University. This study is published by a grant from the Wetmore Colles Fund.

Acknowledgments: We thank J.L. Magnusson for the photos of specimens in preservation and for the preparation of the plates; C.E. Costa-Campos and T.R. Carvalho for cordially providing the calls of *Atelopus hoogmoedi* from Pedra Branca do Amapari (Amapá, Brazil); F.P. Werneck for the access to Coleção Herpetológica of INPA; and N. Higuchi, J.L. Camargo, G. Klein, Mineração Taboca and Tenente S.C. dos S. Gomes and all Army personnel (Centro de Instruções de Guerra na Selva do Exército Brasileiro—CIGS/EB) for logistic support at LBA/ZF-2 scientific station, ARIE PDBFF, REBIO Uatumã, Pitinga River, and CIGS/EB training site, respectively. We thank the Wetmore Colles Fund for supporting the publication's open access.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Frost, D.R. Amphibian Species of the World: An online reference. Version 6.1. New York, NY: *American Museum of Natural History* **2020**. https://doi.org/10.5531/db.vz.0001. Available online: https://amphibiansofthe world.amnh.org/index.php (accessed on 20 11 2019).
2. Lötters, S. *The neotropical toad genus Atelopus*. *Checklist, biology and distribution*; M. Vences & F. Glaw Verlags GbR: Köln, Germany, 1996; pp. 143.
3. Lötters, S.; Van Der Meijden, A.; Coloma, L.A.; Boistel, R.; Cloetens, P.; Ernst, R.; Lehr, E.; Veith, M. Assessing the molecular phylogeny of a near extinct group of vertebrates: the Neotropical harlequin frogs (Bufonidae: *Atelopus*). *Syst. Biodivers.* **2011**, *9*, 45–57.
4. Lötters, S.; Haas, W.; Schick, S.; Böhme, W. On the systematics of the harlequin frogs (Amphibia: Bufonidae: *Atelopus*) from Amazonia. II: Redescription of *Atelopus pulcher* (Boulenger, 1882) from the eastern Andean versant in Peru. *Salamandra* **2002**, *38*, 165–184.
5. Kok, P.J.R. A survey of the anuran fauna of Montagne Belvédère, county of Saül, French Guiana: field list with comments on taxonomy and ecology. *The British Herpetological Society Bulletin* **2000**, *71*, 6–26.
6. Cope, E.D. Ninth contribution to the herpetology of Tropical America. *P. Acad. Nat. Sci. Phila.* **1871**, *November 21*, 200–222.
7. Lescure, J. Contribution a l'etude des Amphibiens de Guyane francaise. VIII. Validation d'*Atelopus spumarius* Cope, 1871, et designation d'un neotype. Description d'*Atelopus spumarius barbotini* nov. ssp. Donnees etho-ecologiques et biogeographiques sur les d'*Atelopus* du groupe *flavescens* (Anoures, Bufonides) *Bulletin du Muséum National d'Histoire Naturelle du Paris* **1981**, *4*, 893–910.
8. Rivero, J.A. More on the *Atelopus* (Amphibia, Salientia) from western South America. *Caribb. J. Sci.* **1968**, *8*, 19–29.
9. Lescure, J. Contribution a l'étude des amphibiens de Guyane Française i. notes sur *Atelopus flavescens* Duméril et Bibron et description d'une nouvelle espèce. *Vie Milieu* **1972-1973**, *23*, 125–141.

10. Lötters, S.; de La Riva, I. Redescription of *Atelopus tricolor* Boulenger from Southeastern Peru and adjacent Bolivia, with comments on related forms. *J. Herpetol.* **1998**, *32*, 481–488. https://doi.org/10.2307/1565201

11. Noonan, B.P.; Gaucher, P. Phylogeography and demography of Guianan harlequin toads (*Atelopus*): Diversification within a refuge. *Mol. Ecol.* **2005**, *14*, 3017–3031. https://doi.org/10.1111/j.1365-294X.2005.02624.x

12. Zimmerman, B.L. A comparison of structural features of calls of open and forest habitat frog species in the Central Amazon. *Herpetologica* **1983**, *39*, 235−246.

13. Zimmerman, B.L.; Bierregaard, R.O. Relevance of the equilibrium theory of island biogeography and species-area relations to conservation with a case from Amazonia. *J. Biogeogr.* **1986**, *13*, 133−143.

14. Zimmerman, B.L.; Simberloff, D. An historical interpretation of habitat used by frogs in a Central Amazonian Forest. *J. Biogeogr.* **1996**, *23*, 27−43.

15. Gascon, C. The tadpole of *Atelopus pulcher* Boulenger (Annura: Bufonidae) from Manaus, Amazonas. *Zoologia* **1989**, *6*, 235–239. https://doi.org/10.1590/s0101-81751989000200007.

16. Boulenger G.A. *Catalogue of the Batrachia Salientia s. Ecaudata in the Collection of the British Museum*, 2nd ed.; London Printed by order of the Trustees: London, England, 1882; pp. 528.

17. Peters, J.A. The Frog Genus *Atelopus* in Ecuador (Anura: Bufonidae). *Smithson. Contrib. Zool.* **1973**, *145*, 1–49.

18. Lima, A.P.; Magnusson, W.E.; Menin, M.; Erdtmann, L.K.; Rodrigues, D.J.; Keller, C.; Hödl, W. *Guide to the frogs of Reserva Ducke – Central Amazonia*, 2nd ed.; Áttema Design Editorial: Manaus, Brazil, 2006; pp. 188.

19. Rojas-Ahumada, D.P.; Menin, M. Composition and abundance of anurans in riparian and non-riparian areas in a forest in central Amazonia, Brazil. *South Am. J. Herpetol.* **2010**, *5*, 157−167. https://doi.org/10.2994/057.005.0210

20. Menin, M.; Waldez, F.; Lima, A.P. Effects of environmental and spatial factors on the distribution of anuran species with aquatic reproduction in central Amazonia. *Herpetol. J.* **2011**, *21*, 255–261. https://doi.org/10.1371/journal.pone.0199852

21. Siqueira, S.; Aguiar Jr, O.; Lima, A.P.; Recco-Pimentel, S.M. Cytogenetics and sperm ultrastructure of *Atelopus spumarius* (Anura: Bufonidae) from the Brazilian Amazon. *Genet. Mol. Biol.* (Impresso) **2013**, *36*, 528−532. http://doi.org/10.1590/S1415-47572013005000038

22. Jorge, R.F.; Simões, P.I.; Magnusson, W.E.; Lima, A.P. Fine-scale habitat heterogeneity explains the local distribution of two Amazonian frog species of concern for conservation. *Biotropica* **2016**, *48*, 694–703. https://doi.org/10.1111/btp.12333

23. Rößler, D.C.; Lötters, S.; Mappes, J.; Valkonen, J.K.; Menin, M.; Lima, A.P.; Pröhl, H. Sole coloration as an unusual aposematic signal in a Neotropical toad. *Sci. Rep.* **2018**, *9*, 1128. https://doi.org/10.1038/s41598-018-37705-1

24. Duellman, W.E.; Lynch, J.D. Descriptions of *Atelopus* tadpoles and their relevance to Atelopodid classification. *Herpetologica* **1969**, *25*, 231–240.

25. Lötters, S.; Mebs, D.; Köhler, G.; Vargas, J.; La Marca E. The voice from the hereafter: vocalisations in three species of *Atelopus* from the Venezuelan Andes, likely to be extinct. *Herpetozoa* **2020,** *32*, 267–275. https://doi.org/10.3897/herpetozoa.32.e39192

26. Kearse, M.; Moir, R.; Wilson, A.; Stones-Havas, S.; Cheung, M.; Sturrock, S.; Buxton, S.; Cooper, A.; Markowitz, S.; Duran, C.; Thierer, T.; Ashton, B.; Meintjes, P.; Drummond, A. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **2012**, *28*, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199.

27. Lanfear, R.; Frandsen, P.B., Wright, A.M., Senfeld, T.; Calcott, B. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Mol. Biol. Evol.* **2017**, *34*, 772–773. https://doi.org/10.1093/molbev/msw260

28. Guindon, S.; Dufayard, J.F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **2010**, *59*, 307–321.

29. Jorge, R.F.; Magnusson, W.E.; Silva, D.A.; Polo, E.M.; Lima, A.P. Urban growth threatens the lowland Amazonian Manaus harlequin frog which represents an evolutionarily significant unit within the genus *Atelopus* (Amphibia: Anura: Bufonidae). *J. Zool. Syst. Evol. Res.* **2020**, *0*, 1−11. https://doi.org/10.1111/jzs.12390

30. da Silva, G.W.B.; Cornélio, G.S.; de Oliveira, E.A.; Trindade, N.G.P.; França, I.; Hernández Ruz, E.J. A candidate species currently classified as *Atelopus hoogmoedi* (Anura: Bufonidae) in the eastern Amazon, Pará, Brazil. *Genet. Mol. Res.* **2020**, *19*, gmr18392. https://doi.org/10.4238/gmr18392

31. Kok, P.J.; MacCulloch, R.D.; Means, D.B.; Roelants, K.; Van Bocxlaer, I.; Bossuyt, F. Low genetic diversity in tepui summit vertebrate. *Curr. Biol*. **2012**, *22*, 589−590. https://doi.org/10.1016/j.cub.2012.06.034

32. Lötters, S.; van der Meijden, A.; Rödder, D.; Köster, T.E.; Kraus, T.; La Marca, E.; Haddad, C.F.B.; Veith, M. Reinforcing and expanding the predictions of the disturbance vicariance hypothesis in Amazonian harlequin frogs: a molecular phylogenetic and climate envelope modelling approach. *Biodivers. Conserv.* **2010**, *19*, 2125–2146. https://doi.org/10.1007/s10531-010-9869-y

33. Van Bocxlaer, I.; Loader, S.P.; Roelants, K.; Biju, S.D.; Menegon, M.; Bossuyt, F. Gradual adaptation toward a range-expansion phenotype initiated the global radiation of toads. *Science* **2010**, *327*, 679–682. https://doi.org/10.1126/science.1181707

34. Frost, D.R.; Grant, T.; Faivovich, J.; Bain, R.; Haas, A.; Haddad, C.F.B.; de Sa, R.O.; Channing, A.; Wilkinson, M.; Donnellan, S.C.; Raxworthy, C.; Campbell, J.A.; Blotto, B.L.; Moler, P.; Drewes, R.C.; Nussbaum, R.A.; Lynch, J.D.; Green, D.M.; Wheeler, W.C. The Amphibian tree of life. *Bull. Am. Mus. Nat. Hist*. **2006**, 297, 1–291. http://dx.doi.org/10.5531/sd.sp.13

35. dos Santos, S.P.; Ibanez, R.; Ron, S.R. Systematics of the *Rhinella margaritifera* complex (Anura, Bufonidae) from western Ecuador and Panama with insights in the biogeography of *Rhinella alata*. *Zookeys* **2015,** *501*, 109–145. https://doi.org/10.3897/zookeys.501.8604

36. Nguyen, L.T.; Schmidt, H.A.; von Haeseler, A.; Minh, B.Q. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* **2015**, *32*, 268–274. https://doi.org/10.1093/molbev/msu300

37. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Sys. Biol.* **2011**, *61*, 539−542. http://doi.org/10.1093/sysbio/sys029

38. Kimura, M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111−120.

39. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA 6: Molecular evolutionary genetics analysis Version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725−2729. http://doi.org/10.1093/molbev/mst197

40. Gray, P.; Cannatella, D.C. A new species of *Atelopus* (Anura: Bufonidae) from the Andes of Northern Peru. *Copeia* **1985**, *4*, 910–917.

41. Coloma, L.A.; Lötters, S.; Salas, A.W. Taxonomy of the *Atelopus ignescens* complex (Anura:Bufonidae): designation of a Neotype of *Atelopus ignescens* and recognition of *Atelopus exiguus*. *Herpetologica* **2000**, *56*, 303–324.

42. Savage, J.M.; Heyer, W.R. Variation and distribution in the tree-frog genus Phyllomedusa in Costa Rica, Central America. *Beiträge zur Neotropischen* **1967**, *5*, 111−131. https://doi.org/10.1080/01650526709360400

43. Savage, J.M.; Heyer, W.R. Digital webbing formulae for anurans: a refinement. *Herpetol. Rev.* **1997**, *28*, 131.

44. Myers, C.W.; Duellman, W.E. A new species of *Hyla* from Cerro Colorado, and other tree frog records and geographical notes from Western Panama. *Am. Mus. Novit.* **1982**, *2752*, 1−32.

45. Jombart, T.; Devillard, S.; Balloux, F. Discriminant analysis of principal components: A new method for the analysis of genetically structured populations. *BMC genetics* **2010**, *11*, 94.

46. Breiman, L. Random forests. *Mach. Learn.* **2001,** *45*, 5–32.

47. R Core Team. *R: A Language and environment for statistical computing*. R Foundation for Statistical Computing: Vienna, Austria, 2018. Available online: https://www.R-project.org (accessed on 17 November 2019)

48. Liaw, A.; Wiener, M. Classification and Regression by randomForest. *R News* **2002**, *2*, 18–22.

49. Center for Conservation Bioacoustics. Raven Pro: Interactive sound analysis software Version 1.6.1. Computer software; The Cornell Lab of Ornithology: Ithaca, NY, 2019. Available online: http://ravensoundsoftware.com (accessed on 15 09 2019).

50. Costa-Campos, C.E.; Carvalho, T.R. The advertisement call of the Hoogmoed's harlequin toad *Atelopus hoogmoedi* Lescure, 1974 from northern Brazil (Anura: Bufonidae). *Zootaxa* **2018**, *4521*, 141–144. https://doi.org/10.11646/zootaxa.4521.1.11

51. Köhler, J.; Jansen, M.; Rodríguez, A.; Kok, F.J.R.; Toledo, L.F.; Emmrich, M.; Glaw, F.; Haddad, C.F.B.; Rödel, M-O.; Vences, M. The use of bioacoustics in anuran taxonomy: theory, terminology, methods and recommendations for best practice. *Zootaxa* **2017**, *4251*, 1–124. https://doi.org/10.11646/zootaxa.4251.1.1

52. Lescure, J. Présence d'une sous-espèce *Atelopus pulcher* (Amphibien, Anoure) dans les Guyanes: *Atelopus pulcher hoogmoedi*. *Bulletin du Muséum National d'Histoire Naturelle du Paris. Zoologie* **1973**, *108*, 997–1005.

53. Lötters, S.; Boistel, R.; Blanc, M.; Haddad, C.F.B.; van der Meijden, A. *Atelopus hoogmoedi* Lescure, 1974. In *Ranas Arlequines*; Almonacid, J.V.R., Mahecha, J.V.R., la Marca, E., Lötters, S., Kahn, T., Angulo, A., Eds.; Conservácion International: Bogotá, Colombia, 2005; pp. 132.

54. Kok, P.J.R.; Kalamandeen, M. Introduction of the taxonomy of the amphibians of Kaieteur National Park, Guyana. *ABC Taxa* **2008**, *5*, 1–278.

55. de La Riva, I.; Castroviejo-Fisher, S.; Chaparro, J.C.; Boistel, R.; Padial, J.M. A new species of *Atelopus* (Anura: Bufonidae) from the Amazonian slopes of the Andes in south-eastern Peru. *Salamandra* **2011**, *47*, 161–168.

56. Lötters, S.; Shulte, R. *Atelopus seminiferus* Cope, 1874. In *Ranas Arlequinas*; Almonacid, J.V.R., Mahecha, J.V.R, la Marca, E., Lötters, S., Kahn, T., Angulo, A., Eds.; Conservácion International: Bogotá, Colombia, 2005; pp. 132.

57. Cope, E.D. On some Batrachia and Nematognathi brought from the Upper Amazon by Prof. Orton. *P. Acad. Nat. Sci. Phila*. **1874**, 26, 120–137.

58. La Marca, E.; Lips, K.L.; Lötters, S.; Puschendorf, R.; Ibáñez, R.; Rueda-Almonacid, J.V.; Schulte, R.; Marty, C.; Castro, F.; Manzanilla-Puppo, J.; García-Pérez, J.E.; Bolaños, F.; Chaves, G.; Pounds, J.A.; Toral, E.; Young, B.E. Catastrophic population declines and extinctions in Neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica* **2005**, 37, 190–201.

59. Lambertini, C.; Missassi, A.F.R.; Jorge, R.F.; Leite, D.A.; Lima, A.P.; Toledo, L.F. Climatic relationships between the killing-chytrid fungus and wild amphibians in the Brazilian Amazonia. Submitted manuscript.

60. Vences, M.; Thomas, M.; van Der Meijden, A.; Chiari, Y.; Vieites, D.R. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Front. Zool.* **2005**, 2, 1−12.

61. Fouquet, A.; Gilles, A.; Vences, M.; Marty, C.; Blanc, M.; Gemmell, N.J. Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *PLoS ONE* **2007**, 2, e1109.

Capítulo 2

Jorge, R.F., Magnusson, W.E., Silva, D.A., Polo, E.M. & Lima, A.P. 2020. Urban growth threatens the lowland Amazonian Manaus harlequin frog which represents an evolutionarily significant unit within the genus *Atelopus* (Amphibia: Anura: Bufonidae). *Journal of Zoological Systematics and Evolutionary Research* 0:1-11.

Title: Urban growth threatens the lowland Amazonian Manaus harlequin frog which represents an evolutionarily significant unit within the genus *Atelopus* (Amphibia: Anura: Bufonidae)

Running Title: Urban expansion threatens an endangered frog

Authors: Rafael Filgueira Jorge[1]*, William Ernest Magnusson[2], Dayse Aparecida da Silva[3], Érico Macedo Polo[4], & Albertina Pimentel Lima[5].

Affiliations: [1]Instituto Nacional de Pesquisas da Amazônia, Programa de Pós-Graduação em Ecologia, Campus III/V8, Avenida André Araújo, nº 2936, Aleixo, Código Postal 2223, CEP 69067-375, Manaus–AM. e-mail: rafajorgebio@gmail.com - *Corresponding Author; [2]Instituto Nacional de Pesquisas da Amazônia, Coordenação de Biodiversidade. e-mail: bill@inpa.gov.br; [3]Universidade do Estado do Rio de Janeiro, Laboratório de Diagnósticos por DNA, Instituto de Biologia Roberto Alcântara Gomes, Rua São Francisco Xavier, nº 524, Maracanã, CEP 20550-900, Rio de Janeiro–RJ; [4]Universidade Federal do Amazonas, Instituto de Ciências Biológicas, Departamento de Genética, Laboratório de Processamento de Dados Genéticos, Avenida General Rodrigo Otávio, nº 6200, Coroado, CEP 69080-900, Manaus–AM; [5]Instituto Nacional de Pesquisas da Amazônia, Coordenação de Biodiversidade, Avenida André Araújo, nº 2936, Aleixo, Código Postal 2223, CEP 69067-375, Manaus–AM. e-mail: lima@inpa.gov.br.

e-mail corresponding author: rafajorgebio@gmail.com

ABSTRACT: The Manaus harlequin frog is an evolutionarily significant clade within the *Atelopus hoogmoedi* species complex. Analyses of 16S and COI concatenated sequences support *Atelopus* from the Manaus region as an evolutionary significant unit, sister of all species of a Guiana Shield clade. A previous study showed that subtle changes in stream characteristics influence the Manaus harlequin frog occurrence and density variation at local-scale in a reserve on the outskirts of Manaus. As deforestation is approaching areas where the Manaus harlequin frog occurs, we asked how site and landscape heterogeneity influence the geographic boundaries, occurrence patterns, and density variation of the Manaus harlequin frog. We searched for the frog in 80 plots that measured 250 m by 4 m on banks of first- to third-order streams during the rainy seasons in 2012–2013 and 2016–2019. The plot distribution covered all likely areas of occurrence of the Manaus harlequin frog and extended to the areas where it is substituted by its geographically closest relative on the Guiana Shield. Ecological drivers related to climate, flooding events, and forest structure apparently restrict the Manaus harlequin frog to a patchy distribution in a narrow portion of the interfluve between the Negro and Uatuma Rivers. Densities of individuals varied in response to subtle changes in floodplain and stream characteristics. The Manaus harlequin frog is associated with a very specific habitat that is directly affected by the growth of Manaus, the largest city in Amazonia. We conclude that it is endangered and urgent actions are required for its conservation.

Keywords: Central Amazonia, environmental heterogeneity, extinction risk, habitat modification, harlequin frog

INTRODUCTION

The genus *Atelopus* is a clade of bufonid frogs that formerly occurred throughout the Andes highlands ranging eastwards into the Guiana Shield and central Amazonian lowlands and north in central America. However, most of the more than 96 species of *Atelopus* are now extinct or in decline, mainly because of infection by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) for those inhabiting mid- to high-elevations and due to habitat loss for lowland species (La Marca et al., 2005). Despite *Bd* being generally less severe in warmer lowland areas (Bacigalupe et al., 2019), these portions of the landscape in Amazonia are often deforested for human use and disturbed areas seem to be suitable for *Bd* invasion in central Amazonia (Becker, Rodriguez, Lambertini, Toledo, & Haddad, 2015), and other tropical regions (Bolom-Huet, Pineda, Días-Fleischer, Muñoz-Alonso, & Galindo-González, 2019).

About ten species of *Atelopus* are known from lowland Amazonia (Lötters, 1996), but the taxonomy of these species is not well established (Lötters, Hass, Schick, & Böhme, 2002; Lötters et al., 2011), and we will provide phylogenetic evidence that *Atelopus* from the Manaus region form an evolutionary significant unit (ESU), sister of all species of the Guiana Shield clade of *Atelopus*. The Manaus harlequin frog also differs considerably from its closest congener *Atelopus hoogmoedi* Lescure, 1974 (Hoogmoed harlequin toad - Uatuma River populations) in morphology (e.g. mean snout-vent length of 11 adult males from Manaus was 21.83 mm, compared to = 29.55 mm for 10 *A. hoogmoedi* adult males) and call traits (e.g. dominant frequency of calls of frogs from Manaus is 3088–3610 Hz, whereas it is 2614–2883 Hz in *A. hoogmoedi*).

Very little is known about local adaptations of the species of *Atelopus* that could affect their distributions. The distribution of the Manaus harlequin frog within Reserva Florestal Adolpho Ducke (hereafter Reserva Ducke), a protected area on the outskirts of Manaus, Amazonas, Brazil, is restricted to the eastern catchments, apparently because tadpoles of the species are sensitive to pH (Jorge, Simões, Magnusson, & Lima, 2016), but the limits to its distribution and the factors that determine its abundance at wider scales are unknown.

The fauna of the Manaus area has elements that do not occur further north on the Guiana Shield. For example, one species of primate, the Pied tamarin *Saguinus bicolor* Spix, 1823, is considered endangered because its distribution coincides almost completely with that of Manaus (Farias, Wagner, Gordo, & Hrbek, 2015). Manaus is the largest city in the Amazonia and has undergone rapid growth in recent years (Puppim de Oliveira et al., 2011). We studied the distribution and abundance of the Manaus harlequin frog to determine the factors that limit

its distribution and to evaluate the threats to its effective conservation. We aimed at answering the following questions: What is the extent of the geographic range of the Manaus harlequin frog? How fragmented are concentrations of the Manaus harlequin frog within its occupancy area? Do the IUCN Red List Categories and Criteria (IUCN, 2012) used to assess species conservation status (extent of occurrence, occupancy area, quality of habitat and number of subpopulations declining) indicate that the Manaus harlequin frog is endangered?

2 MATERIAL AND METHODS

2.1 Study area

The Negro-Uatuma interfluve (Fig. 1) is underlain by sedimentary rocks from the Alter do Chao Formation and Trombetas Group (a geological Group is composed of different formations), and crystalline base rocks from the Guiana Shield, which support heterogeneous soils (Quesada et al., 2010). This Amazonian landscape is covered mainly by submontane (Guiana Shield/Trombetas group) and lowland ombrophilous dense forests (Trombetas group/Alter do Chao) (IBGE, 2004). The interfluve relief comprises alluvial floodplains, plateaus, and small hills ranging from 3 to 230 m a.s.l. (CPRM, 2010). Annual mean temperature ranged from 26.4ºC to 27.6ºC in the period from 1970 to 2000 and rainfall varied from 2,028 to 3,006 mm between localities within the Negro-Uatuma interfluve in the same period (Fick & Hijmans, 2017). This interfluve has been subject to severe forest fragmentation since 1972 (Fig. 2).

2.2 Biological Sampling

Surveys were undertaken in 250-m by 4-m plots extended along stream banks that were divided into 25 subplots (see Magnusson et al., 2013:115-116). Eighty plots were installed on banks of first to third order streams distributed in four tributaries of the Negro River (the lower, middle and upper courses of Taruma-Açu, Cuieiras and Apuau Rivers and the upper course of Camanau River) and four tributaries of the Amazon River (Puraquequara, Preto, Urubu and Uatuma Rivers). We used acoustic and visual searches focused on leaf litter, roots, fallen trunks and the bases of stemless palms and herbs at a maximum distance of four meters from the stream margin. When no individual was detected, recorded calls of the Manaus harlequin frog were played at the middle of each subplot for two minutes (three at 15-s intervals) because observations during previous studies (Lima et al., 2006; Jorge et al., 2016) indicated that Manaus harlequin frog males usually call when another male calls nearby. Only one individual per sub-plot was recorded in order to create an index of density (minimum = 0; maximum =

25). We searched for the frogs between 0700-1000 h and 1500-1700 h, the periods when males call and are more detectable. Sampling was undertaken during the rainy season (December-April) in 2012-2013, 2016-2017, 2017-2018 and 2018-2019.

2.3 Phylogenetic assessment

To confirm the distinct taxonomic status of the Manaus harlequin frog we used fragments of *16S ribosomal RNA gene* (*16S*) and *Cytochrome c oxidase I gene* (*COI*) from four specimens of the Manaus harlequin frog represented by white circles with * in Fig. 1 (two from the middle course of the Cuieiras River − 2°33'32.40" S, 60°13'48.36" W; two from the upper course of the Taruma-Açu River − 2°38'31.20" S, 60°5'45.6" W) and two of the closest populations from the sister clade, *Atelopus hoogmoedi*, from the Reserva Biológica do Uatumã, Presidente Figueiredo, Amazonas, represented by a black square with * in Fig. 1 (1º46'48.07" S, 59º15'4.81" W). Marker sequences of these specimens were obtained in the course of the current study and are referred to by ID numbers one to six in Table 1. We also used 14 sequences of 10 species (four sequences of *Atelopus hoogmoedi* from different localities), obtained from previously published data (ID numbers seven to 20 in Table 1), from the National Centre for Biotechnology Information's GenBank (Table 1 for accession number). Specimens were collected under permission number 56759 from the Sistema de Autorização e Informação em Biodiversidade do Instituto Chico Mendes de Conservação da Biodiversidade (SISBIO/ICMBIO) and were deposited in the herpetological section of the Zoological Collections of the Instituto Nacional de Pesquisas da Amazônia - INPA (Table 1 for specimens' vouchers – ID one to six). This study was approved by the INPA ethics committee (registration number 002/2017 – Comissão de Ética no Uso de Animais – CEUA/INPA).

For our samples (Table 1 – ID one to six), we immersed 5 mg of muscle tissue in phenol/chloroform solution and left it for 24 h in a thermocirculator for DNA extraction, followed by purification procedures using QIamp® DNA FFPE Tissue Kit, following the manufacture's recommendation (Qiagen). *16S* rDNA gene fragments were amplified and sequenced using *16Sar* (forward, 5′-CGCCTGTTTATCAAAAACAT-3′) and *16Sbr* (reverse, 5′-CCGGTCTGAACTCAGATCACGT-3′) primers (Palumbi, 1996). *COI* gene fragments were amplified and sequenced using *AnF1* (forward, 5′-ACHAAYCAYAAAGAYATYG-3′) and *AnR1* (reverse, 5′-CCRAARAATCARAADARRTGTTG-3′) primers (Jungfer et al., 2013; Lyra, Haddad, & Azeredo-Espin, 2017), and *M13F* (forward, 5′ TGTAAAACGACGGCCAGT-3′) and *M13R* (reverse, 5′-CAGGAAACAGCTATGAC-3′) extension tails (Messing, 1983). PCR reactions were carried out with 20 μL final volume [3 μL

DNA (2 ng/μL) + 5 μL 4X Platinum buffer (Applied Biosystems) + 0.2 μL Taq DNA polymerase (5 U/μL) + 5 μL of each primer (2 μM) + 1.8 μL H_2O] under the following amplifications conditions: for *16S* – 3 min hot start at 95°C (denaturation) followed by 35 cycles at 95°C for 30 s (heating), 50°C for 30 s (annealing), and 60°C for 1 min (extension), concluded by 72°C for 10 min (final extension); and for *COI* – 3 min hot start at 95°C (denaturation), 5 cycles at 95°C for 30 s (heating), 48°C for 30 s (annealing), and 60°C for 1 min (extension), followed by 30 cycles at 95°C for 30 s (heating), 50°C for 30 s (annealing), and 60°C for 1 min (extension) with a final extension step at 60°C for 5 min. The *16S* and *COI* PCR products (~ 600 and ~ 700 bp, respectively) were checked on 2% agarose gel electrophoresis to evaluate PCR success and purified using the ExoSAP-IT kit (Ecoli) following the standard protocol. We used 10 μL final volume of purified PCR products [3μL DNA + 3 μL of BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher) + 4 μL H_2O] for forward and reverse terminal reactions following manufacturer recommendations, with 25 cycles at the first stage (95°C for 10 s, 50°C for 5 min, and 60°C for 4 min) and 15°C until the end, following by precipitation according to the standard protocol. Forward and reverse sequences were resolved in an ABI 3500 automatic sequencer (Applied Biosystems).

The extremities of the six alignments obtained in the course of the current study were trimmed to final alignment size of 567 (five sequences) and 512 bp (one alignment) for *16S* and 653 bp for *COI* to avoid errors in phylogenetic reconstruction associated with missing data. All sequences were edited and aligned in Geneious 4.8.2 using its own algorithm (Kearse et al., 2012). The most probable evolutionary model and the best partition schema for the 20 concatenated and aligned *16S* and *COI* sequences (1,274 bp – available in TreeBase under URL http://purl.org/phylo/treebase/phylows/study/TB2:S26036) were selected by Bayesian Information Criteria (BIC) and Akaike Information Criteria (AIC) using PartitionFinder2 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016), as follows: *16S* = TRN + G + X; *COI*\1 = TRNEF; *COI*\2 = HKY + X; *COI*\3 = HKY + X. The gene tree from the *16S* and *COI* concatenated sequences was inferred through Bayesian Inference (BI) using Beast 2.5 (Bouckaert et al., 2019). Four runs of 10 million generations were calculated with the Metropolis coupled Markov chain Monte Carlo algorithm (MCMC), and each run had four Markov chains. Probabilities were sampled every 1,000 generations. We used three priors for divergence times as normal distributions with means and 95% confidence intervals following Kok et al. (2017) to calibrate and estimate divergence times in our phylogeny, as follows: 18.7 (8.5–28.9) million years ago (Mya - Glossary of abbreviations available in Appendix 1) assigned to the root; 5.25 (0–10.5) Mya to the node splitting *Atelopus spurrelli* from *A.*

bomolochos and *A. peruensis*; and 2.51 (0.1–4.9) Mya to the node splitting the two major clades within Guiana Shield. Runs convergence was assessed in Tracer 1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). A consensus tree was calculated after discarding the first 25% of trees using LogCombiner v2 6.0 (Rambaut & Drummond, 2019a), and a Maximum Credibility Clade tree was built in TreeAnotator v2 6.0 (Rambaut & Drummond, 2019b).

2.4 Environmental variables

To represent environmental heterogeneity likely to influence occurrence patterns of the Manaus harlequin frog, we used seven raster layers as landscape-scale variables: (1) precipitation seasonality in percent (PS, 1-km² grain; Fick & Hijmans, 2017); (2) Walsh Index, which describes the duration and severity of the dry season (WI, 1-km² grain; Amaral, Costa, Arasato, Ximenes, & Rennó, 2013); (3) percentage soil silt content (SC, 1-km² grain; Hengl et al., 2014); (4) JERS in square kilometre, which describes flooded-area extent in the high-water seasons (100-m² grain; Hess et al., 2015); (5) live aboveground biomass (MG C ha^{-1})/forest structure (LAB, 500-m² grain; Baccini et al., 2012); (6) altitude (m a.s.l.) (AL, 90-m² grain; Amaral et al., 2013); and height above the nearest drainage (HAND, 30-m² grain; created from a SRTM raster layer using the Hydrology tools of the ArcMap 10.3.1 package Spatial Analyst). All landscape variables were cropped to the study area, projected as Datum WGS 84 and maintained at the original scale for subsequent extraction using the "dismo" R package "extract" tool (Hijmans, Phillips, Leathwick, & Elith, 2017). The values refer to the geographic coordinates obtained at the beginning (1.5 m from stream margin) of each plot using a Garmin 64s GPS (error ± 3 m/Datum WGS 1984).

To represent local heterogeneity of stream banks and streams, we used three variables: (1) pH determined using a calibrated digital pHmeter – the electrode was placed in the middle of the stream and water column, at the beginning of each plot; (2) width (*W*), depth (*D*) and water velocity (*V*) determined at the same point as pH, in order to calculate the stream discharge (SD), by applying the formula SD $m^3/s = W \times D \times V$. Water velocity was estimated from the time a 0.5-g silicon ball took to travel one meter in the stream; and (3) canopy openness (CO) obtained at six equidistant points 1.5 m from the stream margin using a spherical densiometer, following Lemmon (1956).

The local and landscape variables were not highly correlated (Pearson's $r < 0.7$ in all cases), nor did they show multicollinearity (VIF < 3 in all cases). We detected spatial autocorrelation (Moran's Index) associated with CO at distances less than 1.5 km ($p < 0.003$), although it was not detected in the response variable (Manaus-harlequin-frog density) or in

other local predictor variables (pH and stream discharge) at any distance class ($p < 0.05$). As autocorrelation induces type 1 errors in statistical tests only when it occurs in both response and predictor variables (Legendre et al., 2002), no autocorrelation adjustments were necessary. Descriptive statistics and the correlation matrix are given in Tables S1, S2 and S3. All variables, geographic coordinates and metadata are available at https://ppbiodata.inpa.gov.br/metacatui/data/Rafael Jorge.

2.5 Distribution and density-variation analyses

We recorded 22 presences and 58 absences in the surveys for the Manaus harlequin frog. Nevertheless, only 17 presences were included in a logistic model (LM), in order to maintain a minimum distance of 1.5 km among sampling locations. Sites separated by lesser distances are likely not to be independent because of movement of individuals. We defined the occurrence area of the Manaus harlequin frog as that enclosed in a minimum convex polygon containing the occupied sites. Sites outside this area might be unoccupied for biogeographic reasons unrelated to ecological suitability. We used the 30 sampling locations only within this area in a generalized linear model (GLM). As we observed a unimodal relationship between the Manaus harlequin frog's density with pH and SD gradients, we used quadratic transformation ($y = a + b_1 + c_1^2 + e$) of the GLM standard equation, to best fit the data. We applied the LM to estimate how much of the variation in presence (n = 17)/absence (n = 58) of the Manaus harlequin frog could be explained by seven landscape-scale variables (Presence/Absence) = $a + b_1 * PS + b_2 * WI + b_3 * SC + b_4 * LAB + b_5 * JERS + b_6 * AL + b_7 * HAND + e$). We used the GLM, assuming a negative binomial distribution in order to estimate how much of the density variation was explained by stream-bank and stream characteristics (Relative density = $a + b_1 * SD + c_1 * I (SD ^2) + b_2 * pH + c_2 * I (pH^2) + b_3 * CO + e$).

3 RESULTS

3.1 Taxonomic status

Our phylogenetic tree inferred from 1,274 bp concatenated and aligned *16S* (20 individuals) and *COI* (seven individuals) sequences supported the Manaus harlequin frog as an evolutionary significant unit (Posterior Probability = 1), which diverged 3 Mya from of all its sister species of a Guiana Shield clade, including *Atelopus hoogmoedi* (Fig. 3). This confirms that *Atelopus* from the Manaus region represents a candidate species waiting for formal description (Table S4 for genetic distances), but much more information is necessary to

formally describe the species and some of the authors of this paper are in the process of doing so.

3.2 Density variation and geographic range

The relative-density index (number of segments occupied per plot) varied from 0 to 11. The Manaus harlequin frog was encountered only in the Cuieiras-Urubu interfluve, at intermediate altitudes in the Alter do Chao Formation. However, it was not recorded along the entire courses of the five tributaries within its narrow geographic range. It was recorded in the lower, middle and upper course of the Puraquequara River, but only beside headwater streams. It occurred in middle and upper courses of the Preto, Urubu and Cuieiras Rivers and the upper course of the Taruma-Açu River (Fig. 1). Two Negro River tributaries (Curiau and Jauperi) were not sampled, as they are covered mainly by open forests and savanna-like vegetation from which no Manaus harlequin frogs have been recorded.

3.3 Environmental influence on occurrence pattern and density variation

The logistic model explained 51% of occurrences (SE = 18.61, df = 67, $z = 2.77$, $p = 0.005$, *pseudo* $R^2 = 0.51$; Fig. 4). The Manaus harlequin frog was associated with a narrow portion of the Negro-Uatuma interfluve with low precipitation seasonality ($SE_{b1} = 0.30$, $b_1 = -0.79$, $p_{b1} = 0.009$) and less marked dry season ($SE_{b2} = 0.30$, $b_2 = -0.89$, $p_{b2} = 0.003$). The Manaus harlequin frog was only found in sections of streams and rivers with little flooding ($SE_{b5} = 0.09$, $b_5 = -0.16$, $p_{b5} = 0.09$) and in well-structured forests ($SE_{b4} = 0.009$, $b_4 = 0.01$, $p_{b4} = 0.02$). Silt content in soil ($SE_{b3} = 0.21$, $b_3 = 0.11$, $p_{b3} = 0.6$), HAND ($SE_{b7} = 0.05$, $b_7 = 0.02$, $p_{b7} = 0.6$) and altitude ($SE_{b6} = 0.01$, $b_6 = 0.01$, $p_{b6} = 0.29$) did not contribute significantly to the model.

The generalized linear model explained 41% of relative-density variation (SE = 8.9, df = 24, $z = -2.44$, $p = 0.01$, *pseudo* $R^2 = 0.41$). The Manaus harlequin frog was more abundant on banks of streams with pH ranging from 5 to 6 ($SE_{b1} = 3.19$, $b_1 = 7.53$, $p_{b1} = 0.01$; $SE_{c1} = 0.28$, $c_1 = 0.65$, $p_{c1} = 0.02$) and stream discharge between 0.2 and 0.5 m/s^3 ($SE_{b2} = 2.66$, $b_2 = 6.49$, $p_{b2} = 0.01$; $SE_{c2} = 3.83$, $c_2 = -9.79$, $p_{c2} = 0.01$). The lowest densities (0-3) were found at the extreme values of these gradients (Fig. 5). Relative densities were higher in forests with more open canopies within ombrophilous dense forests ($SE_{b3} = 0.02$, $b_3 = 0.04$, $p_{b3} = 0.05$).

4 DISCUSSION

Our phylogenetic assessment shows that an undescribed species is already under extinction risk. The Manaus harlequin frog was considered to be part of a wide-spread species

due to its superficial morphological similarity to an Andean species, the Pebas stubfoot toad (*Atelopus spumarius* Cope, 1871), which was attributed a Vulnerable conservation status due to its broad geographic distribution (Azevedo-Ramos et al., 2010). However, previous studies (Cocroft, Mcdiarmid, & Ruiz-Carranza, 1990; Lötters et al., 2002) suggested that *A. spumarius* should be considered a species complex (Frost, 2020) and our analyses show that the Manaus harlequin frog is more related to species distributed on the Guiana Shield that occupy distinct topographic, climatic and vegetational conditions. This clade seems to be isolated from its closest relatives by unsuitable environments underlain by Trombetas Group soils between the Guiana Shield and the Alter do Chao Formation. The divergence time between Manaus harlequin frog and its sister species from the Guiana Shield (3 Mya) coincides with the end of Miocene glaciations, which may be related with diversification events under a Disturbance-Vicariant hypothesis (Lötters et al., 2010) suggested to explain the diversification of *Atelopus* species from Guiana Shield during the last Glaciations (Noonan & Gaucher, 2005).

4.1 Environmental associations

The Manaus harlequin frog is restricted to a narrow portion of the interfluve with little precipitation seasonality and a short dry season. Generally, *Atelopus* species are very sensitive to high temperatures (Peters, 1973), and severe dry seasons could presumably kill adults or limit egg and tadpole development. Many *Atelopus* species are exclusive to narrow altitudinal zones (Lötters, 1996). These are usually associated with climatic variables, but not in our study area. The Manaus harlequin frog was recorded at altitudes between 61–130 m a.s.l., which is a range too small to be associated with large climatic variation. More likely, it is because lower areas have more sandy soils (Schaefer et al., 2017) and more acid water.

The Manaus harlequin frog inhabits banks of streams with a wide range of pH, but higher densities were observed on banks of streams with less acid waters. Very acid pHs (< 4) are related to reduced growth or even death of larvae and adults of amphibians not adapted to those conditions (Barth & Wilson, 2010). The Manaus harlequin frog is more abundant on stream banks with intermediate discharges; very small streams may not have suitable habitats for its tadpoles and high discharges of large streams may wash tadpoles away. The Manaus harlequin frog occurs only in well-structured forests, but the densities are low where the canopy is continuous.

4.2 Threats and conservation status

The conservation threats associated with a restricted distribution are exacerbated by the fact that the Manaus-harlequin-frog distribution coincides in great part with Manaus, the largest city in Amazonia. Manaus has experienced high rates of urban development and the area occupied by the city and surrounding deforested areas increased by 29% from 2000 to 2010 (IBGE, 2000, 2010). Direct loss of habitat is not the only threat to the Manaus harlequin frog. Pollution and forest-fragment border effects may reduce the capacity of individuals to resist infection by the pathogenic fungus *Bd* (Becker et al., 2015), which has been devastating for other *Atelopus* species (La Marca et al., 2005). Although *Bd* has not been found in the Manaus region, an ongoing study indicates that the Manaus harlequin frog shows high mortality rates in case of *Bd* infection (Lambertini et al., in prep.). There are no published studies of *Bd* distribution in central Amazonia.

Our results indicate that, unless action is taken to minimize threats due to urban expansion, the Manaus harlequin frog is likely to become extinct in the near future. Based on IUCN criteria (IUCN, 2012), it should be assigned the conservation status Endangered, but it is likely to be Critically Endangered in the near future. The extent of occurrence of this clade is less than 5,000 km² (~ 4,500 km² - minimum convex polygon); its occupancy area is severely fragmented by roads, human settlements and unsuitable habitats; its extent of occurrence is inferred to have been reduced and may be undergoing continuing decline in the headwaters of the Urubu and Cuieiras Rivers due to human use; its occupancy area is inferred to be reduced and declining on the right bank of the upper Puraquequara and middle Cuieiras Rivers; the Manaus harlequin frog habitat quality is declining as urban areas are expanding over pristine forests of the headwaters of the catchments occupied by it without any environmental-protection plan. Some areas that we planned to sample repeatedly were deforested during the few years of this study. The right bank of the upper course of the Puraquequara River, which was almost certainly occupied by the Manaus harlequin frog in the past, now has only highly polluted streams within rural unplanned human settlements and land use. Most of the geographic range of the Manaus harlequin frog coincides with that of *Saguinus bicolor*, indicating that the frog is probably as endangered as the primate and should have similar conservation status. Both species are limited to the Alter do Chao Formation and the Manaus harlequin frog has an even smaller distribution than that of *S. bicolor*. Therefore, there is an urgent need to design protective measures for the conservation of the Manaus harlequin frog and the Negro-Uatuma Rivers interfluve landscape as a whole.

ACKNOWLEDGMENTS

The authors would like to thank all the people who aided RFJ in field sampling. Sincere thanks are due to V Albuquerque for taking RFJ to all sampling locations accessed by roads, AJ Stow for reviewing an early draft of the manuscript, M Menin, FRC Costa and J Zuanon for the loans of the pHmeter and spherical densiometer, and N Higuchi, JL Camargo, E Salazar, G Klein and Tenente SC dos S Gomes and all Army personnel (Centro de Instruções de Guerra na Selva do Exército Brasileiro – CIGS/EB) for logistic support at LBA/ZF-2 and ATTO Scientific Stations, ARIE PDBFF, REBIO Uatumã, PARNA Anavilhanas, and CIGS/EB training site, respectively. This work was funded by the Brazilian National Council for Scientific and Technological Development (CNPq Universal Grant nº: 401120/2016-3 to APL) and by the Coordination for the Improvement of Higher Education Personnel through its Support Program for Excellency Centres (CAPES/PROEX Grant nº: 0616/2018). RFJ received a scholarship from CAPES/FAPEAM (Grant nº: 24/2014). WEM was supported by the Program for Biodiversity Research in Western Amazonia (PPBio-AmOc) and the National Institute for Amazonian Research (INCT-CENBAM).

REFERENCES

Amaral, S., Costa, C. B., Arasato, L. S., Ximenes, A. C., & Rennó, C. D. (2013). AMBDATA: Variáveis ambientais para Modelos de Distribuição de Espécies (SDMs). In: Simpósio Brasileiro de Sensoriamento Remoto, 16, Foz do Iguaçu, Paraná, Brasil. Retrieved from http://www.dpi.inpe.br/AmBdata

Azevedo-Ramos, C., Ron, S., Coloma, L. A., Bustamante, M. R., Salas, A., Schulte, R., Lötters, S., Angulo, A., Castro, F., Lescure, J., Marty, C., La Marca, E., & Hoogmoed, M. (2010). *Atelopus spumarius*. In IUCN Red List of Threatened Species. Version 2012.1. Retrieved from http://www.iucnredlist.org

Baccini, A., Goetz, S. J., Walker, W. S., Laporte, N. T., Sun, M., Sulla-Menashe, D., Hackler, J., Beck, P. A. S., Dubayah, R., Friedl, M. A., Samanta, S., & Houghton, R. A. (2012). Estimated carbon dioxide emissions from tropical deforestation improved by carbon-density maps. *Nature and Climate Change*, 2, 182-185. https://doi.org/10.1038/NCLIMATE1354

Bacigalupe, L. D., Vasquez, I. A., Estay, S. A., Valenzuela-Sanchez, A., Alvarado-Rybak, M., Piñafiel-Ricaurte, A., Cunninghan, A. A., & Soto-Azat, C. (2019). The amphibian-killing

fungus in a biodiversity hotspot: identifying and validating high-risk areas and refugia. *Disease Ecology*, 10, e02724. https://doi.org/10.1002/ecs2.2724

Barth, B. J., & Wilson, R. S. (2010). Life in acid: interactive effects of pH and natural organic acids on growth, development and locomotor performance of larval striped marsh frogs (*Limnodynastes peronii*). *Journal of Experimental Biology*, 213, 1293-1300. https://doi.org/10.1242/jeb.028472

Becker, C. G., Rodriguez, D., Lambertini, C., Toledo, L. F., & Haddad, C. F. B. (2015). Historical dynamics of *Batrachochytrium dendrobatidis* in Amazonia. *Ecography*, 39, 954-960.

https://doi.org/10.1111/ecog.02055

Bolom-Huet, R., Pineda, E., Díaz-Fleischer, F., Muñoz-Alonso, A. L., & Galindo-González, J. (2019). Known and estimated distribution in Mexico of *Batrachochytrium dendrobatidis*, a pathogenic fungus of amphibians. *Biotropica*, 51, 731-746. https://doi.org/10.1111/btp.12697

Bouckaert, R., Timothy, G., Vaughan, J., Barido-Sottani, S., Duchêne, M. F., Alexandra, G., Joseph, H., Graham, J., Denise, K., Nicola, de M., Michael, M., Fábio, K. M., Nicola, F. M., Huw, A. O., Louis, du P., Alex, P., Andrew, R., David, R., Igor, S., Marc, A. S., Chieh-His, W., Dong, X., Chi, Z., Tanja, S., & Alexei, J. D. (2019). BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *PLoS Computational Biology*, 15, e1006650. https://doi.org/10.1371/journal.pcbi.1006650

Cocroft, R. B., Mcdiarmid, R. O. Y. W., & Ruiz-Carranza, P. M. (1990). Vocalizations of eight species of *Atelopus* (Anura: Bufonidae) with comments on communication in the genus. *Copeia*, 3, 631-643.

CPRM (Serviço Geológico do Brasil). (2010). Mapa Geodiversidade do Amazonas. Manaus: CPRM - Companhia de Pesquisa de Recursos Minerais. Manaus, Brasil. Retrieve from http://www.cprm.maps.arcgis.com

Farias, I. P., Santos, W. G., Gordo, M., & Hrbek, T. (2015). Effects of forest fragmentation on genetic diversity of the critically endangered primate, the Pied Tamarin (*Saguinus bicolor*): implications for conservation. *Journal of Heredity*, 106, 512-521. https://doi.org/10.1093/jhered/esv048

Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: New 1-Km spatial resolution climate surfaces for global land areas. *International Journal of Climatology*, 37, 4302-4315. https://doi.org/10.1002/joc.5086

Frost, D. R. 2020. Amphibian Species of the World: an online reference. Version 6.1. American Museum of Natural History, New York, USA. https://doi.org/10.5531/db.vz.0001. Retrieve from https://amphibiansoftheworld.amnh.org/index.php

Hengl, T., de Jesus, J. M., MacMillan, R. A., Batjes, N. H., Heuvelink, G. B. M., Ribeiro, E.,Samuel-Rosa, A., Kempen, B., Leenaars, J. G. B., Walsh, M. G., & Gonzalez, M. R. (2014). SoilGrids1km – global soil information based on automated mapping. *PLoS ONE*, 9, e105992. https://doi.org/10.1371/journal.pone.0105992

Hess, L. L., Melack, J. M., Affonso, A. G., Barbosa, C., Gastil-Buhl, M., & Evelyn, M. L. M. (2015). Wetlands of the lowland Amazon basin: Extent, vegetative cover, and dual-season inundated area as mapped with JERS-1 Synthetic Aperture Radar. *Wetlands*, 35, 745-756. https://doi.org/10.1007/s13157-015-0666-y

Hijmans, R. J., Phillips, S., Leathwick, J., & Elith, J. (2017). dismo: Species Distribution Modelling. Retrieve from https://CRAN.R-project.org/package=dismo

IBGE (Instituto Brasileiro de Geografia e Estatística). (2000). Censo Demográfico 2000. Brasília, Brasil.

IBGE (Instituto Brasileiro de Geografia e Estatística). (2004). Mapa da Vegetação do Brasil 1:5.000.000. Brasília, Brasil. Retrieve from http://mapas.ibge.gov.br/tematicos/vegetacao

IBGE (Instituto Brasileiro de Geografia e Estatística). (2010). XII Censo Brasil. Brasília, Brasil.

IUCN. (2012). *IUCN Red List Categories and Criteria: Version 3.1* (2nd ed.). Gland, Switzerland and Cambridge, UK: IUCN.

Jorge, R. F., Simões, P. I., Magnusson, W. E., & Lima, A. P. (2016). Fine-scale habitat heterogeneity explains the local distribution of two Amazonian frog species of concern for conservation. *Biotropica*, 48, 694-703. https://doi.org/10.1111/btp.12333

Jungfer, K-H., Faivovich, J., Padial, J. M., Castroviejo-Fisher, S., Lyra, M. M., Berneck, B. V. M., Iglesias, P. P., Kok, P. J. R., MacCulloch, R. D., Rodrigues, M. T., Verdade, V. K., Gastello, C. P. T., Chaparro, J. C., Valdujo, P. H., Reichle, S., Moravec, J., Gvoždík, V., Gagliardi-Urrutia, G., Ernst, R., De la Riva, I., Means, D. B., Lima, A. P., Señaris, J. C., Wheeler, W. C., & Haddad, C. F. B. (2013). Systematics of spiny-backed treefrogs (Hylidae: *Osteocephalus*): An Amazonian puzzle. *Zoologica Scripta*, 42, 351-380. https://doi.org/10.1111/zsc.12015

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28, 1647-1649. https://doi.org/10.1093/bioinformatics/bts199

Kok, P. J. R., Ratz, S., MacCulloch, R. D., Lathrop, A., Dezfoulian, R., Aubret, F., & Means, D. B. (2017). Historical biogeography of the palaeoendemic toad genus *Oreophrynella* (Amphibia: Bufonidae) sheds a new light on the origin of the Pantepui endemic terrestrial biota. *Journal of Biogeography*, 45, 26-36. https://doi.org/10.1111/jbi.13093

La Marca, E., Lips, K. L., Lötters, S., Puschendorf, R., Ibáñez, R., Rueda-Almonacid, J. V., Schulte, R., Marty, C., Castro, F., Manzanilla-Puppo, J., García-Pérez, J. E., Bolaños, F.,Chaves, G., Pounds, J. A., Toral, E., & Young, B. E. (2005). Catastrophic population declines and extinctions in Neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica*, 37, 190-201. https://doi.org/10.1111/j.1744-7429.2005.00026.x

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2016). PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*, 34, 772-773. https://doi.org/10.1093/molbev/msw260

Legendre, L., Dale, M. R. T., Fortin, M-J., Gurevitch, J., Hohn, M., & Myers, D. (2002). The consequences of spatial structure for the design and analysis of ecological field surveys. *Ecography*, 25, 601-615. https://doi.org/10.1034/j.1600-0587.2002.250508.x

Lemmon, P. E. (1956). A spherical densiometer for estimating forest overstory density. *Forest Science*, 2, 314-320. https://doi.org/10.1093/forestscience/2.4.314

Lima, A. P., Magnusson, W. E., Menin, M., Erdtmann, L. K., Rodrigues, D. J., Keller, C., & Hödl, W. (2006). *Guide to the frogs of Reserva Ducke – central Amazonia* (2nd ed.). Áttema Design Editorial, Manaus.

Lötters, S. (1996). *The neotropical toad genus Atelopus*. *Checklist, biology and distribution* (1st ed.). M. Vences, & F. Glaw Verlags GBR, Köln, Germany.

Lötters, S., Haas, W., Schick, S., & Böhme, W. (2002). On the systematics of the harlequin frogs (Amphibia: Bufonidae: *Atelopus*) from Amazonia. II: Redescription of *Atelopus pulcher* (Boulenger, 1882) from the eastern Andean versant in Peru. *Salamandra*, 38, 165-184.

Lötters, S., van der Meijden, A., Rödder, D., Köster, T. E., Kraus, T., La Marca, E., Haddad, C. F. B., & Veith, M. (2010). Reinforcing and expanding the predictions of the disturbance vicariance hypothesis in Amazonian harlequin frogs: a molecular phylogenetic and climate envelope modelling approach. *Biodiversity and Conservation*, 19, 2125-2146. https://doi.org/10.1007/s10531-010-9869-y

Lötters, S., Van Der Meijden, A., Coloma, L. A., Boistel, R., Cloetens, P., Ernst, R., Lehr, E., & Veith, M. (2011). Assessing the molecular phylogeny of a near extinct group of vertebrates: the Neotropical harlequin frogs (Bufonidae: *Atelopus*). *Systematics and Biodiversity*, 9, 45-57. https://doi.org/10.1080/14772000.2011.557403

Lyra, M. L., Haddad, C. F. B., & Azeredo-Espin, A. M. L. (2017). Meeting the challenge of DNA barcoding Neotropical amphibians: polymerase chain reaction optimization and new *COI* primers. *Molecular Ecology*, 17, 966-980. https://doi.org/10.1111/1755-0998.12648

Magnusson, W. E., Braga-Neto, R., Pezzini, F. F., Baccaro, F., Bergallo, H., Penha, J., Rodrigues, D., Verdade, L. M., Lima, A., Albernaz, A. L., Hero, J. M., Lawson, B. E., Castilho, C., Drucker, D., Franklin, E., Mendonça, F., Costa, F., Galdino, G., Castley, G., Zuanon, J., Vale, J., do Santos, J. L. C., Luizão, R., Cintra, R., Barbosa, R. I., Lisboa, A., Koblitz, R. V., Cunha, C. N., & Pontes, A. R. M. (2013). *Biodiversidade e Monitoramento Ambiental Integrado* (1st ed.). Áttema Editorial: Assessoria e Design, Manaus.

Messing, J. (1983). New *M13* vectors for cloning. *Methods in Enzymology*, 101, 20-78. https://doi.org/10.1016/0076-6879(83)01005-8

Noonan, B. P., & Gaucher, P. (2005). Phylogeography and demography of Guianan harlequin toads (*Atelopus*): Diversification within a refuge. *Molecular Ecology*, 14, 3017-3031. https://doi.org/10.1111/j.1365-294X.2005.02624.x

Puppim de Oliveira, J. A. P. de., Balaban, O., Doll, C. N. H., Moreno-Peñaranda, R., Gasparatos, A., Iossifova, D., & Suwa, A. (2011). Cities and biodiversity: Perspectives and governance challenges for implementing the convention on biological diversity (*CBD*) at the city level. *Biological Conservation*, 144, 1302-1313. https://doi.org/10.1016/j.biocon.2010.12.007

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

Peters, J. A. (1973). The frog genus *Atelopus* in Ecuador (Anura: Bufonidae). *Smithsonian Contribution to Zoology*, 145, 1-49.

Quesada, C. A., Lloyd, J., Schwarz, M., Patiño, S., Baker, T. R., Czimczik, C., Fyllas, N. M., Martinelli, L., Nardoto, G. B., Schmerler, J., Santos, A. J. B., Hodnett, M. G., Herrera, R., Luizão, F. J., Arneth, A., Lloyd, A., Dezzeo, N., Hilke, I., Kuhlmann, I., Raessler, M., Brand,. A., Geilmann, H., Moraes Filho, J. O., Carvalho, F. P., Araujo Filho, R. N., Chaves, J. E., Cruz Junior, O. F., Pimentel, T. P., & Paiva, R. (2010). Variations in chemical and physical properties of Amazon forest soils in relation to their genesis. *Biogeosciences*, 7, 1515-1541. https://doi.org/10.5194/bg-7-1515-2010

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, 67, 901-904. https://doi.org/10.1093/sysbio/syy032

Rambaut, A., & Drummond, A. J. (2019a). Logcombiner v2 6.0. Software development. Part of Beast 2.5.

Rambaut, A., & Drummond, A. J. (2019b). Treeannotator v2 6.0 – MCMC output analysis. Software development. Part of Beast 2.5.

Schaefer, C. E. G. R., Lima, H. N. de., Teixeira, W. G., Vale Jr., J. F. do., Souza, K. W. de., Corrêia, G. R., Mendonça, B. A. F. de., Amaral, E. F., Campos, M. C. C., & Ruivo, M. L. P. (2017). Solos da região amazônica. In N. Curi, J. C. Ker, R. F. Novais, P. Vidal-Torrado, & C. E. G. R. Schaefer (Eds.), *Pedologia - Solos dos Biomas Brasileiros* (pp. 112-167). Sociedade Brasileira de Ciência do Solo. Viçosa, MG.

LIST OF FIGURES

Figure 5. Plots indicating the relationships between local variables and Manaus-harlequin-frog density-index variation: (a) stream pH; (b) stream discharge; and (c) canopy openness.

FIGURES

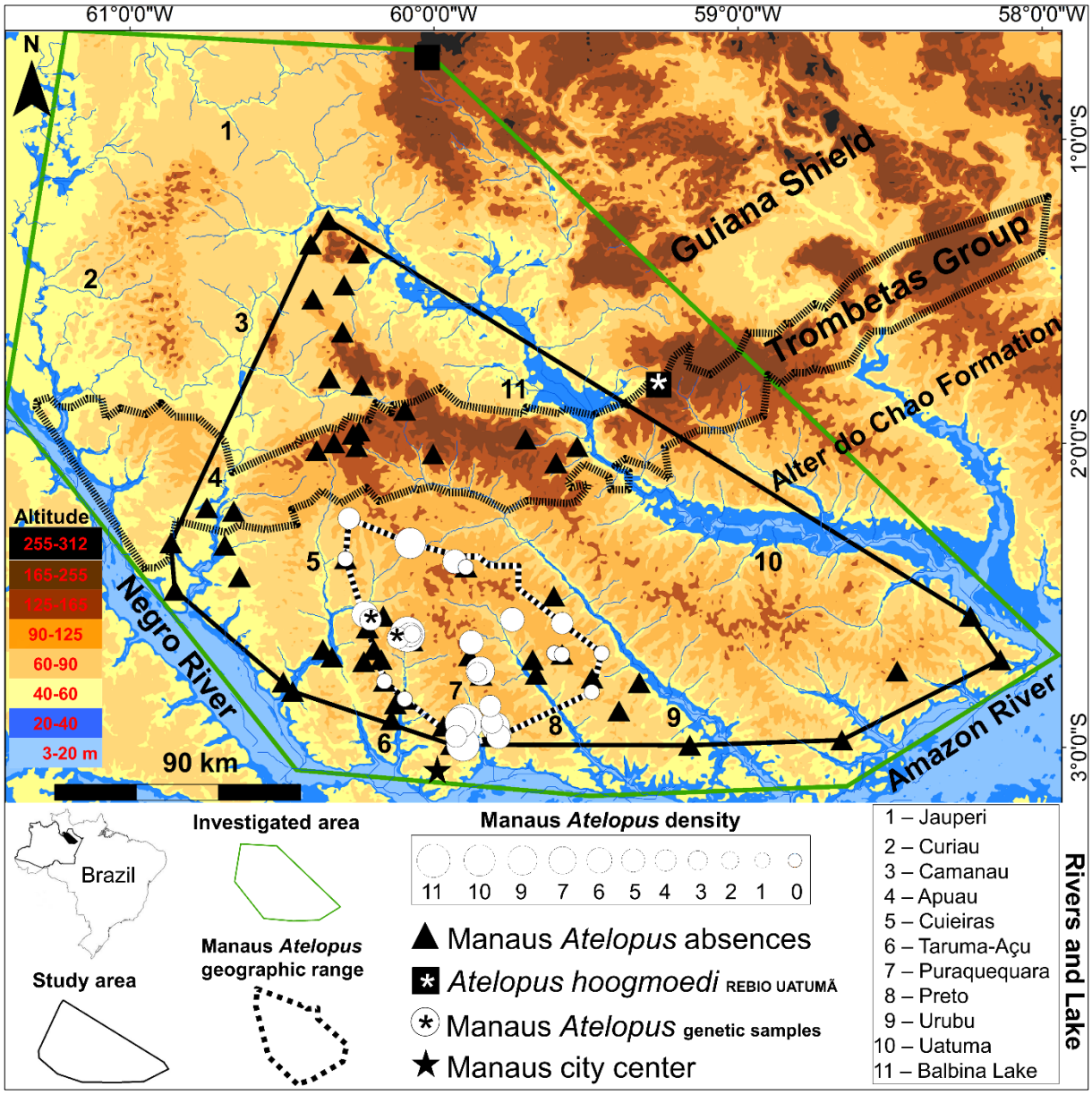


Figure 1.

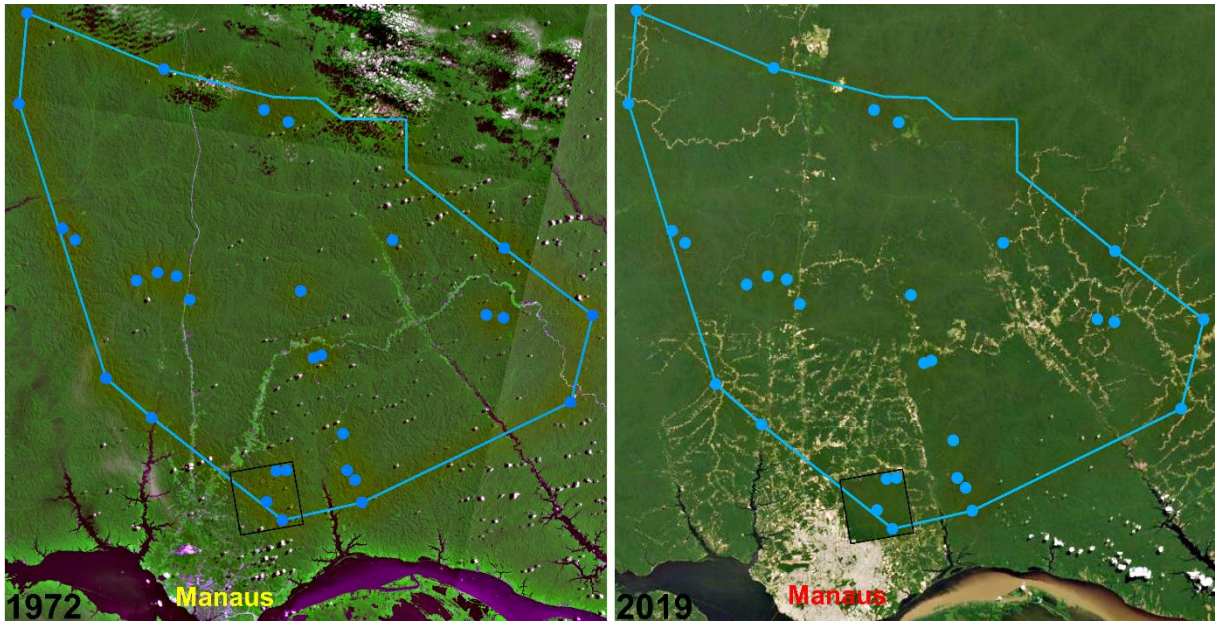


Figure 2.

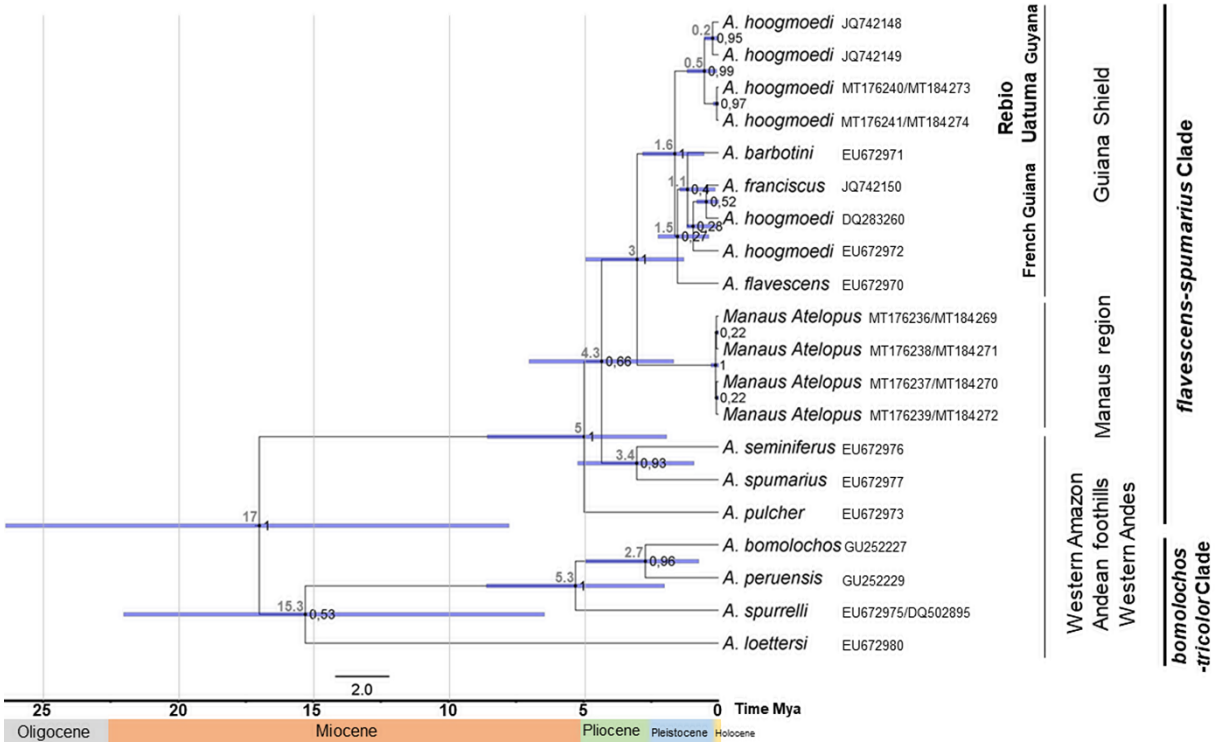


Figure 3.

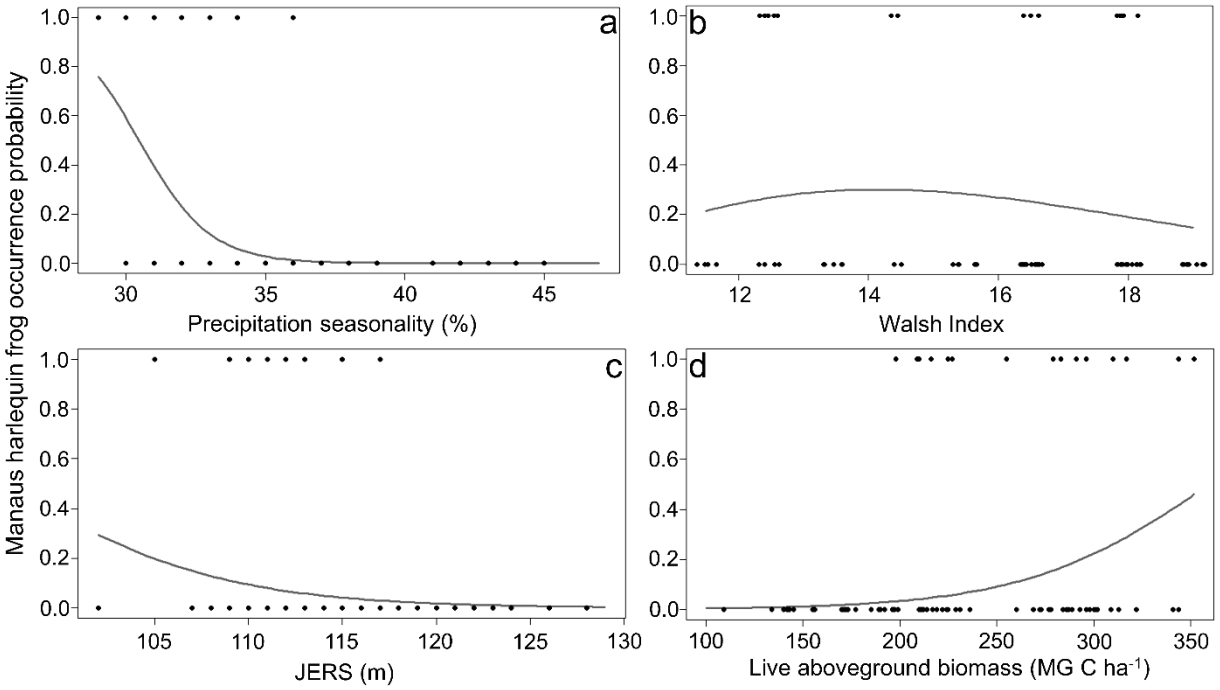


Figure 4.

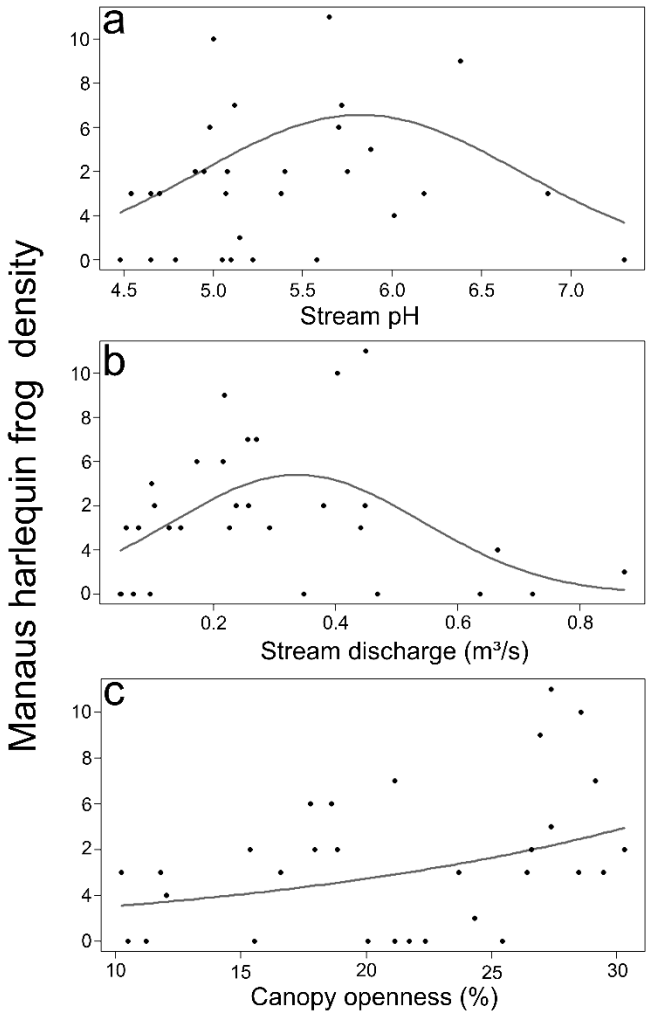


Figure 5.

SUPPORTING INFORMATION

Table S1. Descriptive statistics of seven landscape-scale variables used in the logistic model to estimate the occurrence probability of the Manaus harlequin frog and of four local-scale variables used in the generalized linear models to estimate its density variation, as follows: PS (%) = precipitation seasonality; LAB (MG C ha^{-1}) = live aboveground biomass; AL (m) = altitude; WAL = Walsh Index; silt (%) = percentage soil silt content; JERS (km^2 x 10^3) = flooded-area extent in the high-water seasons; HAND (m) = height above the nearest drainage; pH = pH; SD (m^3/s) = stream discharge; CO (%) = canopy openness. Descriptive statistics abbreviations: CV = Coefficient of variation; SD = Standard deviation.

Table S2. Pearson correlation matrix between landscape-scale variables used in the logistic model to predict the occurrence probability of the Manaus harlequin frog in 75 sampling sites in the study area, as follows: PS (%) = precipitation seasonality; LAB (MG C ha^{-1}) = live aboveground biomass; AL (m a.s.l.) = altitude; WAL = Walsh Index; silt (%) = percentage soil silt content; JERS (m) = flooded-area extent in the high-water seasons; HAND (m) = height above the nearest drainage.

Table S3. Pearson correlation matrix between local-scale variables used in the generalized linear model to predict the density variation for the Manaus harlequin frog in 30 sampling sites within the species geographic range, as follows: Stream pH; Stream discharge (m^3/s); Canopy openness (%).

Table S4. Kimura 2-Parameter (lower diagonal) and uncorrected p–distances (upper diagonal) genetic distances among the taxonomic groups: Manaus harlequin frog (four sequences), the geographically closest congeneric species from Reserva Biológica do Uatumã, Presidente Figueiredo, Amazonas, Brazil (*Atelopus hoogmoedi* – two sequences), six species (plus *Atelopus hoogmoedi* from Guyana and French Guiana populations –10 sequences) from the *flavescens-spumarius* clade distributed on the Guiana Shield, Andean foothills and western Amazonia and four species (four sequences) from the *bomolochos-tricolor* clade from eastern and western Andean foothills and Andean highlands. Genetic distances were estimated based on *16S* rDNA aligned sites.

Table 1. Species used in the phylogenetic assessment, the accession number of *16S* and *COI* sequences in GenBank, the voucher number, and the country where the individuals sequenced were collected. Sequence for specimen one to six were generated in the course of the current study. Sequences of specimens from seven to 20 were obtained from previously published data. GenBank accession number are referred to in Figure 3 after species names and specimens "ID" are referred to by the "ID" column in Table S4.

ID	Species	GenBank *16S*/*COI*	Voucher	Country
1	Manaus halerquin frog *Atelopus* sp.	MT176236/MT184269	INPA-H041289	Brazil
2	Manaus halerquin frog *Atelopus* sp.	MT176237/MT184270	INPA-H041290	Brazil
3	Manaus halerquin frog *Atelopus* sp.	MT176238/MT184271	INPA-H041291	Brazil
4	Manaus halerquin frog *Atelopus* sp.	MT176239/MT184272	INPA-H041292	Brazil
5	*Atelopus hoogmoedi* REBIO Uatumã	MT176240/MT184273	INPA-H041293	Brazil
6	*Atelopus hoogmoedi* REBIO Uatumã	MT176241/MT184274	INPA-H041294	Brazil
7	*Atelopus hoogmoedi* Lescure, 1974	JQ742148	IRSNB15781	Guyana
8	*Atelopus hoogmoedi*	JQ742149	IRSNB14477	Guyana
9	*Atelopus barbotini* Lescure, 1981	EU672971	-	French Guiana
10	*Atelopus franciscus* Lescure, 1974	JQ742150	PK3306	French Guiana
11	*Atelopus* (*spumarius*) *hoogmoedi*	DQ283260	BPN754UTA	French Guiana
12	*Atelopus hoogmoedi*	EU672972	-	French Guiana
13	*Atelopus flavescens* Duméril & Bibron, 1841	EU672970	-	French Guiana
14	*Atelopus seminiferus* Cope, 1874	EU672976	-	Peru
15	*Atelopus spumarius* Cope, 1971	EU672977	-	Peru
16	*Atelopus pulcher* Boulenger, 1882	EU672973	KU 211678	Peru
17	*Atelopus bomolochos* Peters, 1973	GU252227	KU 217468	Ecuador
18	*Atelopus peruensis* Gray & Cannatella, 1985	GU252229	KU 211631	Peru
19	*Atelopus spurrelli* Boulenger, 1914	EU672975/DQ502895	MHNUC 273	Colombia
20	*Atelopus loettersi* De la Riva, Castroviejo-Fisher, Chaparro, Boistel, & Padial, 2011	EU672980	-	Peru

Appendix 1. Glossary of abbreviations

AIC – Akaike Information Criteria

Bd – Batrachochytrium dendrobatidis

BIC – Bayesian Information Criteria

CEUA – Comissão de Ética no Uso de Animais

CPRM – Serviço Geológico do Brazil

ESU – Evolutionary significant unit

GLM – Generalized Linear Model

IBGE – Instituto Brasileiro de Geografia e Estatística

ICMBio – Instituto Chico Medes de Conservação da Biodiversidade

INPA – Instituto Nacional de Pesquisas da Amazônia

IUCN – International Union for Conservation of Nature

LM – Logistic Model

Mya – Million Years Ago

PCR – Polymerase Chain Reaction

SISBIO – Sistema de Autorização e Informação em Biodiversidade

SRTM – Shuttle Radar Topography Mission

VIF – Variance Inflation Factor

Title: Urban growth threatens the lowland Amazonian Manaus harlequin frog which represents an evolutionarily significant unit within the genus *Atelopus* (Amphibia: Anura: Bufonidae)

Authors: Rafael Filgueira Jorge, William Ernest Magnusson, Dayse Aparecida da Silva, Érico Macedo Polo, & Albertina Pimentel Lima

List of supporting information: Supporting Information Table S1; Supporting Information Table S2; Supporting Information Table S3; Supporting Information Table S4.

Table S1. Descriptive statistics of seven landscape-scale variables used in the logistic model to estimate the occurrence probability of the Manaus harlequin frog and of four local-scale variables used in the generalized linear model to estimate its density variation, as follows: PS (%) = precipitation seasonality; LAB (MG C ha^{-1}) = live aboveground biomass; AL (m) = altitude; WAL = Walsh Index; silt (%) = percentage soil silt content; JERS (km^2 x 10^3) = flooded-area extent in the high-water seasons; HAND (m) = height above the nearest drainage; pH = pH; SD (m^3/s) = stream discharge; CO (%) = canopy openness. Descriptive statistics abbreviations: CV = Coefficient of variation; SD = Standard deviation.

	Landscape variables							Local variables		
Descriptive Statistics	PS	LAB	AL	WAL	SILT	JERS	HAND	pH	SD	CO
Range	29–48	109–372	18–178	11.5–19	12–28	102–128	0.0–40.19	4.48–7.3	0.04–0.87	10.22–30.33
Mean	35.24	239.97	85.34	16.08	15.16	113.97	14.99	5.37	0.29	34.81
Median	34	227	77	16.5	14	113	13.82	5.13	0.24	21.43
CV	12.53	25.68	40.13	15.08	21.9	4.43	0.71	12.59	73.54	29.71
Variance	19.5	3799	1173	5.88	11.2	25.49	114.43	0.45	0.047	39.81
SD	4.41	61.64	34.25	2.42	3.32	5.5	10.69	0.67	0.21	6.30

Table S2. Pearson correlation matrix between landscape-scale variables used in the logistic model to predict the occurrence probability of the Manaus harlequin frog in 75 sampling sites in the study area, as follows: PS (%) = precipitation seasonality; LAB (MG C ha^{-1}) = live aboveground biomass; AL (m a.s.l.) = altitude; WAL = Walsh Index; silt (%) = percentage soil silt content; JERS (m) = flooded-area extent in the high-water seasons; HAND (m) = height above the nearest drainage.

Landscape variables	PS	LAB	AL	WAL	SILT	JERS	HAND
PS	1						
LAB	-0.04	1					
AL	0.09	0.40	1				
WAL	-0.24	0.23	0.35	1			
SILT	0.39	0.09	-0.03	0.18	1		
JERS	0.12	-0.09	-0.08	-0.12	-0.04	1	
HAND	0.01	0.20	0.42	0.14	-0.09	0.10	1

Table S3. Pearson correlation matrix between local-scale variables used in the generalized linear model to predict the density variation for the Manaus harlequin frog in 30 sampling sites within the species geographic range, as follows: Stream pH; Stream discharge (m^3/s); Canopy openness (%).

Local variables	Stream pH	Stream discharge	Canopy openness
Stream pH	1		
Stream discharge	-0.10	1	
Canopy openness	0.10	-0.07	1

Table S4. Kimura 2-Parameter (lower diagonal) and uncorrected p–distances (upper diagonal) genetic distances among the taxonomic groups: Manaus harlequin frog (four sequences), the geographically closest congeneric species from Reserva Biológica do Uatumã, Presidente Figueiredo, Amazonas, Brazil (*Atelopus hoogmoedi* - two sequences), six species (10 sequences) from the *flavescens-spumarius* clade distributed in Guiana Shield, eastern Andean foothills and western Amazonia and four species (four sequences) from the *bomolochos-tricolor* clade from western and eastern Andean foothills and Andean highlands. Genetic distances were estimated based on *16S* rDNA aligned sites.

ID	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	Manaus *Atelopus*		0.0	0.0	0.0	2.6	2.7	2.7	2.8	2.8	2.8	3.2	3.1	2.3	3.1	3.8	4.9	9.4	10.0	11.2	10.7
2	Manaus *Atelopus*	0.0		0.0	0.0	2.6	2.7	2.7	2.8	2.8	2.8	3.2	3.1	2.3	3.1	3.8	4.9	9.4	10.0	11.2	10.8
3	Manaus *Atelopus*	0.0	0.0		0.0	2.6	2.7	2.7	2.8	2.8	2.8	3.2	3.1	2.3	3.1	3.8	4.9	9.4	10.0	11.2	10.7
4	Manaus *Atelopus*	0.0	0.0	0.0		2.2	2.2	2.7	2.2	2.1	2.1	2.3	2.5	2.3	3.0	3.9	4.0	10.0	10.2	11.9	10.9
5	*A. hoogmoedi* REBIO Uatumã	2.8	2.8	2.8	2.3		0.0	0.4	0.2	0.4	0.4	0.9	1.3	0.4	2.3	2.9	3.8	9.8	10.4	11.2	10.6
6	*A. hoogmoedi* REBIO Uatumã	2.8	2.8	2.8	2.3	0.0		0.4	0.2	0.4	0.4	0.9	1.3	0.4	2.3	2.9	3.8	9.8	10.4	11.2	10.6
7	*A. hoogmoedi* Guyana	2.8	2.8	2.8	2.8	0.5	0.5		0.5	0.2	0.7	0.2	0.7	0.2	2.6	2.4	3.3	11.6	12.1	13.7	11.8
8	*A. hoogmoedi* Guyana	2.9	2.9	2.9	2.4	0.2	0.2	0.5		0.6	0.2	0.7	1.6	0.6	2.6	3.1	4.1	10.4	11.0	11.7	11.5
9	*A. franciscus*	3.0	3.0	3.0	2.2	0.4	0.4	0.2	0.6		0.7	0.2	1.1	0.2	2.1	2.7	3.6	9.9	10.5	11.2	10.8
10	*A. hoogmoedi* (French Guiana)	3.0	3.0	3.0	2.2	0.4	0.4	0.7	0.2	0.7		0.9	1.5	0.6	2.7	3.1	4.2	10.3	10.9	11.6	11.0
11	*A. hoogmoedi* (French Guiana)	3.4	3.4	3.4	2.5	0.9	0.9	0.2	0.7	0.2	0.9		1.3	0.4	2.3	2.9	3.8	9.8	10.4	11.0	10.6
12	*A. flavescens*	3.3	3.3	3.3	2.6	1.3	1.3	0.7	1.6	1.1	1.5	1.3		0.4	3.1	3.3	4.6	10.5	11.2	12.2	11.1
13	*A. barbotini*	2.4	2.4	2.4	2.4	0.4	0.4	0.2	0.6	0.2	0.6	0.4	0.4		2.2	2.6	2.7	10.3	10.7	11.8	10.8
14	*A. seminiferus*	3.3	3.3	3.3	3.2	2.4	2.4	2.7	2.7	2.2	2.8	2.4	3.2	2.2		2.6	2.7	9.5	9.7	11.2	10.5
15	*A. pulcher*	4.1	4.1	4.1	4.2	3.0	3.0	2.5	3.2	2.8	3.2	3.0	3.5	2.7	2.7		4.3	9.4	9.4	10.8	9.7
16	*A. spumarius*	5.3	5.3	5.3	4.2	4.0	4.0	3.4	4.4	3.8	4.4	4.0	4.9	2.9	2.8	4.6		10.1	10.5	11.2	12.5
17	*A. peruensis*	10.8	10.8	10.8	11.6	11.3	11.3	13.8	12.2	11.6	12.1	11.3	12.3	12.0	11.0	10.9	11.7		2.4	4.6	9.7
18	*A. bomolochos*	11.7	11.7	11.7	12.0	12.2	12.2	14.6	13.0	12.4	12.9	12.1	13.2	12.6	11.3	10.9	12.3	2.5		5.6	10.0
19	*A. spurrelli*	13.3	13.3	13.3	14.3	13.3	13.3	16.8	14.0	13.3	13.8	13.0	14.7	14.1	13.3	12.7	13.3	4.9	6.0		9.5
20	*A. loettersi*	12.7	12.8	12.7	13.0	12.4	12.4	14.1	13.7	12.8	13.1	12.5	13.2	12.8	12.3	11.3	15.1	11.3	11.6	11.1	

Capítulo 3

Jorge, R.F., Lima, A.P. & Stow, A.J. Strong genetic divergence and signatures of selection in an endangered Amazonian frog. Manuscrito em preparação para a revista *Conservation Genetics*.

Authors: Rafael Filgueira Jorge*[1], Albertina Pimentel Lima[2], Adam James Stow[3]

Title: Strong genetic divergence and signatures of selection in an endangered Amazonian frog

Running title: Landscape genomics of an endangered frog species

Affiliations: [1]Programa de Pós-Graduação em Ecologia, Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas, Brazil; [2]Coordenação de Biodiversidade, Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas, Brazil; [3] Department of Biological Sciences, Macquarie University, Sydney, NSW, Australia.

Corresponding author: * rafajorgebio@gmail.com

ORCID: [1] 0000-0001-9803-7546; [2] 0000-0003-4586-5633; [3] 0000-0002-6796-4854.

AKNOWLEGDMENTS

The authors would like to thank all the people who aided RFJ in field sampling. Sincere thanks are due to V. Albuquerque for taking RFJ to all sampling locations accessed by roads; J O'Hare and W Ashley for helping in statistical analyses; A Santana and M Ferrão for suggestions in tissues preparation and shipment; N Higuchi, JL Camargo and Tenente SC dos S Gomes and all Army personnel (Centro de Instruções de Guerra na Selva do Exército Brasileiro – CIGS/EB) for logistic support at LBA/ZF-2, ARIE PDBFF and CIGS/EB training site, respectively.

Abstract. Knowledge of gene flow and dispersal is critical for managing the impact of human activities on species of conservation significance. Here, we use a landscape genomic approach to characterise genetic connectivity and provide useful information to prioritizing conservation measures for an endangered Amazonian frog species, *Atelopus manauensis*. We sampled 94 individuals throughout the upper, middle and lower courses for each of the five catchments within the known geographic range. We used 3,121 putatively neutral single nucleotide polymorphisms (SNPs) to investigate the influence of environmental variables and the role of isolation by geographic distance in shaping the genetic variation of *Atelopus manauensis*. Genetic variation was significantly subdivided into six lineages, and the genetic distances among them were influenced by both isolation by distance and environmental variables - i.e., open forests and extreme altitudes impede genetic connectivity. There is evidence of adaptive differences among the six genetic lineages, suggesting the presence of six distinct population segments (DPSs) to be considered in conservation efforts. We identified 28 SNPs strongly associated with altitude and forest biomass gradients. Overall, the DPSs were characterized by low genetic variation (Observed heterozygosity - $H_o < 0.3$), signals of inbreeding ($Fis < 0.1$) and low effective population size ($N_e < 100$). Despite the limited geographic range of this endangered species we demonstrated distinct and adaptively divergent genetic lineages isolated by aspects of the physical environment related to altitude and forest biomass.

Key-words: Amazonia, endangered frog, connectivity, landscape heterogeneity, conservation

1 | Introduction

Small isolated populations lose genetic variation via random genetic drift which potentially elevates the risk of extinction by inbreeding depression and the loss of evolutionary potential (Keller and Waller 2002; Major et al. 2020). Characterizing genetic variation allows for the identification of Distinct Population Segments (DPSs) for purposes of conservation, defined as reproductively isolated and adaptively distinct lineages within species (Waples 1991; Funk et al. 2012; Supple and Shapiro 2018). Using these properties for delineating DPSs could lead to the protection of their evolutionary potential (Pearse 2016; Funk et al. 2019). Furthermore, characterizing adaptive genetic variation can be used to select the most suitable individuals for translocation (Gebremedhin et al. 2009; Pearse 2016; Fenster et al. 2018). Finally, the maintenance of genetic variation and therefore the evolutionary potential of DPSs can help mitigate the negative effects of changing environments (Moritz 2002; Duckett et al. 2013).

Levels of inbreeding and heterozygosity can be used to identify populations that are suffering the effects of isolation (Fenster et al. 2018). The effective population size (N_e) is the size of an ideal population that would lose heterozygosity at the same rate as the population being evaluated (Reid-Anderson et al. 2019). Therefore, estimates of N_e can be used to assess the risk of deleterious consequences arising from the loss of genetic variation (IUCN 2012; Frankham et al. 2014). The accessibility of large genome-wide SNP data sets has enabled precise estimates of N_e to be calculated, including methods based on levels of linkage disequilibrium from a single sample (Do et al. 2014).

To conserve genetic variation, we need to know its spatial distribution. For example, conservation strategies for the New Zealand Blue Duck were based on the distribution of major historical lineages across the species' geographic range (Grosser et al. 2017). Moreover, knowledge of which landscape characteristics influence dispersal and gene flow can contribute to conservation planning that aims to preserve connectivity among lineages and their genetic variation (Taylor et al. 1993; Beier et al. 2011). Functional connectivity describes the availability of ecologically suitable corridors for dispersal and genetic connectivity relates to the exchange of genetic variants among distinct lineages (Balkenhol et al. 2016). Recently, landscape genomics has been a valuable tool for conservationists and practitioners to evaluate the processes underpinning both genetic and functional connectivity (Balkenhol et al. 2017).

Identifying environmental variables that influence gene flow can be used to predict whether environmental change will alter functional and genetic connectivity (McRae et al.

2008; Wang 2012). For example, gene flow of habitat-specialist species in North American coniferous forests is decreased by deforestation (Emel et al. 2019; Linnell and Lesmeister 2019). Knowledge gained on the processes influencing landscape connectivity can be applied to identify and protect corridors of habitat that facilitate or maintain connectivity (Dickson et al. 2019). This, in turn, can promote the (re)colonization of unoccupied habitats to recover species' historic ranges, allow range expansion of populations threatened by habitat loss or facilitate genetic rescue of isolated populations through corridor restoration (Dickson et al. 2019).

In some Amazon interfluves, stream-breeding anurans are patchily distributed in response to local and landscape heterogeneity (i.e. *Atelopus manauensis*; Jorge et al. 2016; Jorge et al. 2020). *Atelopus manauensis* is a frog species distributed in a small geographic area on the surroundings of the largest city in Amazonia, Manaus, which is growing over the limited geographic area of the species (Jorge et al. 2020). Therefore, *Atelopus manauensis* is a good model to study the influence of landscape characteristics on the distribution of genetic variation and patterns of gene flow that may be useful for its conservation. Here we (1) characterize genetic variation and identify genetically distinct parts of the species distribution; (2) investigate if there is evidence of localised adaptation; and (3) identify which landscape features influence functional and genetic connectivity. This study is the first to use landscape genomic approaches to generate useful information for the conservation of an endangered species in Amazonia. The remarkable environmental heterogeneity of Amazonian landscapes is an important factor generating and maintaining genetic variation of a habitat-specialist frog. Therefore, adaptive genetic variation is useful to identify which lineages should be prioritized in conservation efforts of habitat-specialist species threatened by human impacts in tropical landscapes.

2 | Methods

2.1 | Study species and sampling

The Manaus harlequin frog (Anura: Bufonidae: *Atelopus manauensis*) is a stream-breeding species that inhabits riparian zones of first- to third-order clean-water streams in drainage systems of five central Amazonia catchments between the Cuieiras and Urubu Rivers (Fig. 1). Its occurrence is associated to habitats with more structured forests (live aboveground biomass 200-387 megagrams of carbon per hectare – MG C ha^{-1}) and intermediate altitudes (60-125 meters above sea level – m a.s.l.) that are separated by less structured forests (0-200 MG C ha^{-1}) and by low (3-60 m a.s.l.) and highlands (125-165 m a.s.l.) (Jorge et al. 2020).

We collected 94 individuals (males) of *Atelopus manauensis* in the rainy season (November-March) between 2016 and 2019 from 21 stream banks (numbered 1–21 in Fig. 1) in 14 drainage systems distributed in five catchments/major rivers, as follows: Cuieiras, Taruma-Açu, Puraquequara, Preto and Urubu Rivers (Fig. 1). Geographic coordinates (latitude and longitude) were obtained 1.5 m from stream margin using a Garmin 64s GPS (error ± 3 meters – Datum WGS84; Online Resource 1). Specimens were collected by hand and euthanized with 5% topic Lidocaine. About 5-mg muscle tissue from the thigh of individuals were extracted, preserved in 95% alcohol and stored at -30ºC. Individuals were collected under permission number 56759 from the Sistema de Autorização e Informação da Biodiversidade do Instituto Chico Mendes de Conservação da Biodiversidade (SISBIO/ICMBio). This study was approved by the Instituto Nacional de Pesquisas da Amazônia (INPA) ethics committee (registration number 002/2017 – Comissão de Ética no Uso de Animais – CEUA/INPA).

2.2 | DNA extraction and sequencing

DNA extraction and sequencing was performed at Diversity Arrays Technology sequencing facility (DArTseq – Canberra, Australia). DNA was extracted from 5 μg muscle tissue using the GeneCatchTM Blood & Tissue Geno-246 mic Mini Prep Kit (Epoch Life Science, Inc). SNPs discovery and genotyping was carried out using the standard DartSeq™ protocol. This is a modified double-digest restriction-site associated DNA (ddRAD) sequencing approach described in Kilian et al. (2012), which uses a combination of *Pst*I–*Hpa*II restriction enzymes in library preparation. Respective *Pst*I and *Hpa*II sequence adapters and unique barcodes were ligated to each sample to be amplified and sequenced on an Illumina HiSeq2500. After trimming, 65 bp-long unique sequences were aligned via Basic Local Alignment Search Tool (Blast) in National Center for Biotechnology Information (NCBI) platform. We used *Xenopus tropicalis* (Anura: Pipidae) reference genome with an *E*-value of 5^{e-7} and minimum sequence identity > 70%, as there is no reference genome for species of *Atelopus*. Sequences were also aligned to bacterial and fungal genomes to check for contamination. Further information on SNP extraction, filtering, initial calling and quality control are given in Online Resource 2.

2.3 | Data set filtering

After DArTseq sequencing and filtering, the full data set followed a further two-steps of filtering, as follows: (1) only 100% reproducibility; less than 5% of missing data overall (call rate for SNPs); read depth higher than 10x and lower than 90x; not duplicated; exclusive by

locus; and minimum allele frequency > 0.02; (2) only individuals that had more than 95% of SNPs (call rate for individuals); 97% consistently sequenced among genotypes (RepAvg); and non-monomorphic SNPs. All analyses were performed using the "dartR" package (Gruber et al. 2018) in R software (R Core Team 2020). We used Arlequin 3.5.2 software (Excoffier and Lischer 2010) to identify loci deviating from Hardy-Weinberg Equilibrium (HWE) expectation at 0.05 significant level that were excluded to generate a neutral data set.

2.4 | Summary statistics

Levels of observed (H_o) and expected (H_e) heterozygosity, the number of private alleles, fixation index (F_{is} = 1 - H_o/H_e) and pairwise genetic differentiation (F_{st}) were calculated for samples grouped into each of the 14 drainage systems and also for genetic lineages (K) identified in population structure analyses using the neutral data set (see subsection 2.7). We performed an Analysis of Molecular Variance (AMOVA) using 1,000 permutations to assess levels of genetic variation at different hierarchical levels of structure. All analyses listed above were done in Arlequin 3.5.2 (Excoffier and Lischer 2010). We used pairwise F_{st} to generate a heatmap using the R package "ggplot". We estimated the contemporary effective population size (N_e) for the genetic lineages identified using the molecular co-ancestry method of Nomura (2008), as implemented in N_eEstimator V2.1 (Do et al. 2014.), which is based on linkage disequilibrium (LD). We chose the random mating mode accounting for LD with a cut-off for minimum allele frequency of 0.05 and 0.02. The confidence intervals were obtained by jack-knifing the N_e (Do et al. 2014).

2.5 | Loci deviating from neutrality

We used four methods to detect loci under selection using the 3,859 SNPs data set generated after filtering steps: (1) the R package "pcadapt" (Luu et al. 2017); (2) Arlequin 3.5.2 (Excoffier and Lischer 2010); (3) Linkage disequilibrium network analysis; and (4) Latent factor mixed models (LFMM). "pcadapt" uses PCA scores to identify SNPs excessively associated with population structure that may signal the presence of local adaptation (outliers). In contrast, Arlequin relies on coalescent simulations to assess the *p*-values of loci-specific F_{st} values conditioned to observed heterozygosis (Excoffier et al. 2009). We used the Hierarchical Island Model to assign drainage systems (14) to six genetic lineages identified in population structure analyses in order to account for different patterns of population ancestry and distinct levels of gene flow (Excoffier et al. 2009). From these *p*-values, we calculated the *q*-values using the R package "qvalue" and selected outliers' loci with *q*-value lower than 0.05.

We also searched for loci under selection using the Linkage Disequilibrium approach implemented in the R package "LDna" (na = network analysis) as developed by Kemppainen et al. (2015). The method identifies non-random associations of loci that could be a consequence of different evolutionary forces, such as drift and local adaptation. These are referred to as Single Outlier Clusters (SOCs) and their identification are based on Linkage Disequilibrium thresholds. It starts with high LD threshold values (i.e. 0.9), when only few loci are linked, and as it is lowered more and more loci are added to a cluster. Loci that maintain clustered for a wide range of thresholds are considered "interesting" SOCs (Kemppainen 2014). We used the loci contained in SOCs as an input for PCA analysis and the first PCA axis of each SOC was used as response variable in a multiple linear regression including longitude, latitude, altitude and forest biomass of 21 sampled streams as explanatory variables. The values of altitude and forest biomass were extracted from each geographic coordinate using the R package "dismo" (Hijmans et al. 2017) from raster layers obtained in AmbData website (Amaral et al. 2013).

We searched for associations among loci and environmental gradients (altitude and forest biomass) using latent factor mixed models (LFMM) implemented in the R package "LEA" (Frichot et al. 2013). LFMM identifies significant associations between environmental variables and allele frequencies (signals of selection) while correcting for existent population structure (Latent Factors - LF) (Frichot et al. 2013). We ran LFMM with six LFs, 100,000 interactions and 10,000 burn-in using altitude and forest biomass as environmental factors. The median *z*-scores (environmental association strengthen) of five repetitions was calculated. The Benjamini–Hochberg procedure was applied to obtain candidate loci based on False Discovery Rate (FDR) of 1% using the adjusted *p*-values from model estimation. We used the genomic inflation factor (GIF) calculated over median *z*-scores (Devlin and Roeder 1999) to assess the successful control of FDR (close to 1) as suggested by Frichot and Francois (2015). To look for gene functionality, we selected loci under putative selection by altitude and forest biomass gradients those with *z*-scores greater than 4 ($p < 10^{-5}$) as suggested by Frichot et al. (2013) that matched to those selected based on FDR. Loci identified in above analyses and those deviating from HWE expectation (section 2.4) were excluded to generate a SNPs neutral data set.

2.6 | Gene annotation

We selected DNA sequences (65bp present in the raw DArTseq data set unique RAD tags) from the loci identified in the LFMM analysis with FDR < 0.0001% and *z*-scores > 4. We used these DNA sequences to search for gene annotation associated to altitude and forest biomass gradients. Due to the unavailability of *Atelopus* species' genome, RAD tags were

aligned to the frogs *Naborama parkeri*, *Bufo bufo*, *Xenopus tropicalis*, *X. laevis*, and *Abavorana luctuosa* genomes using the nucleotide Blast mode (*Blastn*) in NCBI platform (Johnson et al. 2008). We selected genes with at least 80% identity to the reference genomes and the lowest *E*-value among those listed in blast search.

2.7 | Population structure

We assessed population structure from the neutral data set (3,121 SNPs) using Discriminant Function of Principal Components (DAPC) in the R package "adegenet" (Jombart 2008). DAPC uses discriminant function to create clusters based on linear combination of alleles that have the largest between- and smallest within-group variance (Jombart and Collins 2012). The best *K* (populations = genetic lineages) is selected using Bayesian Information Criteria. We also used Sparse Non-Negative Matrix Factorization (SNMf) in the R package "LEA" (Frichot et al. 2014). SNMf uses a least-square approach to estimate the probability that a sample is derived from *k*th ancestral gene pools. The *K* that minimizes the cross-entropy is chosen (Frichot et al. 2014). We set SNMf as follows: $\alpha = 10$, $K = 1{:}14$, interactions = 10,000 and tolerance error = 0.00001. Finally, we used the software *Structure* to identify genetic lineages (*K*) and probabilistically assign individuals to them through a Bayesian model-based approach (Pritchard et al. 2000). We selected the optimal *K* based on Evanno et al.'s (2005) ΔK method using Structure Harvester website (Earl and vonHoldt 2012). We set *Structure* to model from 1 to 14 *K*, in order to avoid bias in the optimal *K* estimation (Puechmaille 2016), with 500,000 Markov Chains Monte Carlo (MCMC), 50,000 burn-in and five repetitions per *K*.

2.8 | Intraspecific coalescent tree

We generated a population-level lineage tree with the neutral SNPs data set through a Multispecies Coalescent Model using SNAPP template as implemented in Beast 2.5 software (Bouckaert et al. 2019) in Cipres Science Gateway website (Miller et al. 2010). SNAPP models directly incomplete lineage sorting (Wright-Fisher model), and then uses a coalescent process model to estimate the number of ancestral lineages via Markov process backward in time (Bryant et al. 2012). We ran one million generation sampling every 1,000 generations. Log files were checked for stationarity in Tracer 1.7 software (Rambaut et al. 2018). The final trees were visualized in Densitree v2.2.6. software (Bouckaert 2010). We set SNAPP using BEAUTi, as follows: mutational rates using "Calc mutation rates" box; 14 lineage hypotheses; speciation rate prior Lambda as gamma distribution with alpha 2 and beta 200; and other parameters as default.

2.9 | Influence of isolation by geographic, landscape resistance and environmental distances on genetic variation

2.9.1 | Distance matrices

We generated a pairwise genetic distance matrix for 89 individuals using the neutral SNPs data set with the Genotypic option for codominant data in GenAlEx 6.5 (Peakall and Smouse 2012). We also used GenAlEx to calculate pairwise Euclidean geographic distances (in kilometres). We generated an environmental distance matrix considering the values of altitude and forest biomass of the 21 sampled streams using the R package "stats" (R Core Team 2020).

We calculated pairwise distance matrices based on the resistance that altitude, forest biomass and sixth-order rivers (main catchments) impose to dispersing individuals. To explicitly test which portion of altitude and forest biomass gradients best explained genetic variation across landscape, we created five resistance layers of altitude (0-20, 20-60, 60-90, 90-125, 125-165 m a.s.l.) and four of forest biomass (0-100, 100-200, 200-300, 300-387 MG C ha^{-1}). We then assigned the value of 1000 for the portion of interest (i.e. 0-20 m a.s.l. = 1000) and one for all other portions (i.e. 20-165 m a.s.l. = 1) for each resistance layer. For sixth-order rivers, we assigned 1000 for water courses and one for landscape. We used a circuit-theory algorithm implemented in the Circuitscape software to estimate landscape resistance to gene flow (McRae 2006). Circuitscape evaluates the permeability of alternative pathways from a source to a focal point using random walk movement (McRae et al. 2008). It assigns resistances values to each cell to calculate average cumulative resistance between pair of locations (McRae and Beier 2007).

2.9.2 | Statistical analyses

We assessed the correlation between isolation by geographic distance and total genetic variation of *Atelopus manauensis* using Mantel test in GenAlEX. To further explore this, we used a Moran spatial autocorrelation correlogram to assess the distribution of genetic variation along 100 kilometres divided in even classes. We used geographic, landscape resistance and environmental distances as predictors of genetic variation in a mixed-effects model with maximum likelihood population effects (MLPE; Row et al. 2017) using the "MLPE_rga" function in the "ResistanceGA" R package (Peterman 2018). We built 11 individual models (10 resistance matrices and a null model) and then selected the models that best explained genetic variation using the "MUMIN" version 1.40.0 R package (Barton 2018). We considered the most

parsimonious models those with the lowest change in AICc score and the highest AIC weight (wAIC).

We assessed linear relationships between genetic and geographic (isolation by distance - IBD), landscape resistance (isolation by resistance- IBR) and environment of sample streams (isolation by environment - IBE) distances using redundancy analysis (RDA) in the R package "vegan" (Oksanen et al. 2013). Geographic and all classes of landscape resistance variables were highly correlated (Pearse's $r > 0.8$) and they showed multicollinearity (VIF > 3). Therefore, we built models including IBD and IBR (those selected in MLPE; see results) or IBE variables as predictors of genetic variation separately. Complementary, we built partial models with IBD and IBR or IBE variables conditioned to each other to obtain the individual contribution of each class of variables in explaining genetic variation. We also built a null model with 1 as predictor of genetic variation conditioned to IBD, IBE and IBR variables. We obtained statistical coefficients of each RDA model using ANOVA analysis. As an alternative to RDA analyses, the first axes of PCoA scores from genetic (response) and IBD, IBE and IBR (explanatories) distance matrices were used in multiple linear regression models, as follows: PCo1 genetic distance = $a + b_1$ (PCo1 IBD) + b_2 (PCo1 IBE altitude) + b_3 (PCo1 IBE forest biomass); PCo1 genetic distance = $a + b_1$ (PCo1 IBR 0–20 altitude) + b_2 (PCo1 IBE altitude) + b_3 (PCo1 IBE forest biomass); PCo1 genetic distance = $a + b_1$ (PCo1 IBR 20–60 altitude) + b_2 (PCo1 IBE altitude) + b_3 (PCo1 IBE forest biomass); PCo1 genetic distance = $a + b_1$ (PCo1 IBR 125–165 altitude) + b_2 (PCo1 IBE altitude) + b_3 (PCo1 IBE forest biomass); PCo1 genetic distance = $a + b_1$ (PCo1 IBR 0–100 forest biomass) + b_2 (PCo1 IBE altitude) + b_3 (PCo1 IBE forest biomass); PCo1 genetic distance = $a + b_1$ (PCo1 IBR 300–387 forest biomass) + b_2 (PCo1 IBE altitude) + b_3 (PCo1 IBE forest biomass).

3 | Results

3.1 | Data set features

DArTseq sequencing yielded 38,739 SNPs from 91 individuals (average allelic variation per SNP marker: 0.13; average polymorphic content: 0 to 1). Three individuals were excluded during the sequencing procedures because the poor quality of samples. The sample size of sequenced individuals ranged from three to 14 individuals per drainage system (mean = 6.5 ± 3.4). We obtained a final data set with 3,859 SNPs and 89 individuals after the two-step filtering. We excluded 738 loci identified as outliers, deviating from HWE expectation or associated to environmental variables (161 loci were identified by more than one method) to obtain a final data set of 3,121 neutral SNPs.

3.2 | Genetic diversity and effective population size

Observed and expected heterozygosity were relatively moderate in all 14 drainage systems, with H_o ranging from 0.26 ± 0.17 to 0.42 ± 0.25, and H_e from 0.29 ± 0.14 to 0.41 ± 0.13. *Ho* and *He* of six genetic lineages (identified in population structure analyses; see below) were slightly lower, with H_o varying between 0.18 ± 0.18 and 0.4 ± 0.24 and H_e between 0.2 ± 0.16. Expected heterozygosity was lower than H_o in a sample from the Taruma-Açu River (number 18 in Fig. 1) and in a sample of the upper course of the right headwater of the Cuieiras River (number 21 in Fig. 1) (Table 1). Overall, the fixation index was significantly low, showing strong signals of inbreeding. Considering the 14 drainage systems it varied from -0.06 to 0.14, and in the six genetic lineages from -0.05 to 0.4 (Table 2). This indicates higher gene flow between drainage systems within each of the six genetic lineages than between them.

Analysis of Molecular Variance (AMOVA) using drainage systems as higher level indicated that about 26% of total genetic variation was accounted for between drainage systems, while about 6% was accounted within drainage systems and about 63% between individuals (Table 3). When considering genetic lineages as higher levels, about 29% of the genetic variation was shared between genetic lineages, 5% between drainage systems of each genetic group and 65% between individuals (Table 4). Overall, pairwise genetic differentiation as F_{st} was moderate among the 14 drainage systems (0.3) and genetic lineages (0.29). The highest values (0.4 – 0.5) were found between headwater and lower-course drainage systems, between river banks and between drainage systems separated by high altitudes. The lowest values (0.05 – 0.1) were found between drainage systems from the same river bank, same catchment and in the same portion of altitude or forest biomass gradients (Fig. 2). The pairwise F_{st} between genetic lineages followed the same trend (Fig. 3). The effective population size was lower than 100 in all genetic lineages and the lowest (N_e = 20) in the genetic group with the highest inbreeding coefficient (Table 5), which is composed by sampled streams numbered 14-17-19-20-21 in figure 1 and represented by the blue colour in figures 6 and 7.

3.3 | Environmental Association and Linkage Disequilibrium network analyses

LFMM analysis identified three loci highly associated to forest biomass (*z*-score > 4) and 55 loci putatively under selection along this gradient considering FDR less than 1%, with three loci in common between *z*-score and FDR criteria. LFMM identified 107 loci highly associated to altitude (*z*-score > 4) and 82 are likely to be under selection along this gradient considering FDR less than 1%, with 25 loci in common between *z*-score and FDR criteria. Therefore, we search for gene functionality using these three common loci associated with

forest biomass and 25 common loci associated with altitude. One altitude-related outlier locus is associated to adaptive immune defence (*z*-score = 4.1; *E*-value = 0.5) and one forest biomass-related locus is associated with anion exchange in red blood (*z*-score = 4.3; *E*-value = 6.1). The alleles of these genes changed in frequency across altitude and forest biomass gradients (Fig. 4).

We identified eight Single Outlier Clusters (SOCs) connecting five to 23 loci per SOC by linkage disequilibrium (LD) thresholds ranging from 0.1 to 0.9 (Fig. 5). Multiple linear regression results showed that six SOCs have some degree of association with latitude, longitude, altitude and/or forest biomass. Three SOCs are significantly related to longitude and three to latitude, one of the latter (SOC111) is also related to altitude, which is congruent with the latitudinal change of altitude. SOC12 is related to both altitude and forest biomass of sampled streams, whereas two SOCs are exclusively related to forest biomass (Table 7).

3.4 | Population structure

All three methods identified six ($K = 6$) as the most probable number of genetic lineages. Overall, genetic structure is associated to catchments and both sides of Puraquequara River (Fig. 6). Moreover, our results showed a tendency of genetic structure along the altitudinal gradient, with a core genetic group at catchment headwaters; three genetic (admixture) lineages at intermediated altitudes; and two lower catchment peripheral genetic lineages. It is supported by the SNMf admixture plot and F_{is} statistics that show that gene flow may preferably occur in a hierarchical fashion: first between streams of the same drainage system; second between drainage systems at the same portion of altitudinal and forest biomass gradients within or between catchments; and third from lower to higher altitudes (Fig. 6).

SNAPP analysis supported three main lineages according to the pattern described above: one including all samples from both Puraquequara River banks (left bank streams 1–8 and right bank streams 9–13; Fig. 1); one composed by all samples of the Cuieiras River (streams 19–21) plus all other samples located at the headwaters of catchments (streams 14; 17 and 18); and one compose by sampled streams numbered 15 and 16, sister of the first two lineages cited above. Sampled streams 20 and 21 forms a unique lineage sister of sampled streams 17, 19, 14 and 18, respectively. Within Puraquequara River, both river banks are well supported as distinct lineages. Within the Puraquequara River right bank lineage, there is a lineage composed by sampled streams 12 and 13, that occupy environmentally more similar habitats, sister of the lineage composed by sampled streams 9–12. Similarly, samples from the lower course of the Puraquequara River left bank (streams 7 and 8) plus a sample from the middle course of the

same river bank (stream 6) formed a lineage sister of a lineage composed by samples of the middle (streams 4 and 5) and upper course (streams 1–3) of the Puraquequara River left bank. Samples from the latter lineage (sampled streams 1–5) mostly inhabit similar environmental conditions as well. The geographic distribution of the sampled streams and their referred numbers are showed in Figure 1.

3.5 | Isolation by geographic distance, by landscape resistance and by environment

The mantel test showed that 6% of total genetic variation is positively correlated with geographic distance (Fig. 8A). The spatial autocorrelation of genetic similarity showed a sharply decrease among closest sampled streams (0-20 km), followed by a moderate decrease in the second class of distances (20-40 km), which was similar between 60-80 km and 80-97 km, respectively (Fig. 8B). Distance class 40-60 km did not show spatial autocorrelation of genetic dissimilarity.

The best MLPE models based on AICc and Akaike weighs were those related to the lowest and highest values of both altitude and forest biomass. Altitudes from 0 to 60 and from 125 to 165 m a.s.l. and forest biomass between 0 and 100 MG C ha^{-1} showed the highest resistance for gene flow (Table 8). Forest biomass between 300 and 387 MG C ha^{-1} had the lowest resistance values and is associated to species occurrence, which indicates that more dense forests along intermediate terrain relief might contribute to landscape connectivity (Fig. 9). Altitude was the only environmental characteristic of sampled streams associated to genetic variation of *Atelopus manauensis* (AICc = 61267.07; Weight = 1). Rivers did not show influence on gene flow among sampled streams.

The RDA IBD + IBE model significantly captured 48% of total genetic variation of *Atelopus manauensis*, but only geographic and forest biomass of sampled streams significantly contributed to the model. However, the partial models showed that most of the genetic variation (44%) is explained by geographic distance ($R^2 = 0.44$, $p < 0.001$), whereas 4% is associated with forest biomass of sampled streams (Table 9). The RDA IBR + IBE models explained 37-46% of total genetic variation of the studied species, but forest biomass of sampled streams did not significantly contribute to the model. In partial models, IBR explained 33-41% and IBE 2-5% of total genetic variation. Altitude from 0 to 20 and from 125 to 165 m a.s.l. and forest biomass between 300 and 387 MG C ha^{-1} were the resistance variables that most contributed to the total genetic variation of *Atelopus manauensis*, showing that the lowest and highest altitudes significantly impede gene flow and that more dense forests facilitate gene flow. The multiple linear regressions based on PCoA scores were consistent with the RDA models (Table 10).

Multiple regression models including geographic and environmental variables explained 48% of the total genetic variation ($P < 0.001$), whereas models including landscape resistance and environmental variables explained 14-46% of the genetic variation ($P < 0.003$) (Fig. 10).

4 | Discussion

We identified six genetically distinct lineages and showed that more structured forests facilitate connectivity between areas of suitable habitat. Interestingly, these lineages have been historically and reproductively isolated in different parts of the species distribution despite its small geographic range. Furthermore, our results show signatures for selection associated with altitude and forest biomass gradients. The spatial congruence of reproductively isolated and adaptive lineages indicates the presence of six distinct populations segment (DPSs) within the *Atelopus manauensis* distribution. We observed evidence of inbreeding in some DPSs. Therefore, landscape connectivity is crucial to maintain the present level of genetic variation and viability of *Atelopus manauensis* genetic lineages.

We found relatively strong genetic differentiation between groups of individuals of *Atelopus manauensis* in close proximity, a pattern consistent with the low dispersal capacity of *Atelopus* (Lötters 1996). For example, some *Atelopus* species have small home ranges from which they were shown to be philopatric across consecutive breeding seasons (Crump 1986; Luger et al. 2009). Along with IBD, we show that altitude and forest biomass influenced connectivity, the same as those associated to occurrence and absence data from the study of Jorge et al. (2020). Subtle variations in terrain relief have also been shown to constrain movement and lead to genetic differentiation in Bufonidae frogs (Wang 2009, 2012). Forest cover may facilitate dispersal by providing protection against sun exposure and dehydration, as shown for stream breeding salamanders (Emel et al. 2019).

Localised adaptation was suggested by several analyses with patterns of divergent selection being mostly consistent with historically diverged lineages. The 28 SNPs that highly signalled selection were found to vary in frequency according to changes in altitude or forest biomass. The accumulation of localised adaptive genetic variation can be reinforced by isolation by environment (Wang and Bradburd 2014), a process that explains ~ 4% of the total genetic variation in our RDA model. Moreover, the extent to which altitude or forest biomass influences connectivity might also relate to patterns of localised adaptation. Our SNMf analysis indicates this by revealing higher levels of gene flow between groups of individuals of *Atelopus manauensis* occupying similar environments rather with those occupying distinct ones (Fig. 6).

This is also supported by the positive relationship between pairwise F_{st} and dissimilarity in forest biomass between 21 sampled streams (Fig. 11).

This is also corroborated by the *Blastn* results that revealed gene annotations for the 28 SNPs under selection (25 selected by altitude and three by forest biomass gradients) related to distinct physiologic importance. For example, a gene annotated for an altitude-selected-SNP (Receptor-type tyrosine-protein phosphatase N2 - Ptprn2; Table 6) is required for normal accumulation of secretory vesicles in hippocampus (Universal Protein Research – Uniprot 2019). Hippocampus controls spatial navigation, which is fundamental for frog's orientation when moving between habitats to avoid hostile environments (Mazerolle and Vos 2006). A gene annotated to anion exchange protein 4 (slc4a9) under putative selection by forest biomass is an important molecule in the transportation of a variety of inorganic and organic anions in red blood (Uniprot 2019). Changes in the production of this protein can cause deregulation of intracellular pH and result, for example, in abnormal apoptosis (Lagadic-Gossmann et al. 2004). Finally, a locally adapted SNP across altitude gradient is associated to a gene annotated to vomeronasal type-2 receptor 26-like (Vmn2r26). This gene is related to pheromone receptor and is involved in response in chemical communication. Many amphibians use pheromone to communicate and a variation in pheromone proteins has been shown to result in reproductive isolation and population differentiation in different amphibian species (Houck 2009). However, a more accurate validation of gene functionality and physiologic importance for gene annotations available in Table 6 needs further investigation and is beyond the scope of the present study.

The spatial congruence between genetically distinct lineages and adaptive divergence points to six distinct populations segments (DPSs) in our study area. The persistence of the DPSs in our study system is threatened by habitat loss and fragmentation. A recent assessment of the conservation status of *Atelopus manauensis* showed that the growth of Manaus is reducing its habitat (Jorge et al. 2020). Most of the DPSs have relatively low genetic variation and signals of inbreeding. This may be associated with spatial Wahlund effects (i.e.: reduction of heterozygosity/genetic homogenization) that results from pooling data across genetically distinct parts of a distribution (Wahlund 1928). However, low N_e estimated for these DPSs are consistent with loss of genetic variation by drift.

Low N_e could be exacerbated by the male-biased sex ratio in *Atelopus* species (i.e. 10:1; Crump 1986, 1988). Given fewer females available for reproduction and amplexus lasting at least several weeks with one male, probably resulting in a female reproducing with only that male (Crump 1988). This in turn may be leading to the low genetic variation and strong

464 inbreeding found in some sampled streams in our study system. IUCN's guide and criteria for
465 species conservation assessment suggests that N_e of 50 is sufficient for avoiding inbreeding and
466 N_e of 500 guarantees the preservation of the evolutionary potential of populations (IUCN 2012).
467 More conservative estimates indicate that a minimum 100 N_e is required for maintenance of
468 total fitness of populations and 1000 N_e would retain the evolutionary potential across many
469 generations (Frankham et al. 2014). Despite the estimate criteria, all DPSs in our study system
470 are at risk of inbreed and/or loss of their evolutionary potential ($N_e < 100$). Jorge et al. (2020)
471 suggested the conservation status of "Endangered" for *Atelopus manauensis*, and our results
472 indicate that conservation measures are needed to avoid loss of functional and genetic
473 connectivity between DPSs that would increase the extinction risk of the species, as showed for
474 the island-dwelling Lord Howe woodhen in New Zealand, a species composed of small isolated
475 populations (Major et al. 2020).

476 The identification of adaptive genetic variation among *Atelopus manauensis* genetically
477 structured lineages greatly contributed for the identification of distinct population segments
478 within our study system that may guide future conservation measures. We highlighted the
479 importance of considering both neutral and adaptive genetic variation to identify effective units
480 for conservation. Conservation actions focusing in genetic lineages instead of species may be
481 an effective strategy as resources and lands for conservation purposes are scarce. The protection
482 of headwaters and riparian zones as established by Brazilian Forestry Legislation can minimize
483 the negative effects of changing environment on *Atelopus manauensis*. Conservation measures
484 applied to *Atelopus manauensis* might mitigate the effects of environmental change of other
485 forest-dependent species in the studied landscape.

486 **Declarations**

487 **Funding:** This work was funded by the Conselho Nacional de Desenvolvimento Científico e
488 Tecnológico (CNPq Universal Grant nº: 401120/2016-3 granted to APL) and by the
489 Coordenação de Aperfeiçoamento do Pessoal de Nível Superior Coordination through its
490 Programa de Suporte para Núcleos de Excelência (CAPES/PROEX Grant nº: 0616/2018). RFJ
491 received a PhD scholarship from CAPES and Fundo de Amparo à Pesquisa do Estado do
492 Amazonas (CAPES/FAPEAM Grant nº: 24/2014) and a partial-abroad PhD scholarship
493 (Programa de Doutorado Sanduíche no Exterior - PDSE) to undertake statistical analyses at
494 Macquarie University, Sydney, Australia (CAPES/PDSE Grant nº: 41/2018).

Conflicts of interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethics approval: Specimens used in this research were collected under permission number 56759 from the Sistema de Autorização e Informação em Biodiversidade do Instituto Chico Mendes de Conservação da Biodiversidade (SISBIO/ICMBIO). This study was approved by the INPA ethics committee (registration number 002/2017 – Comissão de Ética no Uso de Animais – CEUA/INPA).

Consent to participate: All authors consented to participate in this research.

Consent for publication: All authors have read and agreed to the final version of the manuscript.

Availability of data: The SNPs dataset generated and/or analysed during the current study are not publicly available due to it is being used in another study, but are available on reasonable request to RF Jorge. Environmental variables used during the current study can be obtained through the methods described in "Material and Methods" section from the coordinates provided in Online Resource 1 and raster layers available in cited literature.

Code availability: R scripts and manuals of software used for analyses are available in cited literature following analyses description and in R Core Team website associated to each package name.

Author contribution: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by RFJ. All authors equally contributed to the writing of the manuscript.

References

Amaral S, Costa CB, Arasato LS, Ximenes AC, Rennó CD (2013) AMBDATA: Variáveis ambientais para Modelos de Distribuição de Espécies (SDMs). In: Simpósio Brasileiro de Sensoriamento Remoto, 16, Foz do Iguaçu, Paraná, Brasil. http://www.dpi.inpe.br/AmBdata. Acessed 15 March 2017.

Balkenhol N, Cushman SA, Storfer AT, Waits LP (2016) Landscape genetics: concepts, methods, applications. Wiley, Oxford.

524 Balkenhol N, Dudaniec RY, Krutovsky KV, Johnson JS, Cairns DM, Segelbacher G, Selkoe
525 KA, von der Heyden S, Wang IJ, Selmoni O, Joost S (2017) Landscape Genomics:
526 Understanding Relationships Between Environmental Heterogeneity and Genomic
527 Characteristics of Populations. In: Rajora D (ed.). Population Genomics. Springer,
528 Switzerland, pp 261–322.

529 Beier P, Spencer W, Baldwin RF, Mcrae BH (2011) Toward best practices for developing
530 regional connectivity maps. Conser Biol 25:879-892. https://doi.org/10.1111/j.1523
531 1739.2011.01716.x

532 Bouckaert R (2010) DensiTree: making sense of sets of phylogenetic trees. Bioinformatics
533 26:1372-1373. https://doi.org/10.1093/bioinformatics/btq110

534 Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, Heled
535 J, Jones G, Kühnert D, De Maio N, Matschiner M, Mendes FK, Müller NF, Ogilvie HA,
536 Du Plessis L, Popinga A, Rambaut A, Rasmussen D, Siveroni I, Suchard MA, Wu C-H,
537 Xie D, Zhang C, Stadler T, Drummond AJ (2019) BEAST 2.5: An advanced software
538 platform for Bayesian evolutionary analysis. PLoS Comput Biol 15:1-28.
539 https://doi.org/10.1371/journal.pcbi.1006650

540 Bryant D, Bouckaert R, Felsenstein J, Rosenberg NA, Roychoudhury A (2012) Inferring
541 species trees directly from biallelic genetic markers: Bypassing gene trees in a full
542 coalescent analysis. Mol Biol Evol 29:917-1932. https://doi.org/10.1093/molbev/mss086

543 Cayuela H, Valenzuela-Sánchez A, Teulier L, Martínez-Solano Í, Léna JP, Merilä J, Muths E,
544 Shine R, Quay L, Denoël M, Clobert J, Schmidt BR (2020) Determinants and
545 consequences of dispersal in vertebrates with complex life cycles: A review of pond-
546 breeding amphibians. Q Rev Biol 95:1-36. https://doi.org/10.1086/707862

547 Crump ML (1986) Homing and Site Fidelity in a Neotropical Frog, *Atelopus varius*
548 (Bufonidae). Copeia 1986:438-444. https://doi.org/10.2307/1445001

549 Crump ML (1988) Aggression in harlequin frogs: male-male competition and a possible
550 conflict of interest between the sexes. Anim Behav 36:1064-1077.
551 https://doi.org/10.1016/S0003-3472(88)80066-6

552 Devlin B, Roeder K (1999) Genomic control for association studies. Biometrics 55:997-1004.
553 https://doi.org/10.1111/j.0006-341X.1999.00997.x

554 Dickson BG, Albano CM, Anantharaman R, Beier P, Fargione J, Graves TA, Gray ME, Hall
555 KR, Lawler JJ, Leonard PB, Littlefield CE, McClure ML, Novembre J, Schloss CA,
556 Schumaker NH, Shah VB, Theobald DM (2019) Circuit-theory applications to
557 connectivity science and conservation. Conserv Biol 33:239-249.
558 https://doi.org/10.1111/cobi.13230

559 Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ, Ovenden JR (2014) N_eEstimator v2: Re-
560 implementation of software for the estimation of contemporary effective population size
561 (Ne) from genetic data. Mol Ecol Resour 14:209-214. https://doi.org/10.1111/1755-
562 0998.12157

563 Duckett PE, Wilson PD, Stow AJ (2013) Keeping up with the neighbours: Using a genetic
564 measurement of dispersal and species distribution modelling to assess the impact of
565 climate change on an Australian arid zone gecko (*Gehyra variegata*). Divers Distrib
566 19:964-976. https://doi.org/10.1111/ddi.12071

567 Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: A website and program for
568 visualizing STRUCTURE output and implementing the Evanno method. Conserv Gen
569 Resour 4:359-361. https://doi.org/10.1007/s12686-011-9548-7

570 Emel SL, Olson DH, Knowles LL, Storfer A (2019) Comparative landscape genetics of two
571 endemic torrent salamander species, *Rhyacotriton kezeri* and *R. variegatus*: implications
572 for forest management and species conservation. Conserv Gen 20:801-815.
573 https://doi.org/10.1007/s10592-019-01172-6

574 Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using
575 the software STRUCTURE: A simulation study. Mol Ecol 14:2611-2620.
576 https://doi.org/10.1111/j.1365-294X.2005.02553.x

577 Excoffier L, Hofer T, Foll M (2009) Detecting loci under selection in a hierarchically structured
578 population. Heredity 103:285-298. https://doi.org/10.1038/hdy.2009.74

579 Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform
580 population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564-567.
581 https://doi.org/10.1111/j.1755-0998.2010.02847.x

582 Fenster CB, Ballou JD, Dudash MR, Eldridge MDB, Frankham R, Lacy RC, Ralls K, Sunnucks
583 P (2018) Conservation and genetics. Yale J Biol Med 91:491-501.

584 Frankham R, Bradshaw CJA, Brook BW (2014) Genetics in conservation management:
585 Revised recommendations for the 50/500 rules, Red List criteria and population viability
586 analyses. Biol Conserv 170:56-63. https://doi.org/10.1016/j.biocon.2013.12.036

587 Frichot E, Schoville SD, Bouchard G, François O (2013) Testing for associations between loci
588 and environmental gradients using latent factor mixed models. Mol Biol Evol 30:1687-
589 1699. https://doi.org/10.1093/molbev/mst063

590 Frichot E, Mathieu F, Trouillon T, Bouchard G, François O (2014) Fast and efficient estimation
591 of individual ancestry coefficients. Genetics 196:973-983.
592 https://doi.org/10.1534/genetics.113.160572

593 Frichot E, François O (2015) LEA: An R package for landscape and ecological association
594 studies. Methods Ecol Evol 6:925-929. https://doi.org/10.1111/2041-210X.12382

595 Funk WC, Forester BR, Converse SJ, Darst C, Morey S (2019) Improving conservation policy
596 with genomics: a guide to integrating adaptive potential into U.S. Endangered Species
597 Act decisions for conservation practitioners and geneticists. Conserv Gen 20:115-134.
598 https://doi.org/10.1007/s10592-018-1096-1

599 Funk W, Chris McKay, J. K., Hohenlohe, P. A., & Allendorf, F. W. (2012). Harnessing
600 genomics for delineating conservation units. Trends in Ecol Evol 27:489-496.
601 https://doi.org/10.1016/j.tree.2012.05.012

602 Gebremedhin B, Ficetola GF, Naderi S, Rezaei HR, Maudet C, Rioux D, Luikart G, Flagstad
603 Thuiller W, Taberlet P (2009) Frontiers in identifying conservation units: From neutral
604 markers to adaptive genetic variation. Anim Conserv 12:107-109.
605 https://doi.org/10.1111/j.1469-1795.2009.00255.x

606 Grivet D, Sork VL, Westfall RD, Davis FW (2008) Conserving the evolutionary potential of
607 California valley oak (*Quercus lobata* Née): A multivariate genetic approach to
608 conservation planning. Mol Ecol 17:139-156. https://doi.org/10.1111/j.1365-
609 294X.2007.03498.x

610 Grosser S, Abdelkrim J, Wing J, Robertson BC, Gemmell NJ (2017) Strong isolation by
611 distance argues for separate population management of endangered blue duck
612 (*Hymenolaimus malacorhynchos*). Conserv Gen 18:327-341.
613 https://doi.org/10.1007/s10592-016-0908-4

614 Gruber B, Unmack PJ, Berry OF, Georges A (2018) dartr: An r package to facilitate analysis
615 of SNP data generated from reduced representation genome sequencing. Mol Ecol Resour
616 18:691-699. https://doi.org/10.1111/1755-0998.12745

617 Hijmans RJ, Phillips S, Leathwick J, Elith J (2017) dismo: Species Distribution Modelling. The
618 R Project for Statistical Computing. https://CRAN.R-proje ct.org/package=dismo.
619 Accessed 28 July 2016.

620 Houck LD (2009) Pheromone communication in amphibians and reptiles. Annu Rev Physiol
621 71:161-176. https://doi.org/10.1146/annurev.physiol.010908.163134

622 IUCN (2012). IUCN Red List Categories and Criteria: Version 3.1. 2nd ed. IUCN, Gland,
623 Switzerland and Cambridge.

624 Jombart T (2008) Adegenet: A R package for the multivariate analysis of genetic markers.
625 Bioinformatics 24:1403-1405. https://doi.org/10.1093/bioinformatics/btn129

626 Jombart T, Collins C (2012) A tutorial for Discriminant Analysis of Principal Components
627 (DAPC) using adegenet 2.0.0. Imperial College London. MRC Centre for Outbreak
628 Analysis and Modelling. http://adegenet.r-forge.r-project.org/files/tutorial-dapc.pdf.
629 Acessed 27 August 2019.

630 Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL (2008) NCBI
631 BLAST: A better web interface. Nucleic Acids Res 36:W5–W9.
632 https://doi.org/10.1093/nar/gkn201

633 Jorge RF, Simões PI, Magnusson WE, Lima AP (2016) Fine-scale habitat heterogeneity
634 explains the local distribution of two Amazonian frog species of concern for conservation.
635 Biotropica 48:694-703. https://doi.org/10.1111/btp.12333

636 Jorge RF, Magnusson WE, Silva DA da, Polo ÉM, Lima AP (2020) Urban growth threatens the
637 lowland Amazonian Manaus harlequin frog which represents an evolutionarily significant
638 unit within the genus *Atelopus* (Amphibia: Anura: Bufonidae). J Zool Syst Evol Res
639 2020:1-11, jzs.12390. https://doi.org/10.1111/jzs.12390

640 Keller LF, Waller DM (2002) Inbreeding effects in wild populations. Trends Ecol Evol 17:230-
641 241. https://doi.org/10.1016/S0169-5347(02)02489-8

642 Kilian A, Wenzl P, Huttner E, Carling J, Xia L, Blois H, et al. (2012) Diversity arrays
643 technology: a generic genome profiling technology on open platforms. Methods Mol.
644 Biol. 888:67-89. https://doi.org/10.1007/978-1-61779-870-2_5

645 Lagadic-Gossmann D, Huc L, Lecureur V (2004) Alterations of intracellular pH homeostasis
646 in apoptosis: Origins and roles. Cell Death Differ 11:953-961.
647 https://doi.org/10.1038/sj.cdd.4401466

648 Linnell MA, Lesmeister DB (2019) Landscape connectivity and conservation prioritization for
649 an old forest species with limited vagility. Anim Conserv 22:568-578.
650 https://doi.org/10.1111/acv.12496

651 Lötters S (1996) The neotropical toad genus *Atelopus*. Checklist, biology and distribution. M.
652 Vences, & F. Glaw Glaw, Köln.

653 Luger M, Hödl W, Lötters S (2009) Site fidelity, home range behaviour and habitat utilization
654 of male harlequin toads (Amphibia: *Atelopus hoogmoedi*) from Suriname: Relevant
655 aspects for conservation breeding. Salamandra 45:211-218.

656 Luu K, Bazin E, Blum MGB (2017) pcadapt: an R package to perform genome scans for
657 selection based on principal component analysis. Mol Ecol Resour 17:67-77.
658 https://doi.org/10.1111/1755-0998.12592

659 Major RE, Ewart KM, Portelli, DJ, King A, Tsang LR, O'Dwyer T, Carlile N, Haselden C,
660 Bower H, Alquezar-Planas DE, Johnson RN, Eldridge MDB (2020) Islands within
661 islands: genetic structuring at small spatial scales has implications for long-term
662 persistence of a threatened species. Anim Conserv 0:0-0
663 https://doi.org/10.1111/acv.12603

664 Mazerolle MJ, Vos AC 2006. Choosing the safest route: frog orientation in an agricultural
665 landscape. J Herpetol 40:435-441.

666 McRae BH (2006) Isolation by resistance. Evolution 60:1551-1561. https://doi.org/10.1554/05-
667 321.1

668 McRae BH, Beier P (2007) Circuit theory predicts gene flow in plant and animal populations.
669 P Natl Acad Sci USA 104:19885-19890. https://doi.org/10.1073/pnas.0706568104

670 McRae BH, Dickson BG, Keitt TH, Shah VB (2008) Using circuit theory to model connectivity
671 in ecology, evolution, and conservation. Ecology 89:2712-2724.

672 Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference
673 of large phylogenetic trees. 2010 Gateway Computing Environments Workshop, GCE
674 2010. https://doi.org/10.1109/GCE.2010.5676129. Accessed 15 September 2019.

675 Moritz C (2002) Strategies to protect biological diversity and the evolutionary processes that
676 sustain it. Syst Biol 51:238-254. https://doi.org/10.1080/10635150252899752

677 Nomura T (2008) Estimation of effective number of breeders from molecular co-ancestry of
678 single cohort sample. Evol Appl 1: 462-474.

679 Barton K (2018) Package 'MuMIn'. R package version 1.40. 4. The R Project for Statistical
680 Computing. https://CRAN.R-proje ct.org/package=MuMIn. Accessed 17 September
681 2019

682 Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara R, Simpson GL, Solymos
683 P, Stevens MHH, Szoecs E, Wagner H (2013) Package 'vegan'. Community ecology
684 package, version, 2(9). The R Project for Statistical Computing. http://vegan.r-forge.r-
685 proje ct.org/package=vegan. Accessed 29 October 2019.

686 Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic
687 software for teaching and research-an update. Bioinformatics 28:2537-2539.
688 https://doi.org/10.1093/bioinformatics/bts460

689 Pearse DE (2016) Saving the spandrels? Adaptive genomic variation in conservation and
690 fisheries management. J Fish Biol 89:2697-2716. https://doi.org/10.1111/jfb.13168

691 Peterman WE (2018) ResistanceGA: An R package for the optimization of resistance surfaces
692 using genetic algorithms. Methods Ecol Evol 9:1638-1647. https ://doi.
693 org/10.1111/2041-210x.12984

694 Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus
695 genotype data. Genetics 155:945-959. https://doi.org/10.1111/j.1471-8286.2007.01758.x

696 Puechmaille SJ (2016) The program structure does not reliably recover the correct population
697 structure when sampling is uneven: Subsampling and new estimators alleviate the
698 problem. Mol Ecol Resour 16:608-627. https://doi.org/10.1111/1755-0998.12512

699 R Core Team (2020) R: A language and environment for statistical computing. R Foundation
700 for Statistical Computing: Vienna, Austria, 2018. Available online: https://www.R-
701 project.org (accessed on 05 March 2020).

702 Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2018) Posterior summarisation in
703 Bayesian phylogenetics using Tracer 1.7. Syst Biol 67:901-904.
704 https://doi.org/10.1093/sysbi o/syy032

705 Reddy PA, Cushman SA, Srivastava A, Sarkar MS, Shivaji S (2017) Tiger abundance and gene
706 flow in Central India are driven by disparate combinations of topography and land cover.
707 Divers Distrib, *23*(8), 863–874. https://doi.org/10.1111/ddi.12580

708 Reid-Anderson S, Bilgmann K, Stow AJ (2019) Effective population size of the Critically
709 Endangered East Australian Grey Nurse Shark. Mar Ecol Prog Ser 610:137-148.
710 https://doi.org/10.3354/meps12850

711 Row JR, Knick ST, Oyler-McCance SJ, Lougheed SC, Fedy BC (2017) Developing approaches
712 for linear mixed modelling in landscape genetics through landscape-directed dispersal
713 simulations. Ecol Evol 7:3751-3761. https://doi.org/10.1002/ ece3.2825

714 Soanes K, Sievers M, Chee YE, Williams NSG, Bhardwaj M, Marshall AJ, Parris KM (2019)
715 Correcting common misconceptions to inspire conservation action in urban
716 environments. Consev Biol 33:300-306. https://doi.org/10.1111/cobi.13193

717 Storfer A, Murphy MA, Spear SF, Holderegger R, Waits LP (2010) Landscape genetics: Where
718 are we now? Mol Ecol 19:3496-3514. https://doi.org/10.1111/j.1365-294X.2010.04691.x

719 Supple MA, Shapiro B (2018) Conservation of biodiversity in the genomics era. Genome Biol
720 19:1-12. https://doi.org/10.1186/s13059-018-1520-3

721 Taylor PD, Fahrig L, Henein K, Merriam G (1993) Connectivity is a vital element of landscape
722 structure. Oikos 68:571-573. http://www.jstor.org/stable/3544927

723 Scrucca L. 2013. GA: a package for genetic algorithms in R. J Stats Softw 53:1–37.

724 Shirk AJ, Wallin DO, Cushman SA, Rice CG, Warheit KI. 2010. Inferring land- scape effects
725 on gene flow: a new model selection framework. Mol Ecol 19:3603–3619.

726 Slatkin M. 1987. Gene flow and the geographic structure of natural populations. Science
727 236:787–792.

728 Smith TB, Calsbeek R, Wayne RK, Holder KH, Pires D, Bardeleben C. 2005. Testing
729 alternative mechanisms of evolutionary divergence in an African rain forest passerine
730 bird. J Evol Biol 18:257–268.

731 Soares-Filho BS, Nepstad DC, Curran LM, Cerqueira GC, Garcia RA, Ramos CA, Voll E,
732 McDonald A, Lefebvre P, Schlesinger P. 2006. Modelling conservation in the Amazon
733 basin. Nature 440:520–523.

734 Steen D. 2010. Snakes in the grass: secretive natural histories defy both con- ventional and
735 progressive statistics. Herpetol Conserv Biol 5:183–188.

736 Stevens VM, Polus E, Wesselingh RA, Schtickzelle N, Baguette M. 2005. Quantifying
737 functional connectivity: experimental evidence for patch- specific resistance in the
738 Natterjack toad (*Bufo calamita*). Landsc Ecol 19:829–842.

739 Stow AJ, Minarovic N, Eymann J, Cooper DW, Webley LS. 2006. Genetic structure infers
740 generally high philopatry and male-biased dispersal of brushtail possums (*Trichosurus*
741 *vulpecula*) in urban Australia. Wildlife Res 33:409–415.

742 Stow AJ, Sunnucks P. 2004. High mate and site fidelity in Cunningham's skinks (*Egernia*
743 *cunninghami*) in a natural and fragmented habitat. Mol Ecol 13:419–430.

744 Stow AJ, Sunnucks P, Briscoe DA, Gardner MG. 2001. The impact of habitat fragmentation on
745 dispersal of Cunningham's skink (*Egernia cunninghami*): evidence from allelic and
746 genotypic analyses of microsatellites. Mol Ecol 10:867–878.

747 Tozetti AM, Vettorazzo V, Martins M. 2009. Short-term movements of the South American
748 rattlesnake (*Crotalus durissus*) in south-eastern Brazil. Herpetol J 19:201–206.

749 Universal Protein Research - Uniprot (2019) UniProt: a worldwide hub of protein knowledge.
750 Nucleic Acids Res 47: D506-515. https://doi.org/10.1093/nar/gky1049.

751 Wahlund S (1928) Zusammensetzung von Population und Korrelationserschei- nung vom
752 Standpunkt der Vererbungslehre aus betrachtet. Hereditasi 11:65-106.

753 Wang IJ (2012) Environmental and topographic variables shape genetic structure and effective
754 population sizes in the endangered Yosemite toad. Divers Distrib 18:1033-1041.
755 https://doi.org/10.1111/j.1472-4642.2012.00897.x

756 Wang IJ, Bradburd GS (2014) Isolation by environment. Mol Ecol 23:5649-5662.
757 https://doi.org/10.1111/mec.12938

758 Waples RS (1991) Pacific salmon, *Oncorhynchus* spp., and the definition of "species'' under
759 the "Endangered Species Act". Mar Fish Rev 53:11-22.

LIST OF FIGURE LEGENDS

Figure 1. Map showing 21 sampled streams (numbers 1-21), where individuals of *Atelopus manauensis* were collected, from 14 drainage systems distributed among five catchments (rivers), as follows: Puraquequara River left bank – upper course (AP1, AP2 and AP3 – 1-3), middle course (MP1, MP2 and MP3 – 4-6), and lower course (BP1 and BP3 – 7-8); Puraquequara River right bank – Tinga (TI1, TI2 and TI3 – 9-11), Ubere (UB – 12), and Ipiranga (IP – 13); Preto River – middle course right bank (PA – 14), and middle course left bank (PM – 15); Urubu River – middle course (UR – 16), and upper course (CF – 17); Taruma-Açu River – upper course (TA – 18); Cuieiras River – middle course (ZF – 19), upper course of the left headwater (DI – 20), and upper course of the right headwater (CU – 21). Colours represent changes in altitudinal gradient from lower (light grey – 3 m a.s.l.) to higher (dark grey – 165 m a.s.l.) altitudes.

Figure 2. Pairwise F_{st} between 14 drainage systems sampled for individuals of *Atelopus manauensis*.

Figure 3. Pairwise F_{st} between six genetic lineages of *Atelopus manauensis* identified in population structure analyses.

Figure 4. Changing in frequency and turnover of alleles across environmental gradients: (A) alleles of a gene associated to immune defence across the altitudinal gradient, and (B) alleles of a gene associated to biological development across the biomass gradient.

Figure 5. Top left shows the limit above when clusters of loci started being considered a SOC and the consistency of formed SOCs along Linkage Disequilibrium gradient. All other graphs represent the first and/or second PCA axes of loci found in each of the eight identified LD clusters labelled according to the individuals carrying clustering loci. On the bottom left of graphs is the identification of each SOC before dash and three last digits after dash the LD threshold from which SOCs formed.

Figure 6. Pie charts showing the geographic distribution of six genetic lineages (different colours) and their genetic admixture found in SNMf analyses. Background colours refer to altitudinal (left) and forest biomass (right) gradients in the study area.

Figure 7. Genetic structure found by *Structure* (A) and DAPC (C) analyses and lineages delimitation by SNAPP analysis (B).

Figure 8. Influence of geography on genetic variation. (A) Mantel correlation test between geographic and genetic distances; and (B) spatial autocorrelation of genetic similarity.

Figure 9. Current flow map between 14 drainage systems sampled for individual groups (21) of *Atelopus manauensis*. Higher current densities (yellow) indicate cells with higher net passage probabilities for random walkers moving from one stream to the other (McRae et al. 2008) and blue colour represents unsuitable patches for dispersal of individuals of the studied species. This map sums altitude and forest biomass current flow maps.

Figure 10. Partial linear models showing the influence of geographic (A), five landscape resistance (B-F) and two environmental (G,H) explanatory variables on total genetic variation of *Atelopus manauensis*.

Figure 11. Plot showing a positive relationship between pairwise F_{st} between 21 individual groups of *Atelopus manauensis* and dissimilarities in forest biomass between 21 sampled streams.

LIST OF FIGURES

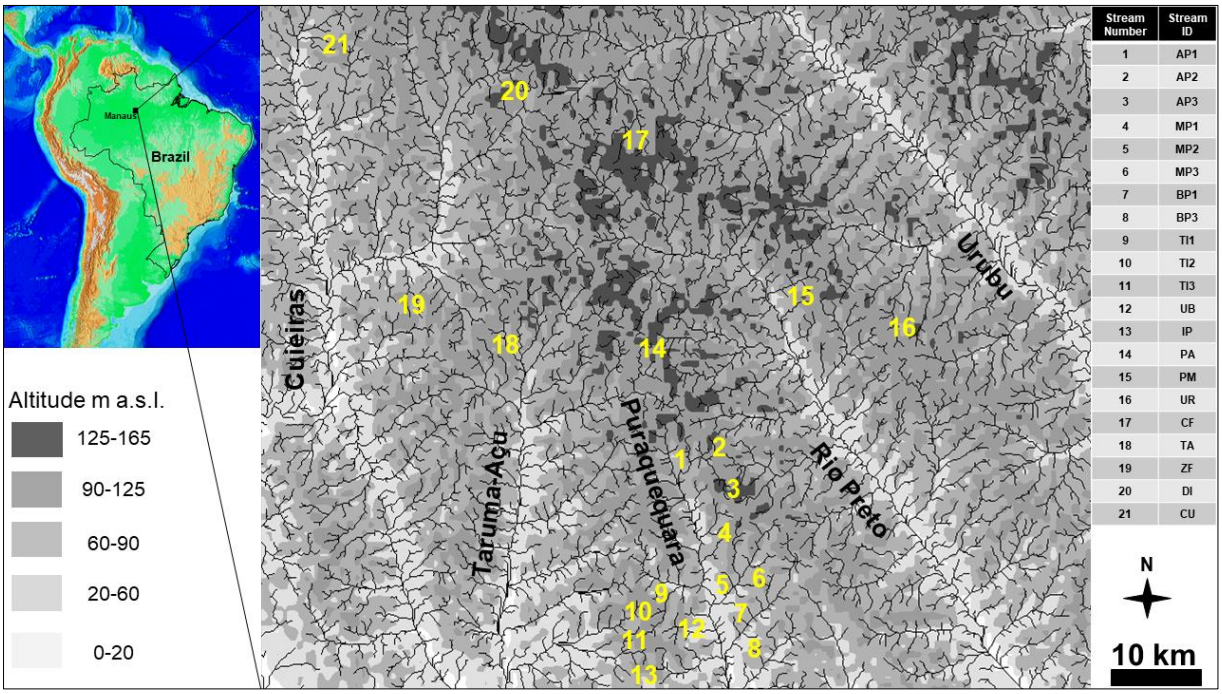


Figure 1.

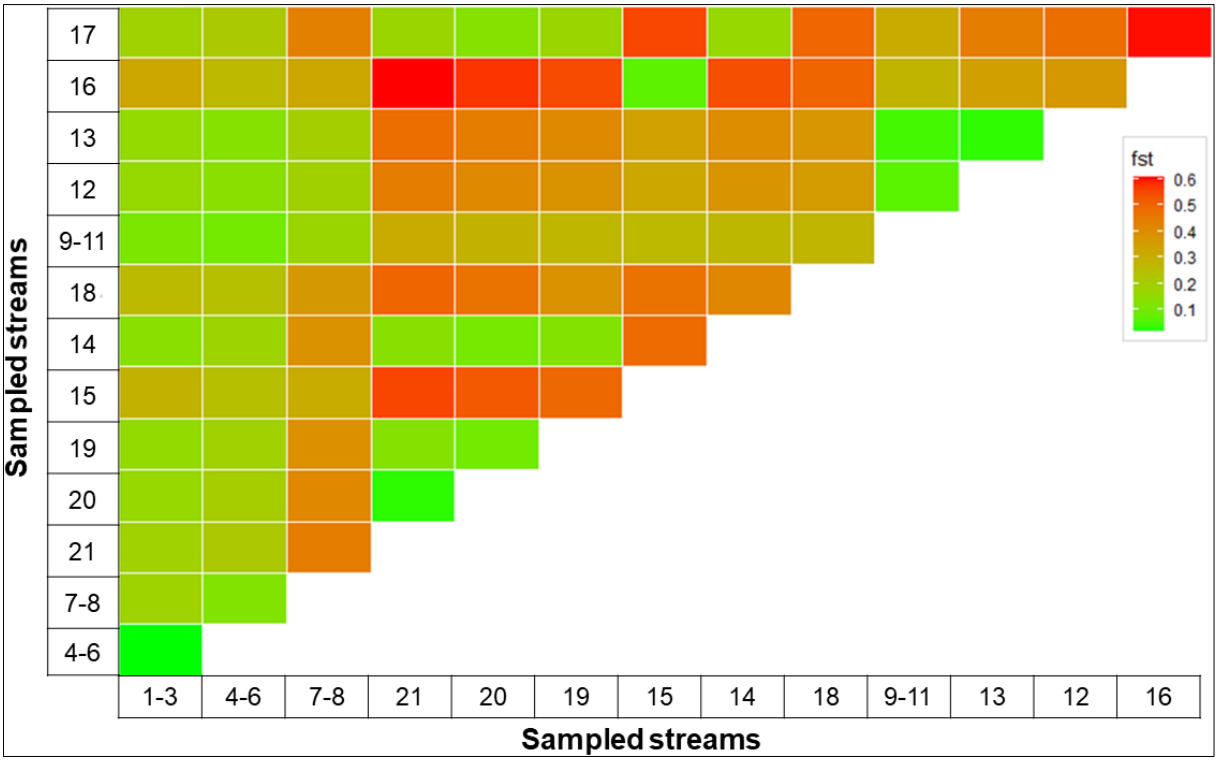


Figure 2.

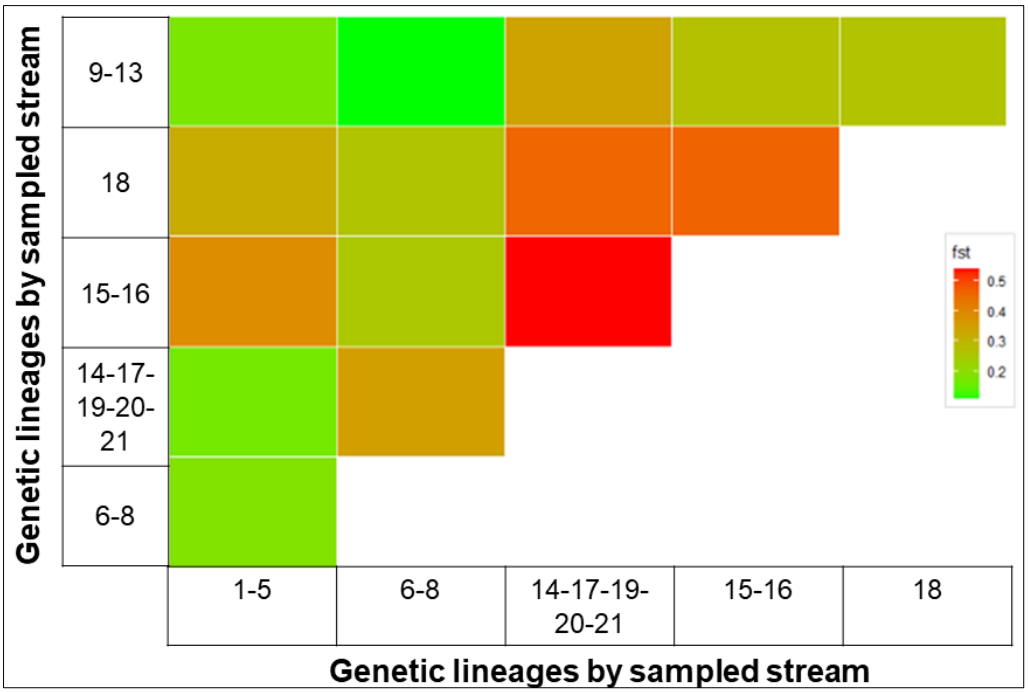


Figure 3.

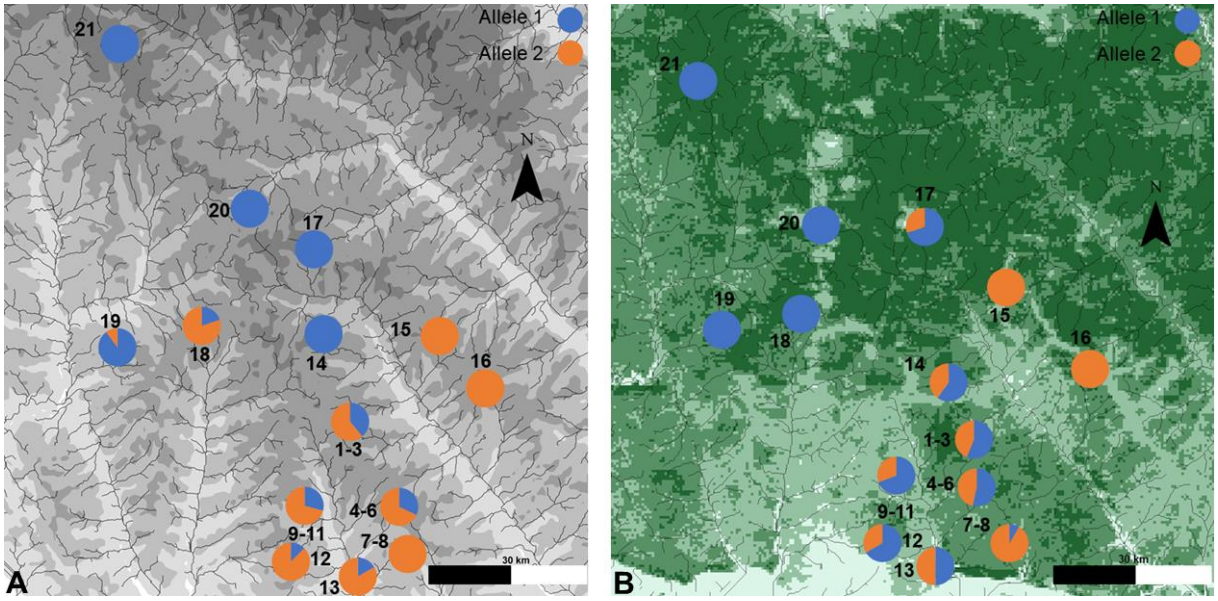


Figure 4.

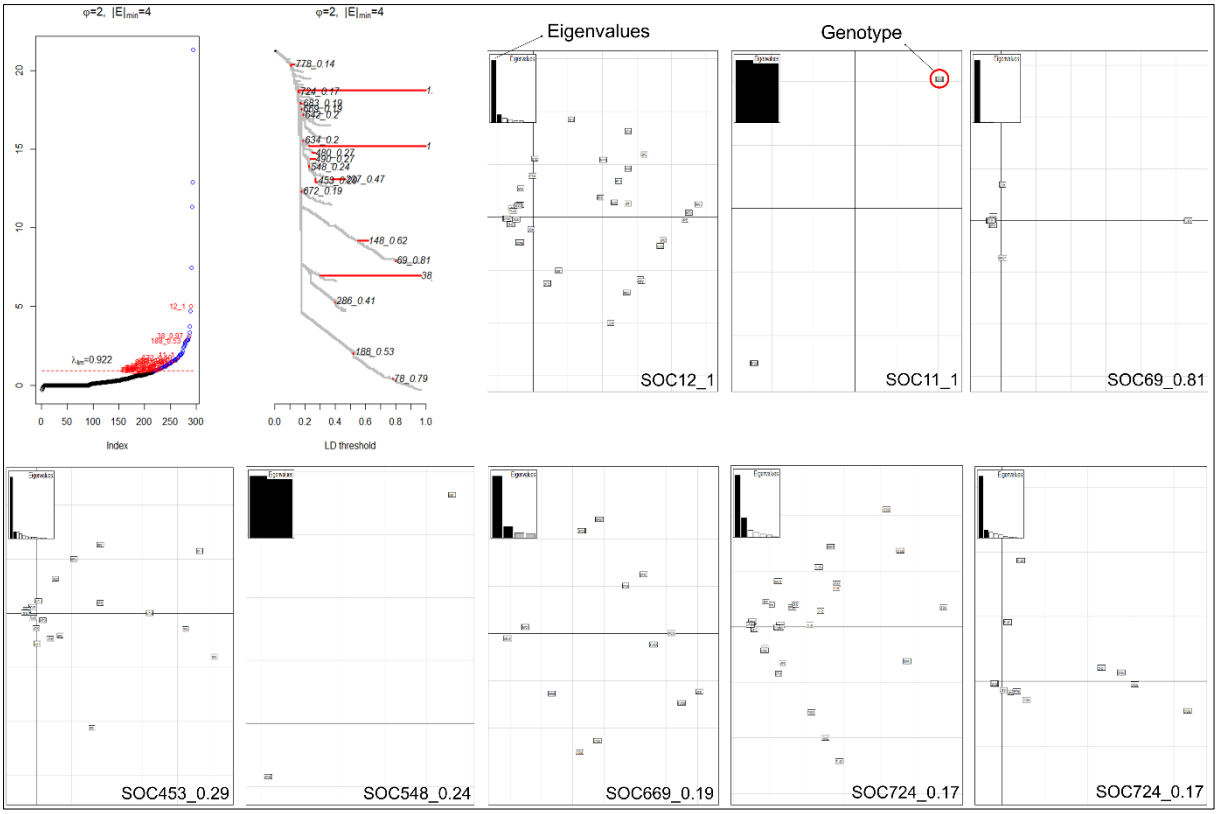


Figure 5.

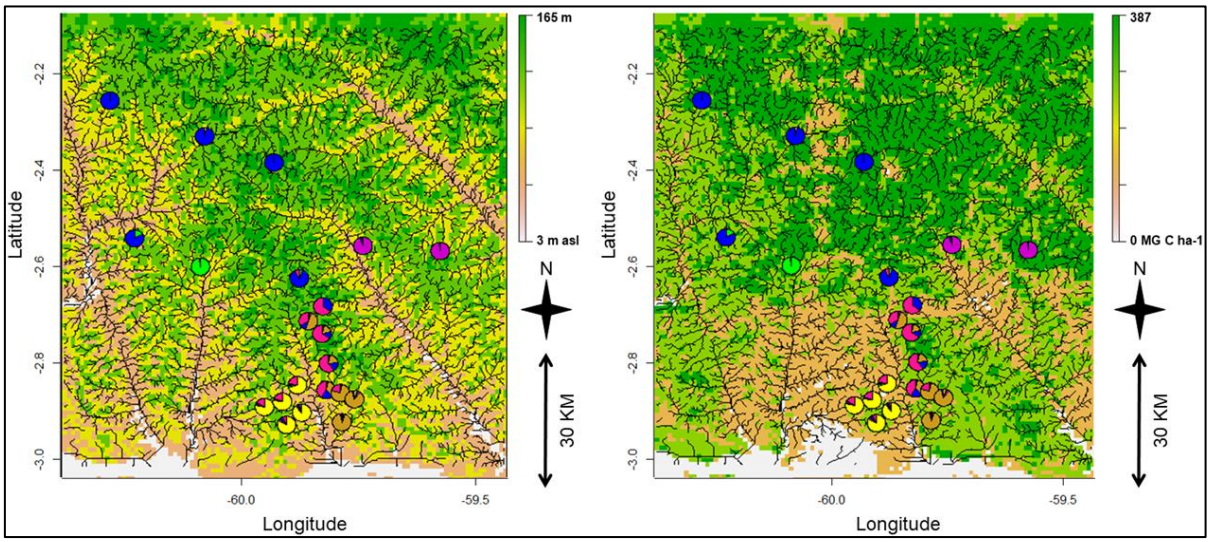


Figure 6.

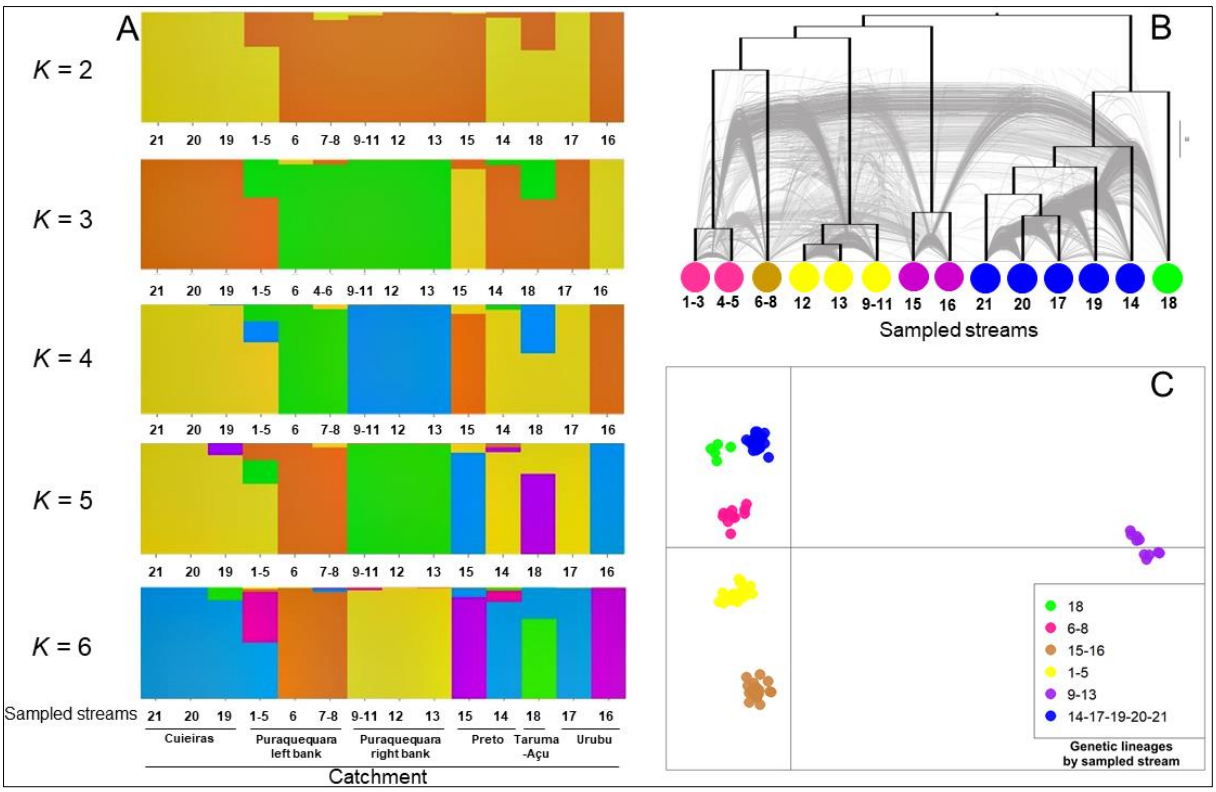


Figure 7.

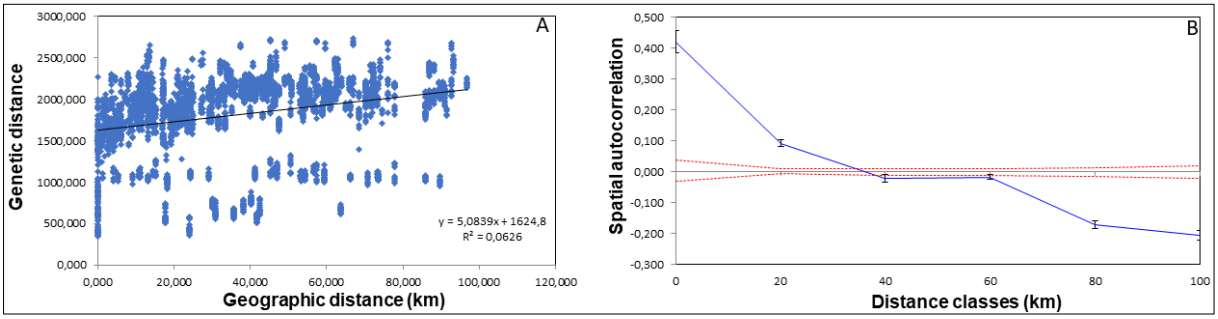


Figure 8.

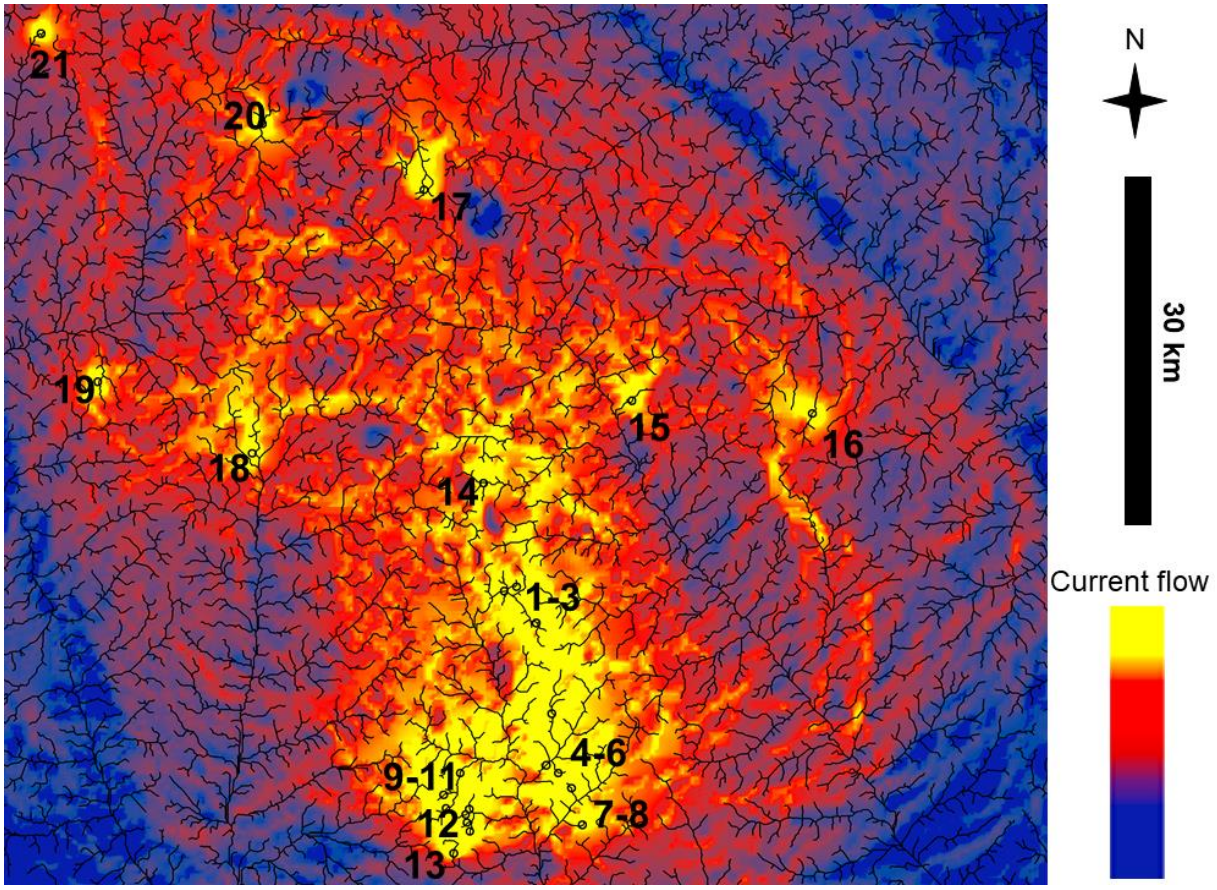


Figure 9.

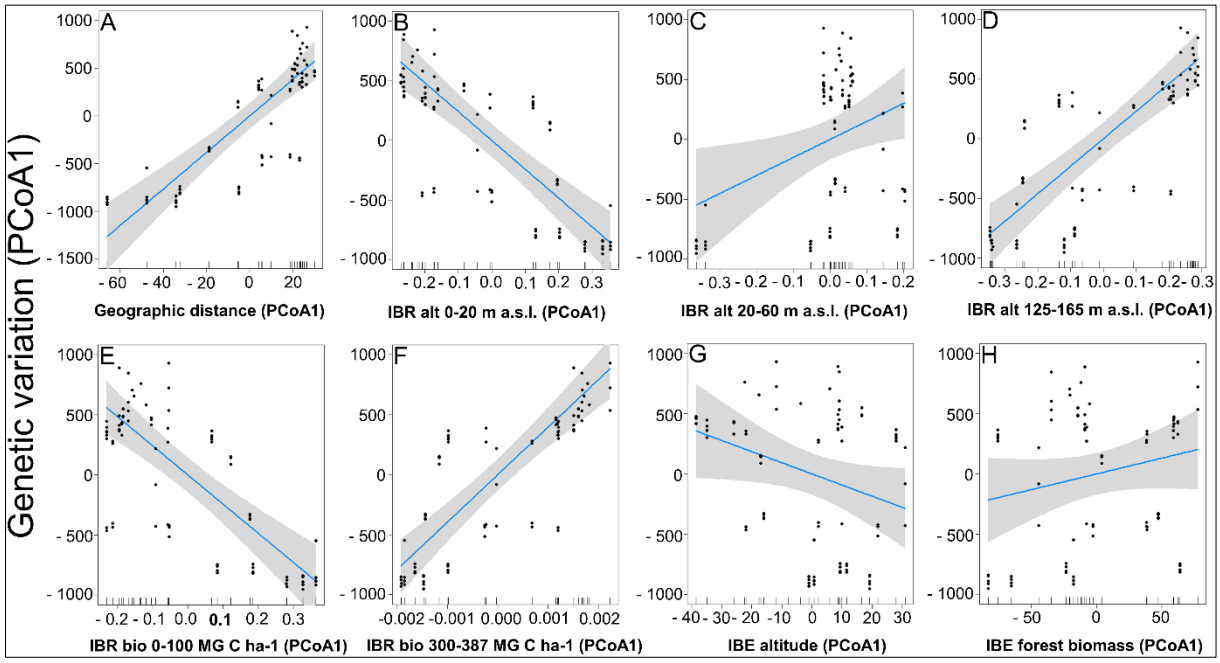


Figure 10.

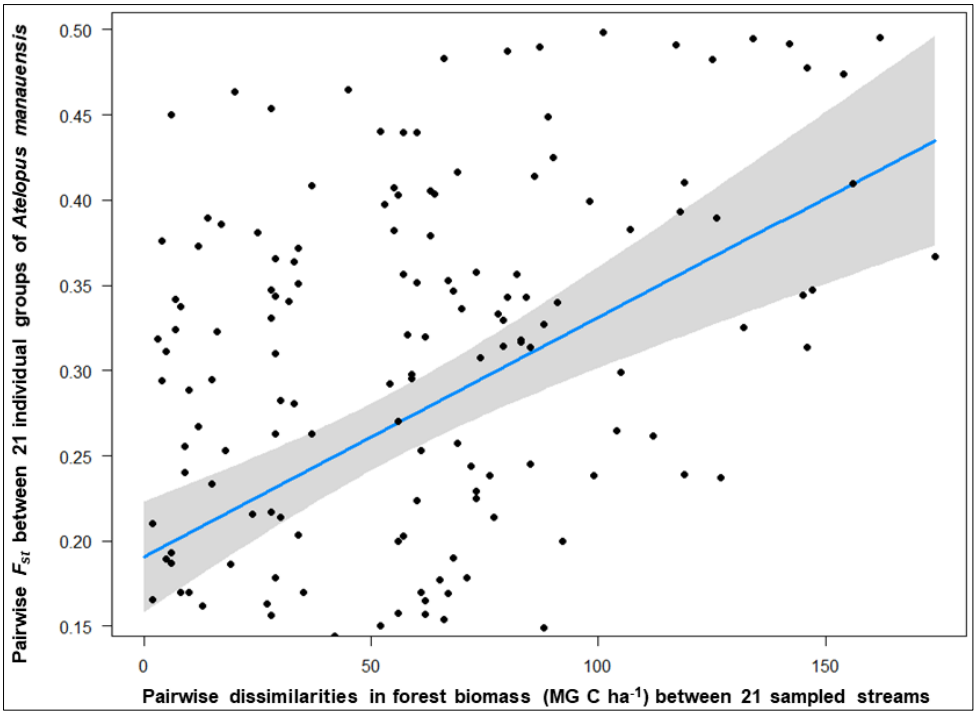

Pairwise F_{st} between 21 individual groups of *Atelopus manauensis*
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0
50
100
150
Pairwise dissimilarities in forest biomass (MG C ha^{-1}) between 21 sampled streams

Figure 11.

LIST OF TABLES

Table 1. Genetic diversity indices for 21 individual groups (sampled streams) of *Atelopus manauensis* distributed in 14 drainage systems. Abbreviations: *n* = number of samples; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{is}, fixation index 1 - (H_o / *He*); SNPs = number of SNPs analysed in drainage system.

Sampled streams	**Drainage System**	***n***	H_o	H_e	F_{is}	**SNPs**	**Private SNPs**
1-3	AP	9	0.26 ± 0.17	0.29 ± 0.14	0.11084	2309	4
4-6	MP	14	0.25 ± 0.15	0.29 ± 0.14	0.14320	2478	8
7-8	BP	6	0.33 ± 0.2	0.37 ± 0.14	0.09296	1848	9
9-11	TI	13	0.27 ± 0.17	0.3 ± 0.14	0.08338	1990	24
12	UB	4	0.33 ± 0.2	0.38 ± 0.13	0.00129	1009	2
13	IP	3	0.38 ± 0.24	0.45 ± 0.11	0.17313	1509	0
14	PA	5	0.36 ± 0.22	0.36 ± 0.13	- 0.01579	897	3
15	PM	5	0.39 ± 0.23	0.39 ± 0.13	0.00712	1286	4
16	UR	5	0.4 ± 0.23	0.41 ± 0.13	- 0.05778	1099	5
17	CF	5	0.42 ± 0.25	0.4 ± 0.13	0.00704	548	3
18	TA	5	0.39 ± 0.23	0.39 ± 0.13	- 0.00524	911	42
19	ZF	5	0.35 ± 0.22	0.37 ± 0.13	0.05544	750	2
20	DI	5	0.34 ± 0.22	0.34 ± 0.14	- 0.02606	770	0
21	CU	5	0.41 ± 0.24	0.39 ± 013	- 0.06800	544	1

Table 2. Genetic diversity indices for six genetic lineages of *Atelopus manauensis* identified in population structure analyses. Numbers 1–21 on the first column refer to individual groups (sampled streams) of *Atelopus manauensis*. Abbreviations: *n* = number of samples; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{is}, fixation index 1 - (H_o / *He*); SNPs = number of SNPs analysed in each group.

Lineages	**Colour***	***n***	H_o	H_e	F_{is}	**SNPs**	**Private SNPs**
14–17–19–20–21	Blue	25	0.18 ± 0.18	0.2 ± 0.16	0.091	1299	38
18	Green	5	0.4 ± 0.24	0.39 ± 0.13	-0.05	926	42
15–16	Purple	10	0.32 ± 0.21	0.34 ± 0.15	0.42	1193	98
1–5	Pink	11	0.28 ± 0.19	0.28 ± 0.15	-0.0001	1718	8
6–8	Brown	18	0.26 ± 0.17	0.29 ± 0.15	0.11	2215	160
9–13	Yellow	20	0.26 ± 0.16	0.29 ± 0.15	0.1	2067	128

* Colour of genetic lineages on Snapp, Structure, DAPC and SNMf plots.

Table 3. Genetic differentiation among 14 drainage systems sampled for individuals of *Atelopus manauensis*. Abbreviation: d.f. = degrees of freedom

Source of variation	d.f	Percentage of variation	Fixation index
Among populations (F_{st})	13	25.69	0.24 (F_{st})
Among individuals (F_{is})	77	6.35	0.09 (F_{is})
Within individuals (F_{it})	91	67.96	0.32 (F_{it})

Table 4. Genetic differentiation among six genetic lineages of *Atelopus manauensis* identified in population structure analysis. Abbreviation: d.f. = degrees of freedom

Source of variation	d.f	Percentage of variation	Fixation index
Among populations (F_{st})	13	28.58	0.28 (F_{st})
Among individuals (F_{is})	77	5.87	0.08 (F_{is})
Within individuals (F_{it})	91	65.55	0.34 (F_{it})

Table 5. Effective population size (N_e) of six genetic lineages considering minimum allele frequency of 0.05 and 0.02. Numbers 1–21 refer to sampled streams where individuals of *Atelopus manauensis* were collected. Abbreviation: *n* = sample size.

Genetic group	Colour*	*n*	N_e 0.05	N_e 0.02
14–17–19–20-21	Green	25	22 (17–29)	21 (15–30)
18	Purple	5	∞	∞
15–16	Pink	10	∞ (163–∞)	∞ (163–∞)
1–5	Brown	11	64 (16 – ∞)	57.6 (19.4 – ∞)
6–8	Yellow	18	41 (21–194)	52 (27–247)
9–13	Blue	20	74 (42–226)	94 (53–326)

*Colour of genetic lineages on Snapp, Structure, DAPC and SNMf plots.

Table 6. Annotation of 28 outlier loci identified by Latent Factor Mixed Effects analyses highly associated to altitude (25) and forest biomass (three) of sampled streams. We used *Naborama parkeri*, *Bufo bufo*, *Xenopus tropicalis*, *Xenopus laevis*, and *Abavorana luctuosa* as reference genome to search for gene annotations in NCBI *Blastn* mode. We selected gene annotations with the lowest *E*-value among those listed.

SNP ID	Environmental association	*z*-score	Reference genome	*E*-value	Identity (%)	Gene annotation	GenBank accession
100031833	Altitude	5.7	*N. parkeri*	1.8	91	FUCA1	XM_018564520
46828174	Altitude	4.6	*X. tropicalis*	6.5	95	NFS1	XM_002932891.5
46829614	Altitude	5.2	*B. bufo*	1.8	88	Rhodopsin	U59921.1
46829632	Altitude	4.1	*X. tropicalis*	1.8	95	LCTL	XM_018253306.1
46830513	Altitude	5.1	*N. parkeri*	6.5	100	Ptprn2	XM_018569937.1
46831161	Altitude	4.2	*X. laevis*	1.8	91	galnt5.L	XM_018235788.1
46831388	Altitude	4.2	*X. laevis*	0.14	92	arhgef40.S	XM_018243980.1
46833712	Altitude	4.5	-	-	-	-	-
46833916	Altitude	4.1	*X. laevis*	1.8	85	camk2a	XM_018254187.1
46834090	Altitude	4.3	*X. tropicalis*	0.5	86	zyg11b	XM_002931422.5
46838777	Altitude	4.3	-	-	-	-	-
46839636	Altitude	4.0	*X. laevis*	6.1	91	Vmn2r26	XM_018262391.1
46840016	Altitude	4.7	*X. tropicalis*	1.8	85	samd11	XM_012965913.3
46840069	Altitude	4.4	*X. tropicalis*	0.5	86	rabgap1l	XM_012961192.3
46840479	Altitude	5.1	*N. parkeri*	6.1	91	SUV39H2	XM_018565041.1
46841657	Altitude	4.1	*X. laevis*	0.5	80	rnf19b	BC130177.1
46841980	Altitude	4.5	*X. laevis*	0.5	95	psmc6.L	BC045087.1
46842319	Altitude	4.8	*X. laevis*	1.8	91	pde4b	XM_018259600.1
46842804	Altitude	4.5	*X. tropicalis*	6.5	91	tmem184a	XM_012970416.3
46843485	Altitude	4.3	*X. laevis*	1.8	91	col20a1	XM_018236804.1
46843555	Altitude	4.3	*X. laevis*	0.012	100	myomegalin	XM_018257111.1
46844215	Altitude	6.4	*A. luctuosa*	0.14	92	NDUFA1	ATB23380
46844510	Altitude	4.2	*X. laevis*	1.8	100	atg2a.L	XM_018257569.1
46845599	Altitude	5.7	*X. tropicalis*	0.5	85	chm	XM_002938547.5
46845622	Altitude	4.4	*X. tropicalis*	1.8	100	fbxl14	XM_031898614.1
100050627	Forest biomass	4.3	*X. laevis*	6.1	100	slc4a9	XM_012959752.3
46829869	Forest biomass	4.6	*X. laevis*	0.5	100	bcl9	XM_018248945
46842854	Forest biomass	5.2	*X. tropicalis*	6.1	100	fn1	XM_012970496.3

Table 7. Coefficients from multiple linear regressions using the first PCA axes representing genetic variation of eight Single Outlier Loci (SOCs) identified in LDna analysis as response variable and the first PCA axes representing geographic (latitude and longitude) and environment of sampled streams (altitude and forest biomass) variation as explanatory variables. Bold values represent significant effects of explanatory on response variable. Abbreviation: *SE* = standard error.

	Residuals	*SE*	*t*-value	*P*
SOC_11_1 $R^2 = 0.10$; $P = 0.05$				
Longitude	0.02	0.6	0.03	= 0.9
Latitude	1.2	0.5	2.2	= **0.02**
Altitude	- 0.0006	0.003	- 2.06	= **0.04**
Forest biomass	- 0.0008	0.006	- 0.1	= 0.9
SOC_12_1 $R^2 = 0.02$; $P = 0.7$				
Longitude	- 0.2	0.8	- 0.3	= 0.7
Latitude	0.5	0.7	0.6	= 0.5
Altitude	0.0006	0.004	0.1	= 0.8
Forest biomass	0.002	0.008	0.2	= 0.8
SOC_69_0.81 $R^2 = 0.09$; $P < 0.06$				
Longitude	- 1.1	0.9	- 1.2	= 0.2
Latitude	- 1.6	0.8	- 2.0	= **0.04**
Altitude	- 0.003	0.004	- 0.8	= 0.3
Forest biomass	0.006	0.009	0.6	= 0.5
SOC_453_0.29 $R^2 = 0.23$; $P < 0.0001$				
Longitude	- 3.5	0.7	- 4.5	< **0.001**
Latitude	- 0.3	0.6	- 0.5	= 0.5
Altitude	- 0.0004	0.003	- 0.1	= 0.9
Forest biomass	0.006	0.007	0.8	= 0.3
SOC_548_0.24 $R^2 = 0.15$; $P < 0.005$				
Longitude	- 2.6	0.7	- 3.7	< **0.0003**
Latitude	- 0.6	0.6	- 0.9	= 0.3
Altitude	0.002	0.003	0.6	= 0.5
Forest biomass	0.0003	0.007	0.04	= 0.9
SOC_634_0.2 $R^2 = 0.15$; $P < 0.005$				
Longitude	- 3.8	1.1	- 3.4	< **0.001**
Latitude	- 1.2	0.9	- 1.3	= 0.1
Altitude	0.006	0.005	1.1	= 0.2
Forest biomass	0.01	0.01	- 1.6	= 0.1
SOC_724_0.17 $R^2 = 0.15$; $P < 0.005$				
Longitude	0.6	0.8	0.7	= 0.4
Latitude	- 1.5	0.7	- 2.1	= **0.03**
Altitude	- 0.0009	0.003	- 0.2	= 0.8
Forest biomass	0.01	0.008	2.1	= **0.03**
SOC_778_0.14 $R^2 = 0.08$; $P < 0.09$				
Longitude	0.08	0.5	0.1	= 0.8
Latitude	- 0.3	0.4	- 0.8	= 0.3
Altitude	- 0.0002	0.002	- 0.1	= 0.9
Forest biomass	0.01	0.005	2.1	= **0.03**

Table 8. Landscape resistance variables that best explained total genetic variation of *Atelopus manauensis* according to the model selection based on AICc and AIC weight in MLPE analysis. These variables represent the especific portion of altitude (meters a.s.l.) and forest biomass (MG C ha^{-1}) gradients that facilitate or impede dispersal and gene flow among the species' individual groups. Abbreviations: d.f. = degrees of freedom; AIC = Akaike Information Criterion.

Resistance variables	Intercept	d.f.	AICc	AIC Weight
Forest biomass 300 - 387	1170.78	4	59297.8	1
Altitude 125 - 165	1258.8	4	59960.7	1.17E-144
Altitude 0 - 20	1155.01	4	59966.3	7.04E-146
Forest biomass 0 -100	1181.81	4	60099.9	6.84E-175
Altitude 20 - 60	1414.19	4	60640.1	3.35E-292

Table 9. Summary of RDA showing the effects of isolation by geographic distance (IBD), isolation by landscape resistance (IBR – altitudes between 0-20, 20-60 and 125-165 m a.s.l. and forest biomass between 0-100 and 300-387 MG C ha^{-1}) and isolation by environment (IBE – altitude and forest biomass of sampled streams) on total genetic variation of *Atelopus manauensis*. The *P* values were obtained by ANOVA separately for IBD (*P* geo), IBR (*P* res 1-5) and IBE (*P* alt and *P* bio) variables. The inertia values are equivalent to variance, and the CP values show the constrained proportion of variance on genetic data captured by RDA. Bold *P* values show significant effects of IBD, IBR and/or IBE on total genetic variation. Abbreviations: example – IBR alt 0-20 – landscape resistance imposed by altitudes between 0 and 20 meters a.s.l.

IBD + IBE					Pure IBD			Pure IBE			
Inertia	CP	*P* geo	*P* alt	*P* bio	Inertia	CP	*P* geo	Inertia	CP	*P* alt	*P* bio
1.54	0.48	**0.001**	0.4	**0.03**	316	0.44	**0.001**	283	0.08	0.4	**0.01**
IBR alt 0 - 20 + IBE					Pure IBR			Pure IBE			
Inertia	CP	*P* res 1	*P* alt	*P* bio	Inertia	CP	*P* res 1	Inertia	CP	*P* alt	*P* bio
307796	0.42	**0.01**	**0.01**	**0.8**	277188	0.38	**0.001**	32102	0.04	0.3	**0.03**
IBR alt 20 - 60 + IBE					Pure IBR			Pure IBE			
Inertia	CP	*P* res 2	*P* alt	*P* bio	Inertia	CP	*P* res 2	Inertia	CP	*P* alt	*P* bio
102782	0.14	**0.007**	**0.04**	0.9	72174	0.10	**0.005**	577	0.08	**0.017**	0.12
IBR alt 125 - 165 + IBE					Pure IBR			Pure IBE			
Inertia	CP	*P* res 3	*P* alt	*P* bio	Inertia	CP	*P* res 3	Inertia	CP	*P* alt	*P* bio
291747	0.40	**0.001**	**0.01**	0.8	261139	0.36	**0.001**	19985	0.02	0.4	**0.06**
IBR bio 0 - 100 + IBE					Pure IBR			Pure IBE			
Inertia	CP	*P* res 4	*P* alt	*P* bio	Inertia	CP	*P* res 4	Inertia	CP	*P* alt	*P* bio
267766	0.37	**0.001**	**0.01**	0.8	237158	0.33	**0.001**	37913	0.05	0.4	**0.01**
IBR bio 300 - 387 + IBE					Pure IBR			Pure IBE			
Inertia	CP	*P* res 5	*P* alt	*P* bio	Inertia	CP	*P* res 5	Inertia	CP	*P* alt	*P* bio
331192	0.46	**0.001**	**0.007**	0.8	300584	0.41	**0.001**	23625	0.03	0.7	**0.03**

Table 10. Coefficients from multiple linear regressions using PCoA scores representing genetic distance (3,121 SNPs data set) as response variable and PCoA scores representing geographic (IBD), landscape resistance (IBR – altitudes between 0-20, 20-60 and 125-165 m a.s.l. and forest biomass between 0-100 and 300-387 MG C ha^{-1}) and environmental (IBE – altitude and forest biomass of sampled streams) distances as predictor variables. Bold *P* values show significant effects of IBD, IBR and/or IBE on total genetic variation. When not specified, IBE includes altitude plus forest biomass of sampled streams as predictor variables in the models.

	Residuals	SE	*t*-value	*P*
IBD + IBE $R^2 = 0.48$; $P < 0.001$				
IBD	23	2.7	8.63	< **0.001**
IBE Altitude	- 14	4.6	- 3.0	= **0.002**
IBE Forest biomass	- 7.8	2.0	- 7.7	= **0.0002**
IBR alt 0 -20 + IBE $R^2 = 0.14$; $P = 0.003$				
IBR alt 0 - 20	-263	342	- 7.6	< **0.001**
IBE Altitude	- 11.3	4.8	- 2.3	= **0.02**
IBE Forest biomass	- 4.9	2.0	- 2.4	= **0.01**
IBR ALT 20 -60 + IBE $R^2 = 0.42$; $P < 0.001$				
IBR alt 20 - 60	227	710	3.1	= **0.001**
IBE Altitude	- 18.7	6.7	- 2.7	= **0.006**
IBE Forest biomass	- 4.5	2.8	- 1.6	= 0.11
IBR alt 125-165 + IBE $R^2 = 0.40$; $P < 0.001$				
IBR alt 125 - 165	240	329	7.31	< **0.001**
IBE Altitude	- 9.1	4.9	- 1.8	= **0.06**
IBE Forest biomass	- 3.7	2.0	- 1.8	= 0.07
IBR bio 0-100 + IBE $R^2 = 0.37$; $P < 0.001$				
IBR bio 0 - 100	- 2760	407	- 6.7	< **0.001**
IBE Altitude	- 11.9	5.13	- 2.3	= **0.02**
IBE Forest biomass	- 5.6	2.21	- 2.5	= **0.01**
IBR bio 300-387 + IBE $R^2 = 0.46$; $P < 0.001$				
IBR bio 300 - 387	42520	5165	8.23	< **0.001**
IBE Altitude	- 8.11	4.73	- 1.71	= 0.09
IBE Forest biomass	- 4.49	1.96	- 2.28	= **0.02**

Online Resource 1. Detailed information of 21 sampled streams from 14 drainage systems distributed in five catchments of the known distribution of the *Atelopus manauensis*. The geographic distribution of the sampled streams is showed in Figure 1 as numbers according to the numbers listed in the column "Stream number".

Stream Number	Stream ID	Longitude	Latitude	nº of samples	Drainage system	Catchments
1	AP1	- 59.829	- 2.782	3	Upper course	Puraquequara River left bank
2	AP2	- 59.858	- 2.753	3		
3	AP3	- 59.847	- 2.749	3		
4	MP1	- 59.815	- 2.864	5	Middle course	
5	MP2	- 59.82	- 2.911	4		
6	MP3	- 59.809	- 2.918	5		
7	BP1	- 59.787	- 2.965	3	Lower course	
8	BP3	- 59.797	- 2.932	3		
9	TI1	- 59.913	- 2.938	4	Tinga	Puraquequara River right bank
10	TI2	- 59.913	- 2.938	5		
11	TI3	- 59.898	- 2.918	4		
12	UB	- 59.89	- 2.951	4	Ubere	
13	IP	- 59.904	- 2.991	3	Ipiranga	
14	PA	- 59.877	- 2.655	5	Middle course right	Rio Preto River
15	PM	- 59.742	- 2.58	5	Middle course left	
16	UR	- 59.931	- 2.389	5	Middle course	Urubu River
17	CF	- 59.578	- 2.592	5	Upper course	
18	TA	- 60.087	- 2.628	5	Upper course	Taruma-Açu River
19	ZF	- 60.227	- 2.563	5	Middle course	Cuieiras River
20	DI	- 60.078	- 2.329	5	Upper course left	
21	CU	- 60.279	- 2.247	5	Upper course right	

Online Resource 2. Methods for DartSeqTM used by Diversity Arrays Technology (Canberra, Australia) to discover and genotype and for quality control and initial calling of SNPs of *Atelopus manauensis*.

To call the SNPs, DNA sequences were aligned via *Blast* using the *Xenopus tropicalis* reference genome with an *E*-value: 5e-7 and minimum sequence identity of > 70%. To check for contamination, sequences were also aligned to bacterial and fungal genomes (NCBI). Furthermore, to call SNPs all tags from all libraries were included in DArTsoft14 proprietary software and were clustered using DArT PL's C++ algorithm at the threshold distance of 3. This was followed by parsing of the clusters into separate SNP loci using a range of technical parameters, especially the balance of read counts for the allelic pairs. In addition, multiple samples were processed from DNA to allelic calls as technical replicates and scoring consistency was used as the main selection criteria for high quality/low error rate markers DNA extraction was performed using the automated DNA extraction protocol on a 'TECAN freedom evo 100' liquid handling robot with a commercially available magnetic bead-based DNA extraction kit (Macherey-Nagel). DNA digestion and ligation reactions were performed as per Kilian et al. (2012). Digested and ligated samples were amplified for 30 cycles using the following PCR conditions: 94°C for 1 min, 94°C for 20 sec, 58°C for 30 sec, 72°C for 45 sec, 72°C for 7 min.

Proprietary analytical pipelines (DArTsoft14 – DArT's proprietary software) were used to process sequences generated from each lane. After quality filtering, approximately 500,000 (± 7%) sequences per sample were retained. DArT uses a reproducibility score (i.e. the proportion of technical replicate assay pairs for which the marker score is consistent) and polymorphism information content (PIC: an index ranging from zero to one and inform allele variations of SNP marker) to assess the quality and information content of SNP calls. The threshold for the reproducibility score is set to 97%. Further quality control was undertaken by removing any bacterial or viral contaminant sequences using alignment to matching sequences within the GenBank and DArT database.

SÍNTESE

No primeiro capítulo desta tese, foi demonstrado que *Atelopus manauensis* é uma unidade evolutivamente significante irmã de um clado de espécies distribuídas no Escudo das Guianas. Foram integrados dados genéticos, morfológicos e bioacústicos para descrever formalmente *Atelopus manauensis*. A espécie apresenta diferenciação genética relativamente elevada (> 4%), mas considerável semelhança fenotípica em relação as espécies a qual foi confundida no passado (*A. pulcher* e *A. spumarius*). *Atelopus manauensis* apresenta traços morfológicos e acústicos que a distingue das espécies com ocorrência no Escudo das Guianas, das quais apresenta em média 2,6% de diferenciação genética, considerada alta uma vez que as outras espécies do complexo *hoogmoedi* apresentam em média < 1%.

No segundo capítulo desta tese, foi demonstrado que fatores ecológicos relacionados ao clima, vegetação e extensão da área de inundação dos rios limitam a distribuição geográfica de *Atelopus manauensis* a machas de ambientes adequados em uma porção restrita entre a margem esquerda do rio Cuieiras e a margem direita do rio Urubu (~ 4,500 Km²), mesmo sem barreiras geográficas evidentes. A expansão urbana de Manaus levará *Atelopus manauensis* a extinção, caso ações conservacionistas não forem tomadas. Com base em critérios da IUCN, a espécie se enquadra na categoria “Em perigo” e é sugerida sua inclusão em listas de espécies ameaçadas.

No terceiro capítulo desta tese, foi demostrado que *Atelopus manauensis* está estruturada em seis grupos genéticos distintos, apesar de sua pequena distribuição geográfica. Estes grupos apresentam diferenças adaptativas em traços genéticos selecionados pelos gradientes de altitude e de biomassa da floresta. A proteção destes grupos garante que toda diversidade genética da espécie e seu potencial evolutivo seja preservado. O fluxo gênico entre os grupos genéticos é facilitado por florestas com maior biomassa florestal em altitudes intermediárias. Estes resultados são fundamentais para guiar ações aplicadas a conservação de *Atelopus manauensis*.

Os resultados obtidos nesta tese avançam no entendimento sobre a escala de influência de processos históricos e ecológicos nos padrões de distribuição geográfica e variação genética de anuros na Amazônia. Foi evidenciada a importância de estudos multidisciplinares para subsidiar ações conservacionistas na região. Assim como *Atelopus manauensis*, outras espécies não descritas podem estar ameaçadas de extinção e o conhecimento taxonômico permite a realização de estudos ecológicos aplicados a conservação de anuros amazônicos. Apesar da importância para conservação, abordagens de genômica de paisagem têm sido pouco utilizadas na Amazônia. Ainda é preciso ser entendido o quanto da variação fenotípica de *A. manauensis* é explicada pela variação ambiental em escala local e pela variação genômica e como processos históricos contribuíram para a variação genética contemporânea da espécie.

REFERÊNCIAS BIBLIOGRÁFICAS

Balkenhol, N.; Dudaniec, R.Y.; Krutovsky, K.V.; Johnson, J.S.; Cairns, D.M.; Segelbacher, G.; Selkoe, K.A.; von der Heyden, S.; Wang, I.J.; Selmoni, O.; Joost, S. 2017. Landscape Genomics: Understanding Relationships Between Environmental Heterogeneity and Genomic Characteristics of Populations. In: Rajora D (ed). Population Genomics. Springer, Switzerland, p. 261–322.

Bueno, A.S.; Bruno, R.S.; Pimentel, T.P.; Sanaiotti, T.M.; Magnusson, W.E. 2012. The width of riparian habitats for understory birds in an Amazonian forest. *Ecological Applications*, 22(2): 722-734. https://doi.org/10.1890/11-0789.1

Coloma, L.A.; Duellman, W.E.; Ana Almendáriz, C.; Ron, S.R.; Terán-Valdez, A.; Guayasamin, J.M. 2010. Five new (extinct?) species of *Atelopus* (Anura: Bufonidae) from Andean Colombia, Ecuador, and Peru. *Zootaxa*, 54(2574): 1-54. https://doi.org/10.11646/zootaxa.2574.1.1

de Fraga, R.; Lima, A.P.; Magnusson, W. E. 2011. Mesoscale spatial ecology of a tropical snake assemblage: the width of riparian corridors in central Amazonia. *Herpetological Journal*, 21(1): 51-57.

de Fraga, R.; Lima, A.P.; Magnusson, W.E.; Ferrão, M.; Stow, A.J. 2017. Contrasting patterns of gene flow for amazonian snakes that actively forage and those that wait in ambush. *Journal of Heredity*, 1085(5): 524-534. https://doi.org/10.1093/jhered/esx051

Espírito-Santo, H.M.V.; Rodríguez, M.A.; Zuanon, J. 2013. Reproductive strategies of Amazonian stream fishes and their fine-scale use of habitat are ordered along a hydrological gradient. *Freshwater Biology*, 58(12): 2494-2504.

Fearnside, P.M. 2020. Oil and gas project threaten Brazil's last great block of Amazon forest (commentary). *Mongabay*, 09 de março de 2020. https://news.mongabay.com/2020/03/oil-and-gas-project-threatens-brazils-last-great-block-of-amazon-forest-commentary/. Acesso: 20/06/2020.

Fernandes, A.M. 2013. Fine-scale endemism of Amazonian birds in a threatened landscape. *Biodiversity and Conservation*, 2211: 2683-2694. https://doi.org/10.1007/s10531-013-0546-9

Ferrão, M.; Colatreli, O.; de Fraga, R.; Kaefer, I.L.; Moravec, J.; Lima, A.P. 2016. High species richness of *Scinax* treefrogs (Hylidae) in a threatened amazonian landscape revealed by

na integrative approach. *PLoS ONE*, 11(11): e0165679 https://doi.org/10.1371/journal.pone.0165679

Ferrão, M.; de Fraga, R.; Moravec, J.; Kaefer, I.L.; Lima, A.P. 2018. A new species of Amazonian snouted treefrog (Hylidae: *Scinax*) with description of a novel species-habitat association for an aquatic breeding frog. *PeerJ*, 6(e4321): 1-34. https://doi.org/10.7717/peerj.4321

Ferrão, M.; Moravec, J.; Moraes, L.J.C.L.; de Carvalho, V.T.; Gordo, M.; Lima, A.P. 2019. Rediscovery of *Osteocephalus vilarsi* (Anura: Hylidae): An overlooked but widespread Amazonian spiny-backed treefrog. *PeerJ*, 2019(12): 1-35. https://doi.org/10.7717/peerj.8160

Frankham, R. 2015. Genetic rescue of small inbred populations: meta-analysis reveals large and consistent benefits of gene flow. *Molecular Ecology*, 24(11): 2610-2618. https://doi.org/10.1111/mec.13139

Frost, D.R. 2020. *Amphibian Species of the World: An online reference. Version 6.1*. New York, NY: American Museum of Natural History. https://doi.org/10.5531/db.vz.0001 (https://amphibiansofthe world.amnh.org/index.php). Acesso: 20/01/2020.

Gascon, C. 1989. The tadpole of *Atelopus pulcher* Boulenger (Annura: Bufonidae) from Manaus, Amazonas. *Zoologia*, 6(2): 235-239.

Godinho, M.B.D.C.; Da Silva, F.R. 2018. The influence of riverine barriers, climate, and topography on the biogeographic regionalization of Amazonian anurans. *Scientific Reports*, 8(3427): 1-11. https://doi.org/10.1038/s41598-018-21879-9

Gotelli, N.J. 2004. A taxonomic wish-list for community ecology. *Philosophical Transactions of the Royal Society B: Biological Science*, 359(1444): 585-597. https://doi.org/10.1098/rstb.2003.1443

Haffer, J. 1969. Hypotheses to explain the origin of species in Amazonia. *Brazilian Journal of Biology*, 68(4): 917-947. https://doi.org/10.1590/S1519-69842008000500003

Hijmans, R.J.; Cameron, S.E.; Parra, J.L.; Jones, P.G.; Jarvis, A. 2005. Very high-resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, 25(15): 1965-1978. https://doi.org/10.1002/joc.1276

Jorge, R.F.; Simões, P.I.; Magnusson, W.E.; Lima, A.P. 2016. Fine-scale habitat heterogeneity explains the local distribution of two Amazonian frog species of concern for conservation. *Biotropica*, 48(5): 694-703. https://doi.org/10.1111/btp.12333

Kaefer, I.L.; Rojas, R.R.; Ferrão, M.; Farias, I.P.; Lima, A.P. 2019. A new species of *Amazophrynella* (Anura: Bufonidae) with two distinct advertisement calls. *Zootaxa*, 4577(3): 316-334. https://doi.org/10.11646/zootaxa.4577.2.5

Kress, W.J.; Heyer, W.R.; Acevedo, P.; Coddington, J.; Cole, D.; Erwin, T.L.; Meggers, B.J.; Pogue, M.; Thorington, R.W.; Vari, R.P.; Weitzman, M.J.; Weitzman, S.H. 1998. Amazonian biodiversity: Assessing conservation priorities with taxonomic data. *Biodiversity and Conservation*, 7(12): 1577-1587. https://doi.org/10.1023/A:1008889803319

La Marca, E.; Lips, K.R.; Lötters, S.; Puschendorf, R.; Ibãnez, R.; Rueda-Almonacid, J.V.; Schulte, R.; Marty, C.; Castro, F.; Manzanilla-Puppo, J.; García-Pérez, J.E.; Toral, E.; Bolãnos, F.; Chaves, G.; Pounds, J.A.; Young, B. 2005. Catastrophic population declines and extinctions in Neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica*, 37(2): 190-201. https://doi.org/10.1111/j.1744-7429.2005.00026.x

Leite, R.N.; Rogers, D.S. 2013. Revisiting Amazonian phylogeography: Insights into diversification hypotheses and novel perspectives. *Organisms Diversity and Evolution*, 13(4): 639-664. https://doi.org/10.1007/s13127-013-0140-8

Lima, A.P.; Magnusson, W.E.; Menin, M.; Erdtmann, L.K.; Rodrigues, D.J.; Keller, C.; Hödl, W. 2006. *Guia de sapos da Reserva Ducke - Amazônia central*. Áttema Design Editorial, Manaus, Amazonas, Brasil. 176pp.

Lötters, S.; Haas, W.; Schick, S.; Böhme, W. 2002. On the systematics of the harlequin frogs (Amphibia: Bufonidae: *Atelopus*) from Amazonia. II: Redescription of *Atelopus pulcher* (Boulenger, 1882) from the eastern Andean versant in Peru. *Salamandra*, 38(3): 165-184.

Maximiano, M.F. de A.; d'Horta, F.M.; Tuomisto, H.; Zuquim, G.; Van doninck, J.; Ribas, C.C. 2020. The relative role of rivers, environmental heterogeneity and species traits in driving compositional changes in southeastern Amazonian bird assemblages. *Biotropica*, 00: 1-17. https://doi.org/10.1111/btp.12793

Menin, M.; Waldez, F.; Lima, A.P. 2011. Effects of environmental and spatial factors on the distribution of anuran species with aquatic reproduction in central Amazonia. *Herpetological Journal*, 21(4): 255-261.

Menin, M.L.; Lima, A.P.; Magnusson, W.E.; Waldez, F. 2007. Topographic and edaphic effects on the distribution of terrestrially reproducing anurans in Central Amazonia: Mesoscale spatial patterns. *Journal of Tropical Ecology*, 23(5): 539-547. https://doi.org/10.1017/S0266467407004269

Moraes, L.J.C.L.; Pavan, D.; Barros, M.C.; Ribas, C.C. 2016. The combined influence of riverine barriers and flooding gradients on biogeographical patterns for amphibians and squamates in south-eastern Amazonia. *Journal of Biogeography*, 43(11): 2113-2124. https://doi.org/10.1111/jbi.12756

Moritz, C. 2002. Strategies to protect biological diversity and the evolutionary processes that sustain it. *Systematic Biology*, 51(2): 238-254. https://doi.org/10.1080/10635150252899752

Moritz, C.; Patton, J.L.; Schneider, C.J.; Smith, T.B. 2000. Diversification of rainforest faunas: An integrated molecular approach. *Annual Review of Ecology and Systematics*, 31: 533-563. https://doi.org/10.1146/annurev.ecolsys.31.1.533

Oliveira, U.; Vasconcelos, M.F.; Santos, A.J. 2017. Biogeography of Amazon birds: Rivers limit species composition, but not areas of endemism. *Scientific Reports*, 7(1): 1-11. https://doi.org/10.1038/s41598-017-03098-w

Pabijan, M.; Palomar, G.; Antunes, B.; Antoł, W.; Zieliński, P.; Babik, W. 2020. Evolutionary principles guiding amphibian conservation. *Evolutionary Applications*, 13: 857-878. https://doi.org/10.1111/eva.12940

Pabijan, M.; Wollenberg, K.C.; Vences, M. 2012. Small body size increases the regional differentiation of populations of tropical mantellid frogs Anura: Mantellidae. *Journal of Evolutionary Biology*, 25(11): 2310-2324. https://doi.org/10.1111/j.1420-9101.2012.02613.x

Quesada, C.A.; Lloyd, J.; Schwarz, M.; Patiño, S.; Baker, T.R.; Czimczik, C.; Fyllas, N.M.; Martinelli, L.; Nardoto, G.B.; Schmerler, J.; Santos, A.J.B.; Hodnett, M.G.; Herrera, R.; Luizão, F.J.; Arneth, A.; Lloyd, G.; Dezzeo, N.; Hilke, I.; Kuhlmann, I.; Raessler M.; Brand, W.A.; Geilmann, H.; Moraes Filho, J.O.; Carvalho, F.P.; Araújo Filho, R.N.;

Chaves, J.E.; Cruz Junior, O.F.; Pimentel, T.P.; Paiva, R. 2010. Variations in chemical and physical properties of Amazon forest soils in relation to their genesis. *Biogeosciences*, 7: 1515-1541. https://doi.org/10.5194/bg-7-1515-2010

Ruland, F.; Jeschke, J.M. 2017. Threat-dependent traits of endangered frogs. *Biological Conservation*, 206: 310-313. https://doi.org/10.1016/j.biocon.2016.11.027

Ruokolainen, K.; Moulatlet, G.M.; Zuquim, G.; Hoorn, C.; Tuomisto, H. 2018. Geologically recent rearrangements in central Amazonian river network and their importance for the riverine barrier hypothesis. *Frontiers in Biogeography*, 11(3): 1-16. https://doi.org/10.21425/F5FBG45046

Santorelli, S.; Magnusson, W.E.; Deus, C.P. 2018. Most species are not limited by na Amazonian river postulated to be a border between endemism areas. *Scientific Reports*, 8(2294): 1-8. https://doi.org/10.1038/s41598-018-20596-7

Silva, E.P.; Borba, G.C.; Magalhães, C.; Zuanon, J.; Magnusson, W.E. 2020. Habitat segregation among freshwater shrimp species in an Amazonian rainforest stream system. *Freshwater Biology*, 65(4): 674-687. https://doi.org/10.1111/fwb.13458

Slatkin, M. 1981. Estimating levels of gene flow in natural populations. *Genetics*, 99:323-335.

Slatkin, M. 1993. Isolation by distance in equilibrium and non-equilibrium populations. *Evolution*, 47(1): 264-279.

Troudet, J.; Grandcolas, P.; Blin, A.; Vignes-Lebbe, R.; Legendre, F. 2017. Taxonomic bias in biodiversity data and societal preferences. *Scientific Reports*, 7(9132): 1-14. https://doi.org/10.1038/s41598-017-09084-6

Vacher, J.; Chave, J.; Ficetola, F.G.; Sommeria-klein, G.; Tao, S.; Thébaud, C.; Blanc, M.; Camacho, A.; Cassimiro, J.; Colston, T.J.; Dewynter, M.; Ernst, R.; Gaucher, P.; Oliveira Gomes, J.; Jairam, R.; Kok, P.J.R.; Dias Lima, J.; Martinez, Q.; Marty, C.; Noonan, B.P.; Nunes, P.M.S.; Ouboter, P.; Recoder, R.; Rodrigues, M.T.; Snyder, A.; Marques-Souza, S.; Fouquet, A. 2020. Large-scale DNA-based survey of frogs in Amazonia suggests a vast underestimation of species richness and endemism. *Journal of Biogeography*, 00: 1-11. https://doi.org/10.1111/jbi.13847

Vogel Ely, C.; Bordignon, S.A. de L.; Trevisan, R.; Boldrini, I.I. 2017. Implications of poor taxonomy in conservation. *Journal for Nature Conservation*, 36: 10-13. https://doi.org/10.1016/j.jnc.2017.01.003

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

Wang, I.J.; Bradburd, G.S. 2014. Isolation by environment. *Molecular Ecology*, 2323: 5649-5662. https://doi.org/10.1111/mec.12938

Wright, S. 1943. Isolation by distance. *Genetics*, 28(2): 114-138.

ANEXOS

Pareceres emitidos pelas bancas examinadoras da aula de qualificação e da versão escrita e defesa pública da tese.

PG·ECO·INPA
PÓS-GRADUAÇÃO EM ECOLOGIA

INPA
INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA

MINISTÉRIO DA CIÊNCIA, TECNOLOGIA, INOVAÇÕES E COMUNICAÇÕES

BRASIL
GOVERNO FEDERAL

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA

AULA DE QUALIFICAÇÃO

PARECER

Aluno (a): **RAFAEL FILGUEIRA JORGE**
Curso: ECOLOGIA
Nível: Doutorado
Orientador (a): Dra. Albertina Pimentel Lima (INPA)

Título:

"O PAPEL DE FATORES ECOLÓGICOS E GEOGRÁFICOS NA VARIAÇÃO FENOTÍPICA E GENÉTICA DO ANURO *ATELOPUS SPUMARIUS* lato sensu (BUFONIDAE) NA AMAZÔNIA CENTRAL"

BANCA JULGADORA:

TITULARES:	**SUPLENTES:**
Mário Eric Cohn-Haft (INPA)	Igor Luis Kaefer (UFAM)
Jansen Alfredo Sampaio Zuanon (INPA)	Fernanda Werneck (INPA)
Marina Anciães (INPA)	

ASSINATURA	PARECER	
MÁRIO ERIC COHN-HAFT	(X) Aprovado ( ) Reprovado	
JANSEN ALFREDO SAMPAIO ZUANON	(X) Aprovado ( ) Reprovado	
MARINA ANCIÃES	(X) Aprovado ( ) Reprovado	Marina Anciães
IGOR LUIS KAEFER	( ) Aprovado ( ) Reprovado	
FERNANDA WERNECK	( ) Aprovado ( ) Reprovado	

Manaus (AM), 31 de Outubro de 2016.

OBS:

PG·ECO·INPA
PÓS-GRADUAÇÃO EM ECOLOGIA

INPA
INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA
UNIDADE DE PESQUISA DO MCTIC

MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E INOVAÇÕES

PÁTRIA AMADA BRASIL
GOVERNO FEDERAL

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA

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

Aos 20 dias do mês de agosto do ano de 2020, às 14:30 horas, por videoconferência, reuniu-se a Comissão Examinadora de Defesa Pública, composta pelos seguintes membros: o (a) Prof (a). Dr (a). **Felipe Toledo**, da Universidade Estadual de Campinas - UNICAMP, o (a) Dr (a). **Ariovaldo Giaretta**, da Universidade Federal de Uberlândia – UFU, o (a) Prof (a). Dr (a). **Fabiane Annibale**, da Universidade Federal de Goiás - UFG, o (a) Prof (a). Dr (a). **Luciana Lourenço**, da Universidade Estadual de Campinas – UNICAMP e o (a) Prof (a). Dr (a). **Marina Anciães**, do Instituto Nacional de Pesquisas da Amazônia – INPA. Tendo como suplentes o (a) Prof (a). Dr (a). Fernanda de Pinho Werneck, do Instituto Nacional de Pesquisas da Amazônia – INPA e o (a) Prof (a). Dr (a). Cintia Cornelius Frische, do Instituto Nacional de Pesquisas da Amazônia – INPA. Sob a presidência do (a) primeiro (a), a fim de proceder a argüição pública do trabalho de **TESE DE DOUTORADO** de **RAFAEL FILGUEIRA JORGE**, intitulada **"ECOLOGIA, GENÔMICA DE PAISAGEM E STATUS TAXONÔMICO DO SAPO *ATELOPUS MANAUENSIS* (BUFONIDAE): UMA ABORDAGEM MULTIDISCIPLINAR APLICADA A CONSERVAÇÃO DE UMA ESPÉCIE EM RISCO DE EXTINÇÃO"**, orientada 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:

[X] APROVADO (A) [ ] REPROVADO (A)

[X] 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). FELIPE TOLEDO

Prof(a). Dr(a). ARIOVALDO GIARETTA

Prof(a). Dr(a). FABIANE ANNIBALE

Prof(a). Dr(a). LUCIANA LOURENÇO

Prof(a).Dr(a). MARINA ANCIÃES

Prof(a).Dr(a). FERNANDA DE PINHO WERNECK

Prof(a).Dr(a). CINTIA CORNELIUS FRISCHE

Coordenação PPG-ECO/INPA